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Evidence for Clonal Origin of Neoplastic Neuronal and Glial Cells in Gangliogliomas

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Gangliogliomas are rare tumors of the central nervous system that account for approximately 1% of all brain tumors. Histologically, gangliogliomas are composed of intimately admixed glial and neuronal components, the pathological origins of which remain to be characterized. Clonal analysis through examination of the pattern of the X chromosome inactivation allows one to distinguish monoclonal differentiation of a genetically abnormal progenitor cell from parallel, but independent, clonal expansion of two different cell types during tumorigenesis in biphasic neoplasms, such as gangliogliomas. In the present study, we investigated the clonality of eight gangliogliomas from female patients using both methylation- and transcription-based clonality assays at the androgen receptor locus (HUMARA) on the X chromosome. Among tumors from seven patients who were heterozygous at the HUMARA locus, five were identified as monoclonal with the methylation-based clonality assay, and the results were confirmed by the transcription-based method, whereas two were shown to be polyclonal by the methylation-based clonality assay but monoclonal by transcription-based clonality analysis. We conclude that the predominant cell types in most gangliogliomas are monoclonal in origin and derive from a common precursor cell that subsequently differentiates to form neoplastic glial and neuronal elements. *(Am J Pathol 1997, 151:565–571)*

Gangliogliomas are rare tumors of the central nervous system (CNS) accounting for approximately 1% of all brain tumors. The proportion in infants and children is as high as 7.6%.[1–3] They occur most commonly within the temporal lobe, where they are associated with temporal lobe epilepsy.[4] Gangliogliomas in other supratentorial areas, in the posterior fossa, and even in the spinal cord also have been reported.[2,5] Histologically, gangliogliomas constitute a type of mixed neoplasm in which both glial and neuronal components are present, although varying in their relative proportions and regional differentiation. The neuronal elements are characterized by large neoplastic neurons (ganglion cells), whereas the glial components are usually of astrocytic or, less frequently, oligodendroglial differentiation.[2,6] They are generally indolent tumors, although they may behave occasionally in a more aggressive fashion.[7–9] Often, it is the glial elements that progress into anaplastic tumors, although malignant transformation of both glial and neuronal components in one ganglioglioma has been described.[10]

Given their mixed composition, identification of clonality could be helpful in understanding the mechanisms of ganglioglioma tumorigenesis. Whether they arise from a single neoplastic precursor cell giving rise to glial and neuronal cell types as a result of divergent differentiation (ie, are monoclonal) or from glial and neuronal cells independently, either synchronously or metachronously producing two different populations of cells within the same tumor (ie, are polyclonal), has been an old question. However, due to the limited incidence of gangliogliomas, very few molecular investigations of their genetic abnormalities have been carried out. We theorized that molecular analysis of such tumors might provide not only further understanding of the mechanism of ganglioglioma pathogenesis but also possibly additional diagnostic and treatment options for patients with gangliogliomas.

Characterization of clonal derivation of human neoplasms has provided important information about etiology and pathogenesis in many human cancers and has practical implications for both diagnosis and subsequent studies of disease progression.[11–13] Analysis of clonality in females heterozygous for specific genes or polymorphic markers on the X chromosome has been widely used for determination of tumor origin.[12] Since the first discovery of an expression clonality assay by Fialkow[14,15] at the glucose-6-phosphate dehydrogenase (G6PD[7]) gene, many molecular genetic approaches

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have been developed to assess the clonality of tumors using DNA restriction fragment length polymorphisms (RFLPs) combined with differential methylation of nearby cytosine residues on the X chromosome, such as hypoxanthine guanine phosphoribosyltransferase, phosphoglycerate kinase, and M27β assays.[16–19] In 1992, Allen et al[20] developed a polymerase chain reaction (PCR) methylation-based clonality assay that takes advantage of a highly polymorphic trinucleotide repeat (CAG) in the coding region of the first exon of the human androgen receptor gene on the X chromosome (HUMARA). This CAG repeat is closely linked to four methylation sites that have served as the basis for studying patterns of X chromosome inactivation in female carriers (Figure 1A). The advantages of this clonality assay include high frequency of heterozygosity (90%) and reliable methylation patterns, and because it is PCR based, small amounts of tumor specimens from OCT or paraffin can be successfully analyzed.[21] By using this technique, monoclonality in Langerhans cell histiocytosis has been documented, and the results were confirmed with the more conventional methylation-based phosphoglycerate kinase and M27β assays.[22]

