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Amplification of the cyclin-dependent kinase 4 (*CDK4*) gene is associated with high cdk4 protein levels in glioblastoma multiforme

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Abstract Genetic alterations on the long arm of chromosome 12, including both gene amplification and allelic loss, are associated with malignant progression of human gliomas. The region of the chromosomal arm 12q that is amplified in malignant gliomas contains the *CDK4* gene, a cell cycle regulatory gene which promotes cell division. To evaluate the frequency of *CDK4* gene amplification, we analyzed a series of 355 brain tumors using a quantitative non-radioactive polymerase chain reaction assay. *CDK4* gene amplification occurred in 9 of 81 glioblastomas (11%), but was rare in other neoplasms, including low-grade and anaplastic gliomas, meningiomas, medulloblastomas and metastatic carcinomas (only 6 of 274 cases). There was no correlation between *CDK4* gene amplification and allelic loss of chromosome 12. To assess the significance of *CDK4* gene amplification, we analyzed protein extracts from 37 glioblastomas by Western blotting with a commercially available polyclonal antibody to cdk4. All tumors with *CDK4* gene amplification showed high cdk4 expression levels, whereas no increased cdk4 expression was seen in glioblastomas without *CDK4* gene amplification. These data support the functional activity of *CDK4* gene amplification in glioblastoma multiforme and point to an important role of *CDK4* gene amplification in a subset of glioblastomas.

Key words *CDK4* · Gene amplification · Protein level · LOH12q · Brain tumors

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Introduction

Gene amplification is an alteration of DNA in which the copy number of a particular gene or series of genes is increased. Gene amplification of oncogenes has been documented in a variety of human malignancies, including the *ERBB2* gene in breast cancer [21], *EGFR* gene in glioblastoma [2, 11] and *cyclinD* gene in esophageal cancer [7]. Recently, an amplification cluster has been described on chromosomal arm 12q13-q14 [8, 14, 16]. This genomic region contains several genes with oncogenic potential, such as the *MDM2* gene (mouse double minute 2), the *SAS* gene (sarcoma amplified sequence), the *GADD 153* (growth arrest DNA damage induced 153), *GLI* (from glioma/glioblastoma) and the *CDK4* gene (cyclin-dependent kinase 4). Interestingly, in gliomas, amplification of the 12q13-q14 region is frequently accompanied by loss of heterozygosity of adjacent regions of chromosome 12q [17].

The *CDK4* gene encodes the cdk4 protein, which belongs to a family of proteins involved in cell cycle regulation in eukaryotic cells [13, 20]. The cdk4 protein has been identified as an inducer of the G1/S transition in the cell cycle. Alterations in cdk4 protein activity may, therefore, play an important role in tumorigenesis [6]. *CDK4* gene amplification on chromosome 12 has been observed in 15% of malignant gliomas [16].

The *CDK4* gene is negatively regulated by the *CDKN2/MTS1* gene, which in malignant gliomas is frequently inactivated by homozygous deletions [22, 24]. In fact, *CDK4* gene amplification, *CDKN2/MTS1* gene deletion and pRb inactivation have been implicated as alternative mechanisms in glioma progression [4, 5, 19, 22]. Furthermore, *CDK4* is the most frequently amplified gene of the 12q13–q14 amplification cluster in gliomas [16]. These observations suggest that *CDK4* amplification plays a role in the malignant progression of astrocytic gliomas.

To investigate the prevalence of *CDK4* gene amplification in human brain tumors we assessed a series of 355 brain tumors for amplification of the *CDK4* gene. The bi-

ological relevance of *CDK4* gene amplification was examined by immunoblotting analysis of the cdk4 protein in a subset of tumors with and without *CDK4* gene amplification. An association of *CDK4* amplification with allelic deletion of chromosome 12q was analyzed by comparing amplification data with the results of studies on loss of heterozygosity using four polymorphic microsatellite markers.

