*BRIEF ARTICLE*

# Loss of chromosome 9p21 and decreased p16 expression correlate with malignant gastrointestinal stromal tumor

Yun Zhang, Hui Cao, Ming Wang, Wen-Yi Zhao, Zhi-Yong Shen, Dan-Ping Shen, Xing-Zhi Ni, Zhi-Yong Wu, Yan-Ying Shen, Yan-Yan Song

Yun Zhang, Hui Cao, Ming Wang, Wen-Yi Zhao, Zhi-Yong Shen, Dan-Ping Shen, Xing-Zhi Ni, Zhi-Yong Wu, Department of General Surgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
Yan-Ying Shen, Department of Pathology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
Yan-Yan Song, Section of Statistics Teaching and Research, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
Author contributions: Zhang Y, Cao H and Wu ZY conceived and designed this study; Zhang Y conducted the experiment and wrote the paper; Shen YY conducted experiment; Song YY dealt with statistical analysis; Wang M, Zhao WY and Shen ZY collected the data; Shen DP and Ni XZ analyzed and interpreted the data; all authors approved the final version of the article.
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Correspondence to: Hui Cao, MD, PhD, Department of General Surgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China. caohuishcn@hotmail.com
Telephone: +86-21-68383751  Fax: +86-21-58394262
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## Abstract

**AIM:** To investigate loss of heterozygosity (LOH) of chromosome 9p21 and the prognostic relevance of p16 expression in gastrointestinal stromal tumor (GIST).

**METHODS:** Fifty-one GIST patients (30 men and 21 women; median age 59 years; range 29-80 years) treated surgically within a 10-year period were grouped by aggressive behavior risk (17 with very low and low, 14 intermediate, and 20 high risk). GISTs were characterized immunohistochemically and evaluated for LOH of 9p21 by microsatellite analysis at D9S1751, D9S1846, D9S942, and D9S1748. LOH of 9p21 and immunohistochemical expression of p16 protein encoded at 9p21 were correlated with clinicopathological parameters, and the prognostic significance of p16 alterations was evaluated.

**RESULTS:** Thirty-one (63.3%) cases showed LOH with at least one microsatellite marker. LOH frequency was 37.0% at D9S1751, 37.5% at D9S1846, 42.1% at D9S942, and 24.2% at D9S1748. There was a higher LOH frequency of D9S942 in high-risk than in non-high-risk tumors ($P < 0.05$, $\chi^2 = 4.47$). Gender, age, tumor size and site were not correlated with allelic loss. Ninety percent (18/20) of the GIST patients in the high risk group showed LOH with at least one of the 9p21 markers, while 57.1% (8/14) in the intermediate risk group and 33.3% (5/15) in the very low and low risk groups, respectively ($P < 0.05$, $\chi^2 = 12.16$). Eight (28.5%) of 31 patients with LOH and 1 (5.6%) of 18 patients without LOH died of the disease during the follow-up period. Loss of p16 protein expression occurred in 41.2%, but in 60% of the high risk group and 23.5% of the very low and low risk groups ($P < 0.05$, $\chi^2 = 4.98$). p16 loss was associated with poor prognosis ($P < 0.05$, $\chi^2 = 4.18$): the 3- and 5-year overall survival rates were 84.8% and 70.8% for p16-negative and 100% and 92.0% for p16-positive patients, respectively.

**CONCLUSION:** LOH at 9p21 appears to play an important role in GIST progression; decreased p16 expression in GIST is highly predictive of poor outcome.

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## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) comprise the largest subset of mesenchymal tumors of the digestive tract. Most GISTs have activating mutations of the c-kit proto-oncogene that have been implicated in their tumorigenesis. They are characterized by the expression of the KIT (CD117, stem cell factor receptor) protein. More recently, activating mutations of platelet-derived growth factor receptor $\alpha$ (PDGFRA) have been identified. Clinically and pathologically, GISTs represent a spectrum of tumors, including benign, malignant, and borderline variants. It is generally recognized that a final consensus on the grading of GISTs has not yet been reached, and their biologic behavior often remains unclear. Most clinicopathological studies have suggested that the tumor site and size and the mitotic index are the most important prognostic indicators of GISTs. However, they do not always reliably predict patient outcomes. The lack of a reliable method for prognosis prediction hampers the selection of patients eligible for imatinib mesylate (Gleevec) therapy. Imatinib was the first targeted therapy approved for the treatment of GIST. The development of imatinib in the treatment of metastatic GIST represents a therapeutic breakthrough in molecularly targeted strategies, while its usefulness in adjuvant setting is under study. Obtaining genetic information of each patient may be critical in tailoring individualized treatment strategies.

Although mutational activation of c-kit or PDGFRA plays an important role in GIST pathogenesis, other cytogenetic alterations, mostly losses of genetic material, have been found, with evidence that losses at chromosome 9p are highly specific for malignant and metastatic GISTs[1]. P16, a cyclin-dependent kinase (CDK) inhibitor, encoded by CDKN2A gene at 9p21 has been shown to be inactivated in a variety of tumors by loss of heterozygosity (LOH), homozygous deletions, or point mutations[2]. P16 as a product of tumor suppressor gene that arrests cells in the $G_1$ phase through reducing the kinase activity of CDKs 4 and 6, thus leaving the retinoblastoma tumor suppressor protein (Rb) in its unphosphorylated active form, which blocks the E2F transcription factor 1 (E2F1), free E2F1 accumulates in the nucleus and initiates S-phase entry *via* transcription of several genes necessary for DNA synthesis and cell cycle progression. Because this situation leads to the management of these tumors, it is helpful to add new molecular markers that may play a role in the diagnosis and treatment of the disease. The aim of this study was to evaluate the status of the LOH at 9p21 and the expression of p16, which controls cell cycle progression in a series of GISTs with diverse biologic aggressiveness, to determine whether alterations in cell cycle regulatory protein can be used as prognostic markers. The identification of an additional criterion for the selection of high-risk cases for treatment with imatinib mesylate was also attempted.

