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Rad51C: A novel suppressor gene modulates the risk of head and neck cancer

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ABSTRACT

We conducted a case–control study to investigate the possible association between the head and neck cancer (HNC) and genetic variability of *Rad51C* tumor suppressor gene. Eight polymorphic sites spanning over non-coding regions of *Rad51C* promoter, exon 1 and intron 1 were genotyped in 81 HNC cases and 156 healthy controls using the real-time PCR technique. One investigated site turned out to be not polymorphic, while among the remaining seven sites a significant HNC risk-increasing effect was found for rs16943176 (c.-118G > A), rs12946397 (c.-26C > T) and rs17222691 (c.145 + 947C > T) on both allelic (OR = 1.8; $p < 0.05$) and genotypic (OR = 2.0; $p < 0.05$) level. Furthermore, our data seem to provide marginal evidence, that this effect might possibly be confined to women only (OR = 2.8; $p = 0.05$ for allelic and OR = 3.7; $p = 0.05$ for genotypic comparisons). These SNPs were found to co-segregate together forming two distinct, HNC risk-modulating haplotypes. The genetic variability of *Rad51C* might thus be of relevance with respect to HNC risk.

1. Introduction

A huge number of studies provide evidence that individual susceptibility to cancer is determined by genetic and environmental factors. The latter include UV light, ionizing radiation and various chemical agents contained for example in tobacco smoke, all of which may lead to double-strand break (DSB) DNA damage. DSB lesions of DNA are toxic and can lead to genome rearrangements resulting in various developmental disorders or cell death and are considered one of the major driving forces in cancer [1]. Recently, there has been a growing evidence in favor of the role of DSBs and the capacity of systems involved in the repair of DSBs in tumorigenesis and cancer therapy (for review see [2–4]). Cells have developed two main pathways crucial for repair of DNA DSBs: the homologous recombination (HR) and non-homologous end joining (NHEJ). While NHEJ is constantly active throughout the whole cell cycle, HR is more important following the DNA replication, as it employs an undamaged homologous sister chromatid strand to assure error-free reconstruction of impaired genetic information. The key roles in the HR machinery are played by Rad51 and its five paralogs: Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3. Biochemical studies have shown that these five proteins form two distinct complexes: Rad51B/Rad51C/Rad51D/Xrcc2 (BCDX2) and Rad51C/Xrcc3 (CX3) [5].

Rad51C (Rad51 homolog C, *Saccharomyces cerevisiae*; 17q25.1) is assumed to play a prominent role among Rad51 paralogs as it is part of both the above-mentioned complexes. Indeed, Rad51C was found to localize to the sites of DNA DSBs in early stage of HR and such localization was revealed to be a prerequisite for Rad51/DNA nucleoprotein filament assembly, a key event in the whole HR reaction [6]. Moreover, this protein is also assumed to facilitate the migration and branch resolution of HR-resultant cross-linked structures (Holliday junctions) in the late stage of HR [7], but several recent studies have identified its additional novel functions related to DNA damage response and checkpoint activation [6], Fanconi anemia pathway-mediated repair of interstrand cross-links (ICLs) [8] and finally a function of tumor suppressor and cancer susceptibility gene [9–12].

Although *Rad51C* reveals a considerable degree of conservatism, its role as a susceptibility gene was first established in hereditary

Abbreviations: 5′UTR, 5′ untranslated region; 95%CI, 95% confidence interval; AC, adenocarcinoma; DSBs, double strand breaks; HR, homologous recombination; HBOC, hereditary breast/ovarian cancer; HNSCC, head and neck squamous cell carcinoma; HWE, Hardy–Weinberg equilibrium; ICLs, interstrand cross-links; IQR, interquartile range; NHEJ, non-homologous end joining; OR, odds ratio; *Rad51C*, *Rad51* homolog C (*S. cervisiae*); SD, standard deviation; SNP, single nucleotide polymorphism.

