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Abstracts of the 2nd Global Congress on Molecular Pathology:
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ORAL PRESENTATIONS

Genetics/Inherited Conditions

OR01. An International Interlaboratory Study of Complex Variant Detection by NGS

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Introduction: Next-generation sequencing (NGS) is a capable technique for detecting single nucleotide variants (SNVs) and small insertions and deletions (indels) in relatively accessible parts of a patient’s genome. However, conventional NGS methods have important limitations. An analysis of over 80,000 patients, tested for constitutional alterations using sensitive methods, showed that variants of other, technically challenging types comprise between 9 and 19% of the reportable pathogenic findings, depending on clinical indication. Approximately 50% of these variants were of challenging types (large indels, single exon copy number variants (CNVs), etc.), 20% were in challenging genomic regions (homopolymers, non-unique sequences, etc.), and 15% were in regions poorly covered by standard commercial kits. A further 15% presented multiple challenges. This study has since been expanded to include over 200,000 patients with diverse indications, and these newer data lead to similar conclusions.

Methods: We developed a synthetic specimen containing 22 challenging variants of diverse types in 7 commonly tested genes. Raw NGS data for these synthetic variants was found to mimic that of the endogenous variants and presented similar technical challenges for NGS. This specimen was sequenced using 10 different NGS tests by an international group of collaborating laboratories. These tests employed different sequencing platforms, library methods, and bioinformatics pipelines.

Results: All 10 tests detected all of the relatively “easy” SNVs and small indels present (with one exception). However, only 10 of the 22 challenging variants were detected by all tests, and just 3 tests detected all 22. Limitations with large indels, homopolymer associated variants, variants in non-unique regions, and other challenges were observed. Many, but not all of these limitations appeared to be bioinformatic in nature. Some of these limitations were not previously known to the respective laboratory directors, demonstrating the utility of this approach.

Conclusions: We believe that both our prevalence data and control specimens such as ours may be a valuable asset to the global AMP community for evaluating and optimizing clinical tests. The particular specimen described is now available to members of the AMP community and we are currently expanding this specimen and others to include additional variants. We are soliciting input from the community on additional variant types and genes to include in new specimens.

OR02. High-Risk Cytogenetics in Multiple Myeloma: Further Scrutiny of Deletions within the IGH Gene Region Enhances Risk Stratification

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Introduction: High-risk (HR) cytogenetics in multiple myeloma are best characterized using fluorescence in situ hybridization (FISH), as low proliferation indices and cryptic genetic aberrations often preclude informative results by standard karyotyping. When combined with the International Staging System, lactate dehydrogenase, and other clinical features, risk can be stratified and treatment
options can be informed by the cytogenetics profile. Certain translocations [i.e., t(4;14), t(14;16), t(14;20)] involving disruption of IGH are in the HR category, while the other IGH-disrupting translocations [i.e., t(11;14), t(6;14)] confer standard-risk (SR). To enhance efficiency, minimize cost, and preserve limited material (CD138+ fraction), laboratories typically use a breakapart probe (BAP) to detect rearrangement of IGH, followed by dual fusion probes to determine the IGH fusion partner. However, there is a paucity of data describing translocations and resultant fusion partners when the BAP for IGH reveals a deletion of part or all of the 5’ (telomeric) or 3’ (centromeric) gene region.

**Methods:** Our laboratory performed a retrospective review of abnormal multiple myeloma FISH studies between July 2012 and December 2017 to identify cases harboring deletions of the IGH region using a BAP. We catalogued these findings in conjunction with the dual fusion studies performed [2012 - 2016: t(4;14), t(14;16), t(11;14); 2017: the aforementioned probes plus t(14;20)] to determine whether these IGH deletions were indicative of clinically-significant translocations associated with HR or SR.

**Results:** Of our 233 patient cohort exhibiting IGH deletions, we identified 73 5’ deletions, 29 3’ deletions, 124 partial 5’ deletions, and 7 partial 3’ deletions with concurrent 3’ deletions. Subsequent dual fusion analysis revealed a fusion partner in 19% (n=44) of cases. Of these fusion-positive (FP) cases, 34% (n=15) exhibited HR translocations, including 26% (n=9) of FP cases with 5’ deletions, 100% (n=8) of FP cases with 3’ deletions, and 66% (n=2) of FP cases with partial 5’ deletions. Of note, patients with 5’ deletions (partial or full) exhibited both HR and SR translocations, while patients with 3’ deletions exhibited only HR translocations [i.e., t(4;14)].

**Conclusions:** Differentiating between IGH translocations that confer HR versus SR is imperative for prognostication and management. Although an IGH BAP strategy is commonly used to screen cases, our data suggest that subsequent analysis with dual fusion probe strategies should not be restricted to cases with traditional IGH BAP rearrangements. Specifically, our data demonstrate that atypical signal patterns (e.g., deletions) are often indicative of clinically-significant IGH-partner gene fusions. Thus, we recommend performing dual fusion FISH testing among cases with deletion of IGH, particularly for assessment of IGH translocations associated with HR gene partners, to ensure appropriate stratification and treatment.

**OR03. The Clinical Utility of Tumour Mutational Signatures for Identifying Hereditary Colorectal Cancer and Polyposis Syndromes**

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**Introduction:** Tumour mutational signatures are derived from the type of nucleotide substitution and their sequence context. Mutational signatures with known aetiology include: A ubiquitous signature associated with spontaneous deamination of 5-methylcytosine and correlated with age (signature 1), and signatures related to defective DNA mismatch repair (MMR; signatures 6, 15, 20, 26), polymerase proofreading exonuclease domain mutations in POLE (signatures 10 and 14) and base excision repair (BER) defects in NTHL1 (signature 30) and MUTYH (signature 18). The aim of this study was to assess the utility of tumour mutational signatures in colorectal cancers (CRCs) for identifying carriers of pathogenic variants and classifying variants of uncertain clinical significance (VUS) in genes underlying hereditary colorectal and polyposis syndromes.

**Methods:** Hereditary CRC and polyposis susceptibility gene mutation and VUS carriers were identified through germline testing of participants from the Australasian Colorectal Cancer Family Registry (MLH1, MUTHY, POLE, POLD1, AXIN2 variant carriers) and from the Genetics of Colonic Polyposis Study (NTHL1 and RNF43 variant carriers). Whole exome sequencing of formalinfixed, paraffin-embedded (FFPE) tumour DNA was performed using Agilent Clinical Research Exome V2 sequenced on Illumina Novaseq. Using the mutational signatures established by the Wellcome Trust Sanger Institute, we calculated the optimal combination of mutational signatures for each tumour sample based on cosine similarity.

**Results:** Two CRCs from MLH1 gene mutation carriers (Lynch syndrome) demonstrated signatures 6 and 15 correlating with defective MMR. The patterns in these two carriers were not ostensibly different to a sporadic CRC with biallelic somatic mutations in MLH1. Three CRCs from homozygous carriers of the MUTHY (p.Gly396Asp) mutation demonstrated a dominant signature 18 associated with defective BER. A CRC from a heterozygous carrier of the MUTHY (p.Gly396Asp) mutation and a VUS in MUTHY (p.Arg304Ser) did not demonstrate signature 18 suggesting the VUS is not pathogenic. The CRC from a biallelic NTHL1 carrier
showed a dominant signature. A MMR-proficient CRC from a carrier of a POLD1 VUS in the exonuclease domain (p.Gly321AlafsTer72) showed no evidence of hypermutation or defective DNA repair signatures. CRCs from a truncating RNF43 (p.Arg132Ter) mutation carrier with Serrated Polyposis Syndrome, and an AXIN2 truncating variant carrier (p.P350LfsX13) also did not demonstrate DNA repair signatures.

**Conclusions:** The tumour mutational signatures observed in CRCs from known pathogenic mutation carriers were consistent with the underlying DNA repair defect. The presence or absence of expected tumour mutational DNA repair signatures in CRCs from individuals with a VUS may help classify rare variants in hereditary CRC and polyposis genes. Further exploration of somatic mutation patterns in CRCs from RNF43 and AXIN2 carriers may identify novel mutational signatures.

**OR04. Prospective Study to Determine the Spectrum of Mutations among Patients with Multiple Endocrine Neoplasia 1 (MEN-1) and to Identify “At Risk”, First-Degree Relatives**

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**Introduction:** Multiple Endocrine Neoplasia (MEN-1) is an autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroid glands, pancreatic islets and the anterior pituitary. About 90% of patients with familial MEN-1 disease are known to carry mutations in the MEN1 gene. However, there is very little information about the spectrum of mutations seen in India.

**Methods:** Twenty-five clinically suspected cases of MEN-1 were enrolled prospectively over a two-year period. Of the 25 clinically suspected cases of MEN-1, 18 patients had definite clinical MEN-1 based on the presence of at least 2 of the endocrine tumours associated with MEN-1; 12 had family history of MEN-1. Further, 16 first-degree relatives of index cases with MEN-1 were screened for mutations that were seen in the index case. The MEN1 gene (exons 2-10), 3’ and 5’ UTR regions of MEN1 gene, CaSR and CDKN1B gene, continued to be negative for mutations. However, certain established polymorphisms in the MEN1 gene, CaSR and the CDKN1B gene were seen among the mutated and mutation negative cases. Interestingly, 7 of the 9 definitive cases of MEN-1 that were MEN1 mutation negative were found to be positive for the p.V109G polymorphism in the CDKN1B gene.

Sixteen first degree relatives including 9 members of a single large family, were screened for the same mutations as seen in the index case. Of the 9 members 6 were found to harbor the same mutations as in the index case (c.1151_1152insGAGG; p.A384fs) while three were negative. Among the other “at risk” families tested 3 of the remaining 7 tested, harboured the same mutations as in the index case.

**Conclusion:** The study has helped to clearly document the spectrum of mutations seen among patients with MEN-1. However, the absence of MEN-1 mutation in ~ 39% of the cases and the presence of the p.V109G polymorphism in the CDKN1B gene among the MEN-1 mutation-negative cases raises the question whether such polymorphisms could be independently contributing to pathogenesis.

**OR05. Five Year Experience of Clinical Next-Generation Sequencing for Somatic Overgrowth and Related Syndromes: Non-PIK3CA Cumulative Findings**

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**Introduction:** A number of oncogenes with recurrent mutations in cancer are now understood to drive segmental overgrowth and related syndromes. Our clinical laboratory has pioneered the sensitive detection of causative mutations in cancer and applied that expertise to mutations associated with overgrowth and related phenotypes. Presented here are our non-PIK3CA cumulative findings.

**Methods:** We use targeted hybridization capture coupled with Next-Generation Sequencing (NGS) for deep and comprehensive coverage. This assay has undergone frequent modifications due to its growing knowledge base. The current assay includes baits targeting exonic regions and selected intronic sequences from 177 genes recurrently mutated across all cancer types (Comprehensive Cancer Gene Set, version 3.2). The 35 genes reported for somatic overgrowth are divided into seven discrete orderable subsets depending on the presenting phenotype. Paired-end 101bp sequencing was performed on Illumina HiSeq 2500 with an average unique on-target coverage depth of >1000x. Variants
were called and analyzed using a combination of multiple software programs, and Clinical Genomics Workspace was used for data visualization and interpretation. Accepted specimen types include fresh and formalin fixed tissues, buccal swabs and fibroblasts. Of note, the somatic analysis of a peripheral blood sample was inappropriate in most cases. 

Results: Clinical NGS analysis was performed on specimens from 306 patients. The age of patients ranges from newborn to 82 years, with an average age of 7.4 years. About half of the patients were under the age of four and 18% were tested within their first year of life. A total of 80/306 patients had reportable genetic variants, 85/180 had non-PIK3CA variants. Among those, 71/85 had a pathogenic (P) or likely pathogenic (LP) variant, and 14/85 had variant of uncertain significance (VUS). A total of 92 non-P2IK3CA variants were identified in 23 genes in those 85 cases: 73/92 were P/LP and 19/92 were VUS. Half of the 92 variants were identified in six genes: GNA11 (12/92), KRAS (8/92), PIK3R2 (7/92), GNAS (7/92), GNAQ (6/92), and MTOR (6/92). Sanger sequencing was performed on a secondary non-affected specimen (peripheral blood) on 62/85 cases as a means to confirm the origin of the causative variant. Among those, 39/62 (63%) cases had negative results, indicating the somatic origin of the variants, 23/62 (37%) cases had positive results, indicating the germline or multi-tissue mosaic origin. In correlation, 63% (37/59) variants are with variant allele frequency (VAF) < 0.2 and 37% (22/59) with VAF>0.2.

Conclusion: Our cumulative clinical experience demonstrates the utility of NGS testing for segmental overgrowth and related syndromes. With new clinical trials for treatment that are often gene or variant specific, somatic testing for causative variants in patients with overgrowth syndromes has become an important part of clinical care.

Hematopathology

OR06. Validation of Immunohistochemistry by a Monoclonal Antibody CAL2 on Trephine Biopsy for CALR Mutation Detection in Myeloproliferative Neoplasms

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Introduction: Calreticulin (CALR) mutation is a major driver genetic event in essential thrombocythemia (ET) and primary myelofibrosis (PMF). Mutations in CALR lead to 1+ base pair frameshifts and generate a novel amino acid C-terminus. The monoclonal antibody CAL2 binds to the novel epitope of CALR mutant protein and allows the recognition of CALR mutations. The objectives of this study were to (1) validate the IHC detection of CALR mutation detection by CAL2 in Myeloproliferative Neoplasm (MPN) cases with known mutation status; (2) to develop an IHC scoring system for CALR mutation detection in the MPNs.

Methods: A total of 70 bone marrow (BM) trephines were selected for paraffin CAL2 IHC, including 31 known CALR mutations (22 ET and 9 PMF) and 31 known JAK2V617F mutations (22 ET and 9 PMF). Molecular testing for CALR mutation was performed by PCR Sanger sequencing. JAK2V617F mutation was confirmed by allele-specific PCR and PCR followed by restriction enzyme digestion analysis (PCR-RFLP). Individual cases were confirmed by fragment analysis or next-generation sequencing as indicated. A known CALR mutated specimen was used as an internal positive control, while 2 normal BMs and 6 cases of other myeloid neoplasms (AML (M5), AML (AML-TMD), MDS-SLD, MDS-MLD and 2 CML-CP) were also used as negative control. Mutant CALR mouse monoclonal antibody (CAL2, Dianova, Germany) was titered at 1/100 by following manufacturer’s instructions. Slides were stained on BOND III automated immunostainer (25 mins epitope retrieval, 30 mins antibody incubation, and 8 mins OptiView detection plus amplification). We developed a scoring system calculated by megakaryocytes (MK) signal strength (0-3+) times positive ratio of index (out of 1.0) for calling CALR mutation.

Results: Among 31 known CALR mutations cases with 9 various types of CALR mutations including 52 bp deletion and 5 bp insertion, 22 positives with strong CAL2 cytoplasmic staining (intensity 2-3/3) and 8 positives with weak CAL2 cytoplasmic staining (intensity 1) were recognizable in the MK. The mean CAL2 IHC score was 1.87, 95% CI [1.56, 2.18]. There were no significant differences between ET and PMF. 1/31 cases with multiple clones of 2 CALR mutations in cis was negative by CAL2 IHC. CAL2 staining was negative in 31 known JAK2V617F cases, as well as in 2 normal BMs and 6 cases of other myeloid disorders. The CAL2 IHC results correlated well with the molecular status.

Conclusions: The specificity and sensitivity of CAL2 IHC were 100% and 97% respectively. CAL2 IHC is an accurate method to identify CALR mutations in MPN. While CAL2 antibody can recognize CALR mutations with 1+ base pair frameshift, a limitation is failure to detect CALR mutation that results in non-canonical amino acid sequence, albeit of uncertain pathogenicity. We have successfully validated CAL2 IHC and the scoring scheme is ready for clinical use on BM trephine. IHC by CAL2 antibody is a simple, quick and cheap method to identify CALR mutation in MPN.

OR07. Comparison of a 640-Gene Next Generation Sequencing Panel to a Virtual 41-Gene Panel for the Diagnostic Utility in Unexplained Cytopenia

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Introduction: The diagnostic utility of somatic mutations for cytopenia is unclear: myeloid neoplasms including myelodysplastic syndrome are clonal, but clonal hematopoiesis can be found in healthy individuals. We examined a large cohort of well-studied patients with cytopenias with next generation sequencing to determine
its diagnostic utility, and also assessed the impact of panel size on diagnostic performance.

**Methods:** This cohort study comprised 207 patients with cytopenias and a specimen (blood or marrow) submitted to a CLIA-certified molecular pathology laboratory for 640-gene NGS from 09/2015 to 03/2018. Patients with diagnoses of acute leukemia, lymphoma, or history of cytotoxic therapy were excluded. Using a combination of marrow morphology and cytogenetics as a diagnostic “gold standard” for MDS, we assessed the diagnostic utility of somatic mutations detected by the 640 gene NGS panel and a virtual panel of 41 genes which are commonly mutated in MDS and aplastic anemia.

**Results:** 586 somatic mutations were identified in 201 patients with definitive diagnosis: 102 of 105 MDS patients, 20 of 34 aplastic anemia patients, and 27 of 52 patients with no evidence of a primary marrow disorder showed somatic mutations. The sensitivity of the large 640-gene NGS panel for MDS was 98.3%. When the variant allele frequency for one or more variants was set at ≥ 20%, the positive predictive value (PPV) for MDS was 94.9% with a specificity of 94.2%. Presence of two or more somatic mutations at any variant allele frequency (VAF) in a specimen showed a PPV of 76% for MDS, which increased to 95.6% when the VAF cutoff was set to 10%. While the virtual 41 gene panel (Figure 1) showed a mild decrease of sensitivity (96.5% versus 98.3%), a better specificity was observed when VAF was set at ≥ 20% (98.8% versus 94.2%). Remarkably, the presence of two or more somatic mutations at VAF ≥ 10% showed 100% specificity for MDS with the 41 gene panel. Mutation analysis can also predict high versus low grade MDS: mutations in TP53 and RUNX1 are associated with high-grade MDS, while SF3B1 mutations are, as expected, associated with MDS with ring sideroblasts. In addition PIGA mutations were present exclusively in aplastic anemia patients.

**Conclusions:** The 41 gene panel showed better diagnostic specificity than the 640 gene panel. Both panels showed high sensitivity in excluding MDS, and the presence of a mutation with a VAF ≥ 20%, or two or more mutations with a VAF≥10%, was highly predictive of MDS. Presence of PIGA mutations was highly predictive of aplastic anemia. Such mutation analysis has the potential to improve the diagnostic approach and accuracy for unexplained cytopenias.

**OR08. T-Cell Clonality Testing by Next Generation Sequencing Facilitates the Initial Diagnosis and Disease Monitoring of Cutaneous T-Cell Lymphomas**

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**Introduction:** The diagnosis of cutaneous T-cell lymphomas (CTCLs) is often challenging based on morphology and immunohistochemistry. Clonality assessment of T cell receptor (TCR) rearrangement is a valuable adjunct in routine clinical work up; however, current gold standard methods by fragment analysis (FA) have distinct limitations due to low sensitivity and specificity. Next generation sequencing (NGS) offers unique advantages over traditional assays as it provides sensitive and sequence specific data. This allows more accurate initial diagnosis of cutaneous lesions as well as highly sensitive assessment of disease involvement beyond skin and the possibility to monitor disease post treatment. Here we describe our clinical implementation of TCR gamma gene (TRG) based NGS clonality testing in the diagnosis and monitoring of CTCLs.

**Methods:** Clinical samples submitted for routine TRG clonality testing were included in this study, including formalin fixed paraffin embedded tissue, peripheral blood and bone marrow. Testing was performed using the LymphoTrack (LT) TRG kits (Invivoscribe) followed by sequencing on illumina MiSeq instrument. Data was analyzed by LymphoTrack MiSeq Software and in-house developed MSK-LymphoClone bioinformatics pipelines. Performance characteristics were evaluated by directly comparing to FA assay (BIOMED-2 primers) and flow cytometry (FC).

**Results:** A total of 47 clinical samples from 33 patients were analyzed, including Sézary syndrome/mycosis fungoides (22), cutaneous gamma-delta T cell lymphoma (3), anaplastic large cell lymphoma/lymphomatoid papulosis (2), CD4+ small/medium T-cell lymphoproliferative disorder (2), CD8+ aggressive epidermotropic T-cell lymphoma (1), primary cutaneous peripheral T-cell lymphoma (1), and CTCL, unclassifiable (2). Among 34 initial characterization samples, 11 (32%) were diagnosed as CTCLs after morphologic and immunophenotypic assessment and a clone was confirmed by both FA and LT. In 23 cases (68%), a diagnosis of atypical lymphohistiocytic infiltrate was rendered. TRG clonal rearrangement was detected in 10/23 (70%) by FA and 22/23 (96%) by LT. Among nine monitoring samples with corresponding FC results, 9/9 and 6/9 were positive by LT and FC, respectively.

**Conclusions:** TRG NGS clonality testing demonstrated higher clonal detection rate compared to FA and FC. Improved clonal detection at initial assessment facilitated the diagnosis of CTCLs in 26% more cases compared to FA. The unique ability to search for diagnostic clonal sequences enables more sensitive and specific staging and monitoring in the subsequent specimens.
OR09: A Noise-Cancelling Method Based on Information Theory for ctDNA Variant Calling
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Introduction: Circulating tumor DNA (ctDNA) is fragmented cell-free DNA (cfDNA) in plasma, originating from tumor cells rather than normal somatic cells. Since it carries great potential for noninvasive early diagnosis, progression monitoring, and prognosis, ctDNA soon arose as one of the hottest topics in cancer research now that NextGen Sequencing makes it feasible to identify ctDNA in clinically acceptable cost and time. However, due to low content of ctDNA in total cfDNA, a challenge we confronted in clinical application was how to identify meaningful low-frequency somatic variants carried by ctDNA from chaotic noise introduced by sample preparation and sequencing.

Information theory has been proven powerful to handle signal-noise problems in various disciplines. Particularly in bioinformatics studies, it has achieved competitive accuracies in challenging signal process problems such as identifying differential gene expression and aligning distantly related protein sequences. Here we implement a method based on knowledge of information theory to cancel the noises in ctDNA sequencing data and amplify the true variant signal.

Methods: Data preparation: SeqMaker (https://github.com/opengene/seqmaker.jl) was used to generate simulated ctDNA sequencing data containing 73 single nucleotide polymorphism (SNP) variants and their brinks of 1000bp in both upstream and downstream with average depth of 1000x. The frequencies of these variants varied from 0.001-0.1 and the sequencing error rate was 0.001. Simulated data were then trimmed by fastp and were then aligned by BWA (0.7.17-r1194-dirty). The output was converted by Samtools into BAM and PILEUP files.

Variant calling and performance benchmarking: The information theory-based algorithm gave each base position a score derived from the base frequencies in the output PILEUP files. The detail of this score will be provided in following publications. Then all base positions were ordered by their score. Applying stepwise cutoffs on these ordered positions, the true positive rate and the false positive rate were determined. The receive operator characteristic (ROC) figure summarizes all results.

Results: The ROC curve of our algorithm is displayed in Figure 1. In this simulated ctDNA data, our algorithm can achieve the true positive rate of 95% with the false positive rate of 0.2% when calling variants. The Area Under Curve (AUC) is 0.986.

Conclusions: Our information theory-based method can cancel sequencing noises and accurately identify low-frequency true variants in ctDNA samples, which could improve diagnosis accuracy in clinical practice.

OR10. Clinical Implications of Cytogenetic Heterogeneity in BCR-ABL1 Fusion Positive Adult B Cell Acute Lymphoblastic Leukemia
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Introduction: Cytogenetic heterogeneity is frequently observed in adults with t(9;22)(q34.1;q11.2)/BCR-ABL1 positive B cell acute lymphoblastic leukemia (Ph+B-ALL). We retrospectively investigated the cytogenetic heterogeneity and correlated additional chromosomal abnormalities (ACAs) with clinical outcomes in 144 adults with Ph+B-ALL.

Methods: There were 85 (59.1%) men and 59 (40.9%) women with a median age of 54-years. Patients were sorted into 6 subgroups based on the karyotypic findings in stemline including: 32 (22.2%) with t(9;22) as the sole aberration, 23 (15.9%) with t(9;22) plus one ACA, 26 (18.1%) with t(9;22) as part of a complex karyotype, 18 (12.5%) showed a variant t(9;22), 30 (20.8%) showed t(9;22) as the stemline with ACAs in the sideline(s), and 15 (10.4%) had the t(9;22) and hyperdiploidy. Of these, 89 cases had one, 41 had two and 14 had ≥ three clones, respectively.

Results: The median follow-up was 25.8 months. The median overall survival (OS) was 25.6 months and the median relapse-free-survival (RFS) was 20.6 months. Patients with variant t(9;22) had a poorer OS and RFS when compared with all other subgroups combined (P = 0.0018 and 0.0049, respectively). In addition, patients with two or more clones had poorer outcomes than those with one clone (P = 0.0427). Multivariate analysis confirmed that variant t(9;22) and clone numbers are the independent negative risk factors.

Conclusion: These data suggest that traditional karyotypic analysis of adult Ph+B-ALL identifying cytogenetic heterogeneity is helpful for the risk stratification of these patients.
Infectious Diseases

OR11. Incidental Detection of Malignancies in Metagenomics Cell-Free DNA Testing

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Introduction: Metagenomic next-generation sequencing (mNGS) is an unbiased test for detecting DNA pathogens through whole genome sequencing of cell-free DNA (cfDNA) in plasma and body fluids. At University of California San Francisco (UCSF) Clinical Laboratories, we launched a clinically validated mNGS test for undiagnosed meningitis and encephalitis using cerebrospinal fluid and are validating a plasma mNGS test for sepsis using cfDNA. In addition, we apply cell-free metagenomics as a general approach to body fluids to test for sepsis using cfDNA. In addition, we apply cell-free metagenomics as a general approach to body fluids to test for sepsis using cfDNA.

Methods: Clinical samples consisting of patient body fluids and plasma were identified due to clinical suspicion or laboratory confirmation of infection or malignancy. In both blood and body fluids, we reduced the majority of leukocyte human by centrifugation and sequenced only the supernatant or plasma. We then created sequencing libraries and performed whole genome sequencing. A similar protocol was used for formalin-fixed, paraffin-embedded (FFPE) material obtained from primary tumor tissue. The fastq output was aligned to hg38 and binned or laboratory confirmation of infection or malignancy. In both blood and body fluids, we reduced the majority of leukocyte human by centrifugation and sequenced only the supernatant or plasma. We then created sequencing libraries and performed whole genome sequencing. A similar protocol was used for formalin-fixed, paraffin-embedded (FFPE) material obtained from primary tumor tissue. The fastq output was aligned to hg38 and binned into segments of the human genome for read counting and normalization.

Results: In the initial set of plasma samples from patients newly admitted for suspected sepsis, 4/9 plasma from metastatic cancer patients showed at least five large (>20 Mb) copy number gains or losses. The positive cases were validated based either on tissue copy number variant (CNV) detection or cytogenetics for hematological malignancies. All body fluid samples (e.g. pleural fluid, peritoneal fluid, spinal fluid) that were confirmed positive for malignancy on cytology also carried at least 5 large (>20 Mb) copy number gains or losses. In addition, we found five examples of positive tumor signatures in body fluid specimens that had resulted ‘benign’ on cytology. For a subset of the samples that had correlated tissue blocks of the tumor available or had cytogenetic data, the same CNV pattern was consistently found in a primary tissue.

Conclusions: We show that incidental data from cfDNA metagenomic testing can pick up large CNVs in cfDNA. This is particularly useful in cases where an undiagnosed infectious and/or malignant etiology overlaps as possibilities on a clinical differential. The high specificity of requiring multiple large CNVs at a high tumor fraction (>5%) will mitigate the risk of false positives. This test may also be useful for monitoring the vast majority of tumors in different body sites, working especially well for metastatic disease with high tumor burdens. Larger studies are needed to validate this finding across a broader range of sample types and tumors and to assess for clinical utility.

OR12. Comparative Whole-Genome Analysis between Pregnant Women-Colonizing and Neonate-Infecting Group B Streptococcus Isolates

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Introduction: Group B Streptococcus (GBS) is a frequent resident of the vaginal tract in pregnant women and a major cause of invasive infection in neonates. GBS is readily transmitted from mother to neonate; however, molecular mechanisms for the transmission and invasion from mothers to their neonates remain unknown.

Methods: In this study, 48 GBS isolates were collected for whole-genome sequencing by using the Illumina next-generation sequencing technologies and Five GBS genomes have been fully assembled by PacBio, including 26 invasive (high-virulence, HV) isolates from bloodstream-infected neonates and/or vaginal/mother milk from colonization mothers, and 22 colonizing (low-virulence, LV) isolates recovered from vaginal colonization mothers without invasive infection in neonates.

We applied Align short reads to reference genome (B111) by bwa, and call variants by samtools mpileup; and annotate and extract single nucleotide polymorphisms (SNPs) by customized BioPerl scripts. The genome sequences between the mothers and neonates in the four pairs were virtually identical. We further compared the genomes between the HV and LV isolates recovered from mothers with and without subsequent neonate infections.

Results: In total, 34,395 variants, and 22,893 SNPs were analyzed, all 48 GBS isolates genomes divided into five major clades, Clade 1 consists of a significantly large number of HV isolates (19 HV isolates included, p=6.7e-4 by Fisher’s test) and other clades consist of more balanced mixed isolates. Opportunistic infections occur in all clades (no fixed diffs between invasive and colonizing isolates) and no single gene or SNP was found to correspond to all HV or LV (no fixed diffs between invasive and colonizing isolates). Recombination (more than mutation) has caused increase of virulence in the HV clade (we used LDhat to run recombination hotspot tests). There are 2-3 loci that stand out as the most promising virulence factor (a large number of nonsynonymous
changes that are statistically significant), including: LPXTG-domain surface protein ("CDH81_02355"), which is a known virulence factor in Gram+ bacteria, and phosphoribosylformylglycinamidine synthase ("CDH81_07310"). This suggests that these are HV factors that could be used as diagnostic markers and that this is deserving of further research.

Conclusion: There are a group of GBS clones circulating exclusively in China. Some clades are associated with high-virulence in neonatal patients. Multiple SNPs and genes are associated with high-virulence, in particular the adhesion known as pullulates.

OR13. Suppressor of Cytokine Signaling (SOCS)-3 Downregulation Is Associated with Increased Proinflammatory Responses in Diabetic Individuals with M. tuberculosis Infection
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Introduction: Pakistan ranks 5th in high tuberculosis (TB) burden countries and 6th in countries with high burden of diabetes mellitus (DM). Individuals with diabetes have been shown to exhibit dysregulation of host protective cytokines such as, IFN-γ, TNFα and IL6. Suppressor of cytokine signaling (SOCS) molecule 3 plays a role in maintaining balance between pro and anti-inflammatory cytokines in patients with TB. Given the co-incidence of both diseases we investigated the role of SOCS3 in modulation of host immunity in individuals infected with the pathogen, Mycobacterium tuberculosis (MTB), comparing latent (LTB) and active disease.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls (EC, n=20), those with LTB (n=10), DM (n=13), DM with LTB (n=15) and TB patients (n=15). PBMCs were stimulated with PPD (10µg/ml) for 18 hours. Cell supernatants were collected and tested for Th1/Th2 Cytokines. RNA was extracted from stimulated PBMCs and mRNA used for RT-PCR based analysis of IFNγ, TNFa, IL6 and SOCS3 genes.

Results: Measurement of stimulated cell supernatants showed increased levels of IFNγ in DM-LTB as compared with EC (p=0.01), LTB (p=0.03), DM (p=0.01) and TB (p=0.03) cases. IL12 and TNFa levels were increased in DM-LTB as compared with LTB (p value: IL12, 0.005; TNFa, 0.006), DM (IL12, p=0.021; TNFa, 0.019). Levels of IL6 were increased in DM-LTB as compared with EC (p=0.001) and LTB (0.021). Coordinately, we observed increased IFNγ (p=0.033) and IL6 (p=0.01) mRNA expression in patients with DM-LTB as compared with EC; also, increased TNFa mRNA expression as compared with LTB (p=0.034) cases. SOCS3 mRNA expression was decreased in DM-LTB as compared with LTB. In patients with TB, IFNγ mRNA levels were increased as compared with EC (p=0.0457), while SOCS3 mRNA levels were decreased as compared with LTB (p=0.0188).

Conclusion: We observed increased pro-inflammatory response in diabetics with latent TB to be associated with a downregulation in SOCS3 expression. As SOCS3 is required for mycobacterial clearance, our data suggests that this could be a mechanism which increases susceptibility to active TB in DM cases who are infected with MTB.

OR14. High Prevalence of NDM-1-Producing Enterobacter cloacae from Three Tertiary Hospitals in China
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Introduction: Enterobacter cloacae has recently emerged as one of the most common carbapenem-resistant Enterobacteriaceae. The emergence and spread of NDM-1-producing E. cloacae have posed an immediate threat globally. Here, we investigated the molecular characteristics of carbapenem-resistant Enterobacter cloacae (CREL) isolates collected from three tertiary hospitals in certain regions of China from 2012 to 2016.

Methods: Eighty-six nonduplicate CREL isolates were collected from Southern (e.g. Grangdong) and Northwestern (e.g. Ningxia) China. Species identification and antimicrobial susceptibility testing were performed using a VITEK-2 system. Susceptibility to carbapenems were also confirmed using broth microdilution method. Isolates were screened for antibiotic resistant genes by PCR, and expression of ompC and ompF was determined by qRT-PCR. Genetic relatedness was performed by pulsed-field gel electrophoresis (PFGE), and selected isolates were subjected to whole-genome sequencing. Multilocus sequence typing (MLST) and plasmid-typing were also mined in our study.

Results: Among the 86 CREL isolates, 50 (58.1%) were detected as carbapenemase producers. NDM-1 was the dominant carbapenemase (46.5%), followed by IMP-26 (4.7%) and IMP-4 (3.5%). Notably, we identified the first NDM-1 and IMP-1 co-producing E. cloacae, carrying plasmids of three incompatibility (Inc) groups, including IncHI2, IncHI2A, and IncN. In addition, Most strains showed decreased expression of ompC and/or ompF, and contained a broad distribution of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamase genes. The high-throughput sequencing demonstrated that seven blαNDM-1-harboring plasmids contained a highly conserved gene environment around blαNDM-1 (blαNDM-1 blαNDM-1 trpF-dsbD-cuA1-groES-groEL). Meanwhile, ltrA and OaeEΔ1 genes were frequently observed.
downstream bla\textsubscript{IMP}-26 and bla\textsubscript{IMP}-4. All the CREL isolates could be divided into 60 PFGE clusters. Nineteen bla\textsubscript{NDM}-1-positive \textit{E. cloacae} isolates obtained from Ningxia had the same PFGE pattern (Type 1) and were classified to ST78 in clonal complex 74 (CC74) by MLST analysis. The replicon typing indicated that IncX3 plasmids mediated the dissemination of bla\textsubscript{NDM}-1 within these homologous strains.

**Conclusions:** These findings suggested that different molecular mechanisms, including carbapenemase, ESBLs and AmpC \(\beta\)-lactamases plus the loss of porins, have contributed to carbapenem resistances in these CREL isolates. We first reported an outbreak of NDM-1-producing \textit{E. cloacae} ST78 with contribution of IncX3 plasmids in northwestern China. There’s an immediate need to to heightened surveillance efforts attentively to prevent and control the further spread of NDM-1 in China. (Y.C., C.C., MZ, and X.Y. contributed equally to this article)

**OR15. Application of a Multiplex PCR to Detect Intestinal Parasite Infections in Antenatal Anemia**

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**Introduction:** Anemia and poor nutrition status, especially in pregnant women, are partially attributable to intestinal parasitic infections. The majority of the global burden is considered due to the five main parasites: \textit{Ascaris lumbricoides}, \textit{Necator americanus}, \textit{Ancylostoma duodanle}, \textit{Trichuris trichiura}, \textit{Giardia duodenalis}, and \textit{Entamoeba histolytica}. Diagnosis of these parasites has always relied on the classical microscopic examination of stool samples as it is relatively simple to perform and does not require expensive laboratory equipment. However, stool microscopy is highly observer dependent and has several shortcomings that greatly affect the efficacy of current management of intestinal parasitic infections in pregnant women. The study aim to assess the utility of multiplex PCR for detection of intestinal parasites as compared to microscopy in establishing intestinal parasitic infections in antenatal anemia.

