A Different View on DNA Amplifications Indicates Frequent, Highly Complex, and Stable Amplicons on 12q13-21 in Glioma

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Abstract
To further understand the biological significance of amplifications for glioma development and recurrences, we characterized amplicon frequency and size in low-grade glioma and amplicon stability in vivo in recurring glioblastoma. We developed a 12q13-21 amplicon–specific genomic microarray and a bioinformatics amplification prediction tool to analyze amplicon frequency, size, and maintenance in 40 glioma samples including 16 glioblastoma, 10 anaplastic astrocytoma, 7 astrocytoma WHO grade 2, and 7 pilocytic astrocytoma. Whereas previous studies reported two amplified subregions, we found a more complex situation with many amplified subregions. Analyzing 40 glioma, we found that all analyzed glioblastoma and the majority of pilocytic astrocytoma, grade 2 astrocytoma, and anaplastic astrocytoma showed at least one amplified subregion, indicating a much higher amplification frequency than previously suggested. Amplifications in low-grade glioma were smaller in size and displayed clearly different distribution patterns than amplifications in glioblastoma. One glioblastoma and its recurrences revealed an amplified subregion of 5 Mb that was stable for 6 years. Expression analysis of the amplified region revealed 10 overexpressed genes (i.e., KUB3, CTDS2, CDK4, OS-9, DCTN2, RAB3IP, FRS2, GAS41, MDM2, and RAP1B) that were consistently overexpressed in all cases that carried this amplification. Our data indicate that amplifications on 12q13-21 (a) are more frequent than previously thought and present in low-grade tumors and (b) are maintained as extended regions over long periods of time. (Mol Cancer Res 2008;6(4):576–84)

Introduction
DNA amplification does not occur in normal human cells but in multidrug-resistant cells and in tumor cells. Numerous studies described gene amplification in various human tumors by cytogenetic and molecular genetic means. The breakage-fusion-bridge cycle (1) is the most popular model to explain intrachromosomal amplifications and many amplified structures like mixed ladders that were found in homogeneously staining regions (2). The “episome model” proposes that epimapses result from excision of small circular DNA that enlarges by overreplication or recombination until it becomes cyogenetically visible as double minute chromosomes (3). Recent results of Tanaka et al. (4) show that large palindromic sequences were present in human cancers and the location of palindromes in the cancer genome serves as a structural platform to support subsequent gene amplification. There is, however, a very preliminary overview picture on where and how amplifications arise in different human tumors. Previous concepts suggested shortening of extended DNA amplifications over time, leaving primarily those genes amplified that contribute to tumor progression. This idea of single selected target genes is likely to hold true for some amplified domains but has been increasingly challenged as a general concept. Glioblastomas were characterized by a severe genomic instability and by frequent amplifications visible predominantly as double minute chromosomes and less frequently as a homogeneously staining region. They show DNA amplifications most often at 7p13 and 12q13-15 (5, 6). Refined mapping of the amplicon at chromosome region 12q13-15 in human malignant glioma suggested CDK4/SAS and MDM2 as independent amplification targets while excluding a common amplification target between CDK4/SAS and MDM2 (7). Previously, we cloned several genes from the amplified domain at 12q13-15 including GAS41 (glioma expressed sequence), KUB3 (Ku70 binding protein 3), and CYP27B1 (8-11). Whereas CYP27B1 was amplified only in glioblastoma, KUB3/XRCC6BP1 was amplified both in glioblastoma and anaplastic glioma and GAS41/YES4 in glioblastoma, anaplastic glioma, and pilocytic astrocytoma. These data indicated a rather complex situation for the amplicon at 12q13-15 in glioma.

Results
Amplicon Array Design and Evaluation
We generated a genomic array for chromosome region 12q13.11-q21.31 containing 39 BAC clones, 2 cosmid clones, and 1 PAC clone with an average coverage of 2 to 3 clones per
megabase. This array allowed detailed analysis of DNA copy number changes within a chromosome region that spans up to 15.4 Mb in length (Fig. 1). We included total genomic DNA as hybridization control. As detailed in Materials and Methods, the experiments were done in triplicates and the mean fluorescence intensity ratios of Cy3/Cy5 (tumor/reference) of each spot were normalized against total genomic DNA. The logarithm (log 2) of the normalized Cy3/Cy5 ratios of 40 glioma, one glioblastoma cell line, and a peripheral blood lymphocyte control is shown in Fig. 2A.

