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Epigenetic Analysis of Long-term Epilepsy-Associated Tumors by Methylation-Specific Multiplex Ligation-dependent Probe Amplification

Análise Epigenética de Tumores Associados a Epilepsia de Longa Duração por Methylation-Specific Multiplex Ligation-dependent Probe Amplification

Joana Jesus-Ribeiro [1,2,*], Ilda Patrícia Ribeiro [2,3], Luis Miguel Pires [2,3], Olinda Rebelo [4], Ricardo Pereira [5], Conceição Bento [6], Francisco Sales [6], Isabel Santana [7,8], António Freire [8,9], Joana Barbosa Melo [2,3]

1-Neurology Department, Leiria Hospital Center, Leiria, Portugal
2-Coimbra Institute for Clinical and Biomedical Research (iCBR) and Center of Investigation on Environment, Genetics, and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, Coimbra, Portugal
3-Laboratory of Cytogenetics and Genomics, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
4-Neuropathology Laboratory, Neurology Department, Coimbra University Hospital Center, Coimbra, Portugal
5-Neurosurgery Department, Coimbra University Hospital Center, Coimbra, Portugal
6-Epilepsy and Sleep Monitoring Unit, Neurology Department, Coimbra University Hospital Center, Coimbra, Portugal
7-Neurology Department, Coimbra University Hospital Center, Coimbra, Portugal
8-Faculty of Medicine, University of Coimbra, Coimbra, Portugal
9-Neurology Department, Luz Hospital, Coimbra, Portugal

*Autor Correspondente / Corresponding Author:
Joana de Jesus Ribeiro
R. de Santo André,
2410-197 Leiria, Portugal
joanajribeiro@gmail.com

Abstract

Introduction: Ganglioglioma and dysembryoplastic neuroepithelial tumor represent the most common neoplasms in the group of long-term epilepsy-associated tumors. Mapping of epigenetic alterations, particularly DNA methylation, has recently been shown to offer promising perspectives in brain tumors, identifying key genes that may serve as potential diagnostic biomarkers. We aim to perform a genetic and epigenetic analysis using long-term epilepsy-associated tumors' tissue, to contribute to the identification of such biomarkers.

Methods: DNA copy number alterations and methylation status in genes relevant to tumorigeneses were analyzed by methylation-specific multiplex ligation-dependent probe amplification using fresh frozen postoperative tissue obtained from epilepsy surgery.

Results: From the six tumors included in the study (three gangliogliomas and three dysembryoplastic neuroepithelial tumors), one ganglioglioma harboring a *BRAF*:p.V600E mutation presented changes in methylation status. This particularly patient had a focal epilepsy with video-electroencephalogram (EEG) reveling an ictal pattern in the right occipito-parietal region. Brain magnetic resonance imaging revealed a right mesial temporal lesion. His seizure frequency increased despite antiepileptic treatment and two years later he underwent his first surgery. Two more surgeries were performed years later due to seizure recurrence associated to an increase of the residual tumor. Postsurgical Engel class is IIA at three years of follow-up. Copy number losses were detected in chromosomes 1p (TP73), 2p (MSH6), 3p (VHL), 10p (CREM), 11q (GSTP1), 12q (CHFR), 14q (MLH3), 16p (PYCARD), 17p (TP53), 17q (BRCA1) and 19p (STK11). Copy number gains were detected in chromosome 11p (CD44). The *MGMT* (58%) and *CD44* (51%) genes were methylated.

Conclusion: A high number of chromosomal aberrations were identified in one ganglioglioma, among which deletions dominated, reinforcing the spectrum of chromosomal abnormalities previously described. We observed copy number gain and methylation in CD44, which contributes to cell-cell/cell-matrix interactions. The methylation of MGMT, involved in DNA repair, is concordant to other studies. Our data highlight the importance of unravel new chromosomal imbalances and the role of DNA methylation in theses tumors, which may provide more arguments in favor of an integrative histological and (epi)genetic classification.

