ABSTRACT

Pilocytic astrocytomas (PAs) are WHO grade I brain tumors that do not typically progress to more malignant grades of astrocytoma. Whereas there have been significant advances in the molecular genetics of high-grade astrocytomas, relatively little is known about the genetic changes associated with PA formation. In an effort to better characterize these low-grade neoplasms, we compared the gene expression profiles of six sporadic and two neurofibromatosis 1-associated PAs with other tissues and cell lines of both astrocytic and oligodendroglial origin. Hierarchical cluster analysis of gene expression data clearly delineated PAs from low-grade oligodendrogliomas and normal white matter. The two NF1-associated tumors and one of the sporadic PAs displayed expression profiles that were more closely related to those of cultured normal human fetal astrocytes. However, PAs also expressed individual genes typically associated with oligodendroglial lineage (e.g., proteolipid protein and PMP-22). The expression patterns of specific genes (e.g., ApoD) were unique to PA tumors, whereas genetic changes characteristic of high-grade astrocytomas were not encountered. Differential expression of two transcripts, neural cellular adhesion molecule and connexin-43, was confirmed at the protein level, suggesting that these cell adhesion molecules might be particularly important in the molecular pathogenesis of these tumors. We conclude that PAs are genetically unique gliomas with gene expression profiles that resemble those of fetal astrocytes and, to a lesser extent, oligodendroglial precursors.

INTRODUCTION

PAs are the most common type of childhood central nervous system astrocytoma. These tumors are classified by the WHO as grade I neoplasms, and they typically behave in a benign fashion (1). PAs appear as relatively discrete lesions on gross neuropathological examination when compared with their more diffusely infiltrative fibrillary counterparts (WHO grades II-IV). Other important features include the presence of Rosenthal fibers, a frequent cystic component, and a lower incidence of malignant progression. These tumors are frequently encountered in the context of NF1, a common inherited tumor predisposition syndrome (2). PAs are observed in 15–20% of children with NF1 and most frequently involve the optic nerve, optic chiasm, optic pathway, and hypothalamus. These tumors are also found in the brainstem, cerebral cortex, and cerebellum, the latter representing a site of reportedly increased aggressiveness when associated with NF1 (3). In the setting of NF1, PAs develop almost exclusively in children with a mean age at diagnosis of 4.5 years (4). As is true for the sporadic PAs, most NF1-associated PAs behave in a clinically benign fashion; however, a small but significant fraction of these tumors will continue to grow and cause loss of vision or dysfunction of the hypothalamus.

It has been reported that PAs express molecular markers shared with oligodendrocytes or O2A precursors. O2A progenitor cells represent multipotent precursor cells that can mature into either type 2 astrocytes or oligodendrocytes (5–7). Low-grade astrocytomas have been shown to express myelin PLP and MBP (8, 9), as well as the PEN-5 epitope (10), which is not detected in high-grade astrocytomas. These proteins are expressed in oligodendrocytes and O2A cells, as well as a large proportion of oligodendrogliomas, suggesting that PAs may arise from a different cell of origin (e.g., O2A progenitor) than the higher-grade fibrillary astrocytoma.

The increased incidence of PAs in NF1 patients suggests that the NF1 tumor suppressor gene is a critical growth regulator for astrocytes. Previous work from our laboratory and others has demonstrated loss of NF1 gene expression in NF1-associated PAs but not in sporadic tumors (11, 12). These results argue that NF1 loss is associated with the development of NF1-associated PAs and that other genes must be important for the pathogenesis of sporadic PAs. Compared with the more malignant fibrillary astrocytoma, relatively little is known about the molecular pathogenesis of PAs. Cytogenetic studies have demonstrated a variety of chromosomal aberrations, including gains of chromosomes 7, 8, 9, and 11 (13–15). Whereas high-grade astrocytomas demonstrate overexpression and/or amplification of the cyclin-dependent kinase 4 and the epidermal growth factor receptor genes, as well as losses of the p16, p53, and PTEN/MMAC1 tumor suppressor genes, these genetic changes have not been identified in PAs (16, 17).

