Gene dosage and mutational analyses of *EGFR* in oligodendrogliomas

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Abstract. We have studied amplification/gene-dosage and sequence variations of the *EGFR* gene in 41 oligodendroglial tumours graded according to the WHO classification (21 oligodendrogliomas grade II, 13 oligodendrogliomas grade III and 6 oligoastrocytomas grade II-III), using multiplex ligation-dependent probe amplification (MLPA), real-time quantitative PCR, and PCR/SSCP techniques. To determine gene-dose we studied exons 11 (extracellular domain) and 25 (intracellular domain) in the *EGFR* gene. Overdose (1- to 5-fold increase) was present in exon 11 in 21 of 41 samples (52.5% of cases) and in exon 25, in 7 of 41 samples (17.5% of cases). Gene amplification (>5-fold increase) was present in exon 11, in 17 of 41 samples (42.5% of cases), and in exon 25 in 6 of 41 samples (15% of cases). Three tumours (two grade II oligodendrogliglioma, one mixed oligo-astrocytoma) displayed high level amplifications: >100 gene copies were identified by both real-time quantitative PCR and MLPA analyses. Gene sequence alterations were identified by PCR/SSCP and sequencing in four cases: two missense mutations: G1051A (Ala351Thr) and G2216A (Arg739Hys); one nonsense mutation: C2934T (Asp978Asp); and an 18 bp deletion in position 2423-2441 of E19. These changes were present only in tumoral DNA, not in the corresponding constitutional patients' DNA. We also found four previously unidentified polymorphic variants: G2025A (Ala675Ala), C2233T (Leu745Leu), C2895T (Treo965Treo) and C3168T (Asp1056Asp), and three previously described polymorphic changes: E12+22 T→A, G1748A (Arg583Lys) and A2547T (Glu849Glu). Our findings demonstrate that mutations and amplification/overdose in the *EGFR* gene are present in low-grade oligodendroglial tumours, and may contribute to the development of these brain neoplasms.

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Introduction

Oligodendrogliomas are tumours composed of cells that histologically resemble the mature oligodendrocyte, occurring predominantly in the cerebral hemispheres (1). Constituting approximately 5% of all intracranial CNS tumours, oligodendrogliomas are most commonly diagnosed during the fourth and fifth decades of life. The mean post-diagnostic survival for low-grade oligodendroglioma is 10 years, although for high-grade tumour survival is only 5 years (1). Cytogenetically over half of oligodendrogliomas show combined loss of 1p and 19q (2,3). At genetic level, the oligodendroglial tumours are characterized mainly by: a) deregulation of the *EGFR* (epidermal growth factor receptor) growth stimulatory pathways; b) deletion of the gene tumour suppressor *p16ink4a* (cyclin-dependent kinase inhibitor 2A) and *PTEN* (phosphatase and tensin homolog); c) mutation in *TP53*.

EGFR is frequently expressed at high levels in both low and high-grade oligodendrogliomas; however, overexpression of *EGFR* in astrocytoma is associated with *EGFR* amplification and occurs primarily in the high-grade tumours (4). In contrast, *EGFR* amplification has rarely been found in oligodendrogliomas (5). *EGFR* is a transmembrane glycoprotein encoded by a gene located on 7p12 (6). This protein consists of an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain and a COOH-terminal regulatory domain containing autophosphorylation sites (7). In regard to exon-intron positions: exon 1 includes the sequence encoding the signal peptide and the first five amino acids of the mature *EGFR*. The extracellular domain is encoded by exons 2-16; as part of this domain, the two cysteine-rich regions are encoded by exons 5-7 and 13-16. The transmembrane domain is encoded

by exon 17. In the intracellular region, the tyrosine kinase domain (TK) is encoded by exons 18-24, and the carboxy terminal region is encoded by exons 25-28 (8). *EGFR* plays a critical role in the control of processes occurring during oncogenesis and tumour progression, such as cell survival, proliferation and metastasis. The receptor is overexpressed in an extensive range of solid tumours, and this alteration has been associated with advanced stage of disease, poor prognosis and development with chemotherapy, radiotherapy and hormone-therapy resistance, but this response also depends on the type of neoplasia (9). Because of its prevalence in malignant disease and its critical role in tumorigenesis, targeting the *EGFR* is a rational approach for cancer treatment (10). Due to the importance of this gene and because scarce data are available for molecular alterations of *EGFR* in oligodendrogliomas, we have completed a mutational and amplification/overdose study of the *EGFR* gene in a series of 41 tumours with a major oligodendroglial component.

