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The different roles of hRAD50 in microsatellite stable and unstable colorectal cancers

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Abstract. RAD50 protein is essential for DNA double-strand break repair and maintaining genomic integrity. In this study, we investigated the clinicopathological significance of hRAD50 expression and mutation in microsatellite stable (MSS) and unstable (MSI) colorectal cancers (CRCs). hRAD50 expression was examined in primary CRC ($n = 268$), the corresponding distant ($n = 69$) and adjacent normal mucosa ($n = 138$), and lymph node metastasis ($n = 44$) by immunohistochemistry. hRAD50 mutation was analyzed in 87 primary CRCs by PCR-SSCP-DNA sequencing. hRAD50 expression was increased in MSS primary CRCs, but not MSI ones, compared with distant/adjacent normal mucosa ($p < 0.05$). There was no difference in the hRAD50 expression between primary and metastatic CRCs. The increased hRAD50 expression in MSS primary CRCs was related ($p < 0.05$) or tended to be related ($p = 0.05$) to early tumor stage, better differentiation, high inflammatory infiltration, p53 overexpression. Frameshift mutations of $(A)_9$ at coding region of *hRAD50* were only found in MSI CRCs. Our results suggest that hRAD50 may play different roles in the development of MSS and MSI CRCs: increased hRAD50 expression in MSS CRCs may be a cellular response against tumor from further progression, while *hRAD50* mutation may be involved in the development of MSI CRCs.

Keywords: Colorectal cancer, hRAD50, immunohistochemistry, microsatellite instability, microsatellite stability

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. It is generally accepted that CRC develops from two different pathways: chromosomal instability and microsatellite instability (MSI) pathway [1]. Chromosomal instability is a common feature of CRCs, accounting for about 85% of CRCs. It refers to numerical and structure chromosomal alterations, and is characterized by a high frequency of allelic losses, deletions and/or mutations of tumor suppressor genes. MSI refers to genome-wide alterations in repetitive DNA sequence and accounts for approximately 15% sporadic CRCs and nearly all hereditary non-polyposis CRCs. It begins with a deficiency in DNA mismatch repair genes, which leads to extensive mutations in the repetitive sequence of downstream target genes with rare alterations of tumor DNA content [1].

RAD50 is one of the RAD52 epistasis group proteins. It is a component of RAD50/MRE11/NBS1 complex that plays an important role in cellular response to DNA double-strand break in mammalian cells, safeguarding genomic integrity and preventing cell transformation [2]. Previous studies in animal models have showed that null mutant in *RAD*50 was lethal [3], while hypomorphic mutant predisposed to cancer [4]. Recently, Shin et al. demonstrated an antiproliferation ac-

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Table 1
hRAD50 expression in the distant normal mucosa, adjacent normal mucosa, primary tumor and lymph node metastasis from colorectal cancer patients

	Total cases			p^*	MSS cases			p^*	MSI cases			p^*
	No.	Weak	Strong		No.	Weak	Strong		No.	Weak	Strong	
Distant normal mucosa	69	67 (97)	2 (3)	0.0001	29	29 (100)	0	0.0008	28	26 (93)	2 (7)	0.49
Adjacent normal mucosa	138	125 (91)	13 (9)	0.0003	84	77 (92)	7 (8)	0.0002	15	11 (73)	4 (27)	0.19
Primary tumor	268	203 (76)	65 (24)		162	115 (71)	47 (29)		41	36 (88)	5 (12)	
Metastasis	44	39 (89)	5 (11)	0.06	26	23 (88)	3 (12)	0.06	8	7 (87)	1 (13)	0.97

MSI, microsatellite instability; MSS, microsatellite stability.
$p = 0.09$, $p = 0.11$, $p = 0.08$ for distant normal mucosa versus adjacent normal mucosa in total, MSS and MSI cases, respectively.
*Comparing with primary tumors.

tivity of overexpression of human RAD50 (hRAD50) in *vitro* and in *vivo* [5]. Moreover, a frameshift mutation at mononucleotide repeats $(A)_9$ between codon 719 and 722 in *hRAD50* has been found in MSI human tumors including CRCs, but not in microsatellite stable (MSS) tumors [6–8], indicating that the frameshift mutation of *hRAD50* may play a role in the tumorigenesis of MSI CRCs. It has been demonstrated that this mutation was related to reduced protein expression and defective non-homologous end joining activity in *vitro* [9]. Together, these results suggest the tumor suppressor function of hRAD50 and that the mutation that impairs hRAD50 expression may contribute to development and progression of MSI CRCs.

