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Phenotypic Heterogeneity in Hereditary Nonpolyposis Colorectal Cancer

Identical Germline Mutations Associated with Variable Tumor Morphology and Immunohistochemical Expression

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ABSTRACT

Background: Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is associated with high risks for colorectal and endometrial cancer, young age at onset and an increased risk of multiple primary tumors. Colorectal cancer in HNPCC is characterized by poor tumor differentiation, an expanding growth pattern, and a pronounced lymphocytic reaction with tumor-infiltrating lymphocytes.

Objectives & Methods: The mutation spectrum in HNPCC is diverse and in order to clarify whether the HNPCC tumor phenotype is influenced by the underlying genetic alteration, we morphologically and immunohistochemically characterized 29 colorectal cancers and 12 adenomas from 24 individuals in two HNPCC families.

Results: The tumor morphology as well as the immunohistochemical expression of β -catenin varied extensively within the families as well as between synchronous/metachronous colorectal cancers from the same individual. Poor tumor differentiation, an expanding growth pattern, and tumor-infiltrating lymphocytes occurred at higher frequencies in proximal tumors, whereas distal colorectal cancers often lacked distinct HNPCC-associated morphological features.

Conclusions: The clinical, morphological and immunohistochemical variability observed within these families indicates that other mechanisms than the underlying germline mutation influence the HNPCC phenotype. Since morphological features linked to HNPCC are less frequent in distal cancers, family history and age of onset may be particularly relevant to obtain in these tumors in order to identify individuals with HNPCC.

Keywords: Hereditary Nonpolyposis Colorectal Cancer, HNPCC, Histopathology, Heterogeneity, MMR

Take home messages

- Tumors that develop as part of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome display extensive heterogeneity, also when caused by identical germline mutations.
- Other mechanisms than the underlying HNPCC-causing mutation are likely to influence tumor development and tumor morphology.
- HNPCC-associated features, e.g. poor tumor differentiation, an expanding growth pattern, and TIL were more common in proximal compared to distal colon cancers.
- Identification of HNPCC cases among distal colon cancers may be challenging since these tumors often lack the specific histopathologic HNPCC-associated features.

INTRODUCTION

Since the identification of mismatch-repair (MMR) gene mutations as the underlying cause of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) close to 500 mutations in more than 700 HNPCC families have been identified worldwide.¹ Mutation carriers are estimated to be at 70-80% life-time risk of colorectal cancer and 40-60% risk of endometrial cancer for female carriers and at increased risks also for ovarian cancer, urothelial cancer, gastric cancer, cancer of the small intestine, sebaceous adenomas/carcinomas, and gliomas.²⁻⁴ Although the median age at development of colorectal cancer among probands in HNPCC families is 44 years, the age at diagnosis is highly variable with about 5% of the patients diagnosed before age 25 and at least one-third of the patients diagnosed after age 60.^{2,3} An increased risk of extraintestinal tumors has been described in *MSH2* mutation carriers, whereas colorectal cancers predominate in individuals with mutations in *MLH1*⁵⁻⁷. A lower incidence of colorectal cancer and later age at onset has been described in families with mutations in *MSH6*.⁸ Despite the large number of HNPCC families identified, no strong genotype – phenotype correlations have been identified and the causes of the phenotypic variability in HNPCC are largely unknown.

A number of histopathological characteristics have been associated with colorectal cancers that develop as part of HNPCC and these characteristics are also overrepresented in the 15% of the sporadic colorectal cancers that develop because of defective MMR due to hypermethylation of the *MLH1* promoter.⁹⁻¹² These characteristics include a proximal tumor location, an expanding growth pattern, poor - sometimes medullary - differentiation, and mucinous histology.^{9,13-15} Additionally, HNPCC tumors tend to show abundant tumor-infiltrating lymphocytes (TIL) and peritumoral lymphocytes, and a Crohn-like lymphocytic reaction.¹⁵⁻¹⁸ Evaluation of these histopathological features, particularly mucinous, medullary, and poor differentiation and presence of TIL, has been shown to be valuable in predicting colorectal cancers with defective MMR.^{10,15} However, these features, although originally associated with HNPCC, may be more

