



Analysis of the Methylation Pattern of *SOX2* and *OCT4* Genes in Astrocytomas

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Abstract

Astrocytoma is a common aggressive intracranial tumor and a formidable challenge in clinic. Association of the altered DNA methylation pattern of the promoter CpG islands has been found in many human tumors. *OCT4* and *SOX2* are essential transcription factors for embryonic development and play key roles in determining the fate of stem cells. In this study, we aimed to investigate the methylation profiles of *SOX2* and *OCT4* genes in astrocytomas samples of Pará state. The methylation status of *SOX2* and *OCT4* genes was examined by methylation-specific polymerase chain reaction (MS-PCR) in 31 samples. At least in the investigated CpG island of *SOX2* and *OCT4* genes, we found that both promoters are methylated. Understanding these epigenetic mechanisms can lead to better prognostic tools and new drug targets for tumors of the central nervous system.

Keywords

Gliomas, MSP-PCR, Tumors of central nervous system

Introduction

Astrocytomas are malignant and prevalent intracranial tumours that comprise the majority of primary central nervous system tumors in adults, account for nearly 75% of neuroepithelial tumors [1]. They are classified according to the WHO malignancy scale, into low-grade astrocytoma (WHO Grade I and II, AI and AII), anaplastic astrocytoma (WHO Grade III, AIII), and glioblastomamultiforme (WHO Grade IV, GBM).

Epigenetic markers, as DNA promoter methylation, can regulate the gene expression without altering the gene coding sequence [2]. One of the features of carcinogenesis is the specific hypermethylation of CpG islands within the promoter of some genes, which commonly results in the silencing of these genes leading to cell growth, proliferation and ultimately to the formation of invasive tumor and metastasis [3,4].

Table 1: Clinical characteristics of patient/tumor samples used for MSP-PCR

Patient	Tumor type	Sex	Age	OMS Grade
1	Subependymal giant cell astrocytoma	M	23	I
2	Pilocytic Astrocytoma	M	13	I
3	Pilocytic astrocytoma	F	16	I
4	Pilocytic astrocytoma	F	3	I
5	Pilocytic astrocytoma	F	27	I
6	Fibrillary astrocytoma	F	12	II
7	Fibrillary astrocytoma	F	52	II
8	Fibrillary astrocytoma	M	26	II
9	Fibrillary astrocytoma	F	34	II
10	Fibrillary astrocytoma	M	64	II
11	Anaplastic Astrocytoma	F	55	III
12	Anaplastic Astrocytoma	M	60	III
13	Anaplastic Astrocytoma	F	31	III
14	GlioblastomaMultiforme	F	68	IV
15	GlioblastomaMultiforme	F	64	IV
16	GlioblastomaMultiforme	M	65	IV
17	GlioblastomaMultiforme	F	7	IV
18	GlioblastomaMultiforme	M	43	IV
19	GlioblastomaMultiforme	F	71	IV
20	GlioblastomaMultiforme	F	51	IV
21	GlioblastomaMultiforme	M	60	IV
22	GlioblastomaMultiforme	M	43	IV
23	GlioblastomaMultiforme	M	78	IV
24	GlioblastomaMultiforme	M	38	IV
25	GlioblastomaMultiforme	M	59	IV
26	GlioblastomaMultiforme	M	42	IV
27	GlioblastomaMultiforme	F	76	IV
28	GlioblastomaMultiforme	F	84	IV
29	GlioblastomaMultiforme	F	72	IV
30	GlioblastomaMultiforme	F	29	IV
31	GlioblastomaMultiforme	F	81	IV

To date, a number of genetics and epigenetics alterations have been correlated with astrocytic tumorigenesis [5-7], however a deep understanding of the molecular basis of this tumour is still far away, and the search for novel prognostic or predictive molecular

indicators are still the primary goal for the improvement of its clinical management [8].

OCT4/POU5F1 (octamer DNA binding transcription factor 4) is an important member of the POU (Pit, Oct, Uncl) domain transcription factors encoded by *POU5F1* gene (6p21.31), with, at least, three variants (A, B, and B1) produced by alternative splicing [9]. *OCT4* performs an important role maintaining the cellular plasticity and promoting the self-renewal and the proliferation of pluripotent embryonic stem and germ cells in collaboration with other proteins, such as *SOX2* (SRX-box 2), *NANOG* (Nanoghomeobox), and *KLF4* (Kruppel-like factor 4) [Burdon, Niwa]. To date, many reports found that *OCT4* is highly expressed in several tumors [10,11] and its expression profile has been correlated with tumor grade and disease progression and is associated with a worse prognosis [12-15]. Therefore, the high expression of *OCT4* is considered as a hallmark of cancer stem cells [16,17].