However, hRAD50 expression during CRC development, especially in MSI versus MSS patients, and its clinicopathological significance, has not been yet studied. In the present study, we investigated hRAD50 protein expression in primary CRC, along with the corresponding distant normal mucosa, adjacent normal mucosa and lymph node metastasis, and the frameshift mutation between codon 719 and 722 in *hRAD50* in CRC as well as their relationships with microsatellite status, biological and clinicopathological variables.

2. Materials and methods

2.1. Material

Paraffin-embedded sections used for immunohistochemistry, including 268 primary CRC, 69 corresponding distant normal mucosa, 138 adjacent normal mucosa and 44 regional lymph node metastasis specimens (Table 1), were collected from 268 patients who underwent surgical resection at Linköping Hospital, Linköping, or Vrinnevi Hospital, Norrköping, Sweden, between 1977 and 2001. The distant normal mucosa was from the distant margin of resections and adjacent normal mucosa was normal mucosa adjacent to primary tumor, and both the normal mucosa was histologically free from pretumor and tumor. The distribution of primary CRC and the corresponding distant, adjacent normal mucosa and metastasis in respective to microsatellite status was shown in Table 1. Genomic DNA used for mutation analysis was extracted from 87 primary CRCs (36 MSI and 51 MSS). The clinicopathological characteristics of the patients and tumors including gender, age (range, 34 to 94, mean age 71 years), tumor location, Dukes' stage, growth pattern and differentiation were obtained from surgical and pathological records and presented in Table 2. The patients were followed up until April 2006, and 108 patients died of CRC by that time. Data of inflammatory infiltration [10], microsatellite status determined by the microsatellite marker Bat 26 [11,12] and p53 determined by PAb1801, DO1 or DO7 antibody [13–15], were taken from our previous studies. There was no information of tumor location in five, Dukes' stage in 12, growth pattern in 16, differentiation in one, inflammatory infiltration in 56, microsatellite status in 65, and p53 expression determined by PAb1801 in 137, DO1 in 147, and DO7 in 145 patients.

Three colon cancer cell lines, KM12C, KM12SM and KM12L4a, kindly provided by Prof. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX), were used to confirm the specificity of the antibody against hRAD50 proteins by Western blotting.

2.2. Immunohistochemistry

Five-micrometer paraffin-embedded sections were deparaffinized in xylene, rehydrated with gradual ethanol to water, and then treated by high pressure-cooking in Tris-EDTA buffer (pH 9.0) for 8 min. Following preincubation in methanol with 0.3% H_2O_2 for 20 min and with protein block for 10 min (Dako, Carpinteria, USA), the sections were incubated with mouse anti-RAD50-13B3 monoclonal antibody (Abcam, UK) in 1:500 at 4°C overnight. After washing

Table 2
The clinicopathological characteristics of patients with colorectal cancers

Variables	No.	%
Gender		
Male	143	53
Female	125	47
Age (yrs)		
< 71	112	42
$\geqslant 71$	156	58
Tumor location		
Colon	144	55
Rectum	119	45
Dukes' stage		
A	33	13
B	91	35
C	81	32
D	51	20
Growth pattern		
Expansive	130	52
Infiltrative	122	48
Differentiation		
Better	183	69
Worse	84	31

in phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with anti-mouse secondary antibody (Dako ChemMate^{TM} EnVision^{TM} Detection) at room temperature for 25 min. Subsequently, the sections were subjected to 3,3'-diaminobenzidine tetrahydrochloride and then counterstained by hematoxylin. Sections known with positive staining for these proteins as positive control and replacement of the primary antibody by IgG_1 as negative control were included in each run.

