The C/A polymorphism in intron 11 of the *XPC* gene plays a crucial role in the modulation of an individual's susceptibility to sporadic colorectal cancer.

Running title:

XPC C/A (i11) polymorphism in sCRC.

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Abstract:

Background: Epidemiological data show that colorectal cancer (CRC) is the second most frequent malignancy worldwide. The involvement of “minor impact genes” such as XME and DNA-repair genes in the etiology of sporadic cancer has been postulated by other authors.

Aim: we focused on analyzing polymorphisms in DNA-repair genes in CRC. We considered the following genes involved in DNA-repair pathways: base excision repair (*OGG1* Ser326Cys, *XRCC1* Trp194Arg and Arg399Gln); nucleotide excision repair [*XPA* (-4)G/A, *XPC* C/A (i11) and A33512C (Lys939Gln), *XPD* Asp312Asn and A18911C (Lys751Gln), *XPF* Arg415Gln, *XPG* Asp1104His, *ERCC1* C118T]; homologous recombination repair [*NBS1* Glu185Gln, *Rad51* 135G/C, *XRCC3* C18067 (Thr241Met)].

Material and methods: The study group consisted of 133 patients diagnosed with sporadic CRC, while the control group was composed of 100 age-matched non-cancer volunteers. Genotyping was performed by PCR and PCR-RFLP. Fisher’s exact test with a Bonferroni correction for multiple testing was used.

Results: We found that: *i) XPC* C/A (i11) heterozygous variant is associated with increased risk of CRC [OR is 2.07 (95% CI 1.1391,3.7782) p=0.038], *ii) XPD* A18911C (Lys751Gln) is associated with decreased risk of CRC [OR=0.4497, (95% CI 0.2215,0,9131) p=0.031] for an individual with at least one A allele at this locus.

Conclusions:

1. the *XPC* C/A (i11) genotype is associated with an increased risk of sporadic colorectal cancer.

2. the NER pathway has been highlighted in our study, as a most important in modulation of individual susceptibility to sCRC.

1. Introduction:

Despite great progress in our knowledge regarding the development and treatment of cancer, sporadic colorectal cancer (sCRC) still remains one of the most common cancers and the cause of a high proportion of cancer-related deaths in developed countries [1,2]. There is a growing body of evidence that an individual's susceptibility to sCRC depends on both his/her an individual's genetic profile (so called "genetic makeup") and exposure to exogenous and endogenous carcinogens (DNA damaging agents) [3,4].

The risk of sporadic CRC is modified mainly (approximately 80%) by environmental and lifestyle factors that could be a source of DNA-damaging carcinogens [6]. Thus, the capacity for DNA-repair, determined by the polymorphisms in DNA-repair genes, seems to be essential in ensuring genomic (molecular and chromosomal) stability, which in turn is strongly associated with the risk of sCRC. DNA-repair genes act on a variety of pathways depending on the type of DNA-damage. The base excision repair (BER) pathway is involved mainly in the removal of oxidative DNA damage induced by reactive oxygen species [5]. The 8-oxoguanine DNA glycosyslase (*OGG1*) gene encodes a DNA repair protein that removes 8-oxo-7,8-dihydroguanine (8-oxoG) [6]. The variant allele of the Ser326Cys polymorphism is associated with higher level of genetic instability and seems to be associated with an increased risk of lung, esophagus, prostate cancer as well as ALL (acute lymphoblastic leukemia) among children [7-9]. Opinion is divided as to whether there is an association between *OGG1*, Ser326Cys and colorectal cancer [8,10,11]. The *XRCC1* gene encodes a protein that interacts with other DNA-repair proteins, such as Lig3, Polβ and PARP, creating a complex involved in short-patch BER [6]. Two polymorphisms: Arg194Trp and Arg399Gln have been widely studied in cancer epidemiology. It has been proven that Arg194Arg individuals have a higher level of DNA damage compared to those with one or two Trp variant alleles, while the highest level of DNA damage is observed in Gln399Gln

homozygotes [12]. The 194Trp variant has also been found to be protective against lung cancer, although it is associated with an increased risk of CRC [8]. The results for the Arg399Gln polymorphism of the *XRCC1* gene in colorectal cancer epidemiology are very ambiguous [13-18].

