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EVALUATION OF *NF2* GENE DELETION IN SPORADIC SCHWANNOMAS, MENINGIOMAS, AND EPENDYMOMAS BY CHROMOGENIC *IN SITU* HYBRIDIZATION

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Abstract

Fluorescent *in situ* hybridization (FISH), loss of heterozygosity (LOH)-testing and comparative genomic hybridization (CGH) have been used to detect *NF2* gene alterations in both sporadic and *NF2*-associated CNS tumors. In this study, we performed chromogenic *in situ* hybridization (CISH) and immunohistochemistry to evaluate for *NF2* gene deletion in a group of sporadic meningiomas, schwannomas, and ependymomas. Twenty-two sporadic tumors, including 9 ependymomas, 10 meningiomas, and 3 schwannomas were studied. CISH and immunohistochemistry were performed utilizing the *NF2* gene deletion probe and *NF2* polyclonal antibody. Deletion of the *NF2* gene was identified in 11 (50%) tumors, including 60% (6/10) of meningiomas, 33% (3/9) of ependymomas, and 67% (2/3) of schwannomas. The remaining 11 (50%) cases were diploid. Overall, immunoeexpression of *NF2* protein was observed in 50% (11/22) tumors and concordance between CISH and immunohistochemistry was observed in 73% of cases. Our results support previous observations that schwannomas and meningiomas, and to a lesser degree, ependymomas express a high incidence of *NF2* gene deletion, which supports the hypothesis that *NF2* gene plays an important role in their tumorigenesis. In addition, we have validated CISH as an efficient, economic and reliable method for routinely assessing *NF2* gene deletion in these tumors.

Keywords

CISH; Ependymomas; Meningiomas; *NF2*; Schwannomas

INTRODUCTION

In 1993, Rouleau and colleagues first characterized the neurofibromin 2 (*NF2*) gene in patients with neurofibromatosis type 2 [1]. This 22q12.2 gene promotes the production of merlin protein, which is found in brain, nerves, eyes, and Schwann cells. It appears to play an important role in many signaling pathways related to cell shape, growth, division, and communication

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between cells. The gene also functions as a tumor suppressor protein, which prevents cells from growing and dividing too rapidly or in an uncontrolled manner [2,3].

Genetic alterations in the *NF2* gene have been reported not only in nervous system tumors but also in mesotheliomas, melanomas, breast and colon carcinomas [4,5], providing evidence that *NF2* gene has a prominent role in the pathogenesis of different tumors. More than 200 mutations in the *NF2* gene have been identified. About 90% of these mutations result in an abnormally shortened version of the merlin protein [6]. Previous studies have suggested that the loss of merlin allows cells, especially Schwann cells, to multiply too frequently, which leads to the formation of schwannomas, meningiomas and ependymomas commonly associated with NF2 syndrome [7].

Recently, somatic mutations of *NF2* gene have been demonstrated in sporadic schwannomas [8], meningiomas [9], and ependymomas [10]. Approximately 60% of sporadic meningiomas show NF2 mutations and deletions [9,11]. Previous studies on schwannomas and ependymomas have reported *NF2* gene deletions in approximately 60 to 80% and 30% of sporadic cases, respectively [12].

To identify genetic abnormalities in the *NF2* gene, many different techniques such as Southern blot [9], semi-quantitative or quantitative PCR [13], fluorescence *in situ* hybridization (FISH) [14], LOH [15], and comparative genomic hybridization (CGH) [16] have been used. However, these tests require expensive equipment for signal detection and recording, and dedicated well-trained personnel for the performance and interpretation of results. Although FISH is an *in situ* method, morphologic features of the tumor are difficult to recognize under fluorescence microscopy. Moreover, the signals cannot be preserved for more than a few weeks owing to fluorescence decay [17].

Chromogenic *in situ* hybridization (CISH) is a sensitive assay that has been used for assessing *HER-2/neu* gene amplification in breast cancer [18]. Recent studies have confirmed a high concordance between FISH and CISH results. In contrast to FISH, CISH signals are permanent and the slides can be read on a regular light microscope in the context of morphologic examination [19,20]. Various studies have demonstrated reliable results using CISH for detection of amplification, translocation, and chromosome number in central nervous system tumors [20-23]. To the best of our knowledge, gene deletion detection by CISH has not already been demonstrated.