The entire area of each section was microscopically examined and assessed independently by two of the authors (one was a pathologist). In order to avoid artificial effect, cells in areas with necrosis, poor morphology or in the margins of sections were not taken into account. Staining intensity was graded as negative, weak, moderate and strong. Percentage of positive tumor cells was classified as $< 25\%$, 25–49%, 50–75% or $> 75\%$. Based on the similarities of some clinicopathological features in the cases with negative, weak and moderate staining versus the cases with strong staining, as well as the most information of hRAD50 expression could provide among the patients, we combined the negative, weak and moderate as one group called weak staining versus strong group, regardless of staining percentage, in statistical analysis. Staining percentage was classified using 75% as a cut-off point, regardless of staining intensity. We did not evaluate the percentage of hRAD50 expression in normal mucosa and metastasis due to the limited size of the sections. There were 12 cases with discrepant scoring, and a consensus score was reached by using a dual-headed microscope after a re-examination and discussion.

2.3. Western blotting

Total proteins were extracted from KM12C, KM12SM and KM12L4a colon cancer cell lines using RIPA buffer (1x PBS, 1% Nonidet P-40 (Amaresco), 0.5% sodium deoxycholate, 0.1% SDS) according to the manufacture's instruction (Santa Cruz Biotechnology). In brief, 20 μg total proteins in loading buffer were heated in the presence of 2-mercaptoethanol at 98°C for 5 min, separated by electrophoresis in gradient (4–15%) Tris-HCL gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Hybond-P, Amersham, UK) in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. After blocking with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), pH 7.4, at room temperature for 1 h, the membranes were incubated with mouse anti-RAD50-13B3 monoclonal antibody (Abcam) at 1:2000 in TBS-T at 4°C overnight and horseradish-peroxidase-conjugated anti-mouse Ig (Dako) at 1:5000 at room temperature for 1 h followed by enhanced chemiluminescence (Amersham). There was only one band showing at 153kDa in all the three cell lines (Fig. 1), which confirmed the specificity of the anti- RAD50-13B3 monoclonal antibody.

2.4. Mutation analysis of hRAD50

hRAD50 mutation was detected by polymerase chain reaction (PCR)-single-strand conformational polymorphism and direct DNA sequencing. Briefly, a DNA segment of 87 base pairs including the $(A)_9$ region in *hRAD50* was amplified by PCR with the primers (forward 5'-AACTGCGACTTGCTCCAGAT-3', reverse 5'-CAAGTCCCAGCATTTCATCA-3') described by Kim et al. [7] in 20 μl mixture containing 50 ng of genomic DNAs extracted from primary CRCs, 1x PCR buffer (Promega, Madison, WI), 1.75 mM $MgCL_2$ (Promega), 0.2 mM dNTP (Invitrogen, Carlsbad, CA), 0.5 μM of each primer and 0.025U/ μl Taq polymerase (Promega). After denaturation at 94°C for 5 min, DNA amplification was performed for 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Following confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide, 1 μl PCR product was reamplified for 15 cycles at the same condition as described above in addition of adding 0.1 μl [α-^{32}P] dATP in-

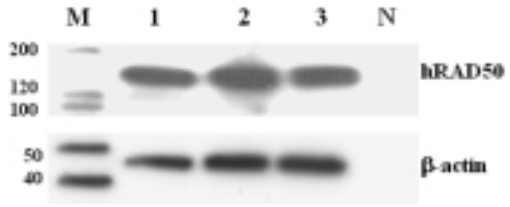

Fig. 1. hRAD50 expression in KM12C (1), KM12SM (2) and KM12L4a (3) colon cancer cell lines was detected by Western blotting. The specific band at 153-kDa, compared to MagicMark^{TM} XP Western Protein Standard marker (M), was corresponding to hRAD50. β-actin as protein loading control and replacement of protein sample by TBS-T as negative control (N) were run together with the protein samples.

stead of dATP and separated in 6% polyacrylamide gel and visualized by autoradiography. *hRAD50* mutation was determined by mobility shift of the PCR products.

