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Investigation of DNA methylation signatures and circulating microRNA expression levels to identify potential prognostic biomarkers for colorectal cancer

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Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide showing a high mortality rate of 694,000 deaths per year with metastasis as the major cause of death. The lack of effective treatment options for the late CRC stages requires a better understanding of distant metastasis formation processes. The gold standard for the prediction of patients' prognosis and therapy response is the tumor-node-metastasis (TNM) staging system. However, the current classification method provides in many cases only limited prognostic information and even patients within the same TNM stage can show a strong heterogeneity for prognosis and treatment response. Therefore, the main aim of this study was to identify efficient and reliable biomarkers that could be incorporated into the traditional clinical classification as a tool for the prediction of patients' prognosis.

The identification of potential prognostic markers for CRC patients was focused on two different types of biomarkers: deoxyribonucleic acid (DNA) methylation markers identified from formalin-fixed and paraffin-embedded (FFPE) tissue samples and circulating micro-ribonucleic acid (miRNA) markers analyzed in the blood plasma. The included patients were all participating in the 'Darmkrebs: Chancen der Verhütung durch Screening' (DACHS) study, an ongoing, long-term population-based case-control study on CRC carried out in the southwest of Germany. CRC patients of all stages (UICC stage I-IV) receiving different therapies were investigated.

The alteration of DNA methylation levels between normal mucosa and CRC tissue have been identified as an early event during CRC tumorigenesis. Increasing evidence suggests that alterations in methylation signatures can not only be applied as cancer risk factors and biomarkers for early detection but also for the prediction of patients' prognosis. In order to detect potential DNA methylation markers for CRC prognosis, the methylation levels of over 450,000 single cytosine-phosphate-guanine dinucleotide (CpG) sites were analyzed in an epigenome wide manner, using the Infinium HumanMethylation450 BeadChip array (450K array) in two patient cohorts, the screening cohort I (n=720) and the validation cohort II (n=480). For the screening procedure in cohort I two independent approaches were conducted, once an epigenome-wide approach using all valid CpG sites on the 450K array and once focusing on CpG sites in 71 selected candidate genes. Using principled sure independence screening for Cox models, the screening for prognostic markers for overall survival

(OS) and relapse-free survival (RFS) revealed 201 candidate CpG sites in the epigenome wide approach and 99 in the candidate-based approach. In a replication analysis in validation cohort II, the prognostic value of 23 and 16 CpG sites, respectively could be successfully validated. After adjustment for multiple testing the methylation levels at specific CpG sites in the genes *ESYT2*, identified with the epigenome-wide approach, and *c-MYC* and *USP22* identified with the candidate-based approach still showed significant associations on the prognosis of metastatic colon carcinoma (CC) patients and were thus regarded as the most promising identified methylation markers. Hypermethylation at cg14395731 in the gene body of *ESYT2*, was associated with a 3.1-5.7 fold decreased OS in metastatic CC patients in both cohorts. Independently, increased methylation levels at cg17505251 in the oncogene *c-MYC* were associated with protective effect (2.1-2.6 fold) as well as increased methylation at cg11691341 in *USP22*, a target gene of *c-MYC*, with a higher risk of death (3.9-6.3 fold) for metastatic CC patients.

A technical verification of the 450K array results from selected CpG sites with the MassARRAY system revealed good correlations.

The second type of biomarkers analyzed in this study, were circulating miRNAs in patients' blood plasma. Their high stability, the ease of access through minimally-invasive techniques and the possibility of repeated sampling makes them almost ideal biomarker candidates for diagnosis, prognosis and monitoring during therapy and follow-up. Several steps during metastasis formation were shown to be associated with changes in miRNA levels. After comprehensive literature study, 95 miRNAs, with a special focus on miRNAs known to be involved in metastasis formation processes were selected for the generation of Custom TaqMan® Array MicroRNA Cards. The profiling cohort (n=40), including 20 primary and 20 metastatic colon cancer patients, each group consisting of 10 samples with good and 10 samples with poor prognosis, was profiled using the array cards. Good prognosis was defined as no event (relapse, metastasis or cancer-related death), occurring for more than 5 years in primary and 2.5 years in metastatic cases and poor prognosis as an event occurring within one year. Identified and selected candidate miRNAs were further validated by RT-qPCR in the whole study cohort of 543 (309 primary and 234 metastatic) CRC patients. The association of the miRNA levels with patients' survival and the prognostic subtypes was analyzed with uni- and multivariate logistic regression and Cox proportional hazards regression models. Potential influencing factors on the miRNA levels, such as hemolysis in the blood plasma and the time-point of blood collection were taken into account for the analysis. Increased miR-122 levels were associated with a poor prognostic subtype in metastatic CRC (Odds ratio: 1.56, 95% confidence interval (CI): 1.04-2.35) and a shorter RFS and OS for non-metastatic (Hazard ratio (HR): 1.37, 95% CI: 1.03-1.83; HR: 1.35, 95% CI: 1.00-1.83) and metastatic (HR: 1.26, 95% CI: 1.05-1.52; HR: 1.29, 95% CI: 1.08-1.55) CRC patients. Additionally, several members of the miR-200 family showed associations with patients' prognosis and correlations to clinicopathological characteristics.

The identified DNA methylation markers as well as the circulating miRNA markers could, in combination with currently used classification methods, improve the prognostic accuracy in the future. Whereas the tumor DNA methylation markers could be valuable for prognosis prediction directly after surgery, the plasma miRNA markers could be of potential use in the development of a molecular multi-marker blood test for monitoring.