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Original Paper

Evaluation of the Replication Error Phenotype in Relation to Molecular and Clinicopathological Features in Hereditary and Early Onset Colorectal Cancer

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Mutations affecting human mismatch repair (MMR) genes (*MLH1*, *MSH2*, *PMS1*, *PMS2*, and *MSH6*) cause tumour predisposition in hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, and an association has been demonstrated with the replication error (RER) phenotype in most colorectal and some extracolonic neoplasms. A pathogenetic model for RER+ tumours through inactivation of suppressor genes has been hypothesised, and *TGFβRII*, *BAX* and *IGFIIR* genes have recently been proposed as targets of such inactivating mutations. In this study, a series of 47 tumours developed in patients with known *MLH1/MSH2* status and a family history of HNPCC and/or early onset colorectal cancer were characterised for the RER phenotype through microsatellite analysis. The RER phenotype, displayed by 17 tumours, was then correlated with the presence of insertions/deletions at the *TGFβRII*, *IGFIIR* and *BAX* gene stretches, confirming that the *TGFβRII* inactivation may be particularly critical for the RER-associated tumorigenesis. RER+ colorectal cancers (CRCs) developed more frequently in patients from HNPCC families (72.7%) than in those from families not fulfilling the Amsterdam criteria (33.3% in suspected HNPCC and 20.8% in early onset CRC patients). A consistent fraction of either Amsterdam and non-Amsterdam patients developed RER– CRCs, pointing to the involvement of other genes not related to the MMR system. The RER phenotype was associated with younger age at diagnosis in familial cases, and there was a trend for an association with proximal CRC localisation and early Dukes' stages. The RER status was also correlated with the presence and type of *MLH1* and *MSH2* alteration.

Key words: colorectal cancer, microsatellite, mismatch repair, mutation, replication error
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INTRODUCTION

HEREDITARY NONPOLYPOSIS colorectal cancer (HNPCC) syndrome is characterised by an increased predisposition to colorectal and extracolonic cancers, particularly of the endometrium, stomach, pancreas, ovary, and biliary tract. Affected individuals often carry alterations at mismatch repair (MMR) genes (*MSH2*, *MLH1*, *PMS1*, *PMS2* and *MSH6*) [1–3]. Defects in MMR genes, and therefore in MMR activity, cause the introduction of small nucleotide insertions/deletions during DNA synthesis and the appearance of the replication error (RER) phenotype, whose major manifestation in eukaryotic cells is the instability of interspersed microsatellite DNA sequences (microsatellite instability) [4].

The RER phenotype has been widely studied in colorectal cancer (CRC), both of familial and sporadic origin, as well as in a variety of extracolonic cancers. In particular, it was present in up to 95% of HNPCC-related CRCs and also in 5–24% of sporadic and APC-related CRCs [5–7]. The RER phenotype is mainly due to constitutional *MLH1* or *MSH2* mutations in CRCs developed in HNPCC patients [8, 9]; it is rarely ascribable to such mutations in sporadic colonic and endometrial tumours [10, 11].

The association of the RER phenotype with many clinicopathological characteristics has been investigated by several authors mainly in CRCs. There was a clear association of

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the RER+ status with features such as age of onset, familiarity, localisation, tumour ploidy, mucinous differentiation, inflammatory reaction, and prognosis [5, 7, 12, 13].

Since MMR gene alterations influence DNA replication fidelity, various studies have been undertaken to verify if specific oncogenes or tumour suppressor genes could be altered as a consequence of such mutational mechanisms. At least in CRCs and gastric cancer, genes such as *p53* and *ras* are less frequently mutated in RER+ than in RER− tumours [6, 14]. Other genes, such as *TGFβRII*, *BAX* and *IGFIIR*, have been found to be inactivated in the presence of microsatellite instability through frameshift mutations at mononucleotide repeat stretches inside the coding sequence. TGFβRII is a receptor whose function is to transduce growth inhibitory signals when bound to TGFβ. IGFII exerts a growth stimulatory effect when bound to IGFIR, whereas IGFIIR, in contrast, makes IGFII unavailable. Thus, the inactivation of *TGFβRII* or *IGFIIR* would lead to the loss of cell growth inhibition. *BAX* is a pro-apoptotic gene whose transcription is regulated by *p53* [15] and whose inactivation