Resumo

Introdução: O ganglioglioma e o tumor neuroepitelial disembrioplásico representam as neoplasias mais comuns no grupo de tumores associados a epilepsia de longa duração. O mapeamento de alterações epigenéticas, particularmente a metilação do DNA, demonstrou oferecer perspetivas promissoras nos tumores cerebrais, identificando genes-chave que podem representar potenciais biomarcadores diagnósticos. O nosso objetivo é realizar uma análise genética e epigenética usando tecido de tumores associados a epilepsia de longa duração, para contribuir na identificação de tais biomarcadores.

Métodos: Alterações no número de cópias de DNA e padrão de metilação em genes relevantes para tumorigénese foram analisados por *methylation-specific multiplex ligation-dependent probe amplification*, usando tecido pós-operatório fresco congelado obtido na cirurgia de epilepsia.

Resultados: Dos seis tumores incluídos no estudo (três gangliogliomas e três tumores neuroepiteliais disembrioplásicos), um ganglioglioma com mutação *BRAF*:p. V600E apresentou alterações na metilação. Este doente em particular tinha uma epilepsia focal, com o vídeo-eletroencefalograma (EEG) a revelar um padrão ictal na região occipito-parietal direita. A ressonância magnética cerebral revelou uma lesão temporal mesial direita. A frequência das crises aumentou apesar do tratamento antiepilético e dois anos depois foi submetido à sua primeira cirurgia. Mais duas cirurgias foram realizadas anos depois devido à recorrência de crises associada ao aumento do tumor residual. A classe de Engel pós-cirurgia é IIA aos três anos de seguimento. Perdas no número de cópias foram detetadas nos cromossomas 1p (TP73), 2p (MSH6), 3p (VHL), 10p (CREM), 11q (GSTP1), 12q (CHFR), 14q (MLH3), 16p (PYCARD), 17p (TP53), 17q (BRCA1) and 19p (STK11). Ganhos no número de cópias foram detetados no cromossoma 11p (CD44). Os genes *MGMT* (58%) e *CD44* (51%) encontravam-se metilados.

Conclusão: Identificou-se um elevado número de alterações cromossómicas num ganglioglioma, com predomínio de deleções, reforçando o espectro de alterações cromossómicas previamente descrito. Observámos um ganho no número de cópias e metilação do CD44, que contribui para interações célula-célula/célula-matriz. A metilação do MGMT, envolvida na reparação do DNA, está de acordo com outros estudos. Os nossos dados destacam a importância de desvendar novos desequilíbrios cromossómicos e o papel da metilação do DNA nesses tumores, fornecendo mais argumentos a favor de uma classificação histológica e (epi)genética integrada.

Introduction

A brain tumor compromising the neocortex or neuronal circuits can cause a seizure and progress into chronic epilepsy. Long-term epilepsy-associated tumors (LEATs) refer to a heterogenous spectrum of generally low-grade brain tumors recognized in patients with long-term medically refractory epilepsy.[1,2] Ganglioglioma (GG), with its biphasic composition of neuronal and glial cell elements, represented the most frequent brain tumor in a European multicentric study analyzing epi-

lepsy surgery brain specimens, followed by dysembryoplastic neuroepithelial tumor (DNT).[3] However, the differential diagnosis and histopathological classification between LEATs entities is sometimes a challenge, due to variable microscopic features and multiple architectural growth patterns.[1,4] Although most cases have indolent clinical behavior, a subgroup of these tumors does recur, and others are unresectable.[1,5] Malignant tumor progression is exceptional and generally restricted to the glial component.[1,5] Thus, it is important to better un-

Table 1. Summary of gene function and chromosomal localization of the 38 genes in study

