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Advances in Brief

Amplification of Multiple Genes from Chromosomal Region 12q13-14 in Human Malignant Gliomas: Preliminary Mapping of the Amplicons Shows Preferential Involvement of *CDK4*, *SAS*, and *MDM2*[1]

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Abstract

We have investigated 234 tumors of the central nervous system for amplification of 9 different loci from 12q13-14 and report that about 15% of the anaplastic astrocytomas and glioblastomas show amplification at this chromosomal region. The genes most frequently amplified were *CDK4* and *SAS* (18 of 19 cases). *MDM2* was coamplified with *CDK4* and *SAS* in 11 tumors while one glioblastoma showed only *MDM2* amplification. Some amplicons additionally included *GADD153* (9 cases), *GLI* (6 cases), *A2MR* (3 cases), and the anonymous locus *D12S8* (2 cases). Either *MDM2* or *CDK4* and *SAS* showed the highest amplification level in each individual amplicon and amplification of these genes was consistently accompanied by strong overexpression. Our results thus suggest *CDK4*, *SAS*, and *MDM2* as main targets for the amplification; however, the possibility exists that all amplicons share a common amplified region between *MDM2* and *CDK4/SAS* which might contain one or more as yet unidentified genes.

Introduction

We have recently shown that 8–10% of human glioblastomas and anaplastic astrocytomas demonstrate amplification and overexpression of the *MDM2* gene (1). *MDM2* has been localized to chromosomal region 12q13-14 (2). Several other genes from this chromosomal segment, including *GLI*, *SAS*, *CDK4*, and *A2MR*, have recently been found amplified in sarcomas of bone and soft tissue origin (3–6). In addition to amplification, the 12q13-14 region is frequently involved in chromosomal translocations associated with certain tumor types and two genes, *GADD153* and *ATF1*, have recently been identified as the targets of such translocations in myxoid liposarcomas (7, 8) and malignant melanomas of soft parts (9). Gene mapping studies have further demonstrated that several other genes of known or suspected oncogenic potential such as *WNT1* (10), *ERBB3* (11), and *CDK2* (12) are located in the 12q13-14 region. In the present study we have investigated a large series of primary tumors of the central nervous system for amplification of 9 different genes/loci from 12q13-14. We report that about 15% of the glioblastomas and anaplastic astrocytomas show amplification and overexpression of one or more of these genes. Preliminary mapping of the amplicons suggests three genes (*CDK4*, *SAS*, and *MDM2*) as likely targets of the amplification event at this chromosomal region.

Materials and Methods

Tumor Material. Tumor tissue was collected from 234 brain tumor patients operated on at the departments of neurosurgery at the Heinrich-Heine-University in Düsseldorf, Germany, the Sahlgrenska Hospital in Gothenburg, Sweden, and the Karolinska Hospital in Stockholm, Sweden. The tumor series consisted of 86 glioblastomas, 29 anaplastic astrocytomas, 27 astrocytomas, 12 pilocytic astrocytomas, 17 anaplastic oligodendrogliomas, 13 oligodendrogliomas, 11 anaplastic mixed gliomas, 8 mixed gliomas, 15 ependymal tumors, and 16 PNETs,[4] and included the 156 cases previously studied for MDM2 amplification (1). Peripheral blood for the extraction of leukocyte DNA was available from 180 cases. All tumors were classified according to the WHO classification of tumors of the central nervous system (13). Histological evaluation of the frozen tumor tissue assured that all specimens studied consisted of at least 75% tumor cells. As control tissue for the expression studies nonneoplastic adult human brain tissue (cortex and white matter) from the temporal lobe of a patient operated on for epilepsy was used.

DNA and RNA Extraction and Analysis. Extraction of high molecular weight DNA and total RNA as well as Southern and Northern blotting were carried out as previously described (1). The blots were hybridized with DNA probes labeled with [^{32}P]dCTP by random priming, exposed to phosphor storage screens (Molecular Dynamics, Sunnyvale, CA), scanned in a Molecular Dynamics PhosphorImager, and densitometrically analyzed by using the ImageQuant software. Quantitative densitometric analysis of Southern blot hybridizations was performed as previously described (1), using the variable number of tandem repeats probe pYNH24 (detects the anonymous locus *D2S44*) as reference. Only a relative increase in gene dosage of more than five times that of constitutional DNA was considered amplification. A synthetic 50-base oligonucleotide probe complementary to bases 101–150 in the glyceraldehyde-3-phosphate dehydrogenase mRNA (EMBL Accession No. XO1677) was used to assess variations in loading of Northern blots.

