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**Translational Database Selection and Multiplexed Sequence Capture for Up Front Filtering of Reliable Breast Cancer Biomarker Candidates**

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**Abstract**

Biomarker identification is of utmost importance for the development of novel diagnostics and therapeutics. Here we make use of a translational database selection strategy, utilizing data from the Human Protein Atlas (HPA) on differentially expressed protein patterns in healthy and breast cancer tissues as a means to filter out potential biomarkers for underlying genetic causatives of the disease. DNA was isolated from ten breast cancer biopsies, and the protein coding and flanking non-coding genomic regions corresponding to the selected proteins were extracted in a multiplexed format from the samples using a single DNA sequence capture array. Deep sequencing revealed an even enrichment of the multiplexed samples and a great variation of genetic alterations in the tumors of the sampled individuals. Benefiting from the upstream filtering method, the final set of biomarker candidates could be completely verified through bidirectional Sanger sequencing, revealing a 40 percent false positive rate despite high read coverage. Of the variants encountered in translated regions, nine novel non-synonymous variations were identified and verified, two of which were present in more than one of the ten tumor samples.

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**Introduction**

Discovery of biomarkers has traditionally been mediated by interpretation of transcriptome data generated using array-based expression profiling platforms [1,2]. This has for instance resulted in better understanding of prostate cancers, where the initial screening for serum PSA has led to earlier detection of the disease, but also to inaccurate diagnosis, necessitating discovery of new and better biomarkers [3,4]. It has earlier been shown that biomarker discovery benefits from integration of genomic and proteomic technologies [3]. Recent data have also demonstrated a stronger correlation between transcripts and proteins than previously anticipated [6,7]. Thus, translating aberrant protein expression patterns to transcript differences is of high interest, and the possibility that some of these changes are encoded in the genome offers new openings to identify causative mutations. Today’s novel technologies and large-scale efforts for proteomic screening are providing the grounds for a great increase of pace in such biomarker discovery. The Human Protein Atlas is one example where the proteome is being screened for differences in expression patterns in a large collection of cancers and corresponding healthy tissues [5,9]. Antibodies are created for each human protein in a mono-specific polyclonal fashion and are used to stain tissue microarrays in order to determine the expression patterns and levels.

In addition to more efficient proteomic screening, the advent of massively parallel sequencing methods [10,11,12] has greatly increased the throughput of genomic data [13] and has also provided new platforms for transcriptomic screening past the traditional array based technologies [14,15]. Whereas genomes of healthy and diseased tissues can be sequenced in full today [16,17], the sequencing throughput is still not able to provide us with enough data to screen vast numbers of genomes in parallel in a cost-efficient manner. The response to this has traditionally been selection of genomic regions of interest by PCR [18], but has lately been replaced by methods for extraction of regions of interest by sequence enrichment strategies [19,20,21].

Here we demonstrate a model for further cost reduction and increased efficiency in biomarker discovery by employing up-front database selection in combination with sample barcoding [22] and multiplexed sequence capture enrichment, to rationally filter out potential biomarkers at an early stage.

**Materials and Methods**

**Ethics statement**

Ethical permission was obtained from the Ethics Committee at Lund University whereby informed consent was deemed not to be required other than by the opt-out method. The study was...
conducted according to Declaration of Helsinki Principles. The data were analyzed anonymously.

Translational database selection

The Human Protein Atlas database [8] was searched for proteins with a clearly differential staining pattern in healthy breast tissue and breast cancer tissue requiring: i) breast glandular cells with no staining and ii) breast cancer tumor cells with at least five patients with strong staining. A complementary search for breast glandular cells with strong staining and breast cancer tumor cells with at least ten patients with weak or no staining was also carried out.

Further, the proteins found through the search results were screened to match a number of criteria of interest. These included overall differential staining of healthy and cancerous tissues for the particular protein and high assay validation scores. Proteins were scored as particularly interesting if they were present in a transmembrane region, and if they contained a signaling peptide. In total, 41 proteins were selected in this way and an additional 10 proteins known to be associated with cancer from the literature were added to the list. (Table S1)

Selection and design of regions for genomic enrichment

The coding exons for the proteins selected through the HPA database were extracted from the UCSC human reference genome (hg18) database. Additionally the 5’UTR and 3’UTR regions were included, as well as 1000 basepairs upstream from the 5’UTR.