Symbol	Name	Function	Chromosomal localization
TP73	Tumor protein p73	Apoptosis related gene	1p36.32
MSH6	mutS homolog 6	DNA mismatch repair	2p16.3
VHL	Von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase	Ubiquitination and proteasomal degradation	3p25.3
RARB	Retinoic acid receptor, beta	Transcription regulation, cell growth and differentiation	3p24.2
CASR	Calcium-sensing receptor	Cellular calcium homeostasis	3q21.1
IL2	Interleukin 2	Immune response	4q27
APC	Adenomatous polyposis coli	Antagonist of the Wnt signaling pathway, cell migration and adhesion, transcriptional activation, and apoptosis	5q22.2
ESR1	Estrogen receptor 1	Transcription regulation, cellular proliferation and differentiation	6q25.1
CDK6	Cyclin-dependent kinase 6	Differentiation and Cell cycle control	7q21.2
CFTR	Cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	Transport of chloride ions	7q31.2
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell cycle control, apoptosis regulation	9p21.3
PAX5	Paired box 5	Regulator in early development	9p13.2
PTCH1	Patched 1	Receptor for sonic hedgehog	9q22.33
CREM	cAMP responsive element modulator	Component of cAMP-mediated signal transduction	10p11.21
KLLN	killin, p53-regulated DNA replication inhibitor	Cell cycle control	10q23.31
PTEN	Phosphatase and tensin homolog	Cell cycle regulation	10q23.31
MGMT	O-6-methylguanine-DNA methyltransferase	DNA repair	10q26.3
CD44	CD44 molecule (Indian blood group)	Cell-cell and cell-matrix interactions	11p13
WT1	Wilms tumor 1	Trasncription factor binding	11p13
PAX6	Paired box 6	Transcription regulation	11p13
GSTP1	Glutathione S-transferase pi 1	Apoptosis regulation	11q13.2
ATM	ATM serine/threonine kinase	Cell cycle control	11q22.3
CADM1	Cell adhesion molecule 1	Cell adhesion	11q23.3
PAH	Phenylalanine hydroxylase	Phenylalanine catabolism	12q23.2
CHFR	Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase	Cell cycle control	12q24.33
BRCA2	Breast cancer 2, early onset	DNA repair, Cell cycle control	13q13.1
RB1	Retinoblastoma 1	Cell cycle control	13q14.2
MLH3	mutL homolog 3	DNA mismatch repair	14q24.3
THBS1	Thrombospondin 1	Cell-cell and cell-matrix interactions	15q14
TSC2	Tuberous sclerosis 2	Cell cycle control	16p13.3
PYCARD	PYD and CARD domain containing	Apoptosis regulation	16p11.2
CDH13	Cadherin 13	Cell adhesion	16q23.3
TP53	Tumor protein p53	Cell cycle control and apoptosis	17p13.1
PMP22	Peripheral myelin protein 22	Growth regulation	17p12
BRCA1	Breast cancer 1, early onset	DNA repair, Cell cycle control	17q21.31
STK11	Serine/threonine kinase 11	Cell metabolism, cell polarity, apoptosis and DNA damage response	19p13.3
KLK3	kallikrein-related peptidase 3	Angiogenesis regulation	19q13.33
GATA5	GATA binding protein 5	Transcription factor binding	20q13.33

derstand the molecular pathogenesis of these tumors, their epigenetic pattern, and risk factors for recurrence or malignant transformation.[1,4]

DNA methylation is one of the main types of epigenetic modifications in humans, and it plays an important part in tumorigenesis.[6] Numerous studies have demonstrated that methylome profiling is a robust approach to central nervous system tumor classification, which sometimes transcend conventional histopathologic diagnosis.[7-11] Aberrant methylation of normally unmethylated CpG-rich areas, also known as CpG (cytidine phosphate guanosine) islands, which are located in or near the promoter region of many genes, have been associated with transcriptional inactivation of important tumor suppressor genes and DNA repair genes.[12] Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) has been accepted as a reliable method for the detection of changes in methylation status as well as copy number quantification in selected genomic DNA sequences in a simple reaction.[12]

In this study, we demonstrate the use of the MS-MLPA assay on DNA samples from LEATs, including GG and DNT tissue, in order to identify potential biomarkers through a genetic and epigenetic analysis.

