Identification of Differentially Expressed Genes in Human Gliomas by DNA Microarray and Tissue Chip Techniques


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ABSTRACT

New genomic large-scale screening techniques have made the task of establishing an accurate molecular fingerprint of cancer cells feasible. Here, we have used a two-phase strategy for identification of molecular alterations in gliomas. First, cDNA microarrays (Clontech Laboratories, Inc., Research Genet[ics), were used to pinpoint differentially expressed genes between normal brain and diffuse astrocytomas (grades II–IV), and between a primary tumor and a later tumor reoccurrence in the same patient. More than 200 gene expression alterations were detected from glioblastomas, whereas relatively few changes were seen in grade II and grade III tumors. The most distinct progression-related expression change was the up-regulation of the insulin-like growth factor binding protein 2 (IGFBP2) gene. Second, a high-density tissue microarray of 418 brain tumors was constructed and used for clinical validation of gene expression changes. Strong expression of IGFBP2 was associated with progression and poor patient survival in diffuse astrocytomas (P < 0.0001). Third, comparisons of the data between (a) multiple spots retrieved from one predefined tumor region (IGFBP2 and vimentin immunohistochemistry, 20 tumors) or between (b) standard slides and arrayed tissues (p53 immunohistochemistry, 42 tumors) revealed very little variation. In conclusion, the combined use of DNA microarrays and tissue microarrays offers a powerful strategy for rapid identification and thorough characterization of differentially expressed genes in gliomas.

INTRODUCTION

Several genetic aberrations and gene expression changes have been shown to occur during malignant transformation, development, and progression of gliomas (1). Characterization and cataloging of the genetic alterations and molecular markers that correlate with the clinical behavior of gliomas are needed to establish the comprehensive molecular fingerprint of these tumors. Such molecular profiling of gliomas may eventually lead to targeted therapeutic approaches that could improve the clinical outcome for patients.

New genomic high-throughput technologies, such as DNA microarrays, may facilitate considerably the molecular profiling of human tumors. Thousands of genes can now be analyzed in a simple microarray hybridization. Low-density nucleic acid arrays (cDNA arrays) have already been used for gene expression analyses of a few glioma samples (2). The expression profile from a single tumor reflects the state of events of an individual malignancy at a certain time point. To generalize the findings and provide conclusive evidence for the involvement of a molecular alteration, it is often necessary to analyze several hundred tumors. Using traditional molecular pathology, such verification could take several months, or even years, to complete. To facilitate translational research in a large-scale manner, recently we developed technology for making high-density arrays of tissue specimens (tissue microarrays, tissue chips; Ref. 3). These arrays can be used for rapid in situ evaluation of gene copy number and expression simultaneously in thousands of tumors.

Here, we have combined DNA microarray and tissue-chip techniques in a rapid two-step screening approach for the identification of differentially expressed genes in human gliomas. Hybridization of labeled cDNAs from tumor samples to cDNA arrays was used for the detection of differences in gene expression between grades II–IV diffusely infiltrating astrocytomas and between the original and the recurrent astrocytoma. Subsequently, a tissue microarray of hundreds of brain tumors, mainly of astrocytic origin, was used for the validation and additional characterization of the aberrant gene expression patterns.

MATERIALS AND METHODS

The brain tumor samples were obtained from patients who underwent surgery at the Tampere University Hospital, Tampere, Finland, during 1983–1998. One neuropathologist (H. H.) evaluated all of the tumors, and the histopathological typing and malignancy grading were done according to the criteria presented by WHO in 1993 (4). In the present context, the term “diffuse” (diffusely infiltrating) astrocytoma refers to grades II–IV astrocytomas [WHO grade II corresponding to grade 2 by the St. Anne-Mayo method, grade III (anaplastic) to grade 3, and grade IV (glioblastoma) to grade 4, respectively]. Diffuse astrocytomas exclude the more circumscribed pilocytic astrocytomas (grade I).