Probes. The *MDM2* probe (pMDM2a) was a 600-nucleotide PCR product amplified by reverse transcription PCR from human glioblastoma cDNA as described previously (1) and subsequently cloned into the pCR™II-vector by using the TA-cloning kit (Invitrogen). The probes for *ERBB3* and *CDK2* were generated in a similar way. The *ERBB3* probe was amplified from cDNA of the MDA-MB361 breast carcinoma cell line (ATCC HTB27) and corresponded to bases 801 to 1402 (EMBL Accession No. M34309). The 778-nucleotide probe for *CDK2* (bases 166 to 943, EMBL Accession No. X61622) was generated by reverse transcription PCR from nonneoplastic human brain cDNA. A 534-nucleotide probe for *CDK4* was generated by PCR of leukocyte DNA by using the primers described by Khatib *et al.* (5). The identity of the probes was ensured by their correct lengths, by partial sequencing (approximately 150 bases at each end), and by their identification of transcripts of correct size on Northern blots. Sequencing was performed by using the Sanger dideoxy method with the USB Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH), using oligonucleotides complementory to the SP6 and T7 promotors of the pCR™II vector.

The probe for *SAS* was an approximately 500-nucleotide genomic DNA fragment amplified by PCR from plasmid pSJP2, a subclone of pSJB2 (4, 14),

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[4] The abbreviations used are: PNET, primitive neuroectodermal tumor; PCR, polymerase chain reaction; cDNA, complementary DNA.

using oligonucleotides complementory to the T3 and T7 promotors of the pBluescript vector as primers. The *GLI* probe (pKK36P1) was a kind gift of Dr. B. Vogelstein, Baltimore, MD, and the *GADD153* probe, a 515-nucleotide mouse cDNA fragment (CHOP10), was kindly provided by Dr. D. Ron, Boston, MA. Plasmid probes for *WNT1* (pAL1), *A2MR* (LRP06), *D12S8* (p7G11), *D2S44* (pYNH24), and *EGFR* (pE7) were obtained from American Type Culture Collection.

Results

All 234 tumors were screened on Southern blots for amplification of 8 different genes located at 12q13-14 (*MDM2*, *CDK4*, *SAS*, *GADD153*, *GLI*, *A2MR*, *WNT1*, *ERBB3*) and the anonymous locus *D12S8*. Amplification of one or more of these loci was found in 19 malignant gliomas including 13 of 86 glioblastomas (15.1%), 5 of 29 anaplastic astrocytomas (17.2%), and 1 of 17 anaplastic oligodendrogliomas (5.9%) (Fig. 1). The size of the amplicon varied from tumor to tumor (Fig. 2). The largest amplicon was found in tumor AA16 and included *D12S8*, *MDM2*, *CDK4*, *SAS*, *GADD153*, *GLI*, and *A2MR*. In contrast, glioblastoma GB80 had the smallest amplicon which consisted of only *MDM2* (Fig. 3, *a–c*). With the exception of GB80, the amplicons in all the other 18 tumors included *CDK4* and *SAS*, 11 of which also showed amplification of *MDM2*. In two tumors, the amplicons extended telomerically and involved the *D12S8* locus at 12q14. In 11 tumors the amplicons comprised genes centromeric to *SAS*, including *GADD153* in 9, *GLI* in 6, and *A2MR* in 3 tumors (Figs. 1, 2, 3, *d–e*).

