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Microsatellite instability: new aspects in the carcinogenesis of colorectal carcinoma

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Abstract Very recently a new molecular mechanism in the tumorigenesis of colorectal carcinoma has been described which is closely linked to hereditary non-polyposis colonic cancer (HNPCC). Ubiquitous changes in the length of simple repetitive DNA sequences between constitutional and tumour DNA occur in about 90% of cases of HNPCC and in about 15% of cases of non-familial, sporadic colorectal carcinoma. Such microsatellite instabilities have been shown to be the phenotypical marker of mutations in the human homologues of prokaryotic mismatch repair genes (*MutS*, *MutL*, *MutH*). These data provide crucial new tools in the detection of patients at high risk of developing colon cancer and other HNPCC-related carcinomas. In addition, these developments provide new insights into a new, presumably primary event in oncogenesis, i.e. the occurrence of mutations in genomic stability genes leading to an increased cellular mutation rate ("mutator phenotype") and thus to cancer.

Key words Colorectal carcinoma · Hereditary non-polyposis colonic cancer · Microsatellite instability

Introduction

Colorectal cancer (CRC) has provided one of the most useful models for a molecular understanding of carcinogenesis. Vogelstein and colleagues have demonstrated that the formation of CRC is a multistep process which involves successive genetic alterations involving oncogenes and tumour suppressor genes (for review see [11]). This concept is now widely accepted and has been established in a series of other malignancies [10, 48]. Furthermore, there is increasing evidence that tumours with a greater number of genetic alterations have in general a more aggressive clinical behaviour [43]. However, the multistep conception has one major limitation. Although several genes that contribute to colon carcinogenesis have been described, none of these has been linked to the most frequent cancer predisposition syndrome, hereditary non-polyposis colonic cancer (HNPCC) [1, 11, 38].

In May 1993 two publications hailed the discovery of a genetic locus predisposing to HNPCC and suggested that this "new colon cancer gene" acts in a novel way, namely by destabilizing the genome in widespread simple repetitive so-called microsatellite DNA [1, 38]. Micosatellite instability (MIN) has since been demonstrated not only in most tumours of the HNPCC syndrome but also in a distinct number of sporadic CRC. These data suggest three major advances: (a) a new mechanism in carcinogenesis, (b) the possibility of identifying high-risk individuals before they develop colon- or other HNPCC-related carcinomas, and (c) the identification of a genotype-specific pathology of a subset of tumours which may have a better clinical outcome.

Familial colon cancer syndromes

CRC is the second most common malignancy, affecting men and women equally. Environmental factors have been suggested as the major aetiological factors and thus most CRCs are considered sporadic. However, a distinct number of genetic syndromes exist which predispose to the development of CRC, and which account for approximately 10–15% of cases, following an autosomal dominant inheritance pattern (Table 1). Two genetic entities – familial adenomatous polyposis (FAP) and HNPCC – are associated with an extremely high risk of developing CRC at a young age. Localization and isolation of the *APC* (adenomatous polyposis coli) gene which causes FAP enabled a detailed analysis of this inherited condition and permits the unambiguous identification of affected patients. FAP accounts, however, for only 1% of CRC cases, and the prevalence is therefore relatively low (1:10 000) [13].

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Table 1 Inherited predispositions to CRC (modified acc. to [45])

Syndromes with pre-existing polyposis	
FAP	colonic polyposis
Gardner's syndrome	colonic polyposis associated with mesenchymal tumours
Oldfield's syndrome	sebaceous cysts
Turcot syndrome	colonic polyposis associated with mal. CNS tumours
Carroli's disease	bile duct and renal anomalies
Syndromes with pre-existing hamartomatous polyps	
Peutz-Jeghers syndrome	abnormal pigmentation of the lips
Ruvalcaba-Myhre-Smith syndrome	macrocephaly, pigmented macules on the penis
Juvenile polyposis	cystic hamartomatous polyps
Cowden's syndrome	multiple hamartomatous lesions, breast and thyroid cancer
Syndromes without pre-existing polyposis	
HNPCC*)	<5 polyps, proximal colon predominant
HNPCC, Lynch type I	CRC in at least 3 first degree relatives, at least one part younger than 50 ys
HNPCC, Lynch II	additional endometrial, gastric, biliopancreatic or urogenital cancers
Muir-Torre's syndrome*	Lynch II with dermatological lesions and laryngeal cancer
Hereditary flat adenoma syndrome	later onset than HNPCC proximal colon predominant

