

Analysis of the polymorphisms XRCC1Arg194Trp and XRCC1Arg399Gln in gliomas

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ABSTRACT. XRCC genes (X-ray cross-complementing group) were discovered mainly for their roles in protecting mammalian cells against damage caused by ionizing radiation. Studies determined that these genes are important in the genetic stability of DNA. Although the loss of some of these genes does not necessarily confer high levels of sensitivity to radiation, they have been found to represent important components of various pathways of DNA repair. To ensure the integrity of the genome, a complex system of DNA repair was developed. Base excision repair is the first defense mechanism of cells against DNA damage and a major event in preventing mutagenesis. Repair genes may play an important role in maintaining genomic stability through different pathways that are mediated by base excision. In the present study, we examined

XRCC1Arg194Trp and XRCC1Arg399Gln polymorphism using PCR-RFLP in 80 astrocytoma and glioblastoma samples. Patients who had the allele Trp of the XRCC1Arg194Trp polymorphism had an increased risk of tumor development (OR = 8.80; confidence interval at 95% (95%CI) = 4.37-17.70; $P < 0.001$), as did the allele Gln of XRCC1Arg399Gln (OR = 1.01; 95%CI = 0.53-1.93; $P = 0.971$). Comparison of overall survival of patients did not show significant differences. We suggest that XRCC1Arg194Trp and XRCC1Arg399Gln polymorphisms are involved in susceptibility for developing astrocytomas and glioblastomas.

Key words: Polymorphism; XRCC1; Astrocytoma; Glioblastoma

INTRODUCTION

Tumors of the central nervous system (CNS) represent approximately 2% of all cancers, with an estimated 4.2 to 5.4 per 100,000 individuals per year (Ohgaki and Kleihues, 2005). Although the incidence of CNS tumors is small compared with other cancers, these are among the most serious human malignancies, since they affect coordination and integration of all organic activities. Moreover, as each region of the brain has a vital function, the therapy used in other cancers (total surgical removal of the organ or tumor with a generous margin of normal tissue) cannot be applied to cure brain tumors (Louis et al., 2002; Ohgaki and Kleihues, 2005). Gliomas are the most common tumors of the CNS. Despite the remarkable progress in the characterization of the molecular pathogenesis of gliomas, these tumors remain incurable and, in most cases, refractory to treatment due to their molecular heterogeneity (Kleihues et al., 2002).

Astrocytomas account for the large majority of gliomas, making up 70% of the total, and can be divided into: pilocytic astrocytoma (grade I), including low-grade astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV) (Kleihues et al., 2002). The relevance of the graduated scheme of malignancy based on histopathology is indicated by patient survival. Patients with low-grade astrocytomas (grade II) have a median survival of about seven years, patients with anaplastic astrocytomas (grade III) have a median survival of half of that time (McCormack et al., 1992), while patients with glioblastomas have an average of 9 to 11 months (Simpson et al., 1993). Unlike grade I astrocytomas, the progression of tumors of grades II and III for most malignant tumors is well documented (Ino et al., 2001; Collins, 2004; Hartmann et al., 2004; Ichimura et al., 2004; Ohgaki, 2005). Ng and Lam (1998) suggested the division of glioblastomas into two clinically and molecularly distinct entities: primary glioblastomas or new ones, which occur in elderly patients and are clinically very aggressive, and secondary glioblastomas, which develop from pre-existing low-grade astrocytomas and have a more prolonged clinical course.

Human tumors may develop through DNA damage caused by ultraviolet rays, ionizing radiation and environmental chemicals. This causes accumulation of DNA damage leading to tumor development and various cellular dysfunctions. The DNA repair system is crucially important for cellular life (Kawabata et al., 2005). To ensure the integrity of the genome, a complex system of DNA repair was developed. Base excision repair is the first defense mechanism of cells against DNA damage and a major event in preventing mutagenesis (Hu et al., 2005). Repair genes may play an important role in maintaining genomic stability through

different pathways mediated by base excision repair (Sreeja et al., 2007).

The repair of single strand breaks induced by a variety of external and internal factors, including ionizing radiation, alkylating agents and reactive oxygen species, occurs through the process of base excision repair, which depends on the function of DNA repair protein XRCC1 (X-ray cross-complementing group), due to its ability to interact with the enzymes DNA polymerase beta, DNA ligase III, APE 1 (apurinic endonuclease 1), PNK (polynucleotide kinase), and poly-ADP-ribose polymerase [PARP - poly-(ADP-ribose) polymerase]. No enzymatic activity for protein XRCC1 has been described, but it interacts with enzymatic components of each stage of the repair of single strand breaks in DNA (Caldecott, 2003). If there is failure at some stage, this defense mechanism is not feasible in the cell (Loizidou et al., 2008).

