Positive effect of single nucleotide RAD51 *135G>C* polymorphism and low Ku70 protein expression on female rectal cancer patients survival after preoperative radiotherapy

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ABSTRACT

Background/Aims: *This is a retrospective analysis of 103 patients having locally advanced rectal cancer who received short-course radiotherapy (SCRT). The objective of the study was to check whether a polymorphism in the* RAD51 *gene (135 G>C), Ku70 protein expression, and tumor microenvironment: proliferation rate measured by BrdUrdLI and Ki-67LI, hypoxia (glucose transporter-1 expression), P53 protein expression, and DNA ploidy can influence DNA repair capacity, the factors contributing to patient overall survival (OS) and the incidence of recurrences and metastases.*

Materials and Methods: RAD51 *(135 G>C) polymorphism was evaluated using restriction fragment length polymorphism polymerase chain reaction, and proteins were identified using immunohistochemistry.*

Results: *There were 3 (2.9%) tumors with* RAD51 *CC, 75 (72.8%) with GG, and 25 (24.3%) with GC genotypes. The median follow-up time was 63.1 months (range 2-120). Patients with CC genotype survived significantly longer than those with GG and GC genotypes and did not develop any recurrences or distant metastases. Female patients with Ku70 expression (≤75.1) or* RAD51*CC genotype (impaired DNA damage repair and radiosensitive) had significantly longer OS (p=0.013) than those with Ku70>75.1 % or* RAD51*GG,GC (radioresistant phenotype) and male patients in the log-rank test. In multivariate analysis, positive prognostic factors for OS in the male patients were grade=1 and ≤17 days break in the treatment, whereas in the female subgroup, only radiosensitive phenotype (Ku70 ≤75.1% or* RAD51*CC genotype).*

Conclusion: *To the best of our knowledge, this is the first study to provide evidence for the positive effect of CC genotype of* RAD51 *or low Ku70 expression on OS in females with rectal cancer after SCRT.*

Keywords: RAD51 *135G>C polymorphism, SNP, rectal cancer, Ku70, preoperative radiotherapy, gender-related differences*

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in men and second in women worldwide. Treatment of locally advanced rectal carcinoma mainly utilizes two modalities of preoperative radiotherapy (RT): (1) long-course radiochemotherapy and surgery after a 4- to 8-week break or (2) short-course radiotherapy (SCRT) with 5×5 Gy followed by immediate or delayed surgery (1). We have previously shown that in SCRT schedule, long intervals between RT and surgery (>17 days) should be avoided. Long interval may result in increased cell proliferation and tumor regrowth (2-5). Concerning DNA repair, we have shown that a large expression of Ku70 in the tumor cells, a protein involved in the repair of DNA double-strand breaks (DSBs) after irradiation (more effective repair of DNA damage), can be associated with poor 10-year survival rates. However, small Ku70 protein expression indicated a significantly higher survival only in women, indicating lower efficiency and/or fidelity of DSBs repair in females than in males. We suggested that the greater ability of tumor cells to repair the radiation-induced damage in men may have a negative impact on patient survival (5).

It is known that DNA DSB is the effect of ionizing radiation, which may result in rearrangement of genome and lead to cell apoptosis. Ku70 protein is involved in the repair of DSBs by non-homologous end joining (NHEJ) of DNA strands and is one of the two main pathways

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for repair of these damages, which plays a dominant role in the G1 to early S phase. The second type of DSBs repair allowing high fidelity of the original DNA sequence is homologous recombination (HR) which operates primarily in the late S-G2 phase or mitosis and may therefore have a major impact on the viability of proliferating cells. In the HR pathway, RAD51 is an important protein forming a heterodimer with a number of proteins (XRCC2, XRCC3, and BRCA2) that play a central role in strand exchange (6,7). There is growing evidence that RAD51 plays a pivotal role in the repair of DSBs and maintenance of genomic stability, which is why genes related to DSBs repair are of considerable importance.

It was shown that in DNA repair genes, there is frequent presence of gene polymorphisms in the form of variants in genetic sequence or single nucleotide variance (single nucleotide polymorphism, SNP) occurring between individuals, contributing to the phenotype variety. Polymorphism of genes involved in the DNA repair pathways can affect sensitivity of cells to radiation and has been shown to exert influence on gene transcription activity (8,9). Among several polymorphisms in the *RAD51* gene, those in untranslated regions are described (UTRs), for example, *5′ UTR G135C*, where a guanine is substituted by cytosine at nucleotide 135 (GenBank accession no. D14134).

To the best of our knowledge, the association between DNA damage repair and *RAD51* SNP in rectal cancer has not yet been studied. The aim of the present study is to check (1) if a polymorphism in the *RAD51* gene (135 G>C), essential for the HR repair, may explain the gender differences in tumor response, overall survival (OS), recurrence-free survival (RFS), and distant metastasis (DM)-free survival (DMFS) in patients with rectal cancer after hypofractionated RT. Furthermore, because DNA repair is a process dependent on tumor microenvironment, we would like to check retrospectively (2) the potential association between the *RAD51* SNP and Ku70 expression, tumor proliferation rate measured by BrdUrdLI, tumor growth fraction (Ki-67LI), hypoxia (glucose transporter through the cell membrane and glucose transporter-1 (GLUT-1) expression), tumor-suppressor gene - P53 expression, and tumor DNA ploidy, all with the purpose of selecting biomarkers that can modify DNA repair and influence the survival of patients with rectal cancer after preoperative RT.