**Methods:** Thirty-four antenatal anemic subjects, enrolled from neighboring villages of Wardha district of India as part of pilot study funded by Public Health Foundation of India in collaboration with Department of Science and Technology, India. Samples were collected and stored in 5% potassium dichromate. DNA extraction was done using conventional protocol. Extracted DNA was subjected to multiplex real time PCR using TaqMan probes, forward, and reverse primers for the following species \textit{Ascaris lumbricoides}, \textit{Ancylostoma duodanle}, \textit{Trichuris trichiura}, \textit{Necator americanus}, \textit{Giardia duodenalis}, and \textit{Entamoeba histolytica} was designed.

**Results:** The age range of antenatal anemic patients was 18-30 years median age 24 years. 16/34 (41%) patients were positive for one or the other parasite. Maximum number 11/34 (32%) had infection with \textit{Ascaris lumbricoides}, 7/34 (12%) were positive for \textit{Ancylostoma duodanle}, 1/34 was positive for \textit{Entamoeba histolytica}. 4/34 had multiple infection with \textit{Ascaris lumbricoides} and \textit{Ancylostoma duodanle}. On microscopy only two cases (\textit{Ascaris lumbricoides} and \textit{Entamoeba histolytica} each) were positive and none was positive for co-infection.

**Conclusion:** The real-time PCR-based diagnosis outperforms microscopy and the multiplex PCR approach was also superior in terms of detection with multiple infections and can be used for detection and management in antenatal anemia.
Informatics

OR16. Identifying True Somatic Variants in Cancer Using Machine Learning
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Introduction: Molecular profiling has become increasingly essential for tumor risk stratification and treatment selection. However, the diversity of somatic events, technical artifacts and genomic complexity make the differentiation of reportable variants from artifacts a challenge. Variant refinement via manual inspection is costly for clinical labs and is subjective to personal bias. Here, we present a machine learning-based classifier developed at Children's Hospital of Philadelphia (CHOP) to distinguish artifacts from bona fide Single Nucleotide Variants (SNVs) of tumor samples.

Methods: All of the SNVs used in this study were from CHOP cancer panels and were manually reviewed and labeled as either true positive or artifact. An array of sequencing attributes were calculated for each SNVs, such as coverage, strand bias and mapping quality. Feature selection was performed to remove attributes that provided little separation between the two classes such as mapping quality. A random forest classifier was trained on a training set of 3367 SNVs from 84 tumor samples. The classifier achieved 100% sensitivity/specificity and 0.98 F-1 score in a 10-fold cross validation. Following this, an independent test set of 2362 SNVs from 66 samples were used to fine-tune the baseline model. During this process, a confidence interval (CI) in the range of [0, 1] was assigned to each of the variants by the model reflecting the certainty of the classification. The range indicates confidence level of the model for a variant being 100% an artifact 0.0 or real 1.0. To achieve optimal performance, different weights were assigned to the two classes to account for different misclassification costs, i.e. a true variant being classified as an artifact (false negative) could lead to a worse adverse clinical consequence than a false positive misclassification.

Results: We identified a model that yielded the optimal performance on the test set. Additionally, a range of CI scores was derived to label SNVs with CI scores within [0.05, 0.9] “uncertain” to meet clinical assurance, which would require manual inspection. The combination of the classifier and the CI thresholds, achieved 100% sensitivity/specificity with 11 SNVs labeled as “uncertain” and zero misclassifications on the test set. We then benchmarked its performance on an independent validation set of 5634 SNVs from 141 samples. As a result, our model achieved 100% sensitivity/specificity with only 3% SNVs labeled as “uncertain” and zero misclassifications.

Conclusions: We presented a computational classifier to identify true positive SNVs from tumor sequencing. The classifier demonstrated high sensitivity/specificity, and utility on a wide range of tumor samples. Overall, 90% of the SNVs detected will receive a definite label and thus be exempt from manual review. Implementing the model can greatly reduce the hands-on time and hence improve the efficiency without compromising the quality of the clinical tests.

OR17. Development and Clinical Validation of a Bioinformatics Pipeline for CNV Detection on Cancer NGS Panels
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Introduction: Copy number variation (CNV) is the most common genomic alteration in pediatric cancer that directly impact patient care. NGS has emerged as the method of choice to analyze a wide range of genes known to be associated with various cancers. We have launched multiple NGS panels targeting childhood cancers including 13 hereditary cancer panels (128 genes), 9 bone marrow failure panels (159 genes), one hematological cancer panel (118 genes), and one solid tumor panel (238 genes). To optimize the detection of CNVs based on the sequencing data, we developed an accurate and efficient bioinformatics pipeline to identify and visualize CNVs allowing simultaneous detection of SNVs, indels and CNVs.

Methods: To overcome the limitation of targeted panel sequencing that lacks continuous coverage required for the detections of CNV and to meet the needs of identifying ploidy changes in cancers, thousands of single nucleotide polymorphism (SNP) probes relatively evenly covering the entire genomic regions of targeted genes were added to each panel as the “backbones”. FastQ files generated by HiSeq sequencer are first aligned to GRCh37 assembly. CNVs are called by VarScan2 on the data from patients and controls sequenced using the same set of baits. B-allele frequency was generated based on SNP calls from the backbone and the regions of interest of the panel for each patient and compared to that of the controls. CNVs were annotated with gene symbols and chromosome locations. CNVs and B-alleles were then post-processed and visualized by home-brew software using R Shiny. The pipeline were designed for analyzing multiple samples in parallel to increase analytic efficiency and accuracy.

Results: To assess the performance of the pipeline, we validated it on 30 different clinical samples with known CNVs. The results showed 100% concordance to that of our previously validated commercial software yet with only 20% of the processing time. Analyzing multiple cases in paralell also improves the accuracy of the CNV calls by eliminating batch effect. In addition to detecting
simple CNVs, the pipeline successfully detected complex genomes with complicated del/dup/amp and ploidy changes in neuroblastoma, rhabdomyosarcoma, and osteosarcoma exemplified by the identification of a pseudo-hyperdiploid genome in a near-haploid ALL patient which would be indistinguishable from true hyperdiploid ALL by cytogenetics or histology/flow cytometry (figure 1).

**Conclusions:** We have developed a clinical bioinformatics pipeline for CNV calling, annotation and visualization for targeted NGS panels. The validation results demonstrate 100% accuracy in identifying cancer associated CNVs with increased efficiency and accuracy.

![figure 1 for caok g-amp 2019 abstract](https://validsplicemut.cytognomix.com/)

**OR18. Pan-Cancer Repository of Validated Natural and Cryptic mRNA Splicing Mutations**

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**Introduction:** Next generation sequencing continues to reveal large numbers of novel variants whose impact cannot be interpreted from curated variant databases or through reviews of peer-reviewed biomedical literature. This has created a largely, unmet need for unequivocal sources of information regarding the molecular phenotypes and potential pathology of variants of unknown significance (VUS); in cancer genomes, such sources are critically needed to assist in distinguishing driver mutations from overwhelming numbers of bystander mutations. VUS classification criteria highlight the limitations in genome interpretation due to ambiguous variant interpretation.

**Methods:** We present a major public resource of mRNA splicing mutations validated according to multiple lines of evidence of abnormal gene expression. Likely mutations present in all tumor types reported in the Cancer Genome Atlas (TCGA) were identified based on the comparative strengths of splice sites in tumor versus normal genomes and then validated by respectively comparing counts of splice junction spanning and abundance of transcript reads in RNA-Seq data from matched tissues and tumors lacking these mutations.

**Results:** The comprehensive resource features 351,423 mutations which strengthen cryptic splice sites (10,943 affect both simultaneously), 27,803 novel or rare flagged variants (with <1% population frequency in dbSNP) were observed in multiple tumor tissue types. Single variants or chromosome ranges can be queried using a Global Alliance for Genomics and Health (GA4GH)-compliant, web-based Beacon “Validated Splicing Mutations” either separately or in aggregate alongside other beacons through the public Beacon Network (http://www.beacon-network.org/#/search?beacon=cytognomix), as well as through our website (https://validsplicemut.cytognomix.com/).

**Conclusions:** The Validated Splicing Mutation resource should substantially contribute to reducing the number of outstanding VUS in tumor (and possibly some germline) genomes, and substantially increases the number of functional variants with previously unappreciated consequences to mRNA splicing, in particular, activation of cryptic splice sites. We previously (doi: 10.1038/srep07063) identified 988 TCGA breast cancer mutations that significantly altered normal splicing (19% of total). This database greatly expands the size of the repository. Here, a higher ratio of rare or novel mutations have been validated (24% of total were flagged). The higher yield could be related to the same mutation being present in multiple samples from the same tumor type and other tumor tissues, which would be expected to increase the probability of observing abnormally expressed splice forms for the mutation.

**OR19. Error Rate Normalization to Establish Position-Specific Limits of Detection in Next Generation Sequencing Assays**

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**Introduction:** Next-generation sequencing (NGS) continues to be the method of choice for high throughput genetic assays. Many assays employ a uniform assay-specific limit of detection (LoD) that tends to be overly conservative due to “worst-case” error and artifact rates (generated by PCR, sequencing and alignment errors, etc.). The process of differentiating sequencing artifacts from real, low-frequency variants is extremely important for clinical utility of NGS-based assays, especially as clinicians push for tracking minimal residual disease (MRD). The motivation for this study was to identify a strategy for finding the inherent error-rate, at single base resolution, for our MyMRD assay. This strategy improved the specificity and sensitivity of our MyMRD assay and can be implemented in any NGS-based assay.

**Methods:** Our study focused on Invivoscribe’s MyMRD assay panel and investigated the observed error rates at all 53,308 targeted bases. We looked at each of these positions independently to evaluate the observed frequency of every possible single nucleotide variation (SNV) and every observed insertion/deletion (indel) in 213 samples (36 samples from reference cell line NA12878 and 177 contrived/clinical samples).
We calculated various inter and intra-sample metrics (e.g., minimums, maximums, average error frequencies) independently for each SNV and indel to understand overall assay trends as well as sample-specific trends at single base resolution.

**Results:**

| Sample Set | Minimum Observed SNV Frequency (%) | Maximum Observed SNV Frequency (%) | Average Observed SNV Frequency (%) | Minimum Observed Indel Frequency (%) | Maximum Observed Indel Frequency (%) | Average Observed Indel Frequency (%) | Highest Freq Indel SNV Type |
|------------|-----------------------------------|-----------------------------------|-----------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|---------------------------|
| NA12878    | 0%                                | 0.30%**                           | 4.4x10^-7                        | 0%                                  | 0.33%**                            | 0.022%                             | C--C                      |
| Contrive d/Clinical | 0%                                | 0.49%                             | 4.8x10^-7                        | 0%                                  | 0.10%                              | 0.002%                             | C--G                      |

Table 1: The analysis showed that error-rates widely vary across different positions and across different variant types (even within a single targeted base).

**Known variants (in NA12878) and high probability germline variants and artifacts were filtered from these analyses to focus on the background error-rate at homozygous reference positions.

**Conclusions:** We were able to increase the sensitivity and specificity of the MyMRD panel by creating non-uniform LoDs that take into account variant specific error rates at every targeted position. We improved the sensitivity of the assay by decreasing the LoD (often dropping from 0.5% to lower than 0.1%) and at >99% of the targeted sites. However, we also identified potential artifacts where the LoD was increased to avoid calling false positives which would have arisen if a uniform LoD was used. By increasing the LoD at these positions, we were also able to improve the assay’s specificity. Overall, performing this analysis makes it easier to differentiate sequencing artifacts from true low-frequency variants, which is particularly important for MRD tracking applications.

**Solid Tumors**

**OR20. Clinical Implication of ctDNA Analysis in Advanced Lung Carcinoma Patients Using Different Technologies: Real-Time PCR and MALDI-TOF**

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**Introduction:** With liquid biopsy (LB) entering the routine in personalized cancer treatment it is important to understand the differences with regards to technical and clinical sensitivity and mutation coverage inherent to technologies for analyzing circulating tumor DNA (ctDNA) and its implication for the patient care.

**Methods:** Circulating cell free DNA (ctDNA) extracted from blood plasma (QaAMP ctDNA, Qiagen) of 100 lung carcinoma patients were assessed for DNA quality and quantity using DNA 3K Assays on the LabChip GX Touch 24 (PerkinElmer). Each 10 ng ctDNA were analyzed for somatic mutations detection in ctDNA using two technologies: Real-time PCR test Cobas EGFR Mutation Test v2 (Roche) that identifies 42 mutations in the EGFR gene (63 patients tested), and UltraSEEK Lung Panel on MALDI-TOF based MassARRAY (Agena Bioscience) that covers 67 mutations in 5 oncogenes: EGFR (43), BRAF (4), ERBB2 (2), KRAS (14) and PIK3CA (4) with a sensitivity down to 0.1% (100 patients tested). All samples were also analyzed with the UltraSEEK EGFR Panel (Agena Bioscience) that surveys the 3 most frequent activating mutations in EGFR and the resistance markers EGFR T790M and EGFR C797S. A set of controls was added to each run: 0.5% positive control (EGFR ctDNA Multiplex), EGFR wild-type control (Horizon Discovery) and no-template control.

**Results:** All ctDNA samples showed the expected fragment-size distribution on LabChip and no significant abundance of genomic DNA.

Four of the 63 (6%) samples tested on Cobas EGFR Mutation Test v2 were invalid for analysis. Concordant results were achieved for 85% of patients (50/59) for overlapping markers in EGFR. With the UltraSEEK Lung Panel 10 additional EGFR mutations (4*Exon 19 deletion, 3*T790M, 1*Exon 20 insertion, 1*L861Q/R, 1*G917A) were detected in 8 of 59 patients (14%). 1 EGFR Exon 19 deletion was detected by Cobas only (2%). EGFR T790M mutation was detected in 7 samples with Cobas and in 10 samples with UltraSEEK (70% concordance). UltraSEEK Lung Panel identified 7 non-EGFR mutations (6*KRAS, BRAF) in 6 patients (10%). Across all 100 patient samples in EGFR 32*Exon 19 deletions, 18*T90M, 17*L858R, 5*G719X, 3*L861X and 1*S768I could be identified with UltraSEEK Lung Panel as well as 9*KRAS and 2*BRAF V600E. The UltraSEEK EGFR Panel confirmed all overlapping mutations in EGFR also assessed with the UltraSEEK Lung Panel.
Conclusion: This study showed that the UltraSEEK Lung Panel improved sensitivity for ctDNA analysis in comparison to Cobas EGFR Mutation Test v2, a method widely used in clinical routine. The UltraSEEK Panel could detect more EGFR primary or secondary resistance mutations. With the use of a panel that covers mutations in multiple genes, additional actionable mutations to EGFR (KRAS, BRAF) could be also detected in LB and used as a guidance for therapy. These new data may lead to improve clinical sensitivity of LB and its clinical utility in the management of lung cancer patients.

OR21. Adjusting for Variation in Tumor Mutation Burden Using a Cancer-Specific Threshold in Whole Exome Sequencing

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Introduction: Tumor mutational burden (TMB) has recently been shown to be an improved biomarker for predicting response to immunotherapy, with high TMB patients responding better to immunotherapy than low TMB patients. Defining a patient as TMB high or TMB low has not been standardized and often uses a pan-cancer threshold. On the other hand, almost all previous publications on TMB cut-off have used comprehensive panel which covers a small portion of the whole genome/exome, and no systematic whole exome sequencing data has been analyzed to analyze the TMB distribution among different cancer types. Hereby, we aim to understand the differences between using a pan-cancer threshold compared to a cancer-specific threshold, as well as understand the clinical context of TMB.

Method: Using whole exome sequencing (WES) data from primary tumors in The Cancer Genome Atlas (TCGA) (n=3886) and advanced Weill Cornell Medicine (WCM) samples (n=979), TMB status was determined by using both a pan-cancer and cancer-specific threshold. Survival curves and number of TMB high classifications were used together to evaluate the differences between the thresholds. Cox regression was performed to understand other clinical variables that may influence the use of TMB as a prognostic biomarker.

Result: The distribution of TMB varied between cancer types. The cancer-specific threshold was able to adjust for the different TMB distributions, while the pan-cancer threshold was often too stringent. The dynamic nature of the cancer-specific threshold resulted in more TMB high classifications compared to the static pan-cancer threshold. Additionally, the survival curves between the cancer-specific and pan-cancer threshold were similar for both TMB high and TMB low groups.

Conclusion: TMB is relative to the context of cancer type, metastatic state, and disease stage. A cancer-specific threshold results in more patients being classified as TMB high while maintaining clinical outcome comparable to pan-cancer threshold.
OR22. Integrated Genomic Profiling of 289 Pediatric Brain Tumors Uncovered Genomic Signatures Significantly Impacting Patient Care

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Introduction: Genomic mutation profiling plays a significant role in brain tumor diagnosis, prognosis, and treatment. Large Next-Generation Sequencing (NGS) panels are rapidly replacing traditional single-gene or focused mutigene testing to provide more comprehensive information about the tumor genome. Here we describe genomic profiles of a large cohort of pediatric brain tumors tested in a single clinical laboratory using a custom-designed NGS panel, CHOP OncoMark Solid, and their impact on patient care.

Methods: Brain tumor samples from patients 0-21 years old over a 33-month period were subjected to NGS testing. Genomic alterations were assessed using the CHOP OncoMark Solid panel to interrogate 238 cancer genes for single nucleotide variants (SNVs), indels, and copy number variants (CNVs), and 110 fusion gene partners for over 600 known and novel fusions. Identified variants were categorized and reported according to the AMP/ASCO/CAP guidelines. The clinical impact of the results were evaluated based on their significance on diagnosis, prognosis and treatment.

Results: 289 consecutive tumors were tested, the most common of which were pilocytic astrocytoma (67/289), medulloblastoma (24/289) and diffuse midline glioma (21/289). Clinically significant genomic alterations were identified in 93% of the tumors. 191 clinically significant SNVs/indels in 73 genes were reported 323 times in 164 cases. The most frequent mutations were BRAF V600E (32/323) in multiple tumor types and H3F3A K28M exclusively in diffuse midline glioma. TP53 and NF1 were second and fourth most mutated genes in this cohort. More than 1500 CNVs were reported in 175 cases with gain of 1q and 7q the most prevalent. 32 fusions were detected in 97 cases with KIAA1549-BRAF the most common. These results provided diagnostic evidence in 73.3% of patients including 9 patients whose diagnoses were changed and 41 patients whose diagnoses were further defined; prognostic evidence in 34.6% of patients, and evidence for targeted therapy in 18% of patients. Distinctive genomic profiles for specific tumor subtypes were observed: 64% of pilocytic astrocytomas harbored KIAA1549-BRAF, all diffuse midline gliomas were H3F3A K28M positive with 52% of them showing H3F3A gain, isochromosome 17q was highly enriched in non-WNT/non-SHH medulloblastomas, and loss/cnLOH of chr22 was observed in 100% of ATRT cases, 42% of which displayed additional SMARCB1 mutations. 71 patients were suspected of carrying germline mutations, of which 25 were evaluated and 11 confirmed. The average tumor mutation burden (TMB) was 3.88 mutations/Mbp and all 5 cases with high TMB (>10 mutations/Mbp) harbored a germline mutation in MSH2 or MSH6.

Conclusion: Integrated genomic profiling of pediatric brain tumors demonstrates significant impact on clinical care in 78.2% of patients in our institution. Broad-base genomic profiling should be implemented as part of the routine patient care for pediatric brain tumors.

OR23. Biomarker Analysis on Limited Biopsy Lung Cancer Samples in Tertiary Care Cancer Centre in South India Basavatarakam Indo American Cancer Hospital, Hyderabad, India

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Introduction: Lung cancer diagnosis and management has undergone a paradigm shift in the last decade with multiple actionable mutations and genetic alterations being detected. Biomarker studies in lung cancers from India are limited owing to the different tissue handling processes and lack of standardisation in the preanalytic phase. We present the data of prevalence of EGFR (Epidermal growth factor), ALKDF53 (Anaplastic lymphoma kinase; clone D5F3) and ROS1 in a cohort from a tertiary care centre in south India seen over the last two years.

Methods: EGFR testing was done by RT-PCR (Qiagen Rotorgene) using Therascreen EGFR RQO PCR kit, ALKDF53 testing was done by immunohistochemistry (IHC) on Ventana Benchmark XT automated immunohistochemistry platform and ROS1 testing by Fluorescence in situ hybridisation (FISH) using Zytolight break-apart probe.

Results: Of a total 515 lung biopsies performed. EGFR mutation testing was done in 269 (52.2%) cases. Morphology included 406 (78.8%) adenocarcinomas, 51 (9.9%) adenosquamous carcinomas, 35 (6.7%) squamous cell carcinomas and 23 (4.4%) of poorly differentiated carcinomas. Overall EGFR mutation was detected in 141(52.4%) patients. The predominant mutations identified were Del 19 in 77 (28.6%) cases, L858R in 52 (19.3%) cases and 102 (37.9%) cases were of wild type. Dual mutations Del19+L858R were noted in two cases, Del19+L861Q in one case, L858R+7790M in one case and 7790M+Del19 in two cases. The biomarkers were mutually exclusive in majority of the cases, however one case showed dual ALKDF53 expression by IHC and EGFR mutation (insertion). One
other case harboured Del19 mutation and ALKD5F3 positivity. 200 patients underwent ALKD5F3 testing by IHC, of which 36 (18%) patients expressed ALK positivity. 195 patients underwent ROS1 testing by FISH of which five (2.56%) patients harboured ROS1 gene rearrangement. T790M testing was done at the time of progression to test for secondary resistance by RTPCR on biopsy samples in 48 patients. Of these 26 patients (54%) harboured T790M mutations indicating secondary resistance pattern. Of these 26 patients, 17(65.3%) harboured Del 19 mutation, six (23%) harboured L858R, two had a report of mutation (done elsewhere) and one was wild type (done elsewhere).

Conclusion: Triaging of tissue samples and coordination between multidisciplinary teams is the key to successful biomarker analysis and patient management in lung cancers.

OR24. Oncologists’ Review of ctDNA EGFR Mutation Testing Reports

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Introduction: GenQA is an External Quality Assessment (EQA) Provider for Genomics which assesses laboratory tests and interpretative content of reports. GenQA provided an EQA for the testing of circulating tumour DNA (ctDNA) for EGFR mutations in lung cancer patients. The submitted reports were anonymously reviewed by a panel of Consultant Oncologists to identify the elements which were clear and unambiguous, and those misleading and may cause patient harm if misinterpreted. The findings were shared with the laboratories to improve the reporting standard of liquid biopsy test results.

Methods: Nine laboratories perform ctDNA testing for EGFR mutations in lung cancer patients in the United Kingdom, and all took part in this EQA and report review. The EQA reports submitted for two clinical cases were anonymised and sent to the reviewers to be rated for ease of finding the result, understanding the test methodology and clarity of the interpretation. Text which the reviewer found comprehensive, and content which was confusing or inaccurate were highlighted. Individual laboratory reviews were provided to the laboratories.

Results: The review identified elements deemed to be essential on the report; a clear statement of the mutation testing result in a format which cannot be misinterpreted, understandable test information and associated limitations, the use of HGVS nomenclature cross referenced to the common names e.g. T790M, a condensed one page report and generic reference to targeted treatment rather than naming individual drugs. The reports submitted for a patient progressing on first line EGFR TKI therapy with the primary EGFR mutation and a resistance mutation detected in the plasma sample, raised comments about reporting allelic frequency and queried the clinical utility for this on the report. The clinicians wished reassurance that ctDNA had been tested as the primary EGFR mutation had been detected.

The second report set was related to a patient relapsing on EGFR TKI therapy with no EGFR mutations detected in the plasma sample. The reviewers’ responses were focussed on the risk of a false negative result and the ways the laboratories reported this varied. If reported at all, and the recommendations on appropriate further testing. The reviewers expressed a wish for a more standardised approach to interpreting such results and to emphasise the importance on highlighting that the primary EGFR mutation had not been detected.

Conclusions: Laboratories report ctDNA EGFR mutation testing in lung cancer patients using a multitude of formats and terminology. The lack of standardisation increases the risk of misinterpreting the result with the potential to harm patients through inappropriate clinical management or treatment. The external oncologist review of the content of reports has provided laboratories with feedback as to the positive and negative elements within their reports and will aid in the improvement in genomic test result reporting.

POSTER PRESENTATIONS

Genetics/Inherited Conditions

P001. The UHN NGS Panel for Hematologic Malignancies: Use for Standard of Care Testing of Acute Myeloid Leukemia in Ontario/Canada

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Introduction: Hematologic malignancies including myeloproliferative neoplasms, myelodysplastic syndromes and acute leukemia are characterized by great clinical and genetic heterogeneity. Genomic profiling is an important component for diagnosis, prognosis and management of patients with these hematopoietic malignancies in the updated 2016 WHO classification of myeloproliferative neoplasms and acute leukemia. AMP recently recommended molecular testing of 34 critical genes as standard of care in chronic myeloid neoplasms. Our previous work on 1,600 patients with various hematopoietic malignancies (Princess Margaret Cancer Center - Advanced Genomics in Leukemia study)
Methods: We developed a custom hybrid-capture NGS panel that targets 28 hotspot gene regions and 21 entire consensus DNA coding sequences of 49 clinically relevant genes in hematologic malignancies.

Results: This clinical test is currently offered as part of a newly funded program for patients with acute leukemia in Ontario/Canada. To date, we have completed molecular profiling on over 500 Acute Myeloid Leukemia (AML) patients. Specific approaches were taken to enable capture of complex gene regions and to identify challenging variants including larger insertions, deletions
duplications in genes such as BRAF, NOTCH1 (KMT2A).

Discussion: Our results demonstrate that this targeted NGS panel has an excellent diagnostic performance and great utility in patients with hearing loss. Deep understanding of the genetic architecture of hearing loss was also of utmost importance, and thus the targeted NGS panel could be used in routine clinical diagnostics with careful interpretation and optimal strategies.

Conclusions: The distinct physical characteristics included a

Introduction: As more genes have been identified as linked to hearing loss and more public databases become available, a substantial proportion of patients with hearing loss are diagnosed for genetic causes using next generation sequencing (NGS) results. In this study, we present clinical and diagnostic utility of a targeted NGS panel in Korean patients with hearing loss, along with several strategies to maximize the diagnostic yield.

Methods: A total of 39 unrelated Korean pediatric patients with moderate to severe nonsyndromic hearing loss (NSHL) at Severance Hospital were tested using a customized NGS panel (Celemics, Korea) targeting all relevant variants (preliminary data on 300 patients) using this NGS assay; minimum: 0, maximum: 13, average 4 per case. NGS detected unsuspected variants in genes (BRAF, NOTCH1) indicating complex clinical presentation of disease; comprehensive NGS testing was also useful in confirmation of diagnosis of AML and in identification of AML patients that were further confirmed to have familial predisposition to myeloid neoplasms (DDX41, RUNX1, MPL mutations).

Conclusions: Molecular profiling by NGS is an effective strategy for proper diagnosis and clinical management of patients with hematologic malignancies. Important considerations for NGS panel testing development and validation in a clinical setting include relevance of hematologic malignancy genes and/or gene regions to be tested, complete gene coverage and uniform coverage of targeted gene regions. Bioinformatics support is required to improve assay performance and analytical sensitivity particularly for detection of large insertions, deletions and duplications.

P002. Real-World Diagnostic Value of Targeted Panel Sequencing in Korean Hearing Loss Patients
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Introduction: As more genes have been identified as linked to hearing loss and more public databases become available, a substantial proportion of patients with hearing loss are diagnosed for genetic causes using next generation sequencing (NGS) results. In this study, we present clinical and diagnostic utility of a targeted NGS panel in Korean patients with hearing loss, along with several strategies to maximize the diagnostic yield.

Methods: A total of 39 unrelated Korean pediatric patients with moderate to severe nonsyndromic hearing loss (NSHL) at Severance Hospital were tested using a customized NGS panel (Celemics, Korea) targeting all coding exons and exon/intron boundaries of 182 deafness-related genes. After target enrichment, sequencing was performed on the MiSeq System (Illumina, USA) according to the manufacturer’s instructions. Our customized bioinformatics pipeline with

Conclusions: The distinct physical characteristics included a

P003. Distinct Craniofacial and Skeletal Features, Keratosis, Hearing Loss, Short Stature, and Complex Congenital Heart Disease with Vasculopathy: Expanding the Phenotype of BRD4-Related Conditions
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Introduction: Patients with point mutations and deletions encompassing BRD4 have been described. Haploinsufficiency of this gene can cause a Cornelia de Lange-like (CdL) syndrome with short stature, microcephaly, characteristic facial features, congenital heart defects, hirsutism, and neurodevelopmental delays. Herein is a detailed description of a new patient with distinct physical characteristics. An updated genetics consultation was requested for a 29 year old male that resulted in a gestalt of a hybrid CdL and Rubenstein Taybi phenotype.

Methods: An exome analysis with trio reference was performed which resulted in a single genetic mutation of interest. A missense mutation in BRD4 (NM_058243.2, c.860G>T (p.Glyc287Val)) was classified as that of uncertain significance. This de novo mutation had a

Results: The distinct physical characteristics included a

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craniofacial appearance reminiscent of the aforementioned syndromes. He had microcephaly, micrognathia, poor dentition, thick and coarse hair with multiple whorls and hirsutism with atypical hair placement and density. He had a very narrow chest wall. There was a single testicle s/p orchiopexy, small hands and feet, hyperkeratosis of the palms, sclerodermoid-like tight skin, and an angiofibromatous persistent rash-like appearance on the face. He had short stature and hearing loss, presumed to be secondary to cholesteatoma. He had a small adrenal mass of no known consequence. He had intellectual disability with a pleasant personality.

Cardiovascular complications included tetralogy of Fallot type double outlet right ventricle, progressive dilation of the ascending aorta and moderate dilation of the celiac artery.

Conclusions: This patient had features of CdL similar to previously reported patients with BRD4 haploinsufficiency. The three individuals previously reported with missense mutations were in a cohort chosen for their CdL phenotype, which was qualified as mild. In contrast, our patient had more distinct features. While his moderate aortic dilation may be at least in part due to his congenital cardiac defect, the celiac artery dilation would support a more systemic vasculopathy. This patient may represent the severe-end of the BRD4-related disorder spectrum and expands the phenotype.

P004. Somatic Mosaic Truncating Mutations in Exon 6 of PPM1D in Leukocytes Are Likely to Be Related to the Effects of Chemotherapy

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Introduction: PPM1D (Protein phosphatase magnesium-dependent 16) plays a key role in the negative regulation of p53 and modulates the DNA damage response pathway. Gain-of-function mutations of the PPM1D gene are associated with a wide variety of cancers including breast cancer and ovarian cancer while over suppressing the function of p53. Reportedly, truncating mutations in exon 6 of PPM1D can cause overexpression of PPM1D and the mutations of mosaic form in peripheral blood are more common in the ovarian cancer or breast cancer group than in the control group. On the other hand, it is reported that PPM1D mosaic truncating mutations in leukocytes are associated with chemotherapy rather than predisposition of solid cancers.

Methods: We examined the PPM1D mutations in next-generation sequencing (NGS) results of patients with suspected hereditary solid tumors. The NGS tests were conducted on hereditary cancer panel with peripheral blood between July 2016 and August 2018. Detected variants were further examined by visual verification using the Integrative Genomic Viewer (IGV). The pathogenicity of variants is classified according to the ACMG criteria. We also examined the mutations in blood and cancer specimen by sanger sequencing in three cases.

Results: A total of 1195 patients were included in the study. Among 1,195 patients, the proportion of patients with breast and/or ovarian cancer (80.3%) was the highest. Truncating mutations in PPM1D were detected in 4 (965, 0.41%) of the patients with breast cancer and/or ovarian cancer. All truncating mutations were in exon 6, in mosaic form. The mean percentage of mutant reads was 11.15% (range= 5.4%-15.4%). All four had a history of chemotherapy. Of the total, 395 were treated with chemotherapy and odds ratio of chemotherapy about PPM1D truncating mutations in exon 6 was 18.4 (95% CI: 0.99-342.67, p=0.051). In three cases, A low percentage of mutations in peripheral blood by NGS were confirmed by Sanger sequencing but no corresponding mutations in the tumor tissues were observed.

Conclusions: In our NGS analysis, PPM1D mosaic truncating mutations in peripheral blood were observed in 0.41% of breast and/or cancer patients, which was similar to that reported previously. However, the mutations were somewhat significant in the group treated with chemotherapy. Clonal hematopoiesis which is related to age cause hematologic cancers according to acquisition of somatic mutations due to chemotherapy. Patients with truncating PPM1D mutations in our study may be subjects with a so-called clonal hematopoiesis of indeterminate potential (CHIP). Nevertheless, it is unclear whether the mosaic truncating mutations of PPM1D in the blood are the cause acting as a driver of solid cancer or the result of chemotherapy. We need to study more samples and make sure that the mutations are in normal tissues.

P005. Molecular Diagnosis of Huntington's Disease in Suspected Cases of Indian Origin

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Introduction: Huntington's disease (HD), an autosomal-dominant, progressive, neurodegenerative genetic disorder is caused by an increase in the number of CAG repeats in the huntingtin (HTT) gene. Genetic tests that accurately determine the number of CAG repeats are performed for confirmation of diagnosis. The aim of our study was to evaluate utility of triplet-primed polymerase chain reaction (TP-PCR) for routine diagnosis of HD in suspected cases from India.

Methods: We ran a combination of CAG flanking PCR and triplet-primed PCR for estimation of CAG repeats in 732 cases with clinical suspicion of HD. Age of onset versus the number of CAG repeats were studied.

Results: There were 363 cases (49.6%) that showed the presence of expanded alleles, with 349 (47.6%) being fully penetrant alleles and 13 (1.77%) in the reduced penetrance category. The remaining suspected cases (N=369) showed CAG repeats in normal range, thereby excluding the diagnosis of HD and these cases should be further evaluated for Huntington's disease-like (HDL) syndromes. There were 13 juvenile cases with an age of onset lower than 20 years, with the longest allele comprising 106 CAG repeats found in an 8-year-old male.
Introduction: Multiple cystic lung disease (CLD) represents a diverse group of uncommon disorders that can present a diagnostic challenge due to the increasing number of diseases associated with this presentation. Among CLD, several diseases have well-defined causative mutations in the relevant genes; e.g., lymphangioleiomyomatosis (LAM), Birt-Hogg-Dube syndrome (BHD), tuberous sclerosis complex (TSC) and cystic fibrosis (CF). Thus far, the molecular diagnosis of CLD is mainly based on Sanger sequencing. As Sanger sequencing of all the candidate genes substantially increase the cost, genetic testing usually starts with the most commonly involved genes and proceeds to less likely genes only when clinical suspicion is very high. In recent years, targeted next-generation sequencing (NGS) platform has been further developed, allowing us to focus specifically on genomic regions of interest for cheaper multiplexed sequencing of more cases. However, inaccuracy in detecting the length of homopolymers repeats and complexity in detecting structural variation became a critical barrier against accurate detection of genomic variations. Herein we seek to evaluate diagnostic yield of customized CLD panel using NGS platform.

