**p16^INK4A (CDKN2A) Gene Deletion Is a Frequent Genetic Event in Synovial Sarcomas**

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**Key Words:** p16^INK4A; Heterozygous deletion; Tissue microarray; Synovial sarcoma; Cell cycle

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**Abstract**

We assessed the frequency of genomic deletion of p16^INK4A (CDKN2A) in synovial sarcomas (SSs) and its possible association with immunoexpression of p16 and cyclin D1 and the Ki-67 proliferation index using dual-color fluorescence in situ hybridization (FISH) on tissue microarray sections of 41 histologically and molecularly confirmed SSs. A heterozygous p16^INK4A gene deletion was identified in 28 (74%) of 38 cases, with 25 (89%) of them showing abnormal p16 protein expression (20 negative and 5 heterogeneous). Of 25 cases, 19 (76%) exhibiting increased cyclin D1 expression also demonstrated heterozygous p16^INK4A deletion. No significant association was observed between p16^INK4A deletion and Ki-67 proliferation index, tumor grade, or histologic subtype. Our results demonstrate that p16^INK4A (CDKN2A) gene deletion is a frequent genetic event in SS.

Alterations involving cell-cycle regulators acting at the G1-S checkpoint, besides being the most frequent molecular targets in tumorigenesis of many human neoplasms, have also been implicated in the pathogenesis and tumor progression of sarcomas, especially those lacking specific genetic alterations.1 Moreover, primary genetic alterations of cell-cycle regulatory proteins are postulated to be less common in translocation-associated sarcomas, in which they are considered as secondary events.2

Synovial sarcomas (SSs) are characterized by recurrent nonrandom chromosomal translocation, t(X;18)(p11.2;q11.2), which is considered the primary genetic event in 90% of cases.3 However, little is known about the tumorigenic role of cell-cycle proteins in SSs. The 9p21 region contains 2 well-known tumor suppressor genes, p16^INK4A and p15^INK4B. p16^INK4A encodes 2 alternatively spliced proteins p16 and p14^ARF. These 2 proteins are encoded in different reading frames and, thus, have no homology. They are, however, important in cell-cycle regulation and cellular senescence. p16 blocks the cell cycle progression at the G1-S transition by interfering with the cyclin D/CDK4 complexes, inhibiting pRb phosphorylation and release of transcription factor E2F, thus forming a critical component of the pRb pathway, whereas p14^ARF is involved in the p53 pathway by inhibiting the binding of MDM2 with p53 and, thus, preventing its degradation in the cytoplasm by proteolysis.4-7

Deletions of the short arm of chromosome 9 have been observed in many human tumors and cell lines.8,9 In the past, several loss of heterozygosity (LOH) assays for loss of the p16^INK4A gene at 9p21 of soft tissue sarcomas, including SSs, have been published. They include multiplex polymerase chain reaction (PCR), Southern blotting, PCR–single-strand

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conformation polymorphism analysis, and differential PCR\textsuperscript{10-13} or microdissection-based microsatellite analysis.\textsuperscript{14,15} Molecular LOH assays assess relative levels of paternal and maternal alleles within a tumor but are unable to determine chromosome number or structure.\textsuperscript{16} Fluorescence in situ hybridization (FISH) represents an enormous advance in the field of molecular cytogenetics for the identification of novel tumor-specific chromosomal alterations and imbalances.\textsuperscript{17} FISH-based chromosomal deletion assays permit investigation of selected nuclei with cytologically atypical features instead of isolating DNA from a mixed cell population. In addition, they help distinguish normal and tumor cells and identify homozygous and heterozygous deletions.\textsuperscript{18} Interphase FISH assays for genomic deletions of \textit{p16\textsuperscript{INK4A}} in soft tissue tumors have been described.\textsuperscript{19,20} However, to our knowledge, FISH testing for deletion of the \textit{p16\textsuperscript{INK4A}} locus in SS has not been reported.

We describe a dual-color interphase FISH analysis using a \textit{p16\textsuperscript{INK4A}} (\textit{CDKN2A}) gene-specific probe to assess the frequency of deletion of \textit{p16\textsuperscript{INK4A}} at the 9p21 region in a tissue microarray (TMA) containing 41 SSs and to determine whether a correlation exists between genomic deletion of \textit{p16\textsuperscript{INK4A}} and immunohistoexpression of p16 and cyclin D1, the Ki-67 index, histologic grade, and subtype.

