ORIGINAL ARTICLE

Phenotypic heterogeneity in hereditary non-polyposis colorectal cancer: identical germline mutations associated with variable tumour morphology and immunohistochemical expression

Britta Halvarsson, Wolfram Müller, Maria Planck, Anna Clara Benoni, Peter Mangell, Johan Ottosson, Magnus Hallén, Anna Isinger, Mef Nilbert

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See end of article for authors' affiliations

Correspondence to:
Dr Britta Halvarsson,
Department of Pathology,
Helsingborg Hospital, 251
87 Helsingborg, Sweden;
Britta.Halvarsson@med.lu.se

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Since the identification of mismatch-repair (MMR) gene mutations as the underlying cause of hereditary non-polyposis 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 also at increased risk 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 tumours 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; these characteristics are also overrepresented in the 15% of sporadic colorectal cancers that develop because of defective MMR due to hypermethylation of the *MLH1* promoter.^{9–12} These characteristics include a proximal tumour location, an expanding growth pattern, poor—sometimes medullary—differentiation, and mucinous histology.^{9–13–15}

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

Aims and Methods: The mutation spectrum in HNPCC is diverse and in order to clarify whether the HNPCC tumour phenotype is influenced by the underlying genetic alteration, 29 colorectal cancers and 12 adenomas from 24 individuals in two HNPCC families were morphologically and immunohistochemically characterised.

Results: The tumour 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 tumour differentiation, an expanding growth pattern, and tumour-infiltrating lymphocytes occurred at higher frequencies in proximal tumours, 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, it may be particularly relevant to obtain family history and age of onset in these tumours in order to identify individuals with HNPCC.

Additionally, HNPCC tumours tend to show abundant tumour-infiltrating lymphocytes (TIL) and peritumoural 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 tumours with a somatically acquired MMR defect.¹¹ We therefore histopathologically characterised all available tumours 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) signalling pathway in HNPCC-associated tumours,¹⁹ we also chose to investigate the variability of β -catenin immunostaining in these tumours.

PATIENTS AND METHODS

Patients

Two HNPCC families that had undergone genetic counselling 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*. Twenty-four of the tumours in family C have previously been characterised regarding somatic frameshift alterations.²⁰ Colorectal tumours, including 29

Abbreviations: APC, adenomatous polyposis coli; HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair; TIL, tumour-infiltrating lymphocytes

colorectal cancers and 12 colorectal adenomas, from 24 family members were analysed. The mean age at development of the first tumour was 49 (range 23–78) years. Colorectal tumours were, according to tumour localisation, classified as proximal (proximal to the splenic flexure), distal, or rectal (tumours located within 15 cm from the anal canal).

Histopathology

H&E-stained slides were re-evaluated by a gastrointestinal pathologist (BH) and the majority of the cases were also reviewed by an additional pathologist (WM). Tumour stage of colorectal cancer was classified according to AJCC/UICC and tumour grade was determined according to the WHO classification.²¹ Tumours with a mucinous or a signet ring-cell component identified in >50% of the tumour 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 tumour area was identified, the tumours were classified as heterogeneous.^{11–12} The tumour growth pattern was classified as expanding or infiltrative.¹³ A Crohn-like infiltrate was defined as three or more nodular lymphoid aggregates deep to the advancing tumour margin within a single $\times 4$ field. Peritumoural lymphocytes were considered to be present when a cap of chronic inflammatory cells was seen in the deep invasive border of the tumour.¹¹ Presence of TIL was defined as ≥ 7 TIL/10 HPF ($\times 40$) on H&E stained slides. Infiltrating components and hot spot areas were primarily analysed. TIL was not classified in early invasive (pT1sm1) tumours. 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 tumours were immunohistochemically stained using antibodies against the MMR proteins MLH1 (clone G168-15, 1:200, BD PharMingen, San Diego, California, USA), PMS2 (clone A16-4, 1:500, BD PharMingen), MSH2 (clone FE-11, 1:100, Oncogene Sciences, San Diego, California, USA), and MSH6 (clone 44, 1:1000, BD Transduction Laboratories, Lexington, Kentucky, USA). The EnVision detection kit (Dako Cytomation, Glostrup, Denmark) was used; the staining procedure has previously been described.²² The immunohistochemical MMR protein expression was classified as retained when nuclear staining in the tumour cells was identified, and as lost when the tumour 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 (labelled 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 adenomatous polyposis coli (APC) directed degradation. β -catenin staining in the cytoplasm was classified as present or absent, and any nuclear β -catenin staining was classified as positive.¹⁹ 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 BH and MN.