To facilitate efficient capture of the targeted genomic regions, the selected regions were expanded to a minimum of 250 basepairs and regions with a resulting overlap were fused together. 479 regions totaling 303,788 basepairs, 89,705 of which were protein coding, were selected in this way.

The selected regions were submitted to the array manufacturer (Nimblegen, Madison, WI, USA) for manufacturing of 385k-feature enrichment arrays. The final design after internal processing and filtering of repetitive regions contained 581 tiled regions spanning a total of 303,986 target bases.

Sampling of tumors and DNA extraction

Tumors were surgically removed from the patients, trimmed for healthy tissue and instantly put into a freezer at −80°C. >99% of the cells were judged to be of tumor origin. For extraction of DNA ten pieces approximately 1 mm³ each were cut out from each tumor and put into a fastprep tube (164102930, Lysing matrix D, Fisher Scientific, Gothenburg, Sweden). 360 μl 3 M NaAc and 500 μl of EB buffer (DNeasy, Qiagen) in separate tubes, 20 μl of proteinase K (Qiagen, DNeasy kit) was added to the tube that was then processed 2 times 60 seconds on a Fastprep FP210 system (Qbiogene, Carlsbad, CA, USA). The homogenized liquid phase was pipetted into a Qiashredder column (Qiagen, Valencia, CA, USA) and centrifuged at 13k rpm. 40 μl of proteinase K (Qiagen, DNeasy kit) was added to the shredded material followed by a 15-minute incubation of the sample at 56°C. 300 μl of buffer AL (DNeasy, Qiagen) and 300 μl 96% ethanol was added to the sample and the resulting mixture was split in half and transferred into 2 DNeasy Mini spin columns.

The columns were processed according to manufacturers instructions (DNeasy, Qiagen) after which each sample was eluted twice with 200 μl of EB buffer (DNeasy, Qiagen) in separate tubes, totaling 4 tubes with 200 μl eluate each for each initial tumor sample.

Following elution each sample was ethanol precipitated by adding 20 μl 3 M NaAc and 300 μl −20°C 96% ethanol, and then incubated at −80°C for 15 minutes. After freezing the sample was centrifuged at 13k rpm for 25 minutes in room temperature. The sample was washed with 500 μl −20°C 70% ethanol and centrifuged at 13k rpm for another 15 minutes in room temperature. The liquid was removed and the sample tubes were dried at room temperature over night with open lids under a protective cover. The following day 20 μl 1xTE buffer was added to each sample tube to dissolve the extracted DNA after which the tubes for each sample were pooled and analyzed for concentration and purity on a NanoDrop N-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Healthy reference tissue from each patient sample was processed in the same way to obtain DNA for validation sequencing of variations indicative of potential biomarkers.

DNA enrichment and sequencing

A total of ten DNA samples from breast cancer tumors were processed into sequencing libraries using an in house developed automated protocol [23] based on the GS FLX titanium Library preparation method (Roche/454, Branford, CT, USA) using multiplex identifier handles (MID 1–10, Roche/454). The samples were then pooled in equimolar ratios into a single tube and enriched for target sequences by hybridizing to the custom Nimblegen 385k array (Roche/Nimblegen, Madison, WI, USA) previously designed and manufactured for the project. Following enrichment the pooled library was titrated and sequenced according to manufacturers instructions (Roche/454) on a GS FLX using long-read titanium chemistry.

Data analysis

The data corresponding to each sample was mapped to the human reference genome (hg19) using the Roche/454 GS Mapper software (Newbler version 2.3), and the file containing the resulting high confidence variants (HGCliffs.txt) was used for further analysis. The single nucleotide variants were annotated using custom perl scripts and the knownGene transcript database (UCSC).

Validation of variations

Following Roche/454 sequencing and data analysis as described above, 15 novel non-synonymous variations were encountered, spread across the different samples (Table S2). These were verified through amplification by PCR followed by bidirectional Sanger sequencing of the corresponding genomic regions. Nine out of these variations were confirmed as heterozygous in both tumor tissue and healthy reference tissue, whereas six of the variants were not visible in either tissue in the validation experiments, hence deemed false positives.

