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Original Paper

Microsatellite Instability: Impact on Cancer Progression in Proximal and Distal Colorectal Cancers

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Whilst individual planning of treatment and follow-up in every colorectal cancer case is an increasing demand, prognostic markers are needed for predicting cancer progression in the primary phase. We studied the effect of replication error (RER)-positivity on colorectal cancer progression by analysing 255 colorectal cancer specimens by polymerase chain reaction (PCR) and fragment analysis and correlating the results with the clinical and histological features of the tumour and with patient outcome. RER-positivity was detected in 12% (28/235) of cases. It was associated with proximal location of the tumour ($P < 0.001$), poor differentiation ($P = 0.001$) and large tumour size ($P = 0.009$). The 5-year cumulative survival rate of the patients with RER-positive cancer of the proximal colon was markedly better (100%) than that of those with RER-negative proximal cancer (74%), whilst in cases of cancer of the distal colon or rectum, RER-positivity (21%) indicated poorer survival than RER-negativity (57%). Thus, it is suggested that RER-positivity has an opposite impact on cancer progression in cases of proximal and distal cancers. RER-positivity appears to indicate improved prognosis only in cases of proximally located cancer, in which it could accordingly be useful as a prognostic marker.

Key words: colorectal cancer, microsatellite instability, prognosis, replication errors, RER, survival
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INTRODUCTION

MICROSATELLITE INSTABILITY (replication-error, RER-positivity) is a phenomenon found in a proportion of colorectal and other cancers, such as endometrial [1], gastric and pancreatic [2] cancer. In this phenomenon, alterations in short repeated (CA) dinucleotide sequences occur in the DNA of tumour cells as a consequence of numerous replication errors [3, 4].

Sporadic colorectal cancers show RER-positivity in 12–20% of cases, while it can be detected in up to 86–95% of hereditary non-polyposis colorectal cancer (HNPCC) cases [3–6]. RER-positivity, in over 70% of hereditary cases [7], is a result of inactivating mutations in one of the known mismatch repair (MMR) genes *MSH2* [8], *MLH1* [9], *PMS1* or *PMS2* [10]. Mutations of *MLH1* and *MSH2* are found in over half the cases, while mutations of *PMS1* and *PMS2* are very rarely seen [7, 10, 11]. In hereditary cases, the mutation leading to inactivation of the first allele of the gene is a germline mutation inherited from either of the parents. Inactivation of the second allele can be a consequence of allelic loss or a somatic mutation [6, 12, 13]. The inactivating mechanism leading to RER-positivity in sporadic cases does not appear to be based on mutational inactivation, since only a few somatic mutations in the four mismatch repair genes have been found in these cases [6, 13–16]. Despite negative mutational status, a notable loss of MLH1 or MSH2 protein expression is obvious in almost all sporadic cases that are clearly RER-positive [13, 17]. Thus, it has been suggested that RER-positivity in some sporadic cases could arise from mutations in genes other than in HNPCC [6, 14, 18]. Additionally, it has been observed that RER-positive sporadic cancers frequently have altered methylation of the promotor area of the *MLH1* gene, and that this consequently leads to

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inactivation of both alleles of the gene and thus to altered expression of the respective protein in the cells. At present, methylation is thought to be the main mechanism leading to inactivation of MMR genes and to subsequent RER-positivity in sporadic cases [19]. Sporadic RER-positive colorectal cancers are suggested to have many features that are common in HNPCC. These tumours often appear right-sided, poorly differentiated, mucinous, diploid, and occur in young patients. RER-positivity is also suggested to be associated with improved prognosis compared with that in other sporadic cases [3–5, 15, 20–22].

The suggested better prognosis of these patients is a matter of interest. Prognostic markers are desperately needed as regards colorectal cancer, as follow-up strategies and adjuvant therapies are rapidly developing. Optimal cancer treatment and post-operative care should be based on individual planning, and in that process reliable markers indicating the outcome of the disease would be very useful. Tumour stage and histological features no longer provide us with enough reliable information concerning the patient's prognosis.

In the present study, primary colorectal cancer specimens from 255 patients were analysed for RER-status in order to clarify the prognostic value of this phenomenon. The RER-status was further correlated with clinicopathological variables of the disease.

MATERIALS AND METHODS

Patients and specimens

A total of 255 patients operated upon for primary colorectal cancer during 1986–1991 and 1993–1996 were included in the study. Blood samples and fresh tissue specimens were consecutively collected at the primary operation from those patients operated upon during 1993–1996. Paraffin-embedded specimens were available from those patients primarily operated upon during 1986–1991.