MATERIALS AND METHODS

Patients

Between November 2003 and January 2006, a total of 103 patients with locally advanced rectal cancer (cT2-4, cN0-1, and stage I-III) were included in the study; a tumor biopsy was performed before preoperative RT.

Treatment

This is a retrospective analysis of two patient cohorts treated with SCRT (5 Gy/5 days) and surgery: one with an interval of ≤17 days (median) and the second with >17 days before surgery. Tumors were classified according to the World Health Organization classification of intestinal carcinoma and clinical and pathological stages according to the American Joint Committee on Cancer tumor-node-metastasis 2010 classification (10,11). The study protocol was approved by the local ethics committee at the Regional Medical Chamber. Written informed consent was obtained from each patient. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Flow cytometric analysis and immunohistochemistry

Tumor samples from each of the 103 patients were obtained before RT. One fragment was used for flow cytometric analysis (BrdUrdLI) and the other for immunohistochemical evaluation of protein expression. The details of flow cytometric analysis (FACSCalibur, Becton Dickinson Immunocytometry Systems, USA) were described in a previous study (3). BrdUrdLI was expressed as a percentage of cells that incorporated BrdUrd. DNA ploidy was evaluated from the DNA profile using the cell cycle analysis ModFit software. Aneuploidy was found if DNA index, i.e., the ratio of the modal DNA fluorescence of abnormal to normal G1/G0 cells, was >1.0.

The expression of the examined proteins was visualized using immunohistochemical staining. Detailed information on the staining procedure was previously presented (2). Immunoreactivity of Ki-67, GLUT-1, P53, and Ku70 was scored as the number of positive tumor cells over total counted tumor cells-labeling index. The P53 positivity was considered when >25% of tumor cells showed immunopositive expression. Immunohistochemical assessments were performed by researchers blinded to the clinical outcome.

RAD51 *135 G>C polymorphism analysis*

Formalin-fixed paraffin-embedded fragments of rectal cancer tissues were used for DNA isolation. *RAD51* (135 G>C) polymorphism was evaluated using restriction fragment length polymorphism polymerase chain reaction method (RFLP-PCR). We analyzed the genotype of *RAD51* on the basis of 157 bp amplicon (surrounding the 135th nucleotide of the gene) in which a single MvaI restriction site is located. It is eliminated in the C135C variant. Hence, in wild-type alleles (135 GG), the amplified fragment was digested using MvaI (Thermo Scientific, Waltham, MA, USA) producing 86 and 71 bp products, whereas in 135 CC homozygotes, it was not digested resulting in a single 157 bp product. Digestion of heterozygous 135 GC variant resulted in all three products (157, 86, and 71 bp).

PCR was performed in an Eppendorf thermal cycler with subsequent primers: 5′-TGGGAACTGCAACTCATCTGG-3′ (forward) and 5′-GCGCTCCTCTCTCCAGCAG-3′ (reverse), which have been already used for the *RAD51* polymorphism assessment (12).

Reactions were performed in a volume of 20 µL, containing 4 µl of genomic DNA (230-4200 ng), 0.3 µM of each primer, 2.5 mM $MgCl_2$, 1 mM dNTPs, and 6 U of TaqNova DNA Polymerase (DNA-Gdansk, Gdansk, Poland). *Thermocycling conditions were* as follows: 94 °C for 10 min; 55 cycles: 94 °C for 30 s, 67 °C for 30 s, and 72 °C for 40 s; and 72 °C for 4 min. After 16 h of digestion with the MvaI, the samples were separated in 6% polyacrylamide gel. Bands were visualized using ethidium bromide staining. Each sample was classified into one of the three possible genotypes: 135 GG, 135 GC, or 135 CC. Figure 1 shows the examples of each genotype.

Overall, 13 (12%) out of 103 DNA samples (representing each genotype) were cross-checked using DNA sequencing (Genomed, Warsaw, Poland), and the results were found to be 100% concordant.

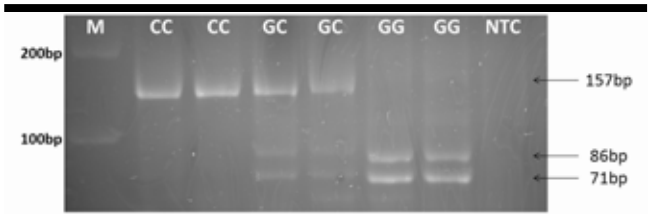

Figure 1. Representative gel of RFLP-PCR results regarding RAD51 135 G>C possible genotypes based on MvaI digestion of PCR products. M, marker; NTC, negative control (no DNA template)

Statistical analysis

Statistical analysis was performed using STATISTICA v.12 (StatSoft Inc., Tulsa, OK, USA). To determine the mean values for variables and standard errors of means (SEs), we used the descriptive statistics. One-way ANOVA test or Student's *t*-test was applied to test intergroup differences in the mean values. The associations between investigated categorical parameters and clinicopathological variables were evaluated using the Chi square test for independence. The difference between male and female subgroups according to all analyzed parameters was assessed using Mann-Whitney U-test. Survival was estimated using the Kaplan-Meier method and tested using the log-rank test. Survival time was calculated from the date of surgery to the date of death, local relapse, metastasis, or last follow-up. A p-value of <0.05 was considered statistically significant. Multivariate analysis to determine independent prognostic factors for OS was performed using the Cox's regression model in which all significant prognostic factors from univariate analysis were entered.