## MATERIALS AND METHODS

### Patients and pathological analysis

A total of 51 cases of GIST, consecutively resected between 1999 and 2007, were retrieved from the archives of our hospital. None of the patients received imatinib therapy. There were 30 males (58.8%) and 21 females (41.2%), aged from 29 to 80 years (median, 59 years). Primary tumors originated from the stomach ($n = 30$), small intestine ($n = 18$), and mesentery ($n = 3$). The tumors were diagnosed as GISTs using previously established histological, immunohistochemical, and molecular genetic criteria[3]. Fifty-one samples of formalin-fixed paraffin-embedded (FFPE) tumor material were examined, and 4-μm-thick sections were initially cut and stained with hematoxylin and eosin. All tumors were positive for CD117. For the purpose of clinicopathological comparison, the GISTs were classified as very low and low ($n = 17$), intermediate ($n = 14$), and high risk ($n = 20$) according to the consensus approach of Fletcher *et al*[4].

### Microsatellite analysis

All cases were positive for KIT, supporting the diagnosis of GIST. Tumor and normal tissue samples were dissected from FFPE tissue blocks. DNA was extracted from FFPE tumor material using a standard extraction protocol (Qiagen, Hilden, Germany). LOH was evaluated by PCR amplification of four microsatellite markers at chromosome 9p21. Primer sequences (provided by Shanghai GeneCore BioTechnologies Co., Ltd. Shanghai, China) were obtained from human genome microsatellite marker databases linked to the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and are shown in Table 1. PCR amplifications were performed in a final volume of 50 μL containing 50 ng sample DNA, GeneAmp 10 × PCR reaction buffer, 25 mmol/L MgCl₂, 5 pmol/L of each primer, 2.5 mmol/L each of dATP, dCTP, dGTP, and dTTP, and 5 U of AmpliTaq DNA Polymerase (Applied BioSystems, Foster City, USA). After denaturation at 95℃ for 10 min, DNA amplification was performed for 40 cycles, consisting of denaturation at 94℃ for 15 s, primer annealing at 50℃ for 15 s, and elongation at 72℃ for 30 s. A final extension step at 72℃ for 30 min completed the reactions. Amplification products were analyzed using the ABI Prism Genetic Analyzer 3730 (Applied Biosystems, Foster City, USA). Data were processed using Genemapper software (Applied BioSystems, Foster City, USA). LOH was defined based on the recommendations by previous studies[5]. The ratio of the

**Table 1  Primer sequences and product size used in polymerase chain reaction for each primer on chromosome 9p21**

| Marker | Forward primer (5'→3') | Reverse primer (5'→3') | PCR product size (bp) |
|--------|------------------------|------------------------|------------------------|
| D9S1751 | TTGTTGATTCTGCCTTCAAAGTCTTTTAAC | CGTTAAGTCCTCTATTACACAGAG | 150-170 |
| D9S1846 | AATGGCTGGTTCTAGGACTG | AAACTGGTCTGGTGTTTGC | 183-197 |
| D9S942 | GCAAGATTCCAAACAGTA | CTCATCCTGCGGAAACCATT | 100-130 |
| D9S1748 | CACCTCAGAAGTCAGTGAGT | GTGCTTGAAATACACCTTTCC | 130-150 |

PCR: Polymerase chain reaction.

peak high values between longer and shorter alleles was calculated for the normal and tumor tissues. To obtain the LOH value, the allele ratio from the normal tissue was divided by the allele ratio from the tumor tissue [allele ratio = $(T_1:T_2)/(N_1:N_2)$]. Values $\leqslant 0.5$ and $\geqslant 1.5$ were considered to represent LOH. In questionable cases, the PCR amplification and LOH analysis were repeated to ensure the consistency in the results.

### Immunohistochemistry

**KIT protein:** All GISTs were immunohistochemically positive for the KIT protein (antibody CD117, GA450202, DAKO, Carpinteria, CA, USA).

**P16 protein:** Briefly, FFPE tissue sections were dewaxed with xylene, dehydrated with an ethanol series (100%, 90%, 70%), and then microwave retrieved in 10-mmol/L citrate buffer, pH 6.0, for 10-20 min. Endogenous peroxidase was blocked with 0.5% $H_2O_2$ for 15 min. The sections were pretreated with blocking serum and washed in Tris-buffered saline (TBS). Each section was incubated with the anti-p16 (M0425, 1:50; Antibody, California, USA) overnight at room temperature. After TBS washing, the sections were incubated with Envision™ (Mouse) (K4001; Dako, Carpinteria, CA, USA) for 30 min at room temperature. Finally, the sections were developed in DAB (Amresco, Ohio, USA) for 5-15 min. Ten high-power fields (HPF) were estimated, and a section was considered to be immunohistochemically positive for p16 if tumor nuclei were stained (with or without cytoplasmic staining), according to a 4-point semiquantitative scale, as follows: negative (-), less than 5% of cells stained; positive (+), 5%-10% stained; positive (++), 11%-50% stained; positive (+++), 51%-75% stained; positive (++++), greater than 75% stained. A cutoff at 10% positivity in at least 10 HPF was used for prognostic analysis. Nontumorous stromal cells showing nuclear reactivity served as an internal control.

