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ORIGINAL ARTICLE

Enhanced Tumoral MLH1-Expression in MLH1-/PMS2-Deficient Colon Cancer Is Indicative of Sporadic Colon Cancer and Not HNPCC

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Abstract

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is caused by germline mutations of mismatch-repair (MMR) genes MLH1, MSH2, MSH6 and PMS2. MLH1-/PMS2-deficient colorectal carcinomas might be HNPCC-associated but also caused by MLH1-promoter methylation in sporadic colon carcinoma. This study analyzed semiquantitatively whether the MLH1 staining pattern might be indicative of sporadic or HNPCC-associated colorectal cancer. Using a semiquantitative score ranging from 0 (negative) to 12 (maximum immunopositivity) we analyzed MLH1 expression patterns in 130 MLH1-/PMS2-deficient colorectal cancers. The collective consisted of 70 HNPCC-associated colorectal cancers and 60 sporadic colon cancers. In tumor cells of 70 HNPCC-associated colorectal cancers, 64 cases (91.43%) showed no MLH1 staining, 5 cases weak (7.14%) and 1 case (1.43%) stronger staining intensity. In contrast, in tumor cells of 60 sporadic colorectal cancers 45 cases (75.0%) showed no MLH1 staining, 10 cases weak (16.67%) and 5 cases (8.33%) stronger staining intensity to a varying extent. In immunopositive cases, MLH1 showed a characteristic dot-like nuclear staining pattern in the tumor cells. We compared cases with absent to weak MLH1-staining (immunoscores 0 to 2) to cases with elevated immunoscores (3 to 12) detecting a statistically significant difference between HNPCC-associated and sporadic colon cancers (p value = 0.0031, Fisher's exact test). Taken together, enhanced tumoral MLH1 expression in MLH1-/PMS2-deficient colorectal carcinomas seems to be indicative of sporadic origin. In contrast, HNPCC-associated colorectal cancer showed absent or very weak MLH1 immunopositivity. Therefore, this semi-quantitative and easy to exert MLH1 immunoscore might help to identify sporadic MLH1-/PMS2-deficient colorectal cancer cases prior to time-consuming methylation analysis.

Keywords Colorectal cancer · HNPCC · Lynch syndrome · MLH1 · Mismatch repair enzymes

Introduction

Colorectal cancer plays a decisive role in cancer mortality and morbidity worldwide [1, 2]. The incidence has increased in the last years with 1.36 million new diagnoses per year [3], being now the third most common cause of cancer in men and the second cause in women [4]. Mortality by colorectal cancer is approximately 694,000 deaths every year [3].

Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also called Lynch syndrome, attributes to approximately 1–7% of all cases of colorectal cancer [2, 5, 6]. HNPCC-associated neoplasias are caused by germline mutations in mismatch-repair (MMR) genes MLH1, MSH2, MSH6 and PMS2 [6–8]. Most HNPCC germline mutations affect *MLH1* or *MSH2* gene followed by *MSH6* and *PMS2* genes [6, 7].

HNPCC diagnosis is based on the patient's family history using well-established clinical Amsterdam- and Bethesda-criteria [5, 9]. These clinical criteria need to be supported by immunohistochemical analyses of MMR protein expression [10] and molecular testing for microsatellite instability (MSI) in the tumor tissue [11]. Immunohistochemistry is a reliable tool to screen for the detection of MMR-deficiency in HNPCC-associated neoplasias [12]. In the literature, differential expression of MMR proteins in cell lines ranging from absent to reduced immunopositivity has been described before [13]. In response to these in-vitro findings other authors tried

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to establish an in-vitro model assessing how reduced MMR expression levels affect repair efficiency [14]. Other studies documented reduced MLH1 expression in-vivo in esophageal cancer concluding that this indicates poor prognosis [15]. Therefore, the present study addressed the question whether differential MLH1 expression is detectable in MSI-H and MLH1-/PMS2-deficient colorectal cancer tissue in-vivo and whether a characteristic expression pattern might be detectable, which could help differentiating between either sporadic or HNPCC-associated colorectal cancer.

Material and Methods

Collective of Tissue Samples

All cases in this retrospective study (Table 1) have been evaluated before in the Institute of Pathology, University of Cologne, which serves as a reference pathology for the German HNPCC Consortium. Immunohistochemical MLH1 stainings of 130 MLH1-/PMS2-deficient colorectal cancers were re-evaluated in order to identify diagnostically relevant MLH1 staining patterns. All cases showed high microsatellite-instability (MSI-H) in the tumor tissue except one case showing microsatellite-stability (MSS) but loss of MLH1- and PMS2-expression and a BRAF V600 mutation, so that we classified this case as sporadic. In addition, BRAF V600 mutational status and MLH1-promoter-methylation status were determined in a subset of cases (supplementary Table 1). 3 cases were not evaluated concerning BRAF V600 mutation due to too small DNA content of the sample. Patient collective (supplementary Table 1) comprised tumor tissue of 70 HNPCC-associated colon cancer cases and 60 sporadic colon cancer cases taken from the archive of the Institute of Pathology (years 2012–2018) and from the archive of the German HNPCC Consortium as part of a larger study on susceptibility to hereditary nonpolyposis colorectal cancer [16].