SOX2 is a transcription factor belonging to the sex determining region Y-box family [18], which is expressed in a wide variety of tissues and play important roles in the regulation of organ development, cell type specificity [19], and in the pluripotency maintenance of cancer stem cells (CSCs) in self-renewal and differentiation [20]. Increased expression of *SOX2* has been reported in a growing list of tumors, including lung cancer, esophageal carcinoma, pancreatic carcinoma, breast cancer, ovarian carcinoma, hepatocellular carcinoma and head and neck cancers [21-26]. In particular, the *SOX2* expression is important for the maintenance and development of the central nervous system tumors [27,28], and some studies present evidences that *SOX2* expression is positively correlated with the malignancy grade in brain tumors [29-31]. Recently, Jesse et al. [27] suggested that an increasing expression of *SOX2* during brain tumor progression are likely to be closely linked with changes in other critical genes that work in concert with *SOX2* to enhance the tumorigenicity of brain tumors.

Although in recent years a considerable number of studies have been carried out on the *OCT4* and *SOX2* expression and methylation

in various tumors and proposed as useful markers of these tumors [32], little is known about their methylation pattern in astrocytomas. In this study, we aimed to identify the *SOX2* and *OCT4* gene promoter methylation signatures in astrocytomas in a population in the northern Brazil (Belém, Pará state) to verify the possible association between the methylation status of these genes with clinicopathological features.

Material and Methods

This study involved 31 tissue samples from astrocytomas (Table 1), obtained by surgical resections from patients who underwent craniotomy at Ofir Loyola Hospital, from 2005 to 2009, in Belém (Pará state). All samples were classified according to the WHO (World Health Organization) classification criteria [33]. All procedures were approved by the Ethics Committee of the involved hospital. All tissue specimens after dissection were snap-frozen and stored with RNeasy Lysis Solution (Qiagen) at -80°C until analysis. Genomic DNA was extracted from tissues using the phenol-chloroform protocol as described by Sambrook and Russell [34].

Bisulfite treatment of DNA samples was performed as previously described by Herman et al. [35]. The methylation and unmethylation-sensitive primers used in this study were previously described [36,37] (Table 2). 1µl of bisulfite-converted DNA was amplified in a 25µl reaction mixture containing 1.25mM dNTPs, 2.5µl of 1x reaction buffer, 2.5mM MgCl₂, 0.5 mM forward and reverse of both genes primers, and 0.03U/µL of Taq DNA polymerase (Invitrogen). Universal methylated human male genomic DNA (Intergen, New York, NY, USA) was used as the positive control.

The MS-PCR profile for both genes was conducted as following steps: pre-denatured for 4 min at 94°C, then at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds for 40 cycles, and finally a 10-min extension at 72°C. Polymerase chain reaction products were separated on 3% Tris-borate EDTA agarose gels, stained with ethidium bromide and visualized under a UV transilluminator. Cases detected with the presence of methylated alleles were repeated once for confirmation.

Table 2: Sequences of primers used in *SOX2* and *OCT4* methylation-specific PCR

Primer	Primer sequence (5' to 3')	Product size (bp)	References
SOX2 promoter MSP-Methylated			
Forward	TGTTTATTTATTTTTTCGAAAAGGCG	206	[36]
Reverse	GAACCCAACCTCGCTACCGAA		
SOX2 promoter MSP-Unmethylated			
Forward	TGTTTATTTATTTTTTTGAAAAGGTG	208	[36]
Reverse	CTCAAACCCAACCTCACTACCAA		
OCT4 promoter MSP-Methylated			
Forward	CGGGATATTTGGTTTCGGATTTC	209	[37]
Reverse	CCCACAAAACCTCATACGACGA		
OCT4 promoter MSP-Unmethylated			
Forward	TGGGATATTTGGTTTTGGATTTT	210	[37]
Reverse	CCCACAAAACCTCATACAACAAA		

Table 3: Associations between demographic and clinical data of patients and methylation of *SOX2* and *OCT4* genes

	SOX2 Gene			OCT4 Gene		
	Methylated group	Unmethylated group	P value	Methylated group	Unmethylated group	P value
Gender						
Male	10	3	0.4171	13	0	0.5806
Female	12	6		17	1	
Age						
< 60	14	5	0.4894	18	1	0.6129
≥ 60	8	4		12	0	
OMS grades						
Low-grades (I and II)	8	3	0.6058	10	1	0.3548
High-grades (III and IV)	14	6		20	0	

