**Original Article**

**Polymorphism within the distal RAD51 gene promoter is associated with colorectal cancer in a Polish population**

Bartosz Mucha¹, Jacek Kabzinski¹, Adam Dziki², Karolina Przybylowska-Sygut¹, Andrzej Sygut³, Ireneusz Majsterek¹, Łukasz Dziki²

¹Department of Clinical Chemistry and Biochemistry, Medical University of Łódz, Poland; ²Department of General and Colorectal Surgery Medical University in Łódz, Poland; ³Department of General and Vascular Surgery, Medical Center of Pabianice, Poland

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**Abstract:** Background: colorectal cancer (CRC) is one of the most common cancers in developed countries. Annually, over one million new cases in the world are recorded. Majority of CRCs occur sporadically with dominant phenotype of chromosomal instability (CIN). Permanent exposure to DNA damaging agents such as ionizing radiation result in DNA double-stranded breaks, which create favorable conditions for chromosomal aberration to arise. Homologous recombination repair (HRR) is the leading process engaged in maintaining of the genome integrity. RAD51 protein was recognized as crucial in HRR. Single nucleotide polymorphisms are the primary source of genetic variation which presence in the RAD51 promoter region can affect on its expression and consequently modulate HR efficiency. Objectives: The aim of this study was to analyze the distribution of genotypes and allele frequencies of -4791A/T and -4601A/G RAD51 gene polymorphisms, followed by an assessment of their relationship with the risk of CRC. Material and methods: The study included 115 patients with confirmed CRC. Control group was consisted of 118 cancer-free individuals with a negative family history. The genotypes were identified by PCR-RFLP method. Conclusion: This study revealed statistically significant association between appearance of G/A genotype in position -4601 of RAD51 gene and CRC risk.

**Keywords:** Homologous recombination repair, DNA double strand breaks, colorectal cancer, single nucleotide polymorphism

**Introduction**

The recent European morbidity registers indicate colorectal cancer (CRC) became a second most frequent type of cancer occur in highly industrialized countries, currently outpaced breast cancer [1]. Similar worrying trends are being observed worldwide [2]. There are several characteristic for modern societies risk factors which contribute to CRC development such as inappropriate fat and red meat rich diet, obesity or sedentary lifestyle. Nearly 1-5% of incidences are associated with inherited genetic mutations whereas 15-20% have family history of cancer unrelated to any syndrome like polyposis or Lynch syndrome. The overwhelming number of cases 70-80% are sporadic CRC [3]. Accordingly, some genetic variations like single nucleotide polymorphism (SNP) may modulate response for particular environmental factors which may underlying the predispose to CRC [4].

Colorectal carcinogenesis can manifest in one of three phenotypes: microsatellite instability (MSI), CpG island methylator (CIMP) and chromosomal instability (CIN) also named suppressor pathway. However, the most common genetic abnormality is CIN (70-85% of all sporadic CRCs), which become apparent as aneuploidy, sub-chromosomal genomic amplifications and a high loss of heterozygosity (LOH). The ongoing several years accumulation of negative genetic alteration can result in transformation of healthy colonic mucosa into tumor and then to malignancy [5].

DNA double strand breaks (DSBs) are common lesion which may originate serious genome changes. It is believed that single unrepair
Table 1. Distribution of age, sex and clinical characteristic in patients group

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Gender</th>
<th>TNM classification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>average</td>
<td>♀</td>
<td>♂</td>
</tr>
<tr>
<td>115</td>
<td>58 ± 9.9</td>
<td>48</td>
<td>67</td>
</tr>
</tbody>
</table>

*T(1-4) size of tumor, N(0-2) degree of spread to regional lymph nodes, M(0-1) presence of metastasis. **Not all have been established.

DSB is able to trigger off cell death or cause chromosome rearrangement [6]. Homologous recombination (HR) repair is one of the major mechanisms responsible for maintaining genome stability. The special feature of this process is capability to error-free restoration of lost due to DSB sequence. This multi-step complex pathway required large set of proteins, which provide recognition of lesion site, anti-exonuclease protection of free DNA ends and re-synthesis based on homology to sister chromatic. The overall course of the HR has been described in certain extent for last 20 years [7, 8]. The essential reaction of HR is seeking out intact homologous region by protein foci formed at the lesion site. RAD51 is ATP-dependent HR protein which exhibit DNA-binding, homologous pairing and DNA strand exchange activity. Both of the 5' ends of the DSB are processed by nuclease in order to obtain 3' single strand DNA overhanging tails. Thus, such DNA arrangement is substrate for RAD51 which accompanied by its paralogues is assembled onto the liberated 3' ends of broken site as helical nucleoprotein filaments [9]. Subsequently, filament complex carries out invasion to undamaged complement homologous dsDNA. Homology allows for creation of DNA heteroduplex being four-stranded “X-like” structure called Holliday Junction. After re-synthesis missed sequence both dsDNA are released [7].

