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Inactivation patterns of *NF2* and *DAL-1/4.1B (EPB41L3)* in sporadic meningioma

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Abstract The molecular basis of tumorigenesis and tumor progression in meningiomas is not fully understood. The neurofibromatosis 2 (*NF2*) locus is inactivated in 50–60% of sporadic meningiomas, but the genetic basis of sporadic meningiomas not inactivated at the *NF2* locus remains unclear. Specifically, there is conflicting data regarding the role of the tumor suppressor gene *DAL-1/4.1B*. Using microsatellite markers, we studied 63 sporadic meningiomas to determine loss of heterozygosity (LOH) at the *NF2* and *DAL-1/4.1B* loci. Array comparative genomic hybridization analysis of 52 of these tumors was performed to determine copy number changes on chromosomes 18 and 22. Forty-one of 62 informative tumors showed LOH at the *NF2* locus (66%) while only 12 of 62 informative tumors (19%) showed LOH of *DAL-1/4.1B*. Eleven of 12 (92%) tumors with *DAL-1/4.1B* LOH also had *NF2* LOH. Monosomy or large deletions of chromosomes 18 and 22 were the main mechanism for LOH in these tumors. These studies implicate the *DAL-1/4.1B* locus in sporadic meningiomas less commonly than reported previously, and suggest that it is a progression rather than an initiation locus. Furthermore, we found the majority of meningiomas developed monosomy rather than isodisomy at the *NF2* and *DAL-1/4.1B* loci as the mechanism for LOH.

1. Introduction

Meningiomas are common neoplasms of the brain and spinal cord, accounting for 15–20% of all tumors of the central nervous system. These tumors arise from the leptomeninges and occur in three genetic contexts: as sporadic tumors in genetically normal individuals, as part of neurofibromatosis 2 (NF2), and in patients with syndrome of multiple meningiomas, which may show autosomal dominant inheritance. Meningiomas are usually benign but malignant progression occasionally occurs.

The molecular origin of meningiomas has not been fully explained yet. Loss of heterozygosity (LOH) of chromosome 22, including the *NF2* region at 22q12, occurs in approximately half of all sporadic meningiomas [1,2]. The genetic basis of sporadic meningiomas not inactivated at the *NF2* locus remains unclear. Losses involving the short arm of chromosome 1 have been described by LOH and comparative genomic hybridization (CGH) analysis in approximately 30% of sporadic meningiomas but were associated with inactivation of *NF2* in the majority of the tumors [3–8]. Other chromosome losses reported include 4q, 6q, 8q, 9p, 10q, 13q, 14q, 15q, 17p, 18, 19p, X, and Y, as well as gains of 12q, 15q, and 18p [4,6,7]. Although several chromosome arms have been implicated in meningioma tumorigenesis by CGH and LOH analysis, only a few genes have been analyzed individually, including *DAL-1/4.1B (EPB41L3)*, *p18*, *TP53*, *PTEN*, *KRAS*, *NRAS*, *HRAS*, *CDKN2A, P14ARF*, *CDKN2B*, and *CDKN2C* [2,9–11].

LOH of *DAL-1/4.1B* was originally reported in 71% (12/17) of sporadic meningiomas [9], but LOH and mutational analysis by exon scanning of multiple meningioma patients

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found that *DAL-1/4.1B* does not act as a tumor suppressor gene in this very particular cohort of tumors [12]. To reconcile these apparently conflicting results regarding the role of *DAL-1/4.1B* in meningiomas, we analyzed the *DAL-1/4.1B* and *NF2* loci in 63 sporadic meningiomas using LOH and CGH analysis, and correlated our findings with tumor histopathologic subtype and grade.