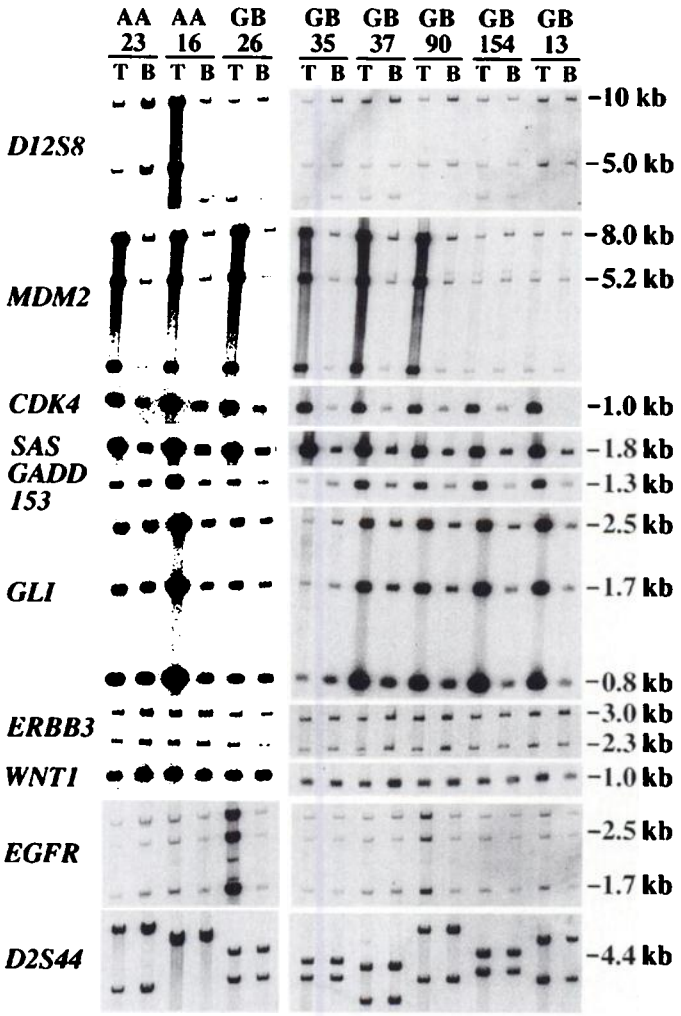

Fig. 1. Representative Southern blot hybridizations of 8 malignant gliomas with 12q13-14 amplification. The case numbers are given on top (*AA*, anaplastic astrocytoma, *GB*, glioblastoma). Tumor (*T*) and peripheral blood (*B*) DNA were digested with the restriction enzyme *Taq*I, electrophoresed, and blotted. The blots were sequentially hybridized with probes specific for the loci indicated on the *left ordinate*. The approximate sizes (in kilobases) of the represented bands are shown on the *right ordinate*. Amplification of *D12S8*, *MDM2*, *CDK4*, *SAS*, *GADD153*, and *GLI* can be easily recognized in the tumor DNA of variable numbers of cases. None of the tumors shows amplification of *ERBB3* and *WNT1* while the *EGFR* gene is coamplified in 2 glioblastomas (GB26 and GB90). As control for the loading amount of DNA the blots were hybridized with the variable number of tandem repeats probe pYNH24 (*D2S44*).

