Role of p16/INK4a in Gastrointestinal Stromal Tumor Progression

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Abstract

Because the p16 locus is involved consistently in chromosomal losses found in malignant gastrointestinal stromal tumors (GISTs), we studied p16 in a series of 21 GISTs with complete follow-up using immunohistochemical analysis, semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and methylation-specific PCR (MSP). A fraction of cells of more than 20% with low or absent p16 immunostaining was detected in 12 GISTs, including all showing malignancy. RT-PCR revealed decreased p16 transcription in all except 2 p16 protein–deficient GISTs. By MSP, 7 cases showed p16 promoter methylation (all hypoexpressing p16; 6 malignant). A fraction of p16-deficient cells of more than 20% was associated with clinical malignancy (P = .003; log-rank test). The percentage of cells underexpressing p16, size, cellularity, mitotic count, and coagulative necrosis were associated with malignancy by Cox proportional hazards univariate analysis; only the former factor was selected by multivariate analysis (P = .039). Thus, p16 down-regulation, partly due to p16 promoter methylation, is implied in GIST progression. Furthermore, p16 immunohistochemical assessment seems a promising method for GIST prognostication.

The relatively recent characterization of the KIT pathway and the exclusion of KIT protein (CD117)-immunonegative neoplasms from the gastrointestinal stromal tumor (GIST) category shed new light on the classification of this formerly confusing group of neoplasms. While this produced immediate therapeutic benefits, with the use of the tyrosine kinase inhibitor imatinib mesylate (STI571; Gleevec), the prognostic approach remains rather elusive. In a recent workshop organized by the National Institutes of Health for reaching a consensus approach to diagnosis and morphologic prognostication of GISTs, a scheme based on mitotic rate and tumor size as features predictive of outcome was proposed, recognizing that none among the several additional parameters considered offered consistent advantages; moreover, because it likely is impossible at present to definitively distinguish benign from malignant cases, the resulting prediction was given only in terms of relative risk.1 The lack of a reliable method of prognostication hampers the selection of patients eligible for imatinib mesylate therapy, a critical step for avoiding waste of resources and unmotivated possible development of resistance of the tumor to treatment, the mechanisms of which have yet to be explained completely.2,3

Among the parameters studied in GISTs, many genetic alterations have been evidenced, with diverse relevance in terms of diffusion or association with aggressiveness; among these, losses in chromosome 9 have been found consistently in malignant GISTs.4,5 These losses often comprise 9p21, the site of p16/INK4a (p16),2 not only as an obvious consequence of whole 9p deletion, but also in cases of very restricted areas of deletion.6 p16 is a tumor suppressor gene that arrests cells in the G1 phase through reducing the kinase activity of cyclin-dependent kinases (CDKs) 4 and 6, thus leaving the
retinoblastoma tumor suppressor protein (RB) in its unphosphorylated active form and preventing the formation of CDK4/6–cyclin D complexes, thereby allowing Cip/Kip CDK inhibitors to exert their role on CDK–cyclin E complexes. The cyclin D–CDK4/6/INK4/Rb/E2F transcription factors pathway has been found to be altered in more than 80% of human neoplasms. With regard to soft tissue tumors, the derangement of this intracellular cascade has been found in human sarcomas, p16 homozygous deletion has been demonstrated to have a role in the development and/or progression of childhood rhabdomyosarcomas, and the targeted deletion of p16 in mice results in an increased occurrence of sarcoma. Recently, p16 genetic alterations also have been demonstrated in GISTs, bearing an unfavorable predictive value on the clinical outcome of affected patients. However, the relationship between p16 gene expression and GIST prognosis remains to be elucidated.

The aim of the present study was to investigate p16 status in a series of GISTs with diverse biologic aggressiveness, focusing on protein and messenger RNA (mRNA) expression; in particular, a possible prognostic approach in GISTs using p16 immunohistochemical analysis, an easily affordable technique, was tested. We also studied the mechanisms regulating p16 expression by investigating p16 promoter methylation, a well-known mechanism of p16 inactivation along with chromosome 9p losses. The results point to a role for p16 underexpression, partly due to promoter methylation, in GIST malignant progression; moreover, p16 immunohistochemical assessment deserves consideration as a possible technique for GIST prognostication, which is affordable for a basic pathology laboratory.