2. Materials and methods

2.1. Samples

Sixty-three individuals undergoing clinically indicated surgery for resection of dural-based tumors were entered in this study. In all cases, the clinical impression of the treating physician was that the patient had a single tumor without evidence of other intracranial neoplasms or NF2, and the treating pathologist classified the tumor as a meningioma. Excess tumor tissue that was unnecessary for diagnosis was flash-frozen on liquid nitrogen. Genomic DNA was extracted from peripheral blood specimens or tumor tissue, as described previously [12]. The Institutional Review Board of Massachusetts General Hospital approved this study, and informed consent was obtained from all study subjects.

2.2. LOH analysis

LOH of the *NF2* region was determined at markers *CRYBB2* and *D22S193* (centromeric to *NF2*), *D22S929* (intragenic), and *D22S268* and *D22S430* (telomeric to *NF2*). The distance from the most centromeric marker to the most telomeric marker is approximately 5 megabases (Mb). LOH of 18p was determined using markers *D18S1412*, *D18S1414*, and *D18S1415*, which lie within intron 1 of the *DAL-1/4.1B* gene, and *D18S1416*, which lies within intron 3 [12]. The distance from the most centromeric marker in intron 1 to the most telomeric marker in intron 3 is 20 kilobases. To exclude the possibility of partial gene deletion telomeric to intron 3, marker *D18S481* [9,13], which lies 2.4 Mb telomeric to the 3′ end of *DAL-1/4.1B,* was used. The location of markers on chromosomes 18 and 22 is shown in Fig. 1; amplification and analysis of microsatellite markers were performed as described previously [12].

2.3. Array CGH

One microgram of tumor or normal genomic DNA (male or female) was digested with *Dpn*II (New England Biolabs, Beverly, MA), purified with DNA Clean & Concentrator (Zymo Research, Orange, CA), and labeled with Cy3-cCTP or Cy5-dCTP (Amersham Bioscience, Buckinghamshire, UK) using the Bioprime DNA Labeling System (Invitrogen Life Technologies, Carlsbad, CA). Labeled DNA was precipitated using isopropanol with Cot-1 DNA (Invitrogen Life Technologies), which was used to block repetitive sequences. The probe pellet was then washed with 70% ethanol, dried, and dissolved in hybridization buffer. Labeled probes were hybridized onto a human cDNA microarray chip (Agilent Technologies, Palo Alto, CA), which contains 12,814 clones. The slides were then air-dried by centrifugation before imaging. Images were collected using an Axon 4000B scanner and processed initially using GenePix Pro 4.1(Axon Instruments, Inc., Union City, CA). Defective spots were flagged by visual inspection of the images and a custom software was used to exclude spots that demonstrated low signal-to-noise ratios [14]. The software calculated the baseline CGH level (two genomic copies) as the median Cy3 and Cy5 ratio of all clones analyzed. The normalized values were transformed to log2 format to ensure equal weighting for gains and losses. The average log2 ratios for all target DNA from chromosomes 22 and X were then calculated for each tumor sample. The X chromosome provided an internal control for large imbalances in the CGH analysis. The expected X chromosome log2 ratios were −0.5 for male versus female comparisons, 0 for sex-matched comparisons, and +0.5 for female versus male comparisons. When X chromosome values differed from the expected ratio by more than 20% (log2 > 0.11), X chromosome CGH data was reviewed to look for whole or partial X chromosome loss or gain. Copy number changes in chromosome X occurred in six samples