RESULTS

Patients

A total of 103 patients were enrolled in the study. There were 73 males and 30 females with a median age of 61.0 (range 30-82) years. Fifty-two patients were treated with ≤17 days break and 51 with >17 days break (well balanced between subgroups). There were 34 (33%) patients who received adjuvant chemotherapy (CHT), and this treatment modality did not differ between male and female patients (Table 1). Clinical and pathomorphological characteristics of the analyzed patients are summarized in Table 1. Majority of patients had moderately differentiated (G2) and moderately advanced tumors (cT2 and pT2-3). The baseline clinical information did not differ significantly for male and female subgroups (Table 1) apart from cN1 where significantly higher (p=0.036) lymph node involvement was indicated in females than in males (Table 1).

In the whole patient group, 17 (16.5%) recurrences were found (9 in male and 8 in female patients) (Table 1). DM developed in 19 patients: 7 (36.8%) in females and 12 (63.1%) in males. There were 12 metastases in the liver (9 in males and 3 in females), 4 in the lung (2 in males and 2 in females), 1 in the brain (1 male), and 2 in the nodules (2 female patients) (Table 1).

Table 1. Baseline clinical information by sex

Characteristics	Total, N (%)	Female, N (%)	Male, N (%)	p*
Age, median (range), years	103 (100)	30 (29.1)	73 (70.9)	0.696
	61.0 (30-82)	30 (57.0)	73 (62.0)	
		(43-82)	(30-77)	
cTNM				
I	29 (28.1)**	7 (23.3)	22 (30.1)	0.046
II	60 (58.2)	15 (50.0)	45 (61.6)	
III	14 (13.6)	8 (26.7)	6 (8.2)	
pTNM				
0	5 (4.8)	2 (6.7)	3 (4.1)	0.644
1	44 (42.7)	10 (33.3)	34 (46.6)	
2	17 (16.5)	6 (20.0)	11 (15.1)	
3	37 (35.9)	12 (40.0)	25 (34.2)	
Clinical tumor category				
cT2	28 (27.2)	5 (16.7)	23 (31.5)	0.106
cT3	68 (66.0)	21 (70.0)	47 (64.4)	
cT4	7 (6.8)	4 (13.3)	3 (4.1)	
Lymph node status				
cN0	90 (87.4)	23 (76.7)	67 (91.8)	0.036
cN1	13 (12.6)	7 (23.3)	6 (8.2)	
Pathological stage				
pT0	5 (4.8)	2 (6.7)	3 (4.1)	0.067
pT1	8 (7.8)	3 (10.0)	5 (6.8)	
pT2	39 (37.9)	7 (23.3)	32 (43.8)	
pT3	44 (42.7)	18 (60.0)	26 (35.6)	
pT4	7 (6.8)	0 (0)	7 (9.6)	
pN0	65 (63.1)	17 (56.7)	48 (65.7)	0.681
pN1	18 (17.5)	6 (20.0)	12 (16.4)	
pN2	20 (19.4)	7 (23.3)	13 (17.8)	
Histological grade				
1	26 (25.5)	9 (31.0)	17 (23.3)	0.694
2	73 (71.6)	19 (65.5)	54 (74.0)	
3	3 (2.9)	1 (3.4)	2 (2.7)	
Locoregional relapse	17 (16.5)	8 (26.7)	9 (12.3)	
Distant metastatic sites	19	7	12	0.169
Liver	12 (63.1)	3 (42.8)	9 (75.0)	
Lung	4 (21.0)	2 (28.6)	2 (16.7)	
Brain	1 (5.3)	0	1 (8.3)	
Nodules (no regional)	2 (10.5)	2 (28.6)	0	
Interval in SCRT				
≤17 days	52 (50.5)	12 (40.0)	40 (54.8)	0.266
>17 days	51 (49.5)	18 (60.0)	33 (45.2)	
Adjuvant chemotherapy				
Yes	34 (33.0)	9 (30.0)	25 (34.3)	0.677
No	69 (67.0)	21 (70.0)	48 (65.7)	

SCRT1: short-course radiotherapy

*Difference between male and female patients (Pearson's $\chi 2$ test)

**Percentage of patients within the subgroup

Association between clinicopathological and biological tumor characteristics

BrdUrdLI was analyzed in all patients, and immunostaining for Ki-67, GLUT-1, Ku70, and P53 was performed on 99, 98, 99, and 94 tumors, respectively. In the whole group, the mean BrdUrdLI was 8.7%±SE 0.5, and the mean Ki-67LI, Ku70LI, and GLUT-1LI were 52.4%±1.3, 75.1%±1.1, 14.0%±1.9, respectively. Fifty-eight (58/94) tumors (61.7%) were P53 positive, and 51.4% (53/103) were aneuploid tumors.

Among all tumor biological parameters analyzed between male and female subgroups, only significantly higher tumor proliferation based on BrdUrd in female than in male tumors was indicated (p=0.048). The expression of Ki-67, Ku70, and GLUT-1 proteins and the percentage of P53 positive and aneuploid tumors were not significantly different with respect to patient gender.