A transcription-based clonality assay was recently developed at the HUMARA locus using a reverse transcriptase (RT)-PCR strategy that allows clonality determination without relying on differential methylation of the X chromosomes (Figure 1B).[23] It permits independent confirmation of results obtained by methylation-based clonality assay at the same locus. However, the technique is limited by the level of transcription of the androgen receptor gene in the tissue of investigation.[24]

In the present study, we investigated the clonal origin of primary gangliogliomas from eight female patients, seven of which were informative. Using both methylation- and transcription-based clonality assays at the androgen receptor locus (HUMARA), we found that the pattern of X chromosome inactivation is nonrandom and is the same in both glial and neuronal cells in five of the seven gangliogliomas.

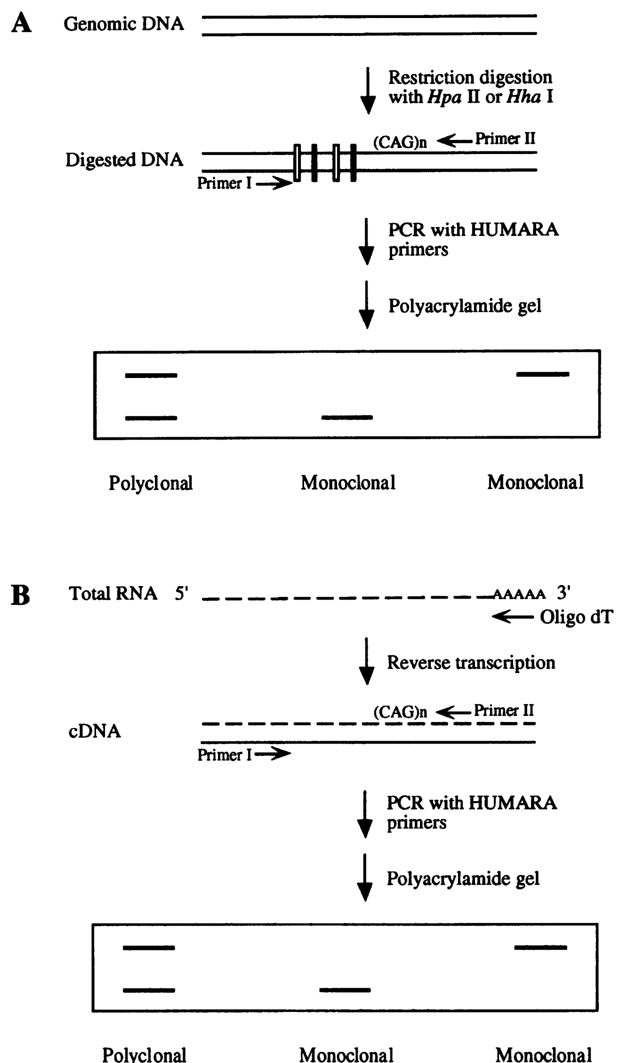

Figure 1. Schematic diagram comparing experimental procedures of the methylation-based (**A**) and transcription-based (**B**) clonality assays at the HUMARA locus. The two assays are based on different principles for identifying the pattern of X chromosome inactivation in female mammals. In the methylation assay, methylation-sensitive restriction enzymes, such as *Hpa*II (**solid vertical bars**) and *Hha*I (**open vertical bars**), are used to distinguish the inactive X chromosome, which is often methylated and resistant to *Hpa*II digestion, from the active X chromosome, which is mostly unmethylated and sensitive to *Hpa*II cleavage (**A**). In contrast, the transcription clonality assay is based on the fact that most X-linked genes, including the androgen receptor gene, are expressed from the active but not the inactive X chromosome (**B**). Therefore, *Hpa*II digestion and reverse transcription were used for methylation and transcription assays, respectively. Subsequent reactions to amplify the products after restriction cleavage or reverse transcription were the same for the two assays. (Reprinted with permission from Ref. 30.)