### Statistical analysis

All statistical analyses were carried out using the $\chi^2$ test or Fisher's exact test in cross tables to assess the relationships between p16 loss and clinicopathological factors. All statistical tests were two-sided. Overall survival curves were drawn according to the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered statistically significant. Calculations were carried out using the SPSS version 13.0 software package (SPSS, Chicago, USA).

## RESULTS

The data for the 51 GISTs are summarized in Table 2. Tumor size ranged from 1.4 to 19 cm in the greatest dimension (mean 6.7 cm). The tumors were histologically classified as predominantly spindled ($n = 32$), epithelioid ($n = 15$), or mixed-spindled epithelioid ($n = 4$). As noted in the methodology, all cases were KIT-positive and showed diffuse strong cytoplasmic and/or membranous staining.

### Genetic studies

A total of four microsatellite markers were used to screen 51 tumors for LOH on chromosome 9p21. Two patients (3.9%) had constitutional homozygosity (noninformative loci) with 4 markers. Overall, 63.3% (31/49) of the tumors showed LOH with at least one locus on chromosome 9p21. The highest frequency of LOH was seen at D9S942 (42.1%, 16/38). The other markers showed the following deletions: D9S1751, 37.0% (10/27); D9S1846, 37.5% (12/32); and D9S1748, 24.2% (8/33). The frequencies of LOH on chromosome 9p21 in the 51 GISTs are shown in Table 3. Representative examples of LOH analysis are shown in Figure 1.

### LOH on chromosome 9p21 and clinicopathological features of GISTs

LOH of 9p21 was compared with the clinical features of the GIST patients. There was no significant difference in LOH frequency by age (< 50 years, $\geqslant$ 50 years), sex, and tumor site and size (< 5cm, $\geqslant$ 5 cm). There were also no substantial differences in LOH frequencies among epithelioid and spindle cell tumors. The LOH frequency increased in accordance with the tumor's risk of aggressive behavior (Table 4). Moreover, GISTs assigned to the high risk group had a higher LOH frequency than the other groups on D9S942 ($P < 0.05$, $\chi^2 = 4.47$). Ninety percent (18/20) of the GIST patients in the high risk group was found to show LOH with at least one of the 9p21 markers, while 57.1% (8/14) in the intermediate risk group and 33.3% (5/15) in the very low and low risk groups, respectively ($P < 0.05$, $\chi^2 = 12.16$). Eight (28.5%) of 31 patients with LOH and 1 (5.6%) of 18 patients without LOH died of the disease during the follow-up period.

### P16 protein expression

In our series, 14 cases were (-), 7 cases were (+), 14 cases were (++), 13 cases were (+++), and 3 cases were (++++) (Figure 2) for p16 immunoreactivity. Adopting a threshold of 10% cells with low to absent p16 immunostaining, Of