closely linked to tumors with a somatically acquired MMR defect¹¹. We therefore histopathologically characterized all available tumors from two HNPCC-families with mutations in *MLH1* and *MSH2*, respectively, in order to further clarify genotypic – phenotypic correlations. Because of reports on frequent activation of the Wingless (Wnt) signaling pathway in HNPCC-associated tumors¹⁹ we also chose to investigate the variability of β -catenin immunostaining in these tumors.

PATIENTS & METHODS

Patients

Two HNPCC families that had undergone genetic counseling and testing at the Oncogenetic clinic, Lund University Hospital were included in the study. Family C carried a c.1586delA (p.529fs) in *MSH2* and family L carried a c.2141G>A 2141 (p.Trp714STOP) in *MLH1*. 24 of the tumors in family C have previously been characterized regarding somatic frameshift alterations.²⁰ Colorectal tumors, including 29 colorectal cancers and 12 colorectal adenomas, from 24 family members were analyzed. The mean age at development of the first tumor was 49 (range 23-78) years. Colorectal tumors were, according to tumor localization, classified as proximal (proximal to the splenic flexure), distal, or rectal (tumors located within 15 cm from the anal canal).

Histopathology

Hematoxylin and erythrosin (H&E) stained slides were re-evaluated by a gastrointestinal pathologist (B.H.) and the majority of the cases were also reviewed by an additional pathologist (W.M.). Tumor stage of colorectal cancer was classified according to AJCC/UICC and tumor grade was determined according to the WHO classification²¹. Tumors with a mucinous or a signet ring-cell component identified in >50% of the tumor area were classified as mucinous or signet ring-cell type and were considered poorly differentiated. If a trabecular, solid or medullary growth pattern or a mucinous or signet ring-cell component encompassing 10-50% of the tumor

area was identified, the tumors were classified as heterogenous.^{11,12} The tumor growth pattern was classified as expanding or infiltrative¹³. A Crohn-like infiltrate was defined as 3 or more nodular lymphoid aggregates deep to the advancing tumor margin within a single x 4 field. Peritumoral lymphocytes were considered to be present when a cap of chronic inflammatory cells was seen in the deep invasive border of the tumor¹¹. Presence of TIL was defined as 7 or more TIL / 10 HPF (x 40) on H&E stained slides. Infiltrating components and hot spot areas were primarily analyzed. TIL was not classified in early invasive (pT1sm1) tumors. In colorectal adenomas the grade of dysplasia was classified as high-grade or low-grade.²¹

Immunohistochemical staining

Fresh 4- μ m sections were obtained for the immunohistochemical stainings. All tumors were immunohistochemically stained using antibodies against the MMR proteins MLH1 (clone G168-15, 1:200, BD PharMingen San Diego, CA, USA), PMS2 (clone A16-4, 1:500, BD PharMingen), MSH2 (clone FE-11, 1:100, Oncogene Sciences, San Diego, CA, USA), and MSH6 (clone 44, 1:1000, BD Transduction Laboratories, Lexington, KY, USA). The EnVision™ detection kit (Dako Cytomation, Glostrup, Denmark) was used and the staining procedure has previously been described²². The immunohistochemical MMR protein expression was classified as retained when nuclear staining in the tumor cells was identified and as lost when the tumor cells showed loss of staining with retained staining in stromal, epithelial, inflammatory, or infiltrating lymphoid cells. The immunostainings for β -catenin (clone 14, 1:5000, Transduction Laboratories) were performed using the LSAB™ (labeled streptavidin-biotin) detection kit (Dako Cytomation). β -catenin staining is normally located to the cell membrane, but translocates to the cytoplasm and further into the nucleus in case of defective APC-directed degradation. β -catenin staining in the cytoplasm was classified as present or absent, and any nuclear β -catenin staining was classified as positive¹⁹. Whenever possible (in 26/29 colorectal cancers) the β -catenin staining was evaluated

at the invasive border. All immunostainings were independently and concordantly evaluated by B.H. and M.N.