cDNA Microarray

Seven primary astrocytomas [two grade II astrocytomas, two grade III astrocytomas, and three glioblastomas (grade IV)] were collected during surgery. In addition, two samples from one patient were collected, the first one from the original occurrence (grade III astrocytoma) and the other sample from the regrowth of the tumor 8 months later (also grade III astrocytoma). All of the samples were freshly frozen in liquid nitrogen and stored at −70°C until use. Total cellular RNA was extracted from the tumor samples using an RNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. For analysis reference, pooled total RNA from normal human brain was used (Clontech Laboratories, Inc., Palo Alto, CA).

cDNA Microarray Hybridization with a Membrane of 588 Individual cDNA Clones as Targets. Equal amounts of total RNA from both of the primary grade II astrocytomas were pooled (5.0 μg total) for the cDNA microarray analysis with a commercially available membrane (Atlas Human Cancer cDNA Expression Array 7742-1; Clontech Laboratories, Inc.). The two primary anaplastic astrocytomas were also pooled together as well as two of the glioblastomas. Additional samples of the original grade III astrocytoma and the recurrent tumor from the same patient were analyzed separately (Atlas Human cDNA Expression Array 7740-1, Clontech Laboratories, Inc.). Single-pass reverse-transcription reaction was used for the preparation of labeled cDNA from the sample RNAs using SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). [α-32P]dCTP was used as a labeled nucleotide. The probes were purified by gel chromatography (BioSpin 6, Bio-Rad, Hercules, CA). The residual RNA was degraded with alkaline hydrolysis (1 M sodium hydroxide at 68°C for 20 min), after which the probes

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2IGFBP, insulin-like growth factor binding protein; VEGF, vascular endothelial growth factor.
were neutralized with 1 M NaH₂PO₄ at 68°C for 20 min. The membranes were prehybridized at 68°C for 2 h in an Express Hybrid solution (Clontech Laboratories, Inc.) containing 100 μg/ml freshly cooked shared salmon sperm DNA, after which the cDNA probes were hybridized onto the membranes at 68°C overnight. Cot-1 DNA was used for blocking of unspecific hybridization (Clontech Laboratories, Inc.). The membranes were washed four times in low stringency wash buffer (2 × SSC-1% SDS) and twice in high stringency wash buffer (0.1 × SSC-0.5% SDS) at 68°C for 20 min each. The membranes were exposed to phosphorimager plates (Phosphorimager 2 SI; Molecular Dynamics, Sunnyvale, CA) for 48 h. Then the plates were scanned with a phosphorimager at a 50-μm resolution and analyzed with Image Quant software from the same manufacturer. In addition, a semiquantitative inspection of the hybridization results was performed for: (a) no signal; (b) a visible signal; and (c) a strong-intensity signal. A gene was regarded as overexpressed if the intensity of the subjectively visible signal in the normal brain membrane was ≥1.8× higher than the signal of the corresponding spot in the normal brain membrane. A gene was regarded as down-regulated if the intensity of the visible signal in the normal brain membrane was ≥1.8× higher than the signal of the corresponding spot in the tumor membrane. A similar comparison was made between the hybridization results of the original tumor case and its reoccurrence in the same patient. From this experiment, one down-regulated (MacMarcks) and one up-regulated (IGFBP2) gene target in the primary tumor were validated by Northern hybridization (Fig. 1).

**cDNA Microarray Hybridization with a Membrane of 5760 Individual cDNA Clones as Targets.** Equal amounts of total RNA from the three glioblastomas were pooled (52.5 μg total) for another cDNA microarray analysis with a commercially available membrane (Human GeneFilter, Release 1, GF200; Research Genetics, Inc., Huntsville, AL). A similar amount of pooled total RNA from normal human brain was used as an analysis reference (Clontech Laboratories, Inc.). The cDNA microarray hybridization was done as described above, with some modifications. [α-33P]dCTP was used as a labeled nucleotide. Hybridization and washings were performed at 60°C. The membranes were exposed to phosphorimager plates for 24 h, scanned with a phosphorimager at a 50-μm resolution and analyzed using Pathways software (Research Genetics, Inc.). Images were normalized using all spots on the membrane as reference points. Cutoff points for up-regulated and down-regulated genes were determined from a histogram analysis of the intensity ratios for all of the spots on the membrane (cutoff, intensity ratio = 1.8).