**Table 2  Clinicopathological data and p16 expression in gastrointestinal stromal tumors**

| Case | Sex | Age (yr) | Risk[1] | Site | OS (mo) | 1751 | 1846 | 942 | 1748 | p16 expression |
|------|--------|----|-----------|----|------------|---|---|---|---|---|
| 1 | Male | 41 | Low | SI | Alive (50) | 2 | 2 | 2 | 3 | N |
| 2 | Male | 73 | Low | S | Alive (47) | 3 | 2 | 4 | 4 | N |
| 3 | Male | 33 | Very low | SI | Alive (21) | 4 | 3 | 2 | 4 | N |
| 4 | Female | 66 | Low | S | Alive (18) | 4 | 4 | 3 | 3 | P |
| 5 | Female | 71 | Low | SI | Alive (17) | 4 | 4 | 4 | 4 | P |
| 6 | Male | 46 | Low | SI | Alive (15) | 4 | 4 | 4 | 3 | P |
| 7 | Female | 59 | Low | SI | Alive (5) | 3 | 4 | 4 | 3 | P |
| 8 | Female | 45 | Low | SI | Alive (13) | 4 | 3 | 3 | 4 | P |
| 9 | Male | 65 | Low | SI | Alive (11) | 3 | 3 | 3 | 4 | P |
| 10 | Male | 66 | Low | S | Alive (11) | 4 | 4 | 4 | 4 | P |
| 11 | Male | 55 | Low | S | Alive (5) | 3 | 2 | 2 | 4 | N |
| 12 | Male | 72 | Low | SI | Alive (102) | 2 | 3 | 4 | 2 | P |
| 13 | Female | 54 | Low | S | Alive (50) | 3 | 3 | 3 | 3 | P |
| 14 | Male | 56 | Low | S | NA | 4 | 4 | 4 | 4 | P |
| 15 | Female | 60 | Low | S | NA | 3 | 3 | 3 | 3 | P |
| 16 | Male | 70 | Very low | S | Alive (45) | 3 | 3 | 4 | 4 | P |
| 17 | Female | 75 | Low | S | Alive (18) | 3 | 3 | 4 | 3 | P |
| 18 | Male | 70 | Intermediate | S | Alive (100) | 4 | 2 | 2 | 3 | N |
| 19 | Female | 50 | Intermediate | SI | Alive (81) | 4 | 4 | 4 | 4 | P |
| 20 | Female | 70 | Intermediate | S | Alive (88) | 4 | 4 | 2 | 2 | N |
| 21 | Male | 72 | Intermediate | SI | Alive (78) | 2 | 4 | 4 | 3 | P |
| 22 | Male | 56 | Intermediate | SI | Dead (60) | 3 | 2 | 2 | 3 | P |
| 23 | Male | 47 | Intermediate | S | Alive (53) | 2 | 3 | 4 | 4 | P |
| 24 | Male | 50 | Intermediate | M | Dead (27) | 3 | 3 | 4 | 4 | P |
| 25 | Female | 73 | Intermediate | S | Alive (21) | 3 | 2 | 2 | 4 | N |
| 26 | Male | 79 | Intermediate | S | Alive (16) | 4 | 3 | 4 | 4 | P |
| 27 | Male | 63 | Intermediate | S | Alive (12) | 3 | 4 | 4 | 4 | P |
| 28 | Male | 68 | Intermediate | S | Alive (12) | 3 | 2 | 4 | 4 | P |
| 29 | Female | 56 | Intermediate | S | Alive (11) | 3 | 4 | 4 | 3 | P |
| 30 | Female | 57 | Intermediate | SI | Alive (9) | 4 | 4 | 3 | 4 | P |
| 31 | Male | 59 | Intermediate | S | Alive (35) | 3 | 4 | 3 | 2 | N |
| 32 | Female | 29 | High | SI | Dead (38) | 3 | 4 | 4 | 2 | N |
| 33 | Female | 77 | High | S | Dead (35) | 4 | 4 | 2 | 4 | N |
| 34 | Male | 64 | High | M | Alive (71) | 2 | 4 | 2 | 3 | N |
| 35 | Male | 47 | High | S | Dead (56) | 2 | 4 | 2 | 4 | N |
| 36 | Female | 50 | High | S | Alive (62) | 3 | 2 | 4 | 2 | N |
| 37 | Female | 36 | High | SI | Dead (11) | 2 | 4 | 4 | 4 | N |
| 38 | Female | 62 | High | S | Alive (55) | 4 | 2 | 3 | 2 | P |
| 39 | Male | 60 | High | S | Alive (54) | 3 | 4 | 3 | 4 | P |
| 40 | Female | 61 | High | S | Alive (63) | 2 | 3 | 3 | 4 | N |
| 41 | Male | 50 | High | S | Alive (51) | 3 | 3 | 3 | 2 | P |
| 42 | Male | 75 | High | S | Dead (18) | 2 | 4 | 2 | 3 | N |
| 43 | Male | 58 | High | S | Alive (46) | 3 | 3 | 3 | 4 | P |
| 44 | Male | 52 | High | S | Alive (31) | 4 | 3 | 2 | 3 | N |
| 45 | Male | 56 | High | SI | Alive (31) | 3 | 2 | 4 | 3 | P |
| 46 | Male | 57 | High | SI | Alive (19) | 2 | 3 | 2 | 4 | P |
| 47 | Male | 67 | High | M | Alive (18) | 3 | 2 | 3 | 2 | P |
| 48 | Female | 47 | High | SI | Dead (5) | 4 | 3 | 2 | 3 | N |
| 49 | Female | 80 | High | SI | Alive (12) | 3 | 3 | 2 | 4 | N |
| 50 | Female | 48 | High | S | Alive (12) | 3 | 3 | 2 | 3 | P |
| 51 | Male | 44 | High | S | Dead (86) | 4 | 2 | 4 | 3 | N |

[1]According to the consensus approach by Fletcher *et al*[4]; [2]Indicates loss of heterozygosity (LOH); [3]Uninformative (homozygosity); [4]Indicates no LOH. S: Stomach; SI: Small intestine; M: Mesentery; NA: Not available; OS: Overall survival; N: Negative expression; P: Positive expression.

**Table 3  Results of loss of heterozygosity analyzed with four microsatellite markers in 51 gastrointestinal stromal tumors**

| Marker | LOH (*n*) | Heterozygosity (*n*) | Frequency of LOH (%) |
|--------|-----------|----------------------|----------------------|
| D9S1751 | 10 | 17 | 37.0 |
| D9S1846 | 12 | 20 | 37.5 |
| D9S942 | 16 | 22 | 42.1 |
| D9S1748 | 8 | 25 | 24.2 |

LOH: Loss of heterozygosity.

the 51 cases of GISTs, p16 protein-negative expression was detected in 21 (41.2%) samples, and p16 protein-positive expression was detected in 30 (58.8%) samples using a threshold of 10% cells with low to absent p16 immunostaining.

### *Correlation of p16 protein expression and clinicopathological factors*

Loss of p16 protein expression was compared with the clinicopathological features of the GIST patients (Table 5).

**Table 4** Rresults of loss of heterozygosity in 51 gastrointestinal stromal tumors according to Fletcher's classification

| Risk classification | 1751 | | | 1846 | | | 942[1] | | | 1748 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LOH (*n*) | Hetero-zygosity (*n*) | Rate (%) | LOH (*n*) | Hetero-zygosity (*n*) | Rate (%) | LOH (*n*) | Hetero-zygosity (*n*) | Rate (%) | LOH (*n*) | Hetero-zygosity (*n*) | Rate (%) |
| Very low and low | 2 | 7 | 22.2 | 3 | 6 | 33.3 | 3 | 9 | 25.0 | 1 | 9 | 10.0 |
| Intermediate | 2 | 5 | 28.6 | 4 | 7 | 36.4 | 4 | 8 | 33.3 | 2 | 8 | 20.0 |
| High | 6 | 5 | 54.5 | 5 | 7 | 41.7 | 9 | 5 | 64.3 | 5 | 8 | 38.5 |