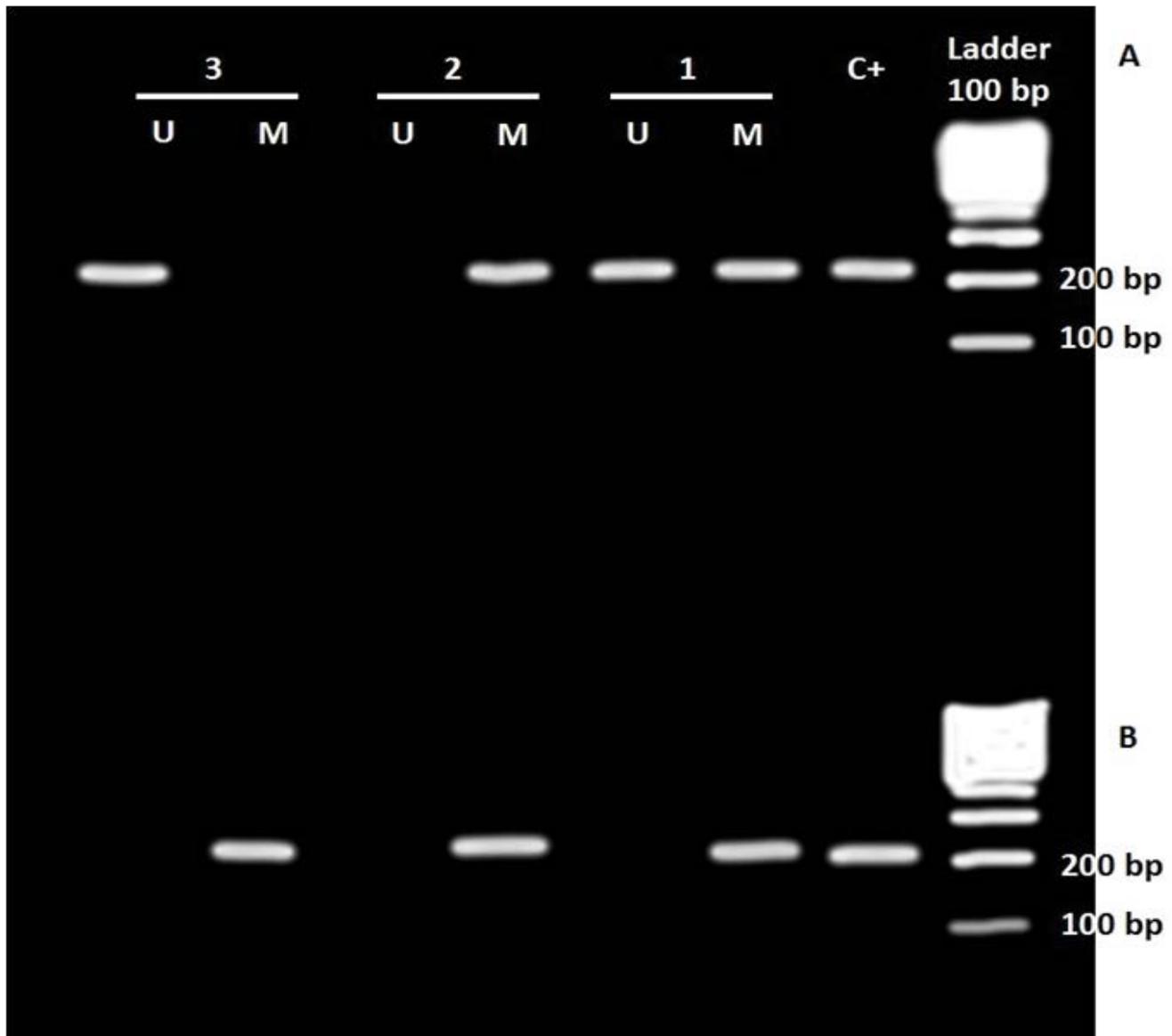


Figure 1: MSP analysis of the promoter CpG islands of *SOX2* (A) and *OCT4* (B) genes in astrocytomas. C+: positive control, U: unmethylated, M: Methylated. Numbers above the figure represent patients 1=patient 3; 2=patient 9; 3=patient 23 (See Table 1)

For statistical analysis, we grouped the samples in data groups based on the histopathological classification of WHO, which were low-grades (I and II OMS grades) and high-grades (III and IV grades). Data were analyzed using Fisher's exact test, with $p \leq 0.05$ being considered as statistically significant and performed with BioEstat 5.0 [38].

Results and Discussion

Of the 31 analyzed samples of astrocytomas patients, 13 were males and 18 females. The median age was 40.36 years (ranging from 3 to 71 years). Table 1 presents a summary of sex, age, tumor stage and histological grade.

For the *SOX2* gene, our results show this gene is methylated in 70.96% of tumor tissues (22/31 cases) (Figure 1). There was no statistically significant difference in the frequencies of hypermethylated *SOX2* gene promoter samples with clinicopathologic variables, age and sex (Table 3).

For the *OCT4* gene, we detected that this gene was hypermethylated in 96.77% of tumor tissues (30 of 31 cases) (Figure 1). Similarly to the *SOX2* gene, there was no statistically significant difference in the frequencies of methylated *OCT4* gene promoter with clinicopathologic variables (Table 3).