Nucleotide excision repair (NER) is involved in the removal of DNA thymidyne dimmers and bulky adducts, induced by UV light, tobacco smoke and environmental xenobiotics [19]. The NER pathway consists of about 30 genes called Xeroderma Pigmentosum (XP) or the excision repair cross-complementing group (ERCC). The *XPA* (-4) G/A variant localized in Kozak sequence near the ATG-start codon may influence the level of the XPA protein [20]. The presence of at least one G-allele is associated with a decreased risk of lung cancer in Caucasians, Koreans and Americans [21]. Nevertheless, Hansen et al. did not observe any statistically significant interaction between this polymorphism and the risk of CRC [22].

The XPC protein is involved in the XPC-HR23B DNA-damage-recognition complex of GG-NER [19]. Three polymorphisms in *XPC* [A33512C in exon 15 (Lys939Gln)], the insertion/deletion poly AT (PAT) in intron 9 and the C/A polymorphism in intron 11 are in high linkage disequilibrium [23]. The *XPC* PAT+/+ variant was shown to be associated with reduced capacity for DNA repair and an increased risk of lung cancer [23,24]. The variant allele of the *XPC* exon 15 polymorphism has been found to be associated with the risk of bladder cancer [25]. The *XPD* gene encodes the helicase participating in the transcription factor IIH. The 312Asn and 751Gln variant alleles of *XPD* correlate with a reduced capacity for repairing bulky adducts [12]. However, the results of studies on *XPD* polymorphisms with regard to a variety of human cancers remain contradictory [22,26,27]. The *XPF* gene encodes a protein which, together with ERCC1, creates the 5'endonuclease [28]. The results of studies on *XPF* and *ERCC1* variants and the risk of cancer are also contradictory. [29-31]. The *XPG*

gene encodes the 3' endonuclease of the NER pathway. The wild type allele of *XPG* (Asp1104His) has been shown to be associated with a decreased risk of lung cancer especially among light smokers and non-smokers [32]. There is a lack of available data on polymorphisms of the *XPF* and *XPG* genes and the risk of sCRC.

Homologous recombination repair (HR) is involved in the repair of double strand breaks (DSBs) [30]. The NBS1 (Nibrin) protein is a component of the MRN-complex, which recognizes DSBs [31]. Most studies investigating the *NBS1* Glu185Gln polymorphism have focused on assessing the risk of breast cancer [32]. There is only one publication on this *NBS1* polymorphism and risk of colorectal cancer [33]. The XRCC3 protein interacts with Rad51-related proteins in the process of homologous recombination. The C18067T (Thr241Met) variant of the *XRCC3* gene in exon 7 modulates its function and is linked to an increased risk of bladder, breast, colorectal, lung and skin cancer [37-41]. Rad51 plays a crucial role in the HR pathway. The increased risk of endometrial cancer for the G135C variant of *Rad51* have been shown as well as for breast cancer among carriers of the *BRCA1* mutation [42,43]. Recently it has been postulated that the above mentioned polymorphism of the *Rad51* gene could be an independent marker of colorectal cancer risk [44].

We aimed to find polymorphisms of the chosen DNA-repair genes which modulate the risk of sCRC. We focused on the following genes: 1) *OGG1* Ser326Cys, *XRCC1* Trp194Arg and Arg399Gln (BER), 2) *XPA* (-4)G/A, *XPC* C/A (i11) and A33512C (Lys939Gln), *XPD* Asp312Asn and A18911C (Lys751Gln), *XPF* Arg415Gln, *XPG* Asp1104His, *ERCC1* C118T (NER), 3) *NBS1* Glu185Gln, *Rad51* G135C, *XRCC3* C18067T (Thr241Met) (HR).