To test for array reliability, we conducted control experiments using normal peripheral blood DNA versus normal peripheral blood DNA. Ratio values ranged from 0.83 to 1.15 with an average of 0.99 for chromosome 12 specific probes. Shapiro-Wilk normality test showed normal distribution of ratio values for control experiments with a P value of 0.78. To distinguish between polysomy, ploidy, and amplifications, we determined the ratio values from tumors with at least a gain of one copy of chromosome 12. These ratio values were elevated for chromosome 12 derived probes with a normal distribution. For example, glioblastoma H1347 showed cytogenetically an additional chromosome 12 in 9 of 10 mitoses. The additional copy was reflected in ratio values ranging from 1.01 to 1.61, with an average value of 1.22. As shown in Fig. 2A, this gain of chromosome 12 was clearly distinguishable from a gain indicated by four BAC clones (RP11-578B16, RP11-366L20, RP11-305O6, and RP11-1029F8).

Prediction of Amplification

A straightforward criterion for amplification is to consider all clones as amplified that show Cy3/Cy5 ratios greater than a given constant threshold. However, despite normalization, some arrays contain only normalized values of <1. Therefore, a more sensitive definition of amplifications that allows for handling normal variations of the experimental data is needed. A more appropriate measure is the z-score, which is defined for clone i and array X as

\[
z_{i,x} = \frac{X_i - \bar{X}}{\sqrt{\frac{1}{n} \sum_{i=1}^{n} (X_i - \bar{X})^2}}
\]

where \(X_i\) is the normalized value of clone i on array X, and \(\bar{X}\) is the mean of all normalized values. We analyzed the z-scores of 36 randomly selected clones. Of the 36 clones, 16 clones were amplified and 20 clones were not amplified as validated by Southern blot (data not shown). The z-scores of the clones that are not amplified ranged between 0.52 and 0.46 with a mean

![FIGURE 1. BAC, PAC, and cosmides clones and their localization on chromosome 12.](image-url)
value of \(-0.23\), whereas the \(z\)-scores of the amplified clones ranged between 2.02 and 4.54 with a mean value of 3.1. The histogram plot for the two distributions of amplified and not amplified clones presented in Fig. 3A shows that amplified regions can be clearly separated from not amplified regions. An unpaired two-sample Wilcoxon Mann-Whitney test for the two distributions yielded a \(P\) value <10^{-10}. Consequently, we could define all clones on array \(X\) with \(z_{i,X}\) above a threshold \(Z\) as amplifications. The \(z\)-score criterion was tested on the above introduced dataset, and a receiver operator characteristic curve with the respective area under the curve was computed. The area under the curve value for a random classification would be 0.5; a classification without any error lead to an area under the curve value of 1. Our predictions of amplifications obviously yielded an area under the curve value of 1 because the histogram of \(z\)-scores shows no overlap of amplified and not amplified clones as presented in Fig. 3A. In summary, we achieve a sensitivity, specificity, and accuracy of the \(z\)-score prediction method of 1 as detailed below.

Amplification Analysis in View of the Distribution of Cy3/Cy5 Ratio Values

Despite the high accuracy of our test set, \(z\)-scores entail some disadvantages. If the variance of the Cy3/Cy5 ratios for a certain tumor is very small, values deviating slightly from the mean of the distribution lead to high \(z\)-scores. In contrast, if the variance and the mean value are high (e.g., due to strong amplifications), the \(z\)-score is very likely to ignore low-level amplifications. To overcome these problems of the \(z\)-score prediction, we took advantage of the normally distributed control comparative genomic hybridization profiles. As mentioned above, the Shapiro-Wilk normality test showed that Cy3/Cy5 ratios of the control experiments with normal DNA versus normal DNA were normally distributed. In contrast, the Shapiro-Wilk normality test of all but one of the 40 glioma arrays yielded significantly decreased \(P\) values ranging from <10^{-14} to 0.93 with a mean value of 0.16, indicating that the Cy3/Cy5 ratios of most tumors do not show a normal distribution. For example, the distribution of the Cy3/Cy5 ratios of tumor H1056 revealed a normal distribution and indicated 14 “outliers” as shown in Fig. 3B. This observation motivated us to implement the following prediction method. We consecutively removed the highest Cy3/Cy5 ratios of a given array as long as the \(P\) values of the Shapiro-Wilk normality test of the remaining ratios indicate a convergence to a normal distribution. All clones removed by this procedure and all clones whose ratios were increased at least 1.2-fold compared with the mean value of the remaining normal distribution are considered as amplified. This refined prediction method performed as well as \(z\)-scores, yielding a specificity of 1 (20 of 20). We predicted 20 BAC clones to be not amplified, and Southern blot analysis revealed no amplifications for these 20 BAC clones. We predicted 16 BAC clones to be amplified, and again Southern blot analysis confirmed these predictions.
Thus, the prediction method yielded a sensitivity, specificity, and accuracy of 1 (36 of 36). The result of the prediction specifying the amplified clones is shown in Fig. 2B. The amplified subregions varied in size from <0.5 Mb (represented by a single BAC clone) to 5 Mb (represented by 12 BAC clones).