* with proven MIN phenotype [1, 2, 18, 26, 42]

In contrast, Lynch and coworkers [28] have delineated another colonic cancer predisposition syndome, that now referred to as HNPCC. This cancer family syndrome is one of the most common genetic diseases in man, affecting as many as 1 in 200 individuals and thus accounting for about 6–10% of CRC. According to the proposals of an international collaborative group (the "Amsterdam criteria") HNPCC should be diagnosed if (a) three or more first-degree relatives are affected with colorectal cancer, and (b) if at least one of these has been diagnosed before the age of 50 years [57]. HNPCC families presenting exclusively CRC have been designated as Lynch syndrome type I. Approximately 30% of HNPCC patients also develop carcinomas of other organs such as endometrium, stomach, biliopancreatic or urinary tract, which is referred to as Lynch syndrome type II [27].

Analysis of a total of 165 kindreds comprising 840 patients with CRC from seven different countries has led to delineation of the main characteristics of HNPCC [58]: an early age of onset (mean 45 years), a proclivity to the proximal colon (60–70%) with no increased adenoma prevalence, and an excess of synchronous and metachronous colonic cancers. Approximately 40% of patients develop second colon carcinomas within 10 years after subtotal colectomy [27, 56]. Histopathologically, there is a preponderance of poorly differentiated and mucinous carcinomas. It has been suggested that patients with HNPCC have a better prognosis, but no controlled prospective study has yet been undertaken [20, 50].

HNPCC, MIN and human mismatch repair genes

Although the familial background of the HNPCC predisposition syndrome was discovered nearly a century ago [59], the molecular basis remained undefined until recently. A fundamental breakthrough in understanding the pathomechanisms underlying inherited CRC susceptibility has been provided by new tools in molecular genetics and by recent advances in the understanding of mismatch-repair mechanisms in bacteria and yeast.

Using the arbitrarily primed polymerase chain reaction (AP-PCR), Peinado et al. [37] were the first to detect widespread subtle genomic alterations in a subset of sporadic CRC cases. Subsequently these alterations have been shown to represent deletions in poly(A) tracts as well as extensions and contractions in the length of simple repeated sequences, particularly of microsatellite sequences consisting of dinucleotide and trinucleotide repeats (Fig. 1) [1, 18, 54]. Screening of human CRC for mutations in simple repetitive DNA sequences disclosed MIN in dinucleotide repeats in about 80–90% of HNPCC tumours and about 12–20% of sporadic CRC [1, 2, 26, 42].

The most common dinucleotide sequence in eukaryotes is the $(CA)_n$ repeat. It is estimated that about 50 000–100 000 $(CA)_n$ repeats are scattered throughout the human genome [15]. Since tumours exhibiting MIN may therefore carry more than 100 000 such mutations at different microsatellite loci, an underyling defect in a DNA replication factor resulting in reduced fidelity of replication or repair was postulated. Tumours with MIN have been suggested to be replication error positive (RER^+), expressing a mutator phenotype [1, 18].

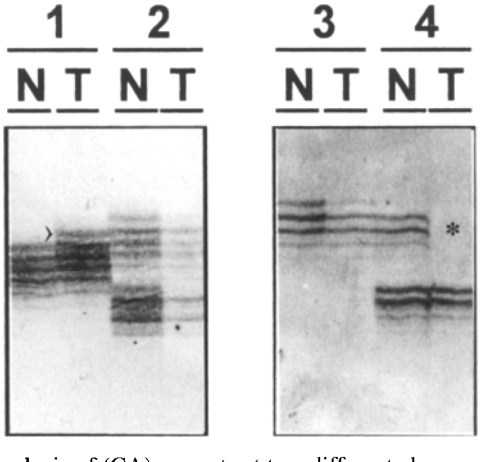

Fig. 1 Analysis of $(CA)_n$ repeats at two different chromosmal loci using microsatellite PCR. *Tumours 1, 2*: microsatellite instability (MIN; *arrowhead*) is defined by an electrophoretic mobility shift of PCR products between pairs of constitutional normal tissue DNA (*N*) and tumour DNA (*T*) (locus D10S89, size 142–156 bp). *Tumours 3, 4*: allelic losses (loss of heterozygosity, LOH; *asterisk*) can be readily demonstrated by the same technique; the mechanisms underlying LOH, however, are totally different from those demonstrated in MIN (locus APC on chromosome 5, size 96–122). (Staining after [44])