Materials and Methods

Tumors
We retrieved 21 GISTs (gastric, 17; small intestinal, 3; large intestinal, 1) from different patients (men, 10; women, 11; age range at diagnosis, 51-87 years) from the surgical pathology files of the Catholic University of Rome, Rome, Italy. GISTs were defined as CD117-immunoreactive mesenchymal lesions with morphologic and clinical features consistent with GIST. The routine H&E-stained sections were reviewed to verify the morphologic diagnosis; sections from the selected representative blocks were cut at a constant thickness of 5 µm for further investigations. Smooth muscle tumors (desmin-positive; CD117–), schwannian tumors (S-100–positive, CD117–), and tumors with positive resection margins were excluded from the study. Morphologic features noted were tumor size, mitotic rate (counted in 50 consecutive high-power fields), spindle and/or epithelioid cellular morphologic features, presence of coagulative necrosis, grade of cytologic pleomorphism, cellular density (expressed as cell numbers per square millimeter), and immunoreactivity for CD117, desmin, S-100, and CD34. Follow-up data for 6 to 193 months (mean, 54.6 months) were available for all patients; no patients received therapy other than surgery.

Immunohistochemical Analysis
Immunohistochemical analysis was performed using antibodies to CD117 (rabbit polyclonal) and desmin (DAKO, Glostrup, Denmark), CD34 and S-100 (YLEM, Avezzano, Italy), and p16 (Santa Cruz, Santa Cruz, CA). For desmin, S-100, and p16, antigen retrieval was accomplished by microwave irradiation at 750 W for 15 minutes in a 10-mmol/L concentration of citrate buffer (pH 6). Specific preimmune serum samples or isotype-specific unrelated primary antibodies were used for control stainings. Immunoreactivity for p16 was nuclear; only in sporadic cases was cytoplasmic staining also detected but was not considered evidence of p16 expression given its nonspecificity.

For a semiquantitative immunostaining evaluation, the slides were screened independently by 3 pathologists (R.R., F.C., and L.M.L.) who were unaware of the clinicopathologic data and counted at least 1,000 neoplastic cells. Nuclear staining was divided into 3 categories: negative, weakly positive, and intensely positive. The latter was considered normal, being detected in the vast majority of nontumoral

**Image 11** Degrees of nuclear immunoreactivity for p16 in gastrointestinal stromal tumors (GISTs). In the context of a GIST heterogeneously expressing p16 at the nuclear level, the elliptic spot evidences paradigmatic examples of absent (top), weak (bottom left), and intense (bottom right) immunostaining with avidin-biotin complex immunoperoxidase method (anti-p16 antibodies, ×400).
stromal, epithelial, and inflammatory cells comprised in the samples, which served as internal positive control samples; the first 2 categories reflected abnormally low (lacking or reduced, respectively) p16 expression, and their cumulated percentage was adopted for scoring the samples. The difference between the extreme counts of the 3 pathologists never exceeded 6%. For the log-rank test, a threshold of 20% of cells with low to absent p16 immunostaining was adopted for dividing the GISTs into high and low p16–expressing cases; a comparable threshold, although in the context of a 2-tiered method of assessment, already had been adopted in works dealing with immunohistochemical evaluation of p16 expression in soft tissue tumors14; interobserver agreement was reached initially in 90% of the cases; for the remaining cases, consensus was reached by joint review of the samples. For the continuous scores used in the Cox proportional hazards regression analysis, the global percentages from cumulated counts of the 3 pathologists were used.

**RNA Extraction and RT-PCR and Semiquantitative Analysis**

RNA was extracted by using TRIzol reagent (Invitrogen-Life Technologies, Paisley, Scotland), following the manufacturer’s instructions. The pathologic areas selected for RNA extraction contained only disease-specific cells to eliminate contamination by normal cells. Total RNA was eluted in RNase-free water and treated with RQ1 RNase-free DNase (1 U/µL; Promega, Madison, WI) at 37°C for 15 minutes to eliminate DNA contamination. RNA then was extracted using standard phenol/chloroform/isoamyl alcohol extraction, precipitated with ethanol, washed with 70% ethanol, and eluted in diethyl pyrocarbonate–treated water.