In the whole patient cohort, BrdUrdLI significantly increased (p=0.027), whereas Ku70 (p=0.040) and GLUT-1 expression significantly decreased (p=0.037) with tumor grade (the last parameter was important in the male subgroup only). Tumor proliferation based on BrdUrdLI significantly increased with clinical tumor (cT) size (p=0.027; lack of difference between male and female subgroups). Pathological nodule (pN) involvement was connected with an increase of P53LI (p=0.007) in the whole cohort and found only in the male subgroup (p=0.009). The percentage of P53 positive cells (mean 47.9%) in the female patient subgroup without nodule involvement (pN0) was higher than that in males (33.8%), which could be the cause of not achieving statistical significance.

Table 2. Genotype frequencies of *RAD51* 135 G>C polymorphism in male and female patients with rectal cancer

		Sex, N (%)	
Genotype/allele	All, N (%)	Male	Female
Homozygote CC	3 (2.9)	1 (1.4)	2 (6.7)
Homozygote GG	75 (72.8)	55 (75.3)	20 (66.7)
Heterozygote GC	25 (24.3)	17 (23.3)	8 (26.7)
Total	103	73 (70.9)	30 (29.1)

RAD51 *135 G>C polymorphism*

In the whole cohort, homozygous 135 CC genotype was found in 3 (2.9%) patients, homozygous GG in 75 (72.8%), and heterozygous GC in 25 (24.3%) (Table 2). A higher frequency of CC genotype (6.7%) was indicated in the female than in the male subgroup (1.4%) (Table 2).

In the whole patient group, *RAD51* polymorphism did not significantly correlate with tumor grade (p=0.214), cT (p=0.064), cN (p=0.467), pT (p=0.121), and pN (p=0.279). However, in one male, CC homozygote was identified in more advanced tumor than in female tumors, and therefore, in the male subgroup, a significant negative correlation with cT (p=0.004) was observed. In the whole patient group, *RAD51* polymorphism was not associated with lymph node invasion (cN); however, in the female subgroup, a significant positive correlation was indicated (p=0.036).

Association between RAD51 *135 G>C polymorphism and tumor biological parameters*

There were differences in the rate of proliferation and expression of proteins between tumors with specific genotype (Table 3). Homozygous CC tumors showed the highest proliferation based on BrdUrdLI and Ki-67 expression, significantly lower Ku70 expression (p=0.014) (Figure 2), low hypoxia (no Glut-1 expression), lower tumor aneuploidy (33.3%), and the highest P53 positivity (66.7%) in comparison with GG and GC tumors (Table 3).

Table 3. The relationship between *RAD51* 135 G>C polymorphism and BrdUrdLI, tumor aneuploidy, and expression of different proteins in rectal cancer

Genotype/allele	BrdUrdLI (%) mean±SE	Ki-67LI (%) mean±SE	Ku70LI (%) mean±SE	GLUT-1LI (%) mean±SE	P53 positivity N (%)	Aneuploidy, N (%)
Homozygote CC	10.7±4.4	56.7±12.2	58.9±4.6	0	2/3 (66.7)	1/3 (33.3)
Homozygote GG	9.3±0.6	52.9±1.7	75.1±1.2	12.4±2.0	43/68 (63.2)	42/75 (56.0)
Heterozygote GC	7.0±0.8	50.3±2.2	77.6±1.3	20.4±2.0	13/23 (56.5)	10/25 (40.0)
p	0.107[a]	0.656[a]	0.014[a]	0.093[a]	0.836[b]	0.310[b]

BrdUrdLI: bromodeoxyuridine labeling index; GLUT-1LI: glucose transporter-1 labeling index; Ki-67LI: Ki-67 labeling index; Ku70LI: Ku70 labeling index; SE: standard error
[a]ANOVA
[b]$\chi 2$ test for independence

Of the 103 patients, 52 received SCRT with a short (≤17 days) and 51 with a long (>17 days) interval between RT and surgery. All patients (3) with CC genotype, 46.7% (35/75) with GG genotype, and 48.0% (12/25) with GC genotype were given preoperative RT with a short break.

Correlation between different proteins expression

In the whole patient cohort, a positive correlation was found between BrdUrdLI and Ki-67 (p=0.027), and a negative correlation between Ku70 and BrdUrdLI (p=0.007). In the male subgroup, Ki-67 correlated positively with BrdUrdLI (p=0.011) and Ku70 (p=0.050); however, in female patients, no association between proteins was indicated.

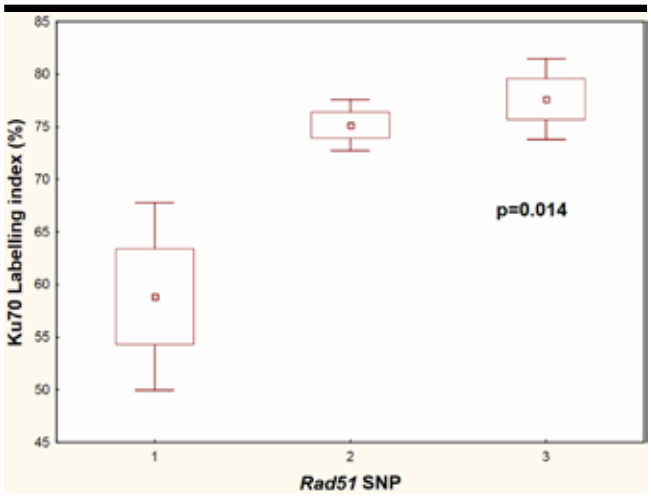

Figure 2. Association between Ku70 protein expression and *RAD51* 135 G>C polymorphism; mean values±SE are shown