**Materials and Methods**

**Tissue Samples and TMA Assembly**

We assembled 41 FISH- and reverse transcriptase–PCR–confirmed (17/41), paraffin-embedded SSs in a TMA consisting of triplicate 1-mm cores using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). The histologic subtypes included 15 biphasic SSs, 24 monophasic...
fibrous SSs Image 1B, and 2 poorly differentiated SSs Image 1C. All cases were graded according to the French sarcoma grading system (Federation Nationale des Centres de Lutte Contre le Cancer): 22 were grade 3 sarcomas, and 19 were grade 2.

*p16INK4A (CDKN2A)-Specific DNA Probe and Dual-Color FISH Assay*

The commercial cocktail DNA probe (Q-BIOgene, Irvine, CA) used in the FISH analysis is composed of a combination of a *p16INK4A (CDKN2A)* gene–specific probe, labeled with rhodamine red and an *ABL* (9q34)-specific probe direct labeled with dGreen for chromosome identification. Pretreatment included pressure cooking in 10 mmol/L of citrate buffer (pH 6.0) for 3 minutes, Proteinase K digestion for 15 minutes in a water bath at 37°C, fixation in 10% buffered formalin, and washing in 2× saline sodium citrate. Addition of 10 µL of the probe to the pretreated slide, simultaneous codenaturation of the probe and the target DNA at 93°C for 4 minutes, overnight incubation at 37°C, posthybridization washes, and counterstaining were carried out according to standard protocol.

The FISH criteria for tumor cells with normal and deleted *p16INK4A* genes at 9p21 loci were defined. Nuclei with a normal *p16INK4A* gene were represented by the presence of 2 red signals for the 2 copies of the *p16INK4A* gene and 2 green signals for the 2 copies of the *ABL* gene Image 2B. Homozygous deletion was defined as the presence of at least 1 green signal (*ABL*) and lacking both signals for the locus-specific probe (*p16INK4A*), and heterozygous deletion was characterized by 2 green signals (*ABL*, 9q34) and 1 red signal (*p16INK4A*, 9p21) Image 2A. Because we observed a very small percentage of homzygous deletions in our cases, we decided to omit the homzygous *p16INK4A* gene deletion.

An average of 500 well-separated nuclei was counted in each core. To define the distribution of interphase FISH signals, normal renal control tissue samples incorporated in the TMA were used for interphase FISH normal value studies. In the control tissues, we established a scoring scheme that placed the cells into 4 distinct groups: 1, cells displaying 2 pairs each of red and green signals (intact *p16INK4A* gene); 2, cells exhibiting 2 green signals and 1 red signal, supposed to represent heterozygous *p16INK4A* deletion; 3, the sum total of the percentage of cells showing FISH signals thought to be possible cutting artifacts and representing the following combinations: cells with 2 red signals and 1 green signal, 1 green and 1 red signal, and either 2 red or 2 green signals (the most frequent pattern encountered in group 3 was cells with 2 red signals and 1 green signal); 4, true percentages of cells showing heterozygous *p16INK4A* deletion (2 green signals and 1 red signal) obtained by subtracting the total percentages of cells with cutting artifacts (group 3) from the cellular population belonging to group 2, thus eliminating all possible false-positives. The percentage of cells obtained in group 4 ranged from 12% to 13%, the mean of which was 12.5% (SD, 0.70%). The mean + 3 SD percentage of the cells in group 4 was 15% or less. A similar scoring scheme was applied to the tumor samples, and heterozygous *p16INK4A* gene deletion was considered present when the total percentage of tumor cells in group 4 exceeded the mean + 3 SD percentage (>15%).

