Identification of targets for diagnosis and therapy in pancreatic neuroendocrine tumors

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Introduction

Alternative splicing is an important mechanism to increase diversity of the proteome in higher eukaryotic organisms. Alternative proteins isoforms are of special interest in tumor biology as they may provide a selective drug target or serve as a marker for cancer diagnosis[1].
While conventional identification of splice variants is labor intensive and generally targets individual genes[2], we present here a custom DNA microarray that allows identification of splice variation and differential expression of 357 G protein-coupled receptors and 11 proteins of the extracellular matrix.

GPCRs like somatostatin and dopamine receptors are established targets in neuroendocrine tumor disease. However, a considerable fraction of patients cannot be treated or diagnosed adequately using currently available agents. The G protein-coupled receptor superfamily is the most important hub for drug therapies[3]. In addition to their widespread expression and their important regulatory properties GPCRs are located on the plasma membrane which makes them easily accessible for both contrast agents and therapeutics.

Methods

We designed approximately 15k 60-mer oligonucleotide probes covering 358 genes (357 GPCRs and 11 surface proteins) and their known variant isoforms on a custom Agilent DNA-microarray. These probes were either homologous to exoninc, exon - exon junction or intron regions (Figure 1). By calculating the splicing index of each gene, targets with high probability of specific alternative splicing were identified[4].

A total number of 16 chips were made, hybridized with 8 pancreatic neuroendocrine tumor and 8 pancreatic control tissue samples. Gene expression results were validated by qRT-PCR, immunohistochemistry or Western blot, while splice variants where amplified by RT-PCR and sequenced afterwards.

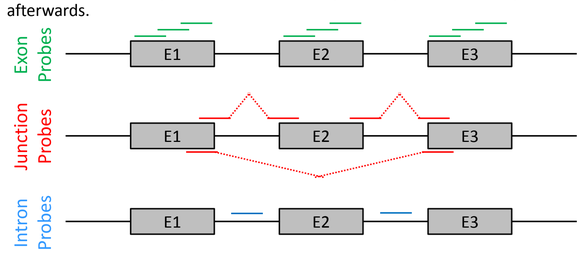

Fig. 1: Schematic representation of probe types used on custom array. A Intron probes (n=816) **B** Exon-exon junction probes (n=5209) **C** Exon probes (n=9173); grey boxes represent exons, lines in between intronic regions

Results

At the level of the complete chip, the control tissue samples are homogenous whereas the different tumor samples are more divergent. This effect is clearly visible in a correlation plot of the raw data since the control samples form a homogenous block (Fig. 2).

The results of the gene expression analysis is visualized in a double volcano plot (Fig. 3). The following two figures are examples for validations of the gene expression results. In Figure 4 an immunohistochemistry staining against Tubulin beta 3 (TUBB3) on control pancreas and pancreatic neuroendocrine tumor cryosections is shown. Quantitative real-time PCR results for six selected genes are shown in Fig. 5.

Melanocortin Receptor 1 was one of the validated targets. It could be shown that the neuroendocrine cell line LCC-18 is expressing the MC1 receptor by functional cAMP assays (Fig. 6).
Splice variants of four selected genes are represented in Figure 7.

Results

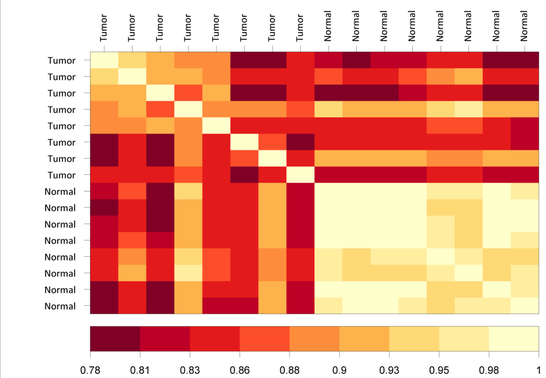

Fig. 2: Correlation Plot of all raw log_2 data; Samples with high similarity are shown in yellow, whereas more divergent samples are depicted in red

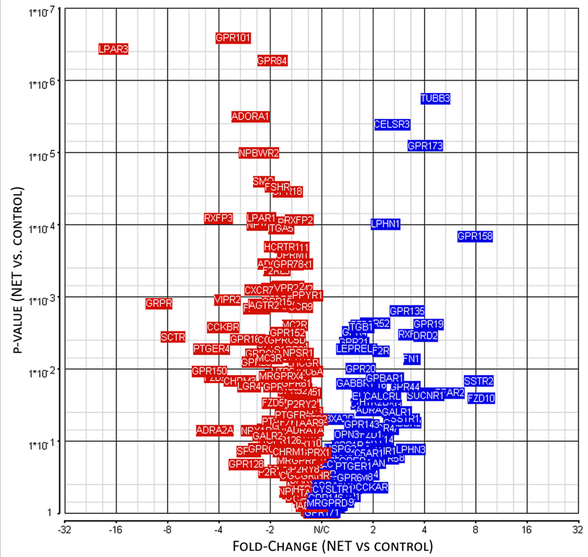

Fig. 3: Double Volcano plot at whole-gene level. Genes displayed in red showed higher signal intensities in control tissues. Blue colored genes were more abundant in pNET samples

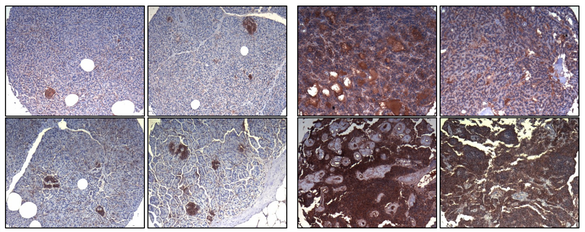

Fig. 4: Immunohistochemistry staining against TUBB3; left hand side control pancreas; right hand side pancreatic neuroendocrine tumor cryosections

Results

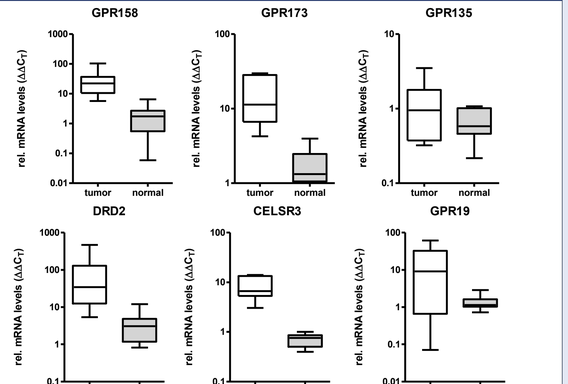

Fig. 5: TaqMan qRT-PCR was performed with 8 NET samples against 8 control samples to validate expression levels of 6 GPCRs. Relative mRNA levels were calculated by Livak method[5].

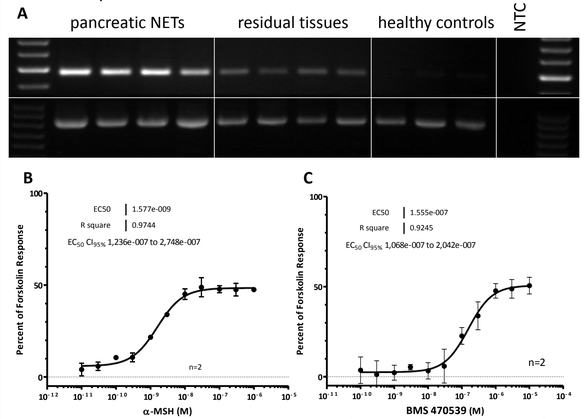

Fig. 6: A semiquantitative RT-PCR; Top: amplification of MC1 Receptor from pNETs, residual pancreatic tissues and from pancreas of non cancer patients; Bottom: beta-Actin control; NTC - no template control **B** Intracellular cAMP levels after stimulation with various α-MSH concentrations **C** Intracellular cAMP levels after stimulation with MC1R specific small molecule agonist[6]; n=2 error bars show 95% confidence interval

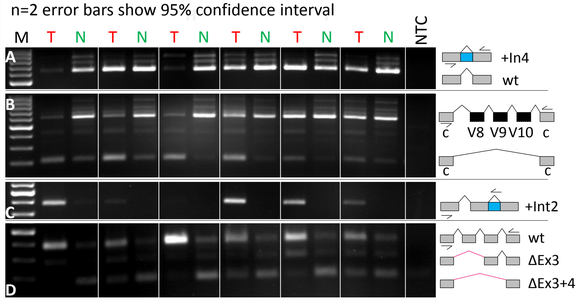

Fig. 7: RT-PCR validation of 4 candidate splicing events **A** CCKBR **B** CD44 **C** GPR179 **D** GIPR; on the right amplified gene regions are shown schematically, arrows symbolize primer positions

Conclusions

The DNA microarray presented here enables the parallel identification of alternative splicing events and gene expression in complex biological samples, providing a powerful tool to identify novel targets for therapy and diagnostic biomarkers.

In the expression level analysis several overexpressed genes were found and validated. While most of them were not known to be differential expressed in pancreatic NETs (e.g. GPR158, TUBB3, MC1R or GIPR) also familiar G-protein coupled receptors were found to be overexpressed (e.g. SSTR2 or DRD2).

By calculating the Splicing Index for all probe sets on the array, genes with a high probability for differentially expressed splice variants were selected and validated in RT-PCR. Novel variants specific for tumor as well as for the control tissues were identified.

References

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Acknowledgements

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