Material and Methods

Six fresh-frozen tissue specimens from LEATs, obtained during epilepsy surgery, were analyzed with MS-MLPA. DNA from brain tissues of patients and controls were extracted using QIAamp DNA mini kit (50) (Qiagen, p/n 51304, Valencia, CA, USA), according to the manufacturer's instructions. The DNAs were quantified by UV spectrophotometric analysis using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA).

MS-MLPA analyses were performed using SALSA MLPA Kit ME002 (MRC-Holland, Amsterdam, The Netherlands), which can simultaneously detect copy number alterations (CNAs) in 38 different tumor-related genes, and aberrant methylation patterns in a subset of 25 of these genes (**Table 1**). All MS-MLPA reactions were performed according to a standard protocol described by Nygren *et al*,[12] with minor modifications. Briefly, 100 ng of each DNA sample was denatured and, after the addition of the probemix, the probes were allowed to hybridize for 15 hours at 60°C. Subsequently, the samples were divided into two groups, i.e., half of the samples was directly ligated and in the other half the ligation was combined with HhaI digestion. Multiplex PCR was carried out for 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 1 minutes at 72°C. All the reactions were carried out in a thermal cycler equipped with a heat lid (ABI 2720, Applied Biosystems, Foster City, CA, USA). The PCR products were heat-denatured and analyzed on a Gene Scan ABI PRISM 3130 capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). Three control specimens (postmortem brain tissue collected upon autopsy of patients without known history of neurological disease), as well as a negative control (without DNA), were always included in each MLPA assay.

Binning of the raw data and comparative analyses were performed using Coffalyser.NET software. For each MLPA probe we determined the specific cut-off values for gain and loss, using 95% confidence intervals as determined on tissues from non-tumor subjects. A copy number gain was scored when a value exceeded 1.2 and a copy number loss was scored when a value was lower than 0.8. A gene promoter was considered

Table 2. Sample characterization

Pt	Histopathological diagnosis	Sex	Age at epilepsy onset (y)	Disease duration at surgery (y)	Age at surgery (y)	Brain location	Type of surgery	Engel surgical outcome (follow-up after surgery)
1	DNT	F	11	8	19	Left frontal	Lesionectomy	IA (4 y)
2	GG	M	1	6	7	Left temporal	ATL + AH	IA (4 y)
3	DNT	M	17	37	54	Right temporal	Lesionectomy	IA (3 y)
4	GG BRAF:p.V600E	M	27	8	35	Right temporal	Lesionectomy	IIA (3 y)
5	DNT	M	6	2	8	Right parietal	Lesionectomy	IA (1 y)
6	GG BRAF:p.V600E	F	24	2	26	Left temporal	ATL	IIIA (1 y)

DNT: dysembryoplastic neuroepithelial tumor; GG: ganglioglioma; F: female; M: male; y: years; ATL: anterior temporal lobectomy; AH: amygdalohippocampectomy

methylated when the methylation dosage ratio was ≥ 0.50, which means that at least 50% of the DNA was methylated. These cut-off values were based on our previous work[13] and more recently recommendations.[14]

All procedures were conducted in accordance with the Declaration of Helsinki and approved by the local