Results

DNA from breast cancer samples and surrounding healthy breast tissue (Figure 1) was isolated from ten surgically removed tumors and selected genes were investigated using array-based enrichment and DNA sequencing. In total, 581 genomic regions corresponding to the 51 selected differentially expressed or literature derived proteins (Table S1) were surveyed for mutations.

Out of the 1,109,321 sequencing reads generated, 22.63% were mapped uniquely to the human genome and overlapped with the 303,936 bases associated with the 581 target regions. Of the generated bases, 69.3 million mapped to the target, corresponding to an average 228 times sequence coverage of the target. >99.9% of the target was covered by at least one read. The distribution of reads between the different samples was good in general (Table 1)
however multiplex identifier 3 (MID 3) proved to have been very inefficiently amplified in the emPCR, as verified later by qPCR of the MIDs [23], and the sample labeled with MID 9 generated very low concentrations at the library preparation, which resulted in a lower molar amount of DNA from the MID 9 tagged sample in the pool.

In total 1,982 single nucleotide variations (SNVs) at 579 unique positions in the target regions were found in the tumor samples when compared to the human reference sequence (hg19). A higher rate of variation, per base, was seen in 5’UTR, 3’UTR, promotor and intron sequence of the target (0.07%), than in protein coding exon sequence (0.05%). Of the 149 unique positions with SNVs in protein coding sequence (488 SNVs total), 66 were subjected to an amino acid change. 15 of these non-synonymous alterations had not previously been reported in dbSNP build 130. All novel SNVs were detected in frequencies above 20% of the reads. Six of these non-previously reported variations were encountered in more than one tumor sample, and were confined to three genes, one in SATB1, four in MUC5AC and one in DDX26B (Table 2).

Confirmatory Sanger sequencing was carried out for all 15 novel SNVs in the tumor samples and normal reference tissue. This resulted in confirmation of nine of the 15 SNVs as heterozygous variations present in both tumor and normal tissue. The six remaining variations turned out to be false positives.

**Discussion**

The percentage of reads mapping to the targeted regions for the used enrichment platform has previously been reported around 60–80% [19]. There are several possible reasons for the modest fraction of sequencing reads that mapped to the targeted regions in the enrichment platform.
regions being able to remain close to the array surface through array may have influenced the result with more non-targeted feasible to believe that the multiplexing of samples on the capture region length as relatively short (average length 521 bp). It is also target regions could be denoted as close to random and the target was as difficult as possible. The design algorithm was the first approach the targeted regions, and the number and spread of the target regions across the genome. From these three aspects our approach variation in enrichment success can always be encountered in the samples, six turned out to be false positives. Two of the remaining non-synonymous SNVs encountered 40% turned out to be false positives. Two of the remaining previously unreported non-synonymous SNVs were found in the genomes of more than one individual. Each was verified as a heterozygous SNV present in both normal and cancerous tissue. Their presence in SATB1, previously linked to breast cancer promotion [26], and DDX26B, to our knowledge previously unreported in relation to breast cancer, provides further support for continued examination of the role of the genes in conjunction to breast cancer.

Lastly, the higher frequency of variations seen in non-coding sequence as compared to coding sequence has previously been reported in healthy [18] and cancerous [17] tissue, strengthening the scientific grounds for the logical reasoning that non-protein-coding regions of the genome are less subjected to evolutionary constraints.

In summary, the employment of a translational database selection strategy in combination with multiplexed enrichment by sequence capture provides a tool for careful biomarker discovery. Given the abundance of false positives generated by massive sequencing approaches, employing a rational selection strategy prior to sequencing can provide an efficient means to limit the number of variants at the end of the pipeline, enabling a complete variant verification process and a more reliable final list of biomarker candidates.

Supporting Information

Table S1 Selected genes. 41 proteins and their corresponding genes were selected through the HPA database and 10 more proteins and their corresponding genes known to be associated with cancer from literature were added to the list.

Table S2 Novel non-synonymous SNVs. Among the novel non-synonymous SNVs encountered 40% turned out to be false positives. Two of the remaining SNVs were present in more than one individual.