Of the patients, 133 (52%) were male and 122 (48%) female. The age distributions were from 12 to 88 years in males (mean 66 years) and from 28 to 93 years in females (mean 68 years). Case records and cancer registry files (Finnish Cancer Registry) were evaluated as regards the medical history of the patients and clinical aspects of the disease. Two of the patients were known to be HNPCC cases with identified mismatch repair gene mutations. Pathological grading and staging were confirmed from the original microscopy slides, reviewed separately by two pathologists. Grading was carried out according to the WHO histological classification system [23] and staging was carried out on the basis of histological and clinical examinations according to the Turnbull modification of Dukes' classification [24]. Tumours located from the caecum to the splenic flexure were considered proximal and those from the descending colon to the rectum, distal [25]. The follow-up time ranged from 2 to 140 months (mean 42 months) and ended on the 30 August 1997 or at the time of death.

Evaluation of the tumour specimens was performed as previously described and the areas with the highest proportions of cancer cells were dissected as samples [26]. Normal tissue or blood were used as normal controls. Extraction of DNA from fresh tissue and blood samples was carried out as described by Elo and associates [27] and from paraffin-embedded tissues according to the protocol described by Wright and Manos [28].

Polymerase chain reaction (PCR) and fragment analysis

Highly polymorphic microsatellite repeat areas from seven different chromosomes (chromosomes 1, 5, 8, 11, 13, 17 and 18) were chosen for PCR amplification and RER identification. The markers, chosen from the Gènèthon human genetic linkage map (ftp://ftp.resgen.com/pub), were *D1S458*, *D5S404*, *D8S255*, *D11S904*, *D13S175*, *D17S787* and *D18S61* [29]. In each primer pair, one primer was fluorescently labelled for allele detection. The PCR reactions contained 100 ng of DNA as template, 0.6 μg of each primer, 200 μM of each dNTP (Pharmacia LKB Biotech, Uppsala, Sweden), 2 μl of 10× reaction buffer IV (200 mM $(NH_4)_2SO_4$, 750 mM Tris–HCl (pH 9.0) at 25°C, 0.1% w/v Tween), 1.75 mM MgCl and 0.5 U of Red Hot DNA polymerase (Advanced Biotechnologies, Surrey, U.K.) in a 20 μl reaction volume. The PCRs were run in the following conditions: denaturation at 95°C for 3 min followed by 35 cycles (fresh tissue and blood) or 45 cycles (paraffin-embedded samples) consisting of 95°C denaturation for 1 min, annealing at 57°C (*D1S458* and *D5S404*) or 53°C (other markers) for 0.45–1.5 min and extension at 72°C for 1 min. Final extension after the cycles was at 72°C for 10 min.

The amplification products of markers *D1S458*, *D5S404* and *D8S255* from individual patients were pooled, as were the products of markers *D11S904*, *D13S175* and *D17S787*. Marker *D18S61* was analysed separately. Each of these was mixed with 0.25 μl of GS500-TAMRA size standard and 3.25 μl of loading buffer (Applied Biosystems, Foster City, California, U.S.A.). Of the mixture, 1.5 μl was loaded onto a Long Ranger 5% gel (FMC BioProducts, Rockland, Maryland, U.S.A.) and the fragments were separated by using an ABI Prism 377 instrument (Applied Biosystems) and further analysed by using GeneScan and Genotyper software (Applied Biosystems). The sample was considered RER-positive when a minimum of two markers showed new alleles in the tumour sample. An example of a representative sample is shown in Figure 1. Samples with a minimum of five informative markers were included in the study.

Statistical analysis

Pearson's χ^2 test or exact tests (with small amounts of data) were used to analyse the statistical correlation between

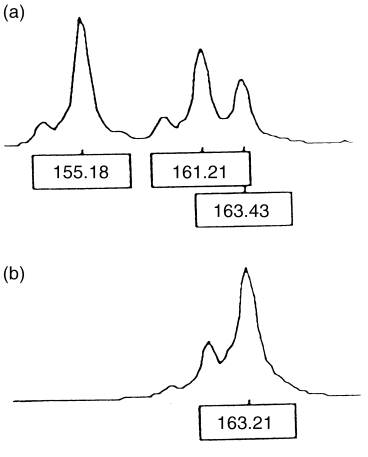

Figure 1. A RER-positive cancer specimen exhibiting characteristic variation in size of a microsatellite area (a) as compared with the respective normal tissue specimen that exhibits a constitutional allele pattern (b). Each peak represents one allele. The allele sizes are given in boxes.