The purpose of this study was to evaluate *NF2* gene deletions in sporadic schwannomas, meningiomas, and ependymomas using *NF2* gene deletion probe for CISH and by immunohistochemical analysis of the NF2 protein expression.

MATERIALS AND METHODS

Tissue specimens

This study was conducted using tissue specimens from 22 sporadic tumors selected from the consultation files of the Armed Force Institute of Pathology (AFIP), Children's Hospital National Medical Center, Washington, DC, and the Laboratory of Pathology at the National Cancer Institute (NCI/NIH). The clinical data were obtained from medical records. Hematoxylin and eosin (H&E)-stained slides were reviewed for confirmation of diagnosis. Histologically, tumor samples were classified according to current World Health Organization (WHO) criteria [24]. The tumors included 9 ependymomas, 10 meningiomas, and 3 schwannomas.

Chromogenic in situ hybridization

Chromogenic *in situ* hybridization (CISH) for the *NF2* gene deletion was performed according to the manufacturer's (Zymed Laboratories Inc., South San Francisco, CA, USA) instructions on 5mm thick unstained sections from archival formalin-fixed paraffin embedded tumor samples. The slides were deparaffinized in xylene and graded ethanols. Heat pretreatment was carried out in the pretreatment buffer (Zymed Laboratories Inc.) at 98-100°C for 15 min. The tissue was digested with the Zymed FFPE digestion enzyme for 10 min. The slides were washed with PBS and dehydrated with graded alcohols. Ready-to-use Zymed SpotLight® digoxigenin-labeled *NF2* deletion probe was applied onto slides, which were covered by CISH™ UnderCover Slips (Zymed); the slides were denatured on a hot plate (98°C) for 5 minutes and hybridization was performed at 37°C for 18 hours. A posthybridization wash was performed in the next day and followed by immunodetection using the CISH™ polymer detection kit (Zymed Laboratories Inc.). A normal brain sample and a tissue known to contain *NF2* gene deletion were used as positive controls. Negative control tissue sections were hybridized in the absence of probe. The Zymed's SpotLight® *NF2* probe is a double stranded DNA probe that has been labeled with digoxigenin. This probe has been demonstrated to bind specifically to the *NF2* gene locus on chromosome band 22q12.2.

Criteria for evaluation of *NF2* gene status was based on previous studies for *NF2* gene deletion by FISH [30]. The probe displayed a single distinct small dark brown signal at the location of each copy of the *NF2* gene. Deletion of *NF2* gene was identified when only one hybridization signal was seen in more than 50% of the tumor cells. Normal diploid was determined when two hybridization signals were observed in more than 50% of the tumor cells. Signal enumeration was performed under a standard light microscope using a 40x objective. At least 200 cells were counted per tissue sample. Only nuclei with histological abnormal appearance were considered and overlapping nuclei or minor hybridization signal was not analyzed.

Immunohistochemistry

Immunostaining for *NF2* protein was performed in 5 µm thick sections of the tumors. Staining was done on paraffin sections using microwave antigen retrieval techniques (95° C for 10 min in citrate buffer pH 6.0). Sections were incubated with rabbit polyclonal *NF2* primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at dilution 1:50 for 60 minutes at room temperature. This antibody recognizes the epitope mapping the N-terminus of *NF2* of human origin. Immunostaining was performed using the biotin-streptavidin-peroxidase procedure (Envision Detection Kit, DakoCytomation, Los Angeles, CA). The peroxidase activity was localized by 0.05 % 3, 3'-diaminobenzidine and 0.03 % hydrogen peroxide in Tris-buffered saline. Slides were counterstained with Mayer's hematoxylin. Tumors were considered positive when >1% of neoplastic cells displayed cytoplasmic staining as previously described [25]. Nuclear staining, although noted in some cases where cytoplasmic staining was present, was disregarded and interpreted as artifact.