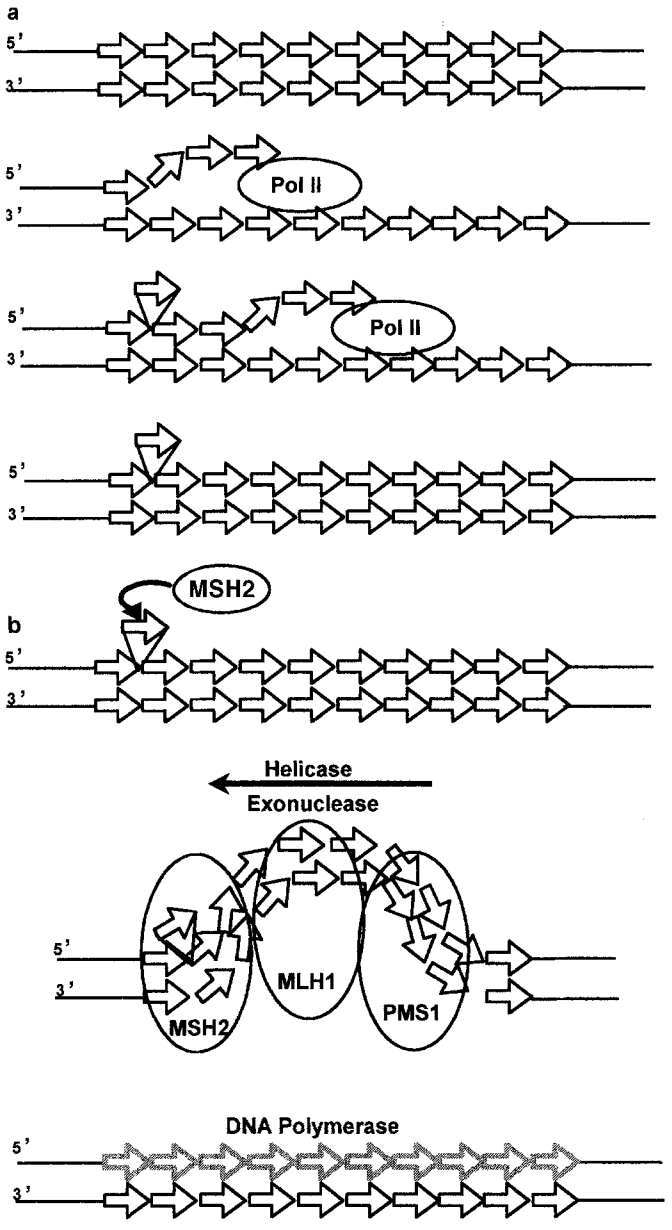

Fig. 2a, b Mechanisms causing dinucleotide repeat instability. **a** DNA polymerase slippage. During DNA replication the newly synthesized primer strand is transiently dissociated from the template strand and reanneals in a misaligned configuration. If the mismatch is located in the primer strand (as shown) an increased tract length results, and vice versa. **b** Model of mismatch repair (in eukaryotes). *MSH2* binds to the mismatched $(CA)_n$ repeat followed by an assembly of a complex of at least two other polypeptides. After excision of the strand containing the mismatched nucleotides the gap is filled by polymerase III. *Arrows* (CA) dinucleotide. (Modified after [19, 51])

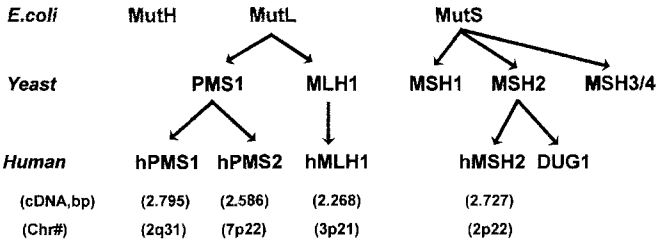

Fig. 3 Human homologues of pro- and eukaryotic mismatch repair genes involved in hereditary non-polyposis colonic cancer (HNPCC). Mutations of *hMLH1* and *hMSH2* are most frequently affected in HNPCC (80–90%). Relationship of DUG1 to HNPCC has not yet been assessed [19]