To better understand the molecular events associated with PA pathogenesis, we used differential expression profiling of eight PAs, including two from individuals affected with NF1. The objective of this study was to define expression profiles characteristic of PAs and to identify potential genes that might be important for PA pathogenesis. In addition, we sought to determine whether PAs had gene expression profiles more similar to those of low-grade oligodendrogliomas and normal white matter as compared with those of committed astrocytic precursors. Such a finding would provide support for the hypothesis of an O2A progenitor cell of origin for PAs.

MATERIALS AND METHODS

Tissue Procurement and Sample Preparation. All tissue samples were collected by the Siteman Cancer Center Tissue Procurement Facility under an approved protocol from the institution’s Human Studies Committee. Resected tumor tissue was immediately snap frozen in liquid nitrogen. Frozen tumor specimens were embedded in freezing medium, sectioned at 5 μm, and stained with H&E. The histopathology of each collected specimen was reviewed to confirm the adequacy of the sample (i.e., minimal contamination with non-neoplastic elements) and assess the extent of tumor necrosis and cellularity. Subsequent 50-μm serial sections from each banked frozen specimen were then cut and placed immediately into TRIzol reagent (Life Technologies, Inc.), and homogenized. RNA was obtained from Clonetics (Walkersville, MD), maintained in the recommended media, and processed according to the manufacturer’s specifications. NHA cells from passages five through eight were used for RNA extraction using the TRIzol reagent. Total RNA was
isolated using the manufacturer’s protocol. Extracted RNA was then further purified by spin chromatography (RNeasy kit; Qiagen) following the manufacturer’s protocol. Purified RNA was quantitated by UV absorbance at 260 and 280 nm and assessed qualitatively using an RNA LabChip and Bioanalyzer 2100 (Agilent).

**Oligonucleotide Array Analysis.** Analysis was performed by the Siteman Cancer Center GeneChip Facility. Purified total RNA (10 μg) was spiked with a set of four synthetic, polyadenylated, and bacterial transcripts (Lys, Phe, Thr, and Trp) diluted to defined copy numbers. Oligonucleotide probes for these transcripts are present on all Affymetrix GeneChips such that monitoring the expression level of these internal standards provides an indication of the total technical variability associated with the experiment. Spiked RNA was converted to cDNA, purified, and then used as a template for in vitro transcription of biotin-labeled antisense RNA. All protocols were performed as recommended by the manufacturer (Affymetrix) and have been described elsewhere (18). Each biotinylated antisense RNA preparation (20 μg) was fragmented, assessed by gel electrophoresis, and placed in hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to Affymetrix Hu95A GeneChip arrays for 16 h. GeneChips were washed and stained using the instrument’s standard Eukaryotic GE Wash 2 protocol, using antibody-mediated signal amplification. The images from the scanned chips were processed using Affymetrix Microarray Analysis Suite 4.0. The image from each GeneChip was scaled such that the average intensity value for all arrays was adjusted to a target intensity of 1500. SADV and Absolute Call Data from each GeneChip were exported as flat text files and used for further analysis. The complete set of raw data will be made available at the Web site.4

**Data Analysis.** Of the total of 12,651 gene sequences represented on the array, hybridization control sequences and sequences scored as “A” (not detected) in all 16 samples were excluded from initial analysis. For the remaining 9,628 genes, all SADVs of <1 were set to 1. For hierarchical clustering, SADVs were normalized to a mean of 0 and an SD of 1. The visualization in Fig. 1 was created using the average linkage of Euclidean distance measures method using the programs CLUSTER and TREEVIEW5 (19). For other comparative analyses of the filtered gene sets, flat text file data of Absolute Calls and SADVs were imported into DecisionSite 6.0 and Array Explorer Software (Spotfire). To distinguish technical variability from biological differences in gene expression, a CV was calculated using SADVs for the 3’ end probe sets directed to the internal standard bacterial transcripts (Lys, Phe, Thr, and Trp). This calculation included values from 11 of the 16 samples, as 5 of the samples were not spiked with the transcript controls. The maximum CV of the control transcripts (0.69) was then used as a threshold value for the genes listed in Fig. 2. To define genes with significant differences between astrocytic and oligodendrocytic specimens and between normal human astrocytes and PAs, SADVs were used in a standard t test, correcting Ps for multiple tests using permutation analysis. For SAGE analysis, the indicated tissue SAGE tag libraries were examined using the Xprofiler tool,6 selecting genes with >90% probability of being differentially represented in the two libraries at ≥3-fold. HTML output from the analysis was imported into Microsoft Access. Unigene IDs corresponding to Affymetrix probe pair sets7 were linked with Unigene IDs from Xprofiler output to create a concordant gene list.