Materials and Methods

Collection of Blood Samples, Tumor, and OCT Tumor Specimens

Eight ganglioglioma specimens were obtained at the time of surgery from seven female patients operated on at the Brigham & Women's Hospital or New England Medical Center. Tumor tissues were evaluated by a neuropathologist. After sampling for pathological evaluation, fresh tissues were snap-frozen and stored in liquid nitrogen, and a small portion of the same tissue was embedded in OCT (optimal cutting temperature) compound (Baxter Scientific, Bedford, MA) and stored at −70°C. A previously studied monoclonal meningioma specimen was used as a positive control (control I). Non-neoplastic brain tissue was obtained from a patient with medically intractable epilepsy after temporal corticectomy and served as a negative control (control II). Peripheral venous blood samples were obtained at the time of tumor resection, with informed consent from every patient.

Sectioning of OCT blocks was carried out in a cryostat at −20°C. A 6-μm section was cut and stained with hematoxylin and eosin (H&E) for histological evaluation (see below) by one neuropathologist (R. D. Folkerth). The adjacent sections were subjected to DNA or RNA isolation. Patient records were reviewed, and tumors were classified clinicopathologically (Table 1) on the basis of presenting clinical signs, tumor location, and pathological examination. Approval for this study was

Table 1. Summary of Clonality Results of Gangliogliomas

Cases	Methylation		Transcription	Age	Tumor	Diagnosis
	Tissue	OCT	OCT	(yr)	location	
Control I	6.0*	----‡	----	39	----	Meningioma
Control II	1.1†	----	1.5§	48	----	Mild Gliosis
G1¶	5.6	3.5	20.1	26	R. frontal	Ganglioglioma
G2¶	3.8	2.7	15.3	28	R. frontal	Recu. G. Ana.
G3	3.2	2.5	6.5	55	R. temporal	Ganglioglioma
G4	1.7	1.4	4.8	31	L. temporal	Ganglioglioma
G5	3.1	----	----	52	CP angle	Ganglioglioma
G6	Homo	Homo	Homo	54	L. temporal	Ganglioglioma
G144	1.4	----	----	30	Ventricle	Ganglioglioma
G104	7.5	----	----	8	L. temporal	Ganglioglioma

Homo, homozygous; R, right; L, left; CP, cerebellopontine; Recu. G. Ana., recurrent ganglioglioma, anaplastic.
*Clonality Ratio, applicable to all the ratios in methylation-based assay unless otherwise specified (see Materials and Methods for detail).
†Corrected ratio for non-neoplastic tissue only.
‡Not done due to lack of OCT tumor specimens.
§Ratio of two alleles in RT^+ lane.
¶Same patient, operated 2 years apart.

granted by the Human Research Committees of the institutions involved.