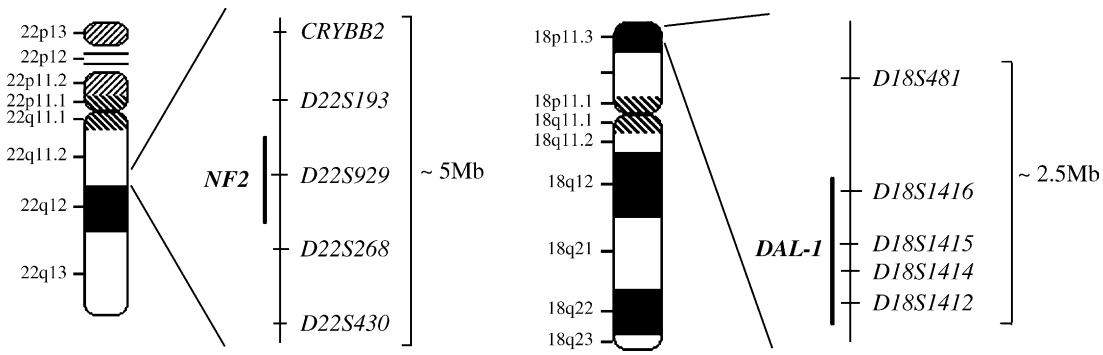

Fig. 1. Location of microsatellite markers used for LOH analysis. Marker positions were established using the resources of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

(12%), and for these, normalization to the average X log2 value was omitted, and log2 ratios were normalized to the median log2 ratio observed for all target clones from the entire genome. When X chromosome losses or gains were not detected, the log2 ratios for chromosome 22 were adjusted according to the expected X chromosome value.

2.4. Histopathology

Hematoxylin and eosin–stained, formalin-fixed, and paraffin-embedded sections of the tumors were evaluated histologically by a neuropathologist (A.S.R.), who was blinded for the genetic analysis results. Tumors were graded as benign, atypical, or malignant meningiomas on the basis of World Health Organization criteria [15] and classified into histologic subtypes (Fig. 2). When malignant features were present in the tumors, subtype classification was based on the better-differentiated areas in which the histologic subtype pattern was best preserved.

3. Results and discussion

Sixty-two of 63 tumor blood pairs studied were informative at one or more *DAL-1/4.1B* markers. Fifty of these tumors showed retention of both alleles in the tumor, while 12 showed LOH in the tumor compared to the blood specimen (19%). LOH of *DAL-1/4.1B* was more common among malignant (seen in 3 of 5 tumors, 60%) and least common among benign meningiomas (1 of 31, 3.2%). Whenever more than one marker was informative, all were either lost or retained, indicating a lack of breakpoints within the small region studied, including marker D18S481. Sixty-two tumor–blood pairs studied were informative at the *NF2* locus for one or more markers. Twenty-one tumors showed retention of both alleles, while 41 tumors showed LOH in the tumor compared to the blood specimen (66%). Sixty-one tumors were informative for at least one marker at each locus. Of the 12 tumors with *DAL-1/4.1B* LOH, 11 (92%) also showed *NF2* loss, while a single tumor had LOH of *DAL-1/4.1B* and retained heterozygosity at the *NF2* locus.

Conflicting results have been reported in the literature regarding the involvement of *DAL-1/4.1B* in meningioma formation. Multiple studies involving non-small cell lung carcinomas, sporadic meningiomas, and multiple meningiomas have failed to identify inactivating mutations in the gene sequence. Methylation of the promoter region has been proposed as an explanation for the decreased protein expression found in tumor tissues without detectable gene mutations [9,12,16]. So far, the presence of LOH in tumors has been the only confirmed inactivation event occurring in this tumor suppressor gene, and additional studies correlating LOH analysis with immunohistochemical and methylation studies in a large cohort of tumors are needed to determine *DAL-1/4.1B* involvement in meningioma formation [9,16].

In 52 of the 63 tumors, adequate-quality DNA was available for array CGH analysis. Three groups of chromosome segments were evident: those with two chromosome copies of the segments examined (log2 CGH ratio from -0.09 to $+0.09$), those with a single copy of the locus tested (log2 CGH ratio < -0.2), and those with amplification of the segments tested (log2 ratio $> +0.2$). Single copies of all chromosome 18 segments, consistent with monosomy, were seen in six of the eight tumors tested by CGH and showing *DAL-1/4.1B* LOH. One tumor with *DAL-1/4.1B* LOH and retention of the *NF2* locus showed two copies of *DAL-1/4.1B* and its surrounding region by CGH examination, consistent with isodisomy of the *DAL-1/4.1B* locus as the apparent mechanism for LOH of *DAL-1/4.1B*. One tumor showed gain on the short arm of chromosome 18 (log2 CGH ratio 0.71) and loss of the long arm. Forty-three of the remaining 44 tumors showing retention at *DAL-1/4.1B* showed two copies of all 18p segments surrounding *DAL-1/4.1B*. One single atypical tumor had an increased copy number for chromosome 18 which involved the whole chromosome. A summary of the