Association of RAD51 *SNP with OS, RFS, and DMFS*

Patient survival

The median followup time for all enrolled patients was 56.0 months (range 2-120). The OS rate based on the Kaplan-Meier analysis was 71.3% at 60 months and 63.2% at 120 months. Adjuvant CHT had no influence on OS (p=0.856). *RAD51* 135 G>C polymorphism had a significant influence on the response to RT and OS. Patients with homozygous CC variant survived longer (mean 89.4 months) than those with homozygous GG (mean 56.3 months) and heterozygous GC (mean 51.3 months) (Table 4). However, in the study, the association of analyzed SNP with OS and the difference in response between patient gender could not be fully informative because of low number of patients with CC genotype and lack of effect on OS in the log-rank test.

In the whole patient group, no difference in OS, time to recurrence, or metastases was observed between patients with tumors of lower (≤75.1%) or higher (>75.1%) Ku70 expression (Table 4). However, when separate analysis for subgroups differing in gender was performed, significantly longer OS for female patients with Ku70 ≤75.1% (63 months) than for those with Ku70 >75.1% (44.4 months) was observed (p=0.013), confirming the results of our previous study (5). In the male patient subgroup, such difference (50.3 vs 57.1 months) was not observed (p=0.755). Because *RAD51* SNP and Ku70 expression influences patient OS, we decided to combine the two parameters. To check the impact of inefficient DNA damage repair on OS, in the log-rank test, we divided patients into two groups: those with radiosensitive phenotype (*RAD51* CC or Ku70 ≤75.1%) and those with radioresistant phe-

Table 4. Overall survival, frequency, and time to appearance of locoregional relapses and distant metastases in patients with rectal cancer differing in *RAD51* polymorphism and Ku70 protein expression

	Genotype/allele			Ku70LI	
Characteristics	Homozygote CC	Homozygote GG	Heterozygote GC	≤75.1%	>75.1%
Overall survival					
N (%)	3 (2.9)	75 (72.8)	25 (24.3)	48 (48.5)	51 (51.5)
months mean±SD	89.4±28.1	56.3±24.1	51.3±20.4	57.1±22.6	53.3±24.9
LR N (%)	0	13 (76.5)	4 (23.5)	8 (47.1)	9 (52.9)
Time to LR months mean±SD	0	18.7±8.7	15.4±6.7	19.5±8.7	16.5±8.0
DM N (%)	0	14 (73.7)	5 (26.3)	9 (47.4)	10 (52.6)
Time to DM months mean±SD	0	21.6±13.2	17.5±8.5	22.4±11.6	18.5±12.8

Ku70LI: Ku70 labeling index; LR: locoregional relapse; DM: distant metastasis; SD: standard deviation

Table 5. Univariate analysis for overall survival for 103 patients with rectal cancer

Variable	All			Male			Female		
	RR	95% CI	p*	RR	95% CI	p	RR	95% CI	p
Age (years)									
≤61.0	1.00	Reference		1.00	Reference		1.00	Reference	
>61.0	3.65	1.55-8.60	0.003	3.88	1.29-11.66	0.015	3.30	0.79-13.88	0.102
Grade									
1	1.00	Reference		1.00	Reference		1.00	Reference	
2-3	4.87	1.15-20.57	0.031	6.63	0.88-49.76	0.065	2.99	0.37-24.34	0.305
Clinical stage (AJCC)									
I	1.00	Reference		1.00	Reference		1.00	Reference	
II-III	2.11	0.80-5.57	0.129	1.97	0.66-5.90	0.224	2.44	0.3-19.84	0.404
Pathological stage (AJCC)									
0-1	1.00	Reference		1.00	Reference		1.00	Reference	
2-3	1.83	0.84-3.96	0.125	1.73	0.71-4.25	0.228	1.87	0.38-9.26	0.423
Break in the treatment									
≤17 days	1.00	Reference		1.00	Reference		1.00	Reference	
>17 days	2.30	1.04-5.10	0.038	3.13	1.20-8.15	0.019	1.02	0.24-4.30	0.969
Ki-67LI									
≤52.4%	1.00	Reference		1.00	Reference		1.00	Reference	
>52.4%	0.86	0.41-1.82	0.701	0.83	0.34-2.01	0.684	1.01	0.27-4.41	0.888
Ku70 ≤74.9% or RAD51 CC	1.00	Reference		1.00	Reference		1.00	Reference	
Ku70 ≤74.9% or RAD51 GG,GC	1.43	0.67-3.05	0.354	0.77	0.32-1.85	0.550	9.12	1.12-74.37	0.039
GLUT-1									
<14.0%	1.00	Reference		1.00	Reference		1.00	Reference	
≥14.%	0.89	0.40-1.97	0.780	0.98	0.39-2.47	0.969	0.58	0.12-2.87	0.502
P53									
≤25.0%	1.00	Reference		1.00	Reference		1.00	Reference	
>25.0%	1.91	0.81-4.52	0.141	1.70	0.65-4.44	0.275	2.94	0.35-24.49	0.317
BrdUrdLI									
≤8.7%	1.00	Reference		1.00	Reference		1.00	Reference	
>8.7%	1.59	0.75-3.37	0.225	1.76	0.72-4.30	0.216	1.41	0.34-5.90	0.638
DNA ploidy									
Diploid	1.00	Reference		1.00	Reference		1.00	Reference	
Aneuploid	0.82	0.39-1.72	0.599	0.80	0.33-1.95	0.622	1.00	0.25-4.03	0.993