Histopathological Evaluation of Tumors

Tissues obtained at surgery were fixed in 10% formalin and processed routinely to H&E-stained slides. Both the formalin-fixed and OCT-embedded cryostat sections from each case had the typical features of ganglioglioma as described in standard texts,[2,25] that is, a gangliocytomatous as well as a glial component (Figure 2). Although gangliogliomas sometimes vary with respect to the type of glial cells, in all of our cases the glial component was astrocytic, with clearly identifiable eosinophilic cytoplasmic processes and oval, fairly uniformly hyperchromatic nuclei. The neoplastic ganglion cells were readily distinguished on the basis of large, round nuclei with vesicular chromatin and prominent nucleoli and slightly basophilic cytoplasm, sometimes with visible fine processes. Differentiation of neoplastic ganglion cells from cortical neurons, when present in the samples, was straightforward, based on the former's atypical cytology, including bizarre shapes and occasional binucleation, and architecture, with haphazardly oriented cells, often in groups or clones having a range of cell diameters (Figure 2). In all cases, there were cells having features intermediate between those of astrocytes and ganglion cells. Cases were immunohistochemically stained with antisera to glial fibrillary acidic protein (GFAP) and to synaptophysin (both Dako monoclonal), according to standard protocols, in one batch. These confirmed the dual phenotypes and illustrated the proportions of each phenotype within and among the tumors; overall, the astrocytic component outnumbered the ganglion cell component by approximately four or five to one, with a range of between three and eight to one. However, in virtually every microscopic field of tumor (away from involved cerebral cortex, when present), examples of each component, as well as intermediate cell types, were readily seen (Figure 2). Anaplastic features were lacking in all but one recurrent tumor (G2), in which pleomorphism and mitotic activity were found. Materials used for all clonality studies corresponded to the deep or central portions of the tumors, away from any adjacent normal cortex.

Isolation of Leukocyte DNA, Tumor DNA from Frozen Tissues, and Tumor DNA and RNA from OCT Specimens (Cryostat Sections)

Blood DNA was extracted from peripheral blood leukocytes by sodium dodecyl sulfate (SDS)/proteinase K digestion followed by phenol and chloroform extraction.[26] Tumor DNA and RNA from frozen tissues were extracted simultaneously from the same sample by a modified procedure as previously described.[27,28] Isolation of tumor DNA embedded in OCT compound was performed according to an existing protocol.[29] Briefly, one 6-μm and four to five 10-μm sections were cut serially at −20°C in a cryostat. The 6-μm section was stained with H&E for pathological examination as described above; the adjacent sections were subjected to DNA isolation with proteinase K digestion followed by phenol/chloroform extractions. Tumor mRNA from OCT sections was extracted by the Micro-FastTrack 2.0 mRNA isolation kit according to the manufacturer's instructions (In-Vitrogen, San Diego, CA).

Methylation- and Transcription-Based Clonality Assays at the Androgen Receptor Locus

Both PCR-based clonality analyses at the human androgen-receptor gene (HUMARA) were performed as previously described.[20,23,30] A schematic diagram comparing experimental procedures of the methylation- and transcription-based clonal assays is presented in Figure 1. Compared with the methylation-based assay (Figure 1A), the transcription-based clonality analysis uses reverse transcription of RNA to cDNA and analyzes the pattern of allelic expression of the androgen receptor gene. When only one of the two alleles is detected on the gel (Figure 1B), it suggests that the tumor is monoclonal. However, a tumor is considered to

be polyclonal when both alleles are observed on the acrylamide gel, with a clonality ratio close to 1 (Figure 1B). For each reverse transcription, identical reactions with and without RT were carried out in parallel, where the RT$^-$ reaction served as a control for possible DNA contamination, which would produce two bands. Methylation-based clonality analysis of DNA from the same patient was performed in each case for comparison with transcription assay results and for identification of positions of the two alleles on gels. A known monoclonal meningioma was used as a positive control (control I) for completion of restriction digestion.[30] Throughout the experiments, one master mix was prepared for all *Hpa*II digestions, and a monoclonal positive control was always included (Figure 3, control I). In addition, each experiment was repeated at least three times, and the results were the same as shown in Figure 3.

Quantitation and Determination of Clonality Ratio (CR)

To obtain a precise determination of the ratio between the two X-linked alleles generated by the HUMARA clonality assays, the same gel after film autoradiography was exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), and the relative amount of PCR products of each allele was measured with ImageQuant software. The clonality of each tumor was determined by the value of the CR as previously described.[30] Briefly, the corrected tumor ratio (ctr) was calculated by the formula: ctr = $(a/b)/(c/d)$, where a/b is the band intensity ratio of upper band over lower band with *Hpa*II precut tumor sample, and c/d is the band intensity ratio of upper band over lower band of the control (non-precut) tumor sample. Corrected blood ratio (cbr) was obtained from blood DNA analysis using the same formula as ctr. Finally, the clonality ratio was obtained by the following formula: CR = ctr/cbr or cbr/ctr, whichever is greater than 1. With these criteria for the clonality assay, a polyclonal sample will have a clonality ratio close to 1.0, and a sample containing an appreciable number of clonal cells (>50%) will have a clonality ratio greater than 3.0. For the transcription-based clonality assay, the clonality ratio equals the band intensity ratio of the two alleles in the presence of RT.