2. Materials and methods

2.1 Patients and controls

The study and the control group consisted entirely of Polish individuals (all Caucasians), who came from the same geographic area (Lower Silesia). The study group consisted of 133 patients diagnosed with sporadic colorectal adenocarcinoma (sCRC). The mean age of the patients was 63.22 with a standard deviation of 11.36. Blood samples from the study group were collected from the 2nd Department of General and Oncological Surgery, Wroclaw Medical University and the Department of General Surgery, Regional Specialized Hospital, Wroclaw.

The control group consisted of 100 patients of the Department of Internal Diseases, Clinical Hospital, Swiebodzice, Poland. Their mean age was 74.89 with a standard deviation of 7.63. We decided to compose the control group of individuals approximately 15 years older on average than the cancer patients, because the risk of sCRC cancer is age-related. None of the control group suffered from cancer or had a family history of cancer.

The design of the study was accepted by the Wroclaw Medical University Ethics Committee and each of individual signed informed consent.

2.2 Analysis of the polymorphisms

Genomic DNA was isolated from peripheral blood lymphocytes using standard phenol-chloroform extraction and ethanol precipitation. The analyses of 14 SNPs in the following DNA-repair genes: *OGG1*, *XRCC1, XPA, XPC, XPD, XPF, XPG, ERCC1, XRCC3, Rad 51* and *NBS1,* were carried out using a polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) assays as we have previously described [45]. To genotype *OGG1* Ser326Cys, we employed the method of Le Marchand et al. [46]. The final products were analyzed using gel electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized directly under UV light.

2.3 Statistical analysis

The chi-squared goodness of fit test was used to test whether the genotype frequencies corresponded to the Hardy-Weinberg equilibrium and to test for linkage disequilibrium between pairs of loci. The frequencies of extended haplotypes were estimated using an iterative procedure based on a standard gene counting procedure [47].

Fisher's exact test was used to assess differences in phenotype distribution between the two groups, firstly under the assumption that the non-wild type allele (assumed to be the least frequent allele) is dominant and secondly under the assumption that the non-wild type allele is recessive [48]. Logistic regression was used to derive of model for the likelihood of developing CRC given the genetic data. Statistical tests were performed using the SPSS and R software packages.

3. Results

We have determined the frequencies of 14 SNPs in 11 genes involved in three DNA repair pathways in sCRC patients compared to the control group. In the first step the loci were tested for agreement with the Hardy-Weinberg equilibrium and linkage disequilibrium.

The *OGG1, RAD51*, *XPF* polymorphisms and the polymorphism at codon 194 Arg/Trp of the *XRCC1* were omitted from the analysis, since there was too little variation at these sites for the approximation used to be reliable. No significant deviations were observed from the Hardy-Weinberg equilibrium in the control group. Significant deviations from this equilibrium were found in the study group at the following loci: *XPC* intron 11 ($p<0.001$), *ERCC1* C118T ($p<0.01$) and *XPG* Asp1104His ($p<0.05$). In the case of *XPC* intron 11, this result is significant at the 1% level, even when the Bonferroni correction is taken into account (based on the 10 sites considered). In both the sCRC-patient and control groups a significant level of linkage disequilibrium was observed for two pairs of polymorphisms *i*) *XPC* A33512C (Lys939Gln) and *XPC* C/A (i11) ($p<0.01$ after the application of Bonferroni correction); *ii) XPD* Asp312Asn and *XPD* A18911C (Lys751Gln) ($p<0.001$ after the application of Bonferroni correction).

The analysis of association between polymorphisms in a single gene and sCRC revealed a significant association between the *XPC* C/A (i11) locus and sCRC risk (recessive model, $p=0.038$). The odds of a heterozygote with an A allele at this locus developing sCRC is estimated to be 2.07 times the odds for an individual with a C/C genotype. The 95% confidence interval for this odds ratio is [1.1391,3.7782]. Moreover, individuals with an A/A were not significantly more likely to develop sCRC.

There was also a significant association between the *XPD* A18911C (Lys751Gln) polymorphism and the risk of sCRC (dominant model, $p=0.031$). The odds of an individual with a C/C (A18911C) genotype developing sCRC is estimated to be 0.4497 times the odds

for an individual with at least one A allele at this locus. The 95% confidence interval for this odds ratio is [0.2215,0.9131] (see Table 1). No other associations were observed (see Table 2, available online).