Statistical analysis

Statistical analysis was performed using the statistics package Stata 9.0 (StataCorp 2005, College Station, TX, USA). Fisher's exact test was used to determine the correlation between morphology and tumor location / age.

RESULTS

Among the colorectal carcinomas, 10/29 (34%) were poorly differentiated, including one mucinous carcinoma, while 3 tumors were undifferentiated of the medullary type (Table 1). Furthermore, 16/29 (55%) colorectal carcinomas were classified as morphologically heterogeneous with areas of mucinous or poor differentiation in less than 50% of the tumor tissue (Table 1). Peritumoral lymphocytic infiltration and TIL were observed in 22/28 (79%) and 16/26 (62%) of the tumors, whereas a Crohn-like lymphocytic reaction was found in 11/25 (44%) tumors. An expanding growth pattern was identified in 17/25 (68%) colorectal carcinomas (Table 1). Several features such as poor tumor differentiation, an expanding growth pattern, and TIL were significantly more often identified in cancers within the proximal colon compared to those in the distal colon or in the rectum (Table 2). Among patients >50 years at diagnosis an expanding growth pattern occurred at increased frequency (13/15 compared to 4/10 in younger patients, $p=0.03$), whereas the other morphological variables did not correlate with age (data not shown). Of the 12 colorectal adenomas investigated, 10 were tubular, 2 were tubulovillous, and 4/12 showed high-grade dysplasia. The adenomas developed at mean age 65 (range 44-78) years and were equally distributed between the proximal and the distal colon.

Although disease-causing germline mutations in *MSH2* and *MLH1* had been identified in all 24 individuals, MMR protein immunostaining was performed in order to confirm a MMR defective phenotype, which was indeed present in all tumors except for one colon cancer and 2 colon adenomas that showed retained expression of all 4 MMR proteins investigated and microsatellite instability (MSI) analysis confirmed a microsatellite stable phenotype in these tumors (Table 1). Cytoplasmic accumulation of β -catenin was identified in 23/29 (79%) colorectal cancers and in all 12 adenomas and nuclear β -catenin accumulation was observed in 4 colorectal cancers and 3 adenomas (Table 1).

DISCUSSION

Genetic as well as phenotypic heterogeneity characterizes HNPCC; mutations in different MMR genes cause morphologically indistinguishable colorectal cancers, but mutations in the same gene may also lead to highly variable phenotypes. In our series, extensive inter- as well as intra-tumor heterogeneity for histopathological and immunohistochemical features was identified. Ages at onset of colorectal cancer varied from 39 to 78 years in the *MSH2* family and from 23 to 61 years in the *MLH1* family with co-existence of early-onset and late-onset cancers in both families. A variable age at cancer development has also been observed in families carrying the two Finnish *MLH1* founder mutations, which emphasizes that factors other than the underlying germline alteration influence the HNPCC phenotype.⁶

Double primary tumors develop in about one-third of HNPCC patients; synchronous colorectal cancers develop in 5-10% of the patients and metachronous colorectal cancers in 20-25% with mean 10 years between the different tumors.²³ Among the 6 individuals who developed at least 2 colorectal cancers, differences in tumor morphology and immunostaining were observed in all cases (Table 1, Fig. 1). Three tumors – one colon carcinoma and two adenomas – showed a MSS

phenotype with retained immunostaining, which may, particularly for the adenomas, reflect that occasional HNPCC tumors escape identification with currently used methods, which identify defective MMR with a sensitivity of about 95% for carcinomas and 60-70% for adenomas²⁴.