**Brain Tumor Tissue Microarray**

The tissue microarray block of 418 formalin-fixed and paraffin-embedded tumor samples of the central nervous system is shown in Fig. 2. A neuropathologist (H. K. H.) evaluated the tumors using H&E-stained standard slides and pinpointed one histologically representative tumor region in each case from which a sample was included in the block. The microarray block was constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) as described earlier (3). The sample diameter was 600 μm, and the spacing between adjacent specimens was 100 μm.

The tissue microarray included 364 gliomas and 54 other types of brain tumors (mainly meningiomas and neuronal or mixed neuronal-glial tumors). In 20 randomly selected tumor cases, multiple samples were collected from different sites of the subjectively most representative tumor region (see above) to evaluate intratumor heterogeneity within one selected tumor area. The gliomas of the tissue microarray (Table 1) comprised 256 primary and 88 recurrent tumors.
High-Throughput Microarray Analysis of Gliomas

Results

cDNA Microarray Analyses

Clontech Laboratories, Inc. (588 Individual Targets). Subjective evaluation of the cDNA microarray hybridization results of pooled samples revealed visible or strong-intensity signals (up-regulated genes) in 55 spots in the normal brain, in 74 spots in grade II astrocytoma, in 77 spots in grade III astrocytoma, and in 149 spots in glioblastoma. The combined visual inspection and quantitative analysis of the grade II astrocytoma resulted in 38 overexpressed and 12 down-regulated genes when compared with the normal brain. Analogous evaluation of the grade III astrocytoma revealed 32 overexpressed and 10 down-regulated genes. The glioblastoma analysis revealed 117 genes that were regarded as overexpressed and 24 that were regarded as down-regulated. Of the 38 overexpressed genes in grade II astrocytoma, 16 genes were found to be overexpressed also in grade III astrocytoma, and 30 genes were found to be overexpressed in glioblastoma. Twenty-six of the 32 overexpressed genes in grade III astrocytoma were found to be overexpressed also in glioblastoma. Of the 12 down-regulated genes in grade II astrocytoma, 7 genes were found to be down-regulated also in grade III astrocytoma, and 7 genes were found to be down-regulated also in glioblastoma. These results have been summarized in Table 2 with respect to the 40 most strongly overexpressed and 10 down-regulated genes detected in glioblastoma. The comparison of the recurrent grade III astrocytoma with the original occurrence of the same brain tumor patient is presented in Table 3.

Research Genetics (5760 Individual Targets). The analysis of pooled glioblastoma samples revealed 107 genes that were regarded as overexpressed and 111 that were regarded as down-regulated when compared with the analysis results of the normal brain (Fig. 2).

Tissue Microarray Analyses

The IGFBP2 immunostaining was technically successful in 95% (346 of 364) and vimentin in 99% (359 of 364) of the gliomas in the array. Analysis of the cases sampled more than once did not show evidence for intratumor heterogeneity within the selected tumor region in either IGFBP2 or vimentin stainings; none of the duplicate samples differed in staining intensity. One duplicate case was excluded because of the destruction of the sample spot in the array. The results of the IGFBP2 and vimentin immunohistochemistry in gliomas are presented in Table 1.