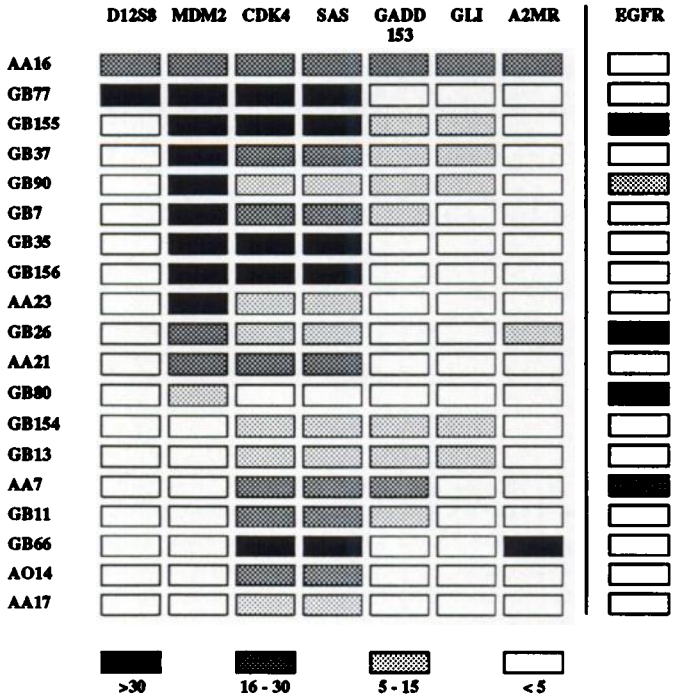

Fig. 2. The 12q13-14 amplicons in 19 primary malignant gliomas. The case numbers are given on the *left ordinate* (*AA*, anaplastic astrocytoma, *AO*, anaplastic oligodendroglioma, *GB*, glioblastoma). The densitometrically determined gene dosages (amplification levels) were subdivided into four categories (*abscissa*). The loci from 12q13-14 are ordered according to their chromosomal location from the most telomeric locus (*D12S8*) to the most centromeric locus (*A2MR*). The positioning of *CDK4* on the telomeric side of *SAS* is provisional and remains to be confirmed by physical mapping. Note the variable sizes of the individual amplicons. Except for GB80 (only *MDM2* amplification), all amplicons contain *CDK4* and *SAS* which are invariably coamplified to similar degrees. *MDM2* is coamplified with *CDK4*/*SAS* in 11 tumors and in all of these *MDM2* was among the genes with the highest level of amplification. The amplicons of tumors GB26 and GB66 were discontinuous between *SAS* and *A2MR*. Five tumors had additionally amplified the *EGFR* gene from 7p12 (*right ordinate*).

Densitometrical analysis revealed that the amplification levels varied not only from case to case but in some tumors also from gene to gene. Amplification ranged from 8- to more than 70-fold. In 12 tumors all genes in the amplicon showed similar amplification levels, whereas the degree of amplification of each gene varied in 7 tumors (Fig. 2). In 5 of the latter, the *MDM2* amplification levels significantly exceeded those of the other genes in the amplicons. In one glioblastoma without *MDM2* amplification (GB11), the amplification levels for *CDK4* and *SAS* were significantly higher than for *GADD153*. Interestingly, *CDK4* and *SAS* were always coamplified to similar levels. *GADD153* and *GLI* were also frequently coamplified and when they were, both were coamplified to the same degree.

In two glioblastomas (GB26 and GB66) the amplicons were discontinuous between *SAS* and *A2MR* (Fig. 2). In both cases, *GADD153* and *GLI* were not included in the amplicons but parts of the *A2MR* gene were coamplified with *SAS*, *CDK4*, and, in GB26, *MDM2* (Figs. 2 and 3, *d–e*). As shown previously (1), GB26 also had a genomic rearrangement of the amplified *MDM2* gene (with overexpression of an apparently normal transcript). Thus, the proximal and distal breakpoints of the amplicon in this case are probably located within *A2MR* and *MDM2*, respectively.

