Association between *XRCC1* Polymorphisms and Head and Neck Cancer in a Hungarian Population

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Abstract. *Background: Head and neck cancer is a significant current health problem in Hungary because the mortality of this cancer has increased by 387% in the last thirty-two years. Because of the important role of the XRCC1 gene in DNA repair, we wanted to test the effects of the Arg194Trp and Arg399Gln polymorphisms of XRCC1 on the clinical outcome of head and neck cancer. Patients and Methods: A polymerase chain reaction-restriction fragment lenght polmorphism (PCR-RFLP) method was used. A total of 108 samples were taken from intraoperatively removed formalin-fixed, and paraffin-embedded blocks of tissue. An age- and sex-matched cancer-free control group was used to compare the frequency of polymorph variants. Results: No significant difference was found between patients and controls in repect of the investigated polymorphisms. A significant difference was found between the patients with different XRCC1 194 polymorph status in clinical stage SIII. The survival proportion of patients with the Arg194Arg genotype was significantly lower than of those with the Arg194Trp genotype. Conclusion: The complex analysis of these factors may provide the basis for personal risk assesement and an opportunity for individualised therapy.*

Head and neck cancer is malignant disease of the upper aerodigestive tract (oral, pharyngeal, and laryngeal regions); histologically it is commonly squamous cell carcinoma (1). In 2006, 70,200 cases were registered in the European Union, which is 3.1% of the total number of cancer cases. The number of fatalites caused by this type of cancer was 25,300 in the same year (2).

This type of cancer is a significant current health problem in Hungary because compared to other European countries, the mortality from head and neck cancer is very high. In 2001, the age-standardised mortality rates (per 100,000 inhabitants per year) was 2.4 in Sweden and 21.2 in Hungary (3). Carcinoma of the head and neck region is the fifth most common newly diagnosed cancer in the Hungarian population, with 3,539 new cases being registered in 2007. Most of the patients were men: of 3,539 patients, 2,677 were men. In 2007, 2,251 patients died in Hungary because of head and neck cancer. All together, cancer of the head and neck region is the third most common cause of cancer death among men in Hungary. Unfortunately, the incidence and mortality of head and neck cancer shows an increasing tendency. In 1975, only 462 patients died from head and neck cancer. This means that the mortality of this cancer has increased by 387% in the last thirty-two years in Hungary (4).

Head and neck cancer is associated with certain typical aetiological risk factors. Tobacco use and alcohol consumption are the most important risk factors of head and neck cancer. Any form of tobacco abuse is a risk factor of head and neck cancer: cigarette smoking, cigar and pipe smoking significantly elevate cancer risk (5). The alternative forms of tobacco abuse are also dangerous. The relationship between using smokeless forms of tobacco and lesions of the mucosa of the oral cavity, startpoints of the development of oral cavity cancer, are well documented (6). Numerous studies have proven that any type of alcohol consumption

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increases the risk of premalignant lesions and cancer of the head and neck region. Smoking and drinking are independently and synergistically associated with the increased risk of head and neck cancer (7). The consumption of alcohol and tobacco in the Hungarian population is higher than the average rate in the EU. In Hungary, the average alcohol consumption is more than 11.6 l/person/year (9.3 in the EU) and tobacco consumption is about 30.5 cigarettes/day (26.5 in the EU) (8).

Carcinogenic metabolites of tobacco and alcohol cause direct DNA damage and, in addition, produce oxidative stress which also contributes to the carcinogenic process. Naturally, genetic susceptibility plays an important role in the development of malignant disease. A multiplex mechanism protects against DNA lesions inside cells. Base damage and single-strand breaks (SSBs) of DNA are the most frequent DNA lesions and they are repaired through base excision repair (BER) mechanisms. BER is a complex mechanism including damage detection, end processing, gap filling and ligation (9).

X-ray repair cross complementing 1 (XRCC1) protein plays an important role in the BER pathway (10). The *XRCC1* gene is located at the long arm of chromosome 19, consists of 17 exons and encodes a 633 amino acid long protein. The XRCC1 protein has a function as a scaffolding protein for other proteins such as DNA ligase III, DNA polymerase β, apurinic/apyridinic endonuclease 1(APE1), polynucleotide kinase/phosphatase, poly(ADP-ribose) polymerase (PARP) (11, 12).