Materials and methods

Tumour samples and DNA preparation. Forty-one tumour biopsy specimens, from 40 patients with oligodendroglial tumours, were obtained during surgical procedures and frozen immediately at -80˚C until use. DNA was prepared from frozen tissue samples and blood samples using standard methods, as described previously (11). All samples were classified by histological examination and graded according to the WHO guidelines (5) and included 21 oligodendrogliomas grade II (O), 13 oligodendrogliomas grade III (AO) and 6 oligoastrocytomas grade II-III (OA).

Real-time quantitative PCR analyses. Real-time quantitative PCR was performed using a Light Cycler (Roche Molecular Biochemical, Mannheim, Germany) to analyze the amplifications status of extracellular (exon 11) and intracellular (exon 25) regions in the *EGFR* gene and 18S gene (used as reference). All the samples were analyzed in duplicate. Genomic amplification of exons was performed with an initial amount of DNA of 50 ng/μl and using the Light Cycler FarstStart DNA Master SYRB Green kit (Roche Molecular Biochemical) (12,13).

Primer sequences for 18S and *EGFR* exons were designed by Primer Premier 5.0 and were the same as those used to perform the PCR-SSCP for these exons (18S: forward, 5'-AGTTGGTGGAGCGATTTG-3'; reverse, 5'-TTGCTCAATCTCGGTGG-3'; exon 11: forward, 5'-TCCTCAGTGGTGTGTGTCTGA-3'; reverse, 5'-CAGGAGCTCTGTGCCCTATC-3'; exon 25: forward, 5'-GCCTCAAAATCTCTGCACCA-3'; reverse, 5'-CAGCTTGAGAGAGAGAGGAACA-3').

PCR was carried out as described (12,13) briefly: after an initial (pre-PCR) 10-min pre-incubation step at 95˚C, 45 amplification cycles were run, each consisting of 95˚C for 10 sec, 63-55˚C for 10 sec, 72-82˚C for 10 sec and (exon 11, exon 25) an additional step for exon 11 and 25, at 80˚C or 85˚C for 2 sec, respectively, was also performed. The relative amounts of exon products were compared with the reference gene (18S) and calculated with Light Cycler Relative Quantification Software (Roche Molecular Biochemicals). To confirm the specificity of the amplification signal, we considered the gene dissociation curve in each case. To obtain gene dosage, the 18S gene was used as a control and the corrected gene dosage for each exon was obtained based on the assumption that the gene ratio in normal tissue was 1.0 [studied exon tumour sample/reference gene (18S) = 1]. A ratio of >5 was considered positive for amplification and values 1-5 positive for overdose. Although ratio values of <0.2 might be compatible with gene deletion, this aspect was not considered in the present study.

Multiplex ligation-dependent probe amplification (MLPA) analysis. For *EGFR* analyses we used the SALSA P105 glioma. In addition to other probes the kit includes probes for exons 1, 8 and 17 of the *EGFR* gene. As a control, it includes 15 probes for different chromosomal locations. Information regarding the probe sequences and ligation sites can be found at www.mlpa.com. MLPA protocol was carried out as previously described by Schouten *et al* (14) using 50 ng for each DNA normal control and tumour sample. One microliter of the amplified sample product was analyzed using ABI 3100. Avant sequencer (Applied Biosystem) and as an internal size standard the ROX-500 Genescan (ABI 401734). Successful ligation reaction and identification of samples with insufficient amounts of DNA were verified using MLPA's internal ligation-independent probes. Data analysis was carried out using the MRC-Coffalyser version 2. Intra normalisation for sample data was carried out on control probes and then each tumour sample was normalized on control probes of 5 control sample data. Single regression for control and tumour data was performed. Normal ratio limits were established between 0.7 and 1.3. Statistical analysis was accomplished using the Coffalyser V2 program.

