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Molecular profiling of signet ring cell colorectal cancer provides a strong rationale for genomic targeted and immune checkpoint inhibitor therapies

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Background: Signet ring cell colorectal cancer (SRCCa) has a bleak prognosis. Employing molecular pathology techniques we investigated the potential of precision medicine in this disease.

Methods: Using test (n = 26) and validation (n = 18) cohorts, analysis of mutations, DNA methylation and transcriptome was carried out. Microsatellite instability (MSI) status was established and immunohistochemistry (IHC) was used to test for adaptive immunity (CD3) and the immune checkpoint PDL1.

Results: DNA methylation data split the cohorts into hypermethylated (n = 18, 41%) and hypomethylated groups (n = 26, 59%). The hypermethylated group predominant in the proximal colon was enriched for CpG island methylator phenotype (CIMP), BRAF V600E mutation and MSI (P < 0.001). These cases also had a high CD3+ immune infiltrate (P < 0.001) and expressed PDL1 (P = 0.03 in intra-tumoural lymphoid cells). The hypomethylated group predominant in the distal colon did not show any characteristic molecular features. We also detected a common targetable KIT mutation (c.1621A>C) across both groups. No statistically significant difference in outcome was observed between the two groups.

Conclusions: Our data show that SRCCa phenotype comprises two distinct genotypes. The MSI+/CIMP+/BRAF V600E+/CD3+/PDL1+ hypermethylated genotype is an ideal candidate for immune checkpoint inhibitor therapy. In addition, one fourth of SRCCa cases can potentially be targeted by KIT inhibitors.

Colorectal cancer (CRC) rates are on the decline in the US and Western Europe, but incidence of signet ring cell colorectal cancer (SRCCa) has remained steady (Gopalan et al, 2011; Arnold et al, 2017). These are highly malignant, dedifferentiated adenocarcinomas and comprise around 0.1–2.4% of all CRC cases (Anthony et al, 1996). Primary SRCCas are most often diagnosed at an advanced stage, and typically have a dismal prognosis with average five year survival rates of around 20% (Nitsche et al, 2013).

Molecular pathology of SRCCa is not well understood and it is unclear whether the signet ring cell phenotype carries a distinct
genotype as well. Because of the rarity of this cancer most published studies are either case reports or retrospective epidemiological and clinicopathological analyses. High frequency of BRAF mutations, microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) have been reported along with predominance in proximal colon and the female gender (Kakar et al., 2012). But to date there has not been any multi-omics study conducted to comprehensively study the molecular pathology of this disease. This is necessary for two reasons: (a) to shed light on whether SRCCA has a molecular profile distinct from other CRC subtypes, and (b) to identify novel biomarkers and therapeutic targets.

MATERIALS AND METHODS

Patient samples. For the test cohort, patients were identified from the pathology archives of Belfast Health and Social Care Trust (BHHSCT) in Northern Ireland and formalin fixed paraffin-embedded (FFPE) tissue blocks were made available by the Northern Ireland Biobank. For the validation cohort, patients were both identified and FFPE tissue blocks made available from the Grampian Biorepository in Scotland. Ethical approval was provided by the Northern Ireland Biobank scientific access group committee (study number–NIB14-0139), the Grampian Biorepository scientific access group committee (tissue request–TR000058) and the NHS Health Research Authority North West–Preston research ethics committee (reference–15/NW/0855). No written consent was required from patients for the use of FFPE tissue blocks and anonymised demographic and clinicopathological data. All identified patients were reviewed by two expert pathologists (MST and MBL for test cohort/GIM and MBL for validation cohort). Only cases that fulfilled the WHO criteria of greater than 50% of the tumour comprising of signet ring cells were selected for the study (Bosman, 2010).

Nucleic acid extraction. Representative normal (furthest from the tumour) and tumour (highest cellularity of signet ring tumour cells) FFPE tissue blocks were selected after haematoxylin and eosin (H&E) slide review. The H&E slides were then annotated for the epithelial layer in normal blocks and signet ring cell rich areas in tumour blocks (MST and MBL). 5 × 10 mm and 5 × 8 mm sections were cut onto glass slides for DNA and RNA extraction, respectively. Annotated areas were macrodissected using sterile scalpels blades into 1.5 ml microcentrifuge tubes. DNA extraction was done using Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega, Southampton, UK), and RNA using the RNeasy FFPE Kit (Qiagen, Manchester, UK). Quantification was conducted using NanoDrop 2000 (Thermo Fisher Scientific Inc., Loughborough, UK) unless mentioned otherwise.