The observation that *XRCC1* deficiency in mice is lethal in the embryonic stage supports the theory that the function of *XRCC1* is a key factor in DNA repair (13). Mutations in the *XRCC1* gene are known to contribute to the development of human tumours. However, the function of *XRCC1* is affected not only by point mutations, but also by single nucleotide polymorphisms (SNP). Of these polymorphisms, around 30 are situated in exons or promoter regions. Based on data from the literature, two SNPs might be associated with the development of malignant diseases: Arg194Trp in exon 6 and Arg399Gln in exon 10 (14-16).

The *XRCC1* Arg194Trp SNP falls within the proliferating cell nuclear antigen-binding region and the Arg399Gln SNP is located in the region of the breast cancer susceptibility gene COOH terminus-1 (BRCT-1) interaction domain of *XRCC1* within a poly (ADP-ribose) polymerase-binding region. Because of the specific functions of these regions, it has been suggested that their polymorphisms are involved in modifying protein-protein interactions during DNA repair. These polymorphisms may be able to modify the risk of cancer development in humans and might also have an effect on the clinical outcome of malignant diseases (17). Our aim was to test this hypothesis in the case of head and neck cancer in a Hungarian population.

Patients and Methods

One hundred and eight patients participated in this study, diagnosed with head and neck cancer between 2000-2003. Sample collection, pathological identification and collection of clinical variables were carried out at the Markusowszky County Hospital Szombathely, Hungary. Men were overrepresented in the population: samples originated from ninety-seven men and only eleven women. The mean age±SD of patients was 56.7±8.9 years. All patients had a history of smoking and all patients had a histological diagnosis of squamous cell carcinoma. The samples were taken from intraoperatively removed, formalin-fixed and paraffin-embedded blocks of tissue.

For staging of the patients, the American Joint Committee on Cancer (AJCC) criteria were used. According to these criteria, 14 SI, 12 SII, 37 SIII, 32 SIVA and 13 SIVB stage patients were identified. Patients with head and neck cancer were followed up, with the endpoint being either an interval of 60 months after diagnosis or the death of the patient.

An age- and sex-matched healthy control group of 102 individuals was used to compare the frequency of polymorphic variants in patients with those of a cancer-free population. A modified protocol of Chan *et al*. was used for extraction of DNA (18). Ten μm thick sections of samples were used for DNA extraction. Samples were placed in a microcentrifuge tube containing 200 μl of digestion buffer (50 mM Tris/HCl, pH 8.5, 1 mM EDTA and 0.5% Tween 20). The tubes were heated in a microwave for 1.5 minutes, with the irradiation time split into 15 s segments to prevent overboiling. The tubes were centrifuged for 10 minutes at 15000 × g, to remove the solid paraffin wax. The tissue was digested in 200 μl of digestion buffer containing 200 μg/ml proteinase K at 56˚C overnight. The samples were centrifuged once more and the supernatant was boiled 10 minutes at 95˚C to denature the proteinase K (18).

For PCR reaction, 5 μl of DNA solution was used. For genotyping of *XRCC1,* a PCR-based restriction fragment length polymorphism (RFLP) analysis was used (19, 20).

Primer sequences for exon 6, Arg194Trp were: 5'- GCC AGG GCC CCT CCT TCA A-3' and 5'- TAC CCT CAG ACC CAC GAG T-3'. The primers for Arg399Gln on exon 10 were: 5'-TGC TTT CTC TGT GTC CA-3' and 5'-TCC AGC CTT TTC TGA TA-3'.

The amplification was performed in a 25 μl reaction volume, with Platinum PCR Supermix (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions.

The restriction enyzyme *Pvu*II was used to distinguish the Arg194Trp polymorphism and *Msp*I enzyme to distinguish the Arg399Gln polymorphism. The restriction products were analyzed by electrophoresis on a 3% agarose gel containing ethidium bromide. The difference between the genotype distribution in patients and controls was tested by Chi-square test (Epi Info 6.0; CDC, Atlanta, GA, USA).

Additionally, we determined whether a subset of these polymorphisms was related to the overall survival of the patients in the hope that genotypes could be used to predict survival of patients with head and neck cancer. Kaplan-Meier survival analyses were performed after the samples were divided into groups according to gene status. A probability of $p<0.05$ was considered as statistically significant.