Single-strand conformation polymorphism (SSCP) analysis and direct sequencing of the EGFR gene. *EGFR* mutations were detected using PCR-SSCP method and direct sequencing of 21 exons. We studied exons that showed a greater rate of mutation according to the literature, including the signal peptide region (exon 1), binding protein domain (exons 2, 7-16), transmembrane domain (exon 17), and TK domain (exons 18-24) and carboxyl terminal (exons 25 and 26). Genomic PCR amplification was performed using intronic primers designed in our laboratory (12). PCR conditions were 35 cycles of 94˚C for 30 sec, 49-65˚C for 30 sec and 72˚C for 90 sec, with a final extension of 7 min at 72˚C using the biotools DNA polymerase kit (Madrid, Spain). PCR products were loaded onto 6-12% non-denaturing polyacrylamide gels (with or without 10% glycerol). All PCR products except the control sample were denatured by heating at 95˚C and immediately chilled on ice. Samples displaying an altered PCR-SSCP pattern were sequenced using the ABI PRISM Big dye terminator cycle sequencing kit (Perkin-Elmer, Alameda, CA). Each amplicon was sequenced bi-directionally.

Results

Gene amplification/overdose status. As shown in Table I, quantitative-PCR analysis showed that of 41 oligodendroglial tumour samples, 21 (12 O; 6 AO; 3 OA) (52.5%)

Table I. *EGFR* amplification values.

Tumour	Exon 11	Exon 25
O-K2	-	-
O-K5	+	-
O-K8	+	-
O-K9T1	+	-
O-K10	++	+
O-K12	+	-
O-K13	++[a]	++
O-K14	+	+
O-K15	+	-
O-K16	+	-
O-K21	++	-
O-K24	++	-
O-K25	+	-
O-K26	+	+
O-K131	+	-
O-K132	++	++
O-K134	++	++
O-K136	++	-
O-K137	+	-
O-K138	++	-
O-K140	+	-
O-K144	++[a]	++
AO-K3	+	-
AO-K9T2	+	-
AO-K11	+	-
AO-K17	++	-
AO-K22	+	-
AO-K23	+	-
AO-K27	++	+
AO-K133	-	-
AO-K139	++	-
AO-K141	++	-
AO-K142	++	+
AO-K143	++	-
AO-K145	+	-
OA-K1	+	-
OA-K4	-	-
OA-K6	+	+
OA-K7	+	+
OA-K28	++	++
OA-K135	++[a]	++

+, 1- to 5-fold; ++, >5-fold. O, low-grade oligodendroglioma (WHO grade II); AO, anaplastic oligodendroglioma (WHO grade III); OA, mixed oligoastrocytoma (WHO grade II and grade III). [a]>100 copies of the *EGFR* gene.

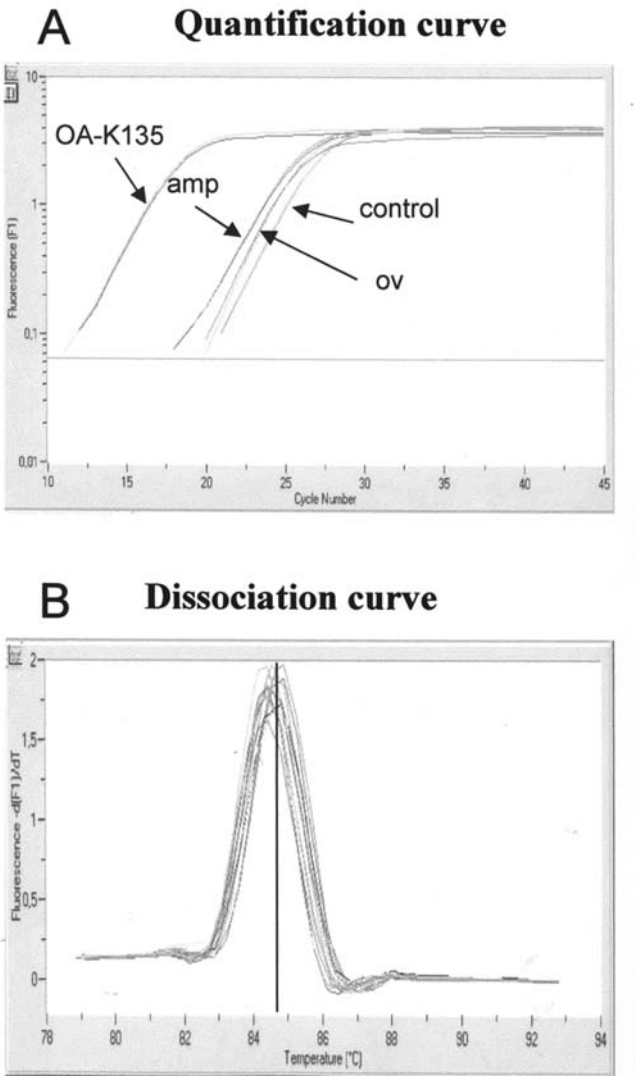

Figure 1. Real-time PCR by Light Cycler (Roche) for amplified samples. (A) Quantification curves for 3 cases with high-level amplification (OA-K135), low amplification (amp) and overdose (ov) of *EGFR* and a normal sample (control). X-axis shows number of cycles and the Y-axis shows the fluore-scence signal. (B) Dissociation curve of the reaction: X-axis represents temperature (in ˚C) and the Y-axis the fluorescence depending on the time of the reaction.