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breast/ovarian cancer (HBOC) by Meindl et al. [9], who identified deleterious frame-shift, splice-site or missense mutations in this gene in HBOC families. Following this report, several other studies reporting mutations in this gene in high-risk HBOC families showed up [11,13–17]. Due to the low frequency of these mutations, studies reporting negative results in HBOC families are also available [12,18–22]. Of note is however the fact, that while all these mutations were present in HBOC or ovarian only cancer families, none of the above cited studies identified any mutations in *Rad51C* in breast cancer only families. As many of HBOC mutation carrier families revealed family history of other cancer types, the link between *Rad51C* genetic variability and colon and prostate cancer has also been investigated, but that study failed to reveal any link between *Rad51C* mutations and these types of cancer [23].

In our recent study we have investigated the association between genetic variability of two other Rad51 family proteins – *Xrcc3* and *Rad51* – and the head and neck cancer (HNC) and have reported a significant association between variability of non-coding regions (promoter, 5′UTR) of the latter gene and HNC risk [24]. In the case of *Rad51C*, the majority of available studies focus on HBOC and missense or deleterious mutations of this gene. To our knowledge, any data concerning the link between *Rad51C* genetic variability and other types of cancer are scarce or completely missing. Therefore, here we report a study in which we used a case–control setup in order to investigate the role of genetic variability of *Rad51C* in HNC. To this end, we selected eight single nucleotide polymorphisms (SNPs) of *Rad51C* and tested the differences in genotype distribution between HNC cases and healthy controls. In addition to this, non-random associations among these SNPs were investigated and found haplotypes tested for association with HNC.

2. Materials and methods

2.1. Subjects

All cancer subjects enrolled in the study were of Caucasian descent and residents of the Lodz district in Poland. The study involved 81 patients (65 men, 16 women) aged 25–74 years (median age at the time of diagnosis 44 years; IQR: 17.5 years) hospitalized in the Department of Oncological Laryngology, Memorial Copernicus Hospital, Lodz, Poland, between February 2005 and September 2006 (49% larynx, 31% pharynx, 16% oral cavity, 4% salivary gland) with diagnosis of head and neck cancer, confirmed histopathologically by two independent histopathologists. Only patients with primary HNC tumor with/without metastases and without any history of previous anti-cancer treatment, undergoing curative resection surgery or radiotherapy were involved in the study. Histopathologically, the group of HNC patients consisted of 80% of patients with head and neck squamous cell carcinoma (HNSCC), 10% of patients were diagnosed with adenocarcinoma (AC), 6% had lymphoepitelioma and 4% of them had other types of cancer.

The control group consisted of 156 healthy volunteers (111 men, 45 women) who agreed to undergo examinations, aged 32–64 years (median age at the time of examination 44 years; IQR: 4.3 years). All control subjects were also of Caucasian descent and residents of the Lodz district.

The characteristics of the two groups investigated in this study are summarized in Table 1. The classification as never- or ever-smokers was performed according to the criterion suggested in the study by Pomerleau et al. [25]. According to this criterion, only those subjects, who have smoked less than 19 cigarettes (a pack) during their lifetime, were classified as never-smokers, while the others were considered as ever-smokers. Unfortunately, no data concerning the alcohol consumption was available for either controls or cases.

Prior to experiments, written and informed consent for participation in this study was obtained from each cancer and control subject enrolled. The study was performed under the guidelines of the Helsinki Declaration for human research and was approved by the Local Bioethics Committee for Scientific Research (resolution no. 8/2004).

2.2. DNA isolation

Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The RNA contamination was removed by digestion with 1 mg/ml RNaze A (Qiagen, Germany) and the obtained DNA was further quantified and analyzed with regard to protein content and purity using an Eppendorf BioPhotometer (Eppendorf, Germany) instrument. Samples were stored at −80 °C for further processing.

Table 1
Characteristics of the sample and control groups involved in the study.