Results

Statistics were calculated for the genotypes for both cases and controls. The distribution of *XRCC1* genotypes among cases and controls are shown in Table I. All possible allelic

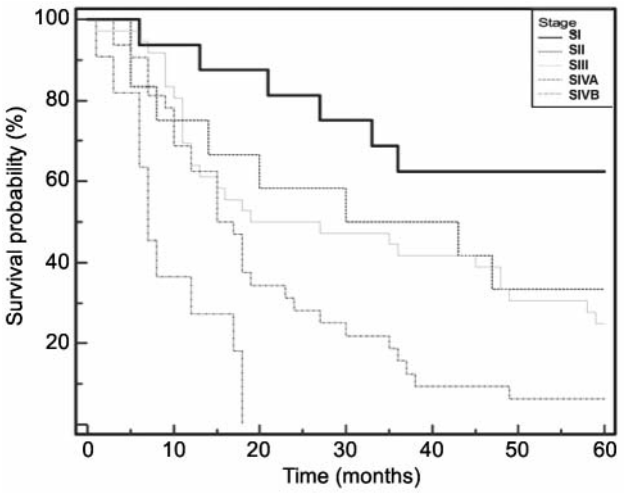

Figure 1. *Kaplan-Meier survival curve selected by stages of head and neck cancer (SI-SIV B).*

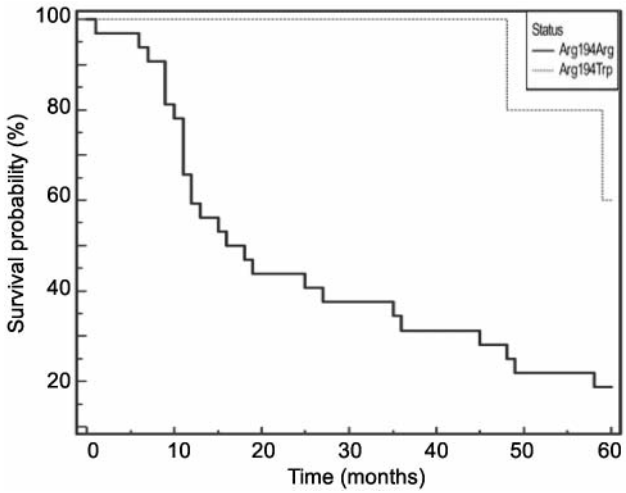

Figure 2. *XRCC1 194 polymorphism in stage SIII patients. Hazard ratio: 3.93, 95% CI: 1.06-6.48, p=0.04.*

variants were represented in both the tumor and the control groups (data in table). The *Arg194Trp* and *Trp194Trp* variants tended to be more frequent among the healthy controls than in cancer patients, although the association was not statistically significant. Similarly, no significant difference was found between patients and controls with respect to the *XRCC1* 399 polymorphism, although the Gln hetero- and homozygote variants were more frequent in the group of cancer patients.

Figure 1 shows the relation between the tumor stage at diagnosis and patient survival. Naturally, the rate of survival was the highest in the group where the disease was discovered at the SI stage (62.5%). In group SII, the survival rate was 33.4%, 25.0% among SIII stage patients, and 6.7% in patients with SIV/A stage disease. None of the patients whose disease was discovered in stage SIVB survived 60 months; the maximal survival was 18 months in this group.

We compared the effect of the wild-type allele (Arg194Arg, Arg399Arg) to the other variants (homo- and heterozygote forms), in regard to Kaplan-Meier survival analysis, in each stage. In the case of SI (p=0.75, 95% confidence interval (CI): 0.05-7.89) and SII stages (p=0.39, CI: 0.39-10.44), we did not find any significant association between the genotype of the *XRCC1* gene and survival; moreover the number of cases was too low for a correct statistical test. For the *XRCC1* 194 polymorphism, in the group of SIII stage, the survival rate was significantly higher in patients with the Arg194Trp genotype (Figure 2, p=0.04, CI: 1.06-6.48).

In groups SIV A (p=0.37, CI: 0.57-4.50) and SIV B (p=0.77, CI: 0.19-9.45), we did not find any statistically significant association between survival and *XRCC1* status. This was also true for the analysis of data of all SIV stage patients together (p=0.31, CI: 0.64-3.93).