In order to detect whether the shifted band represents a frameshift mutation of the *hRAD50* gene, genomic DNA fragments exhibiting shifted bands in polyacrylamide gel were excised, eluted and then PCR amplified using the same primers and PCR conditions above. The PCR product was cleaned up by ExoSAP-IT according to the manufacturer's instructions (Amersham) and subjected to a sequencing reaction in 20 μl reaction mixture containing 7 μl PCR product, 1 μl forward/reverse primer, and 8 μl sequencing reagent premix (Amersham) for 25 cycles at 95 °C for 20 sec, 58 °C for 15 sec and 60 °C for 1 min. After purification, the sequencing reaction mixtures were detected by capillary electrophoresis using the MegaBACE 1000 sequencing instrument and the data were analyzed by MegaBACE 1000 sequencing profiler (Amersham).

2.5. Statistical analysis

The χ^2 test and McNemar's method were used to determine differences in hRAD50 expression among normal mucosa, primary CRC and metastasis, relationship of mutation and protein expression of hRAD50 with microsatellite status, as well as relationship of hRAD50 expression in primary CRC with clinicopathological/biological variables. Cox's Proportional Hazard Model was used to test relationship between the hRAD50 expression and patients' survival with univariate and multivariate analyses. Kaplan-Meier method was used to calculate survival curves. Two-sided p values of less than 5% were considered statistically significant.

3. Results

3.1. hRAD50 expression in normal mucosa, primary and metastatic CRC

hRAD50 was expressed in the nucleus of distant normal mucosa ($n = 69$, weak, 97%; strong, 3%), adjacent normal mucosa ($n = 138$, weak, 91%; strong, 9%), primary CRC ($n = 268$, weak, 76%; strong, 24%) and metastasis ($n = 44$, weak, 89%; strong 11%, Table 1). There were 19 (7%) primary CRCs that had cytoplasmic staining, which was correlated with nuclear expression ($p = 0.03$), and not related to clinicopathological variables ($p > 0.05$). Therefore, in the analyses, we considered only nuclear staining, regardless of the cytoplasmic staining, as positive hRAD50 expression. Regarding staining percentage in primary CRCs, 68% tumors showed $\leqslant$ 75% staining and 32% showed $>$ 75%. Intensity and percentage of hRAD50 expression in primary CRCs were positively correlated with each other ($p < 0.0001$).

Comparing hRAD50 expression among normal mucosa, primary CRC and metastasis, we found that the intensity was increased in primary CRC compared with distant/adjacent normal mucosa ($p < 0.05$) in the whole series of the cases (Table 1) and matched samples from the same patients. There was no difference in the expression between distant and adjacent normal mucosa, or between primary and metastatic CRC ($p > 0.05$). Furthermore, the similar evidence among distant normal mucosa, adjacent normal mucosa, primary and metastatic CRC was found in the MSS cases (Fig. 2), but not in the MSI ones ($p > 0.05$).

3.2. hRAD50 expression in primary CRC in relation to biological and clinicopathological variables

We first analyzed biological and clinicopathological significance of hRAD50 expression in the whole series of primary CRCs. Either strong or high percentage of the expression was related or tended to be related to MSS ($p = 0.03$, $p = 0.06$, Table 3).