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Author Contributions

Conceived and designed the experiments: PLS SH JL. Performed the experiments: PLS HM. Analyzed the data: PLS MKB. Contributed reagents/materials/analysis tools: MKB KJ SH JL. Wrote the paper: PLS MKB JL. Obtained clinical samples: KJ. Obtained permission for clinical samples: KJ.

References

1. Welch JB, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, et al. (2001) Analysis of gene expression profiles in normal and neoplastic ovarian tissue. Proc Natl Acad Sci USA 98: 1176–1181.

Table 2. Encountered single nucleotide variants (SNVs) overview.

| Total unique positions with single nucleotide variants (SNVs) | 579 |
|---------------------------------------------------------------|-----|
| In target genes                                              | 467 |
| In exons (incl UTR)                                           | 266 |
| In introns                                                    | 201 |
| In coding sequence (CDS)                                      | 149 |
| In non-coding sequence                                        | 430 |
| Non-synonymous in CDS                                         | 66  |
| Novel non-synonymous not in dbSNP                            | 15  |
| Verified novel non-synonymous not in dbSNP                   | 9   |
| Novel non-synonymous present in more than one patient         | 6   |
| Verifiable novel non-synonymous present in more than one patient | 2   |

An overview of the single nucleotide variations encountered across the samples. 149 unique positions with single nucleotide variations (SNVs) were found in protein coding sequence, 15 of which gave rise to a different amino acid and were previously unreported in dbSNP (version 130). In total six of these turned out to be false positives when verified by bidirectional Sanger sequencing. Two of the remaining mutations were present in more than one individual and were located in the SATB1 and DDX26 genes.
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The present study. Variation in enrichment success can always be expected and can be coupled to the array-design, the sequence in the targeted regions, and the number and spread of the target regions across the genome. From these three aspects our approach was as difficult as possible. The design algorithm was the first version provided by the manufacturer, the locations of the selected target regions could be denoted as close to random and the target region length as relatively short (average length 521 bp). It is also feasible to believe that the multiplexing of samples on the capture array may have influenced the result with more non-targeted regions being able to remain close to the array surface through binding to the handles of real target fragments hybridized to the actual array.

To determine the usability of the results generated through the translational selection, multiplexed enrichment and sequencing methods, a high level of correlation to dbSNP for the non-synonymous SNVs should give a high validity to the method employed to find the variations. Additionally several of the non-synonymous SNVs (66 in total; Table 2) were present in genes with a previously established connection to breast cancer development through inherited genetic variations such as BRCA1 (seven SNVs) and BRCA2 (two SNVs) [24]. This together with high bidirectional sequence coverage should make the nine verified non-synonymous variations, present in normal and cancerous tissue in their respective individuals and not previously present in dbSNP (version 130), interesting for further analysis.

On the other hand, of the 15 non-synonymous mutations that were encountered in the samples, six turned out to be false positives when verified by bidirectional Sanger sequencing (Table S2). This raises increased concerns relating to the generation of systematic errors by present massive sequencing platforms. Further, comparison of these six seemingly novel non-synonymous mutations to the latest version of dbSNP (version 131) marks two of them as previously reported. Given previous false positive results already raising concerns to the quality of the content of dbSNP [25], this provides even further reason to exercise care when using the current versions of variant databases.

Two of the remaining previously unreported non-synonymous SNVs were found in the genomes of more than one individual. Each was verified as a heterozygous SNV present in both normal and cancerous tissue. Their presence in SATB1, previously linked to breast cancer promotion [26], and DDX26B, to our knowledge previously unreferenced in relation to breast cancer, provides further support for continued examination of the role of the genes in conjunction to breast cancer.

Lastly, the higher frequency of variations seen in non-coding sequence as compared to coding sequence has previously been reported in healthy [18] and cancerous [17] tissue, strengthening the scientific grounds for the logical reasoning that non-protein-coding regions of the genome are less subjected to evolutionary constraints.

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Author Contributions

Conceived and designed the experiments: PLS SH JL. Performed the experiments: PLS HM. Analyzed the data: PLS MKB. Contributed reagents/materials/analysis tools: MKB KJ SH JL. Wrote the paper: PLS MKB JL. Obtained clinical samples: KJ. Obtained permission for clinical samples: KJ.
2. Staal FJ, van der Burg M, Wessels LF, Barendregt BH, Baert MR, et al. (2003) DNA microarrays for comparison of gene expression profiles between diagnosis and relapse in precursor-B acute lymphoblastic leukemia: choice of technique and purification influence the identification of potential diagnostic markers. Leukemia 17: 1324–1332.

