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Mutation analysis of gene *PAX6* in human gliomas

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ABSTRACT. Gliomas are the most common tumors of the central nervous system. In spite of the marked advances in the characterization of the molecular pathogenesis of gliomas, these tumors remain incurable and, in most of the cases, resistant to treatments, due to their molecular heterogeneity. Gene *PAX6*, which encodes a transcription factor that plays an important role in the development of the central nervous system, was recently recognized as a tumor suppressor in gliomas. The objective of the present study was to analyze the mutational status of the coding and regulating regions of *PAX6* in 94 gliomas: 81 astrocytomas (11 grade I, 23 grade II, 8 grade III, and 39 grade IV glioblastomas), 5 oligodendrogliomas (3 grade II, and 2 grade III), and 8 ependymomas (5 grade II, and 3 grade III). Two regulating regions (SX250 and EIE) and the 11 coding regions (exons 4-13, plus exon 5a resulting from alternative splicing) of gene *PAX6* were analyzed and no mutation was found. Therefore, we conclude that the tumor suppressor role of *PAX6*, reported in previous studies

on gliomas, is not due to mutation in its coding and regulating regions, suggesting the involvement of epigenetic mechanisms in the silencing of *PAX6* in these tumors.

Key words: Mutation, *PAX6*, Gliomas

INTRODUCTION

Gliomas are the most common tumors of the central nervous system. In spite of the marked advances in the characterization of the molecular pathogenesis of gliomas, these tumors remain incurable and, in most of the cases, resistant to treatments, due to their molecular heterogeneity (Boudreau et al., 2005). Four degrees of malignancy are recognized by the classification of the World Health Organization (WHO), where grade I tumors are biologically the least aggressive and grade IV the most aggressive. The classification is made according to the origin of the cell assumed to be responsible for the tumor, based on the characteristics displayed by astrocytes, oligodendrocytes or normal ependyma cells, or even by their respective progenitors (Kleihues et al., 2002).

PAX (paired box) genes comprise a small but developmentally crucial gene family that encodes a set of transcription factors, characterized by the presence, in the N-terminal portion, of a paired box DNA-binding domain of 128 amino acids and, in the C-terminal portion, of a transactivation domain (Treisman et al., 1991; Ward et al., 1994). Gene *PAX6*, situated at 11p13, encodes a protein with 422 amino acids that is involved in several developmental processes of the eyes and the central nervous system, and has 14 exons, 11 of which are coding exons (exons 4-13 and an extra exon, 5a, resulting from alternative splicing, which adds 14 amino acids to the paired box domain) (Simpson and Price, 2002).

Several authors have described heterozygous germ cell mutations in gene *PAX6* and their associations with a defect in neural development called aniridia, characterized by hypoplasia of the iris and of the optic nerve, besides foveal dysplasia, cataract, glaucoma, and nystagmus (uncontrolled movement of the eyes) (Glaser et al., 1992; Jordan et al., 1992; Martha et al., 1994, 1995; Azuma et al., 1996; Saunders and Chao, 1998; Wolf et al., 1998; Chao et al., 2000; Song et al., 2005; Neethirajan et al., 2004, 2006; Wang et al., 2006).

There are also studies reporting a close connection of gene *PAX6* with the tumor process. Ballestar et al. (2003) for example, observed the silencing of gene *PAX6* in cell lines and primary breast tumors, and proposed a tumor suppressor role for this gene. Similar conclusions were reached in studies on colorectal, gastric and glial tumors (Toyota et al., 1999; Zhou et al., 2005; Mayes et al., 2006; Yamashita et al., 2006).

High expression levels of *PAX6* have been correlated with a better prognosis in gliomas, and low levels with a poor prognosis (Zhou et al., 2003). Moreover, reduced *PAX6* expression was reported to be a molecular trait that gives glioma cells a true selection advantage over other cell types in surviving stressful conditions, thus resulting in expansion of their population (Chang et al., 2007). These expression data suggest the importance of regulating regions of *PAX6* in the tumor process. Xu and Saunders (1997) analyzed the promoter region of gene *PAX6* and revealed the presence of multiple repressors and activators within this region. One of the repressor elements identified is a powerful silencer (SX250), located between -1518 and -1268, that is able to repress the promoter activity of *PAX6* in cervical carcinoma

and glioblastoma cell lines (Xu and Saunders, 1997). Similarly, Zheng et al. (2001) identified a 57-bp *cis*-regulatory element named EIE (exon 1 enhancer) in the first exon of *PAX6*, which plays an important role in the expression of this gene in glioblastoma cell lines.