Table 1 Patient characteristics of the study collective

Gender	Age (Years)	HNPCC	Sporadic Colon Cancer
Female	0–40	1	0
	41–70	26	18
	71–100	3	27
Male	0–40	5	1
	41–70	34	8
	71–100	1	6
		70	60

Immunohistochemistry

MLH1 immunohistochemistry was carried out as described before [17] using a ready-to-use MLH1 antibody (MLH1, clone M1 #790–4535, Ventana, Basel, Switzerland). MLH1 staining intensity was evaluated using a semiquantitative score as described before [18]: Remmele score was calculated as the product of staining intensity ("0" = absent staining reaction,"1" = weak staining reaction, "2" = intermediate staining reaction and "3" = strong staining reaction) multiplied with the amount of immunopositive tumor cells ("0" = no tumor cells stained, "1" = less than 10% immunopositive tumor cells, "2" = 11–50% immunopositive tumor cells, "3" = 51–80% immunopositive tumor cells and"4" = more than 80% immunopositive tumor cells). Therefore, the applied immunoscore ranged from negative staining reaction (immunoscore 0) to a maximum staining intensity (immunoscore 12); the latter being present in non-neoplastic internal control tissue (crypt epithelia, lymphocytes). All stainings were independently analyzed by M.T-D. and N.F. and five discrepant cases had to be discussed and re-evaluated.

Statistical Analysis

Statistical analyses were performing using Graph Pad Prism software (Graph Pad La Jolla, USA) using two-sided Fisher's exact test.

Results

Diagnostic Stratification of Colorectal Cancer Collective

All 130 cases of the study collective (100.0%) displayed within the tumor cell compartment an immunohistochemical loss or reduction of MLH1 staining and a complete loss of PMS-2 expression. 129 cases showed MSI-H and 1 case was MSS, though, as it clearly showed immunohistochemical deficiency for MLH1 and PMS2 as well as a BRAF V600 mutation we classified this case as sporadic colon cancer. In addition, BRAF V600-mutation analyses were carried out in order to identify sporadic colon cancer cases within the patient collective: 77 carcinomas showed wild type sequence for BRAF V600 indicating a HNPCC-related neoplasia. Though, 7 out of these 77 cases showed a methylation of MLH1 promotor as described in sporadic colorectal cancer before [19]. Therefore, our study collective consisted of 70 HNPCC-associated colon cancers and 60 sporadic colon cancers. Table 1 shows information concerning the patient collective of the study.

Semiquantitative Analyses of MLH1 Expression

Among 70 HNPCC-associated colorectal cancers, majority of cases (64 cases, 91.43%) showed no tumoral MLH1 staining, 5 cases weak (7.14%, Fig. 1e) and only 1 case (1.43%) focally enhanced MLH1 staining intensity.

In contrast, in 60 sporadic colorectal cancers of the collective 45 cases (75.0%) showed no MLH1 staining (Fig. 1f), 10 cases weak (16.67%, Fig. 1c+d) and 5 cases focally enhanced (8.33%, Fig. 1a+b) staining intensity.

Table 2 displays the results of MLH1 immunoscores of sporadic and HNPCC-associated colorectal cancers in detail. None of the analyzed cases showed Remmele scores 9 to 12 in the tumor tissue; these enhanced staining scores were only detectable in non-neoplastic adjacent tissue. In immunopositive tumor tissue, MLH1-immunohistochemistry showed a characteristic dot-like expression pattern in the nuclei of tumor cells (Fig. 1a-e). Adjacent non-neoplastic epithelia or stroma cells showed a strong nuclear staining signal (Fig. 1a). 4 out of 6 MLH1-immuno-positive HNPCC cases showed dot-like expression pattern in the tumor cells while all

Table 2 Distribution of MLH1 immunoscores obtained by semiquantitative Remmele score with regard to sporadic and HNPCC-associated colorectal cancer

MLH1 immunoscores	HNPCC	Sporadic Colon Cancer
0	64	45
1	0	0
2	3	2
3	0	5
4	3	4
5	0	0
6	0	0
7	0	0
8	0	4
Total number of cases	70	60