Statistical analysis

Statistical analysis was performed using the statistics package Stata V9.0 (StataCorp 2005, College Station, Texas, USA). Fisher's exact test was used to determine the correlation between morphology and tumour location/age.

RESULTS

Among the colorectal carcinomas, 10/29 (34%) were poorly differentiated, including one mucinous carcinoma, while three tumours 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 tumour tissue (table 1). Peritumoural lymphocytic infiltration and TIL were observed in 22/28 (79%) and 16/26 (62%) of the tumours, whereas a Crohn-like lymphocytic reaction was found in 11/25 (44%) tumours. An expanding growth pattern was identified in 17/25 (68%) colorectal carcinomas (table 1). Several features such as poor tumour 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 an MMR defective phenotype, which was indeed present in all tumours except for one colon cancer and two colon adenomas that showed retained expression of all four MMR proteins investigated; microsatellite instability analysis confirmed a microsatellite stable phenotype in these tumours (table 1).

Cytoplasmic accumulation of β -catenin was identified in 23/29 (79%) colorectal cancers and in all 12 adenomas; nuclear β -catenin accumulation was observed in 4 colorectal cancers and 3 adenomas (table 1).

DISCUSSION

Genetic as well as phenotypic heterogeneity characterises 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-tumour as well as intra-tumour heterogeneity for histopathological and immunohistochemical features was identified. Age 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 coexistence 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 emphasises that factors other than the underlying germline alteration influence the HNPCC phenotype.⁶

Double primary tumours 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 a mean of 10 years between the different tumours.²³ Among the six individuals who developed at least two colorectal cancers, differences in tumour morphology and immunostaining were observed in all cases (table 1, fig 1). Three tumours—one colon carcinoma and two adenomas—showed a MSS phenotype with retained immunostaining, which may, particularly for the adenomas, reflect that occasional HNPCC tumours escape identification with currently used methods, which identify defective MMR with a sensitivity of about 95% for carcinomas and 60–70% for adenomas.²⁴

Table 1 Clinicopathological data in the colorectal cancers

Causative gene	Patient number	Sex	Age	Tumour location	pTNM	Tumour differentiation	Expanding growth pattern	Morphological heterogeneity	Crohn-like reaction	Tumour infiltrating lymphocytes	Peritumoural lymphocytes	p53 staining	β-catenin cytoplasmic staining	β-catenin nuclear staining
MSH2	C1.1	M	78	Proximal	pT3N2	Poor	+	+	+	+	-	-	+	-
	C1.3	M	78	Proximal	pT1sm1	Moderate	NE	+	NE	NE	+	-	-	-
	C2.1	M	60	Proximal	pT3N0	Undiff*	+	-	-	+	-	-	-	-
	C2.4	M	67	Proximal	pT2NX	Poor	+	+	-	+	-	-	+	-
	C2.5	M	67	Proximal	pT2NX	Poor	+	+	-	+	+	+	+	-
	C2.6	M	67	Proximal	pT2NX	Moderate	+	-	+	-	+	-	-	-
	C3.1	F	44	Proximal	pT3N0	Poor†	+	-	+	-	+	+	+	-
	C3.2	F	50	Rectal	pT2N0	Moderate	+	-	+	+	+	+	+	-
	C4	M	64	Rectal	pT4aNX	Moderate	-	-	-	-	NE	-	+	-
	C5	M	40	Rectal	NE (biopsy)	Moderate	NE	+	NE	-	NE	+	+	-
	C6.1	F	42	Rectal	pT2N0	Moderate	-	+	-	-	+	-	+	+
	C6.2	F	52	Proximal	pT3N0	Undiff*	+	-	+	+	+	+	+	-
	C7.1	M	40	Proximal	pT1sm1	Moderate	NE	-	NE	NE	+	+	+	-
	C7.2‡	M	40	Proximal	pT3N0	Moderate	+	-	+	+	+	-	+	-
	C8.3	F	62	Proximal	pT3N0	Undiff*	+	-	+	+	+	-	+	-
	C9	M	48	Distal	pT3N2	Moderate	-	+	+	-	+	-	+	-
	C11.3	F	63	Distal	pT4aN0	Moderate	-	+	+	-	+	-	+	+
	C12	M	48	Rectal	pT3N1	Poor	+	-	-	+	+	+	+	-
	C14	M	47	Proximal	pT3N0	Moderate	+	+	-	+	+	-	+	-
MLH1	C15	M	39	Rectal	pT1sm1	Moderate	NE	-	NE	NE	+	+	+	-
	C16.1	M	61	Distal	pT2N0	Moderate	+	+	+	-	+	-	+	-
	C16.2	M	61	Proximal	pT3N0	Moderate	+	-	+	+	+	-	+	-
	L1.1	M	53	Proximal	pT3N0	Moderate	+	-	-	+	+	-	+	-
	L2.1	F	47	Proximal	pT3N0	Moderate	+	+	-	+	+	+	+	-
	L3	M	45	Proximal	pT3N0	Poor	-	+	-	+	+	-	-	+
	L4	F	23	Proximal	pT3N2	Poor	-	+	-	+	+	+	-	+
	L5	M	38	Rectal	pT3N0	Moderate	-	+	-	+	+	+	+	-
	L6	M	55	Proximal	pT3N0	Poor	-	+	-	+	+	-	+	+
	L8	M	61	Proximal	pT3N1	Poor	+	+	+	+	+	-	+	-

