Consultations in Molecular Diagnostics

Molecular Diagnosis of Metastasizing Oligodendroglioma

A Case Report

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We report the case of a suspicious parotid mass in which molecular determination of loss of heterozygosity (LOH) of chromosome arms 1p and 19q in combination with cytologic and immunohistochemical analysis defined the tumor to be metastatic oligodendroglioma. The patient was a 41-year-old woman who developed a World Health Organization grade II oligodendroglioma in her right frontal lobe at age 32, for which no adjuvant chemo- or radiotherapy was administered. Five years following this diagnosis, radiological assessment revealed a 10-centimeter mass in the tumor bed, suspicious for a recurrence. Resection of this lesion revealed an anaplastic oligodendroglioma (grade III) and adjuvant radiotherapy was given. Eleven months after this surgery the patient presented with a 5.5-cm subcutaneous, non-mobile, non-tender mass in the region of the right parotid gland. Fine needle aspiration (FNA) yielded highly cellular material, morphologically and immunohistochemically suspicious for oligodendroglioma. Molecular analysis of microsatellite loci residing on chromosome arms 1p and 19q was performed using DNA extracted from the patient's recurrent brain oligodendroglioma and the FNA specimen. This analysis revealed evidence of LOH at all eight of the microsatellite loci tested. The combination of cytologic and molecular findings defined the extracranial tumor to be metastatic oligodendroglioma. (*J Mol Diagn* 2004, 6:52-57)

Extracranial metastasis of a glial neoplasm is a rare occurrence that can potentially create diagnostic problems for both surgical and cytopathologists. Strict pathological criteria for defining extracranial metastasis of primary brain tumors have been previously proposed,¹ however the application of molecular diagnostic techniques to the determination of the relationship of primary and metastatic tumors may be of even greater use in the evaluation of these samples.² Recently, a somatic genetic finding that is common to oligodendrogliomas has been identified: loss of heterozygosity (LOH) of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q, 1p19q LOH) in tumor tissue.^{3,4} The finding of 1p19q LOH in a glial neoplasm has also been shown to be predictive of tumor chemosensitivity and prolonged patient survival.^{5,6,7} 1p19q LOH has been reported to occur in over 85% of oligodendrogliomas, if the tumor is defined by strict histological criteria or a unanimous diagnosis by four neuropathologists.^{3,8,9} Thus, 1p19q LOH has been shown to be a useful marker of the diagnosis of oligodendroglioma when present in the context of appropriate clinical and histopathological features.

Here we report the case of a patient with a well-documented intracranial oligodendroglioma who presented with clinical findings suspicious for extracranial metastasis. The definitive diagnosis of metastasis was made using cytologic and immunohistochemical features in combination with the molecular determination of 1p19q LOH. The molecular determination of 1p19q LOH status in the diagnosis of extracranial metastasis has not previously been reported.

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Table 1. Microsatellite Loci Analyzed for the Diagnosis of 1p19qLOH

	Locus	Chromosome location	Size range (bases)	Label
Mix A	D1S199	1p36.22	85–120	6-FAM (blue)
	D1S162	1p32.3	115–175	TET (green)
	D1S226	1p22.1	69–115	HEX (yellow)
Mix B	D1S186	1p32.3	69–110	TET (green)
	D1S312	1p31.2	135–175	6-FAM (blue)
	D19S112	19q13.31	110–145	TET (green)
Mix C	D19S918	19q13.31	130–185	6-FAM (blue)
	S19S206	19q13.31	95–155	HEX (yellow)

Materials and Methods

Tissue Acquisition, Histological Preparation, Immunohistochemistry, Human Subjects

All tissue samples were processed by standard methods including fixation in 10% formalin, embedding in paraffin and hematoxylin and eosin (H&E) staining. Fine needle aspirate material was fixed in alcohol and air-dried. Diff-quick and PAP staining methods were used. An alcohol-fixed paraffin-embedded cell-block was made of FNA material for immunohistochemistry and molecular analysis. Immunohistochemical studies were performed after antigen retrieval using the avidin-biotin complex method, with diaminobenzidine (DAB) as the chromogen. Appropriate positive and negative controls were included. Antiglial fibrillary acidic protein (GFAP) antibody was used at a dilution of 1:6000 (DAKO, Carpinteria, CA). This case report was classified as exempt from review (exemption number 03–05–20–03e) under 45 CFR 46.101 by the Johns Hopkins University School of Medicine Institutional Review Board.