RER-positivity and other variables. Multivariate analysis was performed by Cox regression analysis in order to recognise independent survival factors. Cumulative survival was assessed by the Kaplan–Meier method and analysed by log-rank analysis. Cases of peri-operative death were excluded from the analysis. Other deaths unrelated to colorectal cancer were censored at the moment of occurrence. In all the analyses, $P < 0.05$ was considered statistically significant, and only the significant *P*-values are mentioned in the text. All the analyses were performed using SPSS for Windows software (SPSS, Chicago, Illinois, U.S.A.).

RESULTS

We studied 255 patients operated upon because of primary colorectal cancer in order to evaluate the prognostic value of RER-positivity, and clinical and histological features related to this phenomenon. Of the patients, 235 were informative regarding RER-status. Twenty paraffin-embedded tissue samples were excluded because of problems with PCR reactions. The patient's characteristics are shown in Table 1.

Altogether, 28 (12%) RER-positive cases were found. RER was more common in females (17/115, 15%) than in males (11/120, 9%), although the difference was not statistically significant. The age distribution at the time of primary operation was from 12 to 86 years (mean 65 years) in the RER-positive group and from 24 to 93 years (mean 67 years) in the RER-negative group.

Proximal tumours (22/28) were RER-positive significantly more often than distal tumours (6/28) (Table 1, $P < 0.001$). Tumours of the caecum were RER-positive in 34% (11/32) of the cases, whilst this was so in only 5% (3/63) of tumours of the distal colon and 3% (3/100) of rectal tumours. The pattern of RER-positivity did not significantly differ in distal and proximal cases. Proximal tumours had a mean of 69% of RER-positive markers, whilst distal tumours had 50%. Tumours with an RER-positive genotype were significantly more often poorly differentiated (Grade III) than RER-negative tumours (Table 1, $P = 0.001$). There was no significant difference regarding the Dukes' stage of the disease between the RER-positive and RER-negative cases, although large tumours were of the RER-positive phenotype significantly more often than small ones ($P = 0.009$). There were 25 (11%) mucinous tumours, 21% (6/28) of RER-positive cancers and 9% (19/207) of RER-negative cancers were mucinous. Thus, mucinous tumours were more often RER-positive, but this did not reach statistical significance.

Table 1. *Clinicopathological features of the patients in relation to RER-status*

	RER – *n* (%)	RER + *n* (%)	*P* value
Dukes' classification			
A	49 (24)	4 (14)	NS
B	79 (38)	14 (50)	
C	53 (26)	5 (18)	
D	26 (13)	5 (18)	
Tumour grade			
I	49 (26)	2 (10)	0.001
II	114 (61)	10 (48)	
III	25 (13)	9 (43)	
Unknown	0	1	
Tumour type			
Mucinous	19 (9)	6 (21)	0.09
Non-mucinous	188 (91)	22 (79)	
Location			
Proximal	50 (24)	22 (79)	< 0.001
Distal	157 (76)	6 (21)	
Tumour size			
< 3 cm	33 (18)	2 (7)	0.009
3–6 cm	131 (69)	14 (52)	
> 6 cm	24 (13)	11 (41)	
Unknown	19	1	

NS, not significant.

During the follow-up period, 67/235 tumour recurrences (29%) were detected among patients informative for RER status. Recurrence was not associated with RER status since it was only slightly more common in RER-negative cases, the percentages being 29% (61/207) compared with 21% (6/28) for RER-positive. There was no correlation with site of recurrence.

Multivariate analysis was performed in order to identify independent factors affecting survival negatively and metastatic Dukes' D cancer had the worst prognosis ($P < 0.001$). In a study of the cumulative 5-year survival rate of the patients, the effect of Dukes' D on the results was also analysed because it was assumed that as a strong factor affecting survival, it might obscure the effect of RER-positivity in the analysis. Because of the small sample size (28 RER positive cases) none of the results below were significant. In the whole group of patients, the 5-year cumulative survival rate was similar in both RER-positive and RER-negative cases (54%). When Dukes' D was excluded from the analysis, the 5-year survival rate of those patients with RER-positive tumours was 72% whilst that of RER-negative patients was 60%.

The effect of RER-positivity on survival was further studied in relation to tumour location. In the group of patients with an RER-positive tumour in the proximal colon, the 5-year survival rate was 80%. In the respective RER-negative group, the survival rate was 63%. When Dukes' D patients