The exact mechanism underlying MIN has only recently been elucidated. Strand et al. [51] have shown that yeast DNA polymerase causes a very high rate of mismatches on templates containing $(CA)_n$ repeats. This process results in length changes of a few base-pairs at a time when it is usually corrected by specific mismatch repair systems. In *Escherichia coli* there are about 20 known genes involved in DNA replication or repair, so-called mutator genes. Whereas excision repair genes deal with oxidative lesions and ultraviolet photoproducts, mismatch repair genes act on single base mismatches and small displaced loops that can occur through slippage during replication of repeat regions [9]. Mutations in the mismatch repair genes such as *MutL* and *mutS* in *E. coli* and of *pms1*, *mlh1* and *msh2* in yeast are followed by a 13-fold elevation in tract instability in *E. coli* and a 100- to 700-fold increase in tract instability in yeast [51]. Thus, it has clearly been shown that MIN is the consequence of DNA polymerase slippage and a decreased efficiency of mismatch repair due to mutations in DNA repair genes (Fig. 2).

In agreement with these data, Parsons et al. [36] demonstrated that the cell line H6 derived from a HNPCC patient is in fact defective in mismatch correction. Transfection of plasmids carrying dinucleotide repeats similar to those found in microsatellite DNA indicated that in H6 cells these sequences acquire mutations at a much higher rate than in the control cell line. These data provide strong evidence for the existence of human homologues of the aforementioned mismatch repair genes in bacteria and yeast. Using probes from *MutL* genes in mice [12] and data bases on cDNA sequences [35], two human homologues of prokaryotic repair genes (*MutS*, *MutL*) were initially detected (*hMSH2*, *hMLH1*); *hMSH2* mapped to chromosome 2p22-21 and *hMLH1* to chromosome 3p21-23 [5, 12, 21, 22, 35]. Most recently two additional genes, *hPMS1* and *hPMS2* (homologues of the prokaryotic *MutL* gene), have been sequenced and localized on chromosome 2q31-33 (*hPMS1*) and chromosome 7p22 (*hPMS2*; Fig. 3) [32].

Linkage analyses and direct sequencing of these genes have clearly established the relationship to HNPCC. Germ-line mutations affecting one allele within one of the mismatch repair genes segregated perfectly with HNPCC; unaffected relatives had no mutations [21]. Most interestingly, lymphoblasts from HNPCC patients had normal repair actvity at the biochemical level although one allele of the repair genes was mutated. In contrast, tumours and tumour cell lines of HNPCC patients studied so far were almost completely devoid of repair activity in the same assay due to the total absence of wild-type alleles in one of the four cloned repair genes. It is therefore concluded that inactivation of both alleles of a mismatch repair gene is required for the ex-

pression of MIN ("mutator phenotype") and thus for tumour formation in HNPCC [35, 36].

These exciting advances in the understanding of familial colon cancer raise several other questions: Is the MIN phenotype a new mechanism of carcinogenesis? How do mutations in mismatch repair genes contribute to tumorigenesis? What are the current perspectives for the screening of HNPCC patients, particularly from the diagnostic pathologist's viewpoint?

MIN: a new mechanism of carcinogenesis

A series of studies have clearly demonstrated that MIN is not associated exclusively with HNPCC. A distinct proportion of carcinomas without known family history exhibit MIN with highest frequency in pancreatic, gastric and bladder cancers (Table 2). These mutations have been found in all neoplastic regions of multiple tumours, including adenomas of the same patient as well as in early-stage bladder and breast cancers [14, 18, 46, 52, 63].These alterations have therefore been considered an early event in tumorigenesis. However, in some other tumours, including gastric carcinoma [8], colitis-associated CRC [53] and renal cancer [55] MIN has been found more frequently in advanced lesions, suggesting that MIN is a later event in tumorigenesis.