Methods: 49 patients with multiple lung cysts was enrolled. Mutations in FLCN gene were characterized through Sanger sequencing, Multiplex Ligation-dependent Probe Amplification (MLPA) and quantitative PCR (qPCR). Evaluation of diagnostic yield of the CLD NGS panel was done using Ion torrent S5 NGS platform. Genomic DNA reference materials obtained from Coriell cell repository and results of Sanger sequencing- dependent Probe Amplification (MLPA) and quantitative PCR (qPCR). Evaluation of diagnostic yield of the CLD panel. Two bioinformatics(BI) pipeline for processing NGS data were used; the Torrent Suite(TSS) with a plug-in Torrent Variant caller (Thermo Fisher Scientific, MA, USA) and NextGENe(NG) software (Softgenetics, PA, USA).

Results: Out of 49 patients with multiple lung cysts, 18 FLCN pathogenic variants and 1 FLCN large deletion were observed confirmed by Sanger sequencing and MLPA respectively. Using CLD NGS panel test, 1 extra pathogenic variant and 3 VOUs were newly observed. In analytical performance evaluation, analytical sensitivity of TSS and NG was 77.1% (59.4-89.0) and 94.3% (79.5-99.0). Specificity of TSS and NG was both 100%. After adapting additional hotspot bed file to TSS, sensitivity went up to 100%. And after adapting IGV visual inspection of the pathogenic variant hotspot list, sensitivity of NG was 100%.

Conclusions: Diagnostic yield using NGS went up from 38.7% to 46.9% compared with FLCN Sanger sequencing alone. Even though the pathogenic hotspot of FLCN is mostly filtered out using TSS BI pipeline due to 8 homopolymers repeat sequences, adjusting BI can dramatically improve the overall performance. Optimization of the BI pipeline is essential when designing difficult NGS panel.

P007. DNA Methylation and Its Correlation with Breast Cancer Pathological Prognostic Staging V. Gupta1,2, P. Deshpande3, A. Blake4
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Introduction: Breast cancer is the most common form of malignant disease in women worldwide and is the major cause of mortality among women. It is a heterogeneous disease with histopathological, genetic and epigenetic characteristics. Epigenetic alterations such as in DNA methylation, characterized by addition of a methyl group at the carbon-5 position of cytosine residue within CpG dinucleotide, are considered an early event in tumor development. However, the changes in DNA methylation and its correlation with the progression of breast cancer is not well studied. The study aims to assess the relationship of DNA methylation in breast cancers presenting at different pathological prognostic stages, for understanding the progressive behavior.

Methods: Cross sectional study on surgically resected mastectomy specimens from breast carcinomas, diagnosed as invasive ductal carcinoma and matched adjacent normal tissues from 63 breast cancer patients were included in the study, conducted in Department of Pathology and Molecular Biology and Epidemiology Laboratory at Jawaharlal Nehru Medical College, Datta Meghe Institute of Medical Sciences, Wardha, India. Clinico-pathological data was obtained from the record in the surgical pathological section. The specimens were collected after pathological examination in phosphate buffer saline.

DNA extraction and methylation: A conventional phenol/chloroform extraction procedure was followed. DNA methylation at CpG islands was studied using ELISA by Epigentek kit.

Statistical analysis: performed using SPSS 23.0. Fisher’s exact tests was used to calculate the agreement from different sources (tumor, and adjacent tissues) among cases.

Results: Sixty-three, breast cancer patients were enrolled in the study. Median age 50 years, range (26 -
Introduction: The etiology of developmental delay (DD) and related disorders is heterogeneous and is therefore still posing diagnostic challenges. With the introduction of next generation sequencing (NGS) techniques, diagnosis and discovery of causative genes have increased tremendously in the past several years. In this presentation, we conducted targeted NGS using various panels for diagnosis and classification of pediatric DD and related conditions.

Methods: The patients were evaluated first by clinical examination at Dong-A University hospital and further enrolled for NGS analysis in the form of targeted sequencing. The panels employed were hypothyroidism panel, proportionate short stature panel, rasopathy panel, skeletal dysplasia panel, congenital adrenal hyperplasia (CAH) panel, and inborn error of metabolism panel, based on the primary clinical and laboratory features of the patients.

Results: Molecular diagnosis was performed for 25 pediatric patients. Eight proportionate short stature panels, 7 hypothyroidism panels, 3 each of CAH and rasopathy panels and 2 each of inborn error of metabolism panel and skeletal dysplasia panels were used. Six pathogenic variants related to the phenotype of patients were identified by mostly hypothyroidism panel in 5 patients. The other patient showed a pathogenic variant of FGF3 gene with skeletal dysplasia panel. Most common variants were obtained with DUOX2 gene. Interestingly 13 patients showed variants of uncertain significance (VUS).

Conclusions: DD patients show variable phenotypes along with genetic heterogeneity. NGS analysis could be the best choice for detection of molecular defects. However, reevaluation of genotype-phenotype data with literature review is still recommended to revise and update the mutation profile especially in patients with VUS.
of patients with CYP2C9+VKORC1 wild-type showed the presence of CYP4F2 variant. Similar association between CYP4F2 variant and supra and sub-therapeutic warfarin dose was observed.

**Conclusions:** The present study, a first from Western India showed the CYP4F2 rs2108622 variant to be clinically relevant and would impact warfarin dose management. Further validation on a large Indian cohort will be required in order to establish the true clinical utility of CYP4F2 variant in management of patients on Warfarin therapy.

**P010. Frequency of the Moyamoya-Related RNF213 p.Arg4810Lys Variant in Patients with Moyamoya Disease**

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**Introduction:** Moyamoya disease (MMD) is a rare, genetically heterogeneous cerebrovascular disease resulting from progressive steno-occlusion of the distal internal carotid arteries accompanied by abnormal collateral vessels. RNF213 was identified as a susceptibility gene for MMD and a variant in the Ring Finger 213 gene (RNF213), altering arginine to lysine at the codon of 4810 (c.14429G>A, p.R4810K) (rs112735431) is associated with MMD in Asian populations. Interestingly, many studies have reported that a certain proportion of the general population in Japan, Korea, and China also has this variant. Patients with the homozygote of the variant were also reported to present more severe form of Moyamoya disease. In this study, we investigated the frequency of the variant including heterozygous and homozygous frequency in Korean MMD patients.

**Methods:** During January 2017 to October 2018, a total of 460 MMD patients were included in this study. MMD was diagnosed as either definite (bilateral) or probable (unilateral) according to published guidelines. We sequenced RNF213 exon 60 (encoding p.R4810K) to determine the genotype of the variant. After obtaining written informed consent from all subjects, peripheral blood samples were obtained. Genomic DNA was extracted from whole blood samples. Rare alleles were not amplified using primer sets designed by the authors (available upon request). The PCR was performed with a thermal cycler (model 2720, Applied Biosystems, Foster City, CA, USA). Direct sequencing was performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on the AB 3730 DNA analyzer (Applied Biosystems).

**Results:** The c.14429G>A (p.R4810K) variant was identified in 44.3% (204/460) of patients with definite or probable MMD. A heterozygote of the variant was observed in 43.0% (198/460) patients and homozygote was identified in 1.3% (6/460) patients. Homozygotes had a slightly earlier age at diagnosis compared with heterozygotes or wild types (median age at Diagnosis 43, 49 and 52 yrs, respectively), which did not achieve the statistical significance. However, the proportion of childhood onset (age under 15 yrs) were significantly higher in patients with homozygotes compared with patients with heterozygotes.

**Conclusions:** In summary, our results confirm that alteration in RNF213 predisposes patients of Korean MMD patients and the homozygous c.14429G>A (p.R4810K) variant in RNF213 could be a useful molecular marker for predicting the severe type of MMD, for which proper medical intervention is recommended, and may provide a better monitoring and prevention strategy. This study will provide the frequency of c.14429G>A (p.R4810K) variant in RNF213, and an effective strategy for the molecular diagnosis of MMD in Koreans.

**P011. Thiopurine S-Methyltransferase (TPMT) Allele Frequencies in Hong Kong Population**

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**Introduction:** Thiopurine drugs, such as Azathioprine, 6-Mercaptopurine and 6-Thioguanine, are effective immunosuppressive agents in the management of inflammatory bowel disease, autoimmune diseases and organ transplantation. It is also used in anti-leukemia therapy. Two modes of action have been proposed for thiopurine. Firstly, the thiopurine metabolite, 6-methyl Thioinosine monophosphate, inhibits the cell proliferation of fast growing lymphocytes by suppressing de novo purine synthesis. In addition, 6-thioguanine nucleotide, another thiopurine metabolites, exerts a pro-apoptotic effect to activated T cells [1]. Thiopurine S-methyltransferase (TPMT) is the key enzyme responsible for catalytic conversion of the thiopurine metabolites to non-cytotoxic products. TPMT variants with reduced enzyme activity may cause accumulation of these cytotoxic active thiopurine metabolites which can lead to myelosuppression [2]. Studies have shown that the TPMT gene is more polymorphic in the Western populations than in the Chinese populations [3-5]. In this study, we investigated the allele frequencies of the most common TPMT variants (TPMT*2, *3A, *3B and *3C) in the Hong Kong population.

**Methods:** Genomic DNA was extracted from whole blood of 300 Hong Kong healthy blood donors. The TPMT*2, *3B and *3C variant alleles were first detected using a modified Amplification-Refactory Mutation System (ARMS) PCR method [6]. Beta-2-microglobulin was used as the internal control. All samples with variants detected were further confirmed by Sanger sequencing of the TPMT exon V, VII and X. Rare TPMT alleles were not determined.

**Results:** TPMT*1/*3C genotype was detected and confirmed in 9 out of 300 healthy subjects. TPMT*2 and TPMT*3 variants were detected in 162 out of 300 subjects. The frequency of TPMT*2 and TPMT*3 variants was 54% and 21% respectively.
TPMT*3B alleles were not detected. The allele frequency of TPMT*3C allele in the Hong Kong population is 1.5%.

**Conclusions:** This study showed the allele frequency of the most common TPMT variants in Hong Kong. Hong Kong as well as the Chinese population has a lower frequency of TPMT*3C variant as compared with the Western populations and only heterozygous TPMT*1/*3C genotype (3%) was found. It was estimated that 3% of the Hong Kong population has an intermediate TPMT enzyme activity. TPMT genotype could be performed for patients requiring thiopurine therapy to guide the reduction of drug dose to minimize the risk of myelosuppression.

P013. Association of Vitamin D Receptor (VDR) Start Codon FokI Polymorphism with Acute Myelogenous Leukemia

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**Introduction:** The role of chromosomal translocations, gene mutations, and polymorphisms in the pathogenesis of leukemia/lymphoma have been extensively studied. Recently, the role of vitamin D and vitamin D receptor (VDR) polymorphisms in hematologic malignancies has been considered. Vitamin D start codon polymorphism has been suggested to be associated with an increased risk of developing different hematological malignancies including AML. The objective of this study was to investigate the association between VDR start codon F/f polymorphism and the risk of AML among Pakistani patients.

**Methods:** A total of 70 Pakistani patients with acute myelogenous leukemia were enrolled in this hospital-based case-control study. In addition, 70 age and sex matched healthy volunteers were included as a control group. Five milliliters (ml) of venous blood were collected and genomic DNA was extracted from all samples using standard methods. This was followed by the detection of VDR Fok-I polymorphism by polymerase chain reaction and restriction fragment length polymorphism.

**Results:** A 280 bp fragment of the VDR gene was amplified by PCR and digested by restriction enzyme (FokI) to detect the polymorphism. The genotype FF was the most frequent (76%) in patients with AML, followed by the genotype Ff (20%) and genotype ff (4%). Similarly in the control group the genotype F/F also was the most frequent (57%) followed by the genotype ff (39%) and F/f genotype (4%). There was statistically significant association between AML and each of the genotypes FF and Ff and F and ff (p=0.0001). No statistically significant correlation between the VDR Fok-I polymorphism and sex/age (P=0.811) was found.

**Conclusion:** Both FF and Ff genotypes are associated with increased risk of AML among Pakistani patients.

P014. Human Genome Reference Assembly for Blood Group Systems

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**Introduction:** Clinical applications of next-generation sequencing (NGS) are continuously expanding. Human reference genome build comparison is a commonly used method for variant detection but such an approach is limited for polymorphic regions encoding blood group antigens. In this study, we analyzed and typed the sequences of two human reference genomes to provide a reference blood group for application of NGS.

**Methods:** Two human reference genome builds, GRCh37 and GRCh38 were analyzed. Representative reference allele sequences were selected for clinical important blood groups: ABO, Rhesus, Kidd, and Kell blood groups (AJ536122.1 for ABO gene; X63094.1 and L08429.1 for RHD gene; DG322275.1 for RHCE gene; NM_015865.7 for SLC14A1 gene; and M64934 for KEL gene). The reference alleles were ABO*A1.01, RH*D01, RH*CE01, JK*02, and KEL*02, respectively. Sequences from the two genome builds were compared to the reference alleles to detect the presence of variants in coding regions.

**Results:** Compared with the reference ABO allele, both reference genome builds had c.261delG, representative
of the O allele. In addition, GRCh37 assembly showed additional variants in coding regions (c.106C>T, c.188G>A, c.189C>T, and c.220C>T). Multiple intronic variants were found in both reference genome builds. For RHD, there was no difference between the two reference genome builds. On the other hand, c.636C>A and c.1036T>C variants were detected when compared to the L08429.1 and X63094.1, respectively. These findings are possibly sequencing artifacts within the reference alleles and require further analysis. There was no difference between the reference alleles and the referenced genome builds for RHCE and SLC14A1. Several intronic differences were found between the two reference builds for KEL.

Conclusion: Several differences between the reference genome build and reference alleles were found. Two reference genome builds also had differences. Findings from our study suggest that caution is necessary when using reference genome builds for polymorphic regions such as blood group antigens.

P015. An Artificial Intelligence Engine for High-Throughput Matching of Genetic Variants to Their ACMG/AMP Classification for Inherited Disease Gene Panels

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Introduction: The ACMG and AMP defined a set of evidence-based guidelines to support variant pathogenicity assessment and reporting in inherited disease diagnostics. The guidelines define several criteria, each assessing particular supporting evidence information that is checked independently for each variant. Criteria are grouped by different levels of evidence and a set of rules combines the evaluated criteria and classifies a variant accordingly as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB), or uncertain significance (VUS). Although widely adopted in clinical interpretation of inherited disease variants, this process has remained a largely manual and time-consuming process in clinical labs that is not scalable to the volume of NGS testing data. The rich evidence needed to properly classify variants is difficult to effectively incorporate in manual classification schemes, resulting in discrepancies of interpretation, excessive specialized reviewing labor, and higher costs. Informatics tools have been proposed to ease the application of the guidelines in clinical practice, such as the web-based ClinGen Pathogenicity Calculator. However, such tools do not solve the issue of interpreting a large set of variants, since they do not automate the entire classification process.

Methods: For these reasons, we developed VariantMatch, an artificial intelligence engine to automatically infer the classification of variants that utilizes a forward-chaining inference engine at its core to implement the ACMG-AMP criteria returning a predicted classification for each variant. The engine is able to incorporate criteria and rule refinements for specific genes and related diseases by separating the rule specification from the code that executes them. A critical input is careful curation of the gene-disease relationship attributes and computed features including variant deleteriousness scores such as VVP. Moreover, a natural language generation module provides a rationale of the classification reached for reference by clinical geneticists when reviewing and reporting cases. To evaluate the performance of our method, we analyzed a set of 2,978 of BRCA1 variants of diverse prior classifications compiled from ClinVar, VarSNP, JapanData, and the Color Database.

Results: Our preliminary results show that VariantMatch was able to correctly classify automatically 96% of 2 stars or more ClinVar variants and was able to reclassify 69% and 76% of Color and JapanData VUS variants. The output provided explanatory text indicating the key rules matched that led to a classification and was easily understandable by clinicians.

Conclusion: We plan to integrate VariantMatch into Fabric Enterprise, a cloud-based software platform for clinical genomic analysis and reporting, to enable high-throughput genetic labs to score variants rapidly, reproducibly, cost-effectively and at scale.

P016. Development of a NGS-based Neotelomere Mapping Method for Detection of Terminal Deletions

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Introduction: Human telomeres are composed of repetitive (TTAGGG)n sequences. Broken chromosomes, when not repaired by homologous recombination or non-homologous end-joining, can be stabilized by telomere capture or by the telomerase-mediated direct addition of telomeric repeats to nontelomeric DNA ends, forming a neotelomere (Flint J et al., 1994 PMID:7521575). This end-repair process is called telomere healing, which occurs in terminal deletions. Neotelomeres are found in human telomere deletion syndromes, which occurs in at least 1/5,000 individuals (Shaffer LG et al., 2000 PMID:11092830; Knight SJL et al., 1999 PMID:10568569). However, it is not easily detected by current assay designs and informatics pipelines, and has not been characterized in a systematic manner.

Methods: We developed a novel NextGen-sequencing (NGS) based method and an informatics algorithm to map neotelomeres attributable to telomere healing. This method uses one telomere-anchored primer and one ligated adaptor primer together for library generation followed by deep sequencing. We applied this assay in a pilot study to human samples with known terminal deletions, and successfully identified telomere junctions for most chromosomes in the initial 4 specimens as well as disease-causing terminal deletions.

Results: The clinical performance and utility of the assay to screen for fetal chromosomal terminal deletions in cell-
can make genetic monitoring easy, efficient, and with the easy workflow and quick turnaround, this panel of cancer risk by using small quantities of genomic DNA.

Methods:

Methods are mainstream for surveying large numbers of specialized equipment. With their time-consuming and cumbersome to perform requiring highly trained operators with specialized equipment.

Methods: We have developed a multiplex PCR-based CleanPlex technology to make it possible to produce high-quality coverage of genomic targets using a fast and sample target enrichment and NGS library construction workflow. Here we present an NGS panel targeting hereditary mutations that covers the full exons of 37 genes using 1445 pairs of PCR primers. In addition, the panel also detects two clinically relevant intronic mutations. This panel paired with the CleanPlex workflow can enable the quick analysis of genes associated with an increased risk of developing hereditary cancers using small amounts of genomic DNA. The CleanPlex 3-step workflow includes a multiplex PCR step with targeted primers, a background cleaning step to remove by-products, and a final PCR to add Illumina adapter sequences and sample indexes. NGS libraries were made using 10 ng of genomic DNA per pool (40 ng total). The CleanPlex Hereditary Cancer Panel contains primers divided into four pools for the multiplex PCR.

Samples were sequenced at ~2,500 read depth on an Illumina NextSeq. Sequenced reads were demultiplexed. Mapping rate and on-target rates were calculated, and variants were identified using Paragon Genomics’ variant calling algorithm.

Results: The samples exhibit >96% uniformity at 0.2X mean, and all exons were sufficiently covered to provide accurate variant calling information. The assay is highly reproducible and sensitive, exhibiting an R2 value of 0.95 between independently prepared replicates, and a detection rate of >99% for single nucleotide variants.

Conclusion: The Paragon Genomics CleanPlex Hereditary Cancer Panel can be used for rapid profiling of cancer risk by using small quantities of genomic DNA. With the easy workflow and quick turnaround, this panel can make genetic monitoring easy, efficient, and economical.

P018. Understanding Next Generation Sequencing for Autosomal Dominant Polycystic Kidney Disease (ADPKD)

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Introduction: ADPKD is a genetically heterogeneous disorder caused by mutations in PKD1 and PKD2, accounting for ~85% and 15% of cases, respectively. PKD1 consists of 46 exons spanning ~52 Kb of genomic DNA, a large part of which is duplicated in six homologue genes (i.e. pseudogenes) showing ~98% homology with PKD1 exons 1-33. PKD2 spans 15 exons and a coding sequence of ~3Kb. Gene-based mutation testing currently is the predominant method of ADPKD genotyping. However, the marked allelic heterogeneity of the disease-associated mutations, the vast majority of which are private, and the duplicated structure of PKD1 together with its large size, make mutation screening a significant technical challenge.

Methods: The key step in ADPKD genotyping procedures is selective amplification of PKD1 sequences, while excluding the pseudogenes. This was traditionally achieved by utilizing long-range PCR (LR-PCR) with primers located to the rare mismatch sites that distinguish PKD1 and the pseudogenes, while the single-copy regions of PKD1 and PKD2 are directly amplified from gDNA. Amplicons are then analyzed by Sanger sequencing. Introduction of next generation sequencing (NGS) has revolutionized the field of molecular genetics and was recently applied to PKD genetic testing. In this method, LR-PCR is coupled with NGS. Bar-coded LR-PCR libraries from individual patients are pooled together and analyzed using paired-end NGS in a single sequencer flow-cell (Illumina). Sequencing results are then sorted according to the sample barcode, aligned against the reference PKD genes and mutations are called using a computational bioinformatics analysis pipeline.

Results: This approach has a sensitivity of 100% compared with Sanger sequencing, while retaining high specificity, enabling identification of single nucleotide variants (SNVs), indel and copy number variation (CNV). Moreover, the high read-depth and average coverage (>400x) obtained over exonic regions significantly increases testing sensitivity compared to Sanger sequencing, allowing detection of low-level mutations (~3% VAF), especially important for the detection of genetic mosaicism. Recently, both WES and WGS have been applied to PKD gene testing, eliminating the need for LR-PCR. However, the sensitivity of these methods varied significantly between the duplicated and single-copy PKD1 compared to LR-PCR based sequencing (30%~80% versus 100% sensitivity), primarily due to low read-depth particularly in GC-rich regions and mapping of reads from duplicated regions.

Conclusion: NGS has the potential to dramatically improve PKD genetic testing by simplifying workflow and allowing simultaneous detection of SNV/indels and CNV
Methods: MFC by testing 101 MM samples. Performance of NGS-based LymphoTrack Assays and report the results of a pilot study comparing the residual disease (MRD) assessment in MM. Here we and ESMO) have recently included NGS for minimum sensitivity, and international organizations (NCCN, IMWG methods have demonstrated advantages with improved patients. Next generation sequencing (NGS) based hematological malignancy. Multiparameter flow cytometry (MFC) is a standard tool used to detect and monitor MM Morrow (BM), represents the second most common the presence of >10% clonal plasma cells in bone marrow (BM), represents the second most common hematological malignancy. Multiparameter flow cytometry (MFC) is a standard tool used to detect and monitor MM patients. Next generation sequencing (NGS) based methods have demonstrated advantages with improved sensitivity, and international organizations (NCCN, IMWG and ESMO) have recently included NGS for minimum residual disease (MRD) assessment in MM. Here we report the results of a pilot study comparing the performance of NGS-based LymphoTrack Assays and MFC by testing 101 MRD MM samples.

Results: Despite the limited DNA available for testing, out of 101 baseline samples, 84 (83%), 30 (79%), 63 (62%) and 87 (86%) samples were detected as clonal positive by IGH FR1, FR2, FR3 and IGK, respectively. When combining FR1 and FR2, 94% (95/101) clonal positivity was achieved. When combining all 4 targets, 100% (101/101) clonal positivity was achieved. Using LymphoTrack MRD Software to identify clonotype sequences, we performed MRD assessment on 91 samples that were positive for IGH FR1 or FR2. 47 (52%) were positive, 43 (47%) were negative, and 1 (1%) was invalid, due to insufficient reads. MRD results by MFC on the same 91 patients showed 45 (49%) positive, 37 (41%) negative, and 9 (10%) invalid samples. Excluding samples with DNA less than 700 ng (~107,000 cell equivalents), concordance was 85.4%. Three samples were detected by MCF but not by NGS while four samples were detected by NGS but not by MCF.

Conclusions: LymphoTrack assays were shown to detect clonal sequences in 100% of MM baseline samples and, despite testing approximately 1/10 the number of cell equivalents, were able to achieve 85.4% agreement with MFC in detecting MRD. This suggests that LymphoTrack assays are a useful tool in identifying and tracking disease status in MM samples. Unlike MFC, the LymphoTrack assays and accompanying bioinformatics software can be submitted for approval to regulatory authorities worldwide.

Hematopathology

P020. Assessment of Minimum Residual Disease Detection in Multiple Myeloma Samples Using Next Generation Sequencing Based LymphoTrack Assays and Flow Cytometry

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Introduction: Multiple myeloma (MM), characterized by the presence of >10% clonal plasma cells in bone marrow (BM), represents the second most common hematological malignancy. Multiparameter flow cytometry (MFC) is a standard tool used to detect and monitor MM patients. Next generation sequencing (NGS) based methods have demonstrated advantages with improved sensitivity, and international organizations (NCCN, IMWG and ESMO) have recently included NGS for minimum residual disease (MRD) assessment in MM. Here we report the results of a pilot study comparing the performance of NGS-based LymphoTrack Assays and MFC by testing 101 MRD MM samples.

Methods: 101 paired BM samples from MM patients were tested in this study. The MFC methods utilize the 8-color direct immunofluorescence technique and test 1.5 million cells. Our NGS based MRD assessment tests only ~1/10 as many cell equivalents of genomic DNA (0.7µg, ~107,000 cell equivalents) from the collected specimens which were blinded prior to testing with four LymphoTrack Assays (IGH FR1, FR2, FR3 and IGK) on a MiSeq. LymphoTrack Software was used to sort data by target and index. The clonal rearrangement (clonotype) identified by LymphoTrack FR1 or FR2 Assay was then tracked to test the 91 subsequent samples. LymphoQuant Internal Control was added to each PCR reaction at 100 cell equivalents to estimate the cell equivalents within each sample. LymphoTrack Software - MiSeq and LymphoTrack MRD software were used to analyze the sequencing results from baseline and follow up samples, respectively.

Results: Despite the limited DNA available for testing, out of 101 baseline samples, 84 (83%), 30 (79%), 63 (62%) and 87 (86%) samples were detected as clonal positive by IGH FR1, FR2, FR3 and IGK, respectively. When combining FR1 and FR2, 94% (95/101) clonal positivity was achieved. When combining all 4 targets, 100% (101/101) clonal positivity was achieved. Using LymphoTrack MRD Software to identify clonotype sequences, we performed MRD assessment on 91 samples that were positive for IGH FR1 or FR2. 47 (52%) were positive, 43 (47%) were negative, and 1 (1%) was invalid, due to insufficient reads. MRD results by MFC on the same 91 patients showed 45 (49%) positive, 37 (41%) negative, and 9 (10%) invalid samples. Excluding samples with DNA less than 700 ng (~107,000 cell equivalents), concordance was 85.4%. Three samples were detected by MCF but not by NGS while four samples were detected by NGS but not by MCF.

Conclusions: LymphoTrack assays were shown to detect clonal sequences in 100% of MM baseline samples and, despite testing approximately 1/10 the number of cell equivalents, were able to achieve 85.4% agreement with MFC in detecting MRD. This suggests that LymphoTrack assays are a useful tool in identifying and tracking disease status in MM samples. Unlike MFC, the LymphoTrack assays and accompanying bioinformatics software can be submitted for approval to regulatory authorities worldwide.
P021. Comparative Analysis of Molecular Approaches Used for Targeted Detection of Mutations in FLT3 and NPM1 from DNA and RNA Templates

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Introduction: FLT3 and NPM1 are key biomarkers in diagnosis, prognosis and disease management of Acute Myeloid Leukemia (AML). WHO recommends molecular profiling of Internal Tandem Duplications in FLT3 (FLT3-ITD), hotspot missense variants in the Tyrosine Kinase Domain of FLT3 (FLT3-TKD) and insertions in NPM1 for standard of care testing in AML. Molecular approaches used to characterize FLT3-ITD, FLT3-TKD and NPM1 mutations differ in performance and analytical sensitivity; there is no consensus on whether levels of FLT3-ITD should be assessed from DNA or RNA templates.

Methods: We developed fluorescent PCR based fragment analysis for targeted detection of FLT3-ITDs, FLT3-TKDs and NPM1 insertions from DNA and RNA; in parallel, we used a Next-Generation Sequencing (NGS) panel for comprehensive analysis of 49 genes altered in hematologic malignancies, which include FLT3 and NPM1. We compared the performance of NGS, DNA and RNA-based fragment analyses approaches on peripheral blood or bone marrow from 100 AML patients. We assessed the diagnostic yield of these different methodologies; variant allelic fraction from DNA versus RNA, length of FLT3-ITD captured, assay sensitivity. Assay limitations were also evaluated in this study.

Results: Preliminary data on 50 patients shows that genotyping calls for FLT3-ITD, FLT3-TKD and NPM1 insertions are concordant in 47/50 of cases analyzed. Discordances were seen for variant with allelic fraction detected below 5% by fragment analysis and missed by NGS. Allelic fraction of variants, particularly FLT3-ITD, FLT3-TKD and NPM1 insertions from DNA and RNA; in parallel, we used a Next-Generation Sequencing (NGS) panel for comprehensive analysis of 49 genes altered in hematologic malignancies, which include FLT3 and NPM1. We compared the performance of NGS, DNA and RNA-based fragment analyses approaches on peripheral blood or bone marrow from 100 AML patients. We assessed the diagnostic yield of these different methodologies; variant allelic fraction from DNA versus RNA, length of FLT3-ITD captured, assay sensitivity. Assay limitations were also evaluated in this study.

Conclusion: NGS and fragment analysis are complementary strategies for detection of FLT3-ITDs, FLT3-TKDs and NPM1 insertions. Fragment analysis is suitable for critical clinical decision making in AML patients due to faster turnaround time; NGS comprehensive molecular profiling aids with diagnosis, prognosis and management of AML. Recent reports are in favor of measurements of FLT3-ITDs by RNA; further investigations and correlation of FLT3-ITDs levels with clinical outcome of patients will be useful in determining the optimal nucleic acid template (DNA or RNA) to use for better stratification of AML patients.

P022. Clinical Application of Next-Generation Sequencing in Myeloproliferative Neoplasms: Genetic Profiles and Prognostic Significance for Personalized Medicine

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Introduction: The mutational landscape of myeloproliferative neoplasms (MPNs) has been discovered including three diagnostic driver mutations, JAK2, MPL, and CALR. Nowadays, next-generation sequencing (NGS), especially targeted panel sequencing, is widely applied in clinical laboratory to identify mutational profile in hematologic malignancies. Herein, we evaluated genetic profiles in MPNs using NGS and explored the clinical application in the single center cohort.

Methods: A total of 432 patients were included; 196 essential thrombocythemia (45.37%), 112 polycythemia vera (25.9%), 72 primary myelofibrosis (16.7%), 14 other MPNs (3.2%) and 38 myelodysplastic/myeloproliferative neoplasms (MDS/MPN, 8.8%). JAK2 V617F, CALR and MPL 515 mutations were firstly analyzed using allele-specific PCR and fragment analysis. Targeted panel sequencing including 86 genes was performed. We analyzed relationships between genetic profiles and clinical outcomes including acute transformation, bone marrow fibrosis and death.

Results: Three driver mutations were detected in 82.4% of patients; JAK2 V617F in 64.8%, CALR in 15.7% and MPL in 1.9%. We also detected mutations in other genes through NGS which included ASXL1 (27.0%), TET2 (7.6%), KMT2C (3.2%), SRSF2 (2.9%), RUNX1 (2.9%), CSF3R (2.5%), ATM (2.2%), SETBP1 (1.8%), DNMT3A (1.8%), TP53 (1.8%), U2AF2 (1.8%), KMT2D (1.8%) and PTPN11 (1.8%). Among the three phenotypic subtypes, PMF showed shorter event free survival (EFS) than the others (hazard ratio: PMF-ET 2.62, PMF-PV and 2.97). In the aspect of genetic profile, TP53 mutation showed worst outcome followed by chromatin or spliceosome mutation (Median EFS: 77 and 99 months, 95% Confidential interval: 5.0-264 and 72-226, respectively).

Conclusions: We demonstrated that NGS effectively identified mutations that were more relevant to the prognosis in MPN patients. Genetic profiles can improve a risk stratification to make efficient therapeutic plan including targeted therapy in MPN patients.
P023. Potential Role of JAK2V617F and ASXL1 Mutations as Biomarkers for Disease Outcomes Amongst Thai Patients with Philadelphia-Negative Myeloproliferative Neoplasms

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Introduction: Polycythemia vera (PV), essential thrombocytopenia (ET), and primary myelofibrosis (PMF) are three main disorders constituting Philadelphia-negative myeloproliferative neoplasms (Ph-negative MPNs). The JAK2V617F mutation is present in the majority of these patients and has been included in diagnostic criteria for these conditions. Furthermore, ASXL1 mutations have been demonstrated as prognostic markers for risk stratification of MPNs. This study therefore aimed to determine the prevalence of JAK2V617F and ASXL1 mutations and their relationship with disease outcomes in Thai Ph-negative MPNs.

Methods: A total of 139 cases (76 male and 63 female) of Ph-negative MPNs, consisting of 51 PV cases, 67 ET cases, 18 PMF cases, and 3 MPN-U cases, were enrolled. All cases were investigated for JAK2V617F and ASXL1 mutations, utilising allele-specific PCR (AS-PCR) and Sanger sequencing, respectively.

Results: The JAK2V617F mutation was detected in 64.8% of Ph-negative MPN cases (72.6% of PV cases, 61.2% of ET cases, 50.0% of PMF cases, and 100.0% of MPN-U cases), whereas ASXL1 mutations were detected in 10.8% of Ph-negative MPN cases (16.3% of ET cases, 0.0% of PV cases, 7.5% of ET cases, and 0.0% of MPN-U cases). ASXL1 mutations were demonstrated in 7 out of 90 JAK2V617F-negative cases (7.8%) and 8 out of 49 JAK2V617F-positive cases (16.3%). Interestingly, JAK2V617-negative ASXL1-positive patients were shown to have distinctive clinical profiles in all groups of patients. However, no difference in thrombosis-free survival was seen amongst MPN patients with different mutations.

Conclusions: JAK2V617F and ASXL1 mutations might become potential biomarkers for disease outcomes in Thai patients with Ph-negative MPNs.

P024. Accurate Detection of Low AF Variants Relevant to AML by Anchored Multiplex PCR and Next Generation Sequencing

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Introduction: Acute Myeloid Leukemia (AML) is clinically and biologically heterogeneous, requiring the detection of multiple mutations for characterization. For instance, FLT3-ITDs and CEBPA mutations represent important markers in AML, however they are difficult to detect by NGS due to the highly variable nature of ITDs, the high GC content of CEBPA, and the difficulty in mapping repeated sequences to a wild-type reference. Tracking low frequency mutations is also of growing importance. The ability to accurately detect variants at low allele fractions (AFs) using a single test can be used to assess treatment efficacy and potential relapse.

Methods: We developed Archer VariantPlex myeloid assays based on Anchored Multiplex PCR (AMP) to detect important mutations in myeloid malignancies. AMP is a target enrichment strategy that uses molecular-barcoded adapters and gene-specific primers for amplification, permitting open-ended capture of DNA fragments from a single end. This approach enables flexible and strand-specific primer design to provide better coverage of ITD-containing and GC-rich regions. We also developed a method to assess SNV sensitivity taking into account both unique coverage depth and noise for single base substitutions. This strategy enables utilization of position-specific detection thresholds and maximizes sensitivity and specificity. We tested this approach using the VariantPlex Core Myeloid panel, by titrating reference inputs into background normal samples to examine detection of low AF variants.

Results: Our assay enables calling of a 30bp FLT3-ITD down to sub-0.05% allele frequencies. Using optimized low AF conditions improves coverage depth, consistency of low AF FLT3-ITD detection, and sensitivity (98.5% of bases are powered to call a true variant at an allele frequency of 3.0% with 1M reads and 200ng of input). We show >1000X unique molecule coverage across the coding region of CEBPA and use this challenging region to visualize the minimum detectable AF (MDAF) at which a variant has a >95% probability of being detected above the noise (95MDAF). Finally, we show consistent single nucleotide variant (SNV), insertion and deletion (indel), and ITD calling at sub-0.5% allele frequencies, and demonstrate the utility of reporting variant-specific MDAFs and normal dataset P-values when analyzing low AF variants.