Further analysis stratified by microsatellite status showed that, in MSS cases (Table 4), strong hRAD50 expression was related to early Dukes' stage (A and B) ($p = 0.04$), high inflammatory infiltration ($p = 0.01$), positive p53 expression (PAb1801, $p = 0.01$ and DO1, $p = 0.05$) and better survival ($p = 0.01$, Fig. 3), and high percentage of hRAD50 expression was related to better differentiation (p = 0.003), high inflammatory infiltration ($p = 0.03$), positive p53 expres-

Table 3
hRAD50 mutation, expression intensity and percentage in MSS and MSI colorectal cancers

Microsatellite status	Mutation (%)		p	Expression intensity (%)		p	Expression percentage (%)		p
	Mut/Wt	Wt/Wt		Weak	Strong		$< 75\%$	$\geqslant 75\%$	
MSS	0	51 (100)	< 0.0001	115 (71)	47 (29)	0.03	106 (65)	56 (35)	0.06
MSI	11 (31)	25 (69)		36 (88)	5 (12)		33 (80)	8 (20)	
Total	11	76		151	52		139	64	

MSI, microsatellite instability; MSS, microsatellite stability; Mut, mutation; Wt, wildtype.

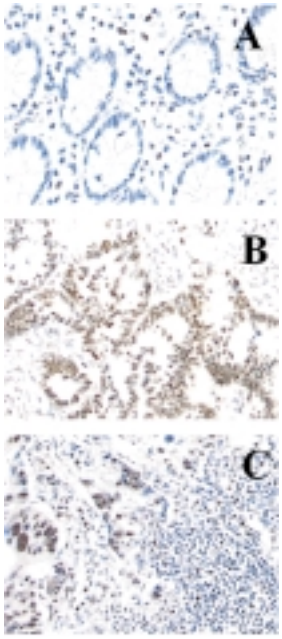

Fig. 2. hRAD50 protein detected by immunohistochemistry was expressed in the nucleus of distant normal epithelial cells (weak, A), primary (strong, B) and lymph node metastatic colorectal cancer cells (strong, C) from one microsatellite stable patient.

sion (PAb1801, $p = 0.04$; DO1, $p = 0.01$ and DO7, $p = 0.01$). However, the prognostic significance of hRAD50 expression was lost after adjusting for tumor location, Dukes' stage, growth pattern, differentiation and inflammatory infiltration ($p = 0.40$). When combining the staining intensity with percentage, hRAD50 overexpression, defined as the cases with both strong and $> 75\%$ staining, was related or tended to be related to better differentiation ($p = 0.05$), high inflammatory infiltration ($p = 0.02$) and positive p53 expression (PAb1801, $p = 0.01$ and DO1, $p = 0.05$), but not to patients' survival ($p > 0.05$). In the MSI cases, there was no association of hRAD50 expression with biological/clinicopathological variables ($p > 0.05$).

3.3. Expression and mutation of hRAD50 in CRCs

Among 36 MSI and 51 MSS CRCs analyzed for the frameshift mutations of $(A)_9$ at the coding region of *hRAD50*, we found a heterozygous 1-bp deletion or 1-bp insertion in 11 (31%) MSI CRCs, but not in any MSS CRCs (Table 3).

To analyze whether there was any impact of the mutation on protein expression, the intensity of hRAD50 expression in tumors having mutation was compared with those without mutation. Of eight cases with the mutation, there were two cases with negative, three weak, two moderate and one strong expression. Among 16 cases without the mutation, there were three negative, five weak, five moderate and three strong expression.

Subsequently, we examined the changed hRAD50 expression in MSI tumors having mutation with those without mutation, when compared to their corresponding distant normal mucosa, based on the cases available for both mutation and protein expression. Among six cases with the mutation, hRAD50 expression was decreased in three (50%) and no change in three (50%) tumors, and of the eight cases without the mutation, hRAD50 expression was decreased in 2 (25%), no change in two (25%) and increased in four (50%) tumors.