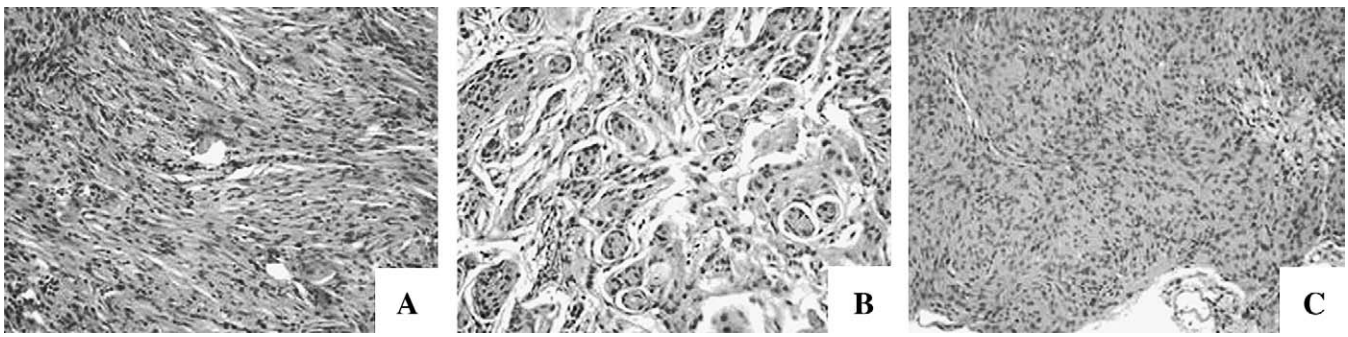

Fig. 2. Histologic characterization of meningiomas. Meningiomas were classified by one of the authors (A.S.R.) blinded for the genetic results. (A) Fibroblastic meningioma showing intersecting fascicles composed of spindle-shaped cells that, at times, resemble schwannomas. (B) Transitional meningiomas, defined as tumors with both components: one fibroblastic, with fascicles of elongated cells, and the other composed of meningothelial cell patterns with the presence of tight, often small whorls. (C) Meningothelial meningioma characterized by ill-defined lobules and whorls and cells with indistinct cell borders (syncytial cells).

Table 1
LOH and CGH results from meningiomas showing DAL-1/4.1B loss

		DAL-1/4.1B		NF2	
Tumor no.	Tumor grade	LOH	CGH	LOH	CGH
1	M	Lost	Single copy	Lost	Single copy
4	M	Lost	Single copy	Lost	Single copy
10	A	Lost	Single copy	Lost	Single copy
17	A	Lost	Single copy	Lost	Single copy
19	M	Lost	NA	Lost	NA
24	A	Lost	NA	Lost	NA
29	A	Lost	Two copies	Retained	Two copies
38	A	Lost	18p - 18q12 amplification, 18q12 - telomere single copy	Lost	Single copy
39	A	Lost	Single copy	Lost	Single copy
60	B	Lost	NA	Lost	NA
76	A	Lost	Single copy	Lost	Single copy
82	A	Lost	NA	Lost	NA

Abbreviations: B, benign; A, atypical; M, malignant; NA, not available.

LOH and CGH results from all tumors showing LOH of chromosome 18 is presented in Table 1. Our findings agree with previous reports of malignant progression of sporadic meningiomas, which showed monosomy of chromosome 18 in three out of four atypical tumors, but large deletions of 18q in only 2 out of 13 benign tumors [5]. Monosomy of chromosome 18, determined by single copy of all chromosome 18 segments by CGH analysis, occurred in 75% of the tumors with LOH of *DAL-1/4.1B* in contrast to the LOH mechanism originally described in sporadic meningiomas, in which only three out of the eight meningiomas informative at marker *D18S452* (centromeric to *DAL-1/4.1B*) were found to have LOH of 18p extending beyond the *DAL-1/4.1B* region [9].