Table 1. *Clinicopathological and genetic features of the tumours*

Tumour	Family history*	Age	Site†	Grading‡	Dukes'	*MLH1*/*MSH2*§
CFS1T	A	52	en	–	–	*MLH1* IVS7-2A>G tr
CFS9T	A A-AV2 (a)	69	dist(II)	P	C	*MLH1* Q301X tr
CFS10T	A A-AV2 (a)	40	dist(II)	P	B	*MLH1* Q301X tr
CFS11T	S A-AV19 (b)	55	dist	–	C	wt
CFS12T	E A-AV12	31	prox	M	C	wt
CFS13T	S A-AV19 (b)	48	dist	–	–	wt
CFS14T	S A-AV21	49	dist	–	B	wt
CFS24T	A A-MD1	43	dist	–	–	*MSH2* 1243del4 tr
CFS25T	E A-AV14	34	prox	W	B	*MLH1* 1783delAG tr
CFS26T	A A-AV1	39	prox	P	B	*MSH2* G322D mss
CFS27T	E A-AV6	38	prox	M	C	wt
CFS28T	E A-AV9	37	prox	M	B	wt
CFS29T	S A-AV20	35	prox	M	C	wt
CFS30T	E A-AV16	39	prox	P	D	wt
CFS31T	A A-AV4	37	prox	–	B	wt
CFS38T	S	42	dist	–	–	wt
CFS39T	E A-AV8	41	prox	M	B	wt
CFS47T	A A-PD1 (c)	41	prox	P	B	*MLH1* 1011delC tr
CFS48T	A A-PD1 (c)	33	dist	–	–	*MLH1* 1011delC tr
CFS53bT	A	39	dist	–	–	*MLH1* 2270insT add
CFS56T	E A-AV23	29	dist	M	B	*MLH1* V716M mss
CFS58T	E A-AV5	36	prox	W	B	wt
CFS59T	E A-AV13	25	dist	M	B	wt
CFS69T	S A-AV17	32	prox	P	B	*MSH2* Q824X tr
CFS70T	S A-AV18	37	dist	P	B	*MLH1* V326A mss
CFS73T	S A-AV22	45	prox	P	C	wt
CFS110T	S	49	prox	P	D	wt
CFS112T	E	35	dist	–	C	*MSH2* A834T mss
CFS113T	E A-AV10	39	dist	P	B	wt
CFS114T	E	28	dist	P	D	wt
CFS116T	A	73	dist	–	–	wt
CFS117T	E	42	dist	P	D	wt
CFS119T	E	25	dist	W	C	wt
CFS120T	E	36	dist	P	D	wt
CFS125T	E	37	dist	P	C	wt
CFS136T	E	38	dist	M	C	*MLH1* V326A mss
CFS137T	E	34	dist	W	B	*MSH2* S473X tr
CFS139T	E	28	dist	M	C	wt
CFS140T	E	41	dist	M	B	wt
CFS141T	E	44	dist	M	D	wt
CFS143T	E	38	dist	P	B	wt
CFS145T	S (d)	40	dist	M	D	wt
CFS150T	A	58	prox(II)	W	B	*MSH2* C778X tr
CFS154T	E	31	dist	M	C	wt
CFS226T	S	38	prox	M	C	*MSH2* delE571 id (som)
CFS291T	E	31	dist	P	C	wt
CFS421T	S (d)	53	dist	M	C	wt

*A, Amsterdam HNPCC; S, suspected HNPCC; E, early onset patient. Family codes used in previous papers [24, 25] are indicated in parentheses. (a), (b), (c), (d), patients belonging to the same family. †en, endometrial cancer; prox, proximal CRC localisation with respect to splenic flexure; dist, distal CRC; dist(II), distal CRC metachronous after a proximal CRC; prox(II), proximal CRC metachronous after a distal CRC. ‡W, well; M, moderate; P, poor differentiation grade. §Constitutional genotype. tr, truncating mutation; add, mutation adding a polypeptide tail; mss, missense variant; id, in-frame deletion; som, somatic; wt, wild type.

might result in the loss of the growth control exerted by the *p53*-programmed cell death pathway. Therefore, it is likely that mutations in these genes are very important in the molecular pathway of RER+ tumour development or progression [6, 16, 17].