4. Discussion

RAD50 protein forms a complex with MRE11 and NBS1, which is essential for the cellular response to DNA double-strand break. Shin et al. [5] have demonstrated that overexpression of hRAD50 had an antiproliferation activity in *vitro* and *vivo*. Increased hRAD50 expression caused cell death by necrosis and apoptosis in cultured cells. They also observed that mice/rat after injected *hRAD50* gene displayed a slower tumor growth rate than controls, and, in hRAD50-delivered tumor section, immune cells, such as macrophages and lymphocytes, were highly infiltrated. In the present study, increased expression of the hRAD50 protein was observed in primary MSS CRCs but not in MSI ones, when compared with their normal mucosa. Further, in MSS primary CRCs, the overexpression of hRAD50 was associated with early stage, better differentiation, high inflammatory infiltration and p53 overexpression.

Table 4
Intensity and percentage of hRAD50 expression in relation to clinicopathological and biological variables in primary MSS colorectal cancer

Variable Category	No.	Intensity Strong	*p*	Percentage ⩾ 75%	*p*	Overexpression Strong/⩾ 75%	*p*
Dukes' stage			0.04		0.19		0.12
A + B	74	27 (36)		29 (39)		22 (30)	
C + D	79	17 (22)		23 (29)		15 (19)	
Differentiation			0.16		0.003		0.05
Better	118	38 (32)		49 (42)		34 (29)	
Worse	43	9 (21)		7 (16)		6 (14)	
Inflammatory infiltration			0.01		0.03		0.02
Low	83	18 (22)		26 (31)		17 (20)	
High	39	17 (44)		20 (51)		16 (41)	
p53 with PAb1801			0.01		0.04		0.01
Negative	44	6 (14)		13 (30)		6 (14)	
Positive	14	6 (46)		8 (62)		6 (46)	
p53 with DO1			0.05		0.01		0.05
Negative	28	3 (11)		6 (21)		3 (11)	
Positive	26	9 (35)		15 (58)		9 (35)	
p53 with DO7			0.25		0.01		0.31
Negative	34	6 (18)		8 (24)		6 (18)	
Positive	19	6 (32)		11 (58)		6 (32)	

MSI, microsatellite instability; MSS, microsatellite stability.

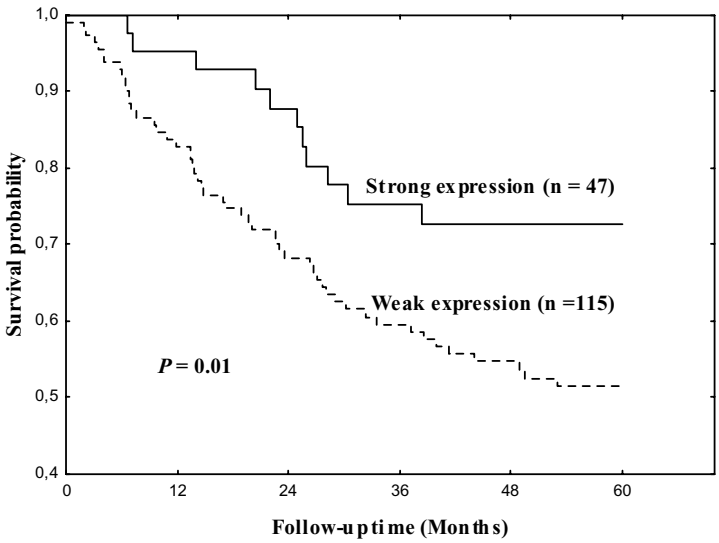

Fig. 3. hRAD50 expression in the nucleus of microsatellite stable primary colorectal cancers in relation to patients' survival.

However, in MSI CRCs, there was no any association between hRAD50 expression and clinicopathological variables. On the other hand, the frameshift mutation of *hRAD50* was only found in MSI, not in MSS CRCs. Taken together, these results suggest that hRAD50 may play different roles in the development of MSS and MSI CRCs. The increased hRAD50 expression in MSS CRCs could be a cellular response against tumor from further progression.