We also investigated the association between an individual's genotype based on a pair of loci and the risk of developing sCRC. A significant association was found for the following seven pairs of loci: *1) XRCC3* C18067T (Thr241Met)*/RAD51* G135C (p=0.01691, here due to their small number 135C/135C individuals were grouped with 135C/wt individuals), *2) XPG* Asp1104His/*ERCC1* C118T (p=0.04297), *3) XPF* Arg415Gln/*XPC* A33512C (Lys939Gln) (p=0.01009), *4) XPC* in(11)/*XPC* A33512C (Lys939Gln) (p=0.009246), *5) XPC* in(11)/*ERCC1* C118T (p=0.04390), *6) XPC* in(11)/XPF Arg415Gln (p=0.01691), *7) XPD* Asp312Asn/*XPD* A18911C (Lys751Gln) (p=0.03596). Several models based on logistic regression were considered. In each case, factors were sequentially added to the model until no factor outside the model was significant. The first model considered sex and all the loci as possible factors. This model stated that taking into account sex (males are more susceptible), individuals with an A allele at *XPC* (i11) are more susceptible to sCRC (p=0.013). In the second model considered, sex, the *XPC* (i11) locus and the *XPD* A18911C (Lys751Gln) were treated as possible factors, together with the genotypes based on the following three pairs of loci not including the two loci above 1) *XRCC3* C18067T (Thr241Met)*/RAD51* G/135C, 2) *XPG* Asp1104His/*ERCC1* C118T and 3) *XPF* Arg415Gln/*XPC* A33512C (Lys939Gln) (the most frequent double homozygote was used as a baseline for comparison in each case). Apart from the significant effect of *XPC* (i11) found in the previous model, this model found the following associations: *XPF* Arg415Gln/*XPC* A33512C (Lys939Gln) – double heterozygotes have a higher susceptibility to sCRC than Arg415Arg/A33512A (Lys939Lys) (the "wild-type" double homozygote, $p<0.01$). *XRCC3* C18067T (Thr241Met)*/RAD51* G135C– CC (18067)/G135C individuals are more susceptible than CC (18067)/G135G individuals

($p<0.05$). *XPG* Asp1104His/*ERCC1* C118T – GG/CT, GC/TT and CC/CT individuals are more susceptible to CRC that GG/TT individuals ($p<0.05$, $p<0.05$ and $p<0.01$, respectively). Due to the relatively low frequency of some of the genotypes, it is difficult to make strong conclusions based on these results. However, one interesting aspect is that for the three pairs considered in the analysis, double homozygotes showed a low level of susceptibility to sCRC.

4. Discussion

The complexity of the process of carcinogenesis, has led to a situation in which, despite tremendous research effort, the genetic etiology of sporadic cancers still remains largely uncovered. Yet the understanding of this complexity resulted in the multi-hit theory of cancer [46], which seems to be more comprehensive with sporadic cancers than double-hit theory, which in turn seems to be more comprehensive with the etiology of hereditary cancers. Finally, an enormous number of papers focusing on associations between the risk of cancer and a variety of polymorphisms/mutations in DNA-repair genes have been published (frequently presenting conflicting results).

In the current study, among the 14 variants in 11 DNA-repair genes analyzed, only variants of two genes: *XPC* C/A(i11) and *XPD* A18911C (Lys751Gln) were shown to be associated with sCRC risk. The carriers of the homozygous C/C A18911C (Lys751Gln) genotype at the *XPD* gene were at decreased (OR 0.4497 CI 95% 0.22 - 0.91), while individuals carrying C/A polymorphism in intron 11 at *XPC* gene at increased (OR 2.07 CI 95% 1.13 -3.77) risk of sCRC.

Previously published data focusing on the role of *XPD* variants in the modulation of an individual's risk of developing various cancers, are scarce and often ambiguous. For example, the results of the study of Skjelbred CF *et al*. studies on Norwegian cohort suggested that *XPD* A18911C (Lys751Gln) variant is associated with an increased risk of low-risk adenomas, but not carcinomas [15], while Hansen's RD *et al*. studies on 160,725 Danish individuals revealed that the *XPD* A18911C (Lys751Gln) variant is not of a major importance in colorectal carcinogenesis [22]. Results similar to those of Hansen et al. [22] were recently published by ref.27 for Polish population Thus, the role of mentioned polymorphism of *XPD* in colorectal carcinogenesis seems to be still uncovered.