Extraction of DNA from Tumor and Normal Tissue

Five- μ m tissue sections were cut from the paraffin-embedded cell-block. The first section was stained with H&E and examined histologically to confirm that tumor cells were present and to identify the region with the highest density of tumor cells for DNA extraction. Tissue microdissection and DNA extraction was performed according to the method of Turbett et al.¹⁰ with modifications as described by Hatanpaa et al.¹¹ In brief, an unstained slide was superimposed on top of the marked stained slide and Pinpoint solution (Zymo Research, Orange, CA) was applied to the area of interest. DNA was released per manufacturer's protocol and no further DNA purification was performed before PCR amplification.

PCR and Capillary Electrophoresis

The PCR primer sequences, fluorescent labeling characteristics and PCR conditions used to amplify microsatellites residing on chromosome arms 1p and 19q were as reported by Hatanpaa et al.¹¹ In brief, eight microsatellite markers were assessed by PCR and capillary electrophoresis, five microsatellite loci on chromosome arm 1p

and 3 on chromosome arm 19q, in three multiplex PCR "mixes" (Table 1). All samples were subjected to PCR analysis in duplicate, with varying amounts of input DNA. Fluorescently labeled PCR products were sized using the ABI 310 Genetic Analyzer and GeneScan Collection software (Applied Biosystems, Foster City, CA). Ideally, the interpretation of LOH is made by comparison of the patterns generated by the tumor tissue to that generated by normal tissue from the patient. Unfortunately, normal tissue is often not available for testing. We previously described a set of criteria specific to this assay for interpretation of 1p19q LOH in the absence of normal tissue.¹¹ In brief, the Hatanpaa criteria require the identification of specific amplicon allelic patterns at each locus for the determination of LOH. Pattern 1 is characterized by the definitive identification of PCR product from only one allele at the locus in question (Figure 1A). This pattern is indistinguishable from the pattern generated by a germ-

Figure 1. Schematic representation of capillary electrophoresis of PCR products resulting from amplification of microsatellite loci. x axis is PCR product size in bases, y axis is fluorescence intensity. **A:** Allelic Pattern 1: PCR product from one allele is identified, associated with ~2 base stutter peaks. It cannot be determined from this pattern if the patient is a germline homozygote for this allele, or if the sample is composed entirely of tumor cells with loss of heterozygosity at the locus tested. **B:** Allelic Pattern 2: two alleles are present at the locus and the longer allele is less than 10% of the height of the shorter allele. This pattern is consistent with LOH at the locus in question. **C:** Allelic Pattern 3: two alleles are present and the shorter allele (in bases) has a lower peak height than the peak height of the longer allele. This pattern is consistent with LOH at the locus in question. Using the 1p19q LOH assay described, the interpretation of 1p19q LOH is made only if all eight of the microsatellite loci tested yield results consistent with one of the three patterns in replicate PCRs.

line homozygote at the locus. In pattern 2, two alleles are generated by the locus (heterozygote), but the peak height of the larger allele (in bases) is less than 10% of the peak height of the shorter allele (in bases) (Figure 1B). In addition, and for any allele to be considered a true allele, the predominant allelic peaks must be accompanied by at least one stutter peak (~ 2 bases for these dinucleotide repeats). In pattern 3, two alleles are generated at the locus and the peak height of the shorter allele (in bases) is lower than that of the longer allele (Figure 1C). Each of the eight loci is evaluated for evidence of LOH. The interpretation of 1p19q LOH requires that all eight loci tested conform to one of the three described allelic patterns in both PCR replicates, and that the patterns of LOH are consistent between loci for a given sample.

Results

Summary of Clinical History

The patient is a 41-year-old female who developed the sudden onset of generalized seizures at age 32 shortly after giving birth to her first child. An MRI scan performed at that time revealed a mass in the right frontal lobe. The patient underwent a right frontal craniotomy and tumor resection shortly after presentation, which revealed histological evidence of a World Health Organization grade II oligodendroglioma.¹² Neither chemotherapy nor radiation therapy was administered at that time. Following resection, the patient was followed with serial MRI scans and 5 years after initial resection, enlargement of the previously stable region of residual tumor was noted. At the time of this imaging study, the patient was 8 weeks pregnant with her second child. At that time, the patient began to experience headaches and dizziness, but received no treatment due to her pregnancy. Eight months later, after delivery, an MRI was performed that demonstrated substantial enlargement of the residual mass. The mass then measured 10 cm in diameter, involved the right frontal lobe and extended across the genu of the corpus callosum. The lesion had cystic regions and demonstrated irregular enhancement, as well as superficial enhancement of the dural surface of the brain in the operative bed. The patient underwent another right frontal craniotomy with total gross resection of the tumor.

