SMP: Epigenetics

Project: Methylation Marker Discovery

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Introduction

There has been a tremendously increased interest in DNA based diagnostics, since the investigation of molecular biological mechanisms has been extended to the genomic level and after the sequence of the human genome has been established. While most research is currently being devoted to the discovery and clinical application of genetic, proteomic and RNA expression markers, DNA methylation is rapidly emerging as a new level of cellular information. DNA methylation is involved in the regulation of gene activity. Aberrant DNA methylation has been associated with a variety of human diseases, and DNA methylation markers hold a great promise as a diagnostic tool, e.g. in early cancer screening [1]. In contrast to expression markers DNA methylation markers are accessible at the DNA level which enables their analysis even in archived paraffin-embedded tissue samples. Methods are in place to detect a few copies of aberrantly methylated DNA in a background of a vast excess of normally methylated DNA which is the precondition for assaying methylation markers also in body fluids that are readily accessible in clinical practice.

Several methods are available for the genome-wide discovery of methylation markers differentiating between different types of tissue samples [1]. They are mainly based on the use of methylation specific enzymes. The most prominent examples are restriction landmark genomic scanning (RLGS), representational difference analysis (RDA), methylated CpG Island amplification (MCA)-RDA, methylation sensitive arbitrarily primed PCR (MS-AP-PCR) and differential methylation hybridization (DMH) (Ushijima, T; 2005). However, non of these published methods fulfil all of the following important requirements:

· usage of small DNA sample amounts;
· viability for analysis of paraffin embedded tissues;
· real genome-wide coverage;
· viability for parallel analysis of unlimited number of samples;
· high reproducibility;
· fast and easy handling.

The focus of our work is in the optimzation and implementation of methylation marker discovery methods fulfilling these criteria as well as of the techniques required to validate multimarker panels on large numbers of clinical DNA samples in a highly standardized fashion.

Results/Project Status

Today DNA methylation analysis is generally accomplished applying two basic principles, i.e. methylation sensitive restriction and bisulfite-mediated conversion of unmethylated cytosine to uracil. Upon methylation sensitive restriction unmethylated doublestranded DNA is digested, whereas methylated DNA remains intact and is subjected to amplification and sequence specific detection. In contrast bisulfite conversion translates the cytosine methylation information into a primary DNA sequence information which is then tracked by one of a plethora of standard techniques, e.g. Sanger sequencing or SNP analysis.

Methylation marker discovery as introduced above has two preconditions: On the one hand the preanalytics workflow needs to optimised towards meeting the different criteria described. This includes the generation of an amplificate with maximum complexity as well as the optimisation towards small sample amounts and different and challenging types of samples. On the other hand an analytical platform must be

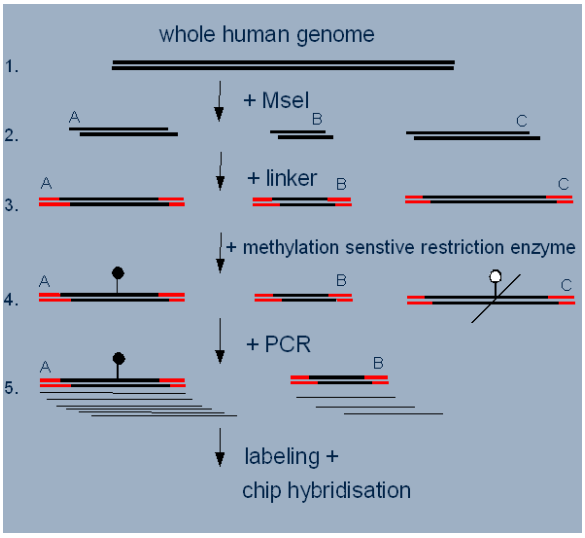

Fig 1: *Workflow of the DMH-amplicon preparation.*

developed which enables a highly sequence specific detection of the labelled amplicons in a quantitative way. In the past year an intensive effort at Epigenomics has led to improvements of the published DMH workflow as presented in Fig. 1 [2]. DMH was chosen because it is not limited to comparative methylation analysis between different samples. Analysis is performed sample by sample and data can be collected in a growing database enabling also retrospective analyses. In particular the mix of methylation sensitive restriction enzymes was designed to give a digest with a narrow fragment length distribution which is a precondition for reproducible and representative amplification (data not shown).

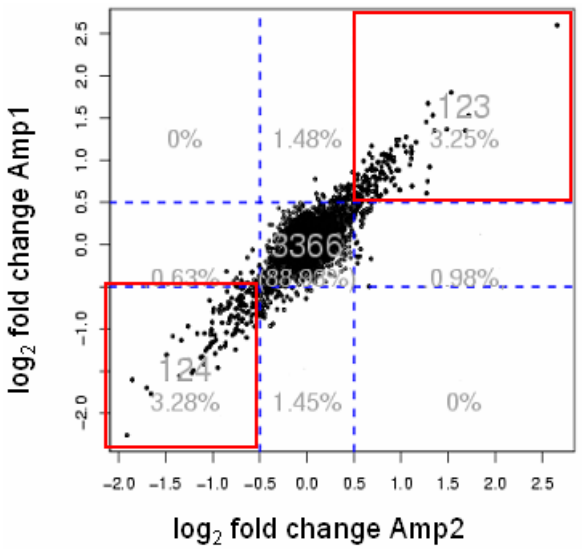

Fig 2: *Correlation plot of two DMH discovery experiments using PBL and breast cancer cell line samples. Spots in the red boxes indicate potential marker fragments reproducibly found in both experiments to be either methylated or unmethylated in breast cancer cell line compared to the PBL.*

Together with an optimised amplification and labelling protocol this workflow was successfully applied to the comparative methylation analysis of peripheral blood lymphocytes and a breast cancer cell line (Fig. 2). A custom made Affymetrix microarray carrying an initial selection of features designed for protocol optimisation was used to analyse the amplicon. First results indicate that the optimised preanalytics workflow generates promising marker candidates and can be conducted in a highly reproducible manner.
In the next step attempts will be made to optimise the preanalytics workflow for analysing DNA from paraffin-embedded tissues which represent a readily available type of sample especially for retrospective studies. In addition an optimised microarray is being designed to ensure that all genomic regions which are expected to be subject to differential methylation are covered by oligonucleotide probes according to state of the art.

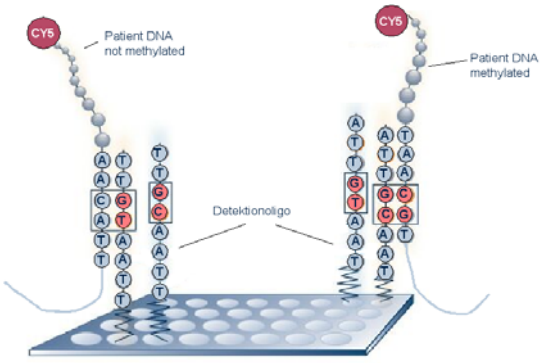

Fig 3.: *Methylation analysis by bisulfite PCR product hybridization onto arrayed oligonucleotide probes.*

Although a DMH utilizing the optimised preanalytics workflow in conjunction with a second generation custom-made microarray is expected to deliver methylation marker candidates at a low false positive rate the validation of these candidates on hundreds of clinical sample is a vital step of the marker development process. Different techniques are available for analysing DNA methylation at individual loci [1], most of them based on bisulfite conversion of sample DNA. At Epigenomics a methodology for the parallel methylation analysis of hundreds of CpG positions based on the hybridisation of bisulfite PCR products onto arrayed oligonucleotide probes has been developed [3] (Figure 3). This method has been successfully applied to the validation of candidate methylation markers in patient sample sets.

Outlook

In the framework of the NGFN2 project it is planned to evaluate different array platforms based on different principles for signal generation for their potential for methylation analysis via hybridisation of bisulfite PCR products. Once a suitable analytical platform has been identified an integrated workflow combining all steps from sample preanalytics to data analysis will be established and optimized using established reference markers. The ultimate goal is to establish a platform which enables clinical studies based on methylation marker analysis to be performed in a highly standardized manner in a sufficient throughput to support the development of diagnostic tests.

Lit.: ***1.*** *Laird PW. The Power and the promise of DNA methylation markers. Nature Rev. Cancer 2003;3:253-66.* ***2.*** *This work was supported by the BMBF Foerderprojekt 0313030C Technologien zur Charakterisierung des humanen regulatorischen Proteom.* ***3.*** *Adorjan P. et al. Tumour class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acids Research, 2002, 30(5), e21.*