Undoubtedly, RAD51 plays central role in HR repair. The process, which every single omission or error may contribute to the chromosomal instability. Recent report has pointed to certain changes in the expression of the RAD51 gene in CRC individuals [10]. That prompted us to explore genetic variation within the promoter sequence of RAD51. For many years, researchers have been focusing their attention particularly on the polymorphisms located at the 5' UTR region RAD51: 135G/C (rs1801320) and 172G/T (rs1801321). It is still valid subject, as evidenced by the constant appearance of new case control study in the context of a variety of cancers [11, 12]. Despite the abundance of results it is still lack of unambiguous explanation for role of RAD51 in carcinogenesis [13]. We suspected that other genetic variability could act additively or independently to 5'UTR polymorphism what might clarify the attitude of RAD51 in cancer development. The presented work is targeted at less well investigated SNP of RAD51 promoter region, rs2619679: -47-19A/T (rs2619679) and -4601A/G (rs5030-789). Furthermore, this is the first study concerning above mentioned SNP in relation to CRC. The goal is to determine the association between occurrence particular genotypes/alleles and the risk of CRC.

Materials and methods

Samples

The peripheral blood samples were collected from 115 patients suffering from CRC, hospitalized in Clinic of General and Colorectal Surgery at Medical University of Lodz. All of cases was histopathologically confirmed and determined in terms of the tumor grade according to TNM classification (Table 1). The control group was consisted of 118 cancer-free individuals who were treated in the same facility for minor gastrointestinal diseases/complaint such as indigestion or food poisoning. The major conditions for including to the control study were cancer negative family history and the distribution of age (average: 58 ± 10.1) and sex (females: 56, males: 62) in order to match with CRC subjects. All patients familiarized themselves with the bulletin about the purpose of the project and signed an informed consent form before entering the study.

Genotyping

Restriction fragment length polymorphism PCR was utilized for genotype detection. Methods were established by Greiner et al. [14]. The proximity of the position both investigated polymorphism allows for analyzing one 402 bp fragment by two different restriction enzymes. Amplification PCR reaction was performed in Termocycler Biorad T-100 under the following conditions: 95°C for 5 min initial denaturation then 34 cycles consisting of denaturation 95°C for 1 min, annealing 60°C for 30 s, elongation 75°C for 45 s and the final elongation 72°C for 10 min. Content of the every single PCR mixture
was 100 ng of genomic DNA extracted from
blood by QiaAmp kit (Qiagen, Valencia, CA), 100
ng both reveres (5-CCGTGCAGGCCTTATATGAT-3)
and forward (5-AGATAAACCTGGCCAACGTG-3)
primers purchased from Sigma Aldrich (Taun-
fkirchen, Germany) and Green Master Mix
(Thermo scientific) polymerase. Total reaction
volume was split into two equal samples and
digested with 1 unit of
Nla
III (rs5030789) and
Hind
III (rs2619679) enzymes (New England
Biolabs Inc. Beverly, MA, USA) overnight. Pa-
r medial DNA fragments were separated on 3%
agarose gel in TAE buffer then stained by ethid-
um bromidium and visualized under UV light.
Obtained band patterns for -4719A/T rs26-
19679 referred to following genotypes 286 bp,
114 bp-A/A; 172 bp, 114 bp-A/T; 286 bp, 172
bp, 114 bp-T/T whereas -4601A/G rs5030789
displayed 168 pb, 35 bp, -A/A; 203 bp, 168 bp,
35 pb-A/G; 203 pb-G/A. The Gel Doc XR Bio-
Rad system was used for photographing and
cataloging gels. To confirm obtained result 10%
of all samples were repeated.

Deviations from Hardy-Weinberg Equilibrium
(HWE) were determined using the Pearson
Chi2-test. Association between a genotypes/
alleles occurrence and an outcome (CRC) was
estimated through calculating odds ratios with
95% confidence intervals. Mathematical opera-
tions were carried out on Statistica software
(V10.0; Statsoft, Tulsa, OK, USA).