The data presented show that gene *PAX6* is involved in the pathogenesis of gliomas; however, there are no studies on these tumors in the literature, as there are for aniridia, demonstrating its structural integrity. Thus, the objective of the present study was to evaluate the mutational status of the regulating and coding regions of gene *PAX6* in a series of gliomas.

MATERIAL AND METHODS

Tumor specimens and DNA extraction

A total of 94 gliomas were analyzed, surgically removed from previously untreated patients under care in the Neurosurgery Department of Fundação Pio XII - Cancer Hospital of Barretos (Barretos, SP, Brazil). The samples were classified according to the WHO criteria (Kleihues et al., 2002) by two experienced pathologists. For DNA extraction, approximately 1 cm^3 of freshly microdissected samples was used. DNA extraction was performed using proteinase K and phenol-chloroform according to routine molecular biology protocols. The collection and use of tissues for this study were previously approved by the appropriate Institutional Ethics Committee.

Polymerase chain reaction amplification

The primers were constructed, using the Gene Runner program (version 3.05, Hasting Software, Inc.), from the genome sequence of the coding and regulating regions of gene *PAX6*, obtained in the databases Ensembl (Accession number: Z83307) and Genbank (Accession number: U63833), respectively. The coordinates of regions SX250 and EIE were obtained from previous studies (Xu and Saunders, 1997; Zheng et al., 2001). The primers chosen for amplification of gene *PAX6* are shown in Table 1.

Table 1. Polymerase chain reaction primers for the amplification of *PAX*6.

Regulatory region/exon	Forward primer (5' → 3')	Reverse primer (5' → 3')	Annealing temperature (°C)	Product size (bp)
SX250	CAGGTGTCTGCCAAGACTTTCC	GATCCCTGGCATTCCCCTGT	62	238
EIE	AGGAGGAAGTGTTTTGCTGG	CCTATGCTGATTGGTGATGG	55	134
4	AGCTGCCCGAGGATTAACTC	CGAAGTCCCAGAAAGACCAG	55	129
5	TCCTCTTCACTCTGCTCTCTTC	ATGAAGAGAGGGCGTTGAGAG	55	258
5a	CAGTAAGTTCTCATACCATTGAAGG	GGGGAAGTGGACAGAAAACCA	58	172
6	TCTGTCCACTTCCCCTATGC	AGGAGAGAGCATTGGGCTTA	55	292
7	GATGGGTGACTGTGTCTTCAGG	TATGGAGAGCTGCGTGGATG	59	263
8	CCAAATTTCTCTTACCATCCTA	GAAGATGTGGCATTTACTTTGA	55	252
9	GGGAACCAGTTTGATGCACA	TGAATCACAAAGTGTGAAACTGC	58	202
10	GCTCGACGTAGACACAGTGCT	GCAAACAGGTTTAAAGACATTG	56	244
11	CGGGCTCTGACTCTCACTCT	GCCACTCCTCACTTCTCTGG	55	220
12	GTGGCTGTGTGATGTGTTCCT	GAGAGATCGCCTCTGTGCAG	56	246
13	CATGTCTGTTTCTCAAAGGGA	TTGTGTCCCCATAGTCACTGA	54	210

Polymerase chain reaction (PCR) was carried out in a final volume of 25 µL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools, Madrid, Spain) with 2 mM $MgCl_2$, 0.4 µM of each primer (Invitrogen, USA), 50 µM dNTPs (Amersham Biosciences), and 0.5 U DNA polymerase (Biotools, Madrid, Spain). Thirty-five cycles were performed in programmable Mastercycler thermocyclers (Eppendorf, Inc.). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 5 min, followed by 35 denaturation cycles of 30 s at 94°C, 30 s of annealing at 55°C (or as specified for primer sets in Table 1), and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min. The amplified PCR products showing a single band of the correct size were stored at -20°C until analysis by electrophoresis.

Screening for mutations

The two regulating (SX250 and EIE) and the 11 coding (exons 4-13, + exon 5a) regions of *PAX6* were screened for mutations by single-strand conformation polymorphism (SSCP), as previously described (Hayashi, 1991). Briefly, approximately 5 µL of the amplified DNA was mixed with 3 µL of denaturing dye (95% formamide, 10 mM NaOH) and run on a 8-12% polyacrylamide gel, at room temperature or at 4°C, with or without 5% glycerol, for 8-14 h, depending on the amplified fragment. The gels were then stained with silver nitrate.