Amplification Examination by Computational Prediction, Fluorescence In situ Hybridization Analysis, and Southern Blot

Using the array and prediction method described above, we analyzed the amplicon structure of 40 glioma samples including 16 glioblastoma (including glioblastoma cell line TX3868), 7 pilocytic astrocytoma, 7 grade 2 astrocytoma, and 10 anaplastic astrocytoma. The WHO grading of the 40 glioma samples and corresponding age and sex distribution of the patients are summarized in Table 1. We found highly variable amplification patterns in glioma with various amplified subregions as shown by the log 2 Cy3/Cy5 ratios of the 40 arrays (Fig. 2A). Analyzing these arrays by the refined prediction method, we detected 179 amplifications (10.7% of all clones), most of them in glioblastoma. Seven glioblastoma showed only one amplicon spanning one to five BAC clones, and the remaining glioblastoma showed two and more amplicons that varied considerably in size (Fig. 2B). Likewise, the majority of pilocytic astrocytoma showed several amplicons. Many regions overlap with regions amplified in anaplastic astrocytoma or glioblastoma. For example, amplifications indicated by clones RP11-305O6, RP11-629N8, RP11-58A17, or RP11-571M6 are found both in glioblastoma and pilocytic astrocytoma. In contrast to the amplicons in glioblastoma, amplicons in pilocytic astrocytoma and astrocytoma WHO grade 2 are more frequently represented by single BAC clones, indicating a smaller average size of those amplicons. Whereas amplicons in glioblastoma preferentially cluster in two chromosome regions, amplicon distribution in low-grade astrocytoma shows a more scattered pattern. Amplifications in astrocytoma WHO grade 2 were less frequently detected in our analysis. In summary, and

Table 1. Additional Information on Glioma Samples

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Sex</th>
<th>Age (y)</th>
<th>WHO Grade</th>
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<tbody>
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<td>39</td>
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<td>1</td>
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<tr>
<td>H22</td>
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<td>1</td>
</tr>
<tr>
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<td>11</td>
<td>1</td>
</tr>
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<td>3</td>
<td>1</td>
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<td>61</td>
<td>1</td>
</tr>
<tr>
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<td>4</td>
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<td>2</td>
</tr>
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</tr>
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</tr>
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</tr>
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<td>55</td>
<td>4</td>
</tr>
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</tr>
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</tr>
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<td>60</td>
<td>4, 4th recurrent</td>
</tr>
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</tr>
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</tr>
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<td>59</td>
<td>4</td>
</tr>
<tr>
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<td>4</td>
</tr>
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</tr>
<tr>
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<td>38</td>
<td>4</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>H465</td>
<td>Female</td>
<td>63</td>
<td>4</td>
</tr>
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</table>
as visualized in Fig. 2B, distribution of amplicons along the analyzed chromosome region seems to be different between glioblastoma/anaplastic astrocytoma and pilocytic astrocytoma/astrocytoma.

We also analyzed amplifications by fluorescence in situ hybridization (FISH). As shown in Fig. 2B, our prediction for case H863 identified two amplified subregions, with one of them represented by BAC clone RP11-58A17. This BAC clone includes the KUB3 gene. Smear preparation of pilocytic astrocytoma H863 identified in 15% of interphase nuclei a KUB3 amplification visible as multiple fluorescence signals of a KUB3 probe and only two to three fluorescence signals of a chromosome 12 probe (Fig. 4A). The remaining 85% of interphase nuclei revealed two fluorescence signals of KUB3 and chromosome 12 (Fig. 4B). In astrocytoma H1631, we predicted an amplification of a region represented by BAC clone RP11-268A19. FISH analysis confirmed this amplification as shown in Fig. 4C. In anaplastic astrocytoma H1381, we detected the predicted amplification of CYP27B1 (Fig. 4F). Our prediction method revealed an additional amplification in the region of RP11-268A19 not included in the homogeneously staining region in the glioblastoma cell line TX3868 (Fig. 4G). Our results from the FISH experiments strongly indicate the formation of double minute chromosomes especially in low-grade glioma and amplicons represented by a single BAC clone. In summary, our FISH analysis confirmed many amplifications and detected further amplifications that were not predicted, indicating an even higher frequency of amplifications in glioma.