Irrespective of these discrepancies, there is general agreement that tumours with MIN have statistically significantly lower rates of loss of heterozygosity (LOH), particularly at the p53 locus (chromosome 17p) [30, 31, 54, 62, 63]. Similarly, a trend towards less frequent p53 gene product overexpression has been found by immunohistochemistry [20, 52]. In addition, tumours as well as tumour cell lines exhibiting the MIN phenotype have been shown often to be diploid or near-diploid [1, 3, 20, 26, 42]. This clearly demonstrates that MIN is not associated with LOH or other gross chromosomal DNA changes that have been described in the multistep conception of carcinogenesis in CRC. These results support the proposal that the development of a mutator phenotype due to intrinstic genetic instability is an early step in the formation of tumours [25].

Most interestingly, MIN-positive colonic and gastric tumours have some morphological features that may be considered as phenotypic markers of genetic change [20]. Both are more frequently high-grade tumours, mucinous type in the colon and non-signet ring cell type in the stomach. They are often located proximal to the splenic flexure (80–94%) [2, 18, 20, 54] and in the distal stomach (83%) [52]. In colonic and gastric carcinomas MIN is correlated with improved survival although this is not an independent prognostic factor [26, 52].

According to these data, a subset of sporadic CRC cases have unique biological and morphological features which are very similar to those described in HNPCC patients. However, the two cancers do not correspond exactly. MIN-positive sporadic tumours seem to be even more homogeneous, or "HNPPC-like", than HNPCC itself, being more right-sided, mucinous and poorly differentiated (Table 3). Explanations for this phenomenon may be related to the clinical definition of HNPCC, which could include some non-hereditary cases [50]. Since the status of the mismatch repair genes in sporadic MIN-positive carcinomas is still unknown, their contribution to these differences in the tumour phenotype cannot yet be assessed.

Table 2 Microsatellite instability in extracolonic cancer

Organ-tumour	Author	Tumours	MIN		Chromosomal loci
		(n=)	pos	(%)	
Breast	Lothe (1993)	84	0	(0)	7 (≥2)
	Han (1993)	26	1	(4)	4 (≥1)
	Yee (1994)	20	4	(20)	7 (≥1)
	Wooster (1994)	104	11	(11)	12 (≥1)
Lung					
-NSCLC	Peltomäki (1993)	85	0	(0)	7 (≥2)
	Shridar (1994)	38	13	(34)	10 (≥1)
-SCLC	Merlo (1994)	33	15	(45)	34 (≥1)
Stomach	Petlomäki (1993)	33	6	(18)	7 (≥2)
	Han (1993)	57	22	(39)	4 (≥1)
	Mironow (1994)	22	5	(23)	10 (≥1)
	Chong (1994)	76	25	(33)	2 (≥1)
	Strickler (1994)	40	6	(15)	3 (≥1)
Pancreas	Han (1993)	9	6	(67)	4 (≥1)
Liver	Han (1993)	29	1	(3)	4 (≥1)
Kidney	Uchida (1994)	36	9	(25)	7 (≥1)
Bladder	Gonzalez (1993)	200	6	(3)	7 (≥1)
	Orlow (1994)	61	25	(41)	1 (≥1)
Testis	Lothe (1993)	86	0	(0)	7 (≥2)
Ovary	Han (1993)	19	3	(16)	4 (≥1)
Cervix	Han (1993)	13	2	(15)	4 (≥1)
Endo-	Peltomäki (1993)	18	4	(22)	7 (≥1)
metrium	Risinger (1993)	36/4*	6/3	(17)/(75)	71 (≥2)
	Burks (1994)	30	7	(23)	8 (≥1)
Brain	Wooster (1994)	54	1	(2)	12 (≥1)
Soft tissue	Wooster (1994)	18	2	(11)	12 (≥1)

* patients without and with HNPCC

Table 3 Microsatellite instability in colorectal cancer – clinical, morphological and molecular findings/characteristics (according to [20, 28, 56])

	Sporadic CRC MIN (–)	Sporadic CRC MIN (+)	HNPCC MIN (+)
Frequency	85%	15%	90%
Age (mean)	66 ys	60 ys	45 ys
Localization (proximal)	30–35%	80–94%	56–62%
Histology			
– grade 3	7%	55%	37%
– mucinous	20–30%	82%	40%
Ploidy (diploid)	25%	80%	60%
LOH (p53)	40–60%	18%	35%

Tumorigenesis and mutations in mismatch repair genes

At present the exact mechanism through which the MIN phenotype contributes to tumorigenesis is unknown. Sev-

eral aspects must be considered. First, the underlying mismach repair deficiency may alter DNA replication and repair, which causes a reduced fidelity of replication. Second, microsatellites are scattered throughout the genome, including both non-coding and coding regions. Thus, MIN may affect the functional activity of genes, such as the androgen receptor, which contains a $(CAG)_n$ repeat [14,63]. Third, since microsatellites play a role in chromatin structure and nucleosome placement, MIN may interfere with proper gene transcription [25].