Feature	Control subjects	Cancer cases
Total number	156	81
Sex [female/male]	45/111	16/65
Age [years]	44 [32–64]	44 [25–74]
Smoking status [never/ever][a]	74/79 (48%/52%)	8/72 (10%/90%)
Tumor localization		
Larynx	–	40 (49%)
Pharynx	–	25 (31%)
Oral cavity	–	13 (16%)
Salivary gland	–	3 (4%)
Tumor histopathology		
Squamous cell carcinoma	–	65 (80%)
Adenocarcinoma	–	8 (10%)
Lymphoepitelioma	–	5 (5%)
Other	–	3 (3%)
Tumor staging[b]		
T [1/2/3/4/x]	–	7/21/13/11/29
N [0/1/2/3/x]	–	20/6/23/6/26
M [0/1/x]	–	49/3/29

[a] $p < 0.0001$; two-sided mid-P test; data available for 80 cases and 153 controls only.
[b] Tumor staging classification: T describes the size of the primary tumor and its invasiveness, N describes the regional lymph nodes involved, M describes the distant metastases; x, data not available.

2.3. Genotyping

In this study, we focused on SNPs occurring predominantly in non-coding regions of *Rad51C*, as it is generally believed that such SNPs may be involved in the regulation of gene expression and, moreover, the missense changes in *Rad51* paralogs are very rare due to the high degree of conservativeness of these genes. We involved 7 single nucleotide polymorphisms (SNPs) localized in gene's promoter, 5′ untranslated region (5′UTR) of exon 1 and intron 1. Only those SNPs with the minor allele frequency (MAF) in the Caucasian population exceeding 10% (according to data contained in dbSNP database [26]) were selected for the study. In addition to this, we also involved one missense SNP occurring in coding region of exon 1 (rs28910276; ^{34}T/C; Trp12Arg), the MAF of which in Caucasian population was unknown. The possible involvement of selected promoter, intronic and 5′UTR SNPs in regulation of *Rad51C* expression was tested by is-rSNP algorithm [27], an in silico method for prediction whether an SNP can be considered as regulatory (i.e. disrupting the transcription factor binding). Exemplary results of such prediction for selected nuclear transcription factors together with other detailed information on SNPs analyzed in this study are provided in Table 2.

All SNPs involved in this study were genotyped by means of the real-time PCR technique using either custom or pre-designed commercially available TaqMan SNP Genotyping assays (Applied Biosystems, USA) according to manufacturer's instructions. Genotyping was performed on BioRad's iQ5 iCycler Multicolor Real Time PCR Detection System (BioRad, USA) in 20-μl aliquots containing 16 ng DNA. The cycling conditions, preceded by polymerase activation (95 °C, 10 min) consisted of 50 cycles involving DNA denaturation at 95 °C for 15 s followed by a combined annealing-elongation step at 61.5 °C for 1 min, during which the fluorescence signal was measured. The genotype recognition of analyzed subjects was performed automatically using an iQ5 Optical System Software ver. 2.0 (BioRad, USA).

Ten percent of the obtained results were verified by repeated genotyping using the same technique.

2.4. Statistical analysis

In the case of all SNPs, both absolute and relative frequencies are provided. The Hardy–Weinberg equilibrium (HWE) for each SNP was tested by a goodness-of-fit chi-square test. In the first part of analysis, all investigated SNPs were tested for possible associations with cancer risk using the logistic regression, at both allelic and genotypic level. To this end, a separate univariate (i.e. single-site) analysis was performed for each SNP, and results were expressed by means of odds ratio (OR) adjusted for age, sex and/or smoking status, where appropriate. Interactions with subjects' sex were analyzed by means of stratified analysis approach. All ORs are provided with corresponding 95% confidence interval (95% CI).

In the second part of analysis, linkage disequilibrium (LD) analysis and haplotype reconstruction were performed using an expectation–maximization algorithm implemented in the Haploview package [28]. LD was expressed by means of a normalized measure of allelic association $|D'|$ [29] and LD blocks were inferred using