Next generation sequencing. Next generation sequencing (NGS) was performed on the entire test cohort tumour samples. TaqMan RNase P Detection Reagents Kit was used to quantify 10 ng of DNA and library prepared using the Ion AmpliSeq Library Kit 2.0 and Cancer Hotspot Panel v2 (Thermo Fisher Scientific Inc.). Sequencing was performed on the Ion PGM System according to manufacturer’s instructions and our previously published protocols (McCourt et al., 2013; Alvi et al., 2015).

DNA methylation. DNA methylation arrays were performed on both test and validation cohort tumour samples and additionally on 10 randomly selected normal samples from the test cohort. We used the Infinium 450k arrays (Illumina Inc., Cambridge, UK) following the manufacturer’s instructions and our previously published protocol (Alvi et al., 2015). Total 200 ng of DNA as quantified using Qubit Fluorometric Quantitation assay (Thermo Fisher Scientific Inc.) was used and arrays were scanned using iScan (Illumina Inc.).

Gene expression. Gene expression arrays were performed on test cohort tumour samples and 10 randomly selected normal samples. The Whole-Genome DSL HT assay was used in combination with the HumanHT-12 v4 BeadChip (Illumina Inc.) according to manufacturer’s instructions and our previously published protocol (Alvi et al., 2015). Around 200 ng of total RNA was used as quantified by Qubit Fluorometric Quantitation assay and chips were scanned using iScan.

Sanger sequencing. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit on the ABI 3500 Genetic Analyzer (Thermo Fisher Scientific Inc.) using manufacturer’s instructions. Primers were designed using NCBI primer designing tool with M13 overhangs. All PCRs were carried out using AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific Inc.), and cleaned using ExoSAP-IT (Affymetrix, UK). Approximately 10–50 ng of DNA was used for each reaction.

Microsatellite instability analysis. MSI status was evaluated using MSI Analysis System, Version 1.2 (Promega) according to the manufacturer’s instructions. We tested five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27), which were co-amplified using fluorescently labelled primers and analysed on an ABI 3500 Genetic Analyzer. Approximately 10–50 ng of DNA was used for each reaction.

BRAF V600E mutation assay. Cobas 4800 BRAF V600 mutation test kit (Roche Molecular Systems Inc., Burgess Hill, UK) was used to look for BRAF V600E mutation according to the manufacturer’s instructions. Around 125 ng of DNA was used for each reaction.

Immunohistochemistry. PDL1 and CD3 immunohistochemistry was carried out on 3 μm full face sections using PD-L1/CD274 (SP142) antibody (Spring Bioscience, CA, USA) at 1: 40 dilution and CONFIRM anti-CD3 (2G6V) rabbit monoclonal antibody (Ventana, UK) respectively. In addition Optiview amplification kit was used for PDL1 antibody. Staining was carried out on Ventana Benchmark XT platform with the Optiview Universal DAB Detection Kit (Ventana Medical Systems, Burgess Hill, UK).

PDL1 scoring was performed separately for peritumoural lymphoid follicles (PLF), intra-tumoural lymphoid cells (ILC) and tumour epithelial cells (TEC). Scoring criteria used was 0 (negative) for no cell staining and 1 (positive) for any number of cells staining. CD3 staining, assessed in ILCs only, was scored semi-quantitatively using a three tiered scoring system (1 for the lowest counts observed and 3 for the highest).

Data analysis. For NGS data vcf files were generated from the torrent server using the variantCaller plugin (Life Technologies, Loughborough, UK) and imported into Ion Reporter 5.0 (Thermo Fisher Scientific Inc.) and imported into Ion Reporter 5.0 (Thermo Fisher Scientific Inc.) for annotation. Methylation and gene expression array data was analysed using GenomeStudio methylation and expression modules version 1.9.0 respectively (Illumina Inc.). Sanger sequencing data were viewed and confirmed with Finch TV version 1.4.0 (Geospiza Inc., WA, USA). Gene set enrichment analysis (www.broadinstitute.org/gsea) was used for pathway analysis using default settings. Assignment of patient samples into their respective consensus molecular subtyping (CMS) groups based on gene expression data was carried out using the ‘CMSclassifier’ package in R version 3.2.4 (The R Foundation for Statistical Computing, Austria; Guinney et al., 2015). For comparing data between groups, using Prism version 5 (GraphPad Software, CA, USA) a t-test was performed for continuous variables and Fisher’s exact test for categorical variables. Cox proportional hazards analysis to look for
associations between molecular and clinicopathological data were conducted using Stata version 11.2 (StataCorp, TX, USA).

**RESULTS**

**Patient cohorts.** Total of 26 and 18 patients were identified from the BHSCT and the Grampian Biorepository, respectively. We did not observe any statistically significant difference between the two cohorts in terms of demographics or clinicopathological data (Supplementary Table 1).