First-strand complementary DNA (cDNA) was synthesized by incubating 0.5-1 µg of RNA at 42°C for 50 minutes in a final volume of 20 µL containing RT buffer 1x (50-mmol/L concentration of tris(hydroxymethyl)aminomethane [Tris] hydrochloride, pH 8.3, 75-mmol/L concentration of potassium chloride, 3-mmol/L concentration of magnesium chloride), 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen-Life Technologies), a 2.5-µmol/L concentration of random hexamers, a 1-mmol/L concentration of deoxynucleoside triphosphate (dNTP) mix, and a 10-mmol/L concentration of dithiothreitol. We amplified 200 ng of cDNA with specific primers for p16 and β-actin in 30 µL of final volume, containing 1 U of Taq DNA polymerase (Invitrogen-Life Technologies), a 1-mmol/L concentration of p16 primers, and a 0.25-mmol/L concentration of β-actin primers (Invitrogen-Life Technologies), a 200-mmol/L concentration of each dNTP, and 10× buffer (100-µmol/L concentration of Tris hydrochloride, pH 8.3, 50-mmol/L concentration of potassium chloride, 1.5 mmol/L of magnesium chloride).

Primers used were as follows: sense, 5′-TTA TTT GAG CTT TGG TTC TG-3′, and antisense, 5′-CCG GCT TTC GTA GTT TTC AT-3′, for p16, with an expected PCR product of 354 base pairs; sense, 5′-TAC ATG GGT GGG GTG TTG AA-3′, and antisense, 5′-AAG AGA GGC ATC CTC ACC CT-3′, for β-actin, with an expected PCR product of 234 base pairs. β-actin was used in each PCR reaction as an internal control according to the method previously described.15

PCR conditions were as follows: 1 cycle of 4 minutes at 95°C, 35 cycles at 95°C for 40 seconds, 58°C for 40 seconds, 72°C for 40 seconds, and a final cycle of 3 minutes at 72°C. cDNA from normal nontumor tissue and ultrapure water were used as positive and negative control samples, respectively. The mixture was separated on a 2% agarose gel, and, after staining with ethidium bromide, the PCR product was visualized under UV illumination.

Densitometric analysis was performed for all the samples using the Molecular Imager (Biorad, Hercules, CA). The level of p16 expression was expressed as the density of the p16 band divided by the band density of β-actin, giving a “normalized” value for p16 transcript levels. This strategy permitted comparisons of the relative p16 transcript level in different specimens and also served as a control for the p16 RT-PCR reaction, because failure to detect β-actin would indicate that the reaction had failed.16

**DNA Isolation and Methylation-Specific PCR**

Three 10-µm slides were cut from paraffin-embedded tissue samples, treated twice with xylene, and washed twice with ethanol. DNA was extracted by using the QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. The pathologic areas selected for DNA extraction contained at least 70% disease-specific cells to minimize contamination by normal cells.

Approximately 1 µg of genomic DNA was subjected to bisulfite modification as previously described.15,17 Methylation-specific PCR distinguishes unmethylated from methylated alleles in a given gene on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil.18 Modified DNA was purified by using Wizard DNA purification resin (Promega) according to the manufacturer’s instructions and then was desulfonated by using sodium hydroxide at a final concentration of 0.3 mol/L for 10 minutes at room temperature, followed by ethanol precipitation, using glycogen as a carrier. DNA was resuspended in water. Next, 100 to 200 ng of bisulfite-modified DNA was amplified with specific primers designed for methylated and unmethylated DNA. Primer sequences of p16 for the unmethylated reaction were sense, 5′-TTA TTA GAG GTG GGG GTG GAT TGT CT-3′, and antisense, 5′-CAA CCC CAA ACC ACA ACC ATA A-3′,
and for the methylated reaction were sense, 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3'; and antisense, 5'-GAC CCC GAA CCG CCG TAA-3'. The PCR mixture contained 1× PCR buffer (20-mmol/L concentration of Tris, pH 8.3, 50-mmol/L concentration of potassium chloride), dNTPs (1.25-mmol/L concentration of each), primers (10 pmol each), modified DNA, and 0.75 U of Taq polymerase platinum (Invitrogen-Life Technologies) in a final volume of 30 µL. The magnesium chloride concentration for unmethylated and methylated reactions was 1.5 mmol/L.