Poor/undifferentiated tumor differentiation has been described in 30-40% of the HNPCC-tumors and was in our series identified in 45% of the tumors.^{9,11,14,25} An expanding tumor margin has also been associated with HNPCC, although not consistently identified as over-represented, and was in our series observed in 68% of the colorectal cancers.^{9,14} Morphologic heterogeneity with mucinous, signet ring-cell, or medullary components has been described as a common characteristic within HNPCC tumors and was in our series present in 55% of the tumors.^{11,15,16} Abundant lymphocytes, which may appear as Crohn-like reactions, as peritumoral lymphocytes or as TIL are among the features most frequently observed in HNPCC-associated colorectal cancers.^{11,12} In our series, TIL were identified in 59% of the colorectal cancers, whereas a Crohn-like reaction was identified in 44% of the tumors. Assessment of TIL has been described to identify MMR-defective colorectal cancers with a sensitivity of 75-90%.^{15,18,25,26} The biological significance of these lymphocytes is still unknown, but may be related to an inflammatory response from cytotoxic T-cells in MMR-defective tumors. Differences exist between sporadic and HNPCC-associated MMR-defective tumors. Serrated polyps, somatic *BRAF* gene mutations, and *MLH1* methylation characterize the sporadic tumors, whereas conventional colorectal adenomas seem to be the precursor lesions in HNPCC with mutations in *APC*, *β-catenin*, and *KRAS* occurring at a higher frequency.²⁷ The morphological parameters investigated herein have been associated with MMR-defective tumors in general, but may be particularly associated with somatic MMR-defects.

Rectal cancer has been reported to be the index cancer in one out of four HNPCC patients, which stands in contrast to the sporadic MMR-defective tumors, 90% of which develop in the proximal

colon.^{16,28} In our series, 19 colorectal cancers developed in the proximal colon and 10 in the distal colon or the rectum. Among the HNPCC-associated features, poor tumor differentiation, an expanding growth pattern, and TIL were significantly more common in proximal than in distal colorectal cancers (Table 2). Hence, identification of HNPCC cases among distal colon cancers based on tumor morphology may be challenging since these tumors often lack distinct HNPCC-associated features, but at the same time identification of a MMR defective phenotype is more likely to reflect HNPCC in the distal tumors because of a low frequency of somatic MMR-defects.

Overactivation of the Wnt signaling pathway favors cell growth. β -catenin is normally directed to ubiquitin-mediated degradation through interaction with the adenomatous polyposis coli (APC) protein. Since mutations in *APC* and *CTNNB1* occur at high frequency in colorectal cancer, defective β -catenin expression with cytoplasmic accumulation and nuclear translocation is common and mutations in the regulatory domain of β -catenin have been described at an increased frequency in HNPCC-associated tumors.³⁰ Cytoplasmic accumulation of β -catenin was identified in 79% of the colorectal cancers and in all adenomas (Table 1). The staining varied in between different tumors from the same individual as well as between different family members with identical mutations and seemed equally common in individuals with *MLH1* and *MSH2* mutations.

We also morphologically and immunohistochemically characterized 9 gynecological cancers from 8 individuals, 7 of whom carried the *MSH2* mutation and 4 of whom also developed colorectal cancers (data not shown). The 7 endometrial cancers developed at a mean age of 49 (range 40-60) years, which is consistent with the reported mean age of 50 years. Poor differentiation, Crohn-like lymphoid reactions, and TIL are overrepresented in MMR-defective endometrial cancers but similarly to colorectal cancer these patterns seem to be more common in

the somatically inactivated tumors, whereas the HNPCC-associated tumors show a more variable morphology.³¹ Only 1/7 endometrial cancers in our series was poorly differentiated, lymphocytic infiltration was identified in 4, and 6 of the 7 tumors showed cytoplasmic accumulation of β -catenin staining (data not shown). Ovarian cancers associated with HNPCC are characterized by early tumor development, mean age 41-43 years, which is consistent with the two cases that developed at ages 40 and 45 years.³² Both patients had clear cell cancers of the ovaries and synchronous early stage endometrial cancers of low grade differentiation and because of the distinct morphologies the tumors were in both cases considered to be separate.