Table 3. MS-MLPA data from GG tissue

Chrom./Genes	Copy number alterations	Chrom./Genes	Methylation dosage ratio
[01p (n=1)].[TP73]	**0.59**	[01p (n=1)].[TP73]	13%
[02p (n=1)].[MSH6]	**0.74**	[02p (n=1)].[MSH6]	7%
[03p (n=2)].[VHL]	**0.78**	[03p (n=2)].[VHL]	0
[03p (n=2)].[RARB]	1.08	[03p (n=2)].[RARB]	3%
[03q (n=1)].[CASR]	1.03	[06q (n=1)].[ESR1]	9%
[04q (n=1)].[IL2]	1.04	[09p (n=2)].[CDKN2A]	8%
[05q (n=1)].[APC]	0.95	[09p (n=2)].[PAX5]	11%
[06q (n=1)].[ESR1]	1.11	[10q (n=4)].[KLLN]	5%
[07q (n=2)].[CDK6]	1.17	[10q (n=4)].[MGMT]	40%
[07q (n=2)].[CFTR]	1.08	[10q (n=4)].[MGMT]	**58%**
[09p (n=2)].[CDKN2A]	1.04	[11p (n=3)].[PAX6]	0
[09p (n=2)].[PAX5]	1.06	[11p (n=3)].[WT1]	11%
[09q (n=1)].[PTCH1]	1.08	[11p (n=3)].[CD44]	**51%**
[10p (n=1)].[CREM]	**0.79**	[11q (n=4)].[GSTP1]	0
[10q (n=4)].[KLLN]	0.94	[11q (n=4)].[ATM]	4%
[10q (n=4)].[PTEN]	1.02	[11q (n=4)].[CADM1]	0
[10q (n=4)].[MGMT]	1.13	[12q (n=2)].[CHFR]	12%
[10q (n=4)].[MGMT]	1.15	[13q (n=3)].[BRCA2]	7%
[11p (n=3)].[PAX6]	1.12	[13q (n=3)].[RB1]	0
[11p (n=3)].[WT1]	1.01	[13q (n=3)].[RB1]	0
[11p (n=3)].[CD44]	**1.21**	[15q (n=1)].[THBS1]	3%
[11q (n=4)].[GSTP1]	**0.79**	[16p (n=2)].[PYCARD]	7%
[11q (n=4)].[ATM]	0.89	[16q (n=1)].[CDH13]	1%
[11q (n=4)].[ATM]	0.87	[17p (n=2)].[TP53]	12%
[11q (n=4)].[CADM1]	1.11	[17q (n=1)].[BRCA1]	4%
[12q (n=2)].[PAH]	1.06	[19p (n=1)].[STK11]	0
[12q (n=2)].[CHFR]	**0.77**	[20q (n=1)].[GATA5]	16%
[13q (n=3)].[BRCA2]	0.95		
[13q (n=3)].[RB1]	1.12		
[13q (n=3)].[RB1]	0.95		
[14q (n=1)].[MLH3]	**0.76**		
[15q (n=1)].[THBS1]	1.13		
[16p (n=2)].[TSC2]	0.91		
[16p (n=2)].[PYCARD]	**0.78**		
[16q (n=1)].[CDH13]	1.13		
[17p (n=2)].[TP53]	**0.77**		
[17p (n=2)].[PMP22]	1.03		
[17q (n=1)].[BRCA1]	**0.70**		
[19p (n=1)].[STK11]	**0.75**		
[19q (n=1)].[KLK3]	0.91		
[20q (n=1)].[GATA5]	1.04		

Ethics Committee. Informed written consent was obtained from all patients.

Results

Six patients were included in the study, four out of six (67%) were male. Neuropathological diagnosis revealed three GGs (two with *BRAF*:p.V600E mutations determined by real-time PCR), and three DNTs, according to the 2021 WHO Classification of Tumors of the Central Nervous System.[14] The median age at epilepsy onset was 14 years (IQR 20), the median of disease duration at surgery was seven years (IQR 13) and the median age at surgery was 23 years (IQR 32). A favorable surgical outcome (Engel class IA) was observed in four out of six (67%) patients. Sample characterization is described in **Table 2**. From the six LEATs analyzed with MS-MLPA, one GG tissue (patient 4) presented changes in methylation status (**Table 3**). We describe in more detail the clinical data of this patient.