The XRCC1 gene is located on chromosome 19 in region 19q13.2 and has 17 exons, with approximately 31.9 kb encoding a protein of 633 amino acids. About 300 polymorphisms (single nucleotide polymorphisms, SNPs) in XRCC1 have been found and are listed in the dbSNP database, of which approximately 35 variants are located in exons or the promoter region of the gene (Wang et al., 2009). Several polymorphisms described in the XRCC1 gene are involved in the formation of various types of tumors. Polymorphisms in exon 6 and exon 10 were described. The most frequent polymorphism in the population is located in exon 10, followed by the polymorphism of exon 6 (Ladiges, 2006).

Polymorphism of exon 6 Arg194Trp (rs179982) C/T is associated with reduced risk for developing tumors. The fragment length polymorphism Arg194Trp is 160 bp; restriction enzyme recognizing the restriction site is CCGG *MspI*. When the T allele is found, the enzyme will not cleave and will yield a fragment of 160 bp, but if the C allele is found, the enzyme cleaves, forming 60- and 100-bp fragments.

The Arg399Gln polymorphism (rs25787) located in exon 10 is the most studied among all polymorphisms of repair genes. In a review, Goode et al. (2002) showed the role of this polymorphism in different types of cancers. In tumors of the bladder, skin and esophagus, it seems that the polymorphism does not exert any function, but for lung, stomach and nervous system cancers, the presence of the polymorphism could be involved in tumor formation. The fragment of the Arg399Gln polymorphism is 180 bp, and the restriction enzyme recognizes the restriction site CCGG *MspI*. When the allele A is found, the enzyme does not cleave and will generate a fragment of 180 bp, but if the allele is G the enzyme will cleave, forming fragments of 55 and 125 bp.

MATERIAL AND METHODS

Study population

Eighty gliomas were analyzed, which had been surgically resected from previously untreated patients under the care of the Neurosurgery Department of Fundação Pio XII, Câncer Hospital of Barretos (Barretos, SP, Brazil). The samples, classified according to WHO criteria, included 43 astrocytomas and 37 glioblastomas. The clinical outcome, including length of survival, was obtained from patient records. For SNP studies, blood samples of 100 healthy individuals were collected for controls. Because of the highly heterogeneous ethnic composition of the Brazilian population, the individuals of the control group were selected from the general population of the São Paulo State, with no family history of cancer in first-degree relatives.

DNA extraction and primer construction

DNA extraction was performed using proteinase K and phenol-chloroform according to routine molecular biology protocols. Primers were constructed using the Gene Runner 3.05 program (Hasting Software, Inc.) from the gene sequence of the XRCC1Arg194Trp and XRCC1Arg399Gln polymorphisms, obtained in the dbSNP of NCBI (accession Nos. rs1799782 and rs25787, respectively). Table 1 shows the primers and polymerase chain reaction (PCR) product sizes.

Table 1. Polymerase chain reaction primers.

SNP	Primer	Sequence	Length (bp)	PCR product (bp)
XRCC1Arg194Trp	Arg164Trp -F	GTG AAG GAG GAG GAT GAG AGC	20	160
	Arg164Trp R	CCC CAG CCC CCT CTA CCC T	19	
XRCC1Arg399Gln	Arg399Gln -F	GCG TAA GGA GTG GGT GCT GG	20	180
	Arg399Gln R	GTC TGC TGG CTC TGG GCT G	19	

PCR = polymerase chain reaction; SNP = single nucleotide polymorphism.