Astrocytic tumors are the most common type of intrinsic brain tumors. They show a tendency for progression toward a more

malignant phenotype [39], and the average survival of patients with aggressive forms of gliomas is less than 2 years [40]. Therefore, anadequate diagnosis and treatment of these brain tumors presents the major challenge in neuro-oncology today.

Promoter CpG methylation has an important role in controlling gene transcription and therefore contributes to the regulation of many biological processes. In cancer, aberrant DNA methylation is associated with initiation and progression of malignant disease. Therefore, the DNA methylation patterns could be used to improve cancer diagnosis and/or prognosis [41]. However, in spite of clinical research progress, there are few epigenetic biomarkers for astrocytoma diagnosis [42].

OCT4 (also known as Oct-3 and POU5F1), is a transcription factor involved in regulation of cell growth and differentiation [43,44]. *OCT4*, as well as *SOX2* and Nanog, plays a pivotal role in the regulation and maintenance of pluripotency. In recent studies, *OCT4* expression has been detected in various carcinomas including breast, prostate, bladder, head and neck squamous cell carcinomas and lung adenocarcinoma, which correlates with an unfavorable prognosis [15,45-47]. Furthermore, considerable studies indicate the DNA methylation of the *OCT4* at the gene regulatory region is a key factor in *OCT4* transcription [48].

Here, our results suggest, at least in the investigated CpG sites of *OCT4* gene promoter, a persistent hypermethylation event in all astrocytomas analyzed. It is well-established that methylation of CpG

dinucleotides is a common mechanism for the silencing of *OCT4* expression within its promoter, the proximal enhancer and distal enhancer regions [49-51]. Lee et al. [51] showed that *OCT4* gene is progressively methylated during the *in vivo* maturation of neural stem cells in the neuroepithelium of the central nervous system, coincident with the downregulation of its expression. It has also been shown previously that *OCT4* expression can be induced by treatment of adult neural stem cells with the DNA methyltransferase inhibitor, 5-azacytidine and histone deacetylase inhibitor [52].

OCT4 promoter demethylation has already been reported to contribute to tumorigenesis [37,53]. In primary gliomas, the methylation levels of the *OCT4* gene is notably reduced as compared to the normal group and is lower in high-grade gliomas than in low-grade ones [54]. On the other hand, the difference between our results and those presented by Shi et al. [54] can be associated with different techniques employed, as well it is also possible that *OCT4* was upregulated by hypomethylation of other CpG islands in the promoter regions of *OCT4* that were not tested in this study.

Another gene evaluated was *SOX2*, a self-renewal transcription factor crucial to pluripotency maintenance in embryonic stem cells (ESCs) [55,56] expressed during various phases of embryonic development, which affects cell fate and differentiation. Increased expression of the *SOX2* has been reported in several tumors and both epigenetic and genetic factors, particularly gene amplification, have been identified as frequent causes of *SOX2* overexpression [57,58]. Schoenhals et al. [59] compared the expression of *OCT4*, *SOX2*, *KLF4* and *C-MYC* in 40 human tumor types and their normal tissue counterparts using publicly available gene expression data, and found a significant overexpression of at least one of the pluripotency factors in 18 out of the 40 cancer types investigated. According to this study, *SOX2* was significantly overexpressed only in grade IV compared to grade II and III of gliomas. This pattern was corroborated by Alonso et al. [58], which evaluated the expression and methylation status of *SOX2* in glioblastomamultiforme (GBM) and found that *SOX2* promoter was hypomethylated in all the patient samples when compared to normal cell lines, correlating this data with high *SOX2* protein levels and mRNA overexpression in 90% of the samples, suggesting that this gene could be used as a therapeutic target in GBM.

Nevertheless, our results suggest that *SOX2* is hypermethylated in 86.36% of the samples, corroborating with other studies in several tumors. Wong et al. [60], who used the MSP-PCR technique to study the methylation profiles of *SOX2* in endometrial carcinomas, observed that this gene was methylated in 37.5% (27/72) of the samples, and with a significant correlation between its mRNA expression, hypermethylation, and shorter survival of patients. *SOX2* hypermethylation and downregulation has been reported in gastric cancers in association with effect on cell growth and patients' survival [61]. Moreover, the hypermethylation in the promoter region of *SOX2* was demonstrated in hydatidiform moles and choriocarcinomas when compared with normal placentas in association with reduced RNA expression [36].

In conclusion, while no statistically significant changes between promoter methylation of both genes with clinicopathological features were found using methylation-specific PCR, we found that both genes are hypermethylated in samples of astrocytomas of the patients of Belém, of Pará state - Brazil. It is clear, however, that more robust techniques such as pyrosequencing or promoter methylation array must be employed to be able to detect marginal but possibly meaningful differences in methylation.

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