Amplifications were performed for 35 cycles (35 seconds at 95°C, 35 seconds at 60°C for unmethylated p16 and 65°C for methylated p16, and 35 seconds at 72°C) after an initial denaturation at 95°C for 3 minutes and followed by a final extension at 72°C for 5 minutes. The PCR products were electrophoresed in a 2.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination. DNA from HL60 and U937 cell lines was used as the positive control for unmethylated and methylated p16, respectively.

Statistical Analysis

The Statistica statistical software package (release 5.5, StatSoft, Tulsa, OK) was used for all calculations. Malignant behavior criteria adopted for uncensored events in survival curves were local recurrence, invasiveness to adjacent organs, peritoneal dissemination, and distant metastases. Low p16 immunoreactivity (cut point at 80% of intensely p16 immunoreactive cells) was tested by calculating cumulative survival rates by the Kaplan-Meier method, followed by comparison with the log-rank test with the Yates correction for continuity. For Cox proportional hazards regression analysis, age at diagnosis, tumor size, mitotic rate, cellular density, and percentage of cells with low to absent immunoreactivity for p16 were considered continuous variables; sex, site (stomach vs other site), morphologic features (spindle cell vs epithelioid or mixed spindle cell and epithelioid), cytologic pleomorphism (low vs high grade), presence of coagulative necrosis, and CD34 positivity were categorical 2-step variables. For multivariate analysis, the prognostic parameters with a P value of less than .05 in the Cox univariate analysis were selected. Any P value of less than .05 was considered statistically significant.

Results

Characterization of GISTs

Data for the 21 GISTs analyzed are summarized in Table 1. Tumors ranged in size from 0.9 to 23 cm (mean, 9.5 cm). The mitotic count ranged from 0 to 70 per 50 high-power fields. Sixteen tumors were spindle-cell type, 3 were epithelioid and 2 mixed spindle-epithelioid. Distant metastases were present in 7 cases; infiltration of adjacent organs was present in 1 case; local relapse was present in 1 case; 3 patients died of disease. All tumors were immunoreactive for CD117 (in 17 the staining was diffuse; in 4 it was limited to a fraction ranging between 12% and 30% of cells); 18 cases (86%) stained for CD34; desmin and S-100 staining was not detected.

p16 Protein Expression

Percentages of tumoral cells with low to absent p16 immunoreactivity are summarized in Table 1. By adopting a threshold of 20% of cells with low to absent p16 immunostaining, similar to that already used in works dealing with immunohistochemical evaluation of p16 expression in soft tissue tumors,14 an abnormally low expression (ie, the fraction of cells underexpressing p16 (20%) of p16) was detected in 11 samples (52% of total); these comprised 100% of the cases with evidence of malignancy (n = 8) and 23% of the cases with unremarkable follow-up (3/13; Table 1).

Correlations

By using the Kaplan-Meier method followed by comparison by the log-rank test with Yates correction for continuity, the presence of a fraction of tumoral cells with low to absent immunoreactivity for p16 higher than 20% was associated with malignancy (P = .003). By using Cox proportional hazards univariate analysis, tumor size, cellular density, presence of coagulative necrosis, mitotic count, and the percentage of cells with low or absent p16 immunoreactivity were associated significantly with malignancy (Table 2). The only prognostic factor selected by multivariate analysis was the percentage of cells with low or absent p16 immunoreactivity, with a relative risk of 0.140 (95% confidence interval, 0.072-0.273; P = .039); the presence of coagulative necrosis was of borderline significance (relative risk, 4.660; 95% confidence interval, −0.063 to 9.382; P = .053) (Table 2).

Mechanism of p16 Modulation

To evaluate whether p16 down-regulation was regulated at the transcriptional level, semiquantitative analysis of p16 transcripts was performed by PCR on cDNA. In all samples with low immunohistochemical p16 expression except 2, the p16 transcript band densitometric value normalized to β-actin was lower than the minimum value found in nontumoral control tissue samples; conversely, all samples with normal immunoreactivity for p16 produced p16 band densitometric values comparable to those of nontumoral control tissue samples (Table 1). By methylation-specific PCR, 7 of 21 GISTs analyzed showed a methylated pattern of p16 (ie, cases 6, 7, 13, 15, 16, 19, and 21) (Table 1). All cases with low p16 expression at the
protein and mRNA levels; 6 had evidence of malignancy in the follow-up. Figure 1 shows p16 expression and p16 promoter methylation in the GISTs studied, divided into benign and malignant according to their clinical behavior.