**Image 2** Representative fluorescence in situ hybridization images of *p16INK4A* heterozygous deletion with tumor nuclei showing 2 green signals and 1 red signal (A) and intact *p16INK4A* gene (B) displaying nuclei possessing 2 green and 2 red signals (DAPI [4'6-diamidino-2 phenylindole] counterstain ×400).
Immunohistochemical Analysis

Immunohistochemical analysis was performed using anti-p16 antibody (clone F12, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution, anti–cyclin D1 antibody (clone SP4, NeoMarkers, Fremont, CA) at a 1:50 dilution, and anti–Ki-67 antibody (MIB-1, DakoCytomation, Glostrup, Denmark) at a 1:50 dilution. Antigen retrieval for p16 and Ki-67 was performed by pressure cooker boiling for 3 minutes in 10 mmol/L of citrate buffer (pH 6.0) and for cyclin D1 by using high temperature retrieval by incubation in high pH buffer (pH 9.0) in a water bath at 100°C for 45 minutes. The LSAB method (DakoCytomation) was used, followed by revelation with 3,3’-diaminobenzidine.

Sections of reactive lymph node were used as positive control samples for all 3 antibodies. Under similar conditions, the primary antibody was replaced by preimmune rabbit serum serving as a negative control sample. Because the antigens under study are nuclear proteins when functional, we scored only the tumor cells displaying nuclear immunoreactivity. Cytoplasmic staining was disregarded and was not scored as a positive phenotype. Sections were examined for nuclear staining, and immunoreactivity for p16 was defined as follows: negative, fewer than 20% of tumor cells were immunoreactive; heterogeneous, 20% to 80% were immunoreactive; positive, more than 80% were immunoreactive. Samples showing negative or heterogeneous staining were considered abnormal, whereas those with positive staining were interpreted as normal.

RESULTS

The results of immunohistochemical and FISH analyses are given in Table I.

FISH Assay for the p16INK4A Gene

FISH scoring was performed independently by 3 investigators (M.M.S., R.N., and M.P.). The observations of the triplicate cores were summed, and their mean was taken into account. Of the total 41 cases, 3 were unscorable owing to the absence of FISH signals. Heterozygous deletion of the p16INK4A gene was detected in 28 (74%) of 38 cases. The percentage of tumor cells showing deletion ranged from 15% to 66%, with a mean of 30% (SD, 11.1%). The remaining 10 cases were devoid of deletion of the p16INK4A gene (range, 2%-14%; mean, 8%; SD, 4.2%).

Immunohistochemical Analysis

All sections were evaluated independently by 3 pathologists (M.M.S., S.N., and A.L.B.). The agreement of staining intensity scoring by the 3 observers was recorded, and in cases of disagreement, intensity was determined by consensus. p16 revealed diffuse positive nuclear staining in 4 (11%) of 38 SSs, heterogeneous expression in 7 (18%) and negative staining in 27 (71%). Cyclin D1 showed increased expression in 25 (66%) of 38 cases, low expression in 4 cases (11%) and negative staining in 9 cases (24%). A high Ki-67 proliferation index was found in 9 (24%) of 38 SSs; 14 (37%) were read as normal, and 15 cases (39%) were negative.

Correlation of Immunoreexpression of p16, Cyclin D1, Ki-67 Proliferation Index, Tumor Grade, Histologic Subtype, and p16INK4A Gene Deletion

Of 28 cases with heterozygous deletion of the p16INK4A gene, 25 (89%) showed abnormal p16 protein expression, of which 20 were negative and 5 revealed heterogeneous staining. Three p16INK4A-deleted SSs (11%) were positive for p16 protein. Among the 10 p16INK4A-intact SSs, only 1 showed diffuse positivity for p16, heterogeneous staining was found in 2 cases, and p16 was negative in 7 cases. Of the 25 cases showing increased expression of cyclin D1, 19 (76%) demonstrated heterozygous deletion of p16INK4A loci, whereas 6 showed intact p16INK4A genes. Of the 28 cases with 9p21 deletion, only 9 (32%) showed high Ki-67 indices, whereas 9 cases were normal and 10 negative, signifying a lack of correlation between heterozygous deletion of p16INK4A and the proliferation index. No significant association between p16INK4A gene deletion and grade or histologic subtype was observed.