3. Ilyin SE, Belkowski SM, Plata-Salaman CR (2004) Biomarker discovery and validation: technologies and integrative approaches. Trends Biotechnol 22: 411–416.

4. Xin W, Rhodes DR, Ingold C, Chinnaiyan AM, Rubin MA (2003) Dysregulation of the annexin family protein family is associated with prostate cancer progression. Am J Pathol 162: 255–261.

5. Nishizuka S, Chen ST, Gwadry FG, Alexander J, Major SM, et al. (2003) Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res 63: 5243–5250.

6. Lundberg E, Fagerberg L, Kèrvebrèng D, Matic I, Geiger T, et al. (2010) Defining the transcriptome and proteome in three functionally different human cell lines. Mol Syst Biol. 450 p.

7. Kèrvebrèng D, Fagerberg L, Lundberg E, Emanuelsson O, Uhlen M, et al. (2010) Analysis of transcript and protein overlap in a human osteosarcoma cell line. BMC Genomics. 604 p.

8. The Human Protein Atlas website. Available: www.proteinatlas.org. Accessed 2011 May 17.

9. Uhlen M, Bjerling E, Agaton C, Szégyarto CA, Amini B, et al. (2005) A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 4: 1920–1932.

10. Margulies M, Egholm M, Altman W, Attiya S, Bader J, et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380.

11. Bentley DR (2006) Whole-genome re-sequencing. Curr Opin Genet Dev 16: 545–552.

12. Mardis E (2008) Next-generation DNA sequencing methods. Annual review of genomics and human genetics 9: 387–402.

13. Shendure J, Mitra RD, Varma C, Church G (2004) Advanced sequencing technologies: methods and goals. Nat Rev Genet 5: 335–344.

14. Shendure J (2008) The beginning of the end for microarrays? Nat Meth 5: 565–567.

15. Cloonan N, Forrest A, Kolle G, Gardiner B, Faulkner G, et al. (2008) Stem cell transcriptome profiling via massive-scale mRNA sequencing. Nat Meth 5: 613–619.

16. Wheeler DA, Srinivasan M, Egholm M, Shen Y, Chen L, et al. (2008) The complete genome of an individual by massively parallel DNA sequencing. Nature 452: 872–876.

17. Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, et al. (2009) A comprehensive catalogue of somatic mutations from a human cancer genome. Nature.

18. Ståhl PL, Stranneheim H, Åsgrum L, Berglund A, Pontén F, et al. (2010) Sun-induced nonsynonymous p53 mutations are extensively accumulated and tolerated in normal appearing human skin. The Journal of investigative dermatology.

19. Albert TJ, Molla MN, Muzzey DM, Nazareth L, Wheeler D, et al. (2007) Direct selection of human genomic loci by microarray hybridization. Nat Meth 4: 903–905.

20. Gnutke A, Meulikov A, Maguire J, Roegg P, LeProust EM, et al. (2009) Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nat Biotechnol 27: 182–189.

21. Porreca GJ, Zhang K, Li JB, Xie B, Austin D, et al. (2007) Multiplex amplification of large sets of human exons. Nat Meth 4: 901–906.

22. Budagalen J, Gilbert MT, Bollback JP, Fanste F, Bennett C, et al. (2007) The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. PLoS ONE 2: e197.

23. Lundin S, Stranneheim H, Peterson G, Kèrvebrèng D, Lundberg J (2010) Increased throughput by parallelization of library preparation for massive sequencing. PLoS ONE 5: e10029.

24. Nathanson KL, Wooster R, Weber BL, Nathanson KN (2001) Breast cancer genetics: what we know and what we need. Nat Med. pp 552–556.

25. Cirulli ET, Singh A, Shinnma KV, Ge D, Smith JP, et al. (2010) Screening the human exome: a comparison of whole genome and whole transcriptome sequencing. Genome Biol. pp R57.

26. Han H, Russo J, Kohwi Y, Kohwi-Shigematsu T (2008) SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature 452: 187–193.