were positive for gene overdose (1- to 5-fold increase) at exon 11 and of 7 of 41 (3 O; 2 OA;2 AO) (17.5%) for exon 25. Also 17 of 41 samples (9 O; 6 AO; 2 OA) (42.5%), were identified as positive for amplification (>5-fold increase) in exon 11 and 6/41 (4 O; 2 OA) (15%) in exon 25. Three of these tumours (two grade II oligodendrogliomas and one oligoastrocytoma) displayed high-level (>100 copies) *EGFR* gene amplification (Fig. 1). MLPA analysis also detected these tumours with high-level *EGFR* amplification, but no insight into gene overdose could be determined by using this methodology (Fig. 2).

EGFR DNA sequence changes. The analysis of the 22 studied exons for the *EGFR* gene with the PCR/SSCP method, allowed us to identify four alterations not previously described. We used X00588 gene bank accession numbers to identify the sequences. The four alterations were not detected

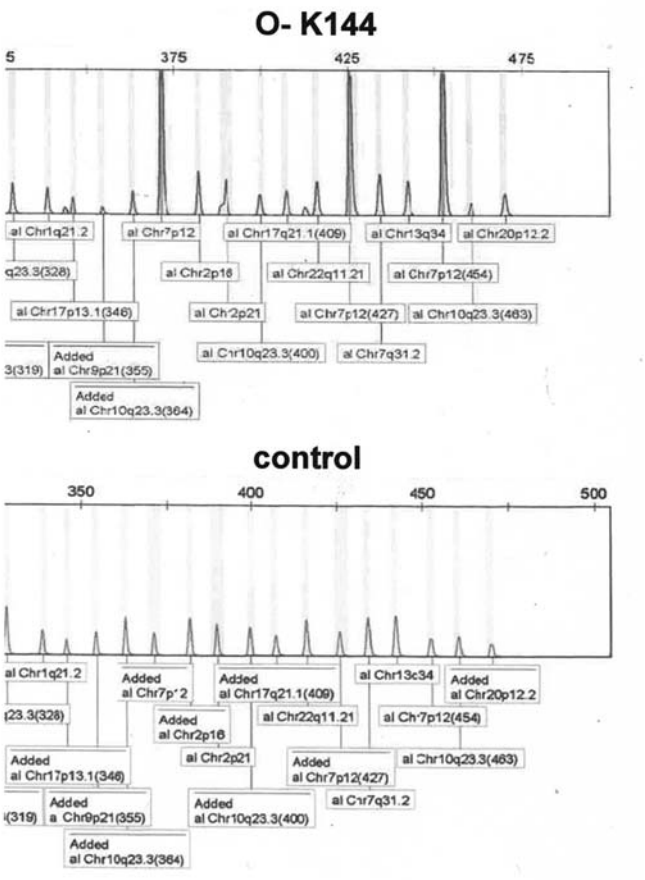

Figure 2. *EGFR* amplification revealed by direct comparison of the MLPA electrophoresis peak patterns. Probe mix P105 glioma contained probes for *EGFR* exons 1, 8 and 17 (marked as Chr7p12 375, 425 and 454), probes for other tumour-related genes and 15 additional control probes (in the X-axis) for other genes on various chromosomes. Amplification is evidenced for all three exons of *EGFR* in tumour O-K144 when compared to control non-tumour DNA.

