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Original Article

Expression of cancer stem cell markers in metastatic colorectal cancer correlates with liver metastasis, but not with metastasis to the central nervous system

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ABSTRACT

Introduction: In colorectal cancer (CRC), metastatic spread is supposed to be mainly driven by tumor cells with stem cell features. Only about 1% of all CRC patients develop metastasis to the central nervous system (CNS). The present study intended to analyze the correlation between the expression of cancer stem cell markers and patterns of liver or CNS metastases.
Material and methods: Immunohistochemistry for β-catenin, CD133, CD44 and the mismatch-repair markers hMLH1 and hMSH2 was applied to primary specimen of two CRC cohorts with CNS ($n=29$) and exclusive liver metastasis ($n=36$). Furthermore, mutation analysis for *KRAS* exon 2 and *BRAF* exon 15 was performed.
Results: The expression of nuclear β-catenin, CD44 and CD133 was associated with the development of liver metastasis, but not of CNS metastasis. CD133 expression was absent in CRC with solitary CNS metastasis. Combination of cancer stem cell markers revealed high discriminatory power for the prediction of different patterns of distant spread. *KRAS* mutation was more frequently detected in patients with CNS metastasis, but the mutational status of *KRAS* and *BRAF* failed to show correlation with clinico-pathological data or the results of immunohistochemistry.
Conclusions: This study demonstrates that deregulation of Wnt/β-catenin-signaling and high-grade expression of cancer stem cell markers correlate with metastasis to the liver, but not to the CNS. These data implicate that in CRC other mechanisms than deregulation of Wnt/β-catenin-signaling and acquisition of cancer stemness are required for formation of CNS metastasis.
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1. Introduction

Approximately 50% of all colorectal cancer (CRC) patients are either diagnosed with primary metastatic disease (20–25%) or develop metastases during their course of disease (25%) [6,7,23,24]. Hematogenous spread to the liver represents the most common type of distant metastases (70–80%), whereas lung (10–35%) and others organs are less frequently affected. Only about 1% of all CRC patients present with CNS metastasis [10,22].

The majority of sporadic CRC develop via the adenoma-carcinoma sequence starting with either loss of APC function or oncogenic β-catenin mutations, triggering a dysregulation of Wnt/β-catenin-signaling and subsequently of Wnt/β-catenin-dependent colonic cancer stem cells (CSCs) [4,5,9,17]. Recently, we could link high nuclear β-catenin expression with poor prognosis and progression in CRC [15]. Furthermore, the cell surface markers CD133 and CD44 have been shown to be associated with colonic CSCs and also poor prognosis in CRC [12–14,16]. Thereupon, we defined an immunohistochemical algorithm indicating an extremely high and low risk of distant metastases in

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right-sided colon cancer [20]. We demonstrated that CRC with a mismatch-repair deficiency (MMRD) proven by loss of hMLH1-protein expression was significantly associated with a very low risk of distant metastases. In contrast, hMLH1-positive cases with combined expression of β-catenin and CD133 are associated with an increased risk of distant metastases to the liver.

Up to now, it is still not known why some CRC metastasize to 'atypical sites' as the brain, whereas in the majority of CRC only liver metastasis is found. The aim of this study was to investigate and to compare the primary CRC tissue from two different metastatic CRC patient groups, namely (1) patients with CNS metastasis, that represent a very exclusive and rare patient population, and (2) patients with the 'typical' metastatic pattern to the liver as 'control group'. In particular, we intended to examine the significance of cancer stem cell markers and Wnt/β-catenin expression in the context of metastasis to the liver and to the CNS on the background of our recently defined algorithm as described above.

2. Material and methods

2.1. Tissue selection

We performed a database query in cooperation with the Munich Cancer Registry (MCR) on the search criteria 'colorectal cancer', 'central nervous system' (CNS) and 'brain metastasis', respectively. Patients with other than colorectal malignancies were excluded. From all selected patients who underwent surgical tumor resection at the Ludwig-Maximilians-Universität (LMU) München between 1999 and 2011, primary tumor tissue was obtained from the archives of the Institute of Pathology. The corresponding clinical datasets were retrieved from MCR.