Results
All members of both control as well as patients
group were genotyped successfully. Conducted
a second set of assays for a 10% of random
samples confirmed the full compliance of
genotypes.

Hardy-Weinberg (HW) chi-square analysis have
shown that distribution and frequency of geno-
types of control group in -4601A/G (rs5030789)
polymorphism (X²=0.26; P =
0.61) is consistent with HW equi-
librium in contrary to CRC patients group
(X²=8.87; P = 0.0029). In the second SNP
-4719A/T (rs5030789), distribu-
tion of genotypes in both control
(X²=2.6; P=0.11) and patients
(X²=1.06; P=0.3) group corre-
sponded to HW equilibrium.

The frequencies of genotypes in
-4601G/A polymorphism of RAD51 was 34% for GG, 61% for
G/A, 9% for AA, allele G 61% and allele A 34%.
The acronym of this polymorphism in the
PubMed databases suggests that the A allele is
native. However, in the detailed description the
G allele is represented as the ancestral there-
fore, it has been used as a reference in our cal-
culations. The odd ratio value indicates an
increased risk of CRC for heterozygous model
A/G (OR=1.854 95% CI= 1.063-2.256). For the
second investigated polymorphism the fallow-
ing frequencies was determined: 22% for A/A,
55% for A/T, 23% for TT and 49% for allele A,
51% for allele T. Statistical analysis have shown
lack of association with CRC. The full outcomes
were detailed in the Table 2 for -4601G/A
(rs5030789) and Table 3 for -4791A/T
(rs2619679).

Calculations with respect to the double combi-
nation of genotypes have not brought any sta-
tistically significant results. It seems to be con-
ditioned by low number of reference genotypes.
It appeared only three GG/AA genotypes in both
group together.

Discussion
DNA DSBs belong to the most harmful and
genotoxic damage in the living environment.
Based on metaphase chromosome and chro-
matid breaks in early passage primary mam-
malian fibroblasts it was estimated that approx-
imately ten DSB occur daily per cell [15]. Other
evaluations point to even fifty DSB per cell per
day [16]. As many as 100000-150000 single
strand breaks (SSB) have the potential to
induce apoptosis in comparison to DSBs, which
only 1-10 bring the same effect [17]. Each day,
human is affected by several damage factors of
endogenous and exogenous origin. There are
two major distinguishable exterior factors, ion-
zizing radiation (IR) and chemical compounds
broadly used as chemotherapeutics such as
mitomycin C, cisplatin, bleomycin, phleomycin

<table>
<thead>
<tr>
<th>genotype/</th>
<th>Patients</th>
<th>Controls</th>
<th>OR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=115</td>
<td>N=118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>allele</td>
<td>no frequency</td>
<td>no frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>35 0.34</td>
<td>51 0.43</td>
<td>1ref</td>
<td>-</td>
</tr>
<tr>
<td>G/A</td>
<td>70 0.61</td>
<td>55 0.47</td>
<td>1.854 (1.063-2.256)↑</td>
<td>0.020</td>
</tr>
<tr>
<td>A/A</td>
<td>10 0.09</td>
<td>12 0.10</td>
<td>1.214 (0.473-3.118) 0.689</td>
<td></td>
</tr>
<tr>
<td>allele G</td>
<td>140 0.61</td>
<td>157 0.66</td>
<td>1ref</td>
<td>-</td>
</tr>
<tr>
<td>allele A</td>
<td>90 0.39</td>
<td>79 0.34</td>
<td>1.278 (0.875-1.865) 0.204</td>
<td></td>
</tr>
</tbody>
</table>

*95% confidence interval; ↑statistical significance.
Table 3. Genotype and allelic frequency distribution of RAD51 -4791A/T (rs2619679) gene polymorphisms and the risk of CRC

<table>
<thead>
<tr>
<th>genotype/allele</th>
<th>Patients N=115</th>
<th>Controls N=118</th>
<th>OR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>25</td>
<td>30</td>
<td>1ref</td>
<td>-</td>
</tr>
<tr>
<td>A/T</td>
<td>63</td>
<td>50</td>
<td>1.512 (0.791-2.890)</td>
<td>0.210</td>
</tr>
<tr>
<td>T/T</td>
<td>27</td>
<td>38</td>
<td>0.853 (0.413-1.760)</td>
<td>0.663</td>
</tr>
<tr>
<td>allele A</td>
<td>113</td>
<td>110</td>
<td>1ref</td>
<td>-</td>
</tr>
<tr>
<td>allele T</td>
<td>117</td>
<td>126</td>
<td>0.904 (0.628-1.300)</td>
<td>0.584</td>
</tr>
</tbody>
</table>

*95% confidence interval.