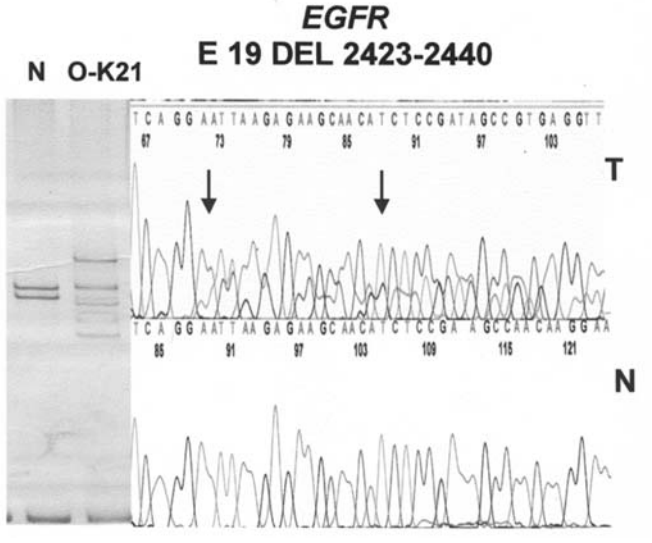

Figure 3. *EGFR* exon 19 deletion of 18 bp (2423-2440) (loss of Glu, Leu, Arg, Glu, Ala, Thr, Ser and insertion of Ala residue). SSCP analysis corresponding to the normal (N) and tumoral (O-K21) DNA. Direct sequencing (forward) analysis of the corresponding tumoral (T) and normal (N) DNA is shown to the right.

by PCR/SSCP and sequencing analysis in the blood DNA samples corresponding to the altered tumour. A missense mutation within exon 7 (G1051A Ala351Thr) was present in the sample O-K2. We also found a missense mutation in exon 17 (G2216A Arg739Hys) in samples OA-K7, O-K9T1, AO-K9T2, O-K10 and AO-K11. A nonsense mutation was found in exon 23 (C2934T Asp978Asp) of sample O-K21. Finally, a deletion was found in exon 19; the deletion starts in position 2423 with an 18 bp loss in sample O-K21. This sequence variation implied a loss of 7 amino acid residues (Glu, Leu, Arg, Glu, Ala, Thr, Ser and insertion of an Ala) (Fig. 3). A summary of these changes is shown in Table II. Seven additional *EGFR* sequence changes were also identified both in blood DNA and tumoral DNA samples, and were considered as gene polymorphisms (Table III). The polymorphic sites were located in *EGFR* gene as follows: intron 12+22, T→A, exon 13, G1748A; exon 15, C2025T; exon 17, C2223T; exon 20, A2547G; exon 23, C2895T and exon 25, 3168 C→T. To determine the constitutional and tumoral genotypes in these polymorphic sites, we also analysed the gene sequences in the cases for which peripheral blood lymphocyte DNA samples were available. Genotype distribution and frequencies are also shown in Table III.

Discussion

We have analysed 41 oligodendroglial tumours for *EGFR* gene-dose/amplification and sequence alterations to determine the possible involvement of this gene in oligodendroglioma development. We used quantitative PCR because it has been demonstrated to be a more sensitive method than other techniques such as Southern blot, which was used earlier to determine gene amplification (6). We studied exon 11 (extracellular domain), and exon 25 (intracellular domain) for *EGFR* gene dosage and we found 52.5% of the samples with gene overdose and 42.5% amplification for *EGFR* exon 11 and lower rates for exon 25 (17.5% overdose and 15% of amplification). The heterogeneous amplification pattern found suggests that the extracellular and intracellular domains were not uniformly amplified, with the extracellular domain presenting a higher gene dosage. Amplification of the intracellular region could be related to increased expression and independent ligand-binding truncated receptor (15), while amplification of the extracellular region could play a role in the development of tumorigenic mutant deletion forms (15). Low-level amplification or overdose could be related to chromosome 7 polysomy in oligodendroglial tumours, a chromosomal variation that is also present in malignant gliomas with astrocytic differentiation (16,17). However, this correlation needs further study to be firmly established. *EGFR* is not the only gene that is amplified in oligodendroglial tumours, as amplification and/or overdose in other genes, such as *MDM2* (p53-binding protein), and *CDK4* (cyclin-dependent kinase 4), have been described (13).

EGFR gene amplification on tumours derived from glial cells, primarily astrocytic cells, was associated with poorer survival (18). This amplification is often linked to structural gene alterations; seven major mutated variants of *EGFR*

Table II. Sequence variations of *EGFR* in 41 oligodendrogliomas.

Location	Sequence variation	Sample
Exon 7	G1051A Ala351Thr	O-K2
Exon 17	G2216A Arg739Hys	OA-K7, O-K9T1, AO-K9T2, O-K10, AO- K11
Exon 19	Deletion 18p.b 2423	O-K21
Exon 23	C2934T Asp978Asp	O-K21

Table III. *EGFR* polymorphisms and allele frequencies in oligodendrogliomas.