RESULTS

CISH and immunohistochemistry were successfully completed in all 22 tumors and positive controls. Results of the *NF2* gene status detected by CISH are summarized in the Table 1. Hybridization signals were easily identified by routine light microscopic observation. Representative cases are illustrated in Figure 1.

NF2 gene deletion was observed in 6/10 (60%) of meningiomas; specifically, in 40% (2/5) of benign, 50% (1/2) atypical, and in 100% (3/3) of anaplastic subtypes (Table 2). The remaining 4 cases (40%) were diploid, exhibiting two hybridization signals in the tumor cells.

Sixty-six percent (2/3) schwannomas showed *NF2* gene deletion. In these cases, more than 50% of tumor cells had a single hybridization signal in the nuclei. Only one schwannoma was diploid for *NF2* gene.

Within the ependymoma group, only 33% (3/9) of cases had an *NF2* gene deletion. The majority of ependymomas (67%) had two hybridization signals in most nuclei. Overall, *NF2* gene deletion was observed in 50% (11/22) cases.

Immunoeexpression of NF2 protein was observed in 50% (11/22) cases, including 60% (6/10) meningiomas, 44% (4/9) ependymomas, and 33% (1/3) of schwannomas (Figure 2). The remaining 11 cases were negative. CISH analyses and immunohistochemistry was concordant for *NF2* gene/*NF2* protein status in 16/22 (73%) cases (Table 3). Six (27%) cases showed discordance between CISH and immunohistochemistry results. Three *NF2* gene deletion associated cases by CISH retained protein expression by immunohistochemistry. On the other hand, three *NF2* gene deletion non-associated cases by CISH had lost of NF2 protein immunoeexpression.

DISCUSSION

The *NF2* tumor suppressor gene is responsible for the inherited disease neurofibromatosis type 2. NF2 patients show germ-line mutations with inactivation of one of the alleles, while inactivation of the second allele leads to the disease [26]. Somatic bi-allelic *NF2* gene mutations with loss or inactivation of the *NF2* gene have been associated with tumorigenesis of many NF2 associated and sporadic nervous system tumors [27,28]. Applying CISH methodology, we demonstrated *NF2* gene deletion in 50% of sporadic central nervous system tumors comprising schwannomas, meningiomas, and ependymomas.

Similar to previous studies, our results showed that 60% of sporadic meningiomas exhibit *NF2* gene deletion. In addition, we observed *NF2* gene deletion with concomitant loss of NF2 protein expression in a higher proportion of grades II or III meningiomas, which support involvement of the *NF2* gene in malignant progression of these tumors [29,30]. Defective *NF2* gene may reduce the activity of NF2 protein at the interface between the plasma membrane and the cytoskeleton, thereby causing dysfunction in adhesion molecules essential to normal cell development and regeneration [31,32]. It has been postulated that this dysfunction causes loss of cell-cell contact growth inhibition resulting in more aggressive tumor growth patterns [2]. Although some of our cases showed histological patterns of aggressiveness, like necrosis or mitotic activity, we did not have patient follow up to evaluate for clinical progression.

Despite the small number of schwannomas, we confirmed the previously described high incidence of *NF2* gene deletion in sporadic lesions [33,34]. Prior studies using LOH in the *NF2* locus and CGH [12] have shown genetic alterations of *NF2* gene in more than 60% of schwannomas. Similar to earlier reports, our results showed deletion of *NF2* gene by CISH in 66% of schwannomas with complete concordance between *NF2* gene deletion by CISH and NF2 protein expression by immunohistochemistry [12].

Most ependymomas exhibit complex cytogenetic changes, which affect a variety of chromosomes. Abnormalities of chromosome 22 have been detected in approximately 30% of ependymal tumors, especially in intramedullary spinal ependymomas [35]. Our results using CISH methods with immunohistochemical confirmation of loss of NF2 protein expression mirrored previous investigations. Previous studies comparing FISH and immunohistochemistry for detection of *NF2* gene deletion and expression in different tumors showed a concordance rate of about 70% [25]. We found a slightly higher concordance of 73% between CISH and immunohistochemistry. This observation is significant, considering that CISH measures gene copy numbers and immunohistochemistry detects protein expression.