Presented paper is a continuation of our screening research on SNP within genes encodes protein participating in the repair of DNA DSBs. Common SNP 135G/C of RAD51 gene has been previously examined by us wherein was no statistically significant association between any allele/genotype and CRC [29]. In subsequent study subjected Thr241Met XRCC3 polymorphism of the HRR gene, we have demonstrated the protective effect for Thr/Met genotype and Met allele [30]. Our recent results presented in herein paper revealed statistically significant increased risk of CRC for G/A genotypes in (rs5030789) simultaneously lack of association for other variants. Both -4719A/T and -4601A/G SNPs have never been genotyped before in context of CRC. So far, they have been screened among the patients from Polish population with the head and neck cancer when authors have reported decreased risk for A/A genotype of RAD51 -4601A/G polymorphism. Additionally it has been found extenuating effect for -4601A/172T haplotype in men's group [14]. Second case-control study was performed on the Korean population with hepatocellular carcinoma but analysis of the genotypes distribution and alleles frequency have shown no relationship with the disease [31].

Composition of RAD51 promoter is characteristic for “housekeeping” genes wherein typical features are lack of TATA-cassette, presence of untranslated first exon for mRNA regulation and CpG-rich region [32]. In our deliberations, we assumed several putative mechanisms linking RAD51 and HRR with CRC occurrence or progression. Firstly, oxidative stress induced by chronic intestinal inflammations may lead to high accumulation of SSD, where some closely situated can be recognized as DSBs what was discussed at the beginning of this section [33]. Low oxygen tension (hypoxia) plays one of the leading role in cancer development and angiogenesis what has been demonstrated in many solid tumors, including CRC [34]. Simultaneously, there is some premises indicate that such oxygen status may down-regulate the RAD51 as well as HR and support...
accumulation of mutations [35]. In another approach, we suggest that certain RAD51 genetic variants may affect drug resistance, promote tumor growth and malignancy. Majority of reports are focused around the regulation of RAD51. It was presented in the recent studies that diminishing of RAD51 expression sensitized many types of cells to radiotherapy [36]. Tennstedt et al. have revealed RAD51 overexpression is associated with poor prediction for CRC patients [10]. It has been established the expression is associated with poor prediction for cancer disease. We believe that our promising results will assuredly be at least doubled. Examined polymorphism are situated at position -4719 and -4601 before translation start site whereas all previous studies on the regulation of RAD51 concerned minimal core promoter covered approximately 500 bp around transcription start site [32, 37]. Possibly, completely different factors may be responsible for the regulation within the region containing the subjected polymorphisms. Hence, our research ought to be extended to the test based on plasmid construct with a reporter gene such as luciferase. Analysis of the promoter in the context of particular haplotypes and new regulatory factors may contribute to elucidate potential functionality of these SNP in CRC or generally cancer disease.

Our research are affected certain limitations. Unquestionably, the size of studied groups may not be sufficient to properly represent the entire Polish population. In our future studies, it will assuredly be at least doubled. Examined polymorphism are situated at position -4719 and -4601 before translation start site whereas all previous studies on the regulation of RAD51 concerned minimal core promoter covered approximately 500 bp around transcription start site [32, 37]. Possibly, completely different factors may be responsible for the regulation within the region containing the subjected polymorphisms. Hence, our research ought to be extended to the test based on plasmid construct with a reporter gene such as luciferase. Analysis of the promoter in the context of particular haplotypes and new regulatory factors may contribute to elucidate potential functionality of these SNP in CRC or generally cancer disease.

Taken together, outcomes of our study indicate an increased risk of CRC appearance in patients with genotype A/G at position -4601. This is one of the first report focused on -4719A/T (rs5030789) and -4601A/G genetic variation. Hopefully, it may contribute to shift scientist attention from heavily exploited topic of 135G/C and 172G/T which for a long time does not bring specific answers about contribution in cancer. We believe that our promising results will be a prelude to further analysis performs by other teams, necessarily for establishing the role of RAD51 in carcinogenesis.

Acknowledgements

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References


-4601A/G RAD51 gene polymorphisms and the risk of colorectal cancer


[44] Chao C, Jamshidi PA, Wang WW and McMasters KM. Colorectal cancer cell adhesion at 4601A/G RAD51 gene polymorphisms and the risk of colorectal cancer