*p-values from log-rank test
RR: relative risk; CI: confidence interval

notype (*RAD51* GG, GC or Ku70 >75.1%). In the whole patient cohort, the association of radiosensitive phenotype (*RAD51* CC or Ku70 ≤75.1%) with OS did not remain univariately significant (p=0.350, Table 5). However, if we analyzed OS in subgroups differing in gender, only female patients with *RAD51* CC or Ku70≤75.1% had significantly (p=0.013) higher 5-year OS (93.3%) than those with *RAD51* GG, GC or Ku70 >75.1% (50.6%, Figure 3). In the male patient subgroup, OS for the first and second group was 78.5%, 74.0%, respectively.

In the whole patient cohort, in univariate analysis, age ≤61.0 years (p=0.003), grade=1 (p=0.031), and break in the treatment ≤17 days (p=0.038) (Table 5) were favor-

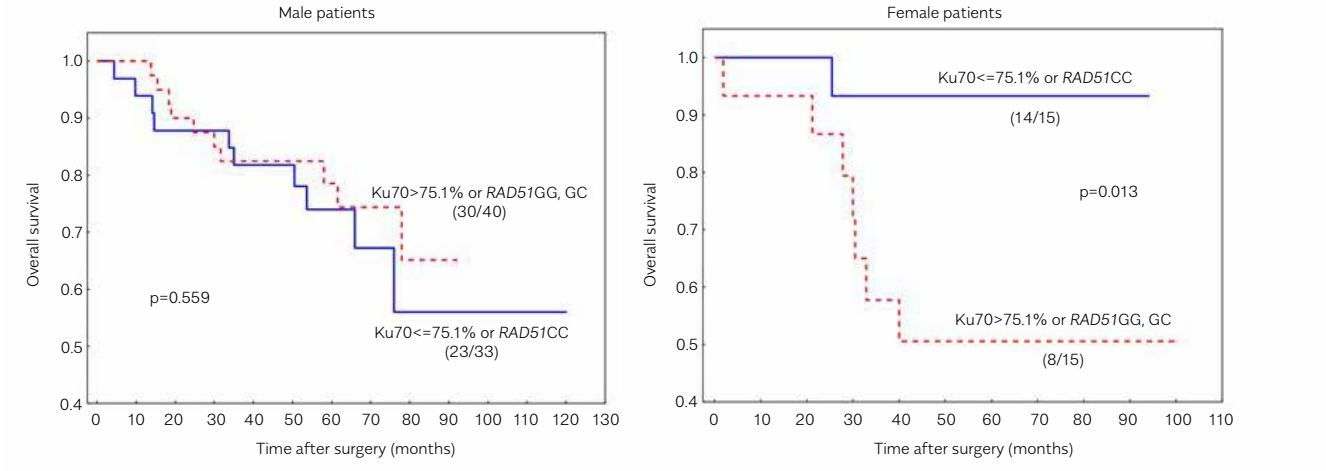

Figure 3. Gender-related differences in overall survival of patients with rectal cancer according to different DNA repair potentials based on Ku70 expression or *RAD51* GC polymorphism

Low potential: Ku70 ≤75.1% or RAD51 CC; High potential: Ku70 >75.1% or RAD51 GG,GC

Table 6. Multivariate Cox analysis performed to determine prognostic factors for overall survival (OS) for rectal cancer patients treated with preoperative short-course radiotherapy

Variable	RR	95 % CI	p
		All patients	
	All patients		
Age (mean)			
≤61 years	1.00	Reference	
>61 years	2.77	1.15-6.67	0.023
Grade			
1	1.00	Reference	
2-3	4.58	1.06-19.72	0.041
Break in the treatment			
≤17 days	1.00	Reference	
>17 days	2.49	1.11-5.56	0.027
	Male patients		
Grade			
1	1.00	Reference	
2-3	8.34	0.10-63.02	0.040
Break in the treatment			
≤17 days	1.00	Reference	
>17 days	3.71	1.42-9.69	0.007
	Female patients		
Ku70 and RAD51 polymorphism			
Ku70≤75.1% or RAD51 CC	1.00	Reference	0.039
Ku70>75.1% or RAD51 GG, GC	9.12	1.12-74.37	

RR: risk ratio; CI: confidence interval

able prognostic factors for OS. Age ≤61.0 years (p=0.015) and ≤17 days break (p=0.019) were important for male patients; however, only tumor radiosensitive phenotype appeared to have an influence on female OS (p=0.039).

RFS and DMFS

In patients with *RAD51* 135 CC homozygous variant, no locoregional relapse (LR) appeared, whereas in GG homozygotes, 13 (76.5%) were indicated (Table 4). Tumors with heterozygous GC genotype developed 4 (23.5%) LR. Mean time to LR was not significantly different between the subgroups (p=0.494) (Table 4).

In the whole patient cohort, 19 developed DM (Table 4). None of the patients with CC genotype developed DM, 14 (73.7%) appeared in homozygote GG, and 5 (26.3%) in heterozygote GC (Table 4). Mean time to DM development did not show significant difference (p=0.805) between the patient subgroups (Table 4).