PCR was carried out in a final volume of 25 μ L containing 50 ng genomic DNA template, 1X PCR buffer with 2 mM MgCl₂, 0.4 μ M of each primer (Invitrogen), 50 μ M dNTPs (Amersham Biosciences) and 0.5 U DNA polymerase (Biotools). The PCR cycling conditions were: 94°C for 5 min, followed by 35 denaturation cycles of 30 s at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C, and a final elongation cycle at 72°C for 5 min. For restriction fragment length polymorphism (RFLP), the PCR products were digested by *MspI* (4 U at 37°C for 4 h - XRCC1Arg194Trp and XRCC1Arg399Gln). *MspI* recognizes a restriction site at Arg194 allele (C▼CGG) and generates two fragments of different sizes (110 and 60 bp), while the Trp194 allele generates only one fragment of 160 bp. For XRCC1, when the polymorphism Arg399Gln occurs, the enzyme *MspI* generates two fragments of different sizes for Gln399 alleles (125 and 55 bp), and only one fragment (180 bp) for the Arg399 allele. DNA fragments were electrophoresed through a 10% acrylamide:bisacrylamide gel (19:1) and stained with silver nitrate.

PCR products were purified and submitted to bidirectional sequencing, to further confirm the reliability of the genotype analysis. The PCR products were purified with ExoSAP (USB), followed by sequencing with the DYEnamic ET Dye Terminator kit (Amersham Bioscience), according to instructions.

Statistical analysis

PCR-RFLP. The independence of alleles (Hardy-Weinberg equilibrium) was ensured using the chi-square test. The distribution of genotype and allele frequencies among patients and controls was compared using the chi-square test and the Fisher exact test. Overall survival curves were obtained using the Kaplan-Meier method and compared with a log-rank test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated using a logistic regression model. Statistical significance was set at $P < 0.05$. Statistical analyses were performed with GraphPad InStat 4.0 and GraphPad Prism 5.0 softwares (GraphPad Software, Inc.).

RESULTS

Analysis of tumors and control populations according to the XRCC1 codon 194 and codon 399 SNPs

Eighty patients and 100 control subjects were included in this study. The patient sample comprised 28 females and 52 males (M/F ratio = 0.65) and the control sample consisted of 63 males and 37 females (M/F ratio = 1.7). Mean age of the patient group was 45 years (range = 1-75) and of the control group 45 years (range = 18-72). Genotype frequencies in controls and patients were in Hardy-Weinberg equilibrium. Allele and genotype frequencies of XRCC1Arg194Trp and XRCC1Arg399Gln in controls and patients are shown in Table 2. The frequencies of Arg/Arg, Arg/Trp and Trp/Trp among controls were 67, 4 and 29%, while in patients the frequencies Arg/Arg, Arg/Trp and Trp/Trp were 18.8, 38.8 and 42.5%, respectively ($P < 0.0001$).

Table 2. Allele and genotype frequencies in case and control groups.

SNP	Genotype	Case group	Control group	P
XRCC1Arg194Trp	Arg/Arg	15 (18.8)	67 (67.0)	<0.001
	Arg/Trp	31 (38.8)	4 (4.0)	
	Trp/Trp	34 (42.5)	29 (29.0)	
	Trp	0.62	0.31	
XRCC1Arg399Gln	Arg/Arg	23 (28.8)	29 (29.0)	0.003
	Arg/Gln	33 (41.3)	20 (20.0)	
	Gln/Gln	24 (30.0)	51 (51.0)	
	Gln	0.5	0.61	

Data are reported as number with percent in parentheses. SNP = single nucleotide polymorphism.

Logistic regression analysis for the investigation of polymorphism association with risk of astrocytomas and glioblastomas is presented in Table 3. Compared to Arg/Arg, the most common genotype of the polymorphism XRCC1Arg194Trp in the study population, the genotypes with the presence of allele Trp revealed an increased risk of tumor development (OR = 8.80; 95%CI = 4.37-17.70; $P < 0.001$). When the polymorphism XRCC1Arg399Gln was analyzed, we observed a small increased risk of tumor development for the presence of the allele Gln (OR = 1.01; 95%CI = 0.53-1.93; $P = 0.971$).

Table 3. Association of XRCC1Arg194Trp and XRCC1Arg399Gln single nucleotide polymorphisms with risk of cancer.

SNP	Genotype	Case/control	OR (95%CI)	P
XRCC1Arg194Trp	Arg/Arg	15/67	1	<0.001
	Arg/Trp	31/4	34.62 (10.61-112.9)	
	Trp/Trp	34/29	5.24 (2.48-11.06)	
	Arg/Trp or Trp/Trp	65/33	8.80 (4.37-17.70)	
XRCC1Arg399Gln	Arg/Arg	23/29	1	0.064
	Arg/Gln	33/20	2.08 (0.95-4.53)	
	Gln/Gln	24/51	0.59 (0.28-1.23)	
	Arg/Gln or Gln/Gln	57/71	1.01 (0.53-1.93)	

SNP = single nucleotide polymorphism. Case/control indicates the number of individuals in each category.