NE, not evaluated; *medullary carcinoma; †mucinous carcinoma; ‡microsatellite stable.

Table 2 Fractions of tumours with features in relation to tumour location

	Proximal colon	Distal colon	p-value
Poorly differentiated/undifferentiated tumours	0.68	0.10	<0.01
Expanding growth pattern	0.88	0.25	<0.01
Morphological heterogeneity	0.53	0.60	NS
Tumour-infiltrating lymphocytes	0.76	0.33	<0.05
Peritumoural lymphocytes	0.84	0.67	NS
Crohn-like reaction	0.41	0.50	NS

Poor/undifferentiated tumour differentiation has been described in 30–40% of the HNPCC tumours and was in our series identified in 45% of the tumours.^{9 11 14 25} An expanding tumour 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} Morphological heterogeneity with mucinous, signet ring-cell, or medullary components has been described as a common characteristic within HNPCC tumours and was in our series present in 55% of the tumours.^{11 15 16} Abundant lymphocytes, which may appear as Crohn-like reactions, as peritumoural 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 tumours. 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 tumours. Differences exist between sporadic and HNPCC-associated MMR-defective tumours. Serrated polyps, somatic *BRAF* gene mutations, and

MLH1 methylation characterise the sporadic tumours, 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 here have been associated with MMR-defective tumours 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 tumours, 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 tumour 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 tumour morphology may be challenging since these tumours often lack distinct HNPCC-associated features, but at the same time identification of an MMR defective phenotype is more likely to reflect HNPCC in the distal tumours because of a low frequency of somatic MMR defects.

Overactivation of the Wnt signalling pathway favours cell growth. β -catenin is normally directed to ubiquitin-mediated degradation through interaction with the 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 tumours.³⁰ Cytoplasmic accumulation of β -catenin was identified in 79% of the colorectal cancers and in all adenomas (table 1). The staining varied between different tumours 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.

Figure 1 H&E stained slides (inserted figures $\times 40$) showing variable tumour morphology in metachronous colorectal cancers from patient C6. (A) Rectal cancer with mucinous areas and infiltrating growth, but no tumour-infiltrating lymphocytes. (B) Proximal, undifferentiated colon cancer with pushing growth and abundant tumour-infiltrating lymphocytes (indicated by arrows in the high-magnification picture).

Take-home messages

- Tumours that develop as part of the hereditary non-polyposis 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 tumour development and tumour morphology.
- HNPCC-associated features, for example poor tumour differentiation, an expanding growth pattern, and tumour-infiltrating lymphocytes are more common in proximal compared to distal colon cancers.
- Identification of HNPCC cases among distal colon cancers may be challenging since these tumours often lack the specific histopathological HNPCC-associated features.

We also morphologically and immunohistochemically characterised 9 gynaecological 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 tumours, whereas the HNPCC-associated tumours 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 tumours showed cytoplasmic accumulation of β -catenin staining (data not shown). Ovarian cancers associated with HNPCC are characterised by early tumour 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; because of the distinct morphologies the tumours 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 tumours in one individual as well as between family members with identical underlying germline mutations. Thus, independent modifiers, e.g. different mutations in, for example *KRAS* or *APC*, or within genes affected by somatic frameshift mutations may influence tumour 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 tumours often lack specific HNPCC-associated features such as TIL, expanding growth pattern and poor tumour differentiation.

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Authors' affiliations

B Halvarsson, M Planck, A C Benoni, A Isinger, M Nilbert, Department of Pathology, Lund University Hospital, Lund, Sweden
W Müller, Gemeinschaftspraxis Pathologie Starnberg, Germany

P Mangell, Department of Surgery, Malmö University Hospital, Malmö, Sweden

J Ottosson, Department of Surgery, Kristianstad Hospital, Kristianstad, Sweden

M Hallén, Department of Surgery, Lund University Hospital, Lund, Sweden

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