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RAPID COMMUNICATION

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Twelve amplified and expressed genes localized in a single domain in glioma

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Abstract Gene amplification has been associated both with tumor stage and progression in human gliomas. Several distinct amplified loci have been identified by comparative genomic hybridization and Southern blot analysis. It has been increasingly recognized that amplified domains comprise multiple genes. Here, we demonstrate amplification of up to 12 different genes from an amplified domain at 12q13–15 that has been found in approximately 15% of astrocytomas and glioblastomas. The amplified genes were GLI, WNT1, MDM2, SAS, CDK4, OS-4, GAS16, GAS27, GAS41, GAS56, GAS 64 and GAS89. In one glioblastoma all 12 amplified genes were also found to be expressed. These results strongly warrant the search for as yet unidentified genes in regions previously reported to be amplified.

Introduction

Gene amplification is a major mechanism of increased gene expression in human neoplasms, including gliomas. Gliomas constitute the most frequent intracranial tumors in man with glioblastoma (GBM) as the most prevalent and malignant type. Approximately 50% of all GBMs exhibit gene amplification with the epidermal growth factor receptor (EGFR) gene amplified in 30% of GBMs, MET amplified in 20% and the mouse double minute (MDM2) gene amplified in 8% (Libermann et al. 1985; Fuller and Bigner 1992; Reifenberger et al. 1994; Fischer et al. 1995). Double minutes (dmin) as cytogenetic manifestations of gene amplification are found in 40–50% of all GBMs (Thiel et al. 1992). Since cytogenetically visible DNA amplifications do not necessarily coincide with the amplification of known genes, dmin and homogeneously staining regions (HSRs) are likely to harbor additional undescribed amplified genes in glioma.

The molecular analysis of amplified DNA domains has been significantly facilitated by chromosomal microdissection, which permits the selective isolation of these chromosomal structures. The chromosomal origin of dmins and HSRs can be determined by fluorescence in situ hybridization (FISH). Most recently, cryptic sites of DNA amplification in breast cancer have been identified by a combination of microdissection and FISH (Guan et al. 1994). To identify genes encoded in amplification units various strategies have been used including exon trapping, zoo blot hybridization, and hybridization of cloned genomic DNA fragments (e.g. yeast artificial chromosomes and cosmids) against cDNA libraries (Lovett et al. 1991; Buckler et al. 1991; Monaco et al. 1986). A combination of microdissection and hybrid selection has successfully been employed to isolate genes from an HSR without cloning of the HSR DNA (Su et al. 1994). Most recently, we cloned novel amplified genes from a glioblastoma by a technique we termed microdissection-mediated cDNA capture (Gracia et al. 1996). In brief, cDNA was generated from tumor cells, hybridized against metaphases of the tumor cells and recovered by microdissection of the HSR. Bound cDNAs were amplified by the polymerase chain reaction.

The increasing number of approaches for the isolation of amplified genes is a necessary prerequisite to analyze the structure and gene content of amplified DNA domains. As yet rather limited data on gene content are available for the majority of the amplification units. In most cases there are at best only one or a few identified genes per amplified domain. In this study, we demonstrate the amplification of up to 12 different genes within a single amplified domain in glioblastoma. The genes were tested for amplification and expression using DNA and RNA from two glioblastoma multiforme and one anaplastic astrocytoma, which were previously identified as containing amplified domains within chromosomal band region 12q13–15.

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Materials and methods

Tumor samples and cell culture

Tumor samples were stored in liquid nitrogen immediately after surgical removal. Following xenografting of tumor cells from the glioblastoma cell line T3868 into nude mice, the glioblastoma cell line TX3868 was established. Cell culture was performed as described previously (Fischer et al. 1994).

DNA extraction

High molecular weight DNA from peripheral blood lymphocytes and tumor samples was isolated according to standard protocols (Sambrook et al. 1989).

RNA extraction

Cytoplasmic RNA was isolated according to Gough (1988). Briefly, cells were lysed with the detergent NP40 and proteins were denatured with sodium dodecyl sulfate and urea. Following phenol-chloroform extraction RNA was precipitated with sodium acetate (0.3 M) and isopropanol.

Southern transfer

Genomic DNA (5 µg) from tumor tissue were completely digested with *Eco*RI, and the fragments were separated by electrophoresis in a 0.8% agarose gel. The DNA was alkali denatured in 0.4 M NaOH, prior to transfer to nylon filters by the method of Southern (Sambrook et al. 1989). After transfer, the blots were neutralized in 0.05 M sodium phosphate buffer, pH 6.5, and the DNA was bound to the membrane by UV-crosslinking.