In summary, the histopathological characteristics and the immunohistochemical staining patterns identified indicate extensive phenotypic heterogeneity, which is pronounced between different tumors in one individual as well as between family members with identical underlying germline mutations. Thus, independent modifiers, e.g. different mutations in e.g. *KRAS*, *APC* or within genes affected by somatic frameshift mutations may influence tumor development as well as morphology in HNPCC. Our results also suggest that identification of HNPCC cases among distal colon cancers or rectal cancers may be challenging since these tumors often lack specific HNPCC-associated features such as TIL, expanding growth pattern and poor tumor differentiation.

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ETHICAL APPROVAL

Ethical approval was obtained from the Ethics Committee at Lund University.

COMPETING INTEREST

No competing interests apply for any of the authors.

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Figure legend

Figure 1

Hematoxylin & Eosin stained slides (inserted figures x 40) demonstrating variable tumor morphology in metachronous colorectal cancers from patient C6. a) Rectal cancer with mucinous areas and infiltrating growth, but no tumor-infiltrating lymphocytes and b) proximal, undifferentiated colon cancer with pushing growth and abundant tumor-infiltrating lymphocytes (indicated by arrows in the high-magnification picture).

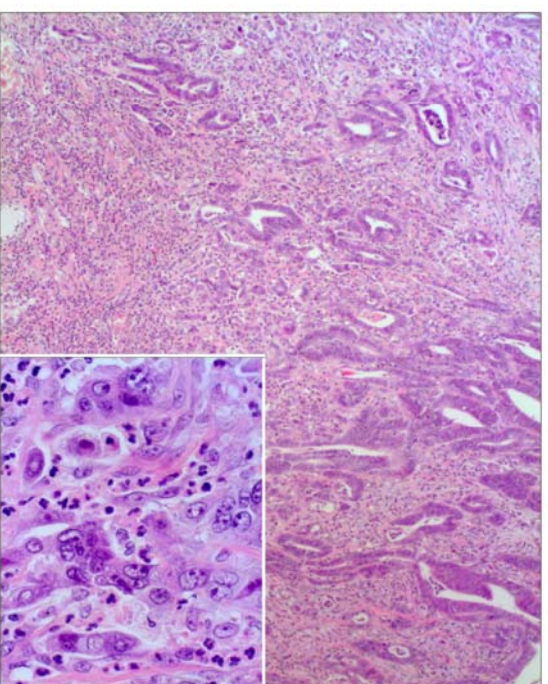
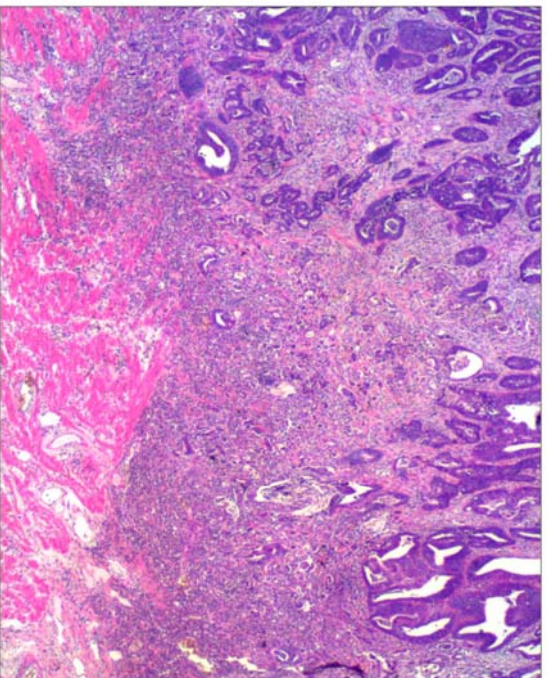
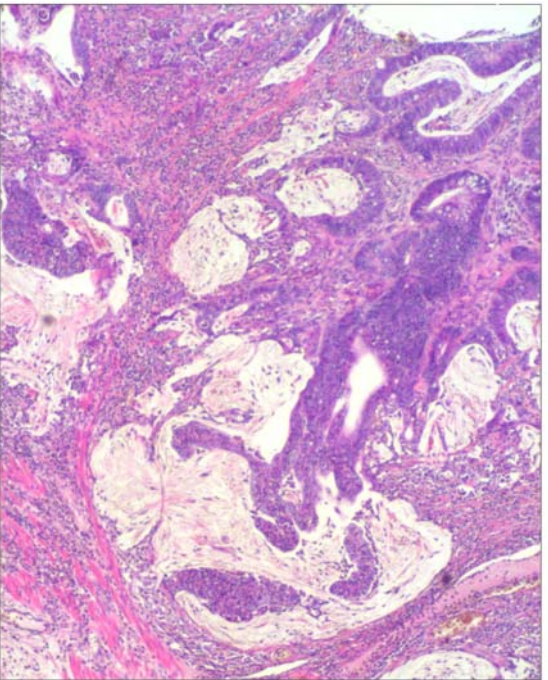
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a