Materials and methods

Tumor material

Surgical biopsy samples of 355 brain tumors from 351 patients were collected at the Massachusetts General Hospital (Boston, Mass.), Zürich University Hospital, and Bonn University Hospital. The specimens were immediately frozen in liquid nitrogen and stored at –80° C for further use. All tumors were classified according to the revised WHO classification of brain tumors [9]. The series of brain tumors contained 81 cases of glioblastoma multiforme WHO grade IV (GBM), 32 anaplastic astrocytomas WHO grade III (A III), 23 astrocytomas WHO grade II (A II), 27 pilocytic astrocytomas WHO grade I (PA I), 1 anaplastic pilocytic astrocytoma WHO grade III (PA III), 1 myxopapillary ependymoma WHO grade I (E I), 6 ependymomas WHO grade II (E II), 3 anaplastic ependymomas WHO grade III (E III), 14 anaplastic oligoastrocytomas WHO grade III (OA III), 19 oligoastrocytomas WHO grade II (OA II), 4 anaplastic oligodendrogliomas WHO grade III (O III), 10 oligodendrogliomas WHO grade II (O II), 10 medulloblastomas WHO grade IV (MB), 2 extracerebellar primitive neuroectodermal tumors WHO grade IV (PNET), 10 gangliogliomas WHO grade I (GG I), 2 anaplastic gangliogliomas WHO grade III (GG III), 55 meningiomas WHO grade I (M I), 8 atypical meningiomas WHO grade II (M II), 6 anaplastic meningiomas WHO grade III (M III), 17 schwannomas WHO grade I (S 1), 1 malignant peripheral nerve sheath tumor WHO grade III (MPNST) and 23 metastatic carcinomas (MET), as summarized in Table 1. Two patients had two M I. Two biopsy samples were included from a patient with an OA II that recurred as an OA III and from a patient with A II that recurred as a GBM. Prior to DNA extraction all tumor tissues were examined by frozen sectioning to exclude the presence of non-tumorous brain tissue.

DNA and protein extraction

DNA from the 355 tumor tissues was obtained by phenol-chloroform extraction as previously described [18]. In addition, DNA and protein were extracted from 37 glioblastomas using the Trizol protocol according to the procedure suggested by the manufacturer (Gibco BCL).

Conditions for differential polymerase chain reaction analysis

For differential polymerase chain reaction (PCR) analysis, a 155-bp fragment of the *CDK4* gene on chromosome 12 was coamplified with a 134-bp fragment of the *APRT* (adenine phosphoribosyltransferase) gene on chromosome 16. The primer sequences and PCR products are shown in Table 2. One primer of each pair was labeled with 5-carboxyfluorescein (FAM; Applied Biosystems) at the 5´ end. Differential PCR was performed in a final volume of 10 µl containing 10 ng DNA, 50 mM KCl, 2.0 mM $MgCl_2$, 10 mM TRIS-HCl pH 8.3, 200 µM of each dNTP, 0.1% gelatin, 1 pmol primer for the *CDK4* gene and 20 pmol primer for the *APRT* gene and 0.25 U Taq polymerase (Stratagene). Initial denaturation at 95° C for 3 min was followed by 27 cycles on a thermocycler (Perkin Elmer 9600). These included denaturation at 95° C for 30 s, annealing at 58° C for 60 s and extension at 72° C for 40 s. A final extension step of 10 min at 72° C was used.

Analysis of the coamplified PCR products

Fluorescent PCR products were separated on a 6% polyacrylamide gel and analyzed on an automated DNA sequencer (Applied Biosystems Model 373A). A quantitative analysis of the signal intensity was carried out with the Genescan program (Applied Biosystems Genescan Version 1.2.1). The technical details of differential PCR have been reported elsewhere [23].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting and immunodetection

Protein concentrations were determined according to the Lowry method using bovine serum albumin as a standard. Corresponding amounts of protein (30 µg) were separated on 12% gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 40 min at 40 mA [10]. Proteins were transferred to a nitrocellulose membrane (Sigma) by semidry blotting and subsequently incubated with the polyclonal anti-human cdk4 antibody (Pharmingen), which was diluted to a concentration of 1 µg/ml. Specific binding of the primary antibodies was detected with an alkaline phosphatase-labeled secondary antibody (Bicol) and 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Sigma) as the chromogen.