Furthermore, we used KUB3, CDK4, and GLI localized at 12q13.3-14 to analyze the amplification in glioblastoma H385 and H981 by Southern hybridization. As predicted by our refined prediction method (Fig. 2B), glioblastoma H385 revealed amplification of the genes CDK4 and GLI without KUB3 amplification, whereas glioblastoma H981 revealed KUB3 and CDK4 amplification without GLI amplification (Fig. 5A).

**Amplicon Maintenance In vivo**

Our analysis included a primary glioblastoma that was initially diagnosed in 1999 (H555) and four recurrent tumors (diagnosed as glioblastoma) that were resected in 1999 (H595), 2000 (H1056), 2002 (H1300), and 2005 (H1453). We were thus able to address the in vivo behavior of the amplicon 12q13.15 over a period of 6 years. As shown in Fig. 2B, the initial tumor H555 displayed two small amplicons represented by a single BAC clone each. The first recurrent tumor, H595, carried six amplified subregions that differed from the amplicons in the initial tumor. The largest amplified subregion of H595 was represented by 12 BAC clones (RP11-186F10 to RP11-754J5) and one smaller amplicon was represented by two BAC clones...
That overall amplicon structure was largely maintained over a period of 6 years as shown by the analysis of the recurrent glioblastoma. Maintenance of the large amplified region at 12q15-21 in four recurrent glioblastoma was confirmed by Southern hybridizations using probes specific for genes LGR5 (12q21), FRS2, GAS41, MDM2, and DYRK2 (12q15; Fig. 5B). Whereas the size of the amplicon remained stable, the copy number varied. The highest copy number was detected in glioblastoma H1056. We found a comparable decrease in the copy number of all analyzed genes in the subsequent two recurrences, H1300 and H1453. Tumor H1453 also showed a deletion of one copy of chromosome 12. The maintenance of a large amplified region, spanning ~5 Mb, supports the idea that several genes within this amplicon contribute to selection advantages for the tumor cells under changing conditions.

**Gene Expression Analysis**

Chromosome region 12q13.3-14 contains more than 50 genes as indicated in GenBank. For the expression analysis, we selected genes mapping within subregions that were frequently amplified as shown by hybridization to BAC clones. We found overexpression of genes KUB3, CTDSP2 (OS-4), CDK4, and OS-9 in three glioblastoma (H981, H346, and H549) as compared with normal brain (Fig. 6A). The level of expression varied among the three glioblastoma: KUB3 expression was highest in glioblastoma H981 whereas CDK4, OS-9, and CTDSP2 revealed the highest expression in glioblastoma H346 and H549. The genes KIF5A and GLI were not consistently overexpressed in the glioblastoma that carried the amplification. We also examined the expression of LGR5, RAB3IP, FRS2, GAS41, MDM2, and RAP1B in three of the recurrences (H595, H1300, and H1453) and two glioblastoma (H17 and H981) that carry a comparably small amplicon in region 12q15 (Fig. 6B). MDM2 expression was highest in glioblastoma H17 and H981 and expression of (RP11-571A2 and RP11-318E11). That overall amplicon structure was largely maintained over a period of 6 years as shown by the analysis of the recurrent glioblastoma. Maintenance of the large amplified region at 12q15-21 in four recurrent glioblastoma was confirmed by Southern hybridizations using probes specific for genes LGR5 (12q21), FRS2, GAS41, MDM2, and DYRK2 (12q15; Fig. 5B). Whereas the size of the amplicon remained stable, the copy number varied. The highest copy number was detected in glioblastoma H1056. We found a comparable decrease in the copy number of all analyzed genes in the subsequent two recurrences, H1300 and H1453. Tumor H1453 also showed a deletion of one copy of chromosome 12. The maintenance of a large amplified region, spanning ~5 Mb, supports the idea that several genes within this amplicon contribute to selection advantages for the tumor cells under changing conditions.

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RAB3IP and RAP1B was highest in the two recurrences H595 and H1300. The MDM2 expression in these two recurrences was higher than in normal brain. LGR5 was the only gene that was amplified but not overexpressed. Our results show that the highly variable expression level of the amplified genes does not depend on the gene localization within this amplicon.