**Western Blot.** Frozen tumor specimens were homogenized in standard protein lysis (radioimmunoprecipitation assay) buffer containing protease inhibitors and the protein concentration determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Total protein (100 μg) was separated by SDS PAGE as described previously (17). Proteins were transferred onto Immobilon membranes for Western blotting. The N-CAM (clone B11; mouse monoclonal antibody; Sigma Chemical Co., St. Louis, MO), WA30 (merlin polyclonal antibody), and connexin-43 (rabbit polyclonal, Sigma Chemical Co.) antibodies were used according to the manufacturer’s recommendations.

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4 Internet address: http://pathbox.wustl.edu/~mgacore.

5 Internet address: http://rana.lbl.gov/EisenSoftware.htm.


7 Internet address: http://www.netaffx.com/index2.jsp.

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Fig. 1. Hierarchical cluster analysis of patient tumor specimens. Gene expression data were scaled, normalized, and subjected to hierarchical cluster analysis as described in the text. The dendrogram at the top displays the relationship of the samples based on their global pattern of gene expression. The samples could be divided into two primary clusters (A), two secondary clusters (B), and two tertiary clusters (C) that were maintained regardless of the clustering algorithm used. Below the dendrogram is a “heat map” representing gene expression with each of the 16 samples represented in a column, each of the 9628 genes represented as a horizontal line, and the relative expression of any one gene in any one sample in continuous linear grayscale from high (black) to low (white) expression. The table (bottom) provides basic diagnostic and demographic information from each of the tumor samples, as well as the number of genes scored as detected (% Genes = P) by the GeneChip software algorithm and the average hybridization signal intensity of detected genes (Avg Diff P).
or our methods published previously (20). Tubulin (Sigma Chemical Co.; clone DM1A) was used as an internal control for protein quality (data not shown). Development was accomplished using appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Arlington Heights, IL).

RESULTS

Sixteen low-grade and nonneoplastic astrocytic and oligodendroglial samples were chosen for analysis (Fig. 1). This included 8 cases of grade I PA, 2 cases of which were from patients with NF1. We chose normal human white matter as a source of brain tissue enriched in mature oligodendrocyte RNA. Cultured NHAs were selected as a model of committed astrocytic precursor cells. Three WHO grade II oligodendrogliomas were chosen to represent a comparable low-grade malignancy of presumed oligodendroglial precursor origin. All PA tumors were carefully reviewed by an experienced neuropathologist (A. P.), and no obvious differences in tumor cellularity, tumor location, patient age, or clinical behavior were observed. In addition, patient 613 has no evidence of NF1 based on the NIH diagnostic criteria at the age of 13 years.

As shown in Fig. 1, the number of genes scored as detected (‘P’) in each sample ranged from 45 to 56%, suggesting that the overall quality of each of the labeled targets and the resulting fluorescent signal obtained from each of the 16 GeneChips was comparable. The expression level of four different internal control transcripts (added to 11 of the 16 RNA samples at four different, defined copy numbers) is shown in Fig. 2. The maximum CV of these values (0.69 for the low copy number Trp transcript) was used as an estimate of the expected technical variability associated with the study. The hierarchical clustering relationship among all 16 specimens is displayed as a dendrogram in Fig. 1. Aligned with this dendrogram is information regarding pathological diagnosis, patient age at resection, gender, tumor location, and NF1 status. As might be expected, the three oligodendrogliomas, three normal white matter specimens, and two cultures of normal human astrocytes each respectively clustered as highly related groups. The clustering relationship among the 16 samples was maintained regardless of the clustering method or metric used (data not shown). Furthermore, all eight PAs were closely related to each other and exhibited profiles of greater similarity to those of the normal human astrocyte cultures as compared with those of either normal white matter or low-grade oligodendrogliomas (Fig. 1A).