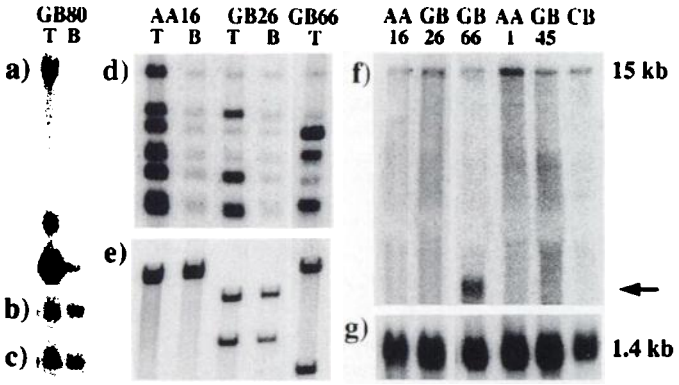

Fig. 3. *a–c*) Amplification of *MDM2* without coamplification of *CDK4* and *SAS* in tumor GB80. Tumor (*T*) and blood (*B*) DNA from this case was digested with *PstI*, electrophoresed, and Southern blotted. The blot was sequentially hybridized with probes for *MDM2* (*a*), *CDK4* (*b*), and *SAS* (*c*). *d–g*) Analysis of amplification and overexpression of *A2MR* in malignant gliomas. *d*) Demonstration of *A2MR* amplification in three malignant gliomas (*AA16*, *GB26*, *GB66*) by Southern blot hybridization with the LRP6 probe. Note that GB26 and G66 show only partial amplification that affects different parts of the *A2MR* gene in each tumor. *e*) Control probing of the same blot with pYNH24 (*D2S44*). *f*) Northern blot analysis of *A2MR* expression in the tumors with amplification (*AA16*, *GB26*, *GB66*), in two tumors without amplification (*AA1*, *GB45*), and control brain (*CB*). The expression level of *A2MR* is not elevated in the tumors with amplification but GB66 shows an aberrant transcript of about 3.5 *kb* (kilobases) (*arrow*) in addition to the normal 15 kilobase mRNA. *g*) Control probing of the same blots for *GAPDH*.

None of the 234 tumors showed evidence of *WNT1* or *ERBB3* gene amplification (Fig. 1). Southern blot analysis of *CDK2* in the 19 cases with 12q13-14 amplification also revealed no amplification (data not shown). *EGFR* amplification was detected in 33 of 234 tumors, including 27 of 86 glioblastomas (31%), 4 of 29 anaplastic astrocytomas (13.8%), 1 of 17 anaplastic oligodendrogliomas (5.9%), and 1 of 11 anaplastic oligoastrocytomas (9.1%). Five tumors with 12q13-14 amplification additionally had amplification of the *EGFR* gene (Figs. 1 and 2). Densitometric analysis of the amplification levels in these cases revealed no relationship between the values obtained for *EGFR* and for the amplified genes from 12q13-14.

Analysis of mRNA expression of *MDM2*, *CDK4*, *SAS*, *GADD153*, *GLI*, and *A2MR* was performed in 18 of the 19 cases with 12q13-14 amplification and an additional series of 134 tumors without 12q13-14 amplification. In total, 44 glioblastomas, 26 anaplastic astrocytomas, 13 astrocytomas, 6 pilocytic astrocytomas, 14 anaplastic oligodendrogliomas, 13 oligodendrogliomas, 10 anaplastic mixed gliomas, 6 mixed gliomas, 11 ependymal tumors, and 9 PNETs were studied. As reported previously (1), *MDM2* amplification was always associated with significant overexpression (Fig. 4). Similarly, all cases with *CDK4* amplification strongly overexpressed this gene (15- to >100-fold higher than control brain) (Fig. 4). In addition, a number of tumors without *CDK4* amplification, particularly among the high-grade gliomas and PNETs, showed increased expression of the *CDK4* transcript (between 2- and 5-fold that of normalized control brain). Amplification of *SAS* was invariably accompanied by increased expression of *SAS* mRNA (Fig. 4) with the 1.8-kilobase transcript being most abundant. Only 2 of 9 tumors with *GADD153* amplification demonstrated strong expression of the 1.1-kilobase transcript. The remaining 7 cases showed only moderate or weak signals on Northern blots and the vast majority of tumors without amplification did not express this gene at levels detectable by Northern blotting (Fig. 4). The tumors with *GLI* amplification showed variable expression of this gene while no expression was found in tumors without amplification (Fig. 4). Exceptional was tumor AA23 which had a 4-fold increase in *GLI* gene dosage (a value just below our 5-fold threshold for amplification) and demonstrated a strong expression of *GLI* (Figs. 1 and 4). *A2MR* was expressed in normal brain as well as in all analyzed tumors. None of the tumors with *A2MR* amplification demonstrated significantly increased expression; however, GB66, which had partially amplified the *A2MR* gene, showed an aberrant transcript of approximately 3.5 kilobases in addition to the normal 15-kilobase mRNA (Fig. 3, *f–g*).

Discussion

Studies using pulsed field electrophoresis have shown that amplicons in human tumor cells usually comprise large regions of genomic DNA which can be up to several megabases in length (15, 16). Thus, not unexpectedly, coamplification of genes located in a limited chromosomal region have been described in human tumors. Examples include the coamplification of *ERBB2* and *ERBBA* (chromosome 17q21) in mammary carcinomas (17) as well as the complex coamplification of multiple genes from 11q13 in breast cancer and certain other types of carcinomas (18).