b

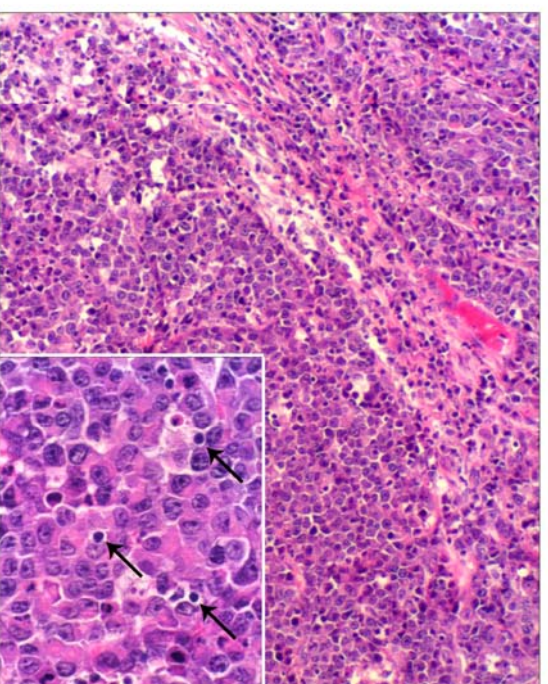
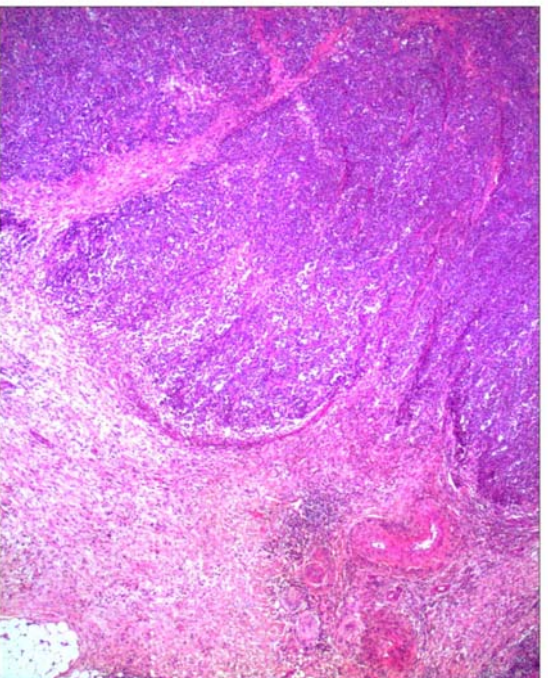
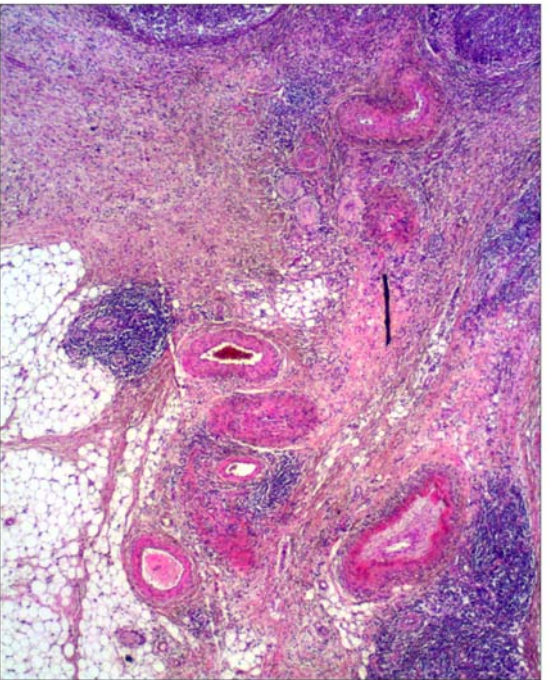