However, the data highlighting the role of *XPC* C/A (i11) in modulation of an individual risk of developing cancers, seems to be much more comprehensive.

The meta-analysis, carried out by Zhang et al. as well as Qiu et al. revealed that *XPC* is one of the most important genes modulating an individual's risk of developing sporadic cancer [48,50]. Moreover, recent data on structure-function relations of the XPC protein suggest that even a single amino acid alteration could be sufficient to compromise XPC function profoundly [51]. The results of our studies showing that *XPC* C/A (i11) individuals are at increased risk of sCRC are in agreement with these observations. An important role of *XPC* C/A (i11) in carcinogenesis probably results from the fact that the *XPC* intron 11 splice acceptor site polymorphism is related to an increased frequency of exon 12 skipping, leading thus to diminished DNA repair. Khan et al. reported that the homozygous variant A/A is associated with an approximately 50% reduction in DNA repair capacity (DRC) of the XPC protein [52]. This is consistent with the observations by Lopez-Cima et al., who found that the PAT+/33512C(939Gln) /intron11A haplotype contributed to reduced DNA repair capacity and thus, to an increased risk of lung cancer [53].

In the current study we did not observe any increased risk of CRC to be associated with the homozygous variant (A/A) in intron 11 of *XPC* gene. This could be explained by the 'greater apoptosis' hypothesis, stating that adverse variants with lower DRCs may have a greater protective effect by increasing apoptosis rate. In fact, the results of our study are in agreement with the results of Huang et al., who found an association between 3 polymorphisms of the *XPC* gene (492R, 499A and 33512C) and the risk of colorectal adenoma, especially in smokers [54]. Tobacco carcinogens (like polycyclic aromatic hydrocarbons) may cause a DNA damage, while the XPC protein plays a crucial role in repairing of this type of damage [54].

The studies of Garcia-Closas et al. and Shen et al. revealed an association between the risk of cancer and a single *XPC* polymorphism, namely: between 33512C and the risk of bladder cancer, as well as the PAT+ polymorphism in intron 9 and the risk of squamous cell carcinoma of head and neck [55,56]. However, taking into account the high LD between these *XPC* variants, it remains to be determined whether a single polymorphism or their association is of most importance in the modulation of cancer risk.

Interestingly, an *in vitro* experiment has confirmed the importance of the *XPC* C/A (i11) genotype in the preservation of genetic stability, by revealing that among young healthy individuals the *XPC* C/A (i11) genotype is associated with the frequency of bleomycin-induced chromosomal aberrations (CA) [45]. This controversial observation was published by Kazimirova et al., who showed that C/A heterozygotes had less chromosomal aberrations than comparing to C/C and A/A homozygotes [57].

This discrepancy could result from an analysis of the relationships between a chromosomal end-point and a single genetic variant [57], but not among a "network" of genetic alterations [45].

We were also searching for an association between pairs of genetic variants and CRC risk. Our results have revealed that NER pathway-genes were present in all but one of gene-variant pairs, highlighted as most important in this process,. All these data taken together, strongly suggest the potential role of the NER pathway (mainly of *XPC*), in ensuring of chromosomal stability and subsequently in the modulation of individual's risk of sporadic colorectal cancer.

5. Conclusions:

On the basis of our study, in regard to relevant literature it can be stated that the *XPC* C/A (i11) is associated with an increased risk of sCRC.

Moreover, in spite of the fact that it is difficult to make strong conclusions based on the results of current studies in regard to the influence of networks of polymorphism in DNA repair genes on an individual's sCRC risk by (because of the relatively low frequency of some of the genotypes), our results strongly supports the thesis that *"it is necessary to access the combined effect of several polymorphisms in the same gene or between different genes that might contribute to cancer risk"* [48].

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