Monosomy of chromosome 22 was seen in 8 out of 34 tumors showing *NF2* LOH (23%). All remaining 26 meningiomas showed decreased copy number of almost all chromosome 22 segments, but had two copies of the most centromeric 15-Mb region and amplification of the same region in a single tumor. Of the 18 tumors without *NF2* LOH, 11 showed 2 copies of all chromosome 22 segments. The remaining seven tumors showed amplification of the most centromeric region of chromosome 22, similar to the findings in tumors with LOH of *NF2*. The *NF2* gene has been well documented as a tumor suppressor gene in 50–60% of sporadic meningiomas [17]. The mechanism by which *NF2* LOH occurs in meningiomas, however, has not been fully described. Our results raise the question of which other genes located on chromosomes 18 and 22 are also being inactivated by this LOH process. Moreover, repetitive elements in the genome are common causes of chromosomal recombination and can lead to deletions, amplifications, or translocations. Palindromic repetitive elements located at the centromeric region of chromosome 22 have been shown to induce genomic instability leading to the rearrangements [18,19]. In this study, the most centromeric portion of chromosome 22 was found to have two or more copies by CGH analysis in 26 meningiomas with *NF2* LOH and more than two copies in seven meningiomas without *NF2* LOH, suggesting that the existence of similar clones located elsewhere in the human genome might be interfering in the analysis of copy numbers of this centromeric region of chromosome 22. It is unclear so far if these repetitive elements influence in the mechanism of chromosome 22 LOH in meningiomas, or whether they represent an artifact of CGH analysis.

Of the 63 tumors studied, the treating pathologist classified 32 as benign (51%), 26 as atypical (41%), and five as malignant (8%). LOH of both loci was more common among malignant tumors than among the atypical and benign. Thirty-three tumors were available for histolopathologic review, with seven tumors classified as meningothelial (21%), 14 as transitional (43%), and 12 as fibroblastic (36%) by the reviewing pathologist. LOH at the *NF2* and *DAL-1/4.1B* loci are shown in Table 2. The fibroblastic subtype of meningiomas has been previously correlated with *NF2* gene inactivation [20,21]. Our studies extend this view, with *NF2* LOH most common in fibroblastic tumors of all grades and least common in transitional tumors. Differences among subtypes were the most prominent in benign tumors, suggesting either that *NF2* LOH occurs during progression or that meningothelial and transitional tumors with *NF2* LOH are most likely to progress. In addition, histologic subtype is at least in part a surrogate for tumor localization [22], which could not be addressed in the current study.

Our analysis has shown that LOH of 18p is not as common in sporadic meningiomas as previously reported, acting more as a progression event than an early event in meningiomas formation. We have also demonstrated that

Table 2
Pathologic and genetic correlation in tumors

	NF2 LOH			NF2 retained			NF2 NI	
Tumor subtype	DAL-1/4.1B LOH	DAL-1/4.1B retained	DAL-1/4.1B NI	DAL-1/4.1B LOH	DAL-1/4.1B retained	DAL-1/4.1B NI	DAL-1/4.1B retained	Total
Meningothelial	1 (M)	4 (3A, 1B)			1 (B)		1 (A)	7 (21%)
Transitional	2 (A)	2 (1A, 1B)		1 (A)	8 (5B, 3A)	1 (A)		14 (42.5%)
Fibroblastic		10 (8B, 2A)	1 (B)		1 (A)			12 (36.5%)

Of the 33 tumors available for subtyping, 30 were informative at both loci. *Abbreviations:* B, benign tumors; A, atypical tumors; M, malignant tumors; NI, not informative.

monosomy or terminal deletion of chromosome 22 is the most common mechanism of *NF2* LOH in meningiomas.

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