In this study, RER status was investigated in a series of neoplasms which developed in patients who fulfilled or did not fulfil the Amsterdam criteria for HNPCC [1, 2]. The RER phenotype was then correlated with various clinical, pathological, and molecular features to evaluate its possible diagnostic significance in hereditary and early onset CRC. In particular, it was correlated with the mutational status of *MLH1* and *MSH2* genes and with the presence of alterations at the polynucleotide repeats of the target genes *TGFβRII*, *BAX*, and *IGFIIR*.

MATERIALS AND METHODS

Patient and tumour collection

Forty-seven frozen or paraffin-embedded tumour samples (46 CRCs and 1 endometrial carcinoma) were collected through pathologists at many North Italian hospitals where patients had undergone surgery. These patients were recruited in the framework of studies on genetic predisposition to HNPCC, and divided into three groups: (1) 11 patients belonging to 9 HNPCC families fulfilling the Amsterdam criteria for definition of the syndrome (Amsterdam HNPCC families); (2) 12 patients belonging to 10 families suspected to be HNPCC, that is, having at least one first-degree relative with CRC or another tumour of the HNPCC spectrum (suspected HNPCC families); and (3) 24 patients having developed CRC before the age of 45 years and with a negative family history for tumours of the HNPCC spectrum (early onset patients).

Some clinicopathological (age at diagnosis, localisation, Dukes' stage, differentiation grade) and genetic (*MLH1* and *MSH2* variants/mutations) features of the tumours are shown in Table 1.

DNA extraction

Genomic DNA was extracted from white blood cell samples, and normal and neoplastic tissues from both frozen or paraffin-embedded samples. An automated extraction from blood cells and frozen tissues was performed with an AB 341 Nucleic Acid Purification System (Perkin Elmer, Foster City, California, U.S.A.); DNA was extracted from slices of paraffin-embedded tissues using a Cleanmix Purification System (Talent, Trieste, Italy) according to the manufacturer's instructions.

Molecular analyses

RER status was determined by analysing 6–8 microsatellite loci, including tetra- (L-*myc*), tri- (*DM*), di- (*D1S170*, *CA21*, *D3S1611*, and *D17S250*) and mononucleotide repeats (*BAT13* and *BAT26*) and by comparing normal and tumour DNA from the same individual. A sample was considered RER+ if at least two markers showed instability in tumour DNA.

TGFβRII, *BAX*, and *IGFIIR* gene analysis specific for the mononucleotide sequence tracts was carried out on tumour DNA samples.

For both RER status assessment and target gene analysis, samples were subjected to two successive PCR reactions. The first was performed under standard conditions, in the presence of 0.5 μM of each primer and 0.2 mM of each dNTP, by an incubation of 30 denaturation/annealing/elongation cycles. The second amplification step was performed from an aliquot of the pre-amplified template in the presence of 2.5 μM of each cold dNTP and 0.1 mCi/ml of α-^{33}P-dATP for 25 cycles. For each locus, primer pairs and annealing temperatures used were as previously described [6, 16–23]. For BAT13 amplification, 5′-GAA CAT GTA ATA TCT CAA ATC-3′ and 5′-GCT CTA TTC TTA TAA ACT TCA AC-3′ were used as sense and antisense primer, respectively, at the annealing temperature of 48°C. Radiolabelled PCR products were run on to 6% polyacrylamide, 8 M urea gel.

Statistical analysis

Two-sided Fisher's exact test, χ^2 test, and Mann–Whitney *U*-test were used when appropriate.

RESULTS

MMR gene mutations

A total of 16 tumours were from patients with constitutional *MLH1* or *MSH2* variants. The mutations/variants of patients CFS9 and CFS10, CFS24, CFS25, CFS47 and CFS48, CFS56, CFS69, CFS70 have been previously described [24, 25], and additional molecular variants were observed in 7 other patients. Of the 13 different variants, 9/11 patients were mutations affecting the predicted protein size and 4/5 patients were alterations causing amino acid substitutions with uncertain pathogenetic significance (Table 1).

Considering only *MLH1* and *MSH2* size-affecting alterations with a clear pathogenetic significance, the frequency of mutation was 66.7% (6/9), 10% (1/10) and 8.3% (2/24) in Amsterdam families, suspected HNPCC families, and early onset patients, respectively [χ^2(trend), $P = 0.001$; χ^2(overall), $P = 0.001$].