### Discussion

The development of a reliable method of GIST prognostication is a major goal for the clinical management of GISTs, given the relevance of proper selection of patients for imatinib mesylate therapy to avoid unmotivated possible development of resistance of the tumor to treatment and waste of resources. Among the numerous parameters studied in GISTs, several genetic alterations have been evidenced, with diverse relevance in terms of diffusion or association with aggressiveness. Losses of chromosome 9 have been demonstrated consistently in malignant GISTs, involving 9p21, the site of p16, a tumor suppressor gene of the cyclin D-CDK4,6/INK4/Rb/E2F transcription factors pathway. Recently, genetic alterations of p16 have been shown to bear a negative prognostic value in GISTs. The emerging promising role of p16 as a prognostic marker in these tumors would be strengthened by further confirmatory studies. A further step would then be the development of an easy, reliable, and affordable method of assessment for the basic pathology laboratory. Although the immunohistochemical assessment of p16 status has been correlated with genetic alterations of p16, this correlation was not total; moreover, the prognostic value of immunohistochemical p16 assessment has not been defined, using only p16 genetic derangements with this aim. Therefore, we studied a
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Selected series of 21 such tumors, well studied both pathologically and clinically, using a 3-tiered p16 immunohistochemical scoring method with results tested against p16 transcript analysis and for which the biologic value was validated by comparison with follow-up data.

We found low to absent p16 immunostaining exceeding 20% in 11 (52%) of 21 cases; this fraction is comparable to the 58.1% of GISTs recently found to bear p16 genetic alterations. Defined in these terms, a low immunohistochemical reactivity for p16 was associated with evidence of malignancy in the survival curve of the GISTs studied ($P = .003$).

A maximum threshold of 20% of p16 hypoeXpressing cells for considering a case as a low p16–expressing case

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Table 2
Statistical Analysis of Potential Prognostic Factors in Gastrointestinal Stromal Tumors

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis ($P$)</th>
<th>Multivariate Analysis ($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low p16 immunoreactivity</td>
<td>.003</td>
<td>.039</td>
</tr>
<tr>
<td>Presence of coagulative necrosis</td>
<td>.006</td>
<td>.053</td>
</tr>
<tr>
<td>Tumor size</td>
<td>.010</td>
<td>.131</td>
</tr>
<tr>
<td>Mitotic count</td>
<td>.003</td>
<td>.169</td>
</tr>
<tr>
<td>Cellular density</td>
<td>.045</td>
<td>.349</td>
</tr>
<tr>
<td>Age</td>
<td>.121</td>
<td>ND</td>
</tr>
<tr>
<td>Morphologic features (spindle cell vs others)</td>
<td>.410</td>
<td>ND</td>
</tr>
<tr>
<td>Sex</td>
<td>.471</td>
<td>ND</td>
</tr>
<tr>
<td>Site (gastric vs others)</td>
<td>.724</td>
<td>ND</td>
</tr>
<tr>
<td>Cytologic pleomorphism (low- vs high-grade)</td>
<td>.871</td>
<td>ND</td>
</tr>
<tr>
<td>CD34 positivity</td>
<td>.881</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.
already was adopted in works dealing with the immunohistochemical evaluation of p16 expression in soft tissue tumors, although in the context of a 2-tiered method of assessment.\textsuperscript{14} However, published data concerning p16 immunohistochemical assessment and its biologic significance in various human stromal tumors are conflicting. This is likely due to heterogeneous methods in terms of techniques used, the use of varied antibodies,\textsuperscript{8,9,19} and the parameters adopted for analyzing the results, which varied from considering only complete negativity\textsuperscript{8,20} to adopting a threshold of “normality,” which, in turn, varied from 10% (with 2 different antibodies) to 80% of stained cells.\textsuperscript{8,19} Finally, many studies dealt with miscellaneous pathologic conditions because they studied “soft tissue sarcomas” as a group,\textsuperscript{14,19} although the frequency of p16 abnormality has been shown to vary with histologic type.\textsuperscript{8} With regard to GISTs, the only existing work used a 4-tiered system based exclusively on the percentage of p16-immunoreactive cells, with no mention of the intensity of staining; the emerging data, although correlated with p16 genetic alterations, were not fully coherent with them and, noticeably, were not mentioned as prognostically valid.\textsuperscript{12}