**DNA methylation.** From the test cohort, based on beta values, most variable probes were identified using a s.d. cut-off of 0.25. This generated a list of 875 probes. These probes were used for unsupervised hierarchical clustering using the manhattan metric and were able to split the 26 sample cohort into distinct hypermethylated ($n=9$) and hypomethylated ($n=17$) groups. The same probes were also able to split the validation cohort into hypermethylated ($n=9$) and hypomethylated ($n=17$) groups. As shown in Figure 1 only 300 (enclosed in red) out of the 875 probes are consistently differentially methylated between the two groups. The full list is available in Supplementary Table 2 and raw data can be obtained from GSE79740.

As shown in Supplementary Figure 1 the hypermethylated group was also CIMP positive. We were also able to identify genes consistently hypermethylated and hypomethylated across all tumour samples compared to normal tissue with potential as diagnostic biomarkers (Supplementary Table 3).

**Mutations.** As shown in Figure 1, compared to the hypomethylated group, we observed the hypermethylated group to be enriched for $BRAF$ V600E mutation ($P<0.001$ in test, validation and both cohorts combined together). This was also confirmed using a PCR based assay. Other mutations were also observed in the test cohort using the 50 gene hotspot panel (PRJNA316428, Supplementary Table 4). According to the COSMIC database, compared to colorectal adenocarcinoma average we observed higher frequencies of $TP53$ (69% vs 44%), $BRAF$ (31% vs 13%) and $KIT$ (34% vs 8%) mutations. At the same time a lower frequency of $APC$ (35% vs 45%), $KRAS$ (12% vs 34%), $PIK3CA$ (4% vs 14%) and $ATM$ (4% vs 18%) mutations was observed (Figure 2 and Supplementary Table 4). The number of mutant genes in each sample also varied ranging between 1 and 11 with an average of 2.7 mutant genes per sample (out of the 50 tested by panel). This average was 3.9 in the hypermethylated and 2.1 in the hypomethylated group ($P<0.05$).

The $KIT$ mutations detected by NGS in the test cohort were similar in eight out of the nine cases (c.1621A>G). This was validated using a Sanger sequencing assay in both the test and validation cohorts and an additional three cases were found in the validation cohort (Forward primer: GTTGTAACACAGGCC-CAGUCGTAAGCTGGATGTTGC, R primer: CACAGGA AACAGCTATGACCTCTGGAGAGAGAACAAATAAATGGT).

**Gene expression.** Gene set enrichment analysis was used to look at pathway enrichment at the gene expression level in the test cohort. Using the ‘hallmark 50 gene sets’ we identified 19 gene sets enriched in the hypermethylated group and 4 in the hypomethylated group ($q<0.05$). The top three were ‘MTOR signalling’, ‘MYC targets V1’ and ‘E2F targets’ in the hypermethylated group and ‘epithelial mesenchymal transition’, ‘myogenesis’ and ‘apical junction’ in the hypomethylated group (Supplementary Figure 2 and Supplementary Table 5).

Gene expression data (GSE79793) was also merged with DNA methylation data using GenomeStudio and spearman correlation coefficients were calculated for every combination of methylation and expression array probes (Supplementary Table 6). We observed 5725 combinations (2088 gene probes) with an inverse relationship in the hypermethylated group and only 753 combinations (439 gene probes) in the hypomethylated group highlighting the impact of differential methylation between the two (spearman

![Figure 1](image_url)

**Figure 1.** Alongside different DNA methylation patterns, data best able to distinguish between the hypomethylated and hypermethylated genotypes are shown in green and red respectively. ILC = intra-tumoural lymphoid cells; PLF = peritumoural lymphoid follicles; TEC = tumour epithelial cells.
Figure 3 we observed a higher infiltration of CD3+ T-lymphocytes and PDL1 expression to evaluate the presence of adaptive immune resistance in our cohorts (Xiao and Freeman, 2015).

Because it has recently been shown that metastatic MSI-high CRCs are good candidates for immune checkpoint inhibitor therapy, we tested for CD3 and PDL1 expression to investigate (Guinney et al, 2015).

Microsatellite instability and PDL1 expression. MSI was called where three or more out of the five loci tested were observed to be aberrant. As shown in Figure 1, we observed most of the MSI cases in both the test and validation cohorts to fall within the hypermethylated group (P<0.001, P=0.06, P<0.001 in test, validation and both cohorts combined together respectively). Because MSI has recently been shown that metastatic MSI-high CRCs are good candidates for immune checkpoint inhibitor therapy, we tested CD3 and PDL1 expression to evaluate the presence of adaptive immune resistance in our cohorts (Xiao and Freeman, 2015).