In patients with homozygous CC genotype, no local recurrence or DM were observed (Table 4). The highest incidence of LR (76.5%) and DM (73.7%) was found in patients with GG genotype (Table 4). In the whole cohort, similar incidence of LR was indicated in patients with lower or higher Ku70 expression, and metastatic potential was also similar in both subgroups differing in Ku70 expression (Table 4).

The association of *RAD51* CC or Ku70 ≤75.1% did not remain univariately significant for RFS (p=0.964) and DMFS

(p=0.931) in the log-rank test. Owing to the low number of female patients and lack of the effect on RFS and DMFS, separate analysis for subgroups differing in gender could not be performed.

Multivariate Cox analysis

In the whole patient cohort, age ≤61.0 years (p=0.023), low grade (p=0.041), and ≤17 days break (p=0.027) appeared to be independent positive factors for OS in multivariate analysis (Table 6). However, in the male patient subgroup, low grade (p=0.040) and short break (p=0.007) were significant. In the female subgroup, only tumor radiosensitive phenotype (*RAD51* CC or Ku70 ≤75.1%) had a significant influence on OS (p=0.039). Owing to the lack of the effect in the subgroup of CC genotype and female patients with Ku70 <75.1%, multivariate analysis for RFS and DMFS for male and female patients, could not be studied.

DISCUSSION

The current study suggested that deficient DNA repair capacity for each specific DNA repair pathway, HR (RAD51) and NHEJ (Ku70), could contribute to better rectal cancer response to RT. *RAD51* CC genotype was more frequent in female (6.7%) than in male patients (1.4%) and connected with longer patient survival and absence of recurrences and DM. Tumors with this genotype were oxygenated (negative for GLUT-1 expression), moderately differentiated, had significantly lower Ku70 expression, lower aneuploidy, and higher P53 protein expression than those with GC and GG variants, suggesting the influence of tumor microenvironment on tumor response. All this may prove that the lack of effective recovery of radiation-induced DNA damage may affect higher mortality of tumor cells, resulting in more effective RT and longer patient survival. We indicated gender-related differences in OS based on Ku70 expression and *RAD51* polymorphism. We demonstrated that women having tumors with radiosensitive phenotype (*RAD51* CC or Ku70 ≤75.1%) had significantly higher 5-year survival (93.3%) than male patients (74.0%), and for these women, the length of the break in the treatment was not significant because of the defect in the repair of DNA damage induced by irradiation. However, for male patients, the length of the break was important, and longer (>17 days) interval between RT and surgery had unfavorable influence on OS, suggesting that efficient repair of DNA damage during the break can cause better tumor cell survival and poor patient OS. This may suggest gender-related difference in tumor cell radiosensitivity and significant role of Ku70 expression and *RAD51* SNP as prognosticators.

In the current study, CC genotype had the lowest frequency (2.9%), which is in agreement with other studies showing 1.5%-4.0% (13-15). However, GG genotypic frequency was found to be predominant (76.5%), being also in accordance with other studies showing 45.6%-86.5% (13-15). Tumors with GG genotype contained higher percentage of hypoxia and Ku70 expression than those with CC genotype, manifested the highest tumor aneuploidy, and developed the highest number of recurrences and metastases. In addition, patients had significantly shorter survival than those with CC genotype. The results are in agreement with the experimental results, proving that in rectal cancer cells, a hypoxic microenvironment might be the cause of a DNA repair-deficient phenotype and genetic instability (16). In hypoxia, a decrease in RAD51 protein expression was observed; however, the expression of the Ku70 protein remained unchanged (17).

In our study, the mean incidence of GC genotype was 24.3%, which is in accordance with the results of other studies (13-15,18) showing 12%-50%, based on the analysis of normal cells from patients with cancer. Tumors had biological characteristics similar to those of GG homozygotes. It was shown that in non-irradiated gut, the GC genotype significantly increases the risk of ulcerative colitis than GG genotype (15).

Searching for the causes of changes in cell phenotype with the *RAD51* SNP, we should look at how a cell detects and signals the presence and repair of DSBs. It is suggested that the location of the C allele in the regulatory element (UTR) of the *RAD51* promoter has a relationship with the transcription factor binding, the stability of mRNA, and the translation process, thus affecting the protein level (9,19,20). Substitution of guanine for cytosine in untranslated 5′ region of the gene (G135C) can lead to unstable mRNA and, in consequence, lower expression of RAD51 protein (13,21). This was confirmed in lymphoblastoid cell lines where cells with the CC genotype synthesized less RAD51 protein (19). Additionally, low Ku70 expression in tumors with this genotype may indicate less effective DSBs repair, and thus, radiosensitivity, which could be in agreement with the experimental study showing the connection between inactivation of Ku70 protein and hypersensitivity to infrared radiation (IR) (22). All of the above findings are in accordance with our results showing that patients presenting tumors with this genotype and low Ku70 expression had longer OS. This was particularly important in female patients who had nine times higher risk in OS than those with radioresistant phenotype (Table 6).

Alternatively, it was suggested that GC genotype enhances the activity of the *RAD51* promoter, resulting in increased RAD51 expression (9). This may be connected with radioresistance (23) which is also consistent with our results showing shorter patient OS.