Within the cluster of eight PAs, both tumors derived from NF1
patients, and a third sporadic tumor (juvenile pilocytic astrocytoma 613) clustered closer to the normal astrocyte cultures than did the five other sporadic tumors (Fig. 1B). Further stratification revealed that NF1-associated tumor #201 was more similar to normal human astrocyte cultures than any of the other primary tumors (Fig. 1C).

Histological review of this NF1-associated tumor did not reveal any differences in cellular composition compared with the other PA tumors or the presence of contaminating nonneoplastic parenchyma to account for its unique clustering profile.

From the complete set of 9628 genes, we next examined a subset of genes identified previously as being either overexpressed or lost in higher grade astrocytomas or those associated with the astroglial phenotype. Fig. 2 illustrates the expression patterns for these genes (arranged by functional category) in each of the 16 samples. First, we observed that many fibrillary astrocytoma-associated genes were neither up- nor down-regulated in the PAs. This included the tumor suppressor genes p53, retinoblastoma, PTEN/MMAC1, p19, p16, mdm2, and cyclin-dependent kinase 4 expression. One exception was merlin (the product of the NF2 gene), which was down-regulated in the PAs relative to normal human astrocyte cultures. This overall pattern was confirmed on the protein level using merlin-specific antibodies on tumors from which high-quality protein lysates could be obtained (Fig. 3).

Second, we evaluated the expression profiles for a number of gene transcripts expressed specifically in subsets of glial cells, such as oligodendrocytes, type 1 astrocytes, and type 2 astrocytes. To explore the possibility that PAs express markers suggestive of an O2A cell, we analyzed transcript expression of specific genes associated with O2A cells. We observed increased expression of PMP-22, PLP, myelin oligodendrocyte glycoprotein, and MBP in many of the PAs. On the basis of histological review of the frozen specimens, these increases in expression did not reflect the presence of contaminating nonneoplastic parenchyma.

Third, a number of genes demonstrated a relatively unique pattern of gene expression in the PA tumors relative to the various controls (NHA cells, oligodendrogliomas, and normal white matter). These genes included GAP43, α-7 integrin, connexin-43, fatty acid binding protein-7, cyclin D2, translation EF1α-2, and lung type-1 cell membrane-associated gp36. Each of these genes was differentially expressed in all of PAs relative to NHA cells and oligodendroglomas, except α-7 integrin (increased in six of the eight PA tumors) and connexin-43 (decreased in five of the eight PA tumors). The decreased expression of connexin-43 was further verified at the protein level in selected tumors for which sufficient material was available for Western blot analysis (Fig. 3). The translation EF1α-2 was overexpressed in the sporadic PA tumors and not the two NF1-associated astrocytommas.

Other transcripts were only differentially expressed in the PA tumors relative to normal human astrocytes. These included N-CAM, L1-CAM, syndecan, ApoD, and SPARC. The overexpression of N-CAM was further confirmed at the protein level by Western blot analysis in selected tumors from which high quality protein lysates could be obtained (Fig. 3).

To assist in determining whether these and other genes were of potential biological significance, we compared the expression profiles of our samples using oligonucleotide array technology with those identified by computational analysis of SAGE tags. Genes on the Affymetrix GeneChip were linked to Unigene ID numbers resulting from Xprofiler analysis of SAGE tags from a PA tumor (34-year-old male, Duke #H-1043) versus a culture of normal human astrocytes (passage 5 NHA). As shown in Fig. 4, many additional genes differentially expressed by GeneChip were similarly represented in the proportion of SAGE tags identified in the Xprofiler output. The expression of ApoD, ApoE, Sparc-like protein, IGFBP-2, and several other transcripts in PA tumors seen in this study was confirmed by SAGE analysis. Although the biological significance of these findings is still uncertain, the concordance of differential gene expression in these cell types by two different analytical methods suggest that these differences are genuine.