Discussion

We describe a dual-color FISH analysis to assess the frequency of p16INK4A gene deletions at 9p21 in SSs and to look for a possible association with other cell-cycle regulatory proteins and histologic grade and subtype. Assessment of chromosome 9 losses can be performed molecularly by LOH studies or cytogenetically by FISH assays. Molecular LOH techniques include conventional DNA-based LOH assays and LOH studies performed on microdissected specimens. Although the conventional LOH tests provide information regarding the relative levels of parental alleles in a tumor, they have the following drawbacks: First, contamination of the tumor cells with a relatively small population of normal cells or tumor heterogeneity in which only a small clone of cells has a homozygous

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Subramaniam et al / HETEROZYGOUS p16INK4A DELETION IN SYNOVIAL SARcoma

Deletion obscures detection of such deletions. Second, molecular studies assess neither the chromosomal structural alterations nor polysomy. The whole genomic amplification of the microdissected tissue allows one to compare the different genetic methods such as FISH, deletion mapping by microsatellite analyses, and mutation analyses of specific genes from small lesions. To date, TMA-based FISH assay for deletion of the p16INK4A gene has not been reported. FISH analysis for deletion of a chromosomal locus is a high-throughput technique for analysis of multiple tumor types; furthermore, inclusion of normal control tissue samples with tumor samples in a TMA format serves as an ideal control for establishing cutoff percentages for deletion of a chromosomal locus in tumor samples. However, it is limited by the inability to optimize tissue preparation and hybridization conditions to suit individual tissue cores and problems associated with truncated nuclei in paraffin sections.

A recent study demonstrated higher sensitivity of FISH for the detection of chromosomal losses than LOH assays.17 FISH, apart from detecting numeric and structural chromosomal alterations, is also a sensitive technique for detecting subtle underrepresentation of a region and for verifying and narrowing the deletions detected by LOH analysis. In addition, interphase FISH on paraffin sections permits direct correlation of the genetic event with the morphologic features. Application of the FISH method to TMA for screening gene amplifications and detecting translocations has been described previously.26-28

**Table 1**

<table>
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<th>Tumor Cells With HD per 100 Tumor Nuclei (%)</th>
<th>p16 Gene Status</th>
<th>p16 Protein Expression (%)</th>
<th>Ki-67 PI (%)</th>
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BSS, biphasic synovial sarcoma; HD, heterozygous deletion; MFSS, monophasic fibrous synovial sarcoma; N, negative; PI, Ki-67 proliferation index.

*p16 expression: H, heterogeneous, 20%-80%; +, p16 positivity, >80%; negative p16, <20%. Ki-67 PI: high, ≥20%; normal, <20%. Cyclin D1 expression: high, >10%; low, 5%-10%; negative, <5%.
Image 3 Immunohistochemical staining. **A**, Diffuse and intense nuclear staining involving almost all tumor cells (p16 immunostain, ×200). **B**, Intense positivity (cyclin D1 immunostain, ×200). **C**, High Ki-67 proliferation index, >20% positive nuclei (Ki-67 immunostain, ×200). **D**, Heterogeneous expression showing alternate positive and negative foci (p16 immunostain, ×200). **E**, Low expression accounting for 5%-10% of tumor nuclei (cyclin D1 immunostain, ×400). **F**, Normal Ki-67 proliferation index (<20%) (Ki-67 immunostain, ×200).
probe combination containing an ABL probe, unlike earlier studies that used α-satellite DNA probes, was to avoid possible cross-hybridization. Moreover, ABL is present in the 9q34, relatively far from the p16INK4A loci, facilitating the easy visualization of FISH signals.

We developed a scoring scheme that screened for all possible permutation combinations of FISH signals resulting from nuclear truncation, differentiating them from the tumor cells containing true deletion of the p16INK4A gene. Inactivation of p16INK4A can occur as a consequence of homozygous deletion, rearrangement, hypermethylation, and point mutation.50-32 Heterozygous p16INK4A deletion was detected in 28 (74%) of 38 cases. Sabah et al15 also observed a high percentage of LOH at 9p21 in their small series of SSs (60%). Although Orlow et al10 proposed that coincident homozygous deletion of p16INK4A and p15INK4B occurs frequently in soft tissue sarcomas, none of the 4 SSs included in their study showed alterations of p16INK4A or p15INK4B. Of 28 cases with heterozygous loss of the p16INK4A gene, 25 (89%) presented p16 protein at abnormal levels, negatively (20 cases) or heterogeneously (5 cases). The latter is thought to be a tumor-associated mutant type and is unable to arrest the normal diploid cells in G1.