The inability to properly respond to DNA damage repair becomes the cause of genetic instability and, in consequence, may enhance the rate of cancer development (24). There is growing evidence that deficiencies regarding DNA damage signaling and repair pathways are highly significant as far as the etiology of majority, if not all, human cancers is concerned.

Many epidemiological studies have been performed to investigate the association between *RAD51* 135 G>C polymorphism and susceptibility to different tumors. However, some of these results are inconsistent. In a previous study, it was shown that CC polymorphism of the *RAD51* gene increases the risk of breast cancer in carriers of mutations in the *BRCA2* gene (20). The BRCA1 and BRCA2 proteins together with RAD51 are involved in DNA repair. In another study, *RAD51* 135 GC was identified as a susceptibility locus in breast cancer (25).

Krupa et al. (13) studied polymorphism of the *RAD51* gene and showed that 135 CC genotype decreases the risk of colorectal cancer. In another study, a 5-fold increase in colorectal carcinoma risk was suggested for individuals carrying the *RAD51* CC genotype compared with subjects carrying the GG, GC genotype (18).

Moreover, our study has shown that tumor biological factors, such as DNA repair, hypoxia, and tumor proliferation, play significant roles in post-RT tumor response. Tumors with CC genotype had higher proliferation rate than tumors with other genotypes, but all were resected in time <17 days after RT, so cells that could survive IR had no time for repopulation (4). They were moderately differentiated and did not show apparent hypoxia (more sensitive to IR than hypoxic cells), and probably, therefore, they responded to RT better than other tumors. They did not develop recurrences and metastases, and patients had the longest OS. In the male subgroup, tumor proliferation correlated positively with Ku70 expression but not in female subgroup. This may suggest gender-related difference in radiosensitivity. We had shown earlier that only female patients with low Ku70 expression had significantly longer OS (5). This may suggest lower NHEJ repair in female patients. This may also confirm the observations from the recent study by Alsbeih et al. (26) showing gender-related difference in radiosensitivity of fibroblasts based on P53 polymorphism (significant only in females). The P53 protein might be the player here. In addition, greater microsatellite instability phenotype in women than in men with colorectal cancer-treated adjuvant CHT was observed to be connected with benefit survival (27).

The role of HR and NHEJ pathways of DNA DSB repair overlaps as concerns the maintenance of chromosomal integrity in cells, and both repair pathways are involved in maintaining chromosomal DNA during the cell cycle (6). A suggestion has been made that RAD51 and Ku70/80, DNA end-binding proteins functioning in NHEJ, are competitors in the process of binding to DSBs and channeling the repair of DSBs into HR or NHEJ, respectively (7,24).

RAD51 interacts with a set of proteins, such as the tumor-suppressor and cell-cycle proteins-P53, BRCA1, and BRCA2 (7). It was indicated that RAD51 operates in concert with these factors to prevent genomic instability and the generation of tumorigenic mutations (7). HR also, directly or indirectly, involves P53, irrespective of the protein's transcriptional activation properties (28). However, it was also shown that the interaction of mutant P53 protein with RAD51 appears to be impossible (29). Therefore, P53 mutations lead to higher level of spontaneous recombination rate and also stimulate radiation-induced recombination, even when its expression is much lower than that of the wild-type P53 protein (28). This may confirm our results showing higher incidence of aneuploidy in the presence of P53 positivity in tumors with GG genotype.

The likelihood of higher spontaneous, radiation-induced recombination is generally observed in cells expressing mutant P53 proteins. It was shown that in the process of HR, the strand invasion mechanism is stimulated, and the type of P53 mutation may modify cell phenotype (28). This may possibly take place in female tumors that had the highest P53 positivity (low binding to RAD51 protein?), perhaps low DNA repair by HR that led to good tumor response, and the longer female OS. The drawback of the current study was that relatively low number of female patients were enrolled in the study. Therefore, to verify our finding, further investigations are necessary with greater number of female patients and investigation of more gene alterations involved in DNA repair. Whether this approach is correct may be confirmed by recently published experimental study that has shown that inactivation of DNA mismatch repair in cancer cells may cause

a hyper-mutation status which increases tumor neoantigens, triggering long-lasting increased immune surveillance that impairs tumor growth (30).

In summary, the present study suggests that low effectiveness of DNA damage repair in patients with rectal cancer treated with preoperative RT may influence patient survival. Our study indicates that 135 CC variant of the *RAD51* gene and low Ku70 protein expression involved in the DNA repair pathways can affect cellular sensitivity to radiation and OS, particularly in female patients. Patients with homozygous CC genotype did not develop any recurrence or DM, contrary to GG and GC genotypes. To the best of our knowledge, this is the first study to evaluate the role of microenvironment on DNA repair capacity in rectal cancer and to show gender-related difference in OS based on RAD51 polymorphism and Ku70 expression. In accordance with the results, we have suggestions for clinical practice: in male patients because of better DNA repair, longer (>17 days) intervals between RT and surgery are not advisable and for female patients with radiosensitive phenotype (*RAD51* CC or Ku70 ≤75.1%) are irrelevant although they are at risk of greater cytotoxicity of normal tissues. The results discussed above may potentially affect the clinical practice with respect to the selection of patients for personalized treatment or those at risk of severe normal tissue complications.

Ethics Committee Approval: Ethics Committee Approval has received for this study from the the Ethical Committee at the Regional Medical Chamber in Cracow (Approval Date: 21/07/2003).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

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