Histopathologically, the resected recurrent tumor was consistent with a World Health Organization grade III oligodendroglioma. Postoperative MRI of the brain showed no evidence of abnormal enhancement in the tumor bed. The patient suffered a post-surgical CSF leak that resolved without intervention. No neurological deficits were identified following surgery. A complete course of radiotherapy was administered. Eleven months after the second craniotomy, the patient presented with a subcutaneous 5.5-cm mass in the region of the right parotid gland.

Histopathology and Molecular Analysis of the Recurrent Brain Tumor

The initial resection of the patient's primary brain tumor was performed at an outside institution. By report, the resection revealed an infiltrating neoplasm composed of highly uniform cells with round nuclei and prominent perinuclear halos. The intracranial tumor recurrence resection was performed at our institution and revealed a glial neoplasm with features similar to the first resection as well as focally increased cellularity, significant cytologic atypia and mitotic activity, and prominent vessels with endothelial cell proliferation. Immunohistochemistry was positive for patchy GFAP staining. These features were histologically consistent with the diagnosis of an anaplastic (World Health Organization grade III) oligodendroglioma (Figure 2A).

DNA was isolated from the patient's recurrent brain oligodendroglioma resection specimen by microdissection. Eight highly polymorphic microsatellite loci residing on chromosome arms 1p and 19q were analyzed using PCR amplification and capillary electrophoresis sizing, as previously described.¹¹ All eight microsatellite loci tested revealed evidence of LOH (Figure 2A), consistent with the diagnosis of oligodendroglioma.

Fine Needle Aspiration and Molecular Analysis of the Parotid Mass

The subcutaneous mass in the region of the right parotid gland was superficially located, non-mobile and non-tender. The lesion was amenable to fine needle aspiration. Multiple needle passes were performed, yielding a highly cellular sample composed of cells occurring both singly and as small tissue fragments in a background of rare vessels and small lymphocytes (Figure 2B). Two distinct morphological types of cells were seen, smaller cells with high nuclear-cytoplasmic ratios, hyperchromatic nuclei and nuclear molding, and larger cells with amphophilic cytoplasm, eccentric nuclei and minimal cytologic atypia. Immunohistochemical analysis revealed focal staining for GFAP (Figure 2B, inset) as well as weak staining for chromogranin and synaptophysin. Flow cytometric analysis of the aspirated cells confirmed the majority of cells to be large "non-hematopoietic" cells, occurring in a background of lymphocytes and occasional granulocytes (data not shown).

A paraffin-embedded cell-block sample of the FNA material was submitted for molecular analysis. As with the recurrent brain sample, all eight microsatellite loci examined demonstrated evidence of LOH. The pattern and intensity of LOH present in the FNA material (Figure 2B) was similar in character to the tumor resection specimen (Figure 2A). These findings strongly suggested that the parotid mass represented extracranial metastasis of the patient's oligodendroglioma.

Figure 2. A: Intracranial oligodendroglioma from second resection, hematoxylin and eosin (H&E) revealing a highly cellular tumor composed of sheets of cells with rounded nuclei and prominent perinuclear halos. The tumor cells showed mitotic activity and were separated by a network of capillaries. **Inset:** immunohistochemistry demonstrating focal GFAP positivity in tumor cells. **At right,** capillary electropherograms generated by sizing of fluorescently labeled PCR products from microsatellite loci D1S226 and D19S206 demonstrate loss of the germline 87 base allele of marker D1S226 and loss of the 142 base germline allele of marker D19S206. **B:** Fine needle aspirate (FNA) sample of the patient's extracranial mass (Diff-Quik). Two cell types were present, small cells with high nuclear-cytoplasmic ratios and nuclear hyperchromasia (**arrow**) and a predominant population of larger cells with amphophilic cytoplasm and eccentric nuclei (**arrowhead**). **Inset:** GFAP positivity seen in the large cells. Capillary electrophoresis analysis of microsatellite loci demonstrates loss of the 87 base germline alleles of markers D1S226 and D19S206, respectively, consistent with that found in the resected oligodendroglioma (**A**). The microsatellite results are representative of the eight loci tested on 1p and 19q.

Resection of Parotid Mass and Molecular Analysis

Following the cytologic and molecular analyses of the parotid mass FNA specimen, resection of the right superficial parotid gland with radical neck dissection was performed. Metastatic malignant oligodendroglioma was identified, involving three intraparotid lymph nodes and 10 of 68 regional (right neck) lymph nodes. The tumor metastases were intranodal with no invasion of the parotid parenchyma noted. The resection specimen revealed classic oligodendroglial features and positivity for GFAP in some tumor cells (Figure 3A). There was no gross or histological evidence of involvement of parotid gland tissue by tumor.