Table 2. Correlations between tumor morphology and tumor location.

	Proximal colon	Distal colon	p-value
Poor tumor differentiation	0.63	0.10	<0,01
Expanding growth pattern	0.88	0.25	<0.01
Morphologic heterogeneity	0.53	0.60	n.s.
Tumor-infiltrating lymphocytes	0.76	0.33	<0.05
Peritumoral lymphocytes	0.84	0.67	n.s.
Crohn-like reaction	0.41	0.50	n.s.

Table 3. Extracolonic tumors associated with HNPCC.

Tumor type	Causative gene	Patient number	Sex	Age	Tumor differentiation	Pushing border	TILs α	Peri-tumoral lymphocytes	p53 staining	β -catenin cytoplasmic staining	β -catenin nuclear staining
Endometrial cancer	<i>MSH2</i>	C6.3	F	43	moderate	-	+	-	-	-	-
Endometrial cancer	<i>MSH2</i>	C13	F	48	moderate	NE	-	-	+	+	+
Endometrial cancer	<i>MSH2</i>	C8.1	F	40	well	-	+	+	-	+	-
Endometrial cancer	<i>MSH2</i>	C10	F	60	moderate	-	+	+	+	+	-
Endometrial cancer	<i>MSH2</i>	C11	F	45	moderate	NE	-	NE	-	+	+
Endometrial cancer	<i>MSH2</i>	C17	F	51	poor	+	+	+	-	+	-
Endometrial cancer	<i>MLH1</i>	L2.4	F	55	moderate	NE	-	-	-	+	-
Ovarian cancer	<i>MSH2</i>	C11.1	F	45	poor	NE	-	NE	-	-	-
Ovarian cancer	<i>MSH2</i>	C8.1	F	40	poor	NE	+	NE	-	-	-
Gastric cancer	<i>MSH2</i>	C8.6 α	F	59	poor	-	-	-	-	+	-
Gastric cancer	<i>MLH1</i>	L1.2	M	72	poor	+	+	+	+	+	-
Urothelial cancer	<i>MLH1</i>	L2.5	F	56	G2	NE	-	-	+	-	-
Urothelial cancer	<i>MSH2</i>	C8.5	F	62	G3-G4	NE	+	+	+	-	-
Sebaceous adenoma	<i>MSH2</i>	C11.2	F	56	-	NE	-	-	+	+	-
Small intestinal cancer*	<i>MSH2</i>	C3c	F	50	poor	-	-	+	+	+	-

NE: not evaluated; α : microsatellite stable; *Crohn-like reaction