Occasional discordant results are to be expected because of technical difficulties. Alternatively, it can be hypothesized that some tumors may delete a genetic region without inactivating the remaining allele, allowing for detection of deletion by CISH with preserved protein expression by immunohistochemistry. In our series, we demonstrated this pattern of discordance in 3 meningiomas (cases 4, 5, and 6). In another study of pediatric meningiomas, this pattern was the most commonly observed, leading the authors to associate these results with higher rates of chromosome 22 loss of heterozygosity (LOH) than with *NF2* mutations [25]. Loss of *NF2* protein expression unassociated with *NF2* deletion by CISH was observed in one meningioma (case 7) and two ependymomas (cases 18 and 19). This observation could be explained by gene inactivation by other mechanisms not detected by CISH, thus leading to loss of expression by immunohistochemistry, despite the lack of a detectable deletion.

In summary, our findings underscore and confirm previous reports regarding the important role that the *NF2* gene plays in the tumorigenesis of sporadic meningiomas, schwannomas, and ependymomas. *NF2* gene deletion appears to be most frequently associated with high grade meningiomas and less so in ependymomas. Our results indicate that CISH appears to be an efficient, economic and reliable method for the evaluation of *NF2* deletion status in CNS tumors. Finally, this method has the advantage of allowing the concomitant assessment of morphologic features in paraffin embedded material.

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List of Abbreviations

AFIP	Armed Forces Institute of Pathology
CGH	comparative genomic hybridization
CISH	chromogenic in situ hybridization
CNS	central nervous system
FFPE	Formalin Fixed Paraffin Embedded
FISH	fluorescent in situ hybridization
H&E	hematoxylin and eosin
LOH	loss of heterozygosity
NF2	neurofibromatosis type II
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction

WHO

World Health Organization

Figure 1: Representative CISH for NF2 gene deletion; normal brain with diploid (A); NF2 deletion in schwannoma (B), meningioma (C), and ependymoma (D).

Figure 1.

Representative CISH for *NF2* gene deletion; normal brain with diploid (A); *NF2* deletion in schwannoma (B), meningioma (C), and ependymoma (D).

Figure 2: Immunohistochemical analysis of merlin protein; H&E meningioma (A), schwannoma (C), ependymoma (E); NF2 protein immunostaining in meningioma (B), schwannoma (D), and ependymoma (F).

Figure 2. Immunohistochemical analysis of NF2 protein; H&E meningioma (A), schwannoma (C), ependymoma (E); NF2 protein immunostaining in meningioma (B), schwannoma (D), and ependymoma (F).

Table 1
NF2 gene deletion in CNS tumors demonstrated using CISH

Tumors	<i>NF2</i> gene deletion
Schwannomas	2/3 (66%)
Meningiomas	6/10 (60%)
Ependymomas	3/9 (33%)
Total	11/22 (50%)

Table 2

CISH results in meningiomas stratified by grade

Meningiomas grade	<i>NF2</i> gene deletion
Grade I	2/5 (40%)
Grade II	1/2 (50%)
Grade III	3/3 (100%)
Total	6/10 (60%)

Table 3
Immunohistochemical and CISH results in sporadic CNS tumors

Case	Diagnosis	<i>NF2</i> gene deletion by CISH	Merlin Immunoreactivity
1	Meningioma	Yes	Negative
2	Meningioma	Yes	Negative
3	Meningioma	Yes	Negative
4	Meningioma	Yes	Positive
5	Meningioma	Yes	Positive
6	Meningioma	Yes	Positive
7	Meningioma	No	Negative
8	Meningioma	No	Positive
9	Meningioma	No	Positive
10	Meningioma	No	Positive
11	Ependymoma	Yes	Negative
12	Ependymoma	Yes	Negative
13	Ependymoma	Yes	Negative
14	Ependymoma	No	Positive
15	Ependymoma	No	Positive
16	Ependymoma	No	Positive
17	Ependymoma	No	Positive
18	Ependymoma	No	Negative
19	Ependymoma	No	Negative
20	Schwannoma	No	Positive
21	Schwannoma	Yes	Negative
22	Schwannoma	Yes	Negative