Discussion

Highly heterogeneous amplicons as a result of extensive genomic rearrangements both in copy number and size have been suggested as early as 1992 (12). Recently, a very complex pattern of amplifications was described on 7p for glioblastoma, raising the possibility that amplified genes of this region, other than EGFR, may be important for tumor formation and progression (13, 14).

One of the concepts about amplification-driving mechanisms postulates single target genes and reduction of the initial amplicon size along with numerical reduction of coamplified genes (15). The diversity of the amplification patterns in the different glioma argues against the idea of single target genes. Eley and coworkers suggest that amplicons can be of either simple or complex nature. Simple amplicons generally retain their native chromosomal configuration and involve a single amplification target whereas complex amplicons undergo substantial reorganization and contain multiple target sequences (16). Our data indicate that the analyzed region has undergone extensive genomic rearrangements with likely more than one initial amplification event and multiple rounds of breakage-fusion-bridge cycles.

Besides the structure of the amplicon, its maintenance over time may shed light on its biological relevance. An amplified region of 5 Mb represented by 12 BAC clones was largely maintained over 6 years in four recurrent glioblastoma. This finding is in contrast to the previous notion that initially large amplified regions will shorten over time. It was hypothesized that mainly a gene that confers growth advantage is maintained in the amplified domain. However, observations supporting this idea were largely based on studies with drug-resistant cell lines. Cells were cultured under selective pressure that drives amplification of a gene that accounts for drug resistance. The scenario in tumor cells is very different with a highly complex environment that affects various genes. The tumor microenvironment-specific requirements for survival and growth advantage define the cancer genes that possess the power to support cancerous growth of the malignant cell when amplified (17). As of yet, however, amplification studies in human tumors were largely restricted to single time points. Recurrent tumors allow for following the development of an amplicon in vivo. As for glioblastoma, two recurrences are extremely rare and thus hampering the analysis. The in vivo maintenance of a large amplicon at 12q14-21.1 strongly indicates that several of the enclosed amplified genes contribute to selective growth advantages. That does not imply that all amplified genes are consistently overexpressed. Although the number of cases studied thus far is small, it is legitimate to speculate that development and maintenance of this amplicon is related to the treatment, including radiotherapy and chemotherapy. While acknowledging the difficulty to identify recurrent tumors with specific amplifications, our results show the usefulness of such an approach to better understand the biology of extended amplicons for tumor growth.

Amplifications are mostly reported in human tumors of advanced stages and are considered late events in tumor progression. Both our computational prediction and our FISH analysis show that amplifications within region 12q13-15 occur in pilocytic astrocytoma and astrocytoma WHO grade 2. The frequency and the pattern of the amplifications are clearly different between glioblastoma and low-grade astrocytoma. With similar approaches, future studies will likely identify frequent and specific amplification patterns in other low-grade tumors.

Our prediction method was able to detect a high-copy amplification in a very heterogeneous cell population of a pilocytic astrocytoma with only 15% of the cells showing this amplification, making it a valuable tool to analyze amplifications in low-grade tumors and to analyze amplification formation and progression. The further analysis of amplifications in tumor recurrences will shed light on the impact of amplification as a mechanism to adopt and to survive.

Our approach also helps to understand the biological effects of the analyzed amplicon in that it defines the most frequently amplified subregions as indicated by BAC and cosmide clones. Chromosome region 12q13.3-14, which was most frequently amplified in our glioma samples, contains various genes including CYP27B1, CDK4, OS-9, TSPAN31, METTL1, TSPM, MARCH9, AVIL, CTDS2P, and KUB3, many of which have recently been associated with tumor development. Expression analysis showed most frequently overexpression of KUB3, CTDS2P, CDK4, and OS-9. The gene KUB3 (XRCC6BP1) encodes a Ku70 binding protein, which is part of the DNA-dependent protein kinase complex involved in double-strand break repair. We have preliminary evidence that KUB3 (XRCC6BP1) gene amplification affects double-strand break repair in glioblastoma cell lines (18). OS-9 may function as an inhibitor of hypoxia-inducible factor 1α–mediated transcription in osteosarcoma. Modulation of OS-9 expression might be a means to alter the set point of the oxygen-sensing system (19). Because both KUB3 and OS-9 also confer properties to survive in hypoxic conditions via altered double-strand break repair and/or altered oxygen sensing, overexpression of KUB3 and OS-9 may contribute to growth advantages in glioblastoma cells. CDK4 overexpression provides advantages for cell proliferation. The functional advantage of CTDS2P overexpression in glioblastoma remains unclear.