Conclusion: AMP provides NGS-based detection of complex mutation types that are relevant in AML. We demonstrate robust calling of FLT3-ITDs and other variants at low AFs. We also demonstrate full coverage of CEBPA with high depth and low noise such that the 95MDAF predicts confident variant calling at low AFs. This approach is accurate and scalable, enabling simultaneous detection of multiple mutation types across multiple target genes in a single assay.
P025. Downregulation of RAG1 Expression Inhibits the Leukemic IK6 Isoform Formation in B-Cell Lymphoblastic Leukemia Cells
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Introduction: The IKZF1 gene isoform, IK6 is a common pathogenic finding in acute lymphoblastic leukemia (ALL) with or without BCR-ABL1 fusion gene. The formation of IK6, however, might be caused by either exon deletion or aberrant RNA splicing. RAG1, one of the key elements for DNA V(D)J recombination, was predicted to be the key player in a previous study (Harvey et al. Blood 2010) but no direct evidence was given. In this study, we examined if down regulation of RAG1 would affect the IK6 formation and what are the other effects of RAG1 in the biology of ALL.

Methods: The RAG1 gene was specifically knocked down by small interference RNA (SiRAG1) or nonspecifically down regulated by givinostat, a type II Histone Deacetylase and potent apoptosis inducer for ALL (Li and Yao et al., 2016 and 2017). The presence IK6 isoform was measured by quantitative RT-PCR. In addition, a next generation sequencing (NGS) was performed to study whole transcription analysis to investigate the differentially expressed whole RNA in the down-regulated RAG1 ALL.

Results: The IK6 isoform was only detected in RNA but not in genomic DNA from this cell line confirmed to be resulted aberrant RNA splicing. The RAG1 was significantly knocked down by both 200 nM SiRAG1 and 25 uM Givinostat, 65% and 90%, respectively, (P < 0.001). Meanwhile, IK6 was co-inhibited, 60% and 75%, respectively (P < 0.001) compared to the controls while wild-type IKZF1 was not significantly affected. Further WTS study showed that knockdown RAG1 only upregulate SOX11 and CCND2 genes and downregulate most CDK genes associated with cell cycle regulation. Finally, the analysis of 150 upregulated genes by SiRAG1 were positively correlated to givinostat treated specimen (P=0.016), indicating these genes might be involved in givinostat induced antineoplastic effect including the apoptosis.

Conclusion: The role of RAG1 in RNA splicing regulation has not been studied and our results in this study were the first to support this hypothesis. In addition, our results provided the evidence that the formation of IK6 in B-cell ALL can be regulated by RAG1 or expression of RAG1 with deacetylation inhibitor, givinostat. Our finding not only revealed the molecular mechanism in IK6 isomform formation but provided evidence of clinical investigation if RAG1 might be a target for personalized medicine in treatment of acute lymphoblastic leukemia.

P026. Panel-Based Next-Generation Sequencing of Chinese Diffuse Large B-Cell Lymphoma
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Introduction: Diffuse large B-cell lymphoma (DLBCL), an aggressive and heterogeneous malignancy, remains a challenging clinical problem, as up to one-third of patients are not cured with initial therapy. Recently, next-generation sequencing (NGS) studies in DLBCL have uncovered recurrent gene mutations. We designed a NGS multi-gene panel to identify genetic characteristics of Chinese DLBCL patients and provide relevant information for panel-based NGS tests to clinical laboratories.

Methods: A panel of 116 DLBCL genes was designed, based on literature, the Catalogue of Somatic Mutations in Cancer (COSMIC) database, and FoundationOne Herne. We studied 96 Chinese DLBCL biopsy samples using targeted sequencing.

Results: We identified the mutation frequency of SPEN (17%) and DDX3X (6%) in our study was higher than Western studies. Despite functional similarities between NOTCH1 and NOTCH2, there was no overlap in cases of NOTCH1 and NOTCH2 mutations, suggesting a distinct pathogenesis of N1 (NOTCH1 mutations) subtype and N2 (NOTCH2 mutations) subtype. KMT2D pathogenic mutations in total patients and patients treated with R-CHOP-like therapy were associated with significantly favorable prognosis. MYD88 L265P mutation, MYD88 pathogenic mutations, TP53 and BCL2 pathogenic mutations were important influence factors of poor prognosis in DLBCL.

Conclusions: This study underlines the genetic heterogeneity of DLBCL and demonstrates the contribution of panel-based NGS to molecular targeted therapy in DLBCL.

P027. Low-Frequency Variant Detection in Cell-Free DNA with Ultrafast Amplicon-Based Targeted Sequencing Using Unique Molecular Identifiers
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Introduction: The use of cell-free DNA (cfDNA) applications has seen tremendous growth in recent years with liquid biopsy being a noninvasive and easily obtainable sample type with several diagnostics and prognostic values. While liquid biopsy can potentially enable applications such as early cancer detection, treatment monitoring, and drug resistance screening, it also presents new challenges to variant detection due to the low fraction of mutant DNA in cfDNA and artifacts from background noise resulting from PCR and sequencing errors. While some success has been made
by using adapters carrying unique molecular identifiers (UMIs) in hybrid capture-based methods and performing deep sequencing, such approaches suffer from long, tricky, and tedious workflows and disappointingly low on-target rates.

**Methods:** We have developed the CleanPlex UMI technology to provide a fast, simple, and reliable NGS solution to low-frequency variant detection. The technology features three simple steps to generate molecular-barcoded and target-enriched NGS libraries in 3.5 hours. This amplicon-based method consists of first, a multiplex PCR reaction for molecular barcoding and target amplification, then followed by a proprietary background removal step, and finally a second round of PCR to add platform-specific adapter sequences and sample indexes. We validated this technology with an NGS panel optimized for cfDNA to target hotspots in 23 genes frequently mutated in lung cancer. NGS libraries were prepared from commercial reference cfDNA at minor allele frequencies (mAF) ranging from 0.1% to 0.5% and sequenced on an Illumina NextSeq to evaluate the performance.

**Results:** Overall, the detection sensitivity for low-frequency alleles is high even at low DNA inputs. We observe significant reductions in the number of false positive calls when analyzing the samples using UMIs. Nearly all known mutations in the reference cfDNA are detected at 0.1% mAF. At an mAF of 0.25%, all known mutants are detected with 100% PPV using 50ng of DNA. Detection of all mutants at mAFs of 0.5% and PPV of 100% require only 20ng or less of DNA. We are also able to accurately quantify the detected allele frequencies due to the high numbers of reference and mutant UMIs identified for each genetic target.

**Conclusion:** The CleanPlex UMI technology demonstrates high sensitivity for the detection of low-frequency alleles with low false positive detection rates.

**P028. Targeted Sequencing Reveals Pathogenic Genes in EBV-associated T/Natural Killer-Cell Lymphoproliferative Disorders**

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**Introduction:** EBV associated T/NK cell lymphoproliferative disorders are characterized by clonal proliferation of EBV positive T or NK cells. Extranodal NK/T cell lymphoma-nasal type (ENKTL) is the most common type of these diseases and it can occur both in the upper aerodigestive tract (nasal-ENKTL) and extranasal sites (extranasal-ENKTL). However, diverse biological behaviors and prognosis were observed for ENKTL involving different sites. The mechanism underlying such heterogeneity remains elusive. Aggressive natural killer cell leukemia (ANKL) and EBV positive T cell lymphoproliferative disorders (EBV+T-LPD) are relatively rare. There are some overlapping clinicopathologic features among ENKTL, ANKL and EBV+T-LPD.

**Methods:** A total of 169 cases of EBV associated T/NK cell lymphoproliferative disorders were collected. This included 123 ENKTL cases (87 nasal-ENKTL and 36 extra-nasal ENKTL), 12 ANKL cases and 34 EBV+T-LPD cases. The targeted sequencing gene panel covers all exons and elected introns of 64 genes involving in the development of lymphoma. The mutation profiles of ENKTL, ANKL and EBV+T-LPD were compared. ENKTL is the most common disease of the three EBV+T/NK-LPD, we further done the analysis of gene mutation of ENKTL cases, Comparison of mutated genes between nasal and extra-nasal ENKTL, protein express detection of KMT2D, TET2, EP300 and NOTCH1, relationship analysis of protein loss expression and gene mutation and prognosis in 123 target sequencing ENKTL cases.

**Results:** STAT3, KMT2D, DDX3X, NOTCH1 and TET2 showed high mutation rates in these three EBV+T/NK-LPD. The comparison of mutated genes of these three disorders showed that the exclusive mutated genes of ENKTL are PRDM1 et al., the ANKL are ITPKB et al., the EBV+T-LPD is MFHAS1. The mutated rates of KMT2D and NOTCH2 in extra-nasal ENKTL are greater than those in nasal-ENKTL.

Further study in ENKTL showed that the loss of KMT2D and TET2 protein were associated with the mutation of KMT2D and TET2 gene respectively. Prognostic analysis showed that the mutation of KMT2D or TET2 gene, the loss of expression of KMT2D or TET2 protein all significantly correlated with inferior overall survival of ENKTL cases.

**Conclusion:** We conclude that the exclusive mutated genes of ENKTL, ANKL and EBV+T-LPD are different, the mutation of KMT2D and TET2 may play an important role in the development of ENKTL.

**P029. Longitudinal Monitoring of AML Tumors with High-Throughput Single-Cell DNA Sequencing Reveals Rare Clones Prognostic for Disease Progression and Therapy Response**

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**Introduction:** AML (acute myeloid leukemia) is increasingly being treated with precision medicine. To better inform treatment, the mutational content of patient samples must be accurately determined. However, current tumor sequencing paradigms are inadequate to fully characterize many instances of the disease. A major challenge has been the unambiguous identification of potentially rare and genetically heterogeneous neoplastic cell populations, capable of critically impacting tumor evolution and the acquisition of therapeutic resistance. Standard bulk population sequencing is unable to identify rare alleles and definitively determine whether mutations co-occur within the same cell. Single-cell sequencing has the potential to address these key issues and transform our ability to accurately characterize clonal heterogeneity
Methods: Previous single-cell studies examining genetic variation in AML have relied upon laborious, expensive and low-throughput technologies that are not readily scalable for routine analysis of the disease. We applied a newly developed platform technology to perform targeted single-cell DNA sequencing on over 140,000 cells and generated high-resolution maps of clonal architecture from AML tumor samples.

Results: Single-cell sequencing of multiple patient samples demonstrated that relapse clones acquired oncogenic RAS mutations. We utilized the high-throughput and sensitivity of our single-cell approach to more definitively assess where in the course of treatment these RAS mutated clones were acquired. Oncogenic RAS harboring clones, comprising between 0.4%, and 0.1% of tumor populations, were identified in patient samples either prior to or shortly after onset of treatment. Significantly, these RAS variant alleles were not detectable with targeted bulk sequencing. Throughout the course of treatment with the FLT3 inhibitor gilteritinib, the RAS mutant clones selectively expanded and were responsible for resistance to therapy and relapse.

Conclusions: These findings point to the presence of underlying genetic heterogeneity in AML that contributes to therapy resistance and relapse. We demonstrate the utility of sensitively assaying clonal architecture with single-cell sequencing to better inform patient stratification and therapy selection.

P030. Determination of Accuracy, Reproducibility, and Repeatability for the Clinical Validation of a 75 Gene Myeloid Mutation Panel

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Introduction: Acute myeloid leukemia is a clinically and biologically heterogeneous disease and the most common cause of leukemia-related mortality in the United States. Multigene mutational analysis of AML and other myeloid disorders provides important information regarding prognosis and treatment. Inter-laboratory, inter-assay, and intra-assay variability measurements are an essential part of the validation process for NGS panels.

Methods: Mutational profiling was performed on blood or bone marrow samples from 50 different patients using the Archer VariantPlex Myeloid gene panel for the MiSeq/NextSeq. For inter-laboratory concordance, 50 samples that had been previously sequenced with an alternative methodology by an outside institution were underwent re-sequencing and analysis. Seven cases were selected for inter-assay reproducibility tests across three different sequencing runs. Duplicates of four cases were sequenced separately for intra-assay comparison. For all three reproducibility studies, detection of variants and their percent variant allele frequency (%VAF) were used as a basis for calculating coefficient of variance percentage (%CV) and average %CV. To consider a variant call positive filtering criteria included: at least 5 reads supporting the variant allele, reads supporting the variant allele must originate from at least unique 3 start sites, the frequency of the variant call in the ExAC global population < 5%, and the %VAF must be >=2.5%.

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P031. Determination of the DNA Input and Limit of Detection in the Clinical Validation of a Next Generation Sequencing (NGS)-Based Myeloid Gene Panel

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Introduction: Next generation sequencing with targeted gene panels has been increasingly applied in clinical practice in the last several years. Genetic aberrations have been heavily integrated particularly into the process of diagnosis, treatment guidance, prognosis and follow-up of myeloid neoplasm. We validated a next generation sequencing-based myeloid gene panels in a diagnostic molecular pathology laboratory. Here, we report the limit of detection (LOD) of our myeloid NGS panel.

Methods: Archer VARIANTPlex NGS Myeloid assay was used for sequencing of 75 genes reportedly involved in myeloid malignancies (Mutational Myeloid Panels NGS-MMP75). The commercially available SeraSeq Myeloid Mutation DNA Mix positive control DNA which includes 23 variants across 18 genes, ABL1, ASXL1, BRAF, CALR, CBL, CEBPA, CSF3R, FLT3, IDH1, JAK2, MPL, MLL, NPM1, SMARCA4, SRSF2 and U2AF1, was utilized in our LOD study. A range of 200 ng, 100 ng, 50 ng and 10 ng of SeraSeq Myeloid Mutation DNA Mix was used to determine the optimal DNA input. Secondly, the LOD was determined by serial dilution at 100%, 70%, 50%, 25%, and 10% SeraSeq relative to NA24365 DNA Correll control background. The admixtures were subjected to library preparation and subsequently sequenced on the Illumina NextSeq sequencer. Using filtering criteria on Archer Virtual Machine (VM), we set various cut-off for our variant detection at variant allele frequencies of 1.5%, 2.0%, 2.5%, 3.0% and 5% to evaluate the lowest possible allele frequency in which all 23 variants could be detected. These parameters were applied to a total of sixty samples analyzed using MMP75 pipeline.

Results: At DNA input of 200ng, 100ng, and 50ng, all 23 variants in the SeraSeq Myeloid DNA Mix were detected. At VAF of 2.5%, 21 variants were detected at 100% sensitivity and 100% specificity. At 70% SeraSeq; 30% NA24365 dilution, NPM1 c.863_864insTCTG (p.W288fs*12) was at a VAF of 2.2%. At DNA input of 50 ng, FLT3 c.1759_1800dup was at a VAF of 1.7%. All 23 variants of the SeraSeq Myeloid Mutation DNA Mix was used to determine the optimal DNA input. Furthermore, the lower limit of detection of FLT3 internal tandem duplication was not determined due to the nature of how structural variants are detected on the Archer VM analysis pipeline. By determining the DNA input and LOD of this assay, we were able to ascertain the filtering criteria for analysis for the myeloid NGS panel.

P032. Validation of an NGS Based Assay for Monitoring FLT3 ITD and TKD Variants in AML Patients

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Introduction: Internal tandem duplication (ITD) and tyrosine kinase domain (TKD) variants are clinically relevant variants in the FMS-like tyrosine kinase 3 (FLT3) that can independently promote constitutive activation of FLT3. AML patients with FLT3 mutations have prolonged overall and event-free survival when kinase inhibitors, such as midostaurin and quizartinib, are utilized during treatment. Invivoscribe currently has an FDA approved (USA), PMDA approved (Japan), CE-marked Leukostrat CDx FLT3 Mutation Assay, which is used to identify ITD and/or TKD variants. Leukostrat is a capillary electrophoresis assay that uses DNA extracted from peripheral blood or bone marrow mononuclear cells to detect FLT3 mutations with a limit of detection (LoD) of 0.05 signal ratio (SR; equivalent to 4.76% variant allele frequency, VAF). Here we describe the development and validation of a simple next-generation sequencing (NGS) assay with high concordance to Leukostrat that is able to detect FLT3 ITD and TKD variants down to 0.5% VAF.

Methods: Cell line and clinical sample DNA was previously assayed for FLT3 mutations using the Leukostrat assay. Contrived samples were generated by diluting genomic DNA with known ITD and/or TKD variants into background DNA with no variants. Contrived and clinical samples were used to generate FLT3 ITD and FLT3 TKD libraries via PCR amplification. Libraries were then pooled and sequenced. Sequencing data was analyzed using proprietary Invivoscribe software.

Results: The limit of blank (LoB) was determined to be 0% for FLT3 ITD, and 0.13% for FLT3 TKD. LoD for both FLT3 ITD and TKD was determined to be 0.5%. The ITD and TKD assays are highly linear, with R^2 values of 0.998 and 0.999, respectively. Variability was low for both assays; samples with expected VAF near the LoD of ~0.5% ranged in CV values from 7.6% to 8.4%. Clinical concordance between the capillary Leukostrat assay and the NGS assay are presented in Table 1. Concordance was 100% for the ITD assay (29 samples) and 97.3% for the TKD assay (38 samples). The discordant sample for the TKD assay was due to the SR near the LoD cutoff of the Leukostrat assay.

Conclusions: NGS based methodology allows for lower LoB and LoD with highly concordant results compared to capillary electrophoretic detection of FLT3 ITDs and TKDs. This assay provides an accurate method for detecting variants in AML patients and can be used to effectively stratify patients for therapies and clinical trials.
**Table 1: FLT3 ITD and TKD Concordance**

| FLT3 ITD | FLT3 TKD |
|----------|----------|
| **Concordance** | **Concordance** |
| CDx Positive | CDx Positive | CDx Positive |
| CDx Negative | CDx Negative | CDx Negative |
| PPA= 100% | PPA= 100% | PPA= 9.1% |

| FLT3 ITD NGS Positive | FLT3 ITD NGS Positive |
|------------------------|------------------------|
| 5 | 0 |
| NPA= 100% | NPA= 100% |

| FLT3 ITD NGS Negative | FLT3 ITD NGS Negative |
|-----------------------|-----------------------|
| 0 | 24 |
| ORA= 100% | ORA= 100% |

| FLT3 TKD NGS Positive | FLT3 TKD NGS Positive |
|------------------------|------------------------|
| 16 | 0 |
| NPA= 100% | NPA= 100% |

| FLT3 TKD ITD Negative | FLT3 TKD TKD Negative |
|-----------------------|-----------------------|
| 21 | 24 |
| ORA= 97.4% | ORA= 97.4% |

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**P033. Implementation of QXDx BCR-ABL %IS Digital Droplet PCR to Clinical Samples**

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**Introduction:** Recent therapeutic issues in the monitoring of patients with chronic myeloid leukemia (CML) are discontinuation of tyrosine kinase inhibitors. Related analytical sensitivities became important to detect low level of BCR-ABL in patients who reached deep molecular response (MR). We implemented QXDx BCR-ABL %IS Kit (BioRad, Hercules, USA) using highly sensitive droplet digital PCR (ddPCR) on the clinical samples.

**Methods:** A total of 20 clinical samples from patients with CML were tested with QXDx BCR-ABL %IS Kit. All ddPCR tests were duplicated as manufacturer’s instruction. Calibration was performed with IS calibrator within the QXDx BCR-ABL %IS Kit in every batch. Automated Droplet Generator, CFX96 thermal cycler and QX200 Droplet Reader (BioRad) were used for ddPCR and interpretation. Results of QXDx BCR-ABL %IS Kit were compared with real-time BCR-ABL Mbr IS-MM R DX Kit (Ipsogen, Lyon, France).

**Results:** QXDx BCR-ABL %IS assay was able to detect and quantify major BCR-ABL up to MR 5.0 (0.001 %IS) and BCR-ABL Mbr IS-MMR DX was able to up to MR 4.0 in analysis of clinical samples. Assay range was MR 1.0-MR 5.0 (10 %IS -0.001 %IS). Imprecision in ten repeats of MR 4.0 was 5.4% CV. Comparison of patient samples showing MR 1.0-MR 4.0 (0.001 %IS) concentrations revealed strong correlation (r =0.71).

**Conclusions:** The first implementation of QXDx BCR-ABL %IS Kit in clinical samples. The results revealed that the QXDx BCR-ABL %IS Kit had better analytical sensitivity than real-time RT PCR. We expect that the QXDx BCR-ABL %IS Kit will be a promising method for minimal residual disease monitoring in patients with CML and that physicians will be more informed in selecting candidates for quitting tyrosine kinase inhibitors.
### Performance of Manual vs Automated Ion Chef Library Preparation

|                      | Manual       | Automated Ion Chef |
|----------------------|--------------|--------------------|
| Total reads          | 18.8M        | 18.8M              |
| Clonality            | 61%          | 64%                |
| Mean read length (DNA)| 227          | 224                |
| Mean read length (RNA)| 113          | 110                |
| Uniformity           | 99%          | 98.4%              |
| Mean depth of coverage| 2652         | 3707               |

[Comparison between manual and automated library preparation]

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**P035. Minimal Residual Disease Monitoring of Rare Fusion Transcripts in Haematological Malignancies Using Droplet Digital PCR: A Step Forward towards Precision Medicine**

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**Introduction:** Minimal residual disease (MRD) detection using real-time quantitative PCR (qPCR) has long been used and is important for clinicians to evaluate the treatment response, risk stratification and prognostic prediction in patients with leukaemia. In ALL and AML patients, positive MRD before bone marrow transplantation has a strong negative prognostic indication for relapse and worse survival. For CML patients, clinicians may consider discontinuation of therapy under defined criteria of continual deep molecular response. Copious labor intensive, time consuming, and costly procedures are required for the development of clinical grade qPCR test. This impedes the monitoring of rare mutations or leukaemic fusion transcripts when commercial qPCR kits are not available. Droplet digital (dd)PCR offers an absolute quantification approach to calculate the mutation burden with high sensitivity by simply a pair of primers and a probe.

**Methods:** Our lab utilized the Bio-rad QX200 ddPCR platform to carry out the MRD monitoring after identifying the driver mutations by Sanger and next generation sequencing (NGS). Plentiful RNA was extracted from the diagnostic specimens of the patients in order to develop personalized assays for MRD monitoring. Primers and probes were designed in-house to detect the level of rare fusion transcripts including atypical BCR-ABL1 (e13a3 and e23a2ins25) in Ph+ ALL and CML patients, as well as NUP98-NSD1 and CBFB-MYH11 (type G) in AML patients. ABL1 was used as an internal reference gene. Serial MRD monitoring of follow-up samples was performed once the assays were established.

**Results:** The linearity of each assay was assessed by a calibration curve of at least 4 points. The limit-of-detection (LOD) was down to ten copies or less in each assay. The maximum copy number hits within linear range were 151,700 for e13a3, 2,740 for e23a2ins25, 38680 for NUP98-NSD1 and 232,000 for CBFB-MYH11 type G respectively. The lowest linear regression is 0.993. The limit-of-blanks (LOB) was determined by no template controls and negative controls, both of which showed zero copy of desired transcript with 95% confidence interval (e13a3 = 0-0.2; e23a2ins25 = 0-0.3; NUP98-NSD1 = 0-0.2; inv16 = 0-0.3).

**Conclusion:** We have demonstrated that ddPCR is deemed applicable to MRD monitoring of rare fusion transcripts for leukaemic patients in a cost effective manner. In order to do so, it is important to collect sufficient diagnostic specimens for the design and validation of the assays. The ddPCR assays can also be easily extrapolated to the field of solid tumour for drug efficacy and molecular relapse monitoring.

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**P036. A Gene Expression Profiling Assay for Widespread Clinical Use in Screening for BCR-ABL1-like ALL: A Single Institution’s Experience**

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**Introduction:** BCR-ABL1-like B-lymphoblastic leukemia (Ph-like-ALL) has a gene expression profile similar to BCR-ABL1 ALL, but lacks the Philadelphia chromosome. It accounts for 10-20% of pediatric and 20-30% of adult ALL. More than 60 gene abnormalities have been identified, making diagnosis a challenge. Precise classification is important because mutations are sensitive to the addition of targeted tyrosine kinase inhibitors to conventional chemotherapy. Clinical trials use a low density array (LDA) assay to screen for the Ph-like signature, with scores from 0-1 and positive when ≥0.5. Additional work up is then needed to identify specific mutations. We report on our experience with validating the LDA assay, demonstrate the ability to translate the methodology into clinical use, and discuss the development of a borderline category.

**Method:** RNA was converted to cDNA and placed on a LDA card with configuration identical to that established by the reference laboratory (RL) used in clinical trials for real-time PCR. Cycle threshold (CT) and delta CT results were analyzed by a modeling program developed by RL to calculate Ph-like signature scores. For optimization and validation, between-lab comparison was done using 54 ALL RNA samples previously run at RL and 16 new samples from our lab. Cases with scores between 0.4-0.6 at RL were enriched to evaluate for accurate classification around the 0.5 cutoff. We also investigated the effect of RNA isolation method (TRIzol, Promega, Qiagen) and collection media (EDTA, sodium heparin, sodium citrate) on score variability using blood from healthy volunteers. Scores were then compared.

**Results:** There was high correlation between RL and our lab (Figure). Variability in extraction methods and media were low. Tech-to-tech analysis showed high reproducibility. In-run reproducibility was high (SD 0.012, CV 2.42%).
Conclusion: The LDA assay is the standard for Ph-like ALL screening in clinical trials. We demonstrate high reproducibility in validating this assay for widespread clinical use. Various extraction methods and media can be used, variability within runs and between technicians performing the LDA was low, and correlation between labs was high. A cutoff of 0.5 was established in RL for clinical trials for high sensitivity, to identify cases that required further testing. Cases with scores near 0.5 may not have targetable mutations and the yield of additional workup is uncertain as they may not be true Ph-like ALL (false positives). A borderline category was established to better capture the biological spectrum and variability associated with the assay and to clarify LDA results for accurate WHO 2017 diagnosis.

Results:

An average of million reads for untreated and treated specimen were obtained with 211 M and 117 M assembled transcripts for control and treated specimen respectively. The low count genes were filtered (< 30 counts) and normalized expression using the Voom transformation. Expression of selected 6 DEGs (n=6) with upregulated and down-regulated expression in ALL from RNA-seq data were also analyzed by qRT-PCR. There were total 700 differentially expressed genes (DEG) identified including 400 upregulated (fold change >4) and 300 down-regulated (Fold-change < -4) genes between control and treated (log2 counts per million reads, CPM).

Novel finding in effect of inhibition of XIAP and BCL2 but upregulation of BCL2L11, TP53INP1 and APAF1 clarified mechanism of Givinostat-induced apoptosis. In HDAC family, Givinostat inhibits expression of HDAC 6 and 8 in family I and II but upregulated SIRT4 and HDAC11 in family III and IV. Finally, givinostat inhibited B-cell signal pathway by down-regulating the expressions of BTK, PLCXD1 and PSTPIP2. Therefore, the RNA-seq analysis of ALL treated with givinostat identifies novel DEG and alternatively spliced genes that are potential prognostic markers and therapeutic targets.

Conclusion: Global comparison of transcriptomes of ALL treated with Givinostat by multidimensional scaling analysis revealed molecular mechanisms of anti-proliferation and induction of apoptosis.

P038. Patterns of BCR/ABL Rearrangements by Interphase Fluorescence In-Situ Hybridization in CML and ALL

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Introduction: The BCR/ABL fusion gene is formed when a region of chromosome 9, c-abl proto-oncogene (ABL) fuses with a region of chromosome 22, breakpoint cluster region (BCR). This is referred to as reciprocal translocation and is represented as t(9:22). The resulting chromosome 22 with BCR/ABL gene sequence is known as Philadelphia (Ph) chromosome. The BCR/ABL gene fusion leads to the formation of an abnormal molecule; tyrosine kinase with increased activity and presumed to be involved in the development of leukemia. Fluorescence in-situ hybridization (FISH) analysis using dual color BCR/ABL translocation probes allows the visualization of BCR/ABL rearrangements in both interphase and metaphase cell, and the presence of the BCR/ABL fusion gene on chromosomes 22.

BCR-ABL testing is used to diagnose Chronic Myelogenous Leukemia (CML) or acute lymphoblastic leukemia (ALL). It is used to determine eligibility for molecular targeted therapy such as imatinib & also monitor response to therapy and disease recurrence. The BCR/ABL fusion gene is present in 90-95% of CML patients at diagnosis. About 25% of adult and 2-4% of pediatric ALL are positive for BCR-ABL fusion. The presence of the BCR/ABL gene in some ALL patients is associated with poor prognosis. Our objective was to determine the diagnostic and prognostic importance of various Fluorescence in situ Hybridization (FISH) signal patterns of dual color dual fusion BCR/ABL probe (D-FISH) in both CML and ALL. The incidence of both classical and variable/atypical signal patterns was
observed.

**Methods:** Samples (734) included in this study were received in the Department of Pathology SKMCH (Lahore, Pakistan) from July 2015 - July 2017 and processed for dual color dual fusion BCR-ABL FISH which was performed on peripheral blood or bone marrow samples.

**Results:** Approximately 428 (58%) were diagnosed as CML and 221 cases (30%) were diagnosed as ALL. Approximately 239 cases (33%) of both CML and ALL showed the classical DF-FISH signal pattern. Whereas 65 (9%) of CML and ALL showed variable DF-FISH signal pattern. In variable DF-FISH different signal patterns were analyzed. In these variable patterns 1F1G1O was 48 (74%), 1F2G1O was 13 (20%), 1F2G2O was 2 (3.1%) and 1F1G2O was 4 (6.2%) in both CML (77%) and ALL (15, 23.1%). Other rare combinations of classical and variable/atypical signal patterns were also observed in both CML and ALL.

**Conclusions:** Our data shows that the classical pattern was typically seen in CML but also reported in ALL. The highest proportion of variable pattern observed was 1F1G1O followed by 1F2G1O. Patients having genetic alteration with the loss of either 9q or 22q sequences may be associated with poor prognosis and the time to disease progression may be shorter. Hence, the establishment of signal pattern with FISH is important as disease progression may be shorter. Hence, the establishment of signal pattern with FISH is important as disease progression may be shorter.

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**P039. Validation of Metal-Conjugated Antibodies on Bone Marrow FFPE Sections Using Imaging Mass Cytometry**

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**Introduction:** Imaging Mass Cytometry (IMC) is a new technology that simultaneously detects more than 40 biomarkers on a histological section with single cell spatial resolution. IMC combines laser ablation of the tissue with inductively coupled plasma mass spectrometry, building on the foundation of the Fluidigm mass cytometry platform. This technology uses formalin-fixed, paraffin-embedded (FFPE) cryosections stained with a cocktail of antibodies tagged with different metal isotopes.

**Methods:** Maxpar Antibodies uses the maleimide conjugation strategy to covalently couple metal-chelating polymers (MCP) to antibodies. Preparation of FFPE bone marrow tissue is a multi-step process that includes the use of bone decalcification reagents. Our goal was to confirm that IMC can be used for multi-parametric analysis of bone marrow sections.

**Results:** Relation of mesenchymal stromal cell (MSC) markers and CD34+ hematopoietic stem / progenitor cells (HSPCs) within paraffin-embedded bone marrow biopsies of benign and neoplastic marrows was demonstrated using immunohistochemistry (IHC) and immunofluorescence (IF) staining, but the relationship to other stromal elements that are also key players of the hematopoietic microenvironment was not assessed due to technical restrictions. Here we demonstrate the validation of various metal-conjugated antibodies for characterizing simultaneously multiple stromal components (trabecules, hematopoietic and endothelial cells, MSCs, osteoblasts, etc.) and secreted factors (Wnt3a and CXCL12) of bone marrow and their relationship to CD34+, lymphoid and myeloid cells. A tissue microarray (1mm cores) of bone marrow from human, mouse and dog was built in order to assess the feasibility of staining bone marrow from different species. Lymph nodes were also included as controls. We compare staining of sequential sections with primary antibodies followed by HRP-labeled secondary antibodies for IHC or by metal-labeled primary antibodies for IMC.

**Conclusion:** We show by IHC that metal-tagged antibodies do not lose antigen specificity when a section is stained with metal-tagged primary antibodies and HRP-tagged secondary antibodies. IMC enables single-cell heterogeneity and functional studies in complex bone marrow tissues.

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**P040. Deep Vein Thrombosis Risk: The First External Physical Marker - Multiple Lipomas Associated with Thrombophilia**

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**Introduction:** Thrombophilia is the increased tendency to form thrombi, often silent, usually due to genetic mutations. Venous thrombosis has an incidence of 0.1% in the general adult population. Deep vein thrombosis (DVT) with pulmonary emboli (PE), collectively called venous thromboembolism (VTE), can be fatal in up to 25% of patients with PE. Case review of two brothers with both familial multiple lipomatosis and personal and family history of multiple DVTs and PEs questioned whether multiple lipomas and venous thrombosis are associated. Thrombophilia is unsuspected unless abnormal clotting occurs. Multiple lipomas, if correlated, would be a first physical finding, other than venous stasis pathology, representing an external marker for VTE risk.

**Methods:** From 2005 to 2016, we retrospectively reviewed one surgeon’s consecutive adult patients with removed lipomas. We recorded available chart history of personal and familial lipomas, personal and familial venous thrombotic events, and thrombophilia testing. Screening for personal and/or familial history of thrombophilia, and laboratory testing for thrombophilia abnormalities (Factor V Leiden, Proteins S and C) were begun when multiple lipomas and thrombophilia were first suspected to be correlated.

**Results:** A total of 153 adults had lipomas removed. Thirty-four had multiple lipomas by history and/or physical examination. The mean age was 55 years, and lipoma numbers ranged from 2 to 21. Of these 34 patients, 8 (20.4%) had had a documented thrombotic event,
compared to the rate of VTE in the general population of 0.1% (95% CI 4.5-24.1%, P< 0.001). Of 11 patients who also had a familial history of multiple lipomas, 2 (23.1%) had a personal VTE event and 3 (20.8%) had a familial VTE event. Of these 54 multiple lipoma patients, nine had prior testing for thrombophilia, of which 4 (46%) were positive.

Conclusion: We suggest that compared to the incidence of venous thrombosis in the adult population, patients with multiple visible lipomas have a previously unknown significantly higher risk of personal and/or familial venous thrombosis. This study suggests a possible genetic link between inherited conditions of multiple lipomatosis and thrombophilia. To our knowledge, this study is the first to identify multiple lipomas as a previously unsuspected, external marker for silent and potentially deadly thrombophilia and increased DVT/PE risk.

P041. Screening of β-Globin Gene (HBB) for Rare Mutations in β-Thalassemia Patients Using Sanger Sequencing
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Introduction: β-thalassemia is an autosomal recessive disorder which results in the formation of abnormal hemoglobin due to a variety of different mutations found in the HBB gene. These mutations render patients incapable of producing correct form of hemoglobin. The aim of this study was to identify HBB gene mutations in β-thalassemic patients, not included in the common-mutation panel of ARMS PCR, by sequencing HBB coding, intronic and promoter.

Methods: A total of 10 samples previously tested for HBB gene mutations by ARMS PCR common-panel (i.e. IVS 1-1, IVS 1-5, Codon 8/9, Codon 41/42 and 619bp deletion) were analyzed by Sanger sequencing. Two healthy subjects were included as negative controls. Genomic DNA was isolated and HBB gene was amplified. Column purified amplified products were utilized for bidirectional cycle sequencing (Big Dye Terminator, ABI, USA).