Formalin fixed-, paraffin-embedded (FFPE) samples of primary colorectal adenocarcinoma from 29 patients with metastatic spread to the CNS were evaluable. For each patient one tumor block, covering representative parts of the adenocarcinoma and adjacent normal colonic mucosa, were selected. Four CRC patients (6.2%) presented with solitary metastatic spread to the central nervous system whereas combined spread to the CNS and other sites was diagnosed in 25 patients (38.5%). As control cohort, 36 patients with exclusive metastatic spread to the liver with preserved nuclear expression of hMLH1 and hMSH2 were selected. Lesions in the CNS and the liver were assumed as metastasis when determined by radiological and clinical assessment by the physicians in charge (cM1) but not necessarily needed to be proven by histology, as this represents the international standard for staging in metastatic disease. The study was approved by the local ethics committee (approval number 505-11).

2.2. Immunohistochemistry

For immunohistochemistry, 5 μm standard tissue sections of representative FFPE tumor samples were used and stainings were done employing a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ) following the manufacturer's instructions. A detailed description of antibodies and protocols used in this study is provided in Table 1. To exclude unspecific reactions of antibodies and/or reagents, isotype and system controls were performed.

2.3. Scoring of immunohistochemistry

All samples were evaluated independently by two investigators (JN and MM), both blinded for the clinical outcome. In case of discrepancy, a consensus agreement was jointly reached. A staining score for nuclear expression of β-catenin was based on the quantity of stained tumor cell nuclei throughout the whole tumor, whereas intensity of staining was not considered. The score was as follows: 0: negative, 1+: <30%, 2+: 30–60%, 3+: >60% positive cells. In addition, the cases were classified into low (scores 0 and 1) and high grade (scores 2 and 3) expression [20]. For CD133, positivity was defined as either staining of apical membranous parts of the cells or of shed cellular debris in the tumor glands. CD133 expression levels were scored as low grade (<50% of positive tumor glands) or high grade (≥50% positive tumor glands) [16]. CD44 staining was semiquantitatively classified based on the percentage of tumor cells with membranous staining, with a scoring similar to that done for CD133: tumors with less than 50% CD44 positive cells were considered as CD44-low, while those with 50% or more positive tumor cells were CD44-high [14]. Since β-catenin, CD133 and CD44 showed heterogeneous expression patterns throughout the tumor percentages were estimated referring to the whole amount of tumor covered on the slide. Loss of hMLH1 and hMSH2 expression (reflecting a mismatch-repair deficiency (MMRD)) was recorded when nuclear staining was absent in malignant cells, but preserved in the stroma cells or normal epithelial cells, respectively. Cases with preserved nuclear hMLH1 and hMSH2 expression in tumor cells were classified as cases without MMRD.

2.4. Mutation analysis

Mutation analysis for *KRAS* exon 2 and *BRAF* exon 15 was performed employing pyro-sequencing according to protocols previously published by our group [21]. In brief, for enrichment of tumor tissue, H&E-stained histological serial sections were inspected, and areas containing tumor cells were defined, marked and used as blue-prints for microdissection. From the resulting tissue, DNA was isolated using DNA-Micro-Amp® Kits (Qiagen, Hilden, Germany) following the user's handbook. For pyrosequencing Hotstar Taq-polymerase (Qiagen, Hilden, Germany) was used together with 1xPCR buffer (1.5 mM $MgCl_2$), 200 μM dNTPs and 400 nM primers. PCR products were analyzed using Pyro-Gold kits (Qiagen, Hilden, Germany) together with 3 nM of the corresponding sequencing primer employing the Biotage™ Q24 device (Qiagen, Hilden, Germany). Finally, data were analyzed applying the PyroMark™ Q24 software (Qiagen, Hilden, Germany). All primer sequences and detailed PCR-condition are reported in Reference [21].

2.5. Statistics

Significance of correlations of the immunohistochemical analyses was tested using the two-sided χ^2-test (SPSS v. 19.0, IBM Inc.,

Table 1
Antibodies used for this study.

Antibody	Company	Source	Clone	Dilution	Detection system
β-catenin	Ventana Medical Systems, Oro Valley, AZ	Mouse monoclonal	Clone 14	Ready to use	Ventana OptiView DAB IHC Detection Kit
CD44	BD Biosience, Franklin Lakes, NJ	Mouse monoclonal	G44-26	1:500	Ventana OptiView DAB IHC Detection Kit
CD133	Miltenyi Biotec, Auburn, CA	Mouse monoclonal	AC133	1:50	Ventana OptiView DAB IHC Detection Kit
MLH1	Leica Biosystems, Newcastle upon Tyne, UK	Mouse monoclonal	ES05	1:200	Ventana OptiView DAB IHC Detection Kit
MSH2	Cell Marque, Rocklin, CA	Mouse monoclonal	G219-1129	Ready to use	Ventana OptiView DAB IHC Detection Kit

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