Of the primary tumors, strong IGFBP2 immunopositivity was significantly associated with the established histopathological malignancy grade of diffuse (grade II–IV) astrocytomas ($P < 0.0001$; $\chi^2$ test). A strong immunopositive expression of the IGFBP2 was associated with poor patient survival of astrocytomas ($P < 0.0001$; log-rank test). Those patients with IGFBP2-negative tumors had a mean survival of 75 months (95% confidence interval for mean survival: 52–97 months), and patients with tumors of strong immunopositivity had a mean survival of 23 months (95% confidence interval for mean survival: 15–31 months). A tendency of IGFBP2 to correlate with poor survival was observed within the grade III astrocytoma category ($P = 0.081; n = 16$), and the univariate survival analysis of the IGFBP expression in the combined group of grade III astrocytomas and glioblastomas (88% positive; Table 1) gained statistical significance ($P = 0.0068$).

Immunohistochemical staining of the p53 protein (negative versus positive) showed a strong correlation with tumor malignancy grade of...
primary astrocytic tumors in the tissue microarray (grades I–II versus grades III–IV; $P < 0.001$; $\chi^2$ test). Good agreement was found between the p53 analysis data of arrayed samples and standard slides in 33 (79%) of 42 cases ($P < 0.001$; $\chi^2$ test). Both strongly p53-positive tumors in the standard slide analysis (distinct immunoreactivity in more than 20% of neoplastic cells) showed an accumulation of p53-positive cells (>20%) in the tissue microarray, whereas one p53-immunonegative diffuse astrocytoma in the array had earlier been regarded as p53-positive.

## DISCUSSION

The development of novel biochip technologies has opened up new possibilities for the high-throughput molecular profiling of human tumors. Microarrays of cDNAs (6) have been introduced recently for gene-expression fingerprinting, and the technique has been used for defining differentially expressed genes in melanomas (7), rhabdomyosarcomas (8), breast cancer (9), and, recently, in gliomas (2). In this study, we used a series of cDNA microarray

<table>
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<tr>
<th>cDNA expression array: Genes$^a$</th>
<th>Normal brain</th>
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<th>Grade III</th>
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<th>GBM vs. normal</th>
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<td>Migration inhibitory factor (MIF)</td>
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$^a$ Atlas Human Cancer; Clontech Laboratories, Inc.

$^b$ Overexpressed genes are presented in roman type and down-regulated genes in italic.

$^c$ Blank, no signal; $\times$, visible signal; $\otimes$, overexpressed; $\otimes$, downregulated. GBM versus Brain shows the intensity ratio of the correspondent signals between the glioblastoma and normal brain.
One such gene was the protein acidic and rich in cysteine (SPARC), a highly conserved metal-binding extracellular matrix glycoprotein, the overexpression of which has been recently suggested to have a role in early neoplastic astrocytic transformation, neovascularization, and tumor invasion (11). Rapid detection of hundreds of differentially expressed genes and expressed sequence tags in this study, with DNA-arrays that cover only about 5% of the genome, demonstrates both the potential and the difficulties associated with DNA chip-based cancer gene categorization approaches. It is likely that cancer-classification DNA chips can be built, but large-scale microarray gene expression data first needs to be sorted, validated, and characterized further in clinical materials to establish cancer markers that distinguish specific tumor subtypes.

Table 3 The ten most strongly down- and up-regulated genes, by cDNA microarray analysis of 588 targets in a recurrent grade II astrocytoma when compared with the original occurrence (grade III) of the same brain tumor