Comparison of overall survival of patients according to XRCC1Arg194Trp genotypes showed a significant difference ($P = 0.20$) when compared to controls, and Arg399Gln genotype did not show significant differences ($P = 0.439$). In the XRCC1Arg194Trp genotype, the median survival of patients with Arg/Arg, Arg/Trp and Trp/Trp was 64 weeks, and in XRCC1Arg399Gln genotype the median survival of patients with Arg/Arg, Arg/Gln and Gln/Gln was 63 weeks (Figures 1 and 2).

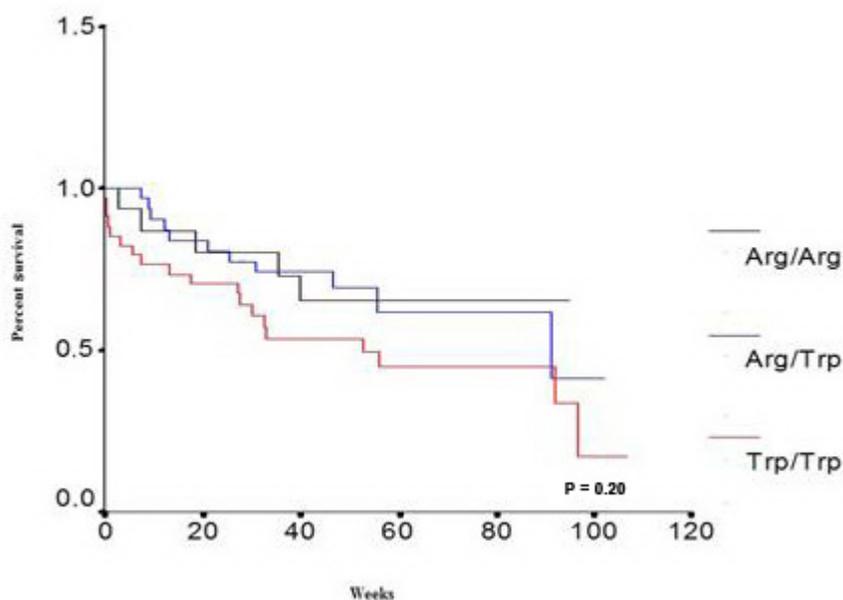


Figure 1. Overall survival in patients according to XRCC1Arg194Trp single nucleotide polymorphism.

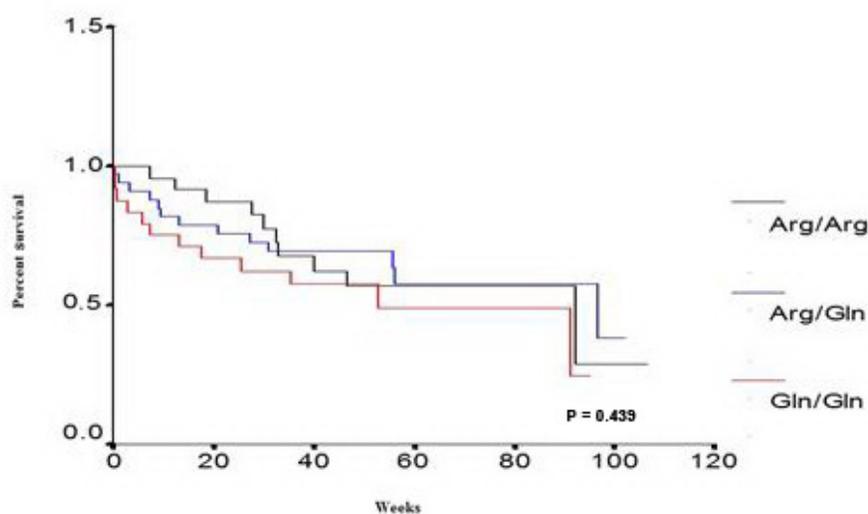


Figure 2. Overall survival in patients according to XRCC1Arg399Gln single nucleotide polymorphism.