RER phenotype

Following the analysis of 6–8 microsatellite sequences (Figure 1), 17 tumours (16 CRCs and 1 endometrial cancer) out of 47 proved to be unstable at two or more microsatellites and were thus defined as RER+ (Table 2). RER+ tumours showed instability at two (3 cases), three (2), four (2), five (5), six (2), seven (2), and eight loci (1). Three CRCs presented instability at only one locus (2 at L-*myc* and 1 at *BAT26*) and were classified as RER− (Table 2). All loci investigated displayed variable frequencies of alteration: 38.2% for L-*myc*, 29.8% for *BAT26*, 25.5% for *D3S1611*, 23.4% for *BAT13* and *D17S250*, 15.2% for *CA21*, 14.9% for *DM*, and 9.1% for *D1S170*. *BAT26* was investigated in all samples and appeared stable in 4 RER+ tumours (CFS13T, CFS25T, CFS39T and CFS59T) and unstable in 1 RER− tumour (CFS31T).

The presence of the RER phenotype was evaluated in relation to clinicopathological and genetic parameters. Eight out of 11 (72.7%) Amsterdam HNPCC patients developed RER+ neoplasms, whereas this occurred in only 4/12 (33.3%) suspected HNPCC and 5/24 (20.8%) early onset patients (Table 3). Constitutional mutations at *MLH1* or *MSH2* were present in 8/8 (100%) Amsterdam HNPCC patients with RER+ tumours but only in 3/9 (33.3%) non-Amsterdam (suspected plus early onset) HNPCC patients with RER+ tumours ($P = 0.009$). Of the six remaining RER+ tumours, only CFS29T and CFS226T could be analysed for *MLH1* and *MSH2* somatic mutations and the latter showed an in-frame deletion at *MSH2* (Table 1).

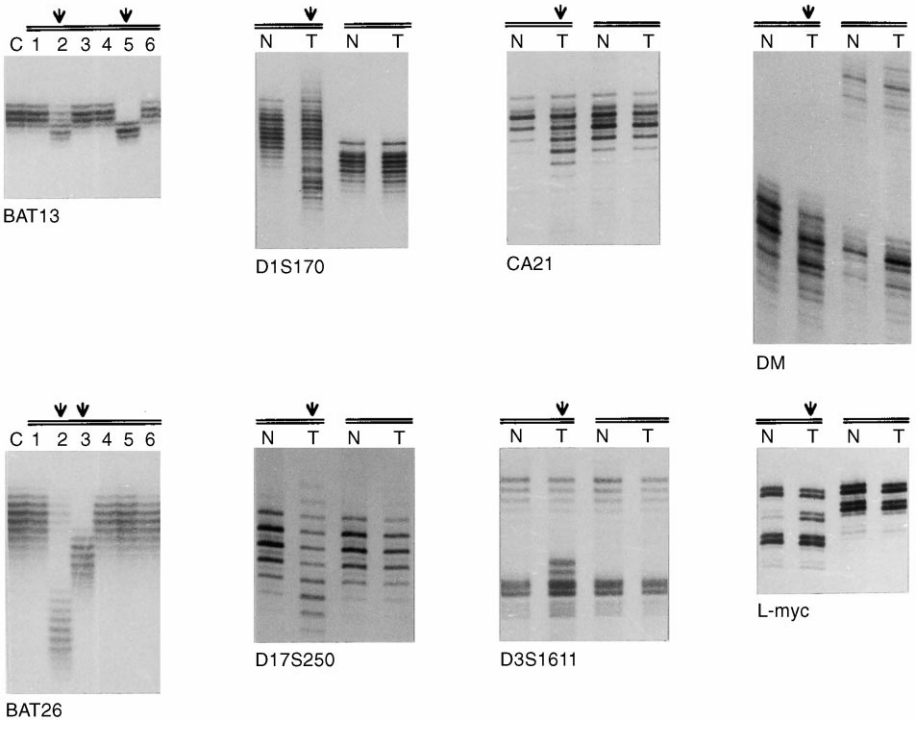

Figure 1. Panel of microsatellites used for RER phenotype assessment. Arrows indicate unstable samples. C, control; N, normal; T, tumour sample.