1p19q LOH analysis of the resected parotid region mass, as would be predicted, demonstrated a pattern of 1p19q LOH that was consistent with that generated by the intracranial oligodendroglioma recurrence and the FNA sample (Figure 3B, resected mass). In addition,

normal parotid gland was microdissected from the metastasis resection specimen, establishing a normal tissue control for comparison with the tumor tissue LOH results (Figure 3B, normal parotid). These findings provided further confirmation that the parotid mass represented extracranial metastasis of the patient's oligodendroglioma.

Discussion

Oligodendrogliomas account for between 10% and 25% of all gliomas and are the third most common CNS tumor of glial origin.^{13,14} Despite the relatively high incidence rate of primary oligodendrogliomas, extracranial metastasis is a rare occurrence that has been reported infrequently.^{15,16} The overall frequency of extracranial metastasis of CNS malignancies in general, has been reported to be as high as 4.3%, while the frequency of metastases to the CNS from extracranial sites is much more common at approximately 10%.¹⁷ Perhaps not sur-

Figure 3. A: Hematoxylin and eosin (H&E) staining of the resected metastasis revealing sheets of oligodendroglioma tumor cells with cleared cytoplasm and central nucleus ("fried-egg" pattern) to the **left** and normal lymphocytes to the **right** with some **central** overlap of lymphocytes and tumor cells centrally. **Inset:** immunohistochemistry demonstrating focal GFAP positivity in tumor cells. **B:** Molecular analysis of the resected mass (**upper**) compared to normal parotid tissue (**lower**) demonstrates loss of the 87 base and 142 base germline alleles of markers D1S226 and D19S206 in the tumor sample compared to normal. These results are consistent with those of the resected intracranial oligodendroglioma and parotid FNA of the mass (compare to Figure 2). The microsatellite results are representative of the eight loci tested on 1p and 19q.

prisingly, shunts, multiple craniotomies, and long survival are factors that have been postulated to predispose gliomas to metastasis.^{16,18,19} The histological diagnosis of primary intracranial oligodendroglioma can be very difficult on morphological grounds alone and there is often significant inter-observer variability in the diagnosis.⁷ The diagnostic features of oligodendroglioma are even harder to appreciate in cytologic specimens, particularly those obtained by aspiration of an extracranial mass. Immunohistochemistry can be helpful in demonstrating the neuroectodermal origin of the neoplastic cells (eg, GFAP, MAP2), but are of limited utility in the specific diagnosis of oligodendroglioma.¹³

Chromosomal alterations have been frequently used as a tool to determine clonality in neoplasms, in fact, certain chromosomal abnormalities or LOH patterns specifically characterize some pathological entities.²⁰ Clonal chromosomal changes have also been used previously to identify the origin of metastatic tumors of unknown primary.^{21,22,23} More recent literature cites the use of

microsatellite loci to identify the presence or absence of clonal LOH relationships between synchronous tumors in an individual^{24,25,26} and to use these findings to define the tumors as either separate primaries or metastases of one tumor. The case presented here builds on these previous analyses by using a newly recognized, specific molecular marker of oligodendroglioma (1p19q LOH) to definitively identify the rare occurrence of extracranial metastasis of this tumor.

In 1955, Weiss proposed rather strict criteria for defining the presence of metastasizing CNS glioma.¹ First, there had to be proven existence of a primary CNS tumor and a clinical patient history indicating that the initial symptoms were due to this tumor. Next, a complete necropsy must have been performed and yielded no suggestion of a primary tumor elsewhere in the body and lastly the morphology of the distant growth had to be consistent with that of the intracranial tumor. Relatively few reported cases of extracranial oligodendroglioma metastasis have completely complied with the criteria listed above, as in many these cases an autopsy was not performed.^{27,28,29} We propose that in the case of an oligodendroglioma, the inclusion of 1p19q LOH molecular analysis in these criteria will increase the accuracy of the diagnosis of extracranial oligodendroglioma metastasis. In the case presented here, 1p19q LOH of tumor tissue in conjunction with the cytologic features and focal GFAP positivity in the cytology specimen by immunohistochemistry, permitted the definitive diagnosis of extracranial metastasis. To our knowledge, this is the first report of the application of this clinical molecular diagnostic approach to the diagnosis of extracranial metastasis of an oligodendroglioma. The use of cytology in combination with the "molecular signature" of oligodendroglioma, 1p19q LOH,^{30,31,32} permitted an accurate, timely, and definitive diagnosis of the tumor.

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