Three p16INK4A-deleted SSs (11%) that were positive for the p16 protein can be explained as follows: These 3 cases (cases 5, 20, and 29) exhibited 26%, 20%, and 23% of cells showing a heterozygous p16INK4A deletion; however, the coexisting normal clone of cells with intact p16INK4A in these cases could function as a compensatory mechanism causing the protein expression. The reason for the presence of abnormal p16 protein in 9 of 10 p16INK4A-intact SSs (2 heterogeneous and 7 negative) is the inability of FISH to test hypermethylation and the promoter or intron mutations as causes for the loss of p16 expression.34 Moreover, posttranslational modifications of p16 protein could also explain this discrepancy. However, we did not perform the methylation and mutational analysis of p16INK4A in our series of cases. Cohen and Geradts34 reported aberrant p16 protein expression in 51% of soft tissue sarcomas, but SSs were not included in their study. Similarly, Yoo et al21 identified altered p16 expression in 94% of sarcomas. In contrast, some authors concluded that p16INK4A alterations were infrequent in soft tissue sarcomas.11,12,35

All of our antibodies, being nuclear proteins, were evaluated on triplicate tissue cores to compensate for tumor heterogeneity because they are better suited for validating 3 category stains such as p16 and pRb and show high concordance and a low tissue loss rate compared with conventional tissue sections.23 Discrepancies in the immunohistochemical results can be explained by the use of different commercially available antibodies, technical variations in immunohistochemical analysis, and variations in the criteria for interpreting results. Alternatively, these same discrepancies can also be explained by the differences in tumor type, histologic grade, and clinical stage of the cases studied. The benefit of p16 protein immunohistochemical analysis over FISH testing is that the absence of functionally significant protein caused by a variety of genetic and epigenetic abnormalities may be detected.34

Cyclin D1 forms active complexes with CDK4 to phosphorylate pRb, allowing entry into the cycle and exerting the early regulation imposed during the G1 phase and also seems to link the Rb and p53 pathways.4,21 We observed increased cyclin D1 expression in 25 (66%) of 38 SSs. Of these 25 cases, 19 (76%) demonstrated heterozygous deletion of p16INK4A, whereas the other 6 cases showed a normal p16INK4A locus. Cyclin D1 overexpression has been reported in SSs.36 However, of the 4 SSs in our series showing low cyclin D1 expression, p16INK4A gene deletion was seen in 3, and 6 of 9 cyclin D1– cases also displayed deletion of p16INK4A. These data suggest a lack of association between high cyclin D1 expression and p16INK4A deletion. A similar finding has been observed in sarcomas and breast carcinomas.33,37 This lack of association can be explained by the fact that transcriptional activation of the cyclin D1 gene due to binding of a complex containing β-catenin and lymphoid enhancing factor (LEF-1) to the LEF-1 binding site on the cyclin D1 promoter causes its overexpression.38 Alternatively, Xie et al39 demonstrated that the SYT-SSX fusion gene, specific for SS, interferes with the ubiquitin-dependent degradation of cyclin D1. These molecular mechanisms are possible explanations for the discrepancy between cyclin D1 expression and p16INK4A deletion; however, because we did not analyze them in our study, we cannot pinpoint the exact reason in our cases.

No significant association was observed between Ki-67 and p16INK4A deletion or between increased cyclin D1 expression and the Ki-67 proliferation index, which can be explained by an unscheduled pattern of cyclin D1 expression aiding in shortening the G1 interval relative to expression of any single cyclin.36

Lack of correlation between heterozygous deletion of the p16INK4A gene and loss of p16 protein expression was observed in grade 2 and 3 SSs. Loss of p16 expression has been associated with histologic grade and tumor progression in chondrosarcomas and malignant melanomas,40,41 although Cohen and Geradts34 did not find any association between loss of p16 expression and overall or mitotic grade in their series of sarcomas.

This high-throughput TMA-based interphase FISH assay and immunohistochemical study strengthens the fact that heterozygous deletion of p16INK4A and loss of p16 protein expression are frequent genetic alterations in SSs. Further investigations are required to identify the role of other unknown tumor suppressor genes at the 9p21 locus in the development of SS to improve our understanding of the biologic behavior of this highly intriguing neoplasm.
References