Results

Analysis of Frozen Tissues and OCT Tumor Specimens by Methylation-Based Clonality Assay

Of the eight ganglioglioma specimens from female patients collected over a five-year period at Brigham & Women's Hospital and New England Medical Center,

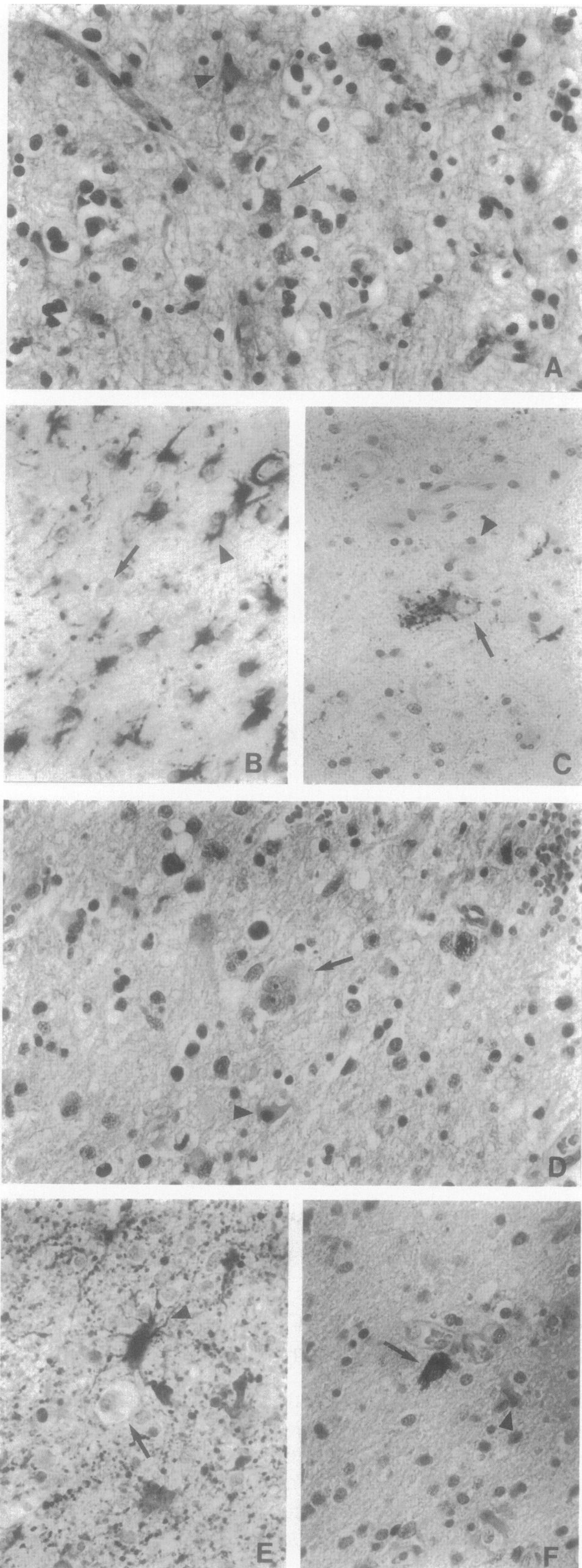

Figure 2. Histopathology of tumors. **A** and **D**: Intermingled populations of cells with hyperchromatic nuclei and dense cytoplasmic processes, representing neoplastic astrocytes (**arrowhead**), and larger, bizarre cells with cleared nucleoplasm and prominent nucleoli, consistent with atypical, occasionally binucleate, ganglion cells (**arrow**). H&E; magnification, ×1000. **B** and **E**: Glial fibrillary acidic protein (GFAP) immunostain highlights the dark, fan-like cytoplasmic processes of the astrocytic component (**arrowhead**), whereas ganglion cells (**arrow**) are unstained. Magnification, ×1000. **C** and **F**: Synaptophysin immunostain results in dot-like positivity over the membrane surface of some of the ganglion-like cells (**arrow**), leaving astrocytes (**arrowhead**) unmarked. Magnification, ×1000. **A** to **C**: From case G3. **D** to **F**: From case G2.

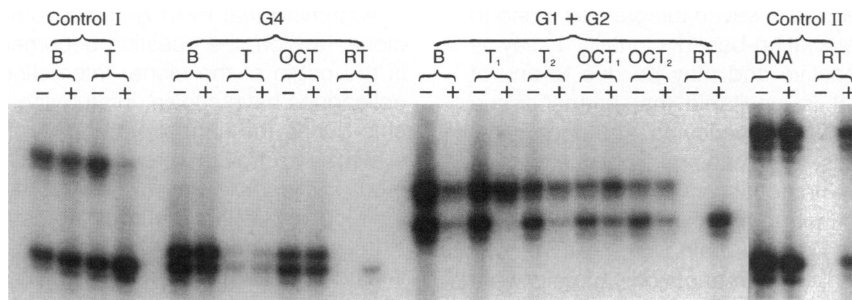

Figure 3. Methylation- and transcription-based clonality analysis of tumors at the HUMARA locus on the X chromosome. The marker is a trinucleotide tandem repeat (CAG), and its PCR products are approximately 280 bp. For the methylation-based clonality assay, parallel DNA samples for blood (B), tumor (T), and OCT were predigested with (+) or without (−) *Hpa*II restriction enzyme followed by PCR amplification with HUMARA primers. The control I was a known monoclonal meningioma and served as a control for complete *Hpa*II restriction digestion. The control II was a non-neoplastic brain tissue and was polyclonal by both methylation- and transcription-based clonal assays. For