[1]There was a higher loss of heterozygosity (LOH) frequency of D9S942 in the high-risk than in non-high-risk tumors ($P < 0.05$, $\chi^2 = 4.47$). There were no substantial differences in LOH frequencies among three groups of 4 markers.
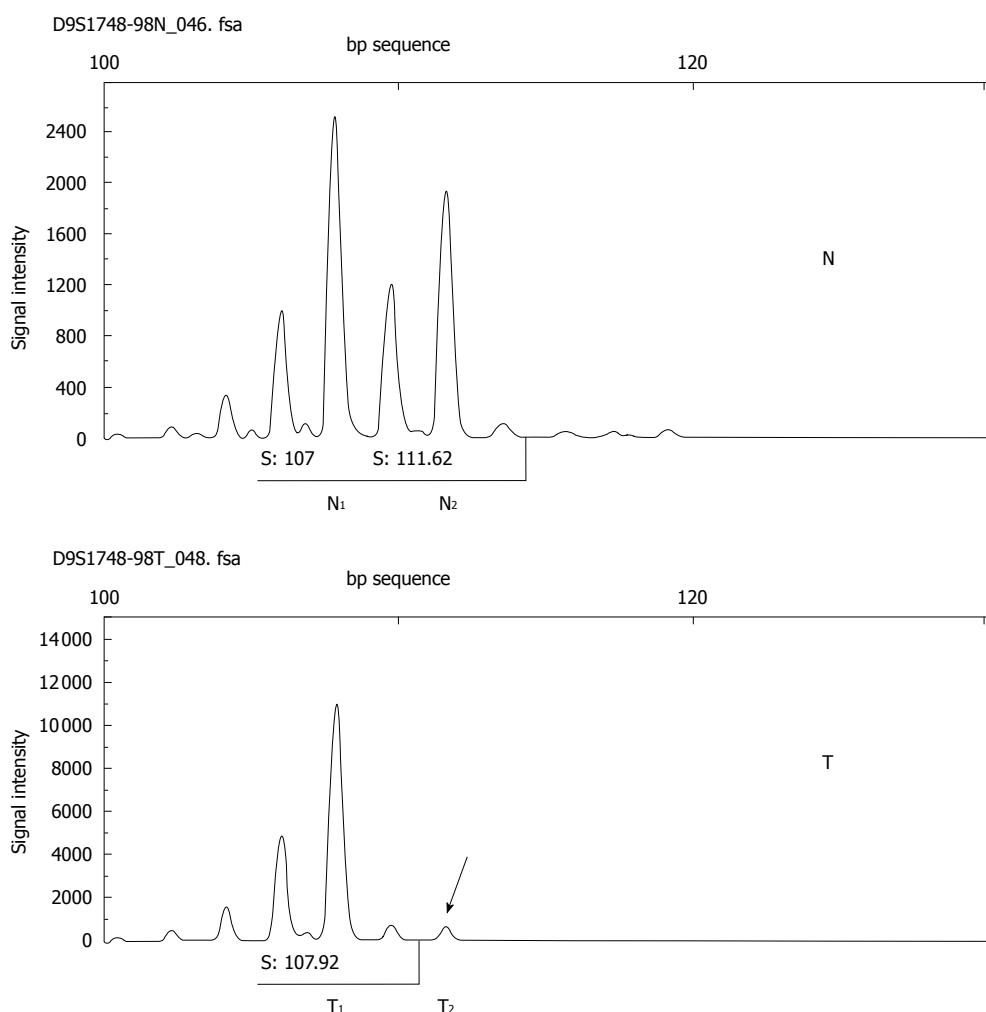


**Figure 1 Representative image of loss of heterozygosity.** Black arrow indicates the lost allele. Allele ratio = $(T_1:T_2)/(N_1:N_2)$. N: Normal; T: Tumor.

Patient age, sex, tumor size and site did not correlate with p16 protein expression. But p16 protein-negative expression had a high mitotic index ($P < 0.05$, $\chi^2 = 5.13$). The rate of p16 protein-negative expression was 60% (12/20) in the high risk group, whereas the rate was 23.5% (4/17) in the very low and low risk group and 35.7% (5/14) in the intermediate risk group. There was a significant difference in p16 down-regulation between the high risk and the very low and low risk groups ($P < 0.05$, $\chi^2 = 4.98$).

### *P16 expression and survival analysis*

Until April 30, 2008, 49 (96.1%) patients had been followed

up. The median follow-up period was 31 mo (range, 5-102 mo). Forty (81.6%) patients were still alive, whereas nine (18.4%) patients died of the disease. Patients who had tumors with p16 protein loss had a worse prognosis than those having tumors without p16 protein loss. Eight (38.1%) of 21 patients with p16-negative expression tumors, but only one (3.6%) of 28 patients with p16-positive expression tumors, died of GIST. The 1-, 3-, and 5-year overall survival rates were 100%, 84.8% and 70.8%, respectively, in the p16 protein-negative expression group. The 1-, 3-, and 5-year overall survival rates were 100%, 100% and 92.0%, respectively, in the p16 protein-positive