In order to prevent false-positive results, all samples were run in duplicate, and the gels analyzed by four researchers. Whenever at least one of the researchers had a divergent opinion regarding the migration pattern of a sample, also in at least one of the gels in duplicate, this sample was purified and submitted to bidirectional sequencing. The PCR products were purified with ExoSAP (USB, Cleveland, OH, USA), followed by sequencing with DYEnamic ET Dye Terminator Kit (Amersham Biosciences), according to the manufacturer's specifications. Sequencing reactions were performed on MegaBACE 1000 (GE Healthcare).

RESULTS

The patients included in the study were 59 males and 35 females (M/F ratio = 1.69), with ages varying from 1 to 75 years (mean: 45 years). Histology results, stage and gender distribution of the sample are shown in Table 2.

Table 2. Clinical samples: tumor histology, stage and gender distribution.

Histology	Patients	Gender		Tumor stage[1]			
		Male	Female	I	II	III	IV
Astrocytoma	81	51	30	11	23	8	39
Oligodendroglioma	5	3	2	–	3	2	–
Ependymoma	8	5	3	–	5	3	–
Total	94	59	35	11	31	13	39

[1]WHO staging.

Once the PCR-SSCP analyses were completed, no abnormal migration pattern was found on the polyacrylamide gels that could be confirmed by sequencing. Of all the 94 glioma DNA samples screened for the 13 segments of *PAX6* analyzed (1222 amplicons screened), only two PCR products were submitted to sequencing, in which no DNA sequence alteration was confirmed.

DISCUSSION

In 1998, Brown et al. built a database (http://pax6.hgu.mrc.ac.uk/) containing details of the 94 mutations described at the time for gene *PAX6*. Currently, the same database contains 309 registers, of which 218 are unique mutations. These mutations are associated with ocular pathologies, among which the most outstanding is aniridia. The most frequently mutated sequences are those of exons 6 and 5 (19.2 and 15%, respectively), and most of the described mutations (42.3%) are located within the region that encodes the paired box domain.

Approximately 92% of the mutations in gene *PAX6* found in patients with aniridia result in a truncated protein, as with missense mutations and splicing site mutations. In addition to these, substitutions have been reported that alter the protein conformation, besides insertions and deletions which originate changes in the reading frame (Brown et al., 1998). There is no doubt that the mutation mechanisms described in gene *PAX6* of patients with ocular disorders are the same as those occurring in genes which are crucial for cell homeostasis and are responsible for the genesis and progression of several types of tumors.

Recently, a tumor suppressor activity has been proposed for gene *PAX6* in studies on gliomas (Zhou et al., 2003, 2005; Mayes et al., 2006; Chang et al., 2007).

In view of the fact that there is a very high frequency of described mutations which alter protein conformation in patients with ocular disorders (Hanson et al., 1999), and that regulating regions have been associated with differential expression in glioma cell lines (Xu and Saunders, 1997; Zheng et al., 2001), it seems reasonable to imagine a possible contribution of the mutation mechanism in regulating and coding regions of *PAX6* in the pathogenesis of these tumors. However, we did not observe any alteration in the 94 gliomas analyzed. For this reason, and because our samples consisted of tumors with different degrees of malignancy, we conclude that the tumor suppressor activity of gene *PAX6* via the mutation mechanism is not related to either the genesis or the progression of gliomas. It should be pointed out here that this is the first study to screen mutations in gene *PAX6* in primary tumors, and that further studies of this kind, with a larger number of samples of the different glioma subtypes, are necessary to corroborate our observations.

Liang et al. (1998) reported methylation of the CpG islands outside the promoter region of gene *PAX6* in cell lines from human tumors. It was later on suggested that *PAX6* silencing does not arise as a result of this methylation outside the promoter region (Salem et al., 2000). Nevertheless, Nguyen et al. (2001) postulated that these CpG islands are more easily methylated, and that methylation can further on spread over the others, including those of the promoter regions. In addition, methylation of the CpG dinucleotides contributes to the great number of C>T transcriptions observed along gene *PAX6* (a result of the deamination of methylated cytosine) (Hanson et al., 1999; Tzoulaki et al., 2005).

In summary, somatic mutations in gene *PAX6* have not been described in tumors so far. However, transcriptional inactivation of *PAX6* as a result of epigenetic events is an alternative and emerging mechanism for the inactivation of its tumor suppression capacity, as has been observed in breast, colorectal and gastric tumors (Toyota et al., 1999; Ballestar et al., 2003; Yamashita et al., 2006). A scenario similar to this one described for gene *PAX6* has been described for other tumor suppressor DNA repair genes, such as *MLH1* and *BRCA1*, which are very rarely mutated in sporadic tumors, but undergo epigenetic inactivation by

hypermethylation of their regulating regions (Herman et al., 1998; Jones and Laird, 1999; Esteller, 2000; Herman and Baylin, 2003).

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