transcription-based assay, mRNA was reverse transcribed with (+) or without (−) reverse transcriptase (RT) followed by PCR amplification with HUMARA. G1 and G2 were from the same patient, and one blood sample (B) was used with T1, OCT1, T2, and OCT2. The RT sample below G1 + G2 was from G1 only. The CRs for frozen tissues, OCTs, and RNA analyses are listed in Table 1. B, blood; T, frozen tissue; OCT, tumor slices cut out of OCT compound; −, no *Hpa*II; +, with *Hpa*II.

seven (88%) are informative at the HUMARA locus after screening for heterozygosity in leukocyte DNA. The clonality ratios of both frozen tissues and OCT-embeded-ded tumors are shown in Table 1. In a non-neoplastic brain specimen (control II) from a patient heterozygous at the HUMARA locus, two bands of close intensities in the *Hpa*II-digested sample were detected with a clonality ratio of 1.1 and was interpreted as polyclonal (Figure 3, control II; Table 1). The known monoclonal meningioma used as a positive control for completion of restriction digestion had a CR value at 6.0 (Figure 3, control I; Table 1).[30] The PCR-based methylation-dependent clonality analysis of the seven blood and tumor pairs showed that five samples were monoclonal in origin with CR > 3, whereas two samples (G4 and G144) were polyclonal with CRs of 1.7 and 1.4 (Figure 3; Table 1). We repeated the clonality analysis on DNA extracted from available OCT samples including G4 and three other tumors (Figure 2). The methylation-based clonality analysis on the OCT sample confirmed the polyclonal pattern obtained from frozen tumor tissue (G4; Figure 3) in three trials with appropriate controls. There were reduced differences of signal intensity between two alleles in the *Hpa*II-treated samples (+) from OCT DNAs *versus* that from fresh-frozen tumor DNA (Figure 3, under G1 + G2, comparing T1 with OCT1), which resulted in reduced CR values (Table 1).