All 5 patients carrying *MLH1* or *MSH2* missense variants developed RER− CRCs. In patients with a positive family history (excluding cases for which only the second primary tumour was analysed), the RER+ phenotype was significantly associated with younger age at diagnosis: 38.62 ± 5.37 years in 8 patients with RER+ tumours compared with 47.18 ± 10.61 years in 11 patients with RER− tumours (Table 3).

Considering only cases for which data were available, differences in relation to CRC localisation and tumour stage were also found, although no statistical significance was reached: 7/15 proximally located versus 6/28 distally located CRCs and 8/17 early stage versus 4/22 advanced stage CRCs were RER+ (Table 3). Finally, no correlation was found regarding RER status and grading.

Target gene mutations

All CRCs (16 RER+ and 30 RER−) and 1 RER+ endometrial cancer were tested for insertions/deletions at the

Table 2. Molecular features of the tumours

Tumour	*MLH1*/*MSH2**	RER n/n†	TGFβRII‡	BAX‡	IGFIIR‡
CFS1T	*MLH1* IVS7-2A>G tr	+5/8	+	−	−
CFS9T	*MLH1* Q301X tr	+5/8	+	−	−
CFS10T	*MLH1* Q301X tr	+3/8	−	+	−
CFS13T	wt	+3/7§	−	−	−
CFS24T	*MSH2* 1243del4 tr	+5/8	−	−	−
CFS25T	*MLH1* 1783delAG tr	+2/8§	+	−	−
CFS29T	wt	+8/8	+	−	−
CFS30T	wt	+2/8	−	−	−
CFS39T	wt	+2/7§	−	−	−
CFS47T	*MLH1* 1011delC tr	+6/7	−	−	−
CFS48T	*MLH1* 1011delC tr	+5/8	−	−	+
CFS53bT	*MLH1* 2270insT add	+7/8	+	−	−
CFS59T	wt	+4/7§	−	−	+
CFS69T	*MSH2* Q824X tr	+5/7	−	−	−
CFS137T	*MSH2* S473X tr	+6/7	+	+	−
CFS150T	*MSH2* C778X tr	+4/7	+	−	−
CFS226T	*MSH2* delE571 id (som)	+7/8	+	−	−
CFS11T	wt	−1/7	−	−	−
CFS31T	wt	−1/8‖	−	−	−
CFS421T	wt	−1/7	−	−	−

*Constitutional genotype. tr, truncating mutation; add, mutation adding a polypeptide tail; mss, missense variant; id, in-frame deletion; som, somatic; wt, wild type. †+, RER positive; −, RER negative; n/n, number of unstable microsatellites/total microsatellites analysed. ‡+, altered mononucleotide stretch size; −, normal size. §RER+ tumours with stability of BAT26. ‖RER− tumours with instability of BAT26.

Table 3. RER phenotype in relation to clinicopathological features

	RER+	RER−	TOT	Statistics
Familiarity				
Amsterdam	8	3	11	
Suspected	4	8	12	$P=0.004$*
Early onset	5	19	24	$P=0.012$†
Age at diagnosis	38.62 ± 5.37 ($n=8$)	47.18 ± 10.61 ($n=11$)	($n=19$)	$P=0.047$‡
CRC localisation				
Proximal	7	8	15	
Distal	6	22	28	$P=0.162$§
Dukes' stage				
A+B	8	9	17	
C+D	4	18	22	$P=0.082$§

*Chi square trend. †Chi square overall. ‡Mann–Whitney U test. §Two-sided Fisher's exact test.

mononucleotide stretches of the *TGFβRII*, *IGFIIR*, and *BAX* genes (Figure 2). The endometrial tumour and 10/16 RER+ CRCs (64.7% of all RER+ tumours) displayed instability in at least one gene (Table 2), whereas none of the RER− CRCs did. Only one tumour (CFS137T) displayed frameshift alteration at *TGFβRII* and *BAX* genes simultaneously. TGFβRII was altered in 8 (47.1%), *IGFIIR* in 2 (11.8%), and *BAX* in 2 (11.8%) out of the 17 RER+ cases. Three CRCs, classified as RER− because of instability at only one microsatellite, had no mutations at any target gene (Table 2).