We initially conducted CD3 IHC to confirm the presence of an immune infiltrate in the test cohort. As shown in Supplementary Figure 3 we observed a higher infiltration of CD3+ T-lymphocytes in both MSI cases compared to microsatellite stable cases (MSS) and also the hypermethylated group compared to hypomethylated group (P<0.001).

We then looked at PDL1 gene expression data and observed a higher expression of PDL1 in MSI cases compared to MSS (Figure 3A, P=0.04). We also observed a similar trend in the hypermethylated group compared to hypomethylated group; however it was not statistically significant (Figure 3A, P=0.07).

This finding was validated at the protein level using IHC in both the test and validation cohorts (representative staining can be seen in Figure 3B). A higher expression of PDL1 was observed in MSI cases compared to MSS cases (P<0.001, P=0.16, P<0.001 in test, validation and both cohorts combined together, respectively, Figure 3C). The trend was consistent across all the three popula- tions we looked at but strongest in the ILCs (P=0.003, P=0.3, P=0.001 in test, validation and both cohorts combined together, respectively, Figure 3C). We also observed a similar trend comparing PDL1 protein expression in hypermethylated vs hypomethylated group (P=0.008, P=0.8, P=0.03 in test, validation and both cohorts combined together, respectively, Figure 3C).

Association between molecular and clinicopathological data. Patients in the hypermethylated group had a higher average age compared to the hypomethylated group (P<0.01 in test, validation and both cohorts combined together, Figure 1). We observed these to be mostly female patients (P=0.10, P=0.15, P=0.01 in test, validation and both cohorts combined together, respectively, Figure 1) and the tumours were mostly in the proximal colon (P=0.01, P=0.13, P<0.01 in test, validation and both cohorts combined together, respectively, Figure 1). We observed no statistically significant link between molecular data and any other clinicopathological parameters including prognosis (overall survival) even when adjusted for age/stage/ gender/MSI/tumour location in a multivariate analysis (Supplementary Table 7).

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(1000 Genomes frequency: 6.45% (The Genomes Project C, 2015), ExAC frequency: 7.89% (Lek et al, 2016), NHLBI ESP European frequency: 11.19% (National Heart, Lung, and Blood Institute). We know that KIT mutant gastrointestinal stromal tumours benefit from treatment with the tyrosine kinase inhibitor imatinib (Siehl and Thiel, 2007) and similarly it has been reported that this mutation not only increases proliferation but also enhances sensitivity to imatinib in certain cancers (Bargou et al, 2006; Masago et al, 2015; Iacono et al, 2016). Also it has not been reported previously in CRC and thus is of potential clinical significance as it may open new targeted approaches to treatment. This finding also fits in with recent studies, where upregulation of mTOR and MYC pathways (as observed in the hypermethylated genotype, Supplementary Figure 2 and Supplementary Table 5) can lead to PDL1 dependant suppression of the immune response (Casey et al, 2016; Lastwika et al, 2016). The immune checkpoint inhibitor therapy clinical trials in CRC have suffered from low-sample numbers because most MSI CRCs are early stage (Xiao and Freeman, 2015). This makes SRCCa hypermethylated genotype an ideal candidate for these trials as these cancers are likely to be both MSI and high stage (Le et al, 2015; Llosa et al, 2015).

Comparing our data to the CMS classification of Guinney et al, 2015 we find our hypermethylated group similar to the CMS1 (microsatellite instability immune, 14%) subtype with a high mutation count, MSI, CIMP, BRAF mutations, immune infiltration (as measured by CD3 IHC) and predominance in the proximal colon and the female gender (Guinney et al, 2015). The hypomethylated group shows similarities to CMS4 (mesenchymal, 23%) subtype in terms of upregulation of epithelial–mesenchymal transition genes, but also to CMS3 (metabolic, 13%) subtype as it contains all of the KRAS mutant tumours.

In summary, SRCCa comprises of two molecularly distinct genotypes. An MSI+/CIMP+/BRAF V600E+/CD3+/PDL1+ hypermethylated genotype predominant in the proximal colon, and a hypomethylated genotype predominant in the distal colon. The high frequency of MSI and PDL1 expression in the hypermethylated genotype makes it a potential target for immune checkpoint inhibitor therapy. In addition, a high-detected frequency of the c.1621A>C (p.M541L) KIT actionable mutation also suggests imatinib as a candidate genomic targeted therapy. Testing tumour tissue for these two molecular aberrations may be
clinically beneficial upon making a diagnosis of SRCCa. Because of the rarity of this disease and the lack of cell line and animal models, the results of this study strongly support the need for an early phase trial aimed at these targets.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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