Results: In the present study, a total of 10 samples were analyzed. Four were males and six females. The mean of the patients was three years. All patients were diagnosed as β-thalassemia major based on their family history, clinical and laboratory findings. On average, patients were receiving transfusions every 2nd week. Seven rare mutations in HBB gene were detected including point mutations. The mutations spanned in the promoter region HBB:c.138C>A (-88 C>A), exon1 HBB:c.17_18 delCT (Codon5 -CT), HBB:c.47G>A (Codon15 G>A), HBB:c.92G>C (Codon30 G>C), HBB:c.50A>C (CAP+1 A>C), exon2 HBB:c.118C>T (Codon39), and intron2 HBB:c.315+1G>A (IVS II-1 G>A). All control subjects showed normal HBB gene sequence. In addition, a polymorphism T>C in codon3 at position HBB:c.59 was detected in majority of the patients and controls.

Conclusion: Although ARMS PCR is a fast and convenient method for detection of common mutations in the HBB gene, a small subset of patients may be missed because of rare mutations, which would require other means for diagnosis. Sanger sequencing is an accurate and robust technique to manage such patients.

(Key words: Thalassemia, β-globin, Sequencing)
Introduction: High survival rates for pediatric leukemia are very promising. With regard to treatment, children tend to be able to withstand a more aggressive treatment protocol than adults. The differences in both treatment modalities and outcomes between children and adults make extrapolation of adult studies to children inappropriate. The higher success is associated with a significant number of children experiencing nutrition-related adverse effects both in the short and long term after treatment. Specific treatment protocols have been shown to deplete nutrient levels, in particular antioxidants. The optimal nutrition prescription during, after and long-term following cancer treatment is unknown. This review article will provide an overview of the known physiologic processes of pediatric leukemia and how they contribute to the complexity of performing nutritional assessment in this population. It will also discuss known nutrition-related consequences, both short and long term in pediatric leukemia patients. Since specific antioxidants have been shown to be depleted as a consequence of therapy, the role of oxidative stress in the pediatric leukemia population will also be explored.

Conclusion: More pediatric studies are needed to develop evidence based therapeutic interventions for nutritional complications of leukemia and its treatment.

Infectious Diseases

P044. Performance Evaluation of the BioFire FilmArray Respiratory Panel 2 Plus (RP2plus) for the Detection of Respiratory Viruses in Swab Specimens

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Introduction: The BioFire FilmArray Respiratory Panel 2 Plus (RP2plus) is a FDA-cleared multiplex panel for the detection of 22 viruses and bacteria on nasopharyngeal swab specimens. In this study, we evaluated the performance of the RP2plus in the detection of respiratory viruses with 313 specimens that were previously tested by the FDA-cleared Luminex NxTAG Respiratory Pathogen Panel (NxTAG RPP).

Methods: A total of 313 frozen respiratory swab specimens, collected between December 2016 and September 2017, were tested with the RP2plus. A result was considered true when the RP2plus and NxTAG results were concordant. To resolve discrepant results, the specimens were extracted and tested using in-house single-plex assays. A discordant result was considered true when it agreed with the single-plex assay, and vice versa. Positive percent agreement (PPA), negative percent agreement (NPA), and kappa coefficient (κ) were determined with respect to the consensus results of the discordance analysis. The significance of differences in the positive detection between RP2plus and NxTAG was checked using the McNemar test. A p-value of < 0.05 was considered as statistically significant. The Middle East respiratory syndrome coronavirus (MERS-CoV), influenza A (H1), and all bacterial targets were excluded from the analyses due to under-represented number of positive specimens.

Results: All specimens were successfully tested by the RP2plus. Result discrepancy between RP2plus and NxTAG was observed for 27 (8.6%) specimens. After discordance analysis, 67 specimens were true negative and 246 specimens were true positive with at least one respiratory pathogen (single infection, n=189; dual infections, n=53; triple infections, n=4), yielding an overall positive rate of 78.6%. Among the positive specimens, 304 viruses (adenovirus, n=31; coronavirus 229E, n=14; coronavirus HKU1, n=5; coronavirus NL63, n=11; coronavirus OC43, n=14; metapneumovirus, n=25; enterovirus/rhinovirus, n=55; influenza A (H1N1/2009), n=26; influenza A (H3), n=28; influenza B, n=21; parainfluenza 1, n=7; parainfluenza 2, n=6; parainfluenza 3, n=29; parainfluenza 4, n=8; and respiratory syncytial virus, n=24) and 3 bacteria (Chlamydia pneumoniae, n=1 and Mycoplasma pneumoniae, n=2) were detected. Bordetella, MERS-CoV, and influenza A (H1) were not detected. With respect to the consensus results, the PPA and NPA of the RP2plus were 98.03% (95% confidence interval (CI), 95.77%-99.27%) and 95.71% (95% CI, 87.98%-99.11%), respectively. Concordance between RP2plus and the consensus results was 97.60% (366/375; κ=0.92, 95% CI, 0.87-0.97).

Conclusions: Overall, RP2plus showed almost perfect agreement with the consensus results. RP2plus has identified more viruses when compared to NxTAG. Notably, more enterovirus/rhinovirus (p-value=0.023) were detected by RP2plus. Also, RP2plus showed higher success rate in influenza A (H1N1/2009) subtyping in comparison to NxTAG (not statistically significant; p-value=0.134).

P045. Assessment and Diagnosis of Entamoeba Hystolytica, E. Dispar and E. Moshkovskii in Stool Samples from A Rural Community of Nepal

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Introduction: Nepal is a developing country which has many health problems. Amebiasis is one of the infectious diseases that is highly seen in rural area of Nepal caused by Entamoeba species. Recent reports show that open defecation, drinking untreated water, unsanitary habits...
and lack of basic health knowledge cause higher mortality and morbidity in our country. *E. histolytica* is an anaerobic pathogenic parasitic. However, *E. dispar* and *E. moshkovskii* are non-pathogenic. Likewise, *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically identical but genetically distinct species.

**Methods:** A total of 270 faecal samples were collected from south eastern terai region of Nepal after the informed consent form. The samples were processed by direct wet smear and formalin ethyl acetate concentration technique. Eventually, microscopic examinations were performed for the detection of *Entamoeba* species along with other intestinal parasites. Furthermore, an enzyme immunassay was executed to detect antigens of *E. histolytica* through ELISA. Additionally, microscopically positive samples for *Entamoeba* species cysts were further characterized using a Nested-PCR targeting 16S-like ribosomal RNA gene. The PCR generate amplicons of 174 bp for *E. dispar*, 439 bp for *E. histolytica* and 553 bp for *E. moshkovskii* were subjected to 2% agarose gels electrophoresis and visualized under UV transilluminator.

**Results:** Of the total collected samples, 8.52% were microscopically positive for *Entamoeba* cysts either singly or in combination with other intestinal parasites. Likewise, 42 samples, viral diarrhoea was the most significant form of diarrhoea found in 76.67% of patients. Among different organisms, *As. lumbricoids* and *E. histolytica*, *G. lamblia* and *H. nana* were identified in most of the patients accounting for 11.11%, 8.52%, 2.59% and 1.11% respectively. However, *Lumbricoides*, *G. lamblia*, *Tenia solium* and *E. histolytica* were present in an individual patient while two patients were found with both *As. lumbricoids* and *G. lamblia*. Among several symptoms, diarrhoea seems to be the common symptom infecting all of the patients which is followed by fever and vomiting which accounts for 55.1 % and 46.2% correspondingly. However, nausea appears to be the least common symptoms infecting only 14.4% of patients. Subsequently, 56 cases were PCR positive, 51 cases were ELISA positive whereas 47 were found to be positive by microscopy.

**Conclusion:** Molecular techniques are indeed promising tools for epidemiological studies, particularly in discriminating the pathogenic from the non-pathogenic species of the *Entamoeba* species. This study reports a new nested multiplex PCR strategy for detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* which is highly rapid, specific and sensitive which is useful for proper diagnosis, immunological assay and drug testing.

**Introduction:** Multidrug resistance in *Enterobacteriaceae* including resistance to quinolones is rising worldwide. Resistance to quinolones in *Enterobacteriaceae* is classically chromosomally mediated. However, several recent studies have indicated that plasmid-mediated resistance mechanisms also play a significant role in fluoroquinolone resistance, and its prevalence is increasing worldwide. Now quinolone resistance determinants (*qnrA*, *qnrB*, *qnrC* and *qnrS*) have been identified in a series of enterobacterial species from the United States, Europe, Japan and Far East. In Pakistan, the presence of the *qnr* gene in the clinical isolates of *Enterobacteriaceae* has not been reported. This study, therefore aimed to investigate the presence of the *qnr* gene in clinical isolates of *E. coli*, *K. pneumoniae*, *Salmonella* sp, *Enterobacter* sp from Pakistan.

**Methods:** A total of 150 non-repetitive, ciprofloxacin resistant *E. coli* (n=60) and *K. pneumoniae* strains (n=40) *Salmonella* sp (n=30) and *Enterobacter* sp (n=20) strains were isolated from clinical specimens of patients from January 2018 to July 2018 using standard microbiological techniques. Minimal inhibitory concentrations (MICs) of the antibiotics were determined according to the Clinical and Laboratory Standards Institute (CLSI). Screening of *qnrA*, *qnrB*, and *qnrS* by was performed by polymerase chain reaction (PCR) amplification.

**Results:** PCR amplification of *qnr* gene in 60 *E. coli*, 40 *K. pneumoniae* isolates, 30 *Salmonella* sp strains, 20 *Enterobacter* sp strains was performed. *qnr* genes was detected in 15% (23 out of 150) strains tested. In *E. coli*, the *qnrB* gene was detected in four out of 60 strains (6%). No *qnrA*, *qnrS* or both were identified in *E. coli* strains. Similarly 11 out of 40 (28%) *Klebsiella* isolates had *qnr* genes where seven (64%) samples showed *qnrB*, 3 (27%) *qnrS* and 1 (9%) showed both *qnrS* and *qnrB* while none showed *qnrA*. In *Salmonella* spp. and enterobacter spp., the prevalence of *qnrB* was3 (10%) and 3 (15%) respectively while 10% strains of *Enterobacter* spp, harbor *qnrS*. None of the isolated strains showed *qnrA* or *qnrA* or *qnrB*.

**Conclusion:** In conclusion, this study reports on the identification of *qnr*-like determinants in *Enterobacteriaceae* from Pakistan. Further studies are needed to document the prevalence of *qnr* in larger number of samples as well as role of chromosomally-mediated or other mechanisms of resistance towards quinolone.
**P047. Development and Evaluation of the Human Papillomavirus Genotype Assay by Next-Generation Sequencing Using Cervicovaginal Swab**  
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**Introduction:** Various human papillomavirus (HPV) genotyping tests were used globally for screening of cervical cancer and precancerous lesion. Recently, the next generation sequencing (NGS) based HPV genotyping assays which could identify the broad spectrum of HPV genotypes has been developed and commercially available. However, the clinical performance of these assays remained uncertain. We developed the NGS based HPV genotyping platform and evaluated its clinical performance using clinical specimens.

**Methods:** A total of 1,022 HPV positive, cervico-vaginal swab samples from women who visited the outpatient clinic and HPV testing by the bead based multiplex assay were used in this study. We performed HPV genotyping using NGS based HPV detection assay (Ezplex HPV NGS kit; Genetree Research Inc., Korea) with the residual same samples, and compared the results with those of liquid-based bead multiplex PCR assay (Osang Healthcare Inc., Korea). Any inconsistent results between two assays were confirmed by Sanger sequencing.

**Results:** Among all 1,022 HPV positive cases by bead array test, overall HPV positivity by the NGS analysis was found in 1,020 (99.8%) cases, leaving two cases. By direct sequencing, these two cases were confirmed as HPV negative group, consistent with the results of NGS test. In comparison of HPV genotypes, the two assays showed highly concordance results (973/1022, 95.2%). Forty-seven cases with discordant HPV genotypes by two assays were verified by Sanger sequencing method and confirmed to be consistent with NGS results. The multiple type HPV infection was found in 112 cases by the NGS assay. In addition, the NGS assay identified additional 10 HPV genotypes (67, 72, 84, 86, 87, 89, 90, 91, 102) that were not identified by liquid bead array test.

**Conclusions:** These results indicated that the HPV NGS assay had high sensitivity for broad spectrum detection of HPV in cervico-vaginal samples. It may be used widely for rapid diagnosis and monitoring of HPV infection.

**P048. Droplet Digital Polymerase Chain Reaction for Detection of Herpes Simplex Virus Type 1 and 2 in Clinical Samples**  
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**Introduction:** Herpes simplex virus 1 (HSV-1) can cause genital herpes as well as oral herpes and herpes simplex virus 2 (HSV-2) infection is sexually transmitted infection. Both HSV-1 and -2 may cause from mild, clinically asymptomatic or life-long latent infection to encephalitis. In this study, droplet digital polymerase chain reaction (ddPCR) were established and evaluated for detection HSV-1 and -2.

**Methods:** Seventy clinical samples from suspected HSV-1 or HSV-2 infected patients were analyzed. The samples had previously been analyzed with a conventional PCR (PCR) and a comparison between QX200 ddPCR platform (Bio-Rad, Hercules, CA) and PCR was performed.

**Results:** For HSV-1 detection, 49 of 70 (70%) samples showed concurrent results (positive: 4 samples, negative: 45 samples) between two methods, but ddPCR showed positive results whereas PCR showed negative results in 21 of 70 samples, and none of samples showed ddPCR negative and PCR positive results. For HSV-2 detection, 66 of 70 (94.3%) samples showed concurrent results, ddPCR showed positive results whereas PCR showed negative results in four of 70 samples. None of samples showed ddPCR negative and PCR positive results. Eight of 70 samples showed dual positive results of HSV-1 and -2 detection in ddPCR.

**Conclusions:** The ddPCR very sensitively detected HSV DNA in clinical samples and could be a useful tool for screening and monitoring of HSV infected individuals.

**P049. Cytomegalovirus Monitoring of Stem Cell Transplantation Recipients by Real-Time Polymerase Chain Reaction**  
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**Introduction:** Cytomegalovirus (CMV) is one of the most important pathogens in transplant patients. Since CMV disease occurs in up to 35% of allogenic bone marrow transplant recipients, early and accurate diagnosis and reliable monitoring methods are mandatory. Use of preemptive antiviral agents depending on copy numbers is sometimes not standardized. Here we studied whether the tendency of viral copy number changes can predict which patients would benefit from preemptive CMV therapy.

**Methods:** Retrospective analysis on allogenic hematopoietic stem cell therapy recipients were done based on medical records from 2016 to 2018. In our institute, ARGENE CMV R-gene Kit (bioMerieux) is used, and range of quantification is 500~10,000,000 copies/mL. 14 selected transplant recipients were reviewed on viral load, ganciclovir treatment, CMV infection or disease, and rate of increase.

**Results:** The 14 studied recipients were diagnosed with acute myeloid leukemia (7), multiple myeloma (2), Hodgkin lymphoma (1), acute lymphoblastic leukemia (2), myelodysplastic syndrome (1), and severe aplastic anemia (1). Eight male patients and six female patients whose average age was 49 years old were studied and their viremia levels were monitored every week by PCR. Most of them were treated with allo-PBSCT, except one
with auto-PBSCT. No antiviral agent was used between the two viral load results. For 9 patients, ganciclovir was used after peak viremia. We could not find a correlation between initial CMV load and peak CMV load in 14 individuals by Spearman’s correlation test showing \( r=0.253 \) and \( p=0.383 \) of statistical significance. No correlation was seen between initial copy numbers and days passed between initial and peak viremia \( (r=0.312, p=0.277) \). In our institute, ganciclovir was generally used when there were two consecutive high copies (>500 copies/ml) or one >10,000 copies/mL on first occasion.

**Conclusion:** PCR assays for CMV DNA detection enable rapid and specific detection prior to clinical symptoms to help improve outcomes, especially for organ transplant/bone marrow allograft patients. Testing helps keep track of the effectiveness of active treatment and can monitor for relapse after treatment. More studies should be done to determine the reliable initial CMV DNA load threshold predicting the eventual need for antiviral therapy.

**P050. Genital Tuberculosis: A Laboratory Diagnosis**

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**Introduction:** Genital tuberculosis is mostly under diagnosed, overlooked, and under reported because of its asymptomatic presentation and pauci-bacillary nature. Although there are multiple microbiological diagnostic modalities available, all exhibit different advantages and disadvantages. When we use these techniques in conjunction with each other, it increases the possibility of better diagnosis of tuberculosis. Here in our study, we have compared three diagnostic modalities namely ZN staining, automated liquid culture (MGIT) and nucleic acid amplification technique (TB-PCR) to ascertain the sensitivity of these tests and their role in diagnosis of genital tuberculosis.

**Methods:** The study was done retrospectively from January 2016 to Aug 2017 on 460 endometrial specimens from females with history of infertility. The samples were received from different centers, associated with Oncquest Laboratory Ltd, New Delhi, as per the laid down collection and transportation policy. All the specimens were tested by direct smear microscopy. Smears were prepared and stained using conventional ZN staining method for detection acid fast bacilli (AFB). MGIT BACTEC 320 liquid culture system was used for culture testing. A single tube Real time PCR was performed using the proprietary IS6110 specific primers and specific probes (Lytex star TB/NTM PCR Kit 2.0) targeting Mycobacterium tuberculosis complex and 16 S r DNA specific primers for Non-Tuberculosis Mycobacteria (NTM).

**Results:** Total 460 endometrial samples were processed for all three tests. Out of these, 18 samples were tested positive by at least one test modality. Out of 16 TB PCR positive samples, 14 samples were Mycobacterium tuberculosis positive and two were Mycobacterium other than tuberculosis (MOTT). Among 7 culture positive samples, four were MTB positive and three were MOTT positive.

**Conclusion:** Overall 3.47% samples were found positive for TB PCR (16 out of 460) and among 18 positive samples, TB PCR accounted for 88.8% positivity. Our values are at variance with other studies wherein the values have ranged between 22.2% and 85.44 %. Even though overall positivity by TB PCR is less, the sensitivity and specificity of TB PCR was found to be 71.43% and 97.54% respectively. This was in line with other studies also. Eleven samples were TB PCR positive, culture negative, this could be due to less bacterial load or dead bacilli that could be detected by TB PCR but not in culture. As we found in our study that sensitivity of both ZN microscopy and culture is very low, TB PCR was good in sensitivity, but overall positivity of samples was very less. TB-PCR can be promising by submitting right sample collected from right site and right time of menstrual cycle and its sensitivity can further be improved. Although it was noted that we cannot completely rely on a single molecular modality for the diagnosis of genital tuberculosis.

**P051. Bioinformatics Pipeline for Predicting Drug Resistant M. tuberculosis from Whole Genome Sequencing Data to Identify the Role Efflux Pumps**

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**Introduction:** Tuberculosis (TB) has a very high global burden; around 10.0 million people developed TB disease in 2017 out of which 5.8 million were men, 3.2 million were women and 1.0 million were children. Pakistan ranks 5th amongst high TB-burden countries. Tackling drug resistance is critical to ending the TB epidemic, hence, there is an urgent need for internationally recognized rules for the unambiguous clinical interpretation of genetic changes that can predict phenotypic resistance to anti-TB drugs. Active efflux of drugs mediated by efflux pumps that confer drug resistance is one of the mechanisms. Whole genome sequencing (WGS) based on target genes allows identification of single nucleotide polymorphisms (SNPs) that may be associated with drug resistance.

**Methods:** To understand SNPs in drug efflux genes we initially focused on 10 genes to pull out SNPs and INDELS (insertion/ deletions) on three M. tuberculosis (MTB) isolates. Further, we will run the same on 25 Efflux pump genes in 800 MTB isolates. The data and its phenotypic drug susceptibility testing (DST) information were identified using ReSeqTB platform and extracted the SRA numbers of these isolates which were than download from ENA database. We developed customized pipeline to identify efflux gene targeted variants.

**Results:** In the preliminary analysis, we were able to get a total of 39 SNPs and 4 INDELS (ranging from 10 - 47bp). The SNPs were then further annotated using...
SNPs in Kelch Protein Propeller Domain from Plasmodium falciparum

Introduction: The spread of artemisinin (ART) resistance in P. falciparum demanded an effective molecular surveillance of ART resistance. Recently, polymorphisms in K13 propeller in Plasmodium falciparum have been found to be a useful marker for ART resistance. We describe genotyping of point mutations in Kelch protein propeller domain of Plasmodium falciparum associated with ART resistance.

Methods: Samples (N=116) were collected from patients with microscopy confirmed P. falciparum malaria attending Aga Khan University Hospital during September 2015-December 2017. DNA was isolated using the whole blood protocol for the QIAmp DNA Blood Kit. The K13-propeller gene (K13) was amplified using nested PCR. Double-strand sequencing of PCR products was performed using Sanger sequencing methodology. Sequences were analyzed with MEGA 6 and Bioedit software to identify specific single nucleotide polymorphism (SNP) combinations.

Results: All isolates analyzed for K13-propeller allele were observed as wild-type in samples collected post implementation of ACT in Pakistan. C580Y, A675V, Y493H and R539T variants associated with reduced susceptibility to ACT were not found. K189T polymorphism was found in 2 isolates not significantly associated with ART resistance.

Conclusion: K13-propeller polymorphism are useful molecular markers for tracking the emergence and spread of ART-resistance in P. falciparum. C580Y polymorphisms are reported from Cambodia and Ghana with rapid invasion of the population and almost near fixation in south East Asia. Surveillance of K13 polymorphism is necessary as recommended partner drug for Pakistan sulfadoxine-pyrimethamine (SP) has shown reduced susceptibility which will compromise the efficacy of fast acting ACT. Surveillance of anti-malarial drug resistance to detect its emergence and spread need to be strengthened in Pakistan.

P054. Evaluation of the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry System for the Identification of Bacteria in Routine Clinical Microbiology

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Introduction: Rapid and accurate identification (ID) of bacteria are essential for the appropriate management of infections. In the clinical microbiology laboratory, bacteria are typically identified using phenotypic and biochemical methods. However, these are time consuming and require subjective interpretation. On the other hand, molecular methods are not suitable for large scale routine identification. The main objective of the study was to evaluate the performance of Vitek MS system for accurate identification of pathogenic microorganisms.

Methods: We analyzed 248 archived microbial isolates on the Vitek MS system. Microbial ID was based on the comparison of the protein spectrum generated from intact whole bacterial cells to a database (v.2.0) of species-specific reference protein profiles using a particular algorithm. A subset of isolates (N=104) were also analyzed by 16s rRNA sequencing to assess the accuracy of data.

Results: Out of 248 isolates analyzed 226 of them were identified by Vitek MS system up to species level. There were about 22 isolates for which no result was obtained, and sequencing of 16s rRNA region revealed that these species were unidentified due to lack of protein spectra in the database. Identification of another 82 isolates by sequencing showed concordance with mass spectrometry data.

Conclusion: In summary, the Vitek MS v2.0 is an accurate and user-friendly system for identifying pathogenic isolates for the organisms present in the database. A microbiology laboratory, however should have an access to 16s rRNA sequencing facility for identification of rarer species.

P055. Customizable Syndromic Panel for Respiratory Tract Pathogen Detection on a Microfluidic Device

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Introduction: In the US alone, uncomplicated respiratory infections account for over 25 million physician visits each year. A wide variety of bacteria and viruses are capable of causing upper and lower respiratory tract infections, but their symptoms are similar or overlapping. Panel based testing using molecular methods to identify these pathogens has clear advantages over non-
molecular or single target molecular testing. However, most current methods are limited to either fixed panels or low target throughput. We have developed a large collection of TaqMan research assays for respiratory tract pathogens detection and leveraged TaqMan Array Card (TAC) technology for high target throughput capability and flexible content. The combination presents a robust research application for respiratory pathogen detection and allows customization of both the size and content of the test panel.

Methods: A novel set of TaqMan qPCR assays was developed to over 40 distinct respiratory pathogen targets, including bacteria, DNA viruses, RNA viruses, and fungi. These FAM dye-labeled assays were pre-loaded and lyophilized onto wells on TaqMan Array Card (TAC), a microfluidic device that enables 8 samples tested for up to 48 targets simultaneously. These assays were evaluated with synthetic DNA or RNA templates and ATCC genomic DNA or RNA controls for sensitivity and specificity on TAC platform. In addition, we also ran ~140 repository samples that were characterized by some existing on-market products to evaluate the concordance.

Results: We report sensitivity, specificity, accuracy of these respiratory tract pathogen assays on the microfluidic TaqMan Array Card (TAC). Analytical sensitivity and linearity was demonstrated with at least 5 log linear dynamic range (with R^2 >0.99), limit of detection (LOD) down to 1-10 copies/µl on TaqMan Array Cards, and high PCR efficiency was observed. For specificity, each assay was tested against the rest of targets on the panel using ATCC gDNA/gRNA controls and no significant cross-reactivity was observed. Greater than 90% concordance with existing on-market product was observed.

Conclusions: We have developed a customizable qPCR assay panel for respiratory tract pathogens, in which any subsets of the targets can be built on the microfluidic TAC. We also have demonstrated excellent analytical performance of these respiratory assays on TAC and high concordance with existing on-market product. The application enables researchers to select their targets of interest and study a large number of respiratory pathogens in a single reaction with a simple workflow and fast turnaround time.

P056. Rapid Detection and Quantification of Epstein-Barr Virus (EBV) by Fully Automated Cassette-Based Real-Time PCR
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Introduction: Epstein-Barr virus (EBV), a herpesvirus leading to latent infections, is principally responsible for infectious mononucleosis, and also plays role in the etiology of various lymphomas and post-transplantation lymphoproliferative disease (PTLD). Especially in the bone marrow or solid organ transplanted, or in otherwise severely immune-suppressed patients, prolonged EBV primary infection or EBV reactivation from latency may be a serious and life-threatening complication. Therefore, direct and quantitative detection of viral DNA are of importance for the diagnosis of serious EBV disease and its monitoring. In this study, we evaluated the performances of fully automated cassette-based Real-Time PCR for EBV DNA in comparison with routine PCR method.

Methods: EBV ELITe MGB assay (ELITechGroup Molecular Diagnostics) is RT-PCR assay based on MGB technology. ELITe InGenius (ELITechGroup Molecular Diagnostics) automatically performs nucleic acid extraction, PCR set-up, PCR amplification and results analysis. Thirteen whole blood and 3 urine samples collected from patients were tested by EBV R-gene PCR kit (bioMérieux) and EBV ELITE MGB kit (ELITech Group). Precision calculations were based on the results of five technical replicate reactions performed on each of five consecutive days.

Results: Eleven (68.7%) samples resulted negative and 5 (31.3%) positive with a quantitative result for EBV ELITe MGB assay and only one sample (6.2%) showed positive with a quantitative result for EBV R-gene assay. In the 16 samples, the coefficient of correlation was 0.949. Four (9.6%) samples were positive with a quantitative result with EBV ELITE MGB assay and negative with EBV R-gene assay. One (6.2%) sample resulted negative with EBV R-gene assay and positive but below the lower limit of quantification (LLQ) with EBV ELITE MGB assay. No samples were positive with EBV R-gene assay and negative with EBV ELITE MGB assay.

Conclusions: The assay showed a good correlation between EBV-DNA levels detected by routine PCR assay and the excellent validation results. Moreover, EBV ELITE MGB assay showed higher sensitivity in samples containing lower amounts of DNA. Therefore, the use of EBV ELITE MGB assay in combination with the ELITE InGenius system allows rapid, sensitive, and reliable detection and the quantification of EBV DNA, making early information-based intervention possible. The clinical value of this assay requires further investigation.

P057. Detection of Commensal and Pathogenic Microbes Associated with Bacterial Vaginosis Using a qPCR Based Spatial Multiplexing Nanofluidics Platform
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Introduction: Bacterial vaginosis is a common condition among women of reproductive age and is associated with potentially serious side-effects, including an increased risk of preterm birth. Recent advancements in microbiome sequencing technologies have produced novel insights into the complicated mechanisms underlying bacterial vaginosis and have given rise to new methods of detecting commensal and pathogenic microbes. Profiling and monitoring vaginal tissue becomes desirable to understand susceptibility and risk
factors. Among the various technologies currently used, some are costly or lack sensitivity/specificity, and others have a complicated workflow. Although 16s rRNA-based sequencing is powerful, it doesn't always provide species level resolution and it cannot detect non-bacterial microbes like *Candida*, protozoa and viruses. PCR-based detection is sensitive and straightforward, but it lacks targeted throughput - only detecting a single or a few species.

**Methods:** To address these unmet needs, we have developed a large collection of TaqMan assays for vaginal health by leveraging our TaqMan technology and high throughput OpenArray platform. First we select different 33 microorganisms (bacteria, fungi, protozoa and even viruses) that are important in vaginal health/diseases, including normal ones present in vaginal flora and pathogenic ones. Then for each of these species, we identify unique gene targets that are species-specific wherever possible. Using our robust and vigorous TaqMan assay design pipeline, we design a collection of assays targeting these signature genes for each of these species. These assays are evaluated with plasmid artificial templates and ATCC gDNA samples on 384 well plates and on OpenArray, a microscope slide-sized plate with 3,072 through-holes (48 subarrays/plate and 64 through-hole/subarray).

**Results:** We first evaluated assay accuracy with plasmids and ATCC controls and all assays show expected on-target signals. We then tested specificity by running each assay with all the plasmids and ATCC samples for 33 species and did not observe any cross-reactivity. We also did serial dilution for each assay and demonstrated 7 log linear dynamic range (with $R^2 > 0.99$) with limit of detection (LOD) down to $\sim 50-100$ copies or even lower. Good PCR efficiency and reproducibility were achieved.

**Conclusions:** We have developed a collection of TaqMan assays for women's health and have demonstrated excellent assay performance - high sensitivity, specificity, accuracy and reproducibility on OpenArray platform. The application provides researchers a powerful and cost-effective tool with simple workflow, fast turnaround time, and high throughput yet essential for the treatment of patients. However, the currently used flow cytometry method can assess only the percentage of infected red blood cells (RBCs). Our aim was to optimize the flow cytometry approach for enumeration of total malaria parasites particles per microliter of the blood specimen.

**Methods:** Specimens were incubated with acridine orange (AO) and an RBC lysis solution. The numbers of malaria parasites were quantitated using the FACSCalibur analyzer and calibrated with size-standard and counting beads. The fluorescence of the AO-stained malaria parasites was examined using a fluorescent microscope. Electron microscopy was also used to study the ultrastructure of the malaria parasites particles in lysed RBC specimens.

**Results:** The number of malaria parasites particles was correlated with the percentage of infected RBCs obtained from manual counting ($r^2 = 0.87, p < 0.0001$). A dilution assay demonstrated that the counting method was linear in the range between 60 to 36,700 particles/µL; however, stored specimens exhibited an increase in the number of malaria particles. The fluorescence of AO-stained malaria parasites particles was confirmed. An electron microscopic study demonstrated that different stages of malaria parasites existed in lysed RBC specimens in the form of membrane-bounded spherical cells.

**Conclusion:** The potential use of flow cytometry for enumeration of malaria parasites particles was demonstrated. The developed approach is reliable and
straightforward for malaria parasite enumeration in the routine laboratory.

**P060. Evaluation of a Rapid and Affordable Modified HCV Genotyping Assay**

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**Introduction:** There are two FDA approved HCV genotyping assays. Siemens HCV Genotyping Assay (LiPA) is a line probe assay which involves handling of PCR product, therefore it is prone to contamination and entire procedure is long (10.5 hours). Abbott HCV Genotyping (GT) is a real time PCR assay in which the process is short (6 hours). However, the GT assay uses m24sp or m2000sp equipment to perform RNA extraction. Both machines are bulky and may not be possible to run in a small lab. Qiagen EZ1 is a generic automated extractor which has been shown to be effective in our lab. Therefore, this study aims to evaluate whether EZ1 extraction works with GT PCR and determine the performance of this modified method.

**Methods:** This is a retrospective study in which LiPA assay was performed as a routine test and residual samples were anonymised for testing by the modified method. For rare genotypes, e.g. 2 & 6, an EQA sample and reference material were collected. RNA extraction was done by EZ1 extractor. Then real time PCR was performed on Abbott m2000rt to detect HCV genotype (1a, 1b and 2 to 6). Common blood born viruses and bacteria were tested for specificity. When results of GT and LiPA were not matched, that sample was further tested by a supplementary test, Abbott HCV Genotyping Plus (GT plus), which can specifically detect HCV genotype 1a, 1b and 6. The agreement of GT/GT plus and the LiPA assay was used as gold standard. Otherwise, sequencing of the NS3 gene was used.

**Results:** As a baseline test, GT was compared with gold standard. 103 of 116 (89%) samples were accurately genotyped, with additional 5 of 31 (16%) genotype 1b samples were detected as genotype 1 no subtype. Of the remaining, 2 of 7 (29%) genotype 6 samples were wrongly detected as genotype 1, 3 of 7 (43%) genotype 6 samples and 3 of 15 (20%) genotype 2 samples were indeterminate. With GT plus added, 113 of 116 (97%) samples were correctly genotyped and left only 3 of 116 (3%) samples as indeterminate. NS3 sequencing confirmed that 2 samples were genotype 2a and another one was genotype 2t. No cross-reactivity to other viruses or bacteria was found. The limit of detection (LOD) of 500 IU/mL in the original assay was valid in the modified assay.

**Conclusions:** It was proved that EZ1 extraction works with GT PCR and maintains the LOD. The assay also displays high specificity. However, GT assay does have its own limitations. The genotype 1 primers are prone to cross react with genotype 6 samples and lead to inaccurate result. In addition, some genotype 2 & 6 samples could not be genotyped by GT assay. In our study, GT plus was performed for sample detected as genotype 1 no subtype and indeterminate sample. However, the necessity of GT plus depends on genotype prevalence in the region. Given genotype 2 and 6 are uncommon in Singapore (<3%), the GT assay is able to accurately genotype the majority of samples, with only a small number of sample need to be tested by GT plus or sequencing.

**P061. Evaluation of the New Cobas 6800 System for the Quantification of HBV, HCV, and HIV Viral Load**

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**Introduction:** The Cobas 6800 system (Roche Diagnostics, Germany) has been recently introduced for HBV, HCV, and HIV viral load assays. We analyzed linearity of the Cobas 6800 system and compared the results with the Cobas Ampliprep/TaqMan (AP/TM) system (Roche Diagnostics, Germany).

**Methods:** We analyzed commercial linearity panels for HBV DNA, HCV RNA, and HIV-1 RNA (Seracare, US) using Cobas 6800. For comparison of Cobas 6800 and Cobas AP/TM, 90 samples of HBV DNA positivity, 70 samples of HCV RNA positivity, and 60 samples for HIV-1 RNA positivity were analyzed using Cobas 6800 and Cobas AP/TM.

**Results:** The results of Cobas 6800 showed good correlations with expected values of linearity panels (R²: 0.9909-0.9976) and the results of Cobas AP/TM (R²: 0.9363-0.9835) for all three viruses. However, Cobas 6800 showed lower values than Cobas AP/TM for all three viruses. The mean differences of Cobas 6800 were -0.13 log10 IU/mL for HBV, -0.19 log10 IU/mL for HCV, and -0.23 log10 IU/mL for HIV.

**Conclusion:** Overall, the linearity of Cobas 6800 and correlation between Cobas 6800 and CAP/CTM systems are satisfactory for the three viruses. Systemic differences were observed between Cobas 6800 and Cobas AP/TM.

**P062. Study on the Clinic Value of qRT-PCR Genotyping for the Detection of Norovirus Infection in Cancer Patients**

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**Introduction:** Using the lab-based quantitative real-time PCR genotyping testing the infective situation in cancer patients, we studied the relationship between viral loads and the risk factors and predicting the prognosis of NoV associated gastroenteritis in cancer patients.

**Methods:** According to the highly conservative of
junction of ORF1–ORF2, we designed type specific primers and probes to detect NoV-GI and GII (GIV was rarely detected). Collection clinical data first option for the first time, we used the related statistic analysis of the data to seek the risk factors and predictor of prognosis.