Conditions for PCR-based analyses of loss of heterozygosity

Assay conditions and allele identification have been described previously [1, 3, 12]. The following polymorphic microsatellite loci were analyzed: D12S373, D12S379, D12S393 and D12S392 (oligonucleotide primers were purchased from Research Genetics, Huntsville, USA). Mapping information was based on the latest update of the Genome Data Base.

Results

Defining limits for gene amplification

To determine the variation in the ratio of the *CDK4* gene to *APRT* gene PCR products in normal DNA, we studied 76 genomic DNA samples from peripheral lymphocytes. Ratios ranged from values of 0.5 to 1.3. The mean ratio and standard deviation of the coamplified *CDK4/APRT* gene fragments were 1.0 and 0.16, respectively. A value of the mean ratio plus three standard deviations was selected as the cut-off level for the normal gene copy number; thus, ratios higher than 1.48 were considered evidence for more than two copies of the *CDK4* gene. To avoid scoring trisomy/tetrasomy of chromosome 12 as gene amplification, a cut-off level of twice the mean ratio plus three standard deviations was selected. Therefore, ratios between 1.48 and 2.48 were assumed to indicate trisomy/tetrasomy of chromosome 12 and ratios higher than 2.48 were taken as evidence of *CDK4* gene amplification. A representative case with *CDK4* amplification is shown in Fig. 1.

CDK4 gene amplification in brain tumors

Of the 355 brain tumors, 15 exhibited *CDK4/APRT* ratios higher than 2.48 in our PCR-based assay and were, there-

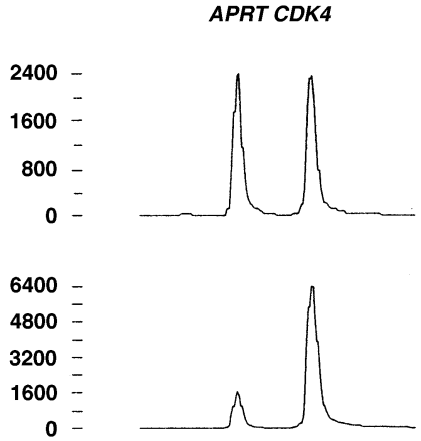

Fig. 1 Differential polymerase chain reaction (PCR) assay for the detection of gene amplification. Fluorescence signal for *APRT* and *CDK4* PCR products in lymphocyte DNA (*upper panel*) and tumor DNA (*lower panel*) of a patient with *CDK4* gene amplification. *CDK4/APRT* ratio equals 5.6. Intensity levels are indicated *at the left*. Note the different scales in the *upper* and *lower graphs*

fore, scored as amplified. These 15 tumors included 9 of the 81 GBM (11%), 2 of the 32 A III (6%), 1 of the 14 OA III (7%), 1 of the 10 O II (10%), 1 of the 10 MB (10%) and 1 of the 23 MET (4%). The *CDK4/APRT* ratios in tumors with amplification varied between values from 2.5 to 21.7. These results are compiled in Table 1.

All tumors exhibiting *CDK4* gene amplification were glial, with the exception of one medulloblastoma and one metastasis. In all but one oligodendroglioma, *CDK4* amplification occurred in highly malignant lesions of WHO grades III or IV.

The *CDK4* gene dose was within normal limits in all samples from the two patients with multiple M I, as well as from the one patient with OA II which progressed to OA III. However, the patient with A II progressing to GBM showed a normal *CDK4* gene copy number in the primary tumor (*CDK4/APRT* = 1.37) but had gene amplification in the recurrent GBM (*CDK4/APRT* = 3.85). The results are compiled in Table 1.