**Table 5  Statistical analysis of p16 expression and clinicopatho-logic factors**

|  | p16 (-) | p16 (+) | *P* value ($\chi^2$) |
|---|---|---|---|
| Sex |  |  |  |
|   Male | 12 | 18 |  |
|   Female | 9 | 12 | 0.838 (0.042) |
| Age (yr) |  |  |  |
|   < 50 | 5 | 4 |  |
|   ⩾ 50 | 16 | 26 | 0.334 (0.933) |
| Size (cm) |  |  |  |
|   < 5 | 4 | 13 |  |
|   ⩾ 5 | 17 | 17 | 0.070 (3.279) |
| Mitotic index |  |  |  |
|   ⩽ 5/50 HPF | 8 | 21 |  |
|   > 5/50 HPF | 13 | 9 | 0.024 (5.126) |
| Risk |  |  |  |
|   Very low and low | 4 | 13 |  |
|   High | 12 | 8 | 0.045 (4.98) |
| Site |  |  |  |
|   Stomach | 13 | 17 |  |
|   Intestine | 7 | 11 |  |
|   Other | 1 | 2 | 0.917 (0.173) |

HPF: High-power fields.



**Figure 2  p16 immunostaining in gastrointestinal stromal tumor.** A: Negative p16 immunostaining in gastrointestinal stromal tumor (GIST) (200 ×); B: p16 immunostaining ++++ in GIST (200 ×).



**Figure 3  Kaplan-Meier plot for overall survival of gastrointestinal stromal tumor patients with p16-negative and p16-positive alterations (*P* < 0.05).**

expression group. There was a strong correlation between p16 alterations and overall survival using the Kaplan-Meier method followed by comparison with the log-rank test ($P < 0.05$, $\chi^2 = 4.18$, Figure 3).

### *Correlation of p16 protein expression and LOH results*

Twenty-one (67.7%) of the 31 patients with 9p21 LOH showed p16 protein-negative expression. The coincident rate between p16 expression and 9p21 LOH was 60% (6/10) on D9S1751, 66.7% (8/12) on D9S1846, 87.5% (14/16) on D9S942, and 70.8% (4/8) on D9S1748.

## DISCUSSION

GISTs comprise the largest subset of mesenchymal tumors of the digestive tract, although they account for < 2% of all gastrointestinal tumors. Before the advent of imatinib (imatinib; Gleevec, Novartis, Switzerland), surgery was the only therapeutic approach for GISTs. However, even after complete resection of a GIST, most patients with advanced disease relapsed, and the prognosis of patients with metastatic and/or recurrent GISTs was extremely poor[6]. Clinically and pathologically, GISTs represent a spectrum of tumors that include benign, malignant, and borderline variants. It is often difficult to predict the malignant behavior of GISTs. Prognostic features indicative of malignancy or high aggressive clinical behavior risk are generally identified by increased tumor size and mitotic activity[4], but this lacks predictive accuracy. Although mutational activation of c-kit or PDGFRA plays an important role in GIST pathogenesis, other changes, mostly losses of genetic material, have been documented in primary tumors[7]. Total or partial loss of chromosome 9 has been found in benign and malignant GISTs, indicating that this change might play a role in GIST tumorigenesis[8]. A CDK
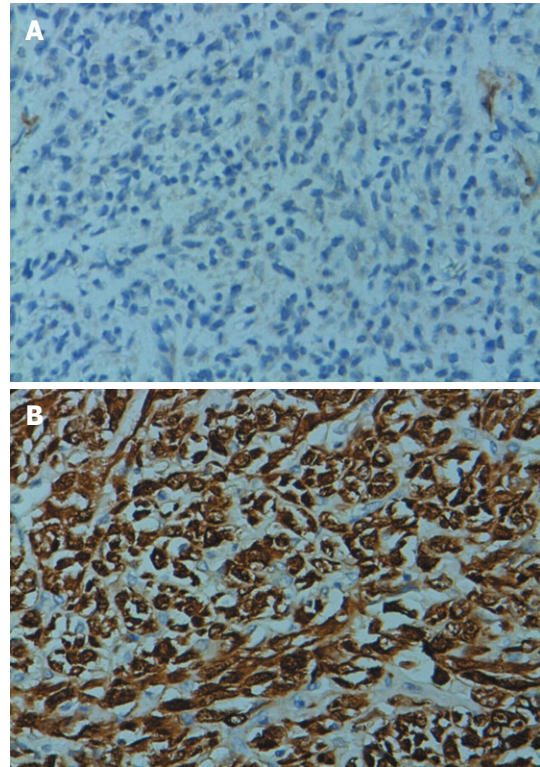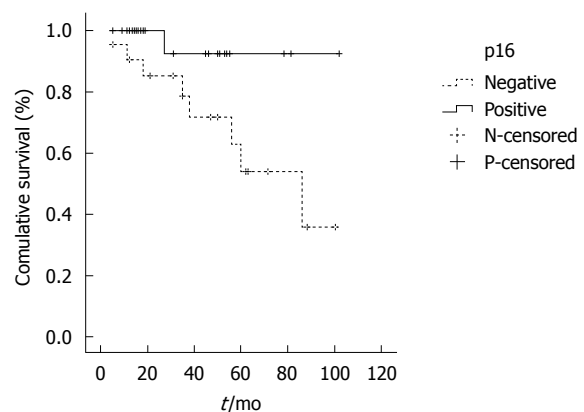
4 inhibitor (p16) gene located at 9p21 has been shown to be inactivated in a variety of tumors. However, the relationship between p16 expression and GIST prognosis is still under debate. For instance, Schneider-Stock *et al*[9] found that aberrant loss of p16 expression was predictive of poor patient survival, but Nakamura *et al*[10] failed to validate its prognostic value in Japanese patients. These discrepant data raised a practical concern about whether p16 can be indiscriminately used as a surrogate marker for various inactivating mechanisms of the p16 gene for prognosis. In this context, p16, as an early G1 phase negative cell-cycle regulator, represents a likely candidate. The aim

of this study was to address the issue of whether alterations in cell cycle regulatory protein can be used as prognostic markers.