Analysis of RNA Isolated from OCT Tumor Specimens by Transcription-Based Clonality Assay

Among seven gangliogliomas in our series, we analyzed four samples (G1, G2, G3, and G4) for which OCT blocks were available and expressed detectable amount of the androgen receptor gene. Although the amount of androgen receptor RNA varies in different tumors, in each of the four tumors, only one allele was observed expressing the gene, which was from the unmethylated allele identified by comparing with the results of the methylation-based assay (Figure 3). Therefore, all four (100%) tumors are monoclonal in origin with clonality ratios over 3.0 by this assay (Figure 3; Table 1). The result of one apparently monoclonal tumor by this assay (G4) is at variance with the previous data derived from the methylation-based clonality assay (Table 1). Non-neoplastic brain tissue expressing a detectable amount of androgen receptor mRNA was used as a polyclonal control for this assay (Figure 3, control II); two bands corresponding to the ones amplified from DNA were observed in RT^+ lanes, whereas no signal was detected in RT^- lanes. The CR for control II was 1.5, indicating polyclonality by the transcription assay (Figure 3; Table 1).

Discussion

Gangliogliomas are mixed tumors containing glial and neuronal elements. The cell of origin of each component has long been a clinicopathological mystery. Many mechanisms could account for the presence of neoplastic glial and neuronal cells in gangliogliomas. These two components could arise independently, with either independent expansions of glioblasts and apolar neuroblasts within the same tumor, or through interaction between these precursors in a paracrine fashion. These processes would be therefore polyclonal, with random inactivation of either parental X chromosome and random expression of the androgen receptor gene from either allele. Alternatively, the two components could be derived from a single abnormal progenitor cell, such as a neuroepithelial cell, which then differentiates into both glial and neuronal elements during pathogenetic development. In such a case, the same parental X chromosome would be inactivated in both types of cells and expression of the androgen receptor gene would be from the same unmethylated allele. By using both methylation- and transcription-based clonality assays, we demonstrate for the first time that the same parental X chromosome was inactivated in the cells making up the majority of our gangliogliomas and that tumor cells were expressing only one allele of the androgen receptor gene. Therefore, we conclude that the two separate cell lineages observed in gangliogliomas are, at least in the majority of cases, developed from a common progenitor cell.

The observation that two of seven tumors was found to be polyclonal by methylation-based clonality assay is interesting and provocative and may be due to any of several possibilities. It is inevitable that normal tissue elements, such as fibroblasts, pericytes, and endothelial cells from blood vessels, leptomeningeal cells, and even the stray normal glial or neuronal cells, be included in any tumor specimen, even though gangliogliomas tend not to be widely infiltrative into normal brain.[2] We believe, however, that in each sample the great majority of cells were neoplastic and not diluted by extraordinary amounts of these inevitable normal tissue elements, although we cannot be certain of this. Given the range of the ratio of ganglion to glial cells in our series, one might wonder whether a greater proportion of a nonclonal element, whether glial or neuronal, would yield a low clonality ratio. A microdissection or *in situ* study would be necessary to address such an issue as we were unable to correlate a difference in histology between those tumors with a low CR and those with a high CR. Alternatively, it is possible that some gangliogliomas are indeed polyclonal, with separately arising glial and neuronal components. Of course, as our methodology can be applied only to female ganglioglioma patients, we cannot speak to the relevance of our findings to males.