DISCUSSION

PAs are low-grade astrocytic tumors that lack many of the genetic changes associated with their high-grade counterparts (16, 17). As these tumors rarely progress to WHO grade II astrocytomas, it has been suggested that they represent a distinct subtype of astrocytoma, perhaps arising from a different precursor cell than the fibrillary astrocytoma. In support of this notion, PAs express the PEN5 protein (10) not expressed in type 1 astrocytes or high-grade astrocytomas but found in oligodendrocyte precursor cells and oligodendrogliomas. To better characterize the specific genetic changes associated with PA tumor formation, we initiated a gene expression profiling study on eight PAs. We proposed to determine whether the overall gene expression profile (“molecular fingerprint”) and specific gene expression patterns of PAs most closely resembled astrocytic or oligodendroglial precursors. In addition, we wanted to identify specific transcripts associated with PAs. Lastly, we planned to determine whether specific gene expression profiles would distinguish NF1-associated from sporadic PAs.

Hierarchical cluster analysis clearly stratified the samples in our series into two subgroups. The first group separated the PA tumors and normal fetal astrocytes from the oligodendrogliomas and white matter samples. This result was obtained using a variety of different mathematical clustering strategies. From these results, the overall pattern of gene expression of the PAs most closely resembles the fetal astrocyte cells, rather than oligodendrocyte-rich white matter or well-differentiated oligodendrogial tumors. The second level of stratification separated out the NHA cells, the two NF1-associated astrocytommas, and one of the six sporadic PAs. The one female patient with the sporadic PA at age 10.5 who clustered with the NF1-associated tumors did not meet diagnostic criteria for NF1 at age 13. Moreover, this tumor was in a typical location for a sporadic PA (posterior fossa). Therefore, it is unclear why this tumor more closely resembled the expression profile of the two NF1-associated tumors or whether this association is significant. Additional studies will be required to de-
increased platelet-derived growth factor-R (PDGF-R) (16). Whereas one report demonstrated this finding of decreased merlin expression relative to NHA cells is intriguing, given the role of this gene in regulating nervous system cell proliferation. Although individuals with NF2 develop astrocytomas at an increased frequency, it is unlikely that merlin plays a significant role in astrocytoma formation, based on previous studies (26–29).

We identified one gene, EF-1α, that was increased in all sporadic PAs relative to the two NF1-associated tumors. Specific antibodies were not available to confirm this observation at present. EF-1α has been implicated in malignant progression of metastatic rat mammary adenocarcinoma (30) and may determine the susceptibility to transformation (31) or apoptosis (32). The observation that EF-1α expression is lower in NF1-associated tumors that are typically less prone to clinical progression is intriguing. We are presently examining a larger series of NF1-associated and sporadic PAs for differences in EF-1α expression.

Several genes that encode cytoskeleton/adhesion-associated molecules demonstrated patterns of expression that differentiated PAs from NHA cells and oligodendroglioma tumors. These included GAP43, connexin-43, ezrin, integrin α-7, and the lung type I-cell membrane-associated gp36. GAP43 was increased in all of the PA tumors in this series. GAP43 is a neuronal protein kinase C substrate expressed in cells of the O2A lineage that has been implicated in regulating actin cytoskeleton dynamics (33–35). We have found recently that GAP43 expression is lost in high-grade astrocytomas, and its replacement in astrocytoma cell lines results in growth arrest (36). Connexin-43 expression was significantly decreased in many of the PA tumors. In support of a growth regulatory role for connexin-43, overexpression of connexin-43 results in decreased C6 cell proliferation (37). Ezrin expression was also found to be decreased moderately in PA tumors compared with normal white matter and NHA cells. This lower expression may contribute to the pathogenesis of both high- and low-grade astrocytomas. Although ezrin associates with the actin cytoskeleton, it may also play an important role in tumor cell motility (38). Integrin α-7 expression was significantly decreased in many of the PA tumors. In support of a growth regulatory role for integrin α-7, knockdown of integrin α-7 results in decreased cell proliferation (39).
membrane-associated glycoprotein (gp36 in humans and gp40 in rodents) is expressed at the apical plasma membrane (40) and has been hypothesized to represent a marker of cellular differentiation in alveolar cells (41). Interestingly, gp36 is expressed in brain and neural derivative tissues beginning after embryonic day 13.5 in the rat but is down-regulated in the adult nervous system (42). Although a precise function for gp36 has not been proven, other similar proteins function to modulate cell adhesion. The identification of several genes involved in adhesion and cytoskeleton-associated processes, whose expression pattern is different from NHA cells and oligodendroglioma tumors, raises the possibility that specific changes in astroglial cell interactions may contribute to the pathogenesis of PAs. In this regard, we have recently found significant alterations in cell adhesion and motility in mouse astrocytes lacking NF1 gene expression (43).