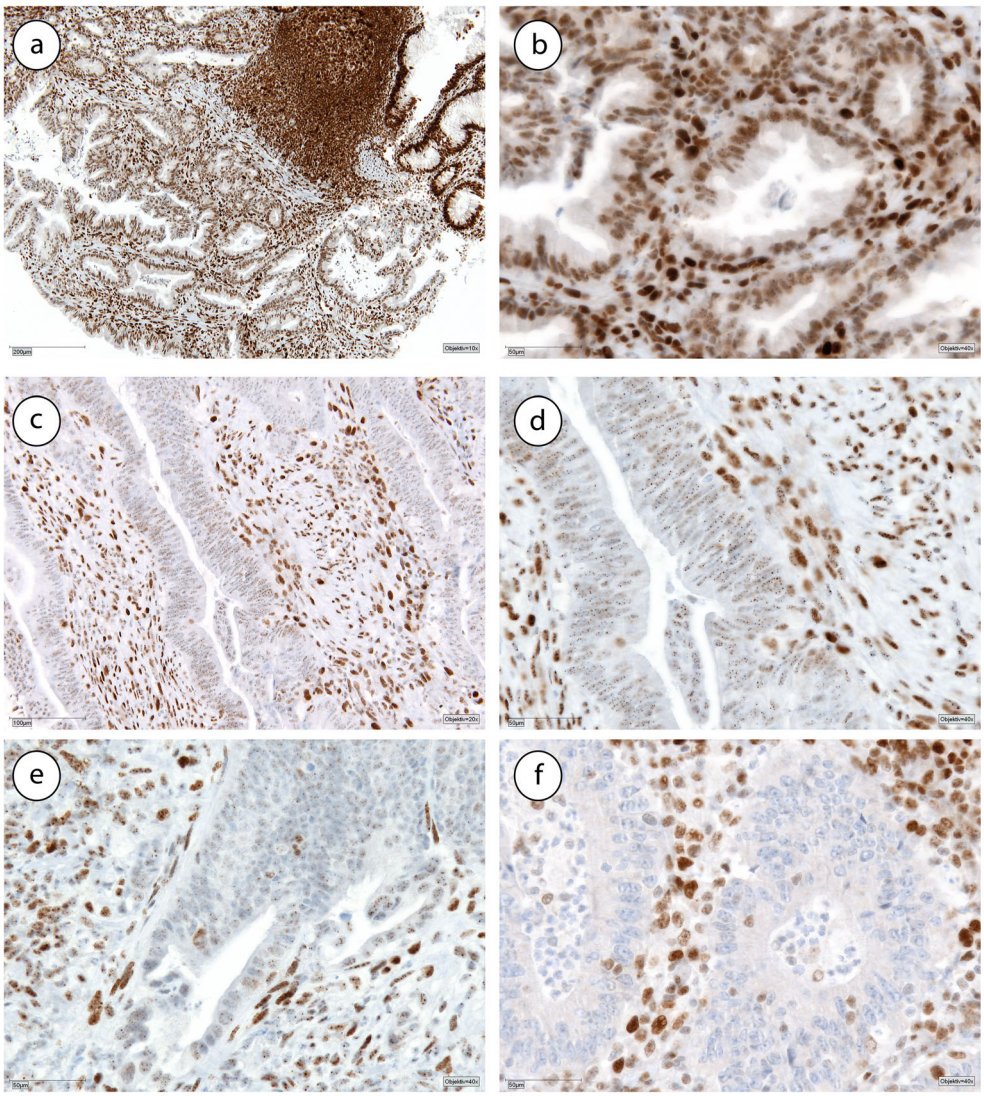

Fig. 1 Differential immunohistochemical staining of MLH1 in sporadic and HNPCC-associated colorectal carcinomas. In (**a**) low-power- and (**b**) high-power-view with enhanced expression of MLH1 in tumor cells of a sporadic colon carcinoma. Note the strong nuclear expression of MLH1 in adjacent normal mucosa in the upper right quadrant of (a). In (**c**) low-power- and (**d**) high-power-view with weak immunopositivity of MLH1 in a sporadic colon carcinoma. Carcinoma cells show a characteristic dot-like staining pattern. Note the strong nuclear expression of MLH1 in adjacent stromal cells. (**e**) shows discrete dot-like MLH1 expression in tumor cell nuclei of a HNPCC-associated colon carcinoma. In (**f**) tumor cells of a sporadic colon cancer immunonegative for MLH1

15 immunopositive sporadic colorectal cancers displayed dot-like expression pattern in the tumor cells.