A 37-years-old, caucasian male, with no risk factors for epilepsy and no relevant family history, which started having seizures at the age of 27. The neurological exam was normal. He was admitted to the Epilepsy and Sleep Monitoring Unit for epilepsy characterization. Video-electroencephalogram (EEG) recorded two seizures arising from the right occipito-parietal region (**Fig. 1A**), characterized by a visual and psychic aura, evolving to a brief automotor component, then progressing to a bilateral tonic-clonic seizure. EEG background activity was normal. Interictal EEG showed rare right temporal epileptiform discharges. Brain magnetic resonance imaging (1.5 Tesla) revealed a right medial temporal lobe tumoral lesion (**Fig.1B**). The patient was treated with up-titrating dose of levetiracetam and clobazam. His seizure frequency increased despite antiepileptic treatment and two years later he underwent brain surgery (lesionectomy), with no postsurgical complications. Two more surgeries were performed years later, the last one at the age of 35, both due to seizure recurrence associated to an increase of the residual tumor. Eslicarbazepine acetate was introduced as a second add-on treatment. The patient has an Engel outcome of IIA since the last surgery (approximately three years of follow-up), with rare seizures now. Microscopy of the paraffin-embedded permanent sections and appropriate histochemical stains and antibody immunoreactivities, confirmed a diagnosis of a GG harboring *BRAF*:p.V600E

mutation (CNS WHO grade 1).

MS-MLPA revealed genetic imbalances in twelve genes (**Table 3**), with a clear predominance of copy number losses. Indeed, copy number losses were detected in chromosomes 1p (*TP73* gene), 2p (*MSH6* gene), 3p (*VHL* gene), 10p(*CREM* gene), 11q (*GSTP1* gene), 12q (*CHFR* gene), 14q (*MLH3* gene), 16p (*PYCARD* gene), 17p (*TP53* gene), 17q (*BRCA1* gene) and 19p (*STK11* gene). On the other hand, copy number gains were identified in chromosome 11p (*CD44* gene).

Regarding methylation analysis, both *MGMT* and *CD44* genes were methylated (**Table 3**). The MGMT presented a methylation dosage ratio of 58% and the *CD44* demonstrated a methylation dosage ratio of 51% (**Table 3**). Although GG is a compound tumor, these results represent the percentage of total methylation for the tissue as a whole, not considering its heterogeneity. Methylation scores below 0.50 were discarded accordingly to the 2021 WHO Classification of Tumors of the Central Nervous System recommendations.[14]

Discussion

Here we describe a MS-MLPA assay, performed with LEATs tissue, for the detection of aberrant methylation patterns of CpG islands and copy number changes of many genes with relevance for oncogenesis, highlighting the results obtained in a GG.

Most LEATs present a flat copy number profile. However, a few common chromosomal alterations have been described in studies including GGs and DNTs, namely gains of chromosomes 5, 6, 7, and 16.[15,16] A high number of chromosomal aberrations were detected in one of our GG tissue samples, among which deletions dominated, reinforcing the spectrum of complete and partial chromosomal abnormalities previously identified applying other cytogenomic techniques.[17-20] We found that none of the genes evaluated exhibited both copy number loss and methylation. In contrast, we observed copy number gain and methylation in *CD44* gene. CD44 is a non-kinase cell surface transmembrane glycoprotein, which is involved in cell activation, cell-cell and cell-matrix adhesion, cell migration, and cell-substrate interaction.[21] CD44 functions as a receptor for hyaluronate and many other extracellular matrix components.[21] Akiyama *et al* demonstrated that the expression of the hyaluronate receptors, CD44 and RHAMM (receptor for HA-mediated motility), is virtually ubiquitous amongst
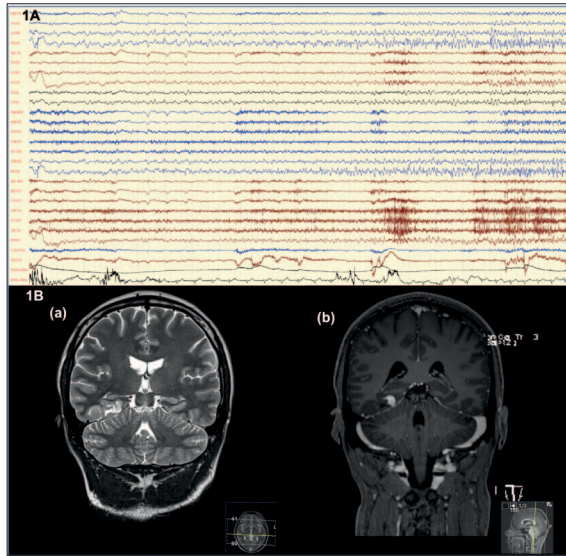
Figure 1. Video-EEG and brain MRI of patient 4.