The same overall results were obtained on OCT analyses as on frozen tumor specimens. The CR values from OCT samples were reproducibly less than that from frozen tissue specimens of the same tumor, a phenomenon that has been observed in clonality analyses of other brain tumors, including sporadic meningiomas.[30] Incomplete digestion due to the presence of enzyme inhibitors is believed to be the cause as the OCT specimens were too small to be subjected to the CsCl method for DNA isolation as performed on frozen tumors. As expression-based clonality methods, such as G6PD assay, have been considered the gold standard for determination of clonal origin, there has been concern that differential methylation at many loci is not a reliable marker for the state of activation of the X chromosome or that variable methylation may occur in association with malignancy.[31–34] Therefore, we checked the clonality of the four of our tumors for which OCT blocks were available with a newly developed transcription-based clonality technique at HUMARA. Using this method, we confirmed monoclonality and concordance with the methylation assay in all but one tumor (G4). We feel that the polyclonal result of G4 and G144 by methylation-based assay is unlikely to be due to experimental artifacts, such as incomplete digestion of DNA from either fresh-frozen tissue or in OCT by *Hpa*II, as complete digestion was observed in control samples run concurrently and repeated in triplicate. Abnormal methylations at the HUMARA locus in tumor G4 and G144 are possible but unlikely as the transcription-based assay demonstrated allelic expression suggesting nonrandom methylation of the X chromosome in G4 as well as in other tumors. Contamination by normal tissue or nontumor tissue intermixed with tumor mass, such as blood vessels, as mentioned above, cannot be completely excluded.

Assuming that most gangliogliomas arise in a monoclonal fashion, the question becomes one of which cells is the origin of the clone. Interestingly, McKay and his colleagues have shown, in studying the lineage relationship during the normal maturation of vertebrate nervous system by retroviral marking, that there is an early common precursor in the rat nervous system that can later develop into either neurons or astrocytes *in vitro*.[35,36] Additional evidence for the presence of pluripotent cells in highly differentiated areas of the adult mammalian CNS was documented by Reynolds and Weiss.[37] Cells isolated from the striatum of adult mouse brain were induced to proliferate *in vitro* by epidermal growth factor. A small number of cells (15 of 1000 cells on average) proliferated and expressed nestin, an intermediate filament found in neuroepithelial stem cells, and subsequently developed the morphology and antigenic properties of both neurons and astrocytes. The existence of dormant, multipotent cells within the adult mammalian CNS would be consistent with the concept that abnormal progenitor cells, or cells of hamartomatous appearance pathologically, may give rise to glial tumors, neuronal tumors, or mixed tumors, such as gangliogliomas.

Immunohistochemical analysis of gangliogliomas has shown that the glial elements in such tumors are typically positive for GFAP, whereas the neuronal components frequently express synaptophysin, a synaptic vesicle membrane glycoprotein.[38] However, a puzzling observation often encountered, not only in gangliogliomas but also in tuberous sclerosis lesions, is an apparent lack of concordance in the immunopositivity for GFAP of cells that in routine stains would be immediately interpreted as glial cells and the apparent presence of the protein in cells that display cellular features more characteristic of neurons.[2] The observation may be explained by the presence of neoplastic, multipotent precursor cells before their terminal differentiation. In a recent study, allelic deletion and clonality analyses of tuberous sclerosis lesions showed that 12 of 13 (92%) hamartomas were monoclonal in origin, confirming the idea that cells in the lesion arose from a pluripotent cell in the hamartoma.[39]

Studies of another mixed human brain tumor, a mixed ependymoma-astrocytoma (subependymoma), have demonstrated the existence of pluripotent progenitor cells that form mixed tumors at the terminal stages of divergent differentiation; ultrastructural evidence suggested neoplastic differentiation into ependymal and astrocytic cells from a common progenitor cells, the ependymoglia cells.[40]

There are many other mixed tumors, such as gliosarcomas and mixed tumors of astrocytomas and oligodendrogliomas of the CNS and carcinosarcomas, lymphoepithelial tumors, and teratomas outside the CNS, the clonal origins of which have been questioned for a long time but have not yet been characterized. With the increasing availability of many clonality assays, these questions will soon be answered.

In conclusion, we provide molecular evidence that monoclonal development is most likely the underlying pathogenesis for gangliogliomas, although additional experiments with a larger number of such tumors will be

necessary to confirm the initial observation. We expect that future molecular study of gangliogliomas will not only shed further light on the mechanism of pathogenesis but in so doing also provide new tools for better detection, diagnosis, and rational treatment of such tumors.

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