<table>
<thead>
<tr>
<th>cDNA expression array: Genes</th>
<th>Expression</th>
<th>Down-regulated</th>
<th>Up-regulated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Original vs. recurrent</td>
<td>Recurrent vs. original</td>
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<td>Platelet-derived growth factor receptor α</td>
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<td>MacMarts</td>
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<td>4.5</td>
<td>4.3</td>
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<td>Receptor tyrosine kinase ligand LERK-4</td>
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<td>3.4</td>
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<tr>
<td>RAS-related protein RAB-3</td>
<td>6.1</td>
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<td>3.1</td>
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<td>Tumor necrosis factor receptor 2</td>
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<td>Thymosin β-10</td>
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<td>Glutathione peroxidase</td>
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<tr>
<td>MAS proto-oncogene</td>
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<tr>
<td>Proto-oncogene tyrosine-protein kinase receptor RET (Papillary carcinoma-encoded protein)</td>
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<td>IGFBP2</td>
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<tr>
<td>IGFBP2</td>
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<tr>
<td>VEGF</td>
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<td>ICH-2 protease</td>
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Efforts to perform high-throughput gene-expression monitoring in brain tumors have been initiated, and results from the Clontech Atlas array survey (2) as well as serial analysis of gene expression tag monitoring have been published recently. IGFBP2 is highly overexpressed in glioblastomas based on our survey of approximately 6000 genes, as well as based on serial analysis of gene expression tags and the cDNA array study by Fuller et al. (2). Therefore, this gene was selected for immunohistochemical validation and characterization of its expression pattern in a large clinical brain tumor material containing 259 astrocytic tumors and 105 other gliomas. Our brain tumor tissue microarray analysis showed a significant difference in the expression pattern of IGFBP2 between low-grade astrocytomas (including pilocytic and grade II diffuse astrocytomas) and high-malignancy (grades III–IV) tumors. The finding was reflected in a strong adverse relationship of the IGFBP2 immunoreactivity status to patient outcome. Furthermore, the reciprocal comparison of cDNA microarray profiles between the original tumor and the recurrent tumor, both expressing similar grade III astrocytoma histology, revealed an activation of the IGFBP2 gene after the first tumor operation. Vimentin, another highly overexpressed gene in cDNA microarray analyses, is a member of a heterogeneous group of intermediate filament proteins exhibiting tissue-specific expression patterns, and our findings are in perfect agreement with the previous knowledge of the antigen immunoreactivity in gliomas (12). Nearly all astrocytic tumors (excluding a few glioblastomas), ependymal, and choroid plexus tumors showed strong vimentin immunopositivity. A large variation in the antigen expression was observed in oligodendroglialomas, and vimentin-negative or weakly -positive mixed gliomas (mixed oligoastrocytomas) were characterized rather by the predominant oligodendroglial than by the astrocytic component. Together, the present IGFBP2 and vimentin experiments demonstrate the capacity of tissue chips in the rapid screening of specific molecular markers in a large-scale manner.

Glioblastomas, especially, may show marked variation in histopathological malignancy between different regions of the tumor sample, and focal expression of potential molecular markers is a potential problem in constructing tissue microarrays (13). Thus, one
aspect of the current study was to investigate the suitability of tissue microarrays for brain tumor analyses. First and most importantly, to ensure accurate sampling from the original “donor” tissue block, a neuropathologist carefully selected the most histologically representative tumor region from which the sample core was retrieved under a dissection microscope. Second, different sites within the subjectively selected tumor region were compared for immunoreactivity in 20 randomly chosen tumors. Third, comparisons were made between conventional whole sections and tissue microarrays for p53 analysis. We observed no difference in either IGFBP2 or vimentin immunostaining between the multiplied samples. However, little variation in p53 labeling indices was detected between the two analysis turns because of the heterogeneous distribution of p53 expression often found in astrocytic tumors. Yet, we could statistically demonstrate with arrayed tumor samples a highly significant increase in aberrant p53 expression in high-malignancy astrocytomas, as earlier reported by us after using corresponding regular sections (5).

The present study demonstrates a strategy for the high-throughput molecular genetic profiling of brain tumors. Large-scale gene expression screening with cDNA microarray analysis presents a new method for the identification of genes potentially involved in tumorigenetic pathways. The high-density tissue microarrays provide an appealing solution for effectively testing all of the emerging candidate genes in a clinical setup of hundreds or thousands of tumors.

ACKNOWLEDGMENTS

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Identification of Differentially Expressed Genes in Human Gliomas by DNA Microarray and Tissue Chip Techniques


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