As a whole, doubts are raised concerning the reliability of the immunohistochemical scoring methods used. This is a crucial point because immunohistochemical analysis is preferable to genetic assessment for prognostication purposes. In fact, events such as some third-base mutations, not determining a change in the amino acid sequence, have no effect on protein antigenicity or on biologic activity. Moreover, immunohistochemical analysis is a technique that is affordable in every pathology laboratory, unlike thorough genetic assessment.

A substantial difference between our study and many others is that we adopted a 3-tiered method of immunohistochemical scoring of nuclear p16 staining based on its intensity, instead of the usual 2-tiered “black or white” method, considering normal only the strongest p16 immunostaining, as observed in the vast majority of nontumoral stromal, epithelial, and inflammatory cells comprised in the samples. In detail, we detected, along with complete negativity and brisk positivity, cells displaying a weak and/or dot-like

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**Image 3** Examples of semiquantitative reverse transcriptase–polymerase chain reaction analysis of p16 messenger RNA (mRNA) expression in gastrointestinal stromal tumors (GISTs). GISTs with high (samples 8 and 12) and low (samples 1, 13, 15, and 17) p16 mRNA expression are shown (compare with nontumoral normal positive control samples: p, placenta; m, bone marrow). p16/β-actin ratios were 1.058, 1.061, 0.973, 0.950, 0.950, 0.943, 1.040, and 1.053 for samples 8, 12, 13, 15, 1, 17, p, and m, respectively. Band length (base pairs), p16INK4A, 354; β-actin, 234. MW, molecular weight marker.

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**Image 4** Examples of gastrointestinal stromal tumors with positive and negative patterns of methylation of the p16 promoter by methylation-specific polymerase chain reaction. The p16 promoter amplification bands were detectable in the presence of unmethylated and methylated sequence-specific primer pairs in samples 13 and 15. Conversely, p16 amplification bands were detectable only in the presence of unmethylated sequence-specific primer pairs in samples 1, 8, 12, and 17. bp, base pairs; M, methylated reaction; MW, molecular weight marker; U, unmethylated reaction.

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**Figure 1** p16 RNA and protein expression, p16 promoter methylation, and malignancy of gastrointestinal stromal tumors (GISTs). RNA p16/β-actin ratios, p16 immunohistochemical reactivity (diamonds, fraction of tumoral cells with intense p16 immunostaining <80%; triangles, same fraction ≥80%), and p16 promoter methylation status (solid symbols, methylated; open symbols, unmethylated) of the 21 studied GISTs, divided into cases with or without evidence of malignancy in the follow-up (referred to as malignant and benign, respectively) are shown. Gray shading shows the range of the p16/β-actin RNA ratio found in nontumoral control tissue samples.
This result is consistent with the possible mechanisms of p16 underexpression, ie, p16 promoter methylation, loss of heterozygosity (LOH) at 9p21 including deletions of the entire 9p, and point mutations affecting p16, all events that can involve one or both p16 loci, resulting in partial or total blocking of p16 expression. The adopted threshold of more than 20% tumoral cells with low or absent p16 immunostaining for considering a case as underexpressing p16 produced data that were confirmed substantially by semiquantitative RT-PCR analysis of p16 mRNA expression and were associated significantly with the biologic behavior of the tumors studied (Tables 1 and 2). In fact, agreement between the detection of a more than 20% fraction of cells with low p16 immunostaining and a low p16/β-actin densitometric rate (with respect to the minimal value found in nontumoral control tissue samples) was found in all cases except 2 (Table 1, Figure 1). The sporadic occurrence of a posttranscriptional down-regulation of p16 could be hypothesized; however, technical variables likely had a role.