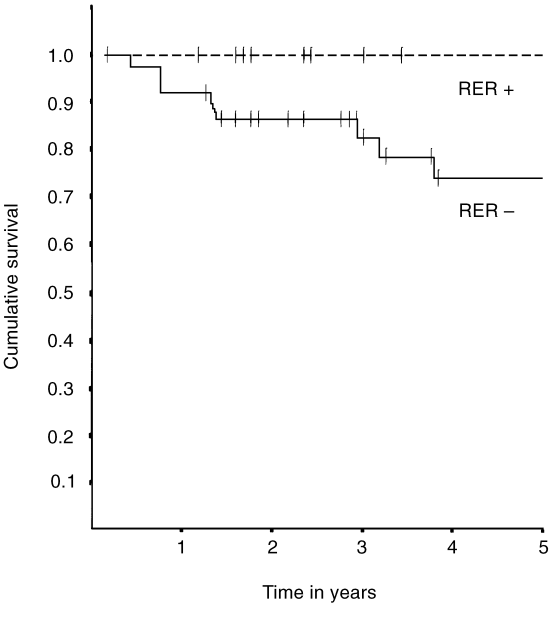

Figure 2. The cumulative survival rates of the patients with proximal RER-positive (*n*=17) and RER-negative (*n*=39) Dukes' A–C tumours.

were excluded from the analysis, the respective cumulative 5-year survival rates in the RER-positive and RER-negative groups were 100% and 74% (Figure 2). Patients with distally located RER-positive tumours had a poorer cumulative 5-year survival rate (21%) than those with distally located RER-negative tumours (52%). When Dukes' D patients were excluded, the percentages were 21% for RER-positive cases and 57% for RER-negative cases.

DISCUSSION

A survival advantage among RER-positive colorectal cancer cases has been suggested in many previous studies [4, 20, 21]. The present results indicate a prognostic role for RER-positivity, but suggest that the effect of RER-positivity on cancer progression is dependent on the location of the cancer, and is not always favourable.

In the present study, patients with RER-positive cancers of the proximal colon showed a markedly better survival rate than those with proximally located RER-negative cancers, with respective cumulative 5-year survival rates of 100% and 74%. This survival advantage associated with proximal RER-positive tumours was seen in Dukes' A–C cases. In contrast, RER-positivity did not improve the prognosis of patients with distally located cancers, but rather indicated poor survival among these patients. The 5-year cumulative survival rate in distal RER-positive cases was only 21%, compared with 57% in distal RER-negative cases. However, because the number of RER-positive patients was only 28, the results were not statistically significant.

It is logical to expect that numerous errors throughout the genome would result in an aggressive rather than a slowly progressing cancer phenotype, as is the case in distal cancers. As regards an aggressive phenotype, RER-positive tumours frequently appear poorly differentiated or mucinous, which are features often related to poor survival [4, 5, 21]. The survival advantage seen in proximal tumours is contradictory in most respects, including histological signs of aggression. Some explanations for this have been proposed.

RER-positive tumours frequently exhibit a Crohn's-like lymphoid reaction, possibly as a result of a provoked immune response against cancer cells. This might have an inhibiting effect on the growth of cancer [5, 30]. It has also been proposed that the high mutation rate caused by replication errors in these cells could lead to subsequent cell death and consequent limitation of cancer growth [31]. In addition, the activation and inactivation of growth-regulating genes by targeted mutations could be one of the growth-limiting factors. Such target genes have been found, all of them growth-promoting when mutated. These are the *TGF-beta receptor II* gene [32], the *BAX* gene [33], the *IGFIIR* gene [34], the β2 *microglobulin* gene [35] and the *MSH3* and *MSH6* genes [36]. Other target genes are likely to exist, some of them possibly growth-inhibiting when mutated.

Although these features might have an inhibitory effect on the course of the disease in proximal cases, this does not appear to be the case in distal ones. None of these proposals explain the difference seen in the behaviour of RER-positive proximal and distal colorectal cancers. The results of many previous studies indicate differences in carcinogenesis, genetic background and progression of proximal and distal colorectal cancers [37–41]. The opposite effect of RER-positivity on cancer progression could be due to different target genes of RER in different parts of the colorectum, or it might also be related to overall differences in carcinogenesis and behaviour of distal and proximal tumours [42, 43].

In our study, RER-positivity was found in 12% of primary colorectal cancers, a frequency similar to that reported previously (12–16.5%) [4, 5, 21]. It was a feature related to proximal location ($P < 0.001$) and poor differentiation ($P = 0.001$) of the cancer, as also noted in previous studies [4, 5, 14, 20, 21]. It was more commonly found in mucinous tumours ($P = 0.09$) and in larger tumours ($P = 0.009$), as noted previously [5, 22]. There was no correlation to the stage of the disease at the time of diagnosis, as also reported by others [21, 22], or to the appearance of recurrence, or the recurrence site.

According to the present results, RER-positivity is related to a subset of colorectal cancers that exhibit certain uniform clinicopathological characteristics. Additionally, the results suggest that the effect of RER-positivity on cancer progression is dependent on the localisation of the tumour, and that RER-positivity appears to indicate improved prognosis only in cases of proximally located cancer, in which it could be useful as a prognostic marker.

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