Northern transfer

Denatured RNA (20 µg) was fractionated by formaldehyde gel electrophoresis according to Sambrook et al. (1989). The RNA size marker used was 16S/23S rRNA of Escherichia coli (Boehringer Mannheim). RNA was transfered to nylon membranes using phosphate buffer, pH 6.5. Prehybridization was carried out in 500 mM phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS at 65°C for 30 min.

Hybridization

The DNA probes (30–60 ng) were radioactively labeled with ^{32}P by using random primers and added to prehybridized filters. The hybridization solution contained 500 mM phosphate buffer, pH 7.2, 1 mM EDTA and 7% SDS at 65°C. Probes used for clone characterization were MDM2, sarcoma amplified sequence (SAS), cyclin-dependent kinase (CDK4), osteosarcoma (OS-4), and glioma (GLI) (pKK36) (Su et al. 1994).

Results

DNA preparations from three glioma samples were hybridized with six different probes previously assigned to chromosomal region 12q13–15. The gliomas included two glioblastomas (T3564, TX3868) and one astrocytoma WHO-Grade III (G1284). The probes included sequences of the genes GLI, WNT1, MDM2, SAS, CDK4, and OS-4. All genes were found to be amplified in tumor T3564. As demonstrated in Fig. 1, the level of amplification was similar between the different genes in tumor T3564. In addition, six novel isolated genes termed “glioma amplified sequences” (GAS16, GAS27, GAS41, GAS56, GAS64

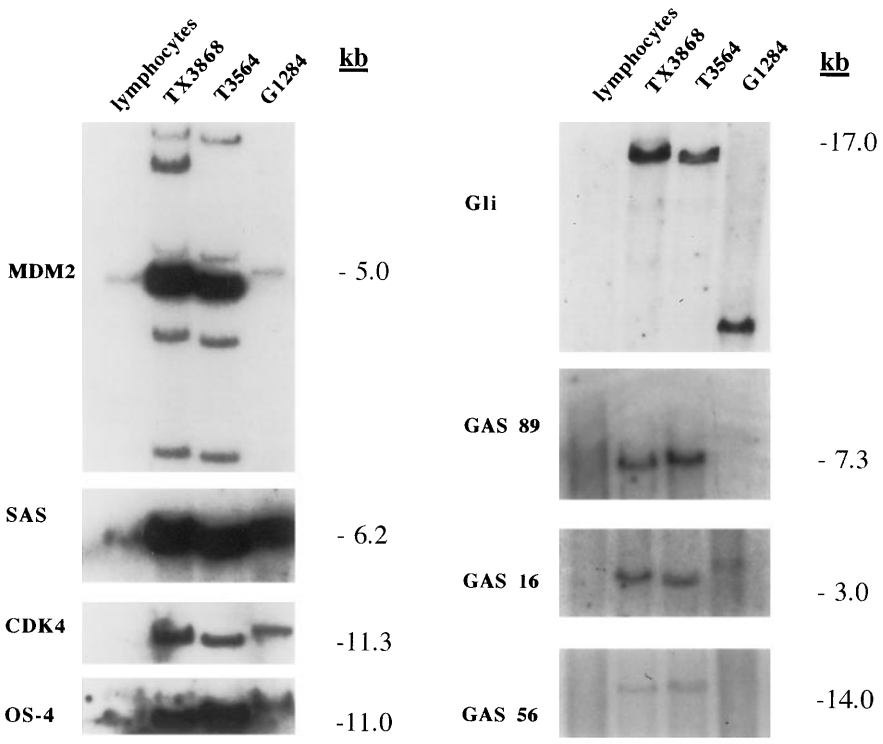

Fig. 1 Amplification pattern of several genes from chromosomal region 12q13–15. DNA 5 µg from normal blood lymphocytes, glioblastoma cell lines TX3868 and T3564, and astrocytoma G1284 was digested with *Eco*RI, separated on a 0.8% agarose gel and transferred to nylon membrane. Southern blots were sequentially hybridized with probes for the genes MDM2, SAS, GLI, CDK4, OS-4, GAS89, GAS16 and GAS56. Sizes of hybridizing restriction fragments are indicated

Table 1 Amplification status of genes at 12q13–15. +++ strong amplification, + weak amplification, – single copy, * rearrangement

Tumor	Gene											
	GLI	WNT1	MDM2	SAS	CDK4	OS-4	GAS16	GAS41	GAS27	GAS64	GAS89	GAS56
TX3868	+++	–	+++, *	+++	+++	+++	+++	+++	+++	+++	+++	+++
T3564	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
G1284	+++, *	–	–	+++	+++	+	+	–	–	–	–	–

and GAS89) localized within 12q13–15 were also amplified in tumor T3564 as shown in Fig. 1. This brings the total number up to 12 amplified genes, all of which localized within the same amplification unit. In tumor TX3868 all but one (WNT1) of the known genes were amplified. All of the GAS genes were also amplified in TX3868 cells (Gracia et al. 1996). In the astrocytoma G1284 we identified amplification of four of the known genes including OS-4, GLI, SAS, and CDK4. The amplification level of the OS-4 gene was significantly lower than the amplification of the genes GLI, SAS, and CDK4. Of the newly isolated genes only the gene GAS16 was amplified in tumor G1284. The results are summarized in Table 1.