Our finding of a methylated pattern of p16 in 7 tumors expressing a low level of p16, representing 33% of the total sample analyzed, points to p16 promoter methylation as one of the mechanisms causing p16 down-regulation in GISTs; this result is consistent with recently published data showing p16 promoter methylation in 13 of 40 cases studied (33%). Allelic loss also is likely involved in p16 deregulation in GISTs and in the progression of these tumors; in fact, not only have losses of chromosome 9 been reported in malignant GISTs and in GIST metastases (with a frequency of 36% and 63%, respectively), but also specific losses of p16 locus on chromosomal arm 9p have been demonstrated. In particular, small areas of deletion combined with complete deletion of the other 9p chromosomal arm were found in 2 cases in a series of 14 GISTs, and LOH at the p16 locus was found in 11 of 43 GIST samples studied, with complete loss of the p16 gene in 2 of these cases.

Unlike chromosomal 9p losses, which can involve other genes whose inactivation could account for malignancy such as p14ARF, our finding of p16 promoter methylation almost exclusively in aggressive GISTs, together with our immunohistochemical and RT-PCR data, supports a role of p16 underexpression in GIST malignant progression. Moreover, the association between GIST aggressiveness and p16 promoter methylation makes this event a good candidate for accounting for the fraction of malignant GISTs with no evidence of losses in the 9p arm. P16 allelic deletion and promoter methylation also can have a combined role, as reported in several diverse tumors and in 1 GIST. A third described way of p16 inactivation, ie, point mutations affecting p16, has been recently described in GISTs (9 of 43 samples); 3 of these cases showed sense mutations, 4 showed missense mutations, and 2 samples (a primitive tumor and its recurrence) showed a T insertion, creating an early stop codon leading to a truncated p16 protein; p16 point mutation was accompanied by simultaneous promoter methylation in 2 cases and by LOH in 4 cases. Thus, GISTs seem to resemble other mesenchymal neoplasms in which no p16 mutations were found in 86 samples from 74 soft tissue tumors.

In multivariate analysis, p16 immunohistochemical underexpression was the only prognostic factor selected (P = .039), coagulative necrosis was of borderline significance (P = .053), and other parameters proposed for and/or commonly used in GIST prognostication, such as size, cellularity, and mitotic count, lost the significance found in univariate analysis (Table 2). This result goes beyond a simple confirmation of the prognostic role of p16 in GISTs emerging in a recent study in terms of genetic alterations; in fact, it opens the way for possible prognostic assessment of GISTs in every pathology laboratory. Our results showing an association between p16 underexpression and clinical malignancy favor a late role for the inactivation of this molecule in GIST progression. The recently reported lack of a role for p16 in GIST proliferation dysregulation supported by the similarity in frequency between p16 genetic alterations found in benign and malignant GISTs, likely is a consequence of considering a morphologic rather than a clinical classification of these tumors. Besides, a link between malignancy and p16 derangement in soft tissue tumors has been already suggested by the finding of p16 alterations in about 30% of cases in a series of sarcomas but not in soft tissue lesions of low malignant potential.

Most GISTs constitutively express activated KIT proteins bearing structural changes that permit receptor oligomerization and cross-phosphorylation in the absence of ligand binding; the activating mechanism in most GISTs is mutation of the KIT gene itself, as demonstrated by studies in which systematic sequencing of the juxtamembrane coding region was coupled with evaluation of the entire KIT coding sequence in cases lacking juxtamembrane region mutation. Evidence of involvement of type D3 cyclin in the cascade triggered by KIT activation has been produced by demonstrating the up-regulation of that type of cyclin by stem cell factor, the ligand of CD117, leading to cell cycle progression through G1/S transition in mouse spermatogonia. Thus, a possible resulting scheme of GIST progression would be the following: a first mutation occurring in the KIT locus would cause the onset of the tumor, resulting in a cyclin D3–driven mitogenic boost whose effect is limited by p16; the inactivation of the latter, through the reported losses involving its locus on chromosome 9 or through promoter methylation, as shown in the present article, could be the
event that ultimately leaves the way open for the development of a malignant phenotype.

We demonstrated that p16 down-regulation is implied in GIST progression, and p16 promoter methylation is one of its causes. Furthermore, our results indicate the immunohistochemical assessment of p16 status as a possible method of GIST prognostication in the immediate future.

References