DNA double-strand break has been proven to trigger activation of ataxia-telangiectasia mutated (ATM) protein kinase to phosphorylate a series of downstream substrates involved in cell-cycle arrest, DNA repair, or apoptosis. Indeed, constitutive activation of several effectors within the DNA double-strand break network, including ATM, Chk2 and p53, have been observed in the early stage of several types of tumors including CRCs, suggesting that the cellular response in early tumorigenesis (before genetic instability and malignant conversion) presented a barrier against tumor progression and genetic instability [16]. Moreover, tumorigenic events, like loss of RB and overex-

pression of cyclin E, occurring early in the progression of major cancer types, might activate ATM- Chk2-p53-regulated checkpoint. Recent evidence suggests that RAD50/MRE11/NBS1 complex can facilitate recruitment of ATM to DNA double-strand break, subsequently, activate ATM, leading to phosphorylation of the downstream cellular targets p53 and Chk2, indicating that RAD50/MRE11/NBS1 complex plays an important role in ATM-Chk2-p53-dependent cellular response to DNA double-strand break [17]. In the present study, we found a positive relationship between the hRAD50 and p53 protein expression determined either by PAb1801, DO1 or DO7 antibody. Similarly, a previous study *in vitro* demonstrated constitutive activation of ATM-dependent phosphorylation of Chk2 and 53BP1 (p53 binding protein 1) in cancer cell lines expressing mutant p53, but not in cells with p53 wildtype [18]. Mechanisms behind the correlation between activated DNA double-strand break response and p53 mutation were unknown. The overexpression of hRAD50 protein was unlikely the cellular response to the p53 mutation, since p53 mutations have been mostly detected in the late-stage of CRCs. Moreover, the antiproliferation activity of hRAD50 protein was p21-dependent but p53-independent [5]. Thus, our data indicated that the overexpression of hRAD50 in the primary MSS CRC might be selected for p53 mutation in a p53-independent way.

Efficiency of cellular response to DNA damage is essential for genomic integrity. Genetic defects that impair the cellular response might lead to genetic instability. It is believed that defects in *hRAD50* could contribute to tumorigenesis by failing to produce proteins concerning DNA double-strand break repair, leading to genetic instability. Susceptibility of hypomorphic mutation in *hRAD50* to cancer has been observed in mice [4], and reduced expression of hRAD50 mRNA and protein induced by frameshift mutations at mononucleotide repeats $(A)_9$ between codon 719 and 722 in *hRAD50* gene was detected in human colon cancer cell lines [9]. In our study, weak expression of hRAD50 protein was more frequently found in MSI CRCs. However, we did not observe a clear relationship between the mutation and protein expression. A possible reason may be due to the limited cases examined for both mutation and protein expression analysis. Another possible reason may be attributable to the limitations in the assay sensitivity of immunohistochemistry for detecting heterozygous mutation of *hRAD50*. As demonstrated by Koh et al. that the heterozygous mutation leaded to impairment, but not abolishment of wild-type hRAD50 expression [9]. A subtle difference in expression of hRAD50 protein that might be induced by mutation may not be detected. It was noted that there was a difference between alterations in the hRAD50 expression in primary MSI CRCs with/without mutation, with respect to their corresponding normal mucosa. In six mutated MSI cases, none of the primary CRCs showed increased expression of hRAD50 (three decreased and three unchanged). However, in eight non-mutated MSI cases, four (50%) of the primary CRCs showed increased expression (two decreased and two unchanged), comparing with their normal mucosa. It seemed that the frameshift mutation in MSI CRC might have an impact on hRAD50 protein expression. Regarding the decreased hRAD50 expression in two non-mutated MSI CRCs, with respect to their normal mucosa, it could be attributable to other mutation within/close to the *hRAD50* gene or other genes regulating the hRAD50 expression, such as *hMRE11* or *hNBS1*. We need a larger number of cases to further investigate this issue.

5. Conclusions

hRAD50 may play different roles in the development of MSS and MSI CRCs: increased hRAD50 expression in MSS CRCs could be a cellular response against tumor from further progression, while *hRAD50* mutation may be involved in the development of MSI CRCs.

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The study has been conducted in accordance with current Swedish law and has been approved by the ethical committee at the Faculty of Health Sciences, Linköping University, Sweden.

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