Expression of the amplified genes GLI, WNT1, MDM2, SAS, CDK4 and OS-4 was analyzed by Northern blotting. All six genes showed expression in tumor TX3868. In tumor T3564 five genes were tested and were also found to be expressed. All of the previously isolated GAS genes, which were amplified in TX3868 and T3564, were expressed in these tumors. Northern blot analysis failed to reveal any expression of the genes CDK4 and OS-4 in tumor G1284. Additional genes including MDM2, SAS, GLI and the newly isolated GAS genes were not tested for expression because RNA was unavailable from tumor G1284.

Discussion

DNA amplification has been reported in multidrug-resistant cells and in human tumors but not in normal human cells. Several lines of evidence indicate rather extended amplified DNA domains, which are likely to harbor numerous as yet unidentified genes. Specifically, many human tumors carry HSRs and d mins that are up to several megabases in length. A more detailed molecular analysis of amplification units has been performed in a few tumor types.

The occurrence of DNA amplification of 12q13–15 has been reported in several human neoplasms including glioblastoma (Khatib et al. 1993; Forus et al. 1993). Previously, we identified an HSR that contains sequences from 12q13–15 in glioma (Gracia et al. 1996). Amplified genes within this chromosomal region include GLI, CDK4, MDM2, and SAS (Reifenberger et al. 1993). The identification of several amplified genes within a single domain leads to the question which genes contribute to tumor development and/or tumor progression. The concept of target genes is based on the assumption that the amplification of one or a few genes has a biological impact whereas other genes are coamplified as bystanders. Generally, genes that are amplified most frequently in a single amplified domain are considered target genes. Recent data, however, indicate that such a simplified concept might be inadequate to describe the in vivo situation.

SAS and CDK4 are most frequently amplified in 12q13–15 in glioblastoma. These data place SAS and CDK4 as target genes in the center of the amplification unit on 12q13–15 (Reifenberger et al. 1993). There are, however, gliomas that carry an amplified MDM2 gene but no amplified SAS and CDK4 genes. A study conducted on a sarcoma cell line with 12q amplification has yielded additional genes that were amplified in sarcoma but not necessarily in glioma-whereas the glioma-derived genes were mostly amplified in glioma and only rarely in sarcoma. Possibly, there are several target genes or even independent amplification units in 12q13–15. The large number of amplified genes identified in this study provides further evidence that several genes at 12q13–15 might be important for tumor progression and development in glioma. Based on the limited knowledge of the physiological functions of SAS, CDK4, MDM and GLI it is legitimate to postulate an amplification-related role for each of these genes in neoplastic pathways. SAS is involved in signal transduction pathways, CDK4 in cell cycle control, GLI has a function as a transcription factor during embryonic development and MDM2 functions as a regulator of p53 (Sherr 1993; Jankowski et al. 1994; Ruppert et al. 1991; Oliner et al. 1992). For the recently cloned subset of genes termed GAS, no information on their potential functions is available.

Expression studies are a necessary step toward the identification of biologically relevant amplified genes. Previous investigations have not shown a consistent correlation between amplification and expression of genes at 12q13–15 in glioma (Reifenberger et al. 1994). Our studies demonstrate expression of all but one gene amplified at 12q13–15 in two glioblastomas. The expression data provide further evidence that more than one amplified gene at 12q13–15 may be important in the pathology of glioma. It is conceivable that gene amplification is necessary but not sufficient for specific disregulation of expression. The impact of amplification might depend on various parameters such as cell cycle, tumor stage and cell environment. To evaluate further the biological role of the amplified genes it will be necessary to identify all genes included in a given amplicon. Subsequently, single genes can be evaluated for their function in neoplastic pathways.

In summary, our study reports two glioblastomas with 12 and 11 genes, respectively, localized in a single amplicon. All tested genes were found to be expressed in both gliomas. Out of the 12 genes only 5 were amplified in an astrocytoma grade III. Based on this study the gene content of additional amplifications frequently found in human tumors warrants further investigation. The increasing number of studies using comparative genomic hybridization will significantly help to establish maps of frequent amplification events in human tumors. These data can in turn be used to search for novel amplified genes and subsequently to determine their biological function.

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