DISCUSSION

The RER phenotype, caused by the inability of the MMR system to correct errors introduced during DNA replication, has been widely studied in relation to clinicopathological and molecular characteristics in CRCs, especially in the HNPCC context. The RER phenotype clearly identifies a subset of CRCs whose pathogenesis involves peculiar molecular events [26] and it appears to be related to a better prognosis [12]. Moreover, the predictive value of the RER status has been discussed, together with the opportunity of considering it as an integral criterion for the identification of an HNPCC and CRC subset more probably due to constitutional MMR gene mutations. To better evaluate this issue in our study, the RER phenotype was examined in a series of tumours with known clinical and pathological features developed in patients evaluated for their tumour family history and with known *MLH1*/*MSH2* genotype.

Given the definition that the RER phenotype is positive when instability is present in at least two markers, 17 out of the 47 tumours we analysed were classified as RER+. For a better characterisation of the RER phenotype in relation to its biological significance, *TGFβRII*, *IGFIIR*, and *BAX* gene status was assessed. The involvement of these genes in RER+ CRC development has already been documented [6, 16, 17, 26].

From a practical point of view, the definition of RER− tumours as tumours that display instability at only one microsatellite finds confirmation in the fact that none of our RER− tumours showed alteration at any of the three target genes analysed. Akiyama and colleagues [26] found *TGFβRII* mutations in a fraction of CRCs showing instability at only one microsatellite. This is apparently in contrast with our findings, but is explainable by the different choice of the microsatellite panel used for RER assessment. Thus, the cut-off of two altered loci for the RER+ definition is justified at least for the set of microsatellites chosen in the present work. Moreover, it has been previously found that CRCs with instability at only one locus had pathological features similar to RER− rather than to RER+ (≥ 2 unstable loci) tumours [12] and that tumours with a low degree of instability have normal expression of MSH2 and MLH1 proteins [27]. In these contexts, the instability at only one locus may be due not to a real MMR failure, but to casual events occurring occasionally and independently of MMR status.

Regarding the now widely used *BAT26* microsatellite, this has been recently proposed as the best marker for a rapid determination of the RER phenotype, because of its sensitivity and specificity [27, 28]. In our series, by limiting the analysis to *BAT26*, there would be 4 false negative and 1 false positive cases. Notably, tumour CFS25T, from a patient with a constitutional *MLH1* mutation, was stable at *BAT26*. The efficiency of this microsatellite appears slightly lower than that reported previously [28] by using different panels of microsatellites for the definition of the RER phenotype.

In our analysis, *TGFβRII* was more frequently mutated (47.1%) than *BAX* (11.8%) and *IGFIIR* (11.8%). These diverse frequencies of alteration could be primarily due to the molecular features of the target repeated tracts, A(10) for *TGFβRII* and G(8) for *BAX* and *IGFIIR*, but a different selective pressure might also occur and favour those clones in which *TGFβRII*, rather than *BAX* or *IGFIIR*, is inactivated. Our study confirms that disruption of the *TGFβ* signalling pathway by inactivation of the *TGFβRII* is a common oncogenic pathway in RER+ tumours [6, 14, 26], although it has to be pointed out that mutations at this A(10) site may or

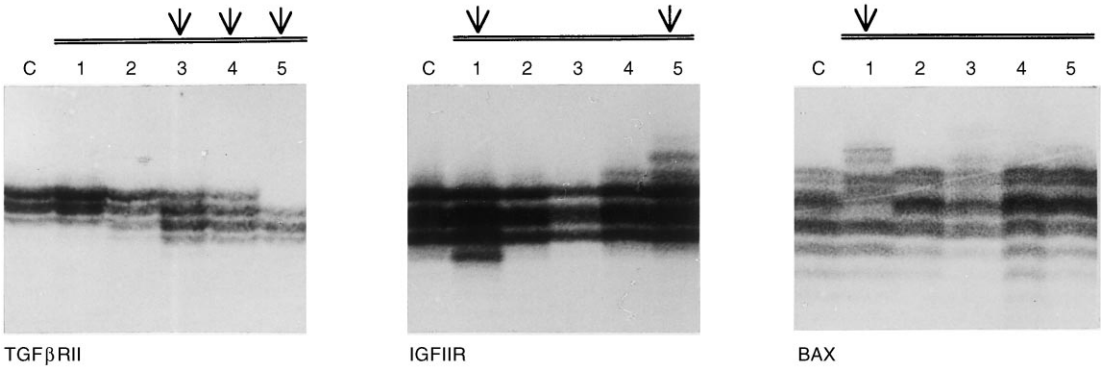

Figure 2. Target gene analysis. Arrows indicate mutated cases. C, control sample.