**Results:** The infection of NoV-GII is higher than GI in cancer patients, but there were lower Ct values by quantitative real-time PCR genotyping testing. It meant that the viral load of GII is higher than GI too.

**Conclusions:** NoV-GII is the main stain of NoV related gastroenteritis in cancer patients. The worse the clinical symptoms, the higher virus carrier. It meant that the patients would be in bad situation in the future.

**Informatics**

**P063. iVarClass: An Intelligent Variant Classification System of Genetic Variants Based on ACMG-AMP Guidelines: Application to BRCA1/2 Variants**

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**Introduction:** Evaluating the pathogenicity of a variant is challenging given that variability between individual interpreters can be extensive because of reasons such as the different understandings of American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG-AMP) guidelines and few methods for implementing them. More recently, studies have also identified clinical significance in germline BRCA1/2 mutations (gBRCA1/2m) as clinically relevant in PARP inhibitors for patients already diagnosed with breast cancer.

**Methods:** This work proposes an intelligent variant classification system named iVarClass to provide the accurate clinical significance of variants to clinical researchers and reviewers. iVarClass integrated more than 50 databases information and developed an optimized pathogenic score to implement ACMG-AMP criteria. A dataset consisting of 7,239 gBRCA1/2m was reviewed by an expert panel. ClinVar (2018Oct) was established to evaluate iVarClass.

**Results:** As a result, iVarClass achieved a 5-class accuracy of 0.93. Comparison of iVarClass with state-of-the-art method InterVar. The result showed that iVarClass enhanced the mean accuracy by 10% and 50% reduction in variants of uncertain significance. In PARP inhibitors treatment, pathogenic and likely pathogenic of gBRCA1/2m are positive, other 3-class of gBRCA1/2m are negative. iVarClass achieved a sensitivity, specificity, and accuracy of 0.98, 0.99, and 0.99. Finally, the system can automatically generate PDF reports with pathogenic variants and variants descriptions.

**Conclusion:** This work demonstrated that iVarClass significantly reduces the time and enhances the accuracy for classifying and interpreting the clinical significance of germline BRCA1/2 variants. The proposed method is suitable for increasing consistency of variant reporting based on ACMG-AMP guidelines.

**P064. A Rigorous Study of the Need for Sanger Confirmation in Clinical Genetic Testing**

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**Introduction:** The use of orthogonal assays (e.g., Sanger sequencing) to confirm variants identified by next-generation sequencing (NGS) is standard practice in many laboratories to reduce the risk of delivering false positive (FP) results. In clinical genetic testing, confirmation may be particularly important for variants that can suggest irreversible interventions or substantial treatment changes for the patient. Moreover, because clinical NGS methods often emphasize sensitivity (to avoid missing clinically important variants), FP rates can be elevated compared with those in research-grade NGS. Published clinical studies have examined this issue, concluding that confirmation of the highest quality NGS calls may not always be necessary. However, these studies are generally underpowered, omit statistical justification, and explore limited aspects of the underlying data. The rigorous definition of criteria that separate high-accuracy NGS calls that do not benefit from confirmation from those of intermediate quality that do, remains a critical and pressing issue.

**Methods:** We examined NGS data from two clinical laboratories. Over 80,000 patient specimens and five well-characterized reference samples (Genome in a Bottle) were analyzed. In total, almost 200,000 variant calls with orthogonal confirmatory data were examined in detail including a diverse set of 1,684 primary variant caller false positives.

**Results:** An optimization algorithm used these data to identify criteria that confidently flag 100% of false positive calls (CI lower bound: 98.5-99.8%, depending on variant type) while minimizing the number of flagged true positives. Rather than relying on one or two quality metrics, as most published methods do, a combination of criteria proved superior, consistent with current clinical NGS guidelines [1]. Indeed, our criteria identify false positives that currently published criteria miss. Consistent with this observation, sampling analysis of our dataset demonstrated that substantially smaller datasets would result in less effective criteria. We also find that historical performance (observing a variant as a true positive some number of times) is often an ineffective criterion.

**Conclusions:** Although we see limitations with the currently published studies, our large multi-laboratory study reaffirms prior findings that high accuracy NGS variant calls can be separated from those of intermediate quality which require confirmation. Our rigorous methodology for determining test and laboratory-specific
Introduction: High TMB is regarded as a predictive biomarker for treatment response with immunotherapy. Targeted next-generation sequencing (NGS) using large gene panels, covering all coding regions of hundreds of cancer-related genes spanning about 1-3Mb, are now being adopted in the routine assessment of TMB. However, the use of large panels is substantially limited by its high cost. In this study, we explored the feasibility of using small panels as a screening method to predict TMB.

Methods: In the training set, TMB derived from 406 non small cell lung cancer (NSCLC) patients using a large panel (OncoScreen Plus) consisting of 520 cancer-related genes, spanning 1.26Mb of the human genome is used to simulate the smaller panel, which consists of 56 lung-cancer related genes, spanning 245kb of human genome. TMB prediction derived from OncoScreen Plus has been validated with TMB estimated from whole exon sequencing. Subsequently, using the cutoffs established from OncoScreen Plus, TMB of an additional 21 NSCLC patients was evaluated using the smaller panel and validated with the OncoScreen Plus panel. TMB was calculated as the ratio of mutation count to the size of the coding region of the panel (1.26Mb or 0.245Mb), excluding copy number variations, fusions, large genomic rearrangements and mutations occurring on the kinase domain of EGFR and ALK.

Results: TMB from 406 NSCLC patients, estimated using OncoScreen Plus, was simulated with the prospective TMB status for the small panel to determine the optimal TMB cut-off for the small panel. By comparing sensitivity, specificity and positive predictive value (PPV) for different cutoffs, a cut-off of 20 mutations/Mb was established, yielding 92.8% specificity, 68.7% sensitivity, and 44% PPV. To validate the results from the simulation data, targeted sequencing using both panels was performed for an additional 21 NSCLC patients. Using the cutoff established previously, we achieved a sensitivity of 42%, specificity of 100% and PPV of 100%. Hence, patients with low TMB (< 20) can be reliably stratified from patients with high TMB (≥20). These data suggest that the small panel with a cut-off of 20 will overestimate TMB, but it is highly unlikely to yield false positives. Therefore, the small panel, more cost-effective, can be used as a screening method to screen patients for low TMB. Patients with TMB≥20 are recommended for further validation using a larger panel.

Conclusion: Our study demonstrated the feasibility of using a small gene panel as an initial screening method to predict TMB status. The use of small gene panel is reliable in distinguishing the subset of patients who will not benefit from sequencing with a large panel, thus providing a cost-effective and convenient screening method. Further studies with a larger cohort are needed to validate the predictive value of data from small gene panel.

P065. Tumor Mutation Burden (TMB) Characterization Using a Small Panel

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Introduction: Comprehensive genomic profiling of myeloid neoplasms by targeted next-generation sequencing (NGS) has become a routine clinical test to inform diagnosis, prognosis, treatment and monitoring. Incorporating molecular barcodes to NGS library preparation is a recent technology advancement to push mutation detection sensitivity to below 1% and to enable robust calling of copy number variants (CNVs). During design and validation of a new myeloid gene panel, we aimed to challenge the panel with a comprehensive spectrum of known and other possible mutations for quality assurance. In addition to using available DNA controls (real clinical specimens or synthetic reference materials), we tested whether in silico simulation could exponentially expand the validated mutation spectrum in a cost-effective manner.

Methods: We ordered a QIAseq Targeted DNA Custom Panel (Qiagen) based on a panel of genomic regions (total length 276 kb, 2317 primers). Over 1 million mutation control samples were simulated, including all possible single-nucleotide variants, short insertions and deletions of varying lengths, hotspot mutations curated by COSMIC, and exon-level CNV. High-throughput simulation using open-source MutationEngineer was performed in a Cray XC30 supercomputer and Microsoft Azure Cloud Virtual Machines. Experimental validation preparation is a recent technology advancement to push mutation detection sensitivity to below 1% and to enable robust calling of copy number variants (CNVs). During design and validation of a new myeloid gene panel, we aimed to challenge the panel with a comprehensive spectrum of known and other possible mutations for quality assurance. In addition to using available DNA controls (real clinical specimens or synthetic reference materials), we tested whether in silico simulation could exponentially expand the validated mutation spectrum in a cost-effective manner.

Results: The key prediction result was that the vendor-designed primer panel would lead to false negative detection of CALR type 1 hotspot mutation (NM_004343.3:c.1099_1150del). Although the hotspot mutation was included as covered genomic region, the primer was too close (17bp) to the long deletion (52bp). Variant calling edge effect persisted even after primer clipping (gene-specific primer removal after mapping) was performed instead of primer trimming (primer removal before mapping). The in silico prediction was confirmed by a real NGS run of a bone marrow DNA with the known CALR type 1 mutation. The primer panel was revised according to simulation of alternative primer
candidates with the highest mutation detection sensitivity for the locus.  

**Conclusions:** A robust clinical test by NGS required extensive validation for quality assurance. *In silico* simulation is an effective stress test to bioinformatics pipelines of sequence mutation and CNV detection. It is a comprehensive, realistic and cost-effective approach for NGS gene panel validation, complementing the conventional approach using real DNA controls.

**P067. Genotyping by Machine Learning for Taqman Genotyping Assays**  
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**Introduction:** The OpenArray technology offers a high-throughput platform for single nucleotide polymorphism (SNP) genotype analysis. The technology uses pre-designed two allele-specific TaqMan MGB probes, covalent with distinct fluoroscent dyes and a PCR primer pair to detect specific SNP targets. The final genotype call, either homozygous or heterozygous for the two alleles, is based on the fluorescent intensities of the two dyes.

**Methods:** We propose to predict the genotype using supervised learning algorithms, specifically Support Vector Machines (SVM). Supervised learning aims to learn an optimized model from a training data set, and further to infer the genotype from uncalled data. We used consistent genotyping instance data to evaluate different standardization methods and to train SVM models. The optimized SVM model is then applied to predict the genotype for instances.

**Results:** Considering the genotyping data that were called by existing software as the baseline, our results show that the optimized SVM model outperforms in terms of prediction accuracy. The results also show that our genotyping algorithm is able to rescue more than half of the uncalled instances that were unable to genotype by the existing Genotypes.

**Conclusion:** Finally, it is worth mentioning that a cross-validation result indicates that our genotyping model is also robust.

**P068. CCKB - a High-Performance and Genome-Scale Informatics Portal for Analysis and Multi-Institutional Sharing of Pediatric Cancer Variants**  
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**Introduction:** Unique challenges exist in interpreting genetic variants in pediatric cancers, which are rare, and more often initiated by germline alterations in cancer susceptibility genes, copy number changes, and gene fusions compared to somatic mutations in adult tumors. In contrast, mutations like TP53 and other cancer gene mutations common in adult cancer are infrequent in young cancer patients. To address the unmet need for a pediatric cancer focused knowledgebase, we have implemented an informatics portal, Childhood Cancer KnowledgeBase (CCKB), to share knowledge regarding DNA and RNA variants across the spectrum of benign and malignant tumors in children. CCKB is cloud-based and web-accessible, and is sequencing-platform and test-agnostic. CCKB takes advantage of the latest computational and bioinformatics advancements to enable genome-scale cancer variant analysis and sharing of data from thousands of patients across multiple institutions.

**Methods:** CCKB is implemented with open-source packages, including OpenCGA; a big-data analytic framework supporting the Genomics England Project, and OncoTree disease ontology. Genomic variants are managed using OpenCGA. CCKB is hosted in the AWS cloud using MongoDB, and web-accessible via a customized Interactive Variant Browser (IVA). Generic annotations are provided via OpenCB. RESTful APIs are leveraged to provide cancer-specific annotations using a variety of genomic resources including Ensembl, ICGC, CIVIC, COSMIC, and Oncotator.

**Results:** CCKB currently hosts genetic data for 400 pediatric cancer patients that we have tested at CHLA with our OncoKids panel, which is based on the Oncomine Childhood Cancer Research Assay (OCCRA) that we developed in collaboration with Thermo Fisher Scientific. The CCKB platform will enable data sharing to support the International Childhood Oncology Network (ICON), initiated by Thermo Fisher Scientific, the Sanford Consortium, AACR GENIE, as well as the larger community of pediatric oncology investigators who participate in the project.

**P070. QCcheck: A Web Server for Quality Check of Next Generation Sequencing Data with Standards and Guidelines**  
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**Introduction:** Next-generation sequencing (NGS) has recently emerged as an essential component of personalized medicine. A bioinformatics pipeline can detect genomics alterations that have significant impacts on disease management. However, there is no comprehensive standards and guidelines provided to
biologists and clinicians for checking the quality of NGS data that is extremely important for meaningful bioinformatics analysis.

**Methods and Results:** This work developed a web server called QCHeck to address critical needs. QCHeck provides: (1) graphic user interface for showing the results of quality check on different sequence data format including FASTQ, BAM and VCF files; (2) flexible checking modular for setting the threshold in different analyses; and (3) comprehensive standards and guidelines including FDA, ACMG, NYSDOH, and CAP for validating NGS data. QCHeck is an efficient and user-friendly web server expected to be useful for biologists and clinicians to get a quick overview of the quality of the raw sequence data obtained from different bioinformatics pipelines.

**Conclusion:** The QCHeck server is available at https://developers.dnarails.com/qcheck_free_demo, and will be continuously updated to reflect the latest guideline information.

**Solid Tumors**

**P071. Is Extra Nodal Rosai Dorfman a Malignant Disease? Exploring BRAF, MYD88, MAPK and AKT-mTOR Pathway in Defining Extra Nodal Rosai Dorfman Disease**

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**Introduction:** Rosai-Dorfman disease (RDD) is an uncommon Non-LCH group of histiocytosis that is traditionally an RDD thought to be a benign non-clonal lesion. However our experience with extranodal RDD indicated otherwise. This study sought to identify mutations in the MAP21K pathway in extra nodal SHML to check if it has different profile from nodal RDD.

**Methods:** formalin-fixed, paraffin-embedded (FFPE) tissues of 22 RDD were evaluated for BRAF, MAP2K1 and MYD88 by Sanger sequencing. Clinical and histological details were noted from Electronic Medical Records. For this study, highly specific Rabbit polyclonal Ab for phosphor-p44/42 MAPK(Thr202/Tyr204) p-ERK(dilution, 1:100, cell signalling), for p4EBP1(Thr37/Thr46) (dilution, 1:100), for phosphor-mTOR(ser2448) (49F9) (dilution, 1:100) CST was used to assess the expression of p-ERK, p4EBP1 and m-TOR C1 complex protein.

**Results:** This study group included 22 cases, where 13 had extra nodal, 8 had nodal and 3 patients with both nodal and extranodal RDD. Of a total of 22 cases, 18 had successful molecular analysis. Mutation analysis showed point mutations in 12 (66.66%) cases, including Missense, Synonymous and intronic regions, mutations were all point mutations in all 12 cases. The patients harboring mutations were two women, ten men with a median age of 38.5 years (range, 11-70yrs). 2/5(40%) had nodal RDD and 9/13 (72.7%) had extra nodal RDD showed mutations. Among the tested cases MAP2K1 mutation in Exon2 was predominant. MAP2K1 mutations: Exon2 mutations (n=7), Exon3 mutations (n=3) and both Exon2 and Exon3 mutations (n=2). MYD88 mutation was observed in only one case and was novel (p.F291I) clinical significance of which is not ascertained. None of the 18 tested cases harbored BRAF mutation.

Immunohistochemical analysis using p-ERK1/2 antibody demonstrated high levels of expression in the nucleus and cytoplasm of the histiocytes in 12 cases all harboring MAP2K1 mutations. Conversely, none of the MAP2K1 mutated and p-ERK1/2 express cases didn't show protein expression of m-TOR and p4EBP1.

Of the 13 extra nodal RDD 10 required steroids or chemotherapy. 9 of these harboured MAP2K1 mutations. Intrinsic mutation 15:66435259, I2, which is near a splice site in MAP2K1 gene were most commonly observed in 6 cases.

**Conclusion:** In summary, detection of somatic mutations in 12 (66.66%) cases both nodal and extra nodal RDD suggests that each of these mutations may function as an initiating mutation, driving clonal proliferation in RDD. A preliminary analysis suggested that extra nodal RDD, especially mutation positive, are more aggressive in behavior and importance of comprehensive genomic analysis. Such cases may benefit from targeted therapy.

**P072. Tumor Immune Microenvironment in Lung Adenocarcinoma (LAC): Relation to Mutations and Expression of COX-2 and STAT6**

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**Introduction:** Various tumor-associated factors including mutations and pro-inflammatory cascade activation can affect tumor immune microenvironment (TIM) composition and tumor progression. It is widely accepted that cyclooxygenase-2 through prostaglandin E2 and IL-4/IL-13 through STAT6 promote tumor progression by polarizing immunity towards a type 2 response. However, there is not enough data on the relations of these signal pathways to TIM in lung adenocarcinoma (LAC). In addition, differences in TIM characteristics in terms of mutations and PDL-1 expression have not been fully characterized. The goal of the study was to evaluate the characteristics of TIM in LAC of different stages in regard with EGFR and ALK mutations.

**Methods:** A total of 320 patients with lung adenocarcinoma were included in the study. All patients underwent EGFR testing by Real-time PCR using EGFR Mutation Analysis Kit (Entrogen). Of these, 208 patients were tested for ALK rearrangement by fluorescence in situ hybridization (FISH) using ZytoLight SPEC ALK Dual Color Break Apart Probe (ZytoVision). Sixty surgically resected samples were selected for immunohistochemical evaluation of tumor associated immune cells (CD3, CD8, CD138, CD163) number and compartmentalization with respect to EGFR/ALK status.
and PDL-1 expression. In addition, expression of COX-2 and STAT6 were assessed. Nodal status and stage were considered for investigation of TIM role in LAC progression.

**Results:** EGFR mutations were detected in 59 patients (18.4%). ALK translocation was detected in 20 patients (9.6%). Assessment of LAC of different stages revealed that LAC progression is associated with higher expression of COX-2 and number of CD163 positive cells (P < 0.05), but varying count of T-cells and plasma cells. Number of CD163-positive cells within the tumor niche tightly correlated with COX-2 but not STAT6 expression that could exclude the role of IL-4 mediating mechanisms in M2-macrophages accumulation in LAC. Interestingly that high CD163 number and COX-2 expression were related to node-positive status (P < 0.05). In addition, most of PDL-1 positive cases were associated with metastatic LAC. Assessment of TIM in respect to PDL-1, demonstrated that PDL-1 expression was associated with increased number of CD163 cells P < 0.05) accompanied with more intensive T-cells infiltration (P < 0.05) and high expression of COX-2 in both tumor and immune cells (P < 0.05). Evaluation of TIM in regard with mutations showed more intensive CD163 infiltration in wild-type EGFR tumors comparing with LAC harboring EGFR mutations. In contrast, ALK-mutation positive tumors did not exhibit significant differences in number of tumor infiltrating macrophages and lymphocytes.

**Conclusion:** Wild-type EGFR lung adenocarcinomas demonstrated significantly higher number of CD163 positive cells and COX-2 but not STAT6 expression. CD163 cells count and COX-2 expression were tightly related with positive node status and distant metastasis.

**P073. Medulloblastoma Molecular Subgroups: Clinical, Histological and Further Molecular Characterisation: A Single Institutional Experience**

**Introduction:** Medulloblastomas (MB), currently are classified into histological and molecular subgroups. This is a single institutional study of evaluation of different molecular subgroups for any distinct clinical, histological and biological features.

**Methods:** Formalin-fixed, paraffin-embedded (FFPE) samples of clinicohistologically diagnosed cases of Medulloblastoma were molecular grouped by Taqman based real time quantitative PCR (qRT-PCR) gene expression profiling of customized 12 protein coding genes. Additionally, MYC amplification by Fluorescence in situ hybridisation (FISH) using ZytoLight SPEC MYC/CEN 8 dual color probe (ZytoVision, Bremerhaven, Germany) was done for non-WNT/non-SHH, group 3 cases. Histological typing was done (on Hematoxylin and Eosin stained, reticulin stained and relevant immunohistochemistry [IHC] slides). The clinical data was recorded from the records.

**Results:** 191 of 197 cases were interpretable, of which 153 (80%) were of pediatric age group (< 18 years) and rest of ≥18 years. Males were predominant (n=150; 76.1%). The age distribution, location, histological findings and presence of metastatic disease of different molecular subgroups were as follows:

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| Molecular subgroups (n=191) | Age range (Mean) | Age distribution (n=191) | Location (n=191) |
|---------------------------|------------------|-------------------------|-----------------|
| WNT (n=32;16.8%)          | 5-40 (14.5)      | <18 years (n=153; 80.1%)| Midline location (n=51; 26.7%) |
| SHH (n=49;25.7%)          | 1-59 (16.7)      | ≥18 years (n=38; 19.9%)  | Lateral location (n=140; 73.3%) |
| Non-WNT/ Non SHH, Group 3(n=28;14.7%) | 2-25 (7.3)    | <18 years (n=26; 13.6%)  | Midline location (n=56; 29.3%) |
| Non-WNT/ Non SHH, Group 4(n=61;31.9%)  | 3-31 (7.3)      | ≥18 years (n=56; 29.3%)   | Lateral location (n=24; 39.3%)  |
| Non-WNT/ Non SHH, NOS (n=13;6.8%)  | 4-18 (9.3)      | ≥18 years (n=12; 6.3%)    | Midline location (n=1; 0.5%)   |
| Unclassified (n=8;4.2%)   | 2-58 (20.2)     | ≥18 years (n=5; 2.6%)     | Lateral location (n=7; 3.6%)   |
```

[Age and location of tumors of various molecular subgroups]
Histological subtype is predominantly SHH-activated and WNT-activated has the widest age range. Desmoplastic common molecular subgroup in the pediatric age-group; S49 jmd.amjpathol.org

Conclusion:
The WNT-activated subgroup and the results will follow.

(ZytoVision, Bremerhaven, Germany) is being studied on ZytoLight SPEC MYB/CEN 6 dual color probe

Clinicopathological features of various molecular subgroups

| Molecular subgroups | Histological subtypes (n=191) | Metastasis (n=47) |
|---------------------|-----------------------------|------------------|
| WNT                | Classic (n=102; 53.4%)       |                  |
|                    | Desmoplastic nodular (n=32; 16.8%) |                  |
|                    | Desmoplastic paucinodular (n=15; 7.8%) |                  |
|                    | MB-EN (n=2; 1%)              |                  |
|                    | Large cell/Anaplastic (n=15; 7.8%) |                  |
|                    | NOS (n=25; 13.2%)            |                  |
|                    | At presentation (n=28)       |                  |
|                    | During follow up (n=19)      |                  |
| Non-WNT/Non-SHH, Group 3 | 18 (9.4%) |                  |
| Non-WNT/Non-SHH, Group 4 | 39 (20.4%) |                  |
| Non-WNT/Non-SHH, NOS | 7 (3.7%) |                  |
| Unclassified        | 4 (2.1%)                     |                  |

[Clinicopathological features of various molecular subgroups]

On IHC, 13 showed diffuse p53 positivity (8 [61.5%] SHH-activated); while 48 showed complete negativity (7 [16.6%] SHH-activated, 22 [45.8%] Group 4). 17 non-WNT/non-SHH, group 3 cases were evaluated for MYC gene amplification, of which 4 (23.5%) were amplified.

Frequency of CTNNBI mutation by direct Sanger sequencing and chromosome 6 alterations by FISH using ZytoLight SPEC MYB/CEN 6 dual color probe (ZytoVision, Bremerhaven, Germany) is being studied on the WNT-activated subgroup and the results will follow.

Conclusion: Non-WNT/Non-SHH, group 4 is the most common molecular subgroup in the pediatric age-group; while SHH-activated is the most common in adults. SHH-activated has the widest age range. Desmoplastic histological subtype is predominantly SHH-activated and rarely non-WNT/non-SHH, group 4 but is never WNT activated and group 3. MYC amplification in group 3 is uncommon.

P074. Audit of Genomic Alterations in 525 Non Small Cell Lung Cancer (NSCLC) Formalin-fixed, Paraffin-embedded Samples by Targeted NGS
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Introduction: Lung cancer is one of the most common malignant diseases with high mortality rates in China. Targeted therapies have been improved dramatically by the development of clinical detection technologies and the accumulating knowledge of genomic alterations for lung cancer patients. Because of low-input DNA, high-throughput and less cost in massively parallel sequencing, next-generation sequencing (NGS) has been performed in clinical detection to identify genomic alterations for the treatment. In this study, 395 cases of adenocarcinoma (ADC) and 130 cases of squamous cell carcinoma (SCC) from West China University Hospital were reviewed respectively to find out the differences and coexistence of genomic alterations.

Methods: Targeted NGS panels were performed on the MiseqDx platform. 230 samples and 295 samples were detected by 8-gene panel and 56-gene panel respectively. 8-gene panel includes EGFR, ALK, ROS1, ERBB2, KRAS, BRAF, MET and RET. 56-gene panel includes the 8 genes and other 48 genes.

Results: With respect to the 8 genes, NGS results of 525 samples showed that mutations detected in ADC were at higher frequency than those in SCC, especially EGFR (55.4% versus 6.9%) and KRAS (14.6% versus 5.4%).

ROS1 fusion mutations were only detected in ADC (1.52%). 295 samples were subjected to the 56-gene panel. In ADC samples, the rate of EGFR mutation was highest, followed by TP53, KRAS, ALK, and STK11. In SCC samples, the rate of TP53 mutation was highest, followed by CDKN2A, PIK3CA, ERBB4, and EGFR. In regard to copy number variations (CNVs), CNVs of ERBB2 were only observed in ADC samples (3.04%), however CNVs of FGFR1 (at 8p11.22) and FGFR19, FGF3, FGF4 (at 11q13.3) were only detected in SCC samples (9.2%~12.64%). Moreover, CNVs of PIK3CA, CCND1, MYC, KRAS and AKT1 were at much higher frequency in SCC than ADC. Totally, both mutations and CNVs of EGFR (54.81% and 9.13%) were at higher frequency in comparison to other genes in ADC samples, and both mutations and CNVs of PIK3CA (12.64%and 36.78%) were at high frequency in SCC samples.

Conclusion: Our data from this cohort of unselected patients confirm genomic alterations are preferentially associated with histological subtype of lung cancer. Mutations are identified more frequently in ADC than SCC, however CNVs are seen more frequently in SCC than ADC. The data unravels that alterations in receptor-tyrosine-kinase genes and gene fusions are enriched in ADC, nevertheless enrichment of alterations in PI3K-Akt-pathway genes and cell-cycle-genes are seen in SCC. Their impact on response to treatment will provide the
P075. Analytical and Clinical Validation of a Capture-Based NGS Assay of Non-Small Cell Lung Cancer (NSCLC)  
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Introduction: Genetic profiling of tissue samples is widely used in clinical settings to guide targeted therapies for cancer patients. In this study, we developed a capture-based next generation sequencing (NGS) assay for detection of single-nucleotide variants (SNVs), insertions/deletions (InDels), genomic rearrangements and copy number alterations (CNAs) in highly important clinically actionable genes in NSCLC, including EGFR, BRAF, KRAS, ALK, ROS1, MET, ERBB2 and RET. Rigorous validation and clinical studies were performed to evaluate analytical and clinical performance for the assay, using authentic formalin-fixed, paraffin-embedded (FFPE) samples or commercial cell lines containing a variety of variants.

Methods: For accuracy analysis, genomic alterations detected by our assay were compared to those detected by orthogonal confirmatory assays including ddPCR or fluorescence in situ hybridization (FISH), using 45 NSCLC FFPE specimens. For sensitivity analysis, the limit of detection (LoD) of alterations binned by type were determined using 3 commercial samples that contain 16 representative mutations at serial frequency levels by Probit approach. And the putative LoD were confirmed using tumor samples diluted with a well-characterized genomic DNA (NA12878). The limit of blank (LoB) was calculated using 15 wild-type FFPE samples and the NA12878. For precision analysis, repeatability and reproducibility performance from library construction to sequence analysis were compared across different reagent lots and days by multiple operators, using 3 commercial samples. For specificity analysis, the potential impact of endogenous and exogenous interfering substances and cross-contamination among adjacent wells were evaluated. Clinical studies were conducted on 1,600 clinical samples using FDA/CFDA approved tests as comparator approaches.

Results: The PPA and NPA for all variants were 100% compared to orthogonal reference assays. No false-positive were detected in any normal FFPE samples. The LoD were 1.67% for SNVs/InDels, 2% for genomic rearrangements, and 2.64 copy numbers for copy number variations (CNVs). All variants had 100% positive call rates in the LOD confirmation study. 100% intra-run and inter-run precision were achieved, with the coefficient of variation (CV%) for the detected frequencies across all replicates being ≤35% for SNVs/InDels, ≤25% for rearrangements, ≤5% for CNVs, respectively. No false positives or false negatives were detected in the presence of the tested interferents and no cross-contamination was observed. In addition, high concordance of our assay with orthogonal references was obtained in clinical studies.

Conclusions: Our assay achieved robust analytical and clinical performance, and thus could serve as an effective tool for NGS-based diagnostics for NSCLC patients.

P076. Identification of AKR1C3 as a Novel Molecular Target for Lung Adenocarcinoma  
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Introduction: Lung adenocarcinoma (LUAD), the most common subtype of lung cancer, remains one of the leading causes of cancer deaths worldwide. With improving knowledge in the molecular aberrations underlying lung carcinogenesis, a number of targeted therapies are being developed to better patient’s survival. Patients harbouring EGFR activating mutations confer sensitivity to EGFR tyrosine kinase inhibitor (TKI) treatment. However, the survival benefit of EGFR-TKI treatment remains modest due to acquired resistance. Recent studies revealed that cancer stem cells (CSCs) endowing with stem cell-like properties are the key drivers of tumor relapse and therapeutic resistance. In this study, we aim to identify pathways crucial for maintaining stemness properties, which might potentially provide novel drug targets to overcome treatment resistance.

Methods: We have exposed two EGFR-mutant (Exon19del) LUAD cell lines to an increasing dose of erlotinib for over 6 months to establish erlotinib resistant sublines. To enrich for CSC population, we have cultured serial passages of spheroids derived from the two parental LUAD cell lines by making use of their self-renewal ability. cDNA microarray (Agilent SurePrint G3 Human gene expression v3 array) was performed to compare the gene expressions between erlotinib resistant sublines and spheroids. Data processing and expression comparison was performed by GeneSpring GX and LIMMA package using R programming.

Results: We have identified aldo-keto reductase family 1 member C3 (AKR1C3), a member of the aldo/keto reductase superfamily, to be commonly upregulated in both erlotinib resistant sublines and self-renewal spheroids. From The Cancer Genome Atlas (TCGA) data, mRNA expression of AKR1C3 is observed to be higher in LUAD tumor when compared with paired normal tissues (p=0.019). High mRNA expression of STK31 was found to be associated with poorer overall survival (p=0.01) in LUAD patients. Consistently, we also observed an overexpression of AKR1C3 in the tumor tissues of our in-house lung cancer cohort. Functional studies using shRNA-based knockdown of AKR1C3 in lung cancer cell lines revealed its regulatory role on cancer and stem cell-like properties in vitro and in vivo including tumorigenicity, self-renewal, drug resistance and metastasis. More importantly, genetic suppression and pharmacological inhibition of AKR1C3 in erlotinib resistant cells re-sensitized the cells to drug treatment.

Conclusions: Our data suggests that AKR1C3 might be a novel therapeutic target for lung cancer. Further studies will be done to delineate the downstream molecular basis to sub-group these genetic changes and catalogue their clinical utility.
mechanism by which AKR1C3 regulates cancer stemness and erlotinib resistance.

P077. Simultaneous Detection of Single Nucleotide Variants, Copy Number and Gene Fusions in a Single Next Generation Sequencing (NGS) Solid Tumour Panel
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Introduction: With advances in cancer genomic profiling and the development of new targeted therapies in solid tumors, comprehensive genomic analysis of clinical samples in patients with solid tumors must identify a range of mutational types including single nucleotide variants (SNVs) and small insertion/deletions (indels), copy number variants (CNVs), and gene fusions. Currently, in non small cell lung cancer (NSCLC), clinically relevant mutations include SNVs and small indels in EGFR, BRAF, KRAS, gene amplification in Her2 and MET, and gene rearrangement in ALK, ROS1, RET, NTRK1,2 and 3. These mutations have been traditionally detected by a combination of techniques including PCR genotyping, DNA sequencing, immunohistochemistry and fluorescent in situ hybridization. An assay capable of simultaneous detection of all three mutation types is imperative for clinical implementation as it will provide rapid and cost effective testing. The oncomine focus assay (OFA) panel can interrogate for these three mutation categories in a single run using RNA and DNA extracts from formalin-fixed, paraffin-embedded (FFPE) samples. This study examines the utility of the OFC panel as a means of simultaneously detection of these of mutation types in a clinical setting.

Methods: 83 previously characterized patient samples and seraSeq RNA and DNA controls were analyzed. Disease entities include 43 NSCLC, 6 Breast cancers, 4 Melanoma, 3 GIST, 2 colorectal adenocarcinoma, 2 neuroblastoma, 1 glioblastoma, 1 lymphoproliferative disorder and 3 controls. Mutations ranged from SNVs/small indels (EGFR, RAS, BRAF, KIT, CTNNB1, PTEN, GNAQ, GNA11, ERBB2, MET, IDH1, AKT1, BRCA2, PIK3CA, CIC, FUBP1, MAP2K), CNVs (ERBB2, myc-N) and gene rearrangements (ALK, ROS1, NTRK1-3). Sequencing was performed using the OFA panel on the ion torrent S5 instrument. NGS library prep, templating, sequencing to reporting was 3 days. Conclusion: The oncomine focus assay on the ion torrent S5 is a rapid, accurate and sensitive NGS method for the simultaneous detection of SNVs, CNVs and fusion genes.

P079. Development of Clinical Epigenome Profiling Assay and Computational Pipeline for FFPE Tumor Tissue
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Introduction: Alteration of chromatin remodeling complexes have been shown to play a central role in the progression of several genetically homogenous tumor types including pancreatic and central nervous system cancers. While the detailed mechanism of this process is not known, it is believed that disrupted regulation of chromatin remodeling complexes in cancers ultimately results in altered chromatin accessibility and gene expression regulatory circuits. Thus, assessing chromatin regulation, accessibility and impact on gene expression may elucidate additional disease-associated signatures. However, clinical epigenome profiling of formalin-fixed paraffin-embedded (FFPE) tissue specimens has been largely ignored due to the practical challenges of using this highly degraded and low molecular weight sample type.

Methods: As a proof-of-principle we have developed a next-generation bisulfite sequencing method for FFPE tissue, which enables genome-scale characterization of DNA hyper- and hypo-methylation status. Additionally, we have developed a computational pipeline to detect epigenome alterations and predict disrupted gene expression pathways.

Results: We have applied our method to 50 clinical pancreatic and central nervous system tumors spanning a broad range of tumor purity to profile epigenetic alterations in the form of DNA methylation signatures. Our method enables comprehensive detection of tumor methylation status in a streamlined workflow and achieves high concordance (>85%) with orthogonal methods.

Conclusions: The deregulation of chromatin accessibility, and DNA expression has been shown to impact tumor grade, tumor stage, and overall patient survival. We demonstrate the ability to identify known epigenomic signatures from highly degraded and sub-optimal FFPE specimens. This advance has broad implications for the correlation of higher-order cellular processes with cancer progression and lays the foundation for targeted clinical molecular diagnostic assays.
Introduction: Precision medicine is an emerging approach which addresses the root cause of a disease rather than the symptoms alone. The need and its potential in our healthcare scenario is high, due to the high disease burden. Here, we review the institutional implementation of next generation sequencing (NGS) on accrual of patients undergoing clinical treatment at our hospital in a cohort of 349 patients.

Methods: NGS was performed using the Ion Torrent platform with the cancer hotspot panel v2. 10ng of DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue as well as peripheral blood for the downstream application. The most common primary tumor types were lung (65.8%), colorectal (15.21%), CNS tumors (7.76%), Acute myeloid leukemia (4.34%), melanomas (2.4%), gastrointestinal stromal tumors-GIST (2.1%) and ovarian (2.1%).

Results: The overall success rate of NGS was at 92.3% (n=322) out of 349 patients with a mean depth of 1098X and 272140 reads. The average turnaround time from sample collection to reporting of the results was 15 days (IQR, 8-32 days). The median patient age was 53 yrs (19-92yrs). Out of 322 reported cases, 99 were wild type (30.7%). 374 mutations were observed within the remaining 223 mutated cases (mean mutation per tumor-1.67). Overall, the most frequently altered genes were TP53 (33.22%), EGFR (23.91%), KRAS (18.32%), CTNNB1 (4.65%), PIK3CA (4.34%), APC (4.03%), PTEN (3.72%), BRAF (4.34%), CDKN2A (4.34%), KIT (2.79%), KDR/PTPN11/FLT3/MET/RET (0.3%) respectively.

Conclusion: Previous precision medicine studies in our center have investigated conventional molecular techniques with limited sets of gene alterations. This study proves the clinical utility of the cancer hotspot panel v2 panel in a tertiary care hospital in eastern India. NGS based extensive molecular profiling using parallel sequencing and high-throughput techniques allows the identification of actionable mutations in majority of cases and is associated with significant clinical benefit in up to four in ten patients.

P081. PD-L1 Expression and Molecular Alterations in Non-Small Cell Lung Cancer

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Introduction: PD-L1 inhibitors are currently part of the treatment of advanced stage non-small cell lung cancer (NSCLC). We explored the association between PD-L1 expression in NSCLC and concomitant molecular alterations including EGFR, ALK (rearrangements and copy number gains), ROS1 and KRAS.

Methods: Pathology reports (Jan 2017-June 2018) of NSCLC with available PD-L1 immunohistochemistry results (using 22C3 pharmDx antibody) were retrieved from our institutional database. Patients’ demographics, NSCLC type, PD-L1 levels and results of molecular studies were recorded. PD-L1 expression was categorized based on the tumor proportion score (TPS) as high (HPD-L1 at least 50%), low (LPD-L1: 1-49%) and negative (NPD-L1 < 1%). Molecular alterations were outlined within each PD-L1 category. In addition, comparison between HPD-L1 versus LPD-L1, HPD-L1 versus NPD-L1 and LPD-L1 versus NPD-L1 was computed for ALK copy number gains (CNG). The PROC Glimmix (SAS 9.3) with logit link function and binary distribution was utilized to calculate odds ratios (OR).

Results: A total of 152 cases were identified: 65.8% adenocarcinomas, 25.7% squamous cell carcinomas and 8.5% other NSCLC (poorly differentiated NS). Average age was 69 years (female to male ratio 1:3.1). PD-L1 positivity was seen in 60% of cases, of which 37% HPD-L1 and 23% LPD-L1. A summary of the molecular alterations for different PD-L1 levels is shown in Table 1. All ALK rearranged cases (2/3 adenocarcinomas, 1/3 squamous cell carcinoma) had at least 90% PD-L1 expression. Interestingly, one squamous cell carcinoma was found to simultaneously harbor an EML4-ALK rearrangement, as well as ALK CNG and a KRAS mutation (c.175G>T).

| Molecular Alterations (n) | PD-L1 Category |
|--------------------------|----------------|
|                          | HPD-L1 n (%) | LPD-L1 n (%) | NPD-L1 n (%) |
| EGFR (13)                | 3 (23.1)     | 4 (30.8)     | 6 (46.2)     |
| KRAS (13)                | 7 (53.8)     | 1 (7.7)      | 5 (38.5)     |
| ALK gene rearrangements (3) | 3 (100.0) | 0 | 0 |
| ALK CNG (94)             | 46 (85.2)    | 20 (71.4)    | 28 (66.7)    |

(Table 1)

Cases with HPD-L1 were more likely to have ALK CNG compared to cases with LPD-L1 [OR = 4.10, 95% CI (1.09, 15.47), p-value 0.04]. Similarly, HPD-L1 cases were more likely to have ALK CNG compared to NPD-L1 cases [OR = 4.56, 95% CI (1.42, 14.68), p-value 0.01].
Conclusion: Our findings suggest patients with HPD-L1 expression are more likely to harbor extra copies of ALK. Although it was previously postulated that ALK CNG could potentially reflect genomic instability, it is not currently understood what therapeutic or prognostic implications this might have. Further studies are needed to show how PD-L1 expression and ALK CNG might impact patients’ response rate to immune checkpoint inhibitors.

P082. Identification of Clinically Significant MUTYH Deletion by Next-Generation Sequencing in Non-Small Cell Lung Cancer

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Introduction: Around 80-85% of lung cancers are diagnosed with non-small cell lung cancer (NSCLC). NSCLC patients, especially non-smokers and the young, often harbor driver mutations. Efficacious therapeutic options can be refined based on the distinct oncogenic drivers as well as the different ethnic populations. Hence the need of finding pathogenic mutations is inevitable. Here, we identified a novel mutation in NSCLC patients in Hong Kong by next-generation sequencing.

Methods: A total of six NSCLC cases including two stage I squamous cell carcinoma (LUSC), two stage I and two stage III adenocarcinoma (LUAD) were sequenced in this study. Genomic DNA was extracted from paired normal-tumor samples. The TOMA OS-Seq 130 cancer gene panel was used to prepare libraries which were then sequenced on the Illumina NextSeq 500 system using a paired end read length of 151 bp. Sequence alignment and variant calling were conducted by integrating TOMA Stratus and in-house pipelines. Total RNA was isolated from 77 paired NSCLC tissue samples, and the expression of MUTYH was measured by RT-qPCR.

Results: DNA samples were sequenced with a mean coverage >1000X with mean aligned read >14 million. Four cases harboring MUTYH deletion were detected. Poorer disease-free survival (DFS) (p=0.0063) and overall survival (p=0.022) of LUAD patients with MUTYH deletion were reported in The Cancer Genome Atlas (TCGA) datasets. Analysis of TCGA RNA-seq data also showed significant correlation between MUTYH copy number and gene expression (r=0.473). LUAD patients with MUTYH mRNA overexpression have better DFS (p=0.0205). However, such correlations were not observed in LUSC patients. In our own cohort, lower expression of MUTYH was found in 77.9% of patients (60/77). MUTYH was significantly downregulated in tumor when comparing to normal (mean relative expression 0.35 versus 0.61; p=0.01).

Conclusions: MUTYH deletion appears to be a clinically significant mutation in LUAD. Our findings demonstrate that MUTYH deletion could potentially be utilized as prognostic and predictive biomarker. In order to further characterize the clinical significance, we are validating the primary result with an independent cohort.

P083. Performance Comparison of DNA Extraction Methods from Formalin-Fixed Paraffin-Embedded Tumor Specimens on PCR Amplification and Sequencing-Based Mutation Analysis

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Introduction: Formalin-fixed paraffin-embedded (FFPE) tissue specimens remain the typical source of nucleic acid for molecular testing in routine oncopathology practice. Harsh conditions and treatment during FFPE tissue fixation, embedding and DNA extraction often contribute to nucleic acid degradation. Given these pre-analytical variables, the need for a well validated method for isolation of high quality DNA is critical. This is a study on evaluation of efficacy of an in-house developed FFPE extraction buffer.

Methods: 100 FFPE specimens from different tumors formed the study sample. DNA extraction was performed using two different methods viz; 1. QIAamp DNA FFPE Tissue Kit (Cat. 56404; Qiagen) and 2. In-house developed FFPE DNA extraction buffer (50 mmol/L Tris-HCl [pH 8.5], 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Tween 20, and 0.5% NP40). The DNA yield was quantified by Nanodrop (Thermo Fisher Scientific) and the DNA purity was measured by the 260/280 nm ratio. The Fragment Analyzer Automated CE System (Agilent Technologies) was used for sizing and quantification of
DNA fragments of FFPE-derived DNA. The effects of the two DNA extraction methods was evaluated by PCR and sequencing by capillary electrophoresis. 

**Results:** The in-house developed FFPE DNA extraction method yielded a higher quantity of DNA compared to the column based QiAamp DNA FFPE Tissue Kit (average DNA yield of 830.65 ng/μL and 266.62 ng/μL respectively). Comparison of DNA fragment quality using fragment analyser also revealed significantly higher DNA quality number (DNQ) using FFPE DNA extraction buffer (P< 0.05). Based on PCR amplification of the ACTB gene, the samples isolated by in-house buffer could be amplified up to 208 bp, 323 bp and 432 bp in 88%, 76% and 72% of samples respectively as compared to the column based Qiagen method with 79%, 68% and 65% samples. Sequencing of samples isolated by in-house buffer yielded a clean readable sequence in 70% of samples as against 62% samples extracted with Qiagen method. Analytically, the in-house FFPE DNA extraction method required shorter hands-on time (~55 min) in comparison to the Qiagen method (~4.5 hrs) and also skipped the deparaffinization step using organic solvent (xylene) thus preventing handling of a hazardous substance. 

**Conclusions:** The in-house FFPE DNA extraction method was shown to perform superior to the column based Qiagen extraction method in terms of suitability for downstream applications, time and cost-efficiency, and ease of performance.

**P084. Clinical Study of a Sensitive Real-Time PCR based Qiagen extraction method in terms of suitability for**

**Methods:** The status of EGFR mutation of exon 19 deletions, L858R and T790M in ctDNA was determined using AmoyDx Super-ARMS EGFR Mutation Detection Kit (Super-ARMS EGFR) and droplet digital PCR (ddPCR) in a cohort of NSCLC patients. The degree of concordance between Super-ARMS EGFR and ddPCR was calculated. Further, the concordance between EGFR mutation status in ctDNA detected by AmoyDx Super-ARMS and that in matched tumor tissue DNA was assessed.

**Results:** In the cohort of NSCLC samples, the sensitivity, specificity and concordance rates for Super-ARMS EGFR test compared with ddPCR were 100%, 88% and 91% for exon 19 deletions (N=113), 96%, 92% and 93% for L858R (N=113), 92%, 100% and 97.8% for T790M (N=86). The sensitivity, specificity and concordance of the Super-ARMS EGFR assay for plasma EGFR mutation detection compared to tumor tissue EGFR mutation status were 82.0% (50/61), 100% (48/48), and 89.9% (98/109), respectively.

**Conclusion:** Super-ARMS EGFR assay is a sensitive and reliable method for the detection of EGFR mutation in lung cancer plasma ctDNA samples.

**P085. Application of Oncomine Lung Cell-Free Total Nucleic Acid Research Assay in Pleural Effusions to Detect Fusion and Copy Number Variation**

**Methods:** cfDNA was isolated from plural fluid of Non-Small Cell Lung Cancer (NSCLC) research subjects (MagMAX Cell-Free Total Nucleic Acid Isolation Kit, Thermo Fisher Scientific). Libraries were prepared according to manufacturer user guide using Oncomine Lung Cell-Free Total Nucleic Acid Research Assay (Thermo Fisher Scientific), and quantified with qPCR (Ion Library Quantitation Kit, Thermo Fisher Scientific). 6 plex libraries were templated with Ion Chef automated templating instrument (Thermo Fisher Scientific) and sequenced in Ion 540 Chip with Ion S5 Sequencing System (Thermo Fisher Scientific). Data was analyzed using customized analysis pipelines.

**Results:** We successfully isolated cell free total nucleic acid (cfDNA) from pleural fluid research samples. cfDNA yield range from 10-50ng from 4ml of fluid. Variants such as EGRF T790M (18.09%), EML4-ALK.E20A20 fusion isoform and MET amplification (2.27 fold) were detected in the cfDNA isolated from pleural fluid of these research samples. All detected variants were confirmed by orthogonal assays. The Oncomine Lung cfDNA assay covers multiple variant types implicated in NSCLC in one single assay. This includes hotspots in 12 genes (ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1), de novo variant calling for TP53, MET copy number amplification, plus detection of nearly 1,000 gene fusions isoforms (ALK, RET, ROS1) and MET exon 14 skipping.

**Conclusion:** Oncomine Lung cfDNA research assay can be applied to other body fluid sample types with analytical testing. The application of this assay to pleural effusions provides a quick and reliable solution to
Introduction: Previously, glioma biopsies at the Department of Pathology, Aarhus University Hospital, Aarhus University, Aarhus University School of Engineering, Aarhus University, and Aarhus University Hospital, Dept. of Laboratory Medicine, Aarhus N, Denmark, Aarhus University Hospital, Dept. of Pathology, Aarhus N, Denmark, have been analyzed using fluorescence in situ hybridization (FISH) for 1p/19q co-deletions, quantitative polymerase chain reaction for IDH1 and IDH2 mutations, immunohistochemistry for TP53 and ATRX, and pyrosequencing for MGMT promoter methylations. This set-up is time- and tissue-consuming and the 1p/19q FISH analyses were especially challenging, requiring frequent re-runs. To improve glioma diagnostics, we have implemented a custom next generation sequencing (NGS) glioma panel including single nucleotide polymorphisms (SNPs) for 1p/19q co-deletion analysis. Methods: A retrospective cohort of 25 archival glioma tissue samples and a prospective cohort of 33 glioma tissue samples were included. All samples were sequenced using a custom NGS panel covering both glioma relevant genes (modified from Zacher A et al) and SNPs covering the 1p and 19q chromosomal arms (SNPs from Dubbink HJ et al.). Using either copy number alteration (CNA) alone or in combination with SNP analysis, the sensitivity and specificity of the NGS based 1p/19q co-deletion were compared with FISH results. All discrepancies were solved using multiplex ligation-dependent probe amplification and mutually exclusive mutations.

Results: The sensitivity, specificity, and accuracy of 1p/19q co-deletion detection (i.e. co-deletion or not) were 75%, 97%, and 91% for FISH; 83%, 94%, and 91% for CNA; and 100% for all three statistical measures using combined CNA and SNP analysis.

Conclusions: For 1p/19q co-deletion detection, we found a combination of CNA and SNP analysis superior to FISH or CNA analysis alone. SNPs can be included in any NGS panel, and the combined analysis of SNP and CNA is easily adaptable in a routine setting. Consequently, the CNA and SNP analysis is now our standard approach to 1p/19q co-deletion detection only supplemented with FISH analysis in rare cases with a low tumor cell count.
P088. Implementation and Validation of MSI Analysis in a Community Cancer Center Setting
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Introduction: Microsatellite instability (MSI) status has been performed in patients with colorectal (CRC) and endometrial cancer (EC) to screen for Lynch syndrome (LS)-associated cancer predisposition. It also was approved by FDA for the selection of patients with metastatic tumors for cancer immunotherapy treatment.

Methods: Reference materials and patient samples (N=62) were utilized to establish the assay's performance characteristics. DNA extraction was performed from formalin fixed paraffin embedded (FFPE) samples using FormaPure Total (Beckman) and from peripheral blood using Maxwell Blood DNA kit. The Promega MSI Analysis System V1.2 assay utilizes fluorescent multiplex PCR-based method to detect MSI with five mono-nucleotide loci (NR-21, NR-24, BAT-25, BAT-26, and MONO-27) and two additional penta-nucleotide loci for sample identity. An internal lane standard is also used for reproducible sizing. Amplified DNA products are separated on a SeqStudio (ThermoFisher). The data files from SeqStudio are loaded in GeneMapper software. The MSI status are determined as MSI-H with at least two of five markers showing significant base shift.

Results: The analytical performance metrics were established with reference material (HCT116 cell line) and clinical FFPE samples. The lower limits of detection (LOD) = 5%. Accuracy and reproducibility are 100%. The clinical validation was conducted with 62 FFPE clinical samples with known MSI status. Sensitivity = 96.6%, Specificity = 100%, Accuracy = 98.4%, Positive Predictive Value (PPV) = 100%, and Negative Predictive Value (NPV) = 97.1%.

Conclusion: Microsatellite instability testing has become an increasingly relevant tool in genetic and immuno-oncology. The PCR-based MSI analysis (Promega) is both sensitive and specific. The assay fits very well with clinical laboratory workflow.

P089. HDAC6 Inhibition Exerts Anti-Neoplastic Effects in Combination with Autophagy Suppression in Diffuse Large B-Cell Lymphoma
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Introduction: Diffuse large B-cell lymphoma (DLBCL) is the major common non-Hodgkin's lymphoma in China. HDAC6 plays a major role in the progression of DLBCL and ACY-1215 is a selective HDAC6 inhibitor that could reduce HDAC6 expression.

Methods: In our study, we found ACY-1215 as a novel anti-neoplastic HDAC6 inhibitor which retarded DLBCL survival. We then profiled downstream genes that altered after HDAC6 overexpression/knockdown with NanoString nCounter assay.

Results: Our results showed that autophagy related genes varied significantly in DLBCL cells. After validation in DLBCL tissues, we found that knockdown of BECN1, a key autophagy regulator, suppressed DLBCL cell lines proliferation, and inhibited nude mice tumor growth. In addition, the combination use of HDAC6 inhibitor ACY-1215 and autophagy inhibitor Chloroquine showed synergetic effect both in vitro and in vivo. Mechanistically, Rel A/p65 was found to increase BECN1 expression by promoting its transcription level, and its nuclear translocalization could be activated by HDAC6. Furthermore, the expression of BECN1 was positively correlated with HDAC6 and DLBCL patients with high expression of BECN1 had a worse overall survival.

Conclusion: Our results demonstrated that the combination strategy of ACY-1215 and Chloroquine may be a potent targeting therapy in DLBCL treatment.
P091. Molecular Screening of aggressive characteristics of BCs.

**Introduction:** Carcinoma of the thyroid represents one of the common malignancies of the endocrine system. The T1799A activating point mutation is detected in >98% of the thyroid tumors and acts as a marker of aggressive disease. The objective of the study was to evaluate the frequency and distribution pattern of BRAF mutation in Indian patients.

**Methods:** 95 thyroid tumors were evaluated for BRAF mutation by pyrosequencing.

**Results:** BRAF mutation was detected in 36/95 (38%) cases. BRAF mutation was predominantly noted in female patients in comparison to males (38.4% versus 36.4%; p=.88). Smaller sized tumors showed increased frequency of BRAF mutation when compared to larger sized tumors. Interestingly, a significantly higher frequency of BRAF mutations was observed in conventional PTC tumor type in comparison to non-conventional and other than PTC tumor type (56% versus 35% versus 4%, p=.0007). Furthermore, a significant correlation between presence of BRAF mutation and extra-thyroidal extension was noted indicating its role as a biomarker for aggressive disease. Also, a higher frequency of BRAF mutation was observed in tumors with no capsular invasion, and in tumors wherein vascular invasion was present.

**Conclusions:** Pyrosequencing assay is a simple, reliable and cost effective method for BRAF mutation analysis. The frequency and distribution pattern of BRAF mutations is similar to global reports.

P092. Clinical Performance of QIAGEN Fibroblast Growth Factor Receptor (FGFR) Assay for the Detection of FGFR Alterations in Erdafitinib-Treated Patients with Metastatic or Locally Advanced, Surgically Unresectable, Urothelial Cancer

**Introduction:** Erdafitinib, a potent pan-fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitor, has shown durable responses in patients (pts) with metastatic or locally advanced, surgically unresectable urothelial cancers (mUC) harboring FGFR alterations. The FGFR inhibitor clinical trial assay (CTA) was used to determine enrollment in a phase 2 trial of erdafitinib (BLC2001; NCT02365597). This bridging study evaluates the concordance between the CTA versus the planned companion diagnostic (CDx) assay developed by QIAGEN. The primary objective was to determine the clinical efficacy of erdafitinib in CDx-confirmed FGFR-positive (FGFR+) pts.

**Methods:** The CTA and the CDx are two-step, multiplex, real-time, reverse transcription polymerase chain reaction (RT-PCR) assays for the detection of nine target alterations in the FGFR2 and FGFR3 genes in RNA derived from formalin fixed paraffin embedded samples. Study samples from the BLC2001 trial included 100 CTA-confirmed FGFR+ samples from 100 pts with mUC enrolled and treated with erdafitinib, plus 200 randomly-selected CTA-confirmed FGFR-negative samples obtained during the screening period of the trial. For the evaluation of concordance, the overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) along with two-sided 95% confidence intervals (CI), were calculated. The primary objective was met if the lower bound of the 95% CI of the objective response rate (ORR) in CDx-confirmed FGFR+ pts was >25%.

**Results:** 292/300 samples (97.3%) yielded valid CDx results. Demographics between the bridging study pts and CTA-screened pts were similar. High values for PPA, NPA and OPA (% [95% CI]) between CDx and CTA assays indicated good analytical concordance (n=292 samples [reference assay: CTA]; PPA: 97.2 [97.0; 97.5]; NPA: 97.0 [93.5; 98.6]; OPA: 93.8 [90.5; 96.1]). After investigation of discordant samples through retesting with a highly sensitive digital droplet (dd)-PCR reference test, the corrected OPA (98.3% [96.1; 99.3]), PPA (97.7% [92.0; 99.4]) and NPA (98.5% [95.8; 99.5]) were high, supporting a positive concordance. Investigator-assessed ORR in the 81 erdafitinib-treated pts identified by the CDx and tested positive for both assays was 45.7% (95% CI: 35.3; 56.5) versus 40.4% (95% CI: 30.7; 50.1) for the CTA (BLC2001 trial) and met the criteria for the primary objective.

**Conclusion:** A high level of concordance was observed between the QIAGEN CDx assay and the CTA assay. The primary objective was met suggesting that the QIAGEN CDx assay could reliably select FGFR+ pts with mUC who could benefit from erdafitinib treatment.

P093. MGMT Promoter Methylation in Glioblastoma: Comparison of Methylation Specific Polymerase Chain Reaction (MSP) and Methylation Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)

**Introduction:** Methylation-specific polymerase chain reaction (MSP) and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) are the most preferred techniques to assess the O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation in glioblastoma (GBM). Methylation-specific PCR (MSP) is the most preferred technique. However, it is majorly limited by inconsistencies of bisulfite conversion.

**Conclusion:** Variable aberrations in copy number and expression of DPPA3, EDR1, and NANG genes in the chromosome 12p13 region are associated with aggressive characteristics of BCs.
Methods: 67 histologically confirmed GBM cases were evaluated for MGMT promoter methylation by MSP (using MethylCode Bisulphite conversion kit) and by MS-MLPA (MRC-Holland). ≥20% methylation for MS-MLPA; while band of any intensity on methylation PCR for MSP was considered as methylated. MS-MLPA kit consists of 5 probes (124, 140, 215, 172 and 190), concordance of all of them together (average) and of 124 nucleotide probe separately with MSP was analyzed.

Result: Age-range: 18-78 years with M:F ratio (2.4:1), 15 were IDH-mutant. Location - temporal (n=13), parietal (n=12), temporo-parietal (n=6), frontal (n=19), occipital (n=2) and others (n=16). 32 (47.7%); 8 were IDH-mutant were methylated by MSP. Of the 67, currently 30 has the MS-MLPA results and rest are being evaluated. 22 were methylated taking all the probes (average) into consideration, of which 16 were methylated and 6 unmethylated by MSP. Of the rest 8 unmethylated, 6 were unmethylated and 2 were methylated by MSP. Thus, the concordance between MS-MLPA average of all the probes was 73.3% (22/30) with MSP. While for 124 nucleotide probe only, 10 were methylated, of which 9 were methylated and one was unmethylated by MSP; while of the rest 20, 11 were unmethylated by MSP. Thus, for 124 probe only, 10 cases were discordant and thus the concordance is 66.67% for 124 nucleotide probe of MS-MLPA with MSP. Of the 18 MSP methylated cases, 16 (89%) were methylated using all the probes and only 9 (50%) were 124 nucleotide methylated on MS-MLPA; while of the rest MSP unmethylated cases, 6 were methylated for all probes and one was 124 nucleotide-only methylated on MS-MLPA.

Conclusion: 73.3% and 66.67% showed concordance between MS-MLPA average and MS-MLPA-124 nucleotide with MSP. 89% showed concurrent result of methylation on MSP and MS-MLPA; however 50% (6/12) of MSP-unmethylated cases showed methylation on MS-MLPA. This study highlights the existence of variation in the results of MGMT promoter methylation for different techniques. Evaluation of the additional 37 DNA samples is currently underway and the final results will be compiled and presented.

P095. The Role of Ki67 Immunopexpression in Predicting the Biological Behaviour of Retinoblastoma Tumour in Tanzania

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Introduction: Retinoblastoma tumour is the most common tumour of the retina and the most common primary intraocular tumour in children with an incidence of 1 in 15,000 to 20,000 live births, and represents approximately 4% of childhood cancers. Prognostication of retinoblastoma prior to treatment by means of markers such as Ki67 will be of importance in preserving vision especially for cases without advanced lesions.

Methods: This is a retrospective study involving 163 eyeball specimens from 158 patients with retinoblastoma tumour diagnosed between 2011 and 2015. The 4μm thickness paraffin embedded tissue sections were incubated in the oven at 8°C using TBS at pH of 6.0 and concentration of 0.01M followed by adding MIB-1 antibody involving horseradish peroxidase (HRP) and 3,3-DAB detection system. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Statistical analyses were done to establish the association between variables.

Results: Males and females were 80(50.63%) and 78 (49.37) respectively. Positively of Ki67 immunopexpression ranged from 3.2% to 70.8% with mean PI of 50.4±45.4%, and higher PI was associated with more advanced tumour stage (P<0.003) and optic
nerve invasion (P =0.027). There was no statistical significance association between Ki67 immunoreexpression with choroid invasion (P= 0.7) and sclera invasion (P=0.8).

Conclusions: Intraocular and differentiated retinoblastoma tumours tend to have lower Ki67 proliferation indexes compared to advanced and poorly differentiated tumours which, in this study, showed higher Ki67 proliferation index. (Keywords: MIB-1, proliferation index, immunoreexpression, retinoblastoma)

P096. Consistent Performance of Highly Multiplexed RNA Fusion Reference Materials across Different NGS Assays in a Multi-Lab Study

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Introduction: Gene fusions, usually the result of chromosomal rearrangements, are frequently associated with many cancer types, and hence clinically actionable, making fusion detection an important part of cancer disease management. We developed a new, optimized version of the Seraseq Fusion RNA Reference Material, and demonstrate its consistent performance across different NGS enrichment assays, sequencing coverage depths, bioinformatics pipelines, and RNA mass inputs from a multi-lab investigation (Sites A, B, C, D and E).

Methods: Biosynthetic DNA was used for transcription of 18 RNA fusions, including more common fusions of ALK, RET, and ROS1, as well as rare fusion events such as PAX-PARP and ETV6-NTRK3. The in vitro transcribed RNAs were mixed with total RNA extracted from GM24385 reference cell line (The 1000 Genomes Project, Coriell). Digital PCR with TaqMan chemistry was used to determine the target fusion RNA concentration and serve as the “truth” data set for comparison to NGS, which can be variable depending on input, assay, and bioinformatics. The fusion-total RNA mix was analyzed by five external laboratories; it was tested using the ArcherDx FusionPlex Solid Tumor Panel (Site A, Site B, Site C), the ArcherDx FusionPlex CTL Panel (Site C), the ArcherDx FusionPlex Lung Panel (Site B), a custom ArcherDx FusionPlex Panel (Site D), and the TruSight Tumor 170 Panel (Site E).

Results: All eighteen (18) fusions in the new Seraseq Fusion RNA Mix v4 reference standard were detected as expected on each NGS platform with an average of greater than 85% of on-target reads across all assays. Even at inputs as low as 20 ng, all 18 fusions were typically detected above fusion-calling thresholds. In general, the results for individual fusions among the different NGS panels and among replicates were concordant, with observed variance in reads across some fusion junctions between assays and replicates. Within FusionPlex assay results, the average percent of reads supporting the fusion call across all fusions was about 63%, regardless of input (a range between 20 to 250 ng). Collectively, the multi-lab results confirm that the Seraseq Fusion RNA Mix v4 reference standard is compatible with both amplicon and hybridization-capture based NGS assays.

Conclusions: Seraseq RNA Fusion Mix v4 has broad NGS assay compatibility and allows for reliable and simultaneous detection of 18 clinically relevant RNA fusions even at low input amounts. The data from a multi-lab study support the use of this reference standard for targeted NGS assay development, assay validation, bioinformatics pipeline optimization, and as positive controls in clinical NGS RNA fusion assays. The biosynthetic manufacturing approach produces reference materials that provide consistent results for a wide variety of common and rare gene fusions.

P097. GenesWell ddEGFR Mutation Test Is a Valuable Diagnostonics to Identify NSCLC Patients for EGFR-TKI Treatment

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Introduction: The GenesWellTM ddEGFR Mutation Test (Gencurix, Inc., Korea) is a droplet digital PCR based test for the qualitative detection of defined mutations of EGFR in DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissue specimens or plasma of non small cell lung cancer (NSCLC) patients. Recently, it was approved as companion diagnostic test (CDx) by MFDS and NECA in Korea.

Methods: In this study, we performed comparative evaluation of the GenesWellTM ddEGFR Mutation Test to identify patients with NSCLC for who are effective in treating EGFR-tyrosine kinase inhibitors (TKIs with the MFDS-approved CDx as a reference method. Also, we examined EGFR mutation status in plasma cfDNA of NSCLC patients to identify the effectiveness as monitoring tool for checking the drug-response. In two retrospective single-center comparison studies, we examined EGFR mutation status in FFPF tissue specimens of NSCLC patients between the GenesWellTM ddEGFR Mutation Test and the reference method. Total 151 specimens were examined for EGFR mutations, especially exon 19 deletion and L858R related with erlotinib treatment. In addition, efficacy for detecting T790M mutation was compared using 161 specimens for osimertinib treatment. In addition, we monitored EGFR mutation status with plasma cfDNA of two NSCLC patients by GenesWellTM ddEGFR Mutation Test.

Results: In this study, the GenesWellTM ddEGFR Mutation Test indicated equivalent clinical performance with the reference method for detection of EGFR mutations.