	Sequence change	No. samples (blood)	Genotype (%)	No. samples (tumour)	Genotype (%)
Exon 12	T→A 12+22	28	AA 2 (7.2) TA 12 (42.8) TT 14 (50)	41	AA 4 (10) TA 14 (34) TT 23 (56)
Exon 13	G1748A Arg583Lys	32	GG 14 (43.7) GA 17 (53.2) AA 1 (3.1)	40	GG 21 (52.5) GA 16 (40) AA 3 (7.5)
Exon 15	C2025T Ala675Ala	32	CC 26 (81.3) CT 6 (18.7) TT 0 (0)	40	CC 32 (80) CT 8 (20) TT 0 (0)
Exon 17	C2233T Leu745Leu	29	CC 28 (96.5) CT 1 (3.5) TT 0 (0)	40	CC 39 (97.5) CT 1 (2.5) TT 0 (0)
Exon 20	A2547G Glu849Glu	23	AA 10 (43.4) GA 10 (43.4) GG 3 (13.2)	41	AA 16 (39) GA 13 (32) GG 12 (29)
Exon 23	C2895T Treo965Treo	28	CC 22 (78.5) CT 6 (21.5) TT 0 (0)	40	CC 28 (70) CT 11 (27.5) TT 1 (2.5)
Exon 25	C3168T Asp1056Asp	28	CC 19 (67.8) CT 9 (32.2) TT 0 (0)	39	CC 28 (72) CT 10 (26) TT 1 (2.5)

have been identified (19). The most common is variant III (*EGFRvIII*), also called del 2-7 *EGFR* or delta EGFR (20) and is present in 20-50% of glioblastomas with EGFR amplification (21,22). We did not study the different variants for *EGFR*, but we thought that some of the samples that present amplification might have variant III (*EGFRvIII*), due to the similar characteristics between oligodendrogliomas and glioblastomas. It is interesting that gene amplification is already present in low-grade samples, suggesting that this molecular abnormality might be a relatively early step in the development of oligodendrogliomas. The high-level *EGFR* amplification we identified in the two low-grade tumours and in one mixed tumour was unequivocally determined, as two distinct methodologies (quantitative-PCR and MLPA) presented positive results and suggested that, most likely, the extra and intracellular domains of *EGFR* are amplified in those tumours. This *EGFR* amplification/overexpression has been shown to correlate with aggressive malignant progression and poor clinical outcome (23,24). Accordingly, the possibility exists that those low-grade oligodendrogliomas with *EGFR* amplification may represent a tumour subset with a more aggressive biological behaviour.

Our studies of mutational analysis suggest that this gene displays alterations of the extracellular (exon 7), transmembrane (exon 17) and intracellular (exons 19, 23) domains. Accordingly, the mutation distribution in our samples appears throughout the gene, showing an absence of hot spots in accordance with previous studies of *EGFR* mutation in glial

tumours and other neoplasms (25). These mutations could be the cause of processes involved in cellular deregulation, apoptosis inhibition or an increase in cellular division, functions that an intact *EGFR* would control. We have not found a relationship between sequence polymorphisms and amplification and/or overdose in our tumour series but the constitutional allelic frequencies in polymorphic sites located in *EGFR* exons 13 [G1748A (G 70%/A 30%)] and 20 [A2547G (A 65%/G 35%)] were similar to those described by Frederick *et al* (26), or by Arjona *et al* (27) in intron 12+22 (T 71%/A 29%). We also found other polymorphisms previously described: exon 15, C2025T (C 90%/T 10%), exon 17, C2223T (C 98%/T 2%), exon 23, C2895T (C 89%/T 11%) and exon 25, C3168T (C 84%/T 16%). The biological significance of these variations regarding the neoplastic development of oligodendrogliomas should be analyzed in depth because a polymorphism at intron 1 has been associated with regulation of *EGFR* expression (28), and also because *EGFR* expression status in lung cancer has been correlated with mutations (29). Knowledge of *EGFR* status is crucial before the establishment of adequate clinical treatment in non-small cell lung cancer (NSCLC) (30). At present, *EGFR* is a target in cancer treatment (31): the presence or absence of mutations or amplification/overdose of *EGFR* are being used to determine treatment (chemotherapy/radiotherapy) in NSCLC (32). One of the drugs being studied is an inhibitor in the tyrosine kinase domain (EGFR TKI) (33). EGRF TKI may be used in the future not only for treatment of lung cancer but also for those malignancies in which the *EGFR* gene is involved (34), as occurs with glial tumours.

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