In fact, Parsons et al. [36] observed a profound defect in strand-specific mismatch repair in MIN-positive tumour cells both in microsatellite $(CA)_n$ repeats and heteroduplexes containing single base-base mismatches. These data provide strong evidence that the mutator phenotype is likely to cause transitions and transversions in addition to microsatellite alterations. Most recently, Bhattacharyya et al. [3] found mutation rates for 6-thioguanine resistance and ouabain resistance to be increased 1000- and 500-fold in a CRC cell line expressing MIN (HCT116) over those observed in normal diploid fibroblasts. This clearly links MIN with mutations other than solely in repetitive sequences which may promote tumorigenesis, for example, by magnification of mutagenic effects of some carcinogens. The question, however, as to why HNPCC kindreds do not develop skin, lung and other non-HNPCC related cancers remains unresolved.

Another group of instability-related diseases must be considered in this regard. Marked increases in length of trinucleotide repeat arrays (from 30 to 2000 repeats) have been shown to be diagnostic of several inherited neurodegenerative diseases such as fragile X syndrome, Kennedy's syndrome, myotonic dystrophy and Huntington's disease [34]. Expansions in unique trinucleotide repeats occur in each of these diseases within or near adjacent genes during meiosis or early embryogenesis which affect all cells. In contrast to MIN, which is restricted to neoplastic tissue, there is no evidence for increased incidence of malignancies in any of the neurodegenerative diseases. Thus, the mechanisms for expansion of repetitive sequences in cancer and neurodegenerative diseases are most likely unrelated [7, 41].

Clinical and pathological implications

In general, these developments are of central importance for both pathologists and clinicians since they may provide new tools for identification of high-risk individuals before they develop colon cancer, other HNPCC-related carcinomas or secondary cancer.

Clinically the diagnosis of HNPCC is often difficult, for several reasons. Most of all, the family history is often incomplete, and due to the small size of many genealogical trees not all families are informative. The minimum requirements proposed by the Amsterdam criteria cannot be assessed in even true HNPCC cases [40]. Therefore the syndrome is rather heterogeneous, and the exact prevalence is difficult to define.

In contrast, the most recent molecular data open a new field in the detection of predispositions for the development of carcinomas, particularly of the HNPCC type. Approximately 90% of all HNPCC patients have a germline mutation within one of the cloned human mismatch repair genes of which *hMLH1* and *hMSH2* are most often affected (70–80%). At present, however, the identification of these mutations is far from being a routinely applicable procedure since the corresponding genes are relatively large, and no clear mutational hotspot has been delineated. Liu et al. [24] proposed a combination of different molecular methods for repair gene analysis, including RT-PCR on mRNA extracts which are then analysed either by SSCP or direct sequencing of RT-PCR or genomic PCR products. However, it must be stressed that mutations in these genes are not necessarily indicative of HNPCC. Missense or inframe deletions as well as mutations in splice signals have been described, suggesting that their effect is not in knocking out protein function completely. In addition, sequence polymorphisms have been observed [12]. To avoid erroneous diagnoses it is therefore important also to study the effect of individual mutations by the biochemical function of the protein [4, 19].

With respect to such sophisticated and tedious molecular methods it is very useful to select those patients for mismatch repair gene analysis who are most probably at risk for HNPCC. Demonstration of the MIN phenotype by PCR thereby meets the basic demands of such a specific and rapid screening method. MIN is considered to be a phenotypic marker of mutated mismatch repair genes since all tumours and tumour cell lines studied so far which have mutations in both mismatch repair gene alleles express the mutator phenotype [2, 3, 36]. Furthermore, we have modified the microsatellite PCR to a rapid non-radioactive technique, which can even be performed in routine histopathological laboratories [44].