Coamplification of multiple genes from 12q13-14 has recently been reported in primary sarcomas and certain sarcoma cell lines (5, 6). Since we observed amplification of the *MDM2* gene from 12q13-14 in a subset of malignant gliomas (1) we decided to further characterize the amplicon in these tumors by studying a number of different loci mapped to 12q13-14. The aim was to identify the genes included in this amplicon, determine the frequency and level of amplification, and thereby more precisely define the actual target(s) of the amplification event.

The two genes most frequently amplified in our series were *CDK4* and *SAS*. Both were coamplified at similar levels in 18 malignant gliomas (12 glioblastomas, 5 anaplastic astrocytomas, 1 anaplastic oligodendroglioma). In a recent study of primary sarcomas and sarcoma cell lines Forus *et al.* (6) also found *SAS* most frequently included in the 12q13-14 amplicons. *CDK4* was not studied by these

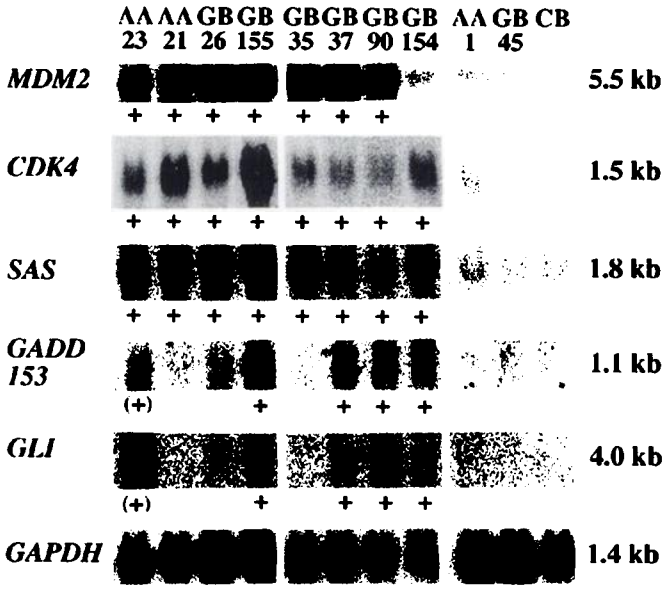

Fig. 4. Analysis of mRNA expression of *MDM2*, *CDK4*, *SAS*, *GADD153*, and *GLI* on Northern blots from malignant gliomas with 12q13-14 amplification (*AA23*, *AA21*, *GB26*, *GB155*, *GB35*, *GB37*, *GB90*, and *GB154*), two control tumors without amplification (*AA1*, *GB45*), and control brain (*CB*). The approximate sizes (in kilobases) of the transcripts are shown on the *right ordinate*. +, amplification of the corresponding gene in that tumor. All cases with amplification of *MDM2*, *CDK4*, or *SAS* demonstrate strong overexpression of the respective transcripts compared to control brain and tumors without amplification. *GLI* is expressed at variable but mostly weak intensities in the tumors with amplification. Tumor AA23, which had a 4-fold increased *GLI* and *GADD153* gene dosage (indicated by +), showed the highest expression of *GLI* among all tumors investigated. *GADD153* is strongly expressed in one tumor with amplification (*GB155*), while three other tumors with amplification show weak signals. The blots were probed for *GAPDH* to assess differences in RNA loading.

authors but its involvement in the 12q13-14 amplicons in two sarcoma cell lines has recently been reported by another group (5). The invariable coamplification of *SAS* and *CDK4* seen in our study indicates that both genes are likely to be located in close proximity to each other. The physical maps of the 12q13-14 region that have been published link *A2MR*, *GLI*, *GADD153*, *SAS*, and *MDM2* (19, 20); however, *CDK4* has only recently been assigned to this region (5, 12). Thus, it remains to be shown whether *CDK4* is located telomerically (as shown in Fig. 2) or centromerically to *SAS*.