The 12 BAC clones that indicate the preserved amplicon in recurrent glioblastoma contain 20 genes including LGR5, RAB3IP, FRS2, GAS41 (Y484T4), MDM2, MDM1, DYRK2, IFNG, and RAP1B. The most elevated expression levels were found for MDM2, RAB3IP, and RAP1B. Overexpression of MDM2 is known to be involved in escape mechanisms of TP53-mediated apoptosis. It is legitimate to speculate that the amplification and overexpression of MDM2 in glioblastoma also contribute to the escape from TP53-mediated apoptosis in glioblastoma. Rap1B, which functions as a positive regulator of angiogenesis (20), may contribute to the high vascularization...
found in glioblastoma. The RAB3IP that we first reported as a glioma-amplified sequence (GAS64; ref. 21) seems to have diverse functions according to the tissue in which it is expressed (22). In summary, gene amplification and overexpression seem to contribute to the various characteristics of human glioblastoma including cell proliferation, radioresistance, and vascularization.

Materials and Methods

BAC, PAC, and Cosmid Clones

BAC and PAC clones were from RP-5 and RP-11 libraries of the Welcome Trust Sanger Institute. Cosmid clones specific for CYP27B1 (LLNLc132M0263Q2) and GLI (LLNLc132M166 2Q2) were obtained from RZPD (German Resource Center for Genome Research).

DNA Preparation

Glioma samples were obtained from the Department of Neurosurgery at the Saarland University. Genomic DNA was extracted from cell cultures, tumor samples, or blood lymphocytes as previously described (8).

Array Preparation, Hybridization, and Detection

Target DNA was amplified by degenerate oligonucleotide–primed PCR using three degenerate oligonucleotide primers as described (23). PCR products from three degenerate oligonucleotide–primed PCRs were pooled and printed in triplicate on PicoArrays by PicoRapid Technologie GmbH. Equal amounts of Cy3-labeled tumor and Cy5-labeled reference DNA were hybridized to arrays for 48 h at 37°C. Slides were scanned and imaged on a ScanArrayLite scanner (Perkin-Elmer) using the ScanArrayExpress software version 2.1.8.

Data Preparation and Normalization

First, we computed intensities of Cy3 and Cy5 by subtracting the median local background intensities from the median local fluorescence intensities for each spot. Second, we calculated fluorescence intensity ratios of Cy3/Cy5 (tumor/reference). Because each clone is represented by three spots, the mean values of the triplicate was computed. If the intensity of the spots deviated from the mean value by >20%, the mean value of the triplicate was computed. If the intensity of the reference (Yo) was less than the background, the background value was used. If the intensity of the reference (Yo) was greater than the background, the background value was subtracted from the reference intensity (Yo) to ensure that the background value was not negative. This resulted in a measured ratio in tumor cells of 6% of all data points. We determined the final quotient of Cy3/Cy5 as the mean value of all three measured ratios in ~94% of the clones, as the mean value of two measured ratios in ~6%, and as single ratio in ~0.1% of the clones. For normalization, all Cy3/Cy5 ratios were divided by the ratio of the genomic control DNA.

FISH

FISH were prepared on either smear preparations or against interphase nuclei from cell culture preparations. The chromosome 12 centromere probe was D12Z1 from American Type Culture Collection and the KUB3 probe was a 3-kb EcoRI fragment from cosmid clone 1F8. The centromere probe was labeled with digoxigenin-11-dUTP and KUB3 was labeled with biotin-16-dUTP. Two hundred fifty nanograms each were coprecipitated with Cot1 DNA and hybridized at 37°C overnight. Biotin-labeled KUB3 probe was visualized by avidin conjugated to FITC. Centromere chromosome 12 probe was visualized with Cy3-conjugated goat-anti-digoxigenin antibodies. The signals were amplified once. Interphase nuclei from cell culture preparations were hybridized with 50 ng of Cy3- or Cy5-labeled BAC or cosmid DNA as described for array hybridization. Denatured FITC-labeled chromosome 12 α-satellite probe (D12Z3) was added to the hybridization mix.

Southern and Northern Hybridization

Hybridizations were done as previously described (10). Hybridization probes for CDK4, CDTSP2, OS-9, and MD2 were kindly provided by Paul Meltzer (NIH, Bethesda, MD). Hybridization probes for KUB3, GAS41, RAB3IP, and GLI (pkk36) were used as previously described (9, 10, 20). Hybridization probes for LGFR, FRS2, and RAP1B were PCR products generated from cDNA sequences.

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