At present, however, there is no standardization regarding either how many microsatellite markers must be tested or how many shifted PCR products are required for the diagnosis of MIN. Accordingly, the frequency of

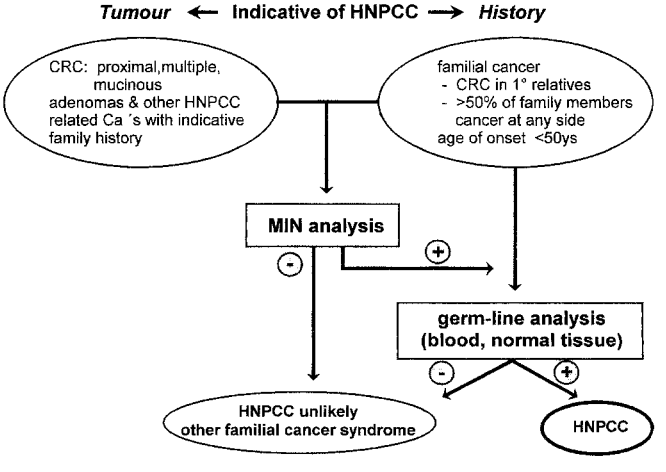

Fig. 4 Strategy of HNPCC screening

MIN phenotype given in the literature varies markedly, even within the same tumour type (Table 2).

Merlo et al. [30] tested more than 5000 markers in about 400 different sporadic tumours and found single somatic alterations of simple repetitive repeats not to be uncommon events (1–3%). The frequency of a new allele for a dinucleotide locus is 0.5% and increases to 0.5–2.6% in tri- and tetranucleotide repeats [61]. Larger tandem repeats seem to be even more susceptible to alterations. Therefore, cases with instability involving only one dinucleotide repeat may fall into a potential background rate. Basically, this has been confirmed by Thibodeau et al. [54], who found that only CRC with MIN at two and more microsatellite loci had a statistically significantly better prognosis than patients with no MIN or only one involved microsatellite locus. Therefore the diagnosis of MIN should be based upon at least two affected loci, and at least five loci should be tested before classifying a case as MIN positive or negative [26, 54]. In addition, there is increasing evidence that, in addition to the number of affected loci, the type of instability with minor alterations (two base-pair changes) or major alterations (more than two base-pair changes) should also be assessed [3, 14]. However, little is known about the relationship between the type of such mutations and the tumour phenotype.

From a diagnostic point of view the most appropriate strategy seems to be a combination of clinical and morphological data suggestive of familial cancer with a stepwise molecular analysis of MIN and underlying mutations in repair genes. Since microsatellite PCR is an easy method to perform [44], screening of all CRC for MIN phenotype has been proposed (Aaltonen, personal communication). Whereas almost all sporadic adenomas are MIN negative [64], the preneoplastic lesions in 57% of HNPCC kindreds are MIN positive [2], indicating a high HNPCC probability. Therefore we propose MIN screening in adenomas and in other HNPCC-related carcinomas particularly in cases of other clinical criteria suggestive of HNPCC as outlined by Ponz de Leon et al. [40]. Those persons who have a familial history of cancer predispostion and/or a tumour expressing the MIN phenotype should then be tested for germline mutations within the predispostion genes (Fig. 4).

Such genetic testing will not only allow a definite assessment of the true frequency of HNPCC. The molecular diagnosis of the syndrome will provide the rationale for an adequate therapy, which is subtotal colectomy in CRC [29] and prophylactic hysterectomy and bilateral salpingo-oophorectomy in females with a syndrome cancer [60]. Relatives at risk can be instructed to take all possible steps towards preventing CRC, including frequent monitoring.

In conclusion, isolation of genes predisposing to cancer opens a new field in the genetic diagnosis of cancer risk. Several cancer predisposition genes have already been identified, such as BRCA1, which appears to be responsible for approximately 40% of hereditary breast cancers [49], and the *ret* oncogene, which allows the unambiguous diagnosis of multiple endocrine neoplasia types 2A and 2B [23]. This clearly shows a potential new role for the pathologist in cancer prevention. In addition, the understanding of such cancer predisposition genes will lead to the development of strategies aimed at blocking the earliest steps in neoplastic development.

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