Most human cancers are characterized by genomic instability, in addition to oncogene activation, the inactivation of tumor suppressor genes has been shown to play an important role in tumorigenesis. Oncogenes obviously play an important role in cell proliferation. Tumor suppressor genes may play important roles in tissue differentiation. LOH is a common form of allelic imbalance, and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages, and progression. In 1987, Hansen *et al*[11] suggested that when there is one gene deletion of both alleles, the other gene appears to be insufficient to carry out its normal functions, i.e. transcriptional transactivation of downstream target genes that regulate the cell cycle and apoptosis. Thus, a tumor may develop. The frequency of LOH always exceeds 20% at some chromosomes where the tumor suppressor gene exists, which means the allele is related to tumorigenesis[12]. Microsatellites are reliable genetic markers for studying LOH. When LOH occurs, microsatellite markers near the allele will be lost. Therefore, microsatellite analysis can be used to score for LOH.

In this study, LOH on chromosome 9p21 was evaluated in 51 well-characterized GISTs using 4 PCR-based microsatellite markers and gel electrophoresis. The results showed that 31 cases (63.3%, two were uninformative cases) had LOH on chromosome 9p21. These results suggest that LOH on chromosome 9p21 is a common phenomenon. With respect to the correlation between clinicopathological features and LOH, Sabah *et al*[1] found no correlation between loss of chromosome 9p and patient age and sex, and site and histological features of the tumor. However, Pylkkänen *et al*[13] validated that loss of chromosome 22 was found more often in the intestine than in the stomach, though a statistically relevant level was not reached. Our results confirmed that the frequency of LOH on chromosome 9p21 increased in a manner consistent with the risk of aggressive behavior of the tumor. Moreover, GISTs had a higher LOH frequency in the high risk group than in the other groups on D9S942 ($P < 0.05$). And there was substantial difference in LOH frequencies with at least one of the 9p21 markers in different risk groups ($P < 0.05$). The death rate with LOH is higher than those without LOH. This suggests that LOH on chromosome 9p may represent possible primary events in the development of GIST.

It is helpful to find the correlation between tumor suppressor gene and tumor progression and unfavorable outcome by LOH analysis. P16, a CDK4 inhibitor, has been shown to be inactivated in a variety of tumors[14-17]. And the cyclin D-CDK4/6/p16 /Rb/E2F1 transcription factors have been found to be altered in more than 80% of human neoplasms and implicated in the pathogenesis and progression of sarcomas[18]. Loss of p16 expression in GIST is described as a significant predictive value in some

but not all studies. Schneider-Stock *et al*[9] reported that p16 alteration was detected in benign, borderline, and malignant GISTs, but it was not considered an independent, poor prognostic factor. Sabah *et al*[1] reported that inactivation of p16 was detected in almost all malignant GISTs. Romeo *et al*[19] also found that impaired p16 expression was common in advanced GISTs. In our study, four microsatellite markers at 9p21 were selected: two were located at the upstream of the p16 gene, and two at the downstream of the p16 gene. D9S942 is the most proximate marker to p16, a distance of less than 1 centimorgan (cM). The highest frequency of LOH on chromosome 9p21 in GISTs was seen at D9S942 (42.1%). Here, we studied the immunohistochemical results for the proposed biomarkers of p16 in GISTs to evaluate their possible usefulness in clinical prognostic assessment. P16 protein-negative expression was detected in 21 (41.2%) samples. Patients who had tumors with p16 loss showed a poor clinical outcome, and had a nearly 11-fold increased risk of dying of the disease (38.1% *vs* 3.6%). The 5-year overall survival probability was 70.8% in the p16 protein-negative expression group. However, the 5-year overall survival probability was 92.0% in the p16-protein positive expression group. Thus, p16 loss may be an important prognostic factor for GISTs. Our LOH and p16 expression results are in agreement with those of previously published studies[1,8,9,20,21]. However, Schmieder *et al*[22] indicated that the expression of p16 was highly predictive of poor outcome. Steigen *et al*[23] also found a positive relationship between p16 immunohistochemical staining and poor prognosis of GIST. The discrepant results should be clarified through further studies in the future.

Generally, p16 under-expression resulted from promoter methylation, LOH at 9p21, and point mutations. In our study, LOH was found in 31 (60.8%) of the 51 GIST cases, and 21 (66.7%) of them showed p16-negative expression. However, no p16-negative expression was found in a few cases of LOH. Multiple genetic and epigenetic alterations of oncogenes and tumor suppressor genes are implicated in the multistep process of human neoplasms[24-26]. LOH is one cause of multiple genetic alterations involved in the under-expression of tumor suppressor genes. In addition to genetic events, epigenetic alterations are also involved in tumor development[27,28]. According to this study, LOH may be a basic event to p16 loss, but epigenetic alterations, such as promoter methylation, may also influence p16 expression. Ricci *et al*[29] reported that p16 down-regulation, partly due to p16 promoter methylation, was implied in GIST progression.