In regard to *XRCC1* 399 polymorphism, we did not register any significant association in the case of SI (p=0.65, CI: 0.11-3.85) and SII stages (p=0.71, CI: 0.32-5.47). In the SIII and SIVA groups, the survival of Gln homo- and heterozygotes was worse, but the relation was not significant (SIII p=0.32, CI: 0.69-3.11; SIV A p=0.24, CI: 0.74-3.32). Between SIVB patients, the same effect was observed (p=0.82, CI: 0.32-4.22). The survival analysis of all stage SIV patients shows similar results (p=0.20, CI: 0.80-2.84).

Table I. *Results of XRCC1 194 and 399 genotype frequencies in patients and controls.*

	Cases (108)	Controls (102)	OR (95% CI)	p-value
Arg194Arg	96 (88.9%)	85 (83.3%)	1.0 (referent)	
Arg194Trp	11 (10.2%)	15 (14.7%)	0.65 (0.26-1.60)	0.30
Trp194Trp	1 (0.9%)	2 (2.0%)	0.44 (0.02-6.37)	0.49
Arg194Trp+ Trp194Trp	12 (11.1%)	17 (16.7%)	0.63 (0.26-1.48)	0.24
Arg399Arg	50 (46.3%)	53 (51.9%)	1.0 (referent)	
Arg399Gln	47 (43.5%)	41 (40.2%)	1.22 (0.66-2.24)	0.50
Gln399Gln	11 (10.2%)	8 (7.9%)	1.46 (0.49-4.38)	0.45
Arg399Gln+ Gln399Gln	58 (53.7%)	49 (48.1%)	1.25 (0.70-2.24)	0.41

OR, odds ratio; CI, confidence interval.

Discussion

Morbidity and mortality of head and neck cancer is very high in Hungary. As risk factors, alcohol and tobacco consumption is substantially higher than in the EU, however, these factors cannot fully explain the high cancer incidence and prevalence alone. In our present study, we aimed to determine the effects of molecular prognostic factors on cancer risk and survival.

DNA repair mechanisms are very important in the maintainance of the genomic integrity and protecting against development of tumours. Polymorphisms of DNA repair genes are important determinants of cancer susceptibility. Our hypothesis was that variance of the DNA repair genes may modify the risk of the development of malignancies and may have an effect on the survival of cancer patients. We investigated the association of polymorphisms of *XRCC1* with head and neck tumours. This gene has a multiplex role in BER and counteracts the effects of exogeneous carcinogens.

In previous years, the association of *XRCC1* gene polymorphsm and head and neck cancer has been investigated by other groups, but the results are still not consistent. Hsieh *et al*. found an association between the *XRCC1* Gln399Gln variant and the risk of oral squamous cell carcinomas in Taiwan (21). Sturgis *et al*. demonstrated that the Arg194Arg genotype was a significant risk factor for cancer of the oral cavity and pharynx (22). Demokan *et al*. did not find a statistical difference between cancer cases and controls in general, but observed a significant association between the Trp194Trp genotype and the risk of cancer for smokers (19).

In our study, we found that the proportion of wild-type Arg194Arg variants was higher in the cancer patients, and the proportion of wild-type Arg399Arg genotypes was higher among the healthy controls. The proportion of polymorphic variants was similar to the results of Juhasz *et al*. who also investigated the effects of *XRCC1* polymorpisms in a Hungarian population (23). We did not find any significant difference between the gene status of tumor cases and controls.

In the second part of our study, we aimed to test the effects of genotype effects on overall survival. Each patient was followed up in the oncological department, and survival data were compared considering *XRCC1* status in each oncological stage. We found a significant difference between the patients with different *XRCC1* 194 polymorph status in clinical stage SIII. The patients with the Arg194Arg genotype had a significantly lower survival than those with the Arg194Trp genotype.

Naturally, the most important factor to obtain good survival is an early diagnosis of the cancer, but low penetrance genetic factors, such as gene polymorphisms may modify the risk of malignant transformation, and influence the chances of survival. The complex analysis of these factors may provide a basis for personal risk assesement and a real opportunity for individualised therapy.

We would like to emphasise the importance of primary and secondary prevention, namely the elimination of the major risk factors such as alcohol and tobacco, and screening. Diagnosis of malignant disease at as early a stage as possible is a pivotal factor in curability.

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