DISCUSSION

SNPs are recognized as important tools in human genetics and medicine and have been widely used in genetic association studies of various complex diseases, such as cardiovascular, psychiatric and autoimmune diseases, obesity, osteoporosis, diabetes, and cancer (Curran et al., 2001; Miller and Kwok, 2001; Lin et al., 2003; Tamura et al., 2003; Yamada et al., 2003; Hirai et al., 2005). In humans, several reviews of SNPs have also been conducted throughout the genome with the intention of determining the patterns of the haplotypes in the populations studied (Daly et al., 2001; Jeffreys et al., 2001; Patil et al., 2001; Reich et al., 2001; Gabriel et al., 2002). Data from these tests are extremely useful for studying the genetic basis of common complex diseases (Phillips et al., 2003). Polymorphisms in DNA repair genes may be associated with differences in the efficient repair of DNA damage and may influence the risk for developing tumors. This can result in subtle changes in the structure of proteins from genes and alter their functions (Sreeja et al., 2007).

In this study, we determined the relationship between XRCC1Arg194Trp and XRCC1Arg399Gln and susceptibility to cancer and patient survival in 80 gliomas. Goode et al. (2002) showed in a review that the polymorphism Arg194Trp in exon 6 is associated with reduced risk for developing tumors.

This was confirmed by an analysis of 38 cases of controls in a Chinese population. A study conducted in Russia with 2000 cases also showed the presence of the polymorphism Arg194Trp associated with reduced risk of lung cancer. These results are surprising considering that the presence of a polymorphism, the exchange of one amino acid, could be deleterious to gene function and could consequently increase the risk of developing tumors (Ladiges, 2006).

There are some explanations considering the presence of a polymorphism not associated with the risk of cancer, one of which is that some studies may present misleading, inaccurate answers.

In the case of this variant, there is strong evidence of a large number of studies suggesting links between its presence and the formation of tumors.

A study by Moullan et al. (2003) revealed that the presence of the polymorphism Arg194Trp in exon 6 may be associated with increased occurrence of breast tumors. Tae et al. (2004) conducted a study of patients with head and neck tumors and found that the presence of this polymorphism was associated with increased risk of tumors.

A study by Wang et al. (2009) showed that the presence of this polymorphism is not related to the risk of developing bladder tumors. The data variation, showing that this polymorphism is involved with the genesis of tumors in some cases and not in others, may be related to the type of tissue considered.

Loizidou et al. (2008) conducted a study of patients with breast cancer and found that the XRCC1Arg194Trp polymorphism showed no association with disease development. Improta et al. (2008), analyzing 104 patients with colorectal and lung cancer, found no changes in the tumors related to polymorphisms XRCC1Arg194Trp and XRCC1Arg399Gln.

A review by Goode et al. (2002) revealed the role of XRCC1Arg399Gln polymorphism in different types of cancer. In tumors of the bladder, skin and esophagus, it seems that the polymorphism does not exert any function, but for lung, stomach and nervous system the presence of the polymorphism could be involved in tumor formation.

The presence of the 399Gln variant was reported to be associated with the risk of head

and neck cancer (Sturgis et al., 1999), breast cancer (Li et al., 2009), and lung cancer (Sreeja et al., 2007; Improtta et al., 2008).

Au et al. (2003) irradiated cells of individuals carrying the Arg399Gln polymorphism with ultraviolet rays and found an increase in chromosomal deletions. These chromosomal deletions resulted from a deficiency of DNA repair, facilitating genetic instability, increased mutations and risk for development of tumors.

Felini et al. (2007) observed no association between XRCC1Arg399Gln polymorphism and gliomas; this agrees with a previous hospital-based case control study. Loizidou et al. (2008) reported that the XRCC1Arg399Gln polymorphism was not associated with risk of development of breast cancer. Wang et al. (2004) did not find any statistically significant differences in the distributions of XRCC1Arg399Gln in 309 patients with gliomas.

Jelonek et al. (2010) observed an association between XRCC1Arg399Gln polymorphism and colorectal cancer: Arg/Arg homozygotes (GG) were more frequent in the control groups, and Gln/Gln homozygotes (AA) were more frequent in the cancer group, while the frequency of heterozygotes (G/A) was similar in patients and the control group. Consequently, the XRCC1399Gln (A) allele was overrepresented in the cancer group and was associated with an increased risk of sporadic colorectal cancer.

In summary, our study provides evidence that the XRCC1Arg194Trp polymorphism may contribute to the etiology of human astrocytomas and glioblastomas, since the allele Trp194 was found more frequently in patients than in controls and because its presence is associated with the genotype and the patients' survival. XRCC1Arg399Gln revealed a small increased risk of tumor development for the presence of the allele Gln and did not show significant differences in patient survival.

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