In summary, LOH on chromosome 9p21 in GISTs could be found in both early and late stages of tumor development in the present study, but the frequency of total gene loss was significantly increased in high-risk GISTs. The p16 protein is encoded by the p16 tumor suppressor gene, which is in the vicinity of the locus with the highest frequency of LOH (D9S942), and its down-regulation is associated with high-risk GISTs. Patients with p16-negative expression had a lower survival rate, therefore ex-

pression of p16 might be a useful prognostic factor. P16 expression in GISTs, combined with Fletcher's aggressive risk scheme, appears to be an accurate evaluation for malignancy risk, particularly in the high-risk recurrent and/or metastatic GISTs. From a clinical perspective, such information can be expected to assist in the selection of cases for adjuvant systemic therapies (i.e. imatinib) after surgery. In addition, other pathogenic mechanisms, besides LOH in the regulation of p16 protein expression, should be the subject of further studies.

## COMMENTS

### Background

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract, previously often diagnosed as benign or malignant smooth muscle tumors. The incidence of GISTs is increasing gradually. Because GISTs have a wide clinical spectrum that ranges from benign to malignant behavior, and GISTs often present with nonspecific symptoms including abdominal pain, anorexia, weight loss, or GI hemorrhage, it is hard to give a prompt treatment. Although mutational activation of c-kit or platelet-derived growth factor receptor α proto-oncogene plays an important role in GIST pathogenesis, other changes, mostly losses of genetic material and the alteration of tumor suppressor gene, have remained unknown.

### Research frontiers

Total or partial loss of chromosome 9p has been found in benign and malignant GISTs, indicating that this change might play a role in GIST tumorigenesis. Whether the expression of tumor suppressor gene at 9p21 is correlated with pathogenesis of GIST is one of the hotspots in recent researches.

### Innovations and breakthroughs

This study has found that loss of chromosome 9p21 and decreased p16 expression are correlated with malignant GIST. The confirmatory result is vital to elucidate the pathogenesis of GIST, and helpful for GIST treatment.

### Applications

Loss of chromosome 9p21 and p16 expression analysis, combined with Fletcher's aggressive risk scheme, appears to be an accurate evaluation for malignancy risk of GIST. Such information can be expected to assist in the selection of cases for adjuvant systemic therapies (i.e. target drug-imatinib) after surgery.

### Peer review

In this study, the authors provide original data showing that loss of heterozygosity of chromosome 9p21 leading to p16INK4A down-regulation, has a high prognostic value in GIST. The study is interesting, well done, methodologically correct and the conclusion drawn fits in with the results shown.

## REFERENCES

1   **Sabah M**, Cummins R, Leader M, Kay E. Loss of heterozygosity of chromosome 9p and loss of p16INK4A expression are associated with malignant gastrointestinal stromal tumors. *Mod Pathol* 2004; **17**: 1364-1371

2   **Ortega S**, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 2002; **1602**: 73-87

3   **Miettinen M**, Lasota J. Gastrointestinal stromal tumors (GISTs): definition, occurrence, pathology, differential diagnosis and molecular genetics. *Pol J Pathol* 2003; **54**: 3-24

4   **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465

5   **Canzian F**, Salovaara R, Hemminki A, Kristo P, Chadwick RB, Aaltonen LA, de la Chapelle A. Semiautomated assessment of loss of heterozygosity and replication error in tumors. *Cancer Res* 1996; **56**: 3331-3337

6   **Miettinen M**, Sobin LH, Lasota J. Gastrointestinal stromal tumors of the stomach: a clinicopathologic, immunohistochemical, and molecular genetic study of 1765 cases with long-term follow-up. *Am J Surg Pathol* 2005; **29**: 52-68

7   **Yang J**, Du X, Lazar AJ, Pollock R, Hunt K, Chen K, Hao X, Trent J, Zhang W. Genetic aberrations of gastrointestinal stromal tumors. *Cancer* 2008; **113**: 1532-1543

8   **Haller F**, Löbke C, Ruschhaupt M, Cameron S, Schulten HJ, Schwager S, von Heydebreck A, Gunawan B, Langer C, Ramadori G, Sültmann H, Poustka A, Korf U, Füzesi L. Loss of 9p leads to p16INK4A down-regulation and enables RB/E2F1-dependent cell cycle promotion in gastrointestinal stromal tumours (GISTs). *J Pathol* 2008; **215**: 253-262

9   **Schneider-Stock R**, Boltze C, Lasota J, Peters B, Corless CL, Ruemmele P, Terracciano L, Pross M, Insabato L, Di Vizio D, Iesalnieks I, Dirnhofer S, Hartmann A, Heinrich M, Miettinen M, Roessner A, Tornillo L. Loss of p16 protein defines high-risk patients with gastrointestinal stromal tumors: a tissue microarray study. *Clin Cancer Res* 2005; **11**: 638-645

10  **Nakamura N**, Yamamoto H, Yao T, Oda Y, Nishiyama K, Imamura M, Yamada T, Nawata H, Tsuneyoshi M. Prognostic significance of expressions of cell-cycle regulatory proteins in gastrointestinal stromal tumor and the relevance of the risk grade. *Hum Pathol* 2005; **36**: 828-837

11  **Hansen MF**, Cavenee WK. Genetics of cancer predisposition. *Cancer Res* 1987; **47**: 5518-5527

12  **Manolaraki MM**, Arvanitis DA, Sourvinos G, Sifakis S, Koumantakis E, Spandidos DA. Frequent loss of heterozygosity in chromosomal region 9pter-p13 in tumor biopsies and cytological material of uterine cervical cancer. *Cancer Lett* 2002; **176**: 175-181

13  **Pylkkänen L**, Sarlomo-Rikala M, Wessman M, Hämäläinen E, Sainio