may not give an important selective advantage, depending on the time at which the replication errors occur in the development of the tumour. With the exception of only one case displaying simultaneous alterations at two target genes, mutations of the three genes analysed appeared mutually exclusive in RER+ CRCs. Although the numbers are small, *TGFβRII* mutations turned out to be common to all tumour types, regardless of hereditary or sporadic nature, MMR gene defect, level of microsatellite instability, tumour site and stage. It is worth noting that all three well differentiated tumours with the RER phenotype (CFS25T, CFS137T, CFS150T), but only 1 (CFS9T) out of the 5 RER+ poor differentiated tumours, had altered mononucleotide repeats of *TGFβRII* gene.

All Amsterdam patients with RER+ tumours carried constitutional mutations at *MSH2* or *MLH1* genes, whereas only 33.3% of non-Amsterdam patients with RER+ tumours did. It appears that RER positivity is a good predictor of the presence of constitutional *MLH1* or *MSH2* mutations, at least in Amsterdam HNPCC families. Conversely, the absence of such constitutional mutations in a considerable fraction of non-Amsterdam HNPCC patients with RER+ neoplasms points to the involvement of somatic events (as in tumour CFS226T) or constitutional alterations at other MMR genes, equally predisposing to the development of genetically unstable tumours but with lower penetrance.

However, 3/11 Amsterdam HNPCC patients did not present constitutional mutations nor RER+ CRCs. In accordance with Moslein and colleagues [8], our data indicate that other genes not related to the MMR system may be involved in a subset of clinically defined HNPCC kindreds. It is also true that in the majority of cases only one proband for family was investigated, leaving open the possibility of selecting phenocopies that might have occasionally developed RER− sporadic neoplasms in the context of an MMR-related HNPCC familiarity.

It is interesting that none of the patients who carried an *MLH1* or *MSH2* missense molecular variant developed RER+ neoplasms, and this may help in the evaluation of their pathogenic potential. *MLH1* Val326Ala has been described by Buerstedde and associates [29] and Liu and colleagues [9] as an alteration with uncertain significance. In the present study, two non-Amsterdam HNPCC heterozygous carriers developed RER− CRCs, indicating that this variant could simply be a rare polymorphism. Another *MLH1* variant, Val716Met, which was carried by a young patient with RER− CRC, has been previously described as a mutation in a patient with a RER+ endometrial cancer [30]. As for *MSH2*, the Gly322Asp variant has been discussed by Froggatt and colleagues [31]; in our study, this variant was carried by an Amsterdam HNPCC patient who developed a RER− CRC. In addition, the *MSH2* Ala834Thr substitution, which has been reported by Wijnen and colleagues [32], was identified in a patient with a RER− CRC.

However, further studies are needed to clarify the role of MMR gene missense alterations, since these might be responsible for a mild level of instability that could generate alterations at genes involved in tumorigenesis in the absence of a clearly defined RER phenotype. In these cases a segregation analysis would be helpful.

The RER phenotype was also significantly related to an earlier age of onset in familial cases, suggesting that two kinds of HNPCC might exist due to different molecular causes, one predisposing to the development of early onset RER+ tumours and the other one predisposing to RER− tumours at a more advanced age. This confirms an analogous observation made by Pensotti and colleagues [33] in relation to the *MLH1* and *MSH2* mutational status.

Finally, there was a trend for an association of RER+ status with CRC proximal localisation, in agreement with previous reports [5, 7, 34], and a tendency of A and B Dukes' stage tumours to be more frequently RER+. This might be due to the presence of familial cases and to a higher sensibility of these patients to their health problems, resulting in an earlier diagnosis, rather than to a real distinctive feature.

In conclusion, the RER phenotype is a good predictor of *MLH1* and *MSH2* constitutional mutations, better for Amsterdam HNPCC families than non-Amsterdam cases. RER+ tumours developed in non-Amsterdam patients may also be due to somatically acquired *MLH1*/*MSH2* alterations or to not fully penetrant constitutional mutations at other MMR genes. Finally, a fraction of both Amsterdam and non-Amsterdam cases seems to be due to defects at genes not involved in the MMR system, but an HNPCC MMR-related family history is characterised by an earlier age of CRC onset.

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