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Investigation of DNA methylation signatures and miRNA expression levels to identify potential prognostic and/or predictive biomarkers for colorectal cancer

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DNA methylation signatures were identified as potential early detection markers in colorectal cancer. Moreover, they can indicate different phenotypes of the primary tumor and thereby influence the prognosis or even the response to an anticancer therapy of the patient. To date, the stage in which CRC is diagnosed is used for the estimation of prognosis, furthermore it designates the treatment of the patient with only little or no respect to other predictive factors. Another class of alterations influencing CRC development and progression is the dysregulation of microRNAs (miRNAs). Their role in several cellular functions e.g. cell differentiation, proliferation and apoptosis lead to an intensive research to compare the expression levels between normal colonic mucosa and CRC tumor tissue and their impact on patients prognosis and therapy response. However, only few miRNAs differentially expressed in the tumor tissue could be identified to have a prognostic or predictive information.
The purpose of the present study was to elucidate if the methylation levels at distinct cytosine-guanine dinucleotides (CpG loci) or differentially expressed miRNAs can be associated with the prognosis or predict survival under chemotherapy in patients diagnosed with stage II or III colon cancer. The study population comprised of formalin-fixed, paraffin-embedded primary tumors of patients who either received a combined chemotherapy with 5-Fluorouracil (5-FU) and folic acid or no chemotherapy.
Two independent approaches to quantify methylation levels at distinct CpG loci were performed. The GoldenGate Methylation Cancer Panel I (Illumina) was used to analyse 1505 CpG loci in 807 cancer related genes. In addition, a total number of 51 candidate genes not represented on the array were selected based on their role in 5-FU and folic acid metabolism, or DNA repair as well as a known influence in the prognosis of CRC. In total, 75 distinct CpG loci in 18 out of the 51 selected candi-date genes were analysed for their methylation levels using mass spectrometric analysis (MassARRAY, SEQUENOM). To identify potential prognostic or predictive biomarkers, methylation levels were separated using two different thresholds with respect to tumor suppression or patients overall survival, either tumors showing high methylation levels over 90% were compared to those with lower methylation levels (<90%) or tumors showing low methylation levels (<10%) to those with higher methylation levels (>10%).
Concordance regression analysis revealed two independent prognostic CpG loci in the EGF and MPO genes, both identified with the GoldenGate Methylation Cancer Panel I. Both potential prognostic CpG loci were associated with improved overall survival (OS) and progression free survival (PFS) if they were methylated >90%.
Independently, a CpG locus in CSF3R and HYAL2 were observed to have predictive biomarker poten-tial while no prognostic effect was observed. CSF3R was identified using the GoldenGate Methylation Cancer Panel I and HYAL2 with the candidate gene approach. For the CpG in CSF3R, microsatellite (MS) stable colon cancer patients who received a 5-FU chemotherapy combined with folic acid had better OS if the methylation levels were <10% compared to those patients with higher methylation levels. Interestingly, methylation levels >10% at this distinct CpG locus was strongly associated with dramatic worse OS and PFS under therapy compared to

patients with lower methylation levels. This indicates that patients with a MS stable primary tumor with methylation levels >10% should not be recommended for a combined chemotherapy of 5-FU and folic acid. Independently, low methylation levels (<10%) at the CpG locus in HYAL2 predicted better OS and PFS under chemotherapy. Further-more, if a chemotherapy is applied patients with low methylation levels (<10%) in the primary tumor showed better benefit from the therapy than those with higher methylation levels. Thus, patients with these features should be suggested to therapy even in stage II.

The biomarkers identified as the most potential ones exhibit prognostic and predictive information. While high methylation levels (>90%) at CpG loci analysed in the FRZB gene were associated with worse OS and also tend to indicate worse PFS of stage II and III CRC patients, the on the other hand a methylation level >90% predicted an improved OS and PFS under 5-FU chemotherapy combined with folic acid. The same observations were made for methylation levels of CpG loci localized in the promoter region of the candidate gene DKK2. The proteins encoded by both genes are involved in the inhibition of the wnt signaling pathway and were identified separately by the GoldenGate Methylation Cancer Panel I and MassARRAY analysis. The observed prognostic impact of the CpG loci in the DKK2 gene was even stronger since almost all CpG loci in the analysed region showed a significant association with OS and PFS as well as survival under therapy. Further potential prognostic and predictive biomarkers were the CpG loci analysed in the MAL promoter region. High methylation levels (>90%) were associated with worse OS and PFS of stage II and III CRC patients. Furthermore, low methylation levels (<10%) showed the inverse association. In respect to a given chemotherapy consisting of 5-FU and folic acid, patients with methylation levels less than 10% showed bigger benefit than those with higher methylation levels.

Due to the enormous stability of miRNAs in FFPE tissue, differentially expressed miRNAs were iden-tified and their usage as prognostic or predictive biomarkers was evaluated using Human microRNA Microarray and qRT-PCR. With the microarray 38 miRNAs were shown to be differentially expressed between patients who survived at least five years after diagnosis and patients who died within the first three years. Out of these, six miRNAs could be verified to be differentially expressed. However, according to the results obtained in the present study none of them could be suggested as potential prog-nostic or predictive biomarker.

In conclusion, we investigated CpG loci in 807 cancer related genes using the GoldenGate Methy-lation Cancer Panel I and further 18 candidate genes using mass spectrometric analysis to identify independent potential biomarkers: Methylation of CpG loci in EGF and MPO as potential prognos-tic biomarkers, in CSF3R and HYAL2 as potential predictive biomarkers and furthermore CpG loci in FRZB, DKK2 and MAL as potential prognostic and predictive biomarkers.

Using the methylation levels of one or more of the potential prognostic CpG loci in clinical routine could lead to an improved understanding and classification of patients survival. In addition, the methy-lation levels at potential predictive biomarkers identified in the present study could be used to select or reject patients for chemotherapy as a supporting factor to the stage at diagnosis. Thus, a more personalized therapy can be achieved. As most chemotherapeutic strategies for CRC are based on 5-FU, the findings of the present study might have the potential to be translated into clinical applications. However, further studies including more patients are necessary to support the potential usage of the identified biomarkers of this study. In addition, the identified biomarker candidates should be analysed for different anticancer therapy strategies and drugs to investigate their negotiability on predicting therapeutic outcome of colorectal cancer patients.