Conclusion: Based on these results, the GenesWellTM ddEGFR Mutation Test is an effective in vitro diagnostics for EGFR-TKIs treatment in NSCLC patients as a follow-on CDx by providing quantitative and accurate information on the mutations with relatively less DNA samples compared to other products. Moreover, we detected acquired EGFR mutations in cfDNA related to EGFR-TKI treatment.
P098. Clinical Significance and Role of TK1, CEA, CA 19.9, CA 72.4 Levels in Diagnosis of Gastric and Colorectal Cancers
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Introduction: Despite extensive progress in treatment for cancer in recent decades, the early diagnosis for gastric cancer (GC) and colorectal cancer (CRC) remains poor. In this study we explored the diagnostic value of joint detection of Thymidine Kinase 1 (TK1), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9) and carbohydrate antigen 72-4 (CA 72-4) in the diagnosis of GC and CRC, and evaluated the relationship between TK1 expression and clinical pathological characteristics in the patients.

Methods: Pre-operative serum TK1, CA 19-9, CA 72-4 and CEA levels were measured in 85 patients with GC, 172 patients with CRC and 75 healthy controls. Tumor staging was classified according to the AJCC (American Joint Committee on Cancer Staging), Tumor, Lymph nodes, Metastasis (TNM) staging classification. Sandwich Elisa, biotin-labeled antibody kit was used for TK1, and other tumor markers were measured using electro-chemiluminescence. Normal reference values for TK1, CA 19-9, CA 72-4 and CEA were assumed to be 0-2.0 pmol/L, 0-37 U/mL, 0-5.7 U/mL and 0-6.5ng/mL respectively.

Results: The TK1 concentration was significantly higher in patients with cancer than in healthy controls and patients with clinical stage I + II had higher TK1 levels than clinical stage I + I (P < 0.05). The levels of TK1 was significantly associated with tumor stage, lymph node metastasis, distant metastasis, tumor differentiation and age (P < 0.01). When the tumor markers (TK1, CA 19-9 and CA 72-4) were detected respectively, the area under receiver operating characteristics curve (AUC) of TK1 for the cancers was the highest (0.823-0.895). However, the combination of AUC was higher than that for each tumor marker detected respectively (0.935-0.954), and the receiver operating characteristics curve (AUC) of TK1 for the cancers was the highest (0.823-0.895). However, the combination of AUC was higher than that for each tumor marker detected respectively (0.935-0.954), and the Hosmer-Lemeshow test showed an adequate model of calibration. Moreover, the AUCs varied significantly between the combination tests and single biomarker tests (Z test, P < 0.01).

Conclusion: Serum TK1 may be an independent tumor marker for GC and CRC patients, and the combination of TK1, CA 19-9 and CA 72-4 and CEA performed even better. This study suggests that combination detection of four tumor markers may prove to be useful for early diagnosis of GC and CRC. (Keywords: Gastric cancer; Colorectal cancer; TK1; CA 19-9; CA 72-4; CEA)

P099. A Comparative Study of Immunohistochemical Expression of Engrailed 1 (EN1) Protein on Normal and Cancer Cells in Primary Invasive Breast Carcinoma Tissue
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Introduction: The Engrailed 1 (EN1) gene has been reported to show gain in a human basal-like breast carcinoma cell line. The objective of this study was to first evaluate an immunohistochemical expression of EN1 protein on normal mammary epithelial cells (NMECs) and malignant cells (MCs) of primary invasive (infiltrating) breast carcinoma (PIBC) tissue.

Methods: The deparaffinized and rehydrated formalin-fixed, paraffin-embedded tissue sections of 40 PIBC cases were optimally treated in 1x 10 mM sodium citrate buffer solution of pH 6.0 with a microwave antigen retrieval at 700 watts for 10 minutes. Mouse and Rabbit Specific Horseradish Peroxidase / 3,3’-Diaminobenzidine (Avidin-Biotin Complex) Detection Immunohistochemistry Kit with the anti-human EN1 mouse monoclonal primary antibody (clone number 1F5) (ab119111) concentrations of 10.0 µg/mL were precisely used for determining EN1 protein expression. The NMECs and MCs were independently evaluated the nuclear immunostaining intensity (i) [0 (none), 1 (weak), 2 (moderate), and 3 (strong)] and a percentage of immunostained nuclei at each intensity level (Pi). The immunohistochemical scoring (H-score) was calculated as (0 x P0) + (1 x P1) + (2 x P2) + (3 x P3).

Results: At the 95% confidence interval, the H-score of NMECs [mean = 219.3, standard deviation (SD) = 96.16] was significantly higher than MCs (mean = 108.9, SD = 90.25).

Conclusions: The PIBCs show higher expression of EN1 protein in the NMECs compared to their corresponding MCs. Therefore, altered expression of EN1 gene at the protein level in normal breast tissue may correlated with breast carcinogenesis.

P100. Clinicopathological Findings of Retinoblastoma Tumour: a 10-Year Experience from a Tertiary Hospital in Kampala, Uganda
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Background: Retinoblastoma tumour (RBT) is one of the most common cancers occurring in young children in Sub-Saharan Africa. The incidence rate reported in the literature is 9,000 new cases per year, which corresponds to 1 in 15,000 births. This study aimed at
analyzing the clinicopathological findings in children with RBT in Uganda. The purpose of this study was to describe and analyze the clinicopathological findings of the patients with RBT patients.

Methods: This was a cross-sectional analytical study involving 234 eyeball surgical specimens from 214 patients with RBT diagnosed between January 2010 and December 2015.

Results: Invasion of choroid, sclera, anterior chamber and optic nerve was found in 26.5% (n = 58), 51.2% (n = 88), 26.2% (n = 45) and 29.2% (n = 49) respectively. Twenty-six per cent (n = 56) of the cases with intraocular tumour had stage IA and all patients with metastasis 5.1% (n = 11) had stage V. Metastasis was found in 5.1% (n = 11) of the cases. The correlation between optic nerve invasion and metastasis was not statistically significant (P = 0.39) whereas the correlation between poorly differentiated tumour and metastasis was statistically significant (P = 0.001).

Conclusion: The majority of children with RBT in Uganda present clinically with leukokoria and their parents or guardians seek medical intervention at a late stage. Moreover, there is a noticeable significant lag period for the patients to begin treatment even if diagnosis has been done. (Keywords: Retinoblastoma tumour, clinicopathological, Flexener-Wintersteiner, Azzopardi phenomenon)

P101. A Non-Small Cell Lung Cancer Reference Material
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Introduction: Lung cancer is the leading cause of cancer death in the Asia as well as globally. The major diagnosis (~85%) of lung cancer patients is non-small cell lung carcinoma (NSCLC). About 10-50% of NSCLCs have several activating EGFR driver mutations which have been clinically validated as therapeutic biomarkers for anti-EGFR drugs. Identifying these driver mutations through liquid biopsies is a promising alternative to traditional biopsies; however, the precision of mutation analysis for some of these targets, e.g., T790M, especially in advanced-stage metastatic NSCLC patients remains a challenge. To address this, we developed a platform of single and low complexity, highly patient-like circulating tumor DNA (ctDNA) EGFR reference standards designed for PCR assays that are used for EGFR-based patient testing.

Methods: We mixed genomic DNA from the highly characterized human cell line (GM24385) with four synthetic EGFR variant-containing DNA sequences: T790M, L858R, G719S, and an ex19 deletion, in both individual and combined format. Using digital PCR we were able to precisely blend the material at the defined variant allele frequencies of 1% and 0.1%. In addition, wild-type reference material without any synthetic EGFR variants was also created as a comparable negative control. The materials were size-selected (160-170bp) after fragmentation for more patient-like ctDNA size characteristics. After size-selection the material is further refined to improve conversion efficiency. In order to make the material comparable to native ctDNA, such as that isolated from cancer patients, we encapsulated the DNA fragments into a synthetic plasma matrix. The process of DNA size selection followed by encapsulation and formulation into a synthetic plasma matrix enhances the commutability of these reference standards and improves the long-term stability of the samples to at least two years.

Results: The allele frequencies of the reference materials were confirmed by both dPCR and NGS. The Archer Reveal ctDNA 28 Kit was used for next generation sequencing (NGS) library preparation while an illumina MiSeq, using v2 (2x150 bp) PE chemistry reagents, was used for sequencing. Additional evaluation of these materials was conducted by laboratories that provide EGFR RT-PCR tests for patient anti-EGFR therapy stratification.

Conclusions: The data from the dPCR, NGS and RT-PCR assays were comparable and highlight the broad compatibility of these EGFR ctDNA reference standards on a variety of assay testing platforms for NSCLC.

P102. Considering Prevalence of B-RAFV600E and PI3KCA Gene Mutations in Iranian Colorectal Cancer Patients: Full COLD PCR and HRMA
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Introduction: Colorectal cancer is the third most common cancer in the world. Mutations detection of B-RAF and PI3KCA genes is resolvable for choosing a target Anti-EGFR therapy. The aim of study is considering the mutation prevalence in B-RAF gene of by using Full-COLD PCR method and HRMA and in the PI3KCA gene by using HRMA.

Methods: DNA of 27 formalin-fixed, paraffin-embedded (FFPE) samples of Colorectal Cancer was extracted. Therefore, for prevalence in exon 15 of B-RAF gene with using 2 methods combining of Full-COLD and HRMA was determined and for mutations in 2 exons 9 and 20 of PI3KCA gene by HRMA was used. Sensitivity of Full-COLD and HRMA by serial dilution of A-375 cell-line was determined. Sensitivity of this cell-line by serial dilution in Genomic DNA background with ARMS PCR method was considered. HCT-116 cell-line was chose as positive control for PI3KCA gene mutations.

Results: Sensitivity of A-375 and HCT-116 cell-lines was 270 cell/µM. Sensitivity of combining 2 methods of Full-COLD and HRMA was %1. Sensitivity range of A-375 in Genomic DNA background by ARMS PCR was also %1. Although, HCT-116 cell-line was confirmed as positive control for mutations of 2 exons 9 and 20 of PI3KCA gene by using HRMA.

Conclusions: No detectable mutation in B-RAF and exon 20 of PI3KCA in extracted DNA from FFPE was found, but in exon 9 of this gene %3.7 was mutated. It seems
P103. Tumor Mutational Burden (TMB) Analysis Using a Fast, Ultra-High Multiplexed 20,000-Amplicon NGS Panel
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Introduction: Tumor mutational burden (TMB) is currently of high interest in the field of immuno-oncology due to its correlation with patient response to checkpoint inhibitor chemotherapy. Traditionally, TMB is calculated using whole exome sequencing via lengthy hybrid-capture based methods; however, more targeted sequencing approaches provide better coverage of the genetic regions of interest at lower costs. Here we present CleanPlex technology - a fast, 4-hour method for preparing target enriched NGS libraries for assessing TMB for clinical research to streamline and lower the cost of immuno-oncology studies. This multiplex PCR-based technology provides a highly efficient, accurate and robust method for unbiased enrichment of tens of thousands of target regions while minimizing non-specific primer interactions and GC bias and maximizing coverage uniformity. We demonstrate this using a highly-multiplexed prototype NGS panel that contains ~20,000 amplicons and covers 355 genes for assessment of TMB.

Methods: Sequencing-ready NGS libraries were prepared using a 3-step workflow that combines target enrichment and next generation sequencing (NGS) library preparation. The protocol includes an ultra-high multiplex PCR step to amplify ~20,000 regions of interest with target-specific primers, a background cleaning step to remove non-specific PCR products, and a final PCR to add illumina sequencing adapter and sample indexes. Libraries were made using 20ng of genomic DNA and sequenced on an illumina NextSeq platform. Sequencing metrics such as on-target rates were calculated, and variants were identified using Paragon Genomics’ variant calling algorithm.

Results: Using the CleanPlex technology, this prototype TMB panel exhibits >95% uniformity at 0.2Xmean, limited GC bias, and >94% detection rate for mutants with 5% allele frequencies. The CleanPlex background cleaning step was essential for removing the undesirable byproducts of this highly multiplexed reaction step.

Conclusion: We demonstrate that CleanPlex technology is capable of creating high quality amplicon libraries with high uniformity, low GC bias, and sensitive variant calling even when using a ultra-high multiplexed panel with ~20,000 amplicons.

Full-COLD plus HRMA method is more robust and sensitive method for detection or Screening of PI3KCA and B-RAF mutations. (Keywords: Colorectal cancer, B-RAF, PI3KCA, Full-COLD PCR, HRMA)
are eluted off the beads following wash steps and the PCR is done using the bead-free eluate. This study was designed to determine whether performing the PCR directly on the bead/eluate mix resulted in improved pre-capture library yields when using formalin-fixed, paraffin-embedded (FFPE) derived tumour samples.

**Methods:** KAPA Hyper DNA libraries were prepared as per the manufacturer’s instructions apart from the pre-capture PCR step. Following fragmentation, end repair/A tailing and adapter ligation steps, SPRI (Ampure) purification was performed without the final separation of eluate from beads. PCR enriched libraries were purified by adding 90µl of Ampure bead buffer (no beads) in order to “reactivate” the beads already present. Libraries were then assessed using Agilent TapeStation. SureSelectXT hybridization was used for target enrichment. Pooled libraries were sequenced on an Illumina NextSeq500 using paired 75bp reads. Three tests were undertaken in order to determine yield change, minimum DNA input, effect on variant detection and effect on sequencing QCs.

**Results:** Results for Test 1 revealed that the use of an on-bead PCR increased the pre-capture library yield by an average of 4.15 × (range: 2.7 - 5.4). Two samples using low input gDNA (50ng and 18ng) both produced enough yield for the hybridisation step when using the on-bead PCR but not when using an off-bead PCR run in parallel. Test 2 results showed that for 4 FFPE tumour samples use of an on-bead PCR increased the pre-capture yield by 1.8 × (range: 0.88 - 2.39) even when 1 less PCR cycle was used. Following sequencing, 66 previously detected variants from these samples, including SNVs, indels and fusions, were detected at the same allele frequency as previously found using on-bead PCR. Sequencing sample QCs were concordant except that the percentage mapped read duplicates value was reduced by an average of 0.54 × (range: 0.34 - 0.65). Test 3 results showed that at 100ng input gDNA 4 FFPE tumour DNAs produced enough pre-capture library for hybridisation. At 60ng input, 3 samples passed and 1 sample was borderline. At 30ng input 2 samples passed, 1 sample was borderline and 1 sample failed.

**Conclusions:** The use of on-bead pre-capture library enrichment dramatically increased the library yields. In our laboratory this has resulted in more FFPE tumour samples being able to undergo hybridisation and less sample failures. In addition, the reduction in PCR cycles has resulted in improved sequencing quality with a significant reduction of mapped read duplicates. This method should be applicable to many NGS protocols including those that are amplicon based.

P106. A One-Step, Highly Efficient Sample Prep Method from FFPE and Rare Precious Specimens for NGS and Real-Time PCR

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**Introduction:** Formalin-Fixed, Paraffin-Embedded (FFPE) is one of the most important DNA and RNA sources for cancer clinical diagnosis by next-generation sequencing (NGS) and real-time PCR. Typically, a slide of FFPE sample has only about a 4µm x 0.2-0.5cm² volume, which is very small and delicate to handle. In tumor diagnosis, low frequency somatic mutations are key to cancer progression in order to offer desirable biomarkers for ultimately improving clinical outcomes for a wide spectrum of cancers. However, the limited quantity and poor quality of DNA obtained from FFPE samples are major hurdles to the discovery of somatic mutations using NGS. Here we report a one-step, highly efficient FFPE sample preparation method to solve these problems.

**Methods:** Currently, FFPE DNA library preparation is a tedious and time-consuming process. It involves following steps: 1. Deparaffinization with xylene; 2. Wash in ethanol; 3. Protease K digestion; 4. Lysis; 5. DNA extraction and purification by columns or beads; and 6. PCR amplification of the purified DNA to yield DNA libraries for sequencing. We have developed a new method to amplify DNA from FFPE tissue directly without the pre-treatment steps as described above. The amplified DNA was successfully used for targeted DNA sequencing in NGS.

**Comparison of two methods:**

**Results:** DNA was amplified by PCR from FFPE tissues directly without any pre-treatment, resulting in high quality DNA libraries for next generation sequencing. The DNA libraries were successfully sequenced either by Illumina sequencing platforms or Ion Torrent sequencers. Advantages of the new method include:

- No deparaffinization or any pre-treatment needed
- Require only a sample of 1/8 to 1/4 of a FFPE slide
- One tube reagent does it all, and directly generate high quality DNA library from FFPE tissue
- Greatly simplify the FFPE NGS work flow with low cost and fast turn-around time

**Conclusions:** NGS has become an very important tool for clinical diagnosis, especially for cancers. However, sample preparation in some aspects is still challenging, such as FFPE. FFPE is one of the most important DNA and RNA sources for NGS cancer diagnosis. Current FFPE DNA library workflow is tedious, time-consuming and labor intensive. It significantly contributes to the total
cost of doing NGS (1/3 to 1/2). We have developed a new method to make FFPE DNA library preparation much easier, simpler and low cost. This new method will make NGS more straightforward and cost effective. In cancer clinical diagnosis and treatment. It should become a very useful tool for molecular biology research and clinical diagnostics.

P108. Development of a Highly Customizable Platform for Variant Detection in Circulating Tumor DNA
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Introduction: Variant detection in circulating tumor DNA (ctDNA) provides an unprecedented opportunity to study the mutational landscape of cancers with minimal patient invasiveness. Meanwhile, new variants providing information about drug resistance and disease prognosis are being rapidly discovered. Therefore, to fully harness the power of ctDNA variant detection it is critical to be able to rapidly modify the set of variants targeted in an assay. Here we present a platform for next generation sequencing-based ctDNA assays that allows researchers to readily customize their panel targets.

Methods: Anchored Multiplex PCR (AMP) is a molecular biology technique that allows for near infinite modularity of assay targets while maintaining variant detection sensitivity. In order to ensure assay specificity, AMP utilizes molecular barcoded adapters (MBC) to uniquely label input molecules prior to amplification, enabling post-sequencing error correction. This increases analytical sensitivity by reducing background noise. We also developed bioinformatics methods, which allow the characterization of position-specific error levels. By combining molecular barcode-based error correction and error profile characterization, we can reliably detect low frequency variants with high specificity.

Results: We tested the modularity of the AMP ctDNA platform by creating ctDNA panels targeting either 6 kb of cancer variant hotspots or 34 kb of tumor suppressors plus the same 6 kb of cancer hotspots (40 kb total target sequence). We then prepared and sequenced libraries using ctDNA-like inputs containing important cancer variants at low allele fractions (AF). We used these data to characterize the noise inherent in AMP library preparation and Illumina sequencing. We then performed a power analysis to measure the lowest AF at which we could detect variants at each target position given the noise level and sequencing coverage. Our results show that we can confidently detect variants with an AF of less than 0.18% in 50% of bases and 0.47% in 95% of bases covered in the 6kb panel. Variant detection sensitivity remained consistent over the 6kb of hotspot targets in the context of the 40kb panel (50% of bases powered to detect 0.17% AF variants and 95% powered to detect 0.45% variants). Across the full 40kb of the larger panel we had statistical power to call 0.23% AF variants at 50% of target bases and to call 0.56% variants at 95% of target bases. These data demonstrate that ctDNA panels can maintain assay performance upon panel modifications.

Conclusions: The modular nature of AMP ctDNA panels allows for endless combinations of targeted genes to maximize detection sensitivity in relevant regions while also maintaining assay performance. Our experience with AMP assays indicates that the range in the number of targets that can be included in a panel is quite large, allowing broad implementation of the assays developed on the AMP platform.

P110. TCR Sequencing from Tissue Micro-Regions and Single Cells Utilizing RareCyte CytePicker and Archer Immunoverse Technologies
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Introduction: T-cell receptor (TCR) based immunotherapies are becoming an important cornerstone of immuno-oncology. Complete TCR sequencing requires single-cell resolution to capture both α and β chains. There is great interest in obtaining single-cell TCR sequences from archived tumor tissue. This task requires technology that can not only retrieve single cells but also sequence degraded RNA from archived tissues samples at the single cell level. The RareCyte CytePicker platform provides integrated multi-parameter imaging and retrieval capabilities for identification and isolation of rare cells and microscopic regions of interest (ROI) for molecular analyses. Archer has developed the Immunoverse platform of targeted Next-Generation Sequencing (NGS) assays to characterize the human immune repertoire from partially degraded RNA inputs. Combining these two technologies affords the unique potential to accelerate engineered cell-based therapeutic by sequencing TCR from individual cells.

Methods: 40 μm ROI containing 1 to 10 T-cells were isolated from fresh/frozen (OCT) melanoma or OCT and formalin-fixed, paraffin-embedded (FFPE) tonsil using the CyteFinder system. In addition, single flu antigen-specific T-cells were retrieved from live cell preparations. OCT tonsil sections were stained with a 6-color panel to discriminate T-cell and B-cell zones, and OCT tumor sections were stained with a 6-color panel to identify immune infiltrate, while FFPE sections were stained with a nuclear marker. The Archer Immunoverse TCR All Chains library preparation kit was used to generate libraries which were then sequenced on the Illumina NextSeq platform. The resultant library sequences were then analyzed to reference V, D, J and C regions of TCRs, and assembled to identify clonotypes from α and β chains using the Archer Analysis tool.

Results: TCR transcripts were observed from RNA isolated from as few as one cell. CDR3 sequences with V and J segments from single flu-targeting T-cells were obtained and found to match previously published sequences. TCR transcripts were observed in all ROI retrieved from OCT and FFPE tonsil when processed with the Immunoverse TCR assay. The numbers of transcripts observed correlated with the number of...
isolated cells analyzed. The sample source also affected the number of observed clones with more clones observed from cells isolated from fresh/frozen tissue.

**Conclusions:** These results support the utility of the RareCyte platform and Archer Immunoverse TCR assay for profiling RNA derived from low numbers of cells in either fresh/frozen tissue, FFPE tissue, or live cells. A workflow that combines RareCyte and Archer technologies shows promise as a method for pairing α and β chain TCR sequences from RNA isolated from a single cell.

**P111. A New Highly Efficient, Sensitive and Specific NGS Library Preparation Technology Allows for Error Correct Detection of Ultra-Low Frequency Mutations in both Liquid Biopsies and FFPE DNA Samples**

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**Introduction:** We have developed an innovative and novel method for preparing targeted next generation sequencing (NGS) libraries that is highly resilient to inefficiencies associated with low integrity DNA, such as from liquid biopsies or formalin-fixed, paraffin-embedded (FFPE) DNA. This technology, called ATOM-Seq (Adaptor Template Oligo Mediated Sequencing), fundamentally differs from the most frequently used library preparation techniques which use ligation, PCR and/or capture. ATOM-Seq is based on a novel method for generating unique molecular identifiers (UMIs) directly to the 3’ end of starting material with very high efficiency which allows mutation hotspots to be interrogated with ultra-high sensitivity. ATOM-Seq’s advantages over exiting methods make it uniquely suited for clinical mutation testing. Here we present technical data showing the efficacy of the ATOM-Seq approach on a range of control samples, reference standards and clinical samples showing that this technology is perfectly suited for clinical sample testing.

**Methods:** The ATOM-Seq technology forms the bases for generating targeted amplicon NGS libraries which initially adds both a UMI and a universal priming site to the 3’ ends of single stranded DNA. This material is then used as a template for two rounds of target specific PCR. Two strands of each target region are specifically amplified and sequenced separately. We set out to determine the following; sensitivity limits testing down to 0.1% allele frequency using normal and FFPE reference material; the ability to use cfDNA as a starting material; the ability to use different quantities of starting material; the ability to use cfDNA as a starting material; the ability to use different quantities of starting material; the ability to use different quantities of starting material; the ability to use different quantities of starting material; finally, we assessed performance with a range of clinical samples.

**Results:** Sequenced libraries have very high genome mapping rates and on target rates of up to 96%. We detected reference material mutations at both a 1% and 0.1% allele frequency. Our protocol works with gDNA between 1-50 ng, with peak conversion efficiency above 50% when only counting ‘biologically relevant’ UMI families. When using clinical material we found that the ATOM-Seq technology is highly sensitive and robust when used for low frequency mutation detection.

**Conclusions:** These data we show that ATOM-Seq libraries are both specific and sensitive. High accuracy is achieved because of sequencing two original strands of each target region. Libraries also show primer uniformity, >90%. The whole procedure is simple and takes less than 6.5 hours. This technology is perfectly suited for processing fragmented gDNA, FFPE material, as well as DNA extracted from liquid biopsies. Using reference and clinical samples we show sensitivity of down to 0.1% allele frequency using relatively small quantities of starting material. This data shows ATOM-Seq is uniquely suited for processing clinical material such as from liquid biopsies due to its high conversion rate and sensitivity.

**P112. Validation of Antibody Panels for High-Plex Immunohistochemistry Applications**

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**Introduction:** Characterization of the spatial distribution and abundance of key proteins within tissues enables a deep understanding of biological systems. However, it has proven difficult to perform such studies in a highly-multiplexed manner on formalin-fixed, paraffin-embedded (FFPE) tissue sections. There has been significant progress in developing technologies with expanded capabilities to analyze higher numbers of proteins, however, the validation of these technologies and their associated affinity reagents remains a significant barrier to adoption. We have developed a validation pipeline that ensures optimal sensitivity and specificity for high-plex antibody panels for the analysis of FFPE sections using the NanoString Digital Spatial Profiling (DSP) platform. The DSP is designed to simultaneously analyze up to 96 proteins by detecting oligos conjugated to antibodies that can be released via a UV-cleaveable linker.

**Methods:** Antibodies targeting immuno-oncology proteins were tested for specificity and sensitivity by immunohistochemistry on FFPE human tissues, as well as human cell line pellets to evaluate binding specificity of both unconjugated and oligo-conjugated antibodies. The sensitivity and dynamic range were tested using FFPE cell pellets with target-specific positive and negative cells at different ratios. An interaction screen was performed to evaluate potential deleterious effects of multiplexing antibodies, and a human tissue microarray (TMA) containing normal and cancer tissues was employed to assess assay robustness. The reproducibility of the panel on DSP was tested on serial FFPE tumor specimens by correlating the expression of all markers across 24 spatially-registered regions of interest (ROI) as well as the ability to reveal biological heterogeneity within lymphoid tissue by characterizing the expression of 40+ proteins in a spatial grid of 100um x 100um ROIs.

**Results:** Immunohistochemical analysis of unconjugated and oligo-conjugated antibodies displayed indistinguishable staining patterns on control tissues and cell lines. Mixed cell pellet assays revealed strong
correlations between observed counts and positive cell numbers. Antibody interaction studies showed similar count values for antibodies alone or in combination, and TMA hierarchical clustering analysis demonstrated expected patterns of expression across tissue types. Analysis of all markers across 24 registered regions of interest across serial FFPE sections were highly correlated. Spatial analysis of lymphoid tissue revealed high levels of biological heterogeneity across multiple germinal centers.

**Conclusion:** These results demonstrate the validation and application of high-plex protein panels to accurately interrogate the immune biology within FFPE tissue using the NanoString DSP platform.

**P113. Business Case Validation of the High Throughput Promega Viral Total Nucleic Acid Automation**

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**Introduction:** Justification of capital expenses for new equipment is an ongoing challenge for molecular diagnostic laboratories necessitating the use of business models. The following presentation will provide validation data for the model and identify gaps or errors for replacing the manual Maxwell 16 nucleic acid extraction instruments with a high throughput extraction system.

**Methods:** Workflow analysis was performed to identify operational bottlenecks in the workflow for a molecular laboratory. Testing is performed 20 hours per day, seven days a week. Workflow analysis included the schedule for sample receipt from healthcare providers via local couriers and FedEx shipments, delivery of samples for extraction, performance of PCR and result reporting. The business justification included cost per extraction (consumables and reagents), impact on patient testing, evaluation of quality and service, labor impact, and other efficiencies created with automation for a presumed instrument life span of 5 years. Some of these factors were used to calculate a theoretical return on investment (ROI) along with the capital expense payback period. The theoretical ROI was compared with a real time cost analysis for validating the financial model.

**Results:** Analysis of workflow determined that the largest bottle neck for performing laboratory developed tests (LDTs) was the DNA extractions processing step that necessitated using multiple Maxwell 16 instruments. The high throughput extraction includes liquid handling instrumentation, Fluent 780 from Tecan (Mannedorf, Switzerland), and the automation of the Promega Corp (Madison, WI, USA) Total Viral Nucleic Acid Kit (AS1150) with additional proprietary steps. Some costs such as training and validation of the high throughput process were not included in the model and represent additional fixed cost in the initial ROI model. Equipment redundancy required maintaining the two smaller capacity whose operational expense was not captured in the original ROI analysis. Maxwell16. Some cost savings, such as decreased plastic waste with the high throughput system, were difficult to assign a value. The Helix on-demand inventory system continued to yield savings by eliminating the need for inventory counts.

**Conclusions:** In this study, we developed and validated an ROI model for the justification of a large capital expense. Gaps in the model were identified resulting in improvements. Validation of the ROI facilitates improved budget management, optimize labor planning and scheduling in the clinical laboratory.

**P114. Reference Materials for Development, Validation, and Quality Control of Circulating Tumor DNA (ctDNA) Assays**

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**Introduction:** Liquid biopsy testing has emerged as an important tool for clinical management of patients with cancer. However, lack of proper reference materials and the limitations of existing solutions hinder the clinical implementation. Here we developed a promising method to produce ctDNA-like reference materials that can be used in assay validation and routine performance monitoring from sample extraction to result reporting.

**Methods:** To closely resemble ctDNA extracted from human plasma, wild-type background cell lines with diploid status of all targeted genes were cultured and digested with endonuclease in cellular state. The digested nucleosome-bound DNA fragments then are purified and size-selected. For mutational allele, similar approach can be done with the mutation harboring cell line, preferably derived from the wild type line aforementioned. Alternatively, synthetic harboring desired mutation with proper enzymatic fragmentation and size-selection can be used. Both wild-type and mutational fragment DNA were quantified carefully before mixing to create artificial reference ctDNA material ranged from 0.1% to 2%. The reference materials can be delivered either in form of purified DNA mixture or by spiking DNA in synthetic plasma.

**Results:** The reference materials exhibit similar fragment length distribution patterns, sequence complexity and NGS library construction efficiency, compared to authentic ctDNA. The reference materials are easy to source, produce and cost-effective because of the high recovery rate. The AF of each mutation was determined by both ddPCR and NGS-based method, and achieved high consistency.

**Conclusions:** The reference materials have superior performance, and are useful for development, validation, and quality control of ctDNA-based assays.

**P115. How Pathology Is Integrated within the Cancer Care Team - Landscape Analysis from the Association of Community Cancer Centers**

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Introduction: In an era of precision medicine the role of pathology in the diagnosis/management of cancer is evolving. Pathologists are positioned at the intersection of multiple points along the cancer care continuum. Starting at the point of cancer diagnosis, pathologists provide expert interpretation and may recommend biomarker testing to guide treatment decisions.

Methods: ACCC joined with partner organizations AMP, ASCP, and CAP, administered a survey in June 2018. There were 37 questions and 659 responses were received. Responses came from a multidisciplinary group including physicians, lab scientists and technicians, administrators, and other members of the cancer care team. Cancer program settings represented included community, hospital, integrated so that patients are receiving appropriate and timely care.

Results: Respondents report that gaps or breakdowns in communication are most likely to occur when selecting and ordering biomarker tests (78%) and when reporting the results of the tests (34%). The top five challenges reported were coverage and reimbursement, insufficient quantity of material, turnaround time, test selection/ordering, and communication across the multidisciplinary team. There were sizable gaps in regular ordering of NGS between those with 1 tumor board (28%), 2-3 tumor boards (42%) and 4 or more tumor boards (64%). The current use of liquid biopsy remains low, with the majority (52%) reporting that clinicians rarely order ctDNA testing (12% "routinely"). When tests were sent out-of-house the majority (58%) took 5-10 business days to receive results. 19% took < 5 business days and 24% took >10 business days. 43% indicated that pathologists are authorized to order all types of cancer biomarker tests. 62% indicated that pathologists have access to all inpatient records. 24% reported that rapid onsite evaluation during biopsy procedures was performed by pathologists in their organization regularly. There is a general lack of standardization for testing procedures (see chart). 

Conclusions: Success stories from programs that feel their pathology program is integrated include:

- Pathologists leading/driving institutional biomarker testing protocols/policies, involved in leadership roles
- Standardized reflexive testing pathways that reduce waste and turnaround time

Methods: The automated Illumina TruSight Tumor 170 workflow supports the construction of 3 to 64 libraries in a single run. A maximum of 32 RNA samples derived from FFPE or cell lines and a maximum of 32 DNA samples (either genomic DNA or DNA isolated from the same FFPE samples as the RNA) can be prepared simultaneously into Illumina libraries using two TruSight Tumor 170 kits. Features of the automation workflow include a modular method design for customized deployment and operation, an HTML driven interface that allows for simplified operation, and Guided Labware Setup to reduce setup errors.

Results: The automated Illumina TruSight Tumor 170 workflow supports the construction of 3 to 64 libraries in a single run. A maximum of 32 RNA samples derived from FFPE or cell lines and a maximum of 32 DNA samples (either genomic DNA or DNA isolated from the same FFPE samples as the RNA) can be prepared simultaneously into Illumina libraries using two TruSight Tumor 170 kits. Features of the automation workflow include a modular method design for customized deployment and operation, an HTML driven interface that allows for simplified operation, and Guided Labware Setup to reduce setup errors.

Reference:
[Standardization for Testing Procedures]

P116. Automation of the TruSight Tumor 170 Assay on the Biomek i5 Span-8 Liquid Handler
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Introduction: The TruSight Tumor 170 sequencing assay is a qualitative oncology test optimized for the detection of gene variants across 170 gene targets in nucleic acids (DNA and RNA) extracted from formalin-fixed, paraffin-embedded (FFPE) cancer tissue samples. Here we describe an automation solution for performing the TruSight Tumor 170 sequencing assay on the Beckman Coulter Biomek i5 Span-8 automated liquid handler.

Methods: The automated Illumina TruSight Tumor 170 workflow supports the construction of 3 to 64 libraries in a single run. A maximum of 32 RNA samples derived from FFPE or cell lines and a maximum of 32 DNA samples (either genomic DNA or DNA isolated from the same FFPE samples as the RNA) can be prepared simultaneously into Illumina libraries using two TruSight Tumor 170 kits. Features of the automation workflow include a modular method design for customized deployment and operation, an HTML driven interface that allows for simplified operation, and Guided Labware Setup to reduce setup errors.

Results: The automated Illumina TruSight Tumor 170 workflow supports the construction of 3 to 64 libraries in a single run. A maximum of 32 RNA samples derived from FFPE or cell lines and a maximum of 32 DNA samples (either genomic DNA or DNA isolated from the same FFPE samples as the RNA) can be prepared simultaneously into Illumina libraries using two TruSight Tumor 170 kits. Features of the automation workflow include a modular method design for customized deployment and operation, an HTML driven interface that allows for simplified operation, and Guided Labware Setup to reduce setup errors.

Reference:
[Standardization for Testing Procedures]
number of in-house samples. 108 DNA samples and 80 RNA samples were processed on the Biomek i5 Span-8 over the course of 13 runs. All DNA libraries passed the minimum quality threshold (median insert length ≥ 79bp and Percent Exon Bases ≥ 100X greater than 95%). All RNA libraries passed the minimum quality thresholds (Median Insert Size ≥ 63bp and RNA Median CV Coverage ≥ 1000X less than or equal to 0.88).  

**Conclusion:** The automation workflow for performing the TruSight Tumor 170 sequencing assay on the Beckman Coulter Biomek i5 Span-8 automated liquid handler is able to provide high quality sequence ready libraries across a variety of sample types.

**P117. Development and Validation of a Novel, Automated, High-Sensitivity NGS-Based Liquid Biopsy Assay**  
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**Introduction:** It is now well established that many solid tumors release detectable levels of mutation containing DNA into a patient's blood stream. This circulating tumor DNA (ctDNA) has been the focus of much research over recent years and significant progress has been made in assessing the spectrum of possible applications for detection and quantification of ctDNA in different cancer types. Clinically, molecular profiling of Circulating tumor DNA (ctDNA) is increasingly utilized to guide treatment decisions, especially in settings where tissue is limited or there is a need to utilize a less invasive method for biopsy.

**Methods:** We have developed a novel, automated, high-sensitivity NGS-based liquid biopsy assay that profiles all four classes of variants across 36 genes commonly mutated in non-small cell lung cancer (NSCLC) and other cancer types. The InVisionFirst assay has been developed to detect point mutations, indels, amplifications and gene fusions from cell-free DNA. Following the development of this novel, automated, high-sensitivity next generation sequencing (NGS)-based liquid biopsy, we conducted an analytical validation to demonstrate the performance of the test. For analytical validation of InVisionFirst-Lung, two 10mL blood tubes were collected from NSCLC patients and healthy volunteer donors. In addition, contrived samples were used to represent a wide spectrum of genetic aberrations and VAFs. Samples were analyzed by multiple operators, at different times and using different reagent Lots. Results were compared with digital PCR (dPCR).

**Results:** The InVisionFirst assay demonstrated an excellent limit of detection, with 99.48% sensitivity for SNVs present at VAF range 0.25%-0.33%, 92.46% sensitivity for indels at 0.25% VAF and a high rate of detection at lower frequencies while retaining high specificity (99.9997% per base). ALK and ROS1 gene fusions, and DNA amplifications in ERBB2, FGFR1, MET and EGFR were also detected with high sensitivity and specificity. Comparison between the InVisionFirst assay and dPCR in a series of cancer patients showed high concordance.

**Conclusion:** This analytical validation demonstrates that the InVisionFirst assay is highly sensitive, specific and robust, and meets analytical requirements for clinical applications.

**P118. LymphoTrack Low Positive Control and LymphoQuant Internal Control for MiSeq and S5/PGM LymphoTrack Assays**  
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**Introduction:** The LymphoTrack Assays and the associated LymphoTrack software, that have been developed for either MiSeq or Ion S5 platforms, are able to detect immunoglobulin (Ig) and T-cell receptor (TCR) clonal rearrangements in suspected lymphoproliferative disease (LPD) clinical samples. The detected sample specific clonal V-(D)-J sequence in baseline samples can be tracked in the follow-up samples with a potential application to identify and monitor disease status. Typically, the clonal molecules in follow-up samples are presented at very low level (10^4 or below). When testing these samples, it is important to include a low positive control (LPC) in the run to report the estimated clonal cells in a sample. We report the design and development of these controls for LymphoTrack Assays: the LPCs as run quality controls, and the LymphoQuant internal controls (IC) for estimating clonal cells within a sample.

**Methods:** Two types of LPC and IC were developed, one for Ig (IGHV Leader, IGH FR1, IGH FR2, IGH FR3, and IGK) and one for TCR (TRG and TRB). Each LPC consists of clonal positive cell line DNA diluted in clonal negative DNA at 10^-4 level. Each IC is diluted clonal positive cell line DNA at concentration of about 50 cell equivalent per µL. All LPCs and ICs can be detected by the LymphoTrack Assays either on Illumina MiSeq or Thermo Fisher Ion S5/PGM platforms. Multiple lots of LPCs and ICs were made and lot-to-lot variations were evaluated using surrogated samples that were prepared by 10-fold serial dilution of clonal positive cell line DNA into clonal negative DNA at 10^-2 to 10^-5.

**Results:** Using the LPCs and ICs, estimated %clonal cells within surrogated samples (10^-2 to 10^-5) or follow up samples were able to be detected by LymphoTrack Assays on both MiSeq and S5/PGM platforms.

**Conclusion:** The LPC and IC described have demonstrated to monitor LymphoTrack run performance and to estimate clonal cells present in the sample for both baseline and follow up samples, suggesting potential applications in detect and monitor minimal residual disease.

**P119. Assessing the Level of Knowledge and Mode of Awareness for Infectious Diseases among Primary Level Children in under Developed Country, Pakistan**  
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Introduction: Infectious diseases are a major problem of the underdeveloped countries with children more vulnerable due to under-developed immune system, lack of understanding and inadequate sources of knowledge for the infectious diseases. To the best of our knowledge preschool and primary level children get little knowledge of basic hygiene via the curriculum. This study was therefore designed to collect data from different primary level schools to determine the understanding of children with respect to daily hygiene activities.

Methods: In this study five primary schools were visited and data was collected from a total of 224 students from the age of 3 to 9 years. The students were divided into 5 groups. Data was collected by personally asking questions of each individual regarding basic hygiene such as hand washing, covering face during sneezing and coughing, dental health and hygiene, cleaning of genital areas, and regular baths. Moreover, each student was asked for their source of basic knowledge of infectious diseases and hand hygiene.

Results: A total of 224 children participated in this study. They were distributed into different groups. 40 ranged from the age of 3 to 5 years, 38 from 5 to 6 years, 47 from the age of 6 to 7 years, 58 from the age of 7 to 8 years and 41 from 8 to 9 years. Only 35% children from the age of 3 to 9 years have learned the basics of personal hygiene. The best source for awareness was electronic media.

Conclusion: Teaching of personal hygiene is important for keeping children healthy and clean. Considering the unsatisfactory level of awareness among pre-school children, there is a strong need to develop modules for educating pre- and primary-level children in the importance of proper hygiene. Hygiene education in form of poems, cartoons, songs and video clips need to be introduced at pre- and primary-level schools.