1A: Video-EEG showing an ictal pattern in the right occipito-parietal region (20 mm/sec, 70 µV/cm, HFF: 70 Hz, LFF: 1,6 Hz).
1B: Coronal T2 (a) and Coronal Reformatted 3D T1 post-gadolinium (b) showing a right medial temporal lobe tumoral lesion, involving the cortex of parahippocampal and fusiform gyrus, but sparing the hippocampus, with approximately 2 cm of maximum diameter. The tumor is heterogeneous, with a lateral cystic area and a medial solid component avidly enhancing with gadolinium.

glioma cell lines, and surgical specimens of human brain tumors, including two GGs.[21] There was a gradient of expression amongst gliomas, with high grade gliomas expressing more RHAMM and CD44 than lower grade lesions or non-neoplastic specimens of human brain.[21]

The methylation of *MGMT* in our GG tissue is concordant to previous studies.[22,23] MGMT, known as O-6-methylguanine-DNA-methyltransferase, is a DNA repair enzyme that repairs damaged guanine nucleotides by transferring the methyl at O^6 site of guanine to its cysteine residues.[24] The expression of MGMT is governed by epigenetic gene silencing, which means that when the CpG island of *MGMT* promoter is methylated, the MGMT protein expression should be low.[22,23] The level of MGMT varies widely according to the type of tumor, and even varies among tumors of the same type.[25] Wang and coworkers showed that 20% (5/25) of the GGs (WHO grade I) analyzed by pyrosequencing (PSQ) harbored *MGMT* promoter methylation.[22] Liu *et al* reported that methylation-specific PCR (MSP) analysis revealed *MGMT* promoter methylation in all the 3/3 (100%) cerebellar GGs.[23] On the other hand, in a study with nine WHO grade I GGs from cerebral and extrac-

erebral locations, 56% (5/9) of the tumors exhibited nuclear staining for MGMT protein.[5] Tumors with more intensive MGMT protein expression tended to recur more frequently, corresponding to the worse prognostic predictive value.[5] This data suggested that the status of MGMT protein expression may have prognostic value for WHO grade I GGs.[5] Moreover, MGMT methylation has been detected in other LEATs, namely 25% (1/4) of pilocytic astrocytoma and 75% (3/4) papillary glioneuronal tumors.[22,26] While *MGMT* has been deeply investigated in diffuse infiltrative gliomas and related drug response to alkylating agents, its role in low-grade tumors is less clearly understood, and further studies are needed.[24,25]

DNA methylation profiling is highly robust and reproducible even from small samples and poor quality material.[7] A number of different methods and platforms, including PSQ, MSP, methylation-sensitive high-resolution melting, next generation sequencing, and MS-MLPA have been used to detect promoter methylation in tumors.[22] Due to its simplicity, the MS-MLPA method described here may have potential as a screening tool to identify specific epigenomic alterations, helping tumor classification.[12] The main advantages of MS-MLPA are: (1) a large number of genes can be studied using a minimum amount of DNA; (2) owing to its simple procedure, large number of samples can be analyzed simultaneously; and (3) MLPA is quantitative and can discriminate between methylation of one, both or none of the alleles.[12]

In conclusion, our data highlight the importance of identify chromosomal regions for further fine mapping and epigenetically assess LEATs tissue, in order to unravel key genes that may serve as potential diagnostic biomarkers, contributing to an integrative tissue-based histological and (epi)genetic classification. ∎

CB: Study execution, analysis and interpretation of the clinical data, manuscript review and final approval.

FS: Study execution, analysis and interpretation of the clinical data, manuscript review and final approval.

IS: Design of the work, supervision of the work, manuscript review and final approval.

AF: Design of the work, supervision of the work, manuscript review and final approval.

JBM: Design of the work, analysis and interpretation of the data, manuscript review and final approval.

Responsabilidades Éticas

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