Other gene expression changes were observed between PA tumors and NHA cells. These include N-CAM, L1-CAM, and syndecan. N-CAM is also expressed in a subpopulation of astrocytes in normal and glaucomatosus human optic nerve (44). In tumors, expression of N-CAM has been correlated inversely with astrocytic malignancy grade (45). It has been implicated in intracellular signaling pathways involved in the negative regulation of astrocyte proliferation (46, 47). Another related CAM (L1-CAM) was also increased in the PA tumors in our series. L1-CAM has been reported to be overexpressed in malignant gliomas (48). The increase in syndecan expression in the PA tumors is intriguing given a recent report describing this transmembrane proteoglycan as a specific NF1 gene product (neurofibromin) interacting protein (49). Because these particular gene expression changes were observed when PAs were compared with human fetal astrocytes grown in vitro, additional experiments will be required to exclude the possibility that these alterations reflect the differences between tumors in situ and cultured astroglial cells.

PAs express some proteins that suggest an O2A lineage. On histopathological examination, they typically exhibit a more astrocytic phenotype with long, hair-like cytoplasmic processes, which account for the name “pilocytic.” However, a significant subset appears remarkably oligodendrocyte-like and is occasionally misdiagnosed as oligodendroglioma (1). Therefore, to determine whether PA tumors express transcripts typically associated with O2A cells, we examined oligodendrocyte-specific genes in our sporadic and NF1-associated PAs. Although the overall expression pattern most closely resembled fetal astrocytes, we observed the expression of oligodendrocyte-associated transcripts, such as PLP, PMP-22, MBP, and oligodendrocyte myelin glycoprotein, suggesting that these tumors have at least some gene expression features of O2A cells. In contrast, increased expression of PLP and MBP has not been observed in high-grade astrocytomas (50). The observation that the PEN5 marker of an oligodendrocyte precursor cell population (10) is expressed in PAs, but not high-grade astrocytomas, also supports a separate lineage for PAs compared with fibrillary astrocytomas. The expression of genes more typically associated with oligodendrogliomas or O2A progenitor cells raises the possibility that PA tumors arise from precursor cells of an intermediate differentiation stage. Future work will be required to definitively establish the cell of origin relevant to the histogenesis of PA tumors.

In summary, we identified transcripts that were uniquely expressed in PAs relative to oligodendrogliomas, NHA cells, or normal white matter. In addition, we found one potential gene (EF-1α) that may distinguish NF1-associated from sporadic PA tumors. Future studies on larger cohorts of PA tumors will be required to validate these initial gene expression profiles and enable a more detailed functional analysis of these genes to define their respective roles in astroglial cell growth regulation and tumorigenesis. Similarly, the finding that PAs express molecular markers of oligodendrocytes and O2A progenitors will necessitate additional studies on the contribution of dysregulated O2A lineage cell growth to the molecular pathogenesis of grade I PAs. Lastly, because PAs exhibit clinical behavior distinct from other astrocytic tumors, the preliminary insights provided by this study may serve to identify targets for PA-specific tumor therapies.

**ACKNOWLEDGMENTS**

We thank Dr. William Shannon and Maciej Faifer for providing access to the Multitest tool for statistical analysis; the technical assistance of Sarah Kunz, Victoria Holtscogl, and Kate Hamilton; and the secretarial support of Nancy North during the preparation of this work.

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Comparative Gene Expression Profile Analysis of Neurofibromatosis 1-associated and Sporadic Pilocytic Astrocytomas

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