In a first statistical analysis we compared cases with absent tumoral MLH1 staining (immunoscore 0) to cases with detectable MLH1 staining (immunoscores 1 to 12): This difference between HNPCC-associated colon cancers and sporadic colon cancers was statistically significant (p value = 0.0159, Fisher's exact test).

As some HNPCC-associated colon cancers showed a dot-like tumoral MLH1 staining we recalculated the statistical analysis comparing cases with negative to very weak MLH1-immunopositivity (immunoscores 0 to 2) to cases with stronger MLH1 expression (immunoscores 3 to 12). With this approach, a statistically more significant difference between HNPCC-related cancers and sporadic cancers was detectable (p value = 0.0031, Fisher's exact test). Therefore we conclude that an intermediate or strong MLH1 staining of the tumor cell nuclei in MLH1-/PMS2-deficient cancers is indicative of sporadic colon cancer origin.

Discussion

Mutations of mismatch repair genes like MLH1 and MSH2 play a significant role in carcinogenesis [20, 21]. Hereditary Non-Polyposis Colorectal Cancer (HNPCC) / Lynch syndrome is caused by germline mutations in MMR genes MLH1, MSH2, MSH6 or PMS2 inducing various neoplasias like carcinomas of the ovary, small bowel, pancreas, stomach, biliary tract, brain as well as colorectal and endometrial cancers [5, 22].

Diagnostic detection of HNPCC-associated neoplasias is based on clinical Amsterdam- and Bethesda-criteria [23] but also on tumor tissue analyses like microsatellite-status and immunohistochemical detection of MMR expression [10, 12]. Colorectal cancer with high microsatellite instability (MSI-H) and loss of MLH1- and PMS2-expression in the tumor cells can be a HNPCC-associated neoplasia but also a sporadic, non-hereditary colon cancer induced by MLH1 promoter methylation [24]. MLH1 promoter methylation analyses help distinguishing both origins but are nevertheless a time consuming step in diagnostics [25].

In our collective of 130 colorectal cancers MLH1 immunhistochemistry occasionally showed a weak to enhanced staining in MLH1-/PMS2-deficient tumor cell nuclei compared to the internal positive control (normal colon mucosa, immune cells). Immunopositive carcinoma cells showed a characteristic dot-like nuclear staining pattern for MLH1. This dot-like staining pattern has been observed before in analyses on serrated polyps of the intestine [26]. Furthermore, reduced MLH1 staining in MLH1-/PMS2-deficient tumor cells has been observed in HNPCC-associated cancers before [27], though a systematic correlation to findings in sporadic colon cancer has not been performed to our knowledge, yet.

In the present study, we analyzed 70 HNPCC-associated colon cancer cases and 60 sporadic colon cancer cases. We observed weak to elevated MLH1 expression with complete PMS2 loss in 15 out of 60 sporadic colon cancers (25%) but only in 6 out of 70 HNPCC-associated cancers (8.5%). Reduced tumoral MLH1 expression has been observed before in studies on esophageal cancer [15], thyroid cancer [28] and colon cancer [27] in-vivo. In-vitro, reduced immunopositivity of MLH1 has been found in various cell lines [13]. Therefore we conclude that differential MLH1 expression is a frequent finding in malignant tumors. As Kansikas et al. reported that a reduction of MLH1- and MSH2-gene-expression induces a loss of MMR expression in colorectal cancer cells in an in-vitro study [14] we conclude that the assessment of tumoral MLH1 staining in routine immunohistochemistry might be diagnostically relevant.

We found in 130 MLH1-/PMS2-deficient colon cancer cases a statistically significant difference between HNPCC-associated colorectal cancers and sporadic colon cancers with regard to MLH1 staining intensities. HNPCC-associated colon cancers rarely showed enhanced MLH1 staining. In contrast, a significantly higher number of sporadic colorectal cancers displayed weak to strong MLH1 immunopositivity in the tumor cell nuclei. In the literature, low MLH1 expression indicated poor prognosis in esophageal cancer [15]. In thyroid cancer, a decrease of MLH1 expression was found to be associated with BRAF mutations [28]. In colon cancer, reduced MLH1 staining of the tumor cells was not linked to in-frame or missense-mutations [27]. Though, Springuel and co-workers demonstrated in-vitro a causal relationship between MLH1-deficiency and incidence of oncogenic point mutations in tyrosine kinases driving cell transformation and acquired resistance to kinase-targeted cancer therapies [29].

Therefore, we conclude that according to the data of the present study elevated MLH1 expression in MLH1-/PMS2-deficient colorectal cancers is indicative of a sporadic cancer origin. In turn, HNPCC-associated neoplasias might be identified immunohistochemically by entirely negative or very weak MLH1 staining prior to time-consuming MLH1-promoter-methylation analyses.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

For this type of study formal consent is not required.

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