Several lines of evidence suggest that the amplification and consequent overexpression of *CDK4* could be of pathogenetic significance for neoplastic growth. *CDK4* belongs to the family of cyclin dependent kinases which, together with the cyclin proteins, plays a crucial role in the regulation of cell cycle progression in eukaryotic cells (21). Under physiological conditions, Cdk4 becomes activated upon complex formation with cyclin D1, proliferating cell nuclear antigen, and p21 protein, and this interaction has been shown to be seriously altered in many types of transformed cells (22). Overexpression of cyclin D1 has already been found in different human tumors (23) and transfection experiments have recently shown that overexpression of this gene can result in neoplastic transformation (24, 25). In addition, recent studies suggest that different types of human tumors, including malignant gliomas, frequently show inactivating mutations and/or deletions of a tumor suppressor gene on chromosome 9p21 (*MTS1* or *CDK4I*) which encodes a physiological inhibitor of Cdk4 (26–28). Furthermore, Waf-1 (also known as p21 or Cip1) (29–31), another important inhibitor of Cdk4, is induced by p53 as a principal downstream target (29). Thus, it is very likely that amplification and overexpression of *MDM2* or *CDK4*, or the lack of expression of *MTS1/CDK4I*; or the noninduction of Waf-1 expression by a functionally defect p53, all may have similar cell cycle effects and promote neoplastic cell growth.

Less is known about the physiological functions of *SAS*. This gene encodes a protein of the transmembrane 4 protein superfamily (TM4SF) (32). The precise functions of TM4SF proteins is unknown at present but several studies have provided evidence that members of this family might play a role in signal transduction and growth control [reviewed in Jankowski *et al.* (32)]. Therefore, *SAS* amplification and overexpression could result in growth alterations; however, additional studies will be necessary to clarify the normal function of *SAS* and to prove a potential relationship between *SAS* overexpression and neoplastic growth.

According to our results, the *MDM2* oncogene can be regarded as the third possible target selected for by the 12q13-14 amplification. Although *MDM2* was less frequently included in the 12q13-14 amplicons than *CDK4* and *SAS*, it generally demonstrated the highest levels of amplification when coamplified and was the only amplified gene from 12q13-14 in one glioblastoma. *MDM2* amplification without coamplification of *CDK4* or *SAS* has also been documented in one sarcoma (6). Furthermore, we have recently identified two glioma cell lines with *MDM2* amplification, only one of which has coamplified *CDK4* and *SAS* (33). Taken together, these findings suggest that in some tumors amplification of *MDM2* may provide the selective growth advantage.

In addition to *CDK4*, *SAS*, and *MDM2*, the 12q13-14 amplicons in malignant gliomas frequently contained other genes from this region such as *GADD153*, *GLI*, and *A2MR*. *GLI* has been shown to be a cellular oncogene that can transform cells in cooperation with the adenovirus *E1A* gene (34). A role for *GADD153* in oncogenesis has recently been shown by the finding of its consistent disruption in myxoid liposarcomas with the characteristical t(12;16)(q13;p11) translocation (7, 8). Nevertheless, our present study provides several lines of evidence suggesting that these genes are not the primary target of the amplification. (*a*) They are included in only a minority of the amplicons; (*b*) they were invariably coamplified with *CDK4* and *SAS* and never showed the highest amplification levels in the individual tumors; (*c*) although the tumors with amplification of *GLI* and/or *GADD153* showed elevated expression compared to control brain, the expression levels varied considerably with only a fraction of tumors demonstrating strong overexpression. Furthermore, none of the tumors with *A2MR* amplification revealed significantly increased expression. As has been reported in sarcomas (6), our data therefore suggest that *GADD153*, *GLI*, and *A2MR* are just incidentally included in some 12q13-14 amplicons.

In conclusion, the present study shows that a subset of human malignant gliomas is characterized by amplification of multiple genes from the chromosomal segment 12q13-14. Our preliminary mapping of the 12q13-14 amplicons in 19 malignant gliomas shows that they consistently include either *CDK4* and *SAS* or *MDM2*. It is conceivable that in the individual case amplification and overexpression of one or more of these three genes may provide a selective growth advantage. However, it is also possible that all amplicons share a common amplified region located between *MDM2* and *CDK4/SAS* which might contain one or more as yet unidentified genes.

Acknowledgments

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