Comparison of *CDK4* gene amplification and cdk4 protein levels

DNA and frozen tumor tissue from 37 GBM patients were available for study. These cases were examined for both *CDK4* gene amplification by differential PCR and cdk4 protein expression by Western blotting. *CDK4* gene amplification in this subset was observed in 4 of the 37 samples.

Western blot analysis revealed cdk4 signals at 32 kDa and a non-specific band at 40 kDa as described by the manufacturer (Pharmingen). The non-specific 40-kDa signal served as an indicator for the amount of protein applied. High cdk4 protein levels were detected only in the 4 tumors with *CDK4* gene amplification. The remaining 33 samples exhibited clearly weaker cdk4 signals. Representative data are depicted in Fig. 2.

Loss of heterozygosity on chromosomal arm 12q

In our series, 206 patients showed evidence of at least one polymorphic marker on the long arm of chromosome 12. Of these 206 cases, LOH was seen in ten tumors including one PA I, two OA III, two MET, one M I, three GBM and

Table 1 Primer sequences used for the differential polymerase chain reaction (PCR) assay. The primers indicated by *FAM* were labeled at the 5´ end with 5-carboxyfluorescein

CDK4 (155 bp)	5´TGTAAGTGCCATCTGGTAGC
	5´FAM ATGCGCCAGTTTCTAAGAGG
APRT (134 bp)	5´TGGGAAAGCTGTTTACTGCG
	5´FAM CAGGGAACACATTCCTTTGC

Table 2 Survey of *CDK4* gene amplification using a PCR-based assay in 355 different brain tumors (*GBM* glioblastoma multiforme WHO grade IV, *A III* anaplastic astrocytoma WHO grade III, *A II* astrocytoma WHO grade II, *PA I* pilocytic astrocytoma WHO grade I, *PA III* anaplastic pilocytic astrocytoma WHO grade III, *E I* myxopapillary ependymoma WHO grade I, *E II* ependymoma WHO grade II, *E III* anaplastic ependymoma WHO grade III, *OA II* oligoastrocytoma WHO grade II, *OA III* anaplastic oligoastrocytoma WHO grade III, *O II* oligodendroglioma WHO grade II, *O III* anaplastic oligodendroglioma WHO grade III, *MB* medulloblastoma WHO grade IV, *PNET* extracerebellar primitive neuroectodermal tumor WHO grade IV, *M I* meningioma WHO grade I, *M II* atypical meningioma WHO grade II, *M III* anaplastic meningioma WHO grade III, *GG I* ganglioglioma WHO grade I, *GG III* anaplastic ganglioglioma WHO grade III, *S I* schwannoma WHO grade I, *MPNST* malignant peripheral nerve sheath tumor WHO grade III, *MET* metastatic carcinoma)

Tumor entity	Number of cases	Amplified cases
GBM	81	9 (11%)
A III	32	2 (6%)
A II	23	0
PA I	27	0
PA III	1	0
E I	1	0
E II	6	0
E III	3	0
OA III	14	1 (7%)
OA II	19	0
O III	4	0
O II	10	1 (10%)
MB	10	1 (10%)
PNET	2	0
GG I	10	0
GG III	2	0
M I	55	0
M II	8	0
M III	6	0
S I	17	0
MPNST	1	0
MET	23	1 (4%)

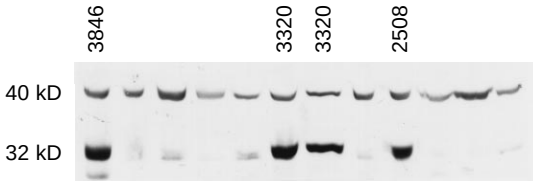

Fig. 2 Detection of cdk4 protein. The 32-kDa band corresponds to cdk4 protein. The 40-kDa band is due to cross-reaction with an unknown protein (according to the instructions of the manufacturer). All lanes show a similar protein load as indicated by the 40-kDa band. Cases 3846, 3320 and 2508 exhibit elevated cdk4 protein levels. Two independently extracted samples of case 3320 were loaded

one A III. LOH12q was thus not associated with a distinct histopathological tumor entity. Two of the tumors with LOH12q also exhibited *CDK4* gene amplification (one OA III and one A III). No significant association was found between LOH12q and *CDK4* gene amplification (Yates' χ^2 test).

Discussion

In our series of brain tumors, *CDK4* gene amplification was observed predominantly in high-grade astrocytic gliomas. These data confirm previous studies demonstrating that *CDK4* gene amplification is primarily restricted to higher-grade lesions. The results also indicate that *CDK4* gene amplification is exceedingly rare in non-glial tumors. Our observed frequency of 11% for *CDK4* amplification in GBM is comparable to the 15% previously reported; however, we noted only 6% of A III with *CDK4* amplification, as opposed to 17% in a previous study [16]; this difference, however, was not significant (Yates' χ^2 test).

The observation that *CDK4* gene amplification only occurs in highly malignant gliomas suggests that *CDK4* gene amplification is a late event in astrocytic tumorigenesis and is primarily associated with the development of GBM. This hypothesis is supported by the findings for one of our patients, who was initially diagnosed with A II, which later progressed to GBM. In the primary tumor the *CDK4/APRT* ratio was 1.37, while the GBM had a ratio of 3.85, illustrating *CDK4* gene amplification in the transformation of A II to GBM. This hypothesis is further supported by recent studies showing that other proteins in the cell cycle regulatory pathway that includes cdk4 are also altered in the transition of A II to A III and GBM [5, 22].

The differential PCR assay, which allows semiquantification of gene copy number, also shows a correlation between gene copy number and histopathological grade in gliomas. The average ratio of *CDK4/APRT* amplification was 6.21 in nine GBM, but only 4.31 in two A III. Interestingly, each of the three non-astrocytic tumors with *CDK4* amplification (one O II, one MB and one MET) had relatively low *CDK4/APRT* ratios (2.94, 2.56 and 2.64, respectively). Given the association between *CDK4* gene amplification and cdk4 protein overexpression, this may imply that cdk4 overexpression confers a distinct growth advantage in astrocytic tumors; indeed, recent studies have shown that the *p16* gene, and presumably the p16-cdk4-pRb pathway, is rarely altered in medulloblastomas [15].

A number of observations point to *CDK4* as the primary glial oncogene on the chromosome 12q amplicon. In gliomas, *CDK4* is the most commonly amplified gene from this region, although at least one case has been described with *MDM2*, and no *CDK4*, amplification [16]. Alterations in *CDK4* typically occur in those tumors without inactivation of the p16 or pRb proteins, which operate in the same cell cycle regulatory pathway [5, 22]; such an inverse correlation suggests that *CDK4* amplification is a functionally important event. Increased CDK4 mRNA levels have been noted in those tumors harboring the 12q amplification, implying that the amplified gene is expressed in gliomas. Our data of elevated cdk4 protein levels only in those tumors exhibiting *CDK4* gene amplification further support a critical role for *CDK4* gene amplification in glioma progression. Furthermore, a close correlation between *CDK4* gene amplification and overexpression of cdk4 protein has also been demonstrated recently on Western blots [5].

LOH12q may accompany amplification of the oncogene cluster on chromosome 12q13-14 [17]. In our series, however, only 10 of 206 tumors exhibited LOH12q. Of these tumors, 2 showed both LOH12q and *CDK4* gene amplification. Among the 196 tumors without LOH12q, 7 had amplification of the *CDK4* gene. The relationship between LOH12q and *CDK4* amplification was not significant. Thus, it remains to be resolved whether an increased LOH12q frequency is specific for gliomas with *CDK4* gene amplification, or whether higher rates of LOH12q simply reflect the increased genomic instability in a malignant tumor which also exhibits *CDK4* gene amplification.

In conclusion, *CDK4* gene amplification correlates with elevated cdk4 protein levels in malignant gliomas. This supports an active role of the cdk4 protein in the process of tumor progression in a subgroup of astrocytomas, and further implicates cdk4 overexpression as a functional consequence of chromosome 12q13-q14 amplification.

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