Evaluation of different approaches to analyze *de novo* methylation of *MGMT*

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Introduction

Resistance to chemotherapy is a major complication during treatment of cancer patients. Hypermethylation of the *MGMT*-gene alters DNA repair and is associated with longer survival of glioblastoma patients treated with alkylating agents (Esteller 2000, Hegi 2005). Methylation of *MGMT* is also found in several other cancer entities. Therefore *MGMT* plays an important role as a predictive epigenetic marker for chemotherapy resistance. To adopt this established correlation into a molecular diagnostic procedure, we compared different technologies with regard to their sensitive and reproducible detection of methylation in frozen and paraffin embedded tissues.

Material and Methods

DNA samples.

Genomic DNA was extracted either from frozen or formalin-fixed, paraffin-embedded (FFPE) glioblastoma multiforme (GBM) samples. For this purpose we used standard proteinase K digestion followed by phenol/chloroform extraction and the QIAamp DNA Mini Kit, respectively.

Bisulfite treatment and bisulfite sequencing.

Genomic DNA was subjected to bisulfite conversion with the EpiTect Bisulfite Kit. Following bisulfite PCR the products were cloned by using the TOPO TA Cloning Kit and the clones were subjected to sequencing employing the BigDye V.1.1 Cycle Sequencing chemistry and separated on a 3130 Genetic Analyzer. Single clone sequences (Fig. 1) were analyzed and visualized with the BiQ Analyzer software (Bock 2005).

Combined Bisulfite Restriction Analysis (COBRA; Xiong 1997).

The labeled PCR products generated by a pseudo-nested PCR from bisulfite treated tumor samples and control DNA were digested with the restriction endonucleases *TaqI* and *BstUI* and loaded onto an ABI 377 sequencer (Fig. 2a). *TaqI* has the recognition site TCGA, whereas *BstUI* cuts the site CGCG. The restriction sites are conserved when the respective samples were methylated at these sites. Unmethylated samples are changed by bisulfite treatment into TTGA for *TaqI* and TGCG, CGTG, and TGTG for *BstUI*, respectively, and are not recognized by these enzymes. The electropherograms were analyzed and the methylation levels were calculated.

SIRPH (SNuPE IP-RP HPLC; El-Maarri 2002).

PCR products were treated with ExoSAP-IT and added to the primer extension mix. Extension was performed by ddCTP and ddTTP. Subsequent separation of the extended primer products utilized dHPLC (Transgenomics) (Fig. 2b).

Pyrosequencing (Tost 2003).

The PCR products, containing the informative region, were subjected to Pyrosequencing. The sequencing reaction was performed on an automated PSQ 96MA System (Biotage) using the Pyro Gold Reagents Kit. After purification and subsequent processing of the biotinylated single-strand DNA the resulting data was analyzed and quantified with the PSQ 96MA 2.1 software (Fig. 2c).

Results and Conclusion

We identified most informative CpG positions in the *MGMT* promoter region discriminating glioblastoma DNA from normal brain tissues. As shown in Fig. 1 the tumor samples show methylation pattern with significant variation regarding to the content of methylated alleles as well as to the positions of methylated CpG sites. Technologies that rely on the amplification of methylated and unmethylated alleles in separate reactions, may lead to false positive/negative results due to the mosaicsm methylation pattern of the target, heterogenous cell populations and mis-priming and therefore should be used with great caution especially if a therapeutic decision is desired.

The results of a comprehensive investigation of individual clones from 22 GBM cases and age matched normal brain tissues were used for development of three different assays to assess the methylation state at these positions. We constructed candidate methylation markers which can be readily analyzed by COBRA, SIRPH or Pyrosequencing. Each epigenetic marker candidate was statistically evaluated on the full methylation profiles, and for each method optimal marker candidates were selected based on two criteria: strong correlation with overall promoter methylation and suitability/availability for testing by a given method. All selected markers were validated on a set of tumor samples with predefined methylation status for their experimental accuracy. Finally, we compared these approaches with regard to their robustness, significance and reproducibility on frozen as well as FFPE clinical specimen. For both the COBRA (5 CpG sites) and the Pyrosequencing marker candidate (4 CpG sites), logistic regression led to correct classification of all 13 tumor samples (100% test set accuracy as determined by leave-one-out cross-validation). For the SIRPH marker (1 CpG site), 12 out of 13 tumor samples were classified correctly (92% test set accuracy). The Pyrosequencing assay allowed best separation of methylated and unmethylated samples, as well as correct classification of borderline cases (Fig. 3, sample 16) which show few methylated alleles in a large number of unmethylated alleles (Fig. 1, sample a and c). The marker's robustness was also confirmed on FFPE specimen, opening up the possibility to accurately investigate *MGMT* methylation in archival tissues. A comparison of the pyrograms obtained from matched frozen and FFPE tissues showed virtually identical results (Fig. 4). Only little variation with regard to the methylation degree of individual positions was observed, which may be explained by slight heterogeneity of *MGMT* methylation pattern in spacial separated regions in the same tumor. However, most importantly the overall methylation score as determined by the interrogated CpG positions led to the same molecular diagnostic decision.

The COBRA marker also provides a good separation of methylated and unmethylated samples but the classification of borderline cases is less accurate than for the Pyrosequencing marker. For the SIRPH marker the separation was generally low due to high score variance within tumor subclasses and the fact, that only one CpG position is interrogated.

We conclude that the established Pyrosequencing assay is suitable for a clinical application and allows accurate, sensitive, and quantitative identification of *MGMT* methylation in clinical samples.

Fig.1

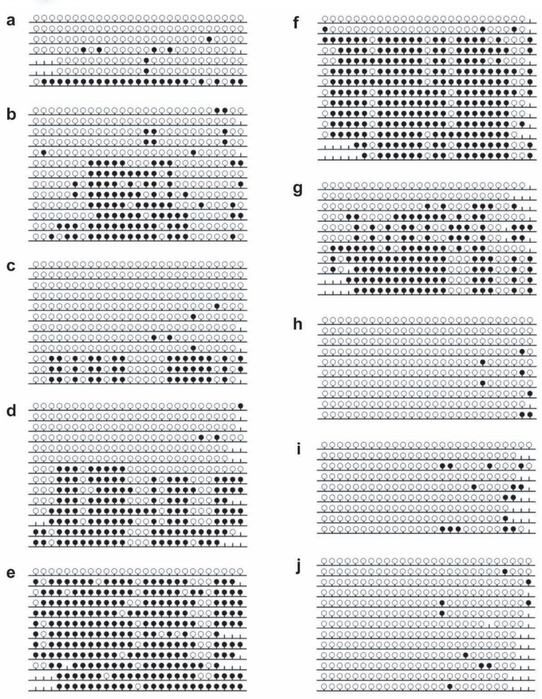

Fig. 1: Methylation pattern obtained by bisulfite sequencing of single clones. a-i: tumor samples, j: control sample. Filled circles correspond to methylated CpG positions, unfilled circles correspond to unmethylated CpG positions, and the vertical lines without a circle corresponds to CpG positions not determined in the sequence.

Fig.2a

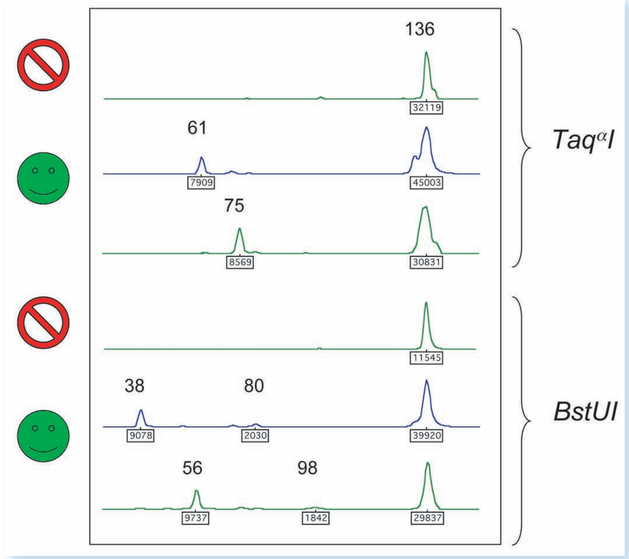

Fig. 2a: Restriction pattern obtained in the COBRA assay. An unmethylated sample shows only a signal for the undigested PCR product (red symbol), while a methylated sample is cut by the restriction endonucleases at different sites generating fragments of different size (green symbol).

Fig.2b

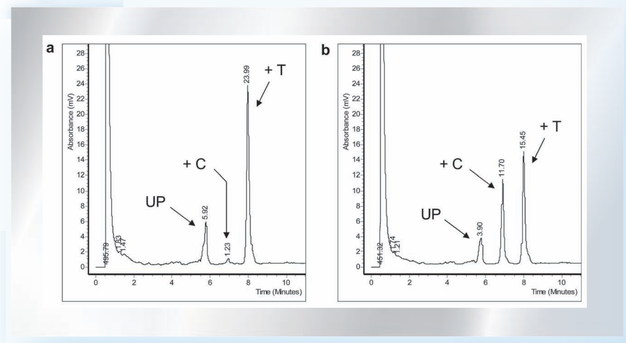

Fig. 2b: Typical chromatograms obtained in the SIRPH assay for an unmethylated sample (a), and for a methylated sample (b). UP: signal of the unextended primer; +C and +T: signal of the ddCTP and ddTTP extended primer, respectively. The signal seen in the beginning of the chromatogram is due to loading the sample on the dHPLC column.

Fig.2c

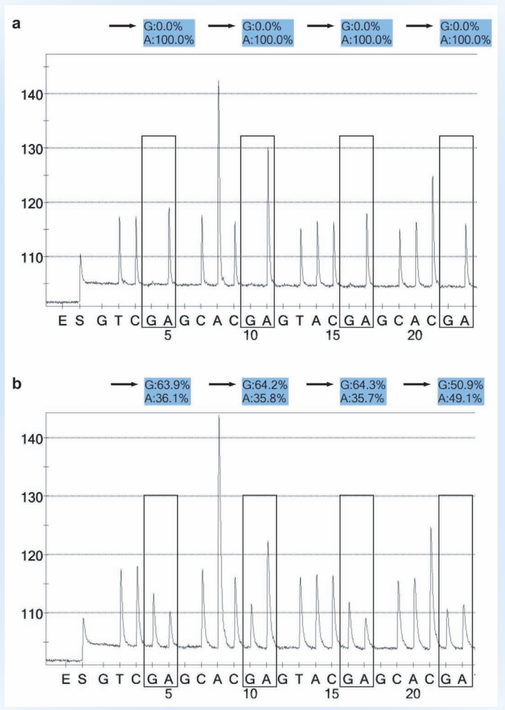

Fig. 2c: Typical pyrograms obtained for an unmethylated sample (a), and for a methylated sample (b). Each box represents one of the four CpG positions interrogated by the Pyrosequencing assay. Due to reverse sequencing the upper strand of the PCR product a C/TpG position appears as a CpG/A. The incorporation of the base guanine in this context represents the methylated fraction (arrows) and the base adenine the unmethylated fraction, respectively. Sample (b) shows, that more than 50% of the alleles present in the tumor DNA are methylated at all 4 positions.

Fig.3

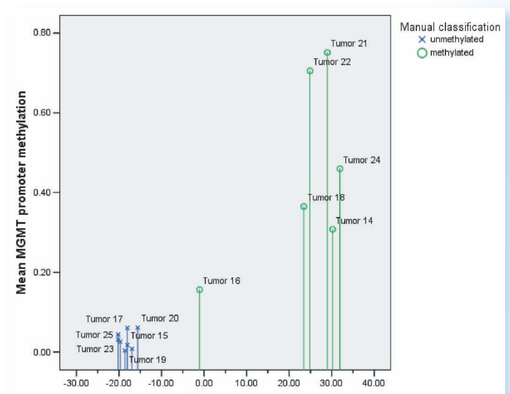

Fig. 3: Marker score for the Pyrosequencing assay plotted against overallmethylation level.

Fig.4

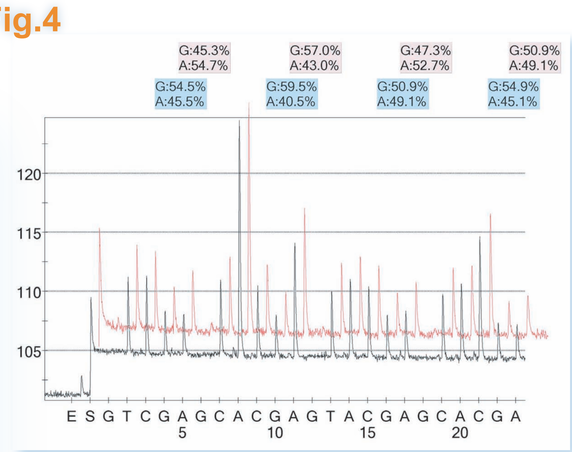

Fig. 4: Pyrograms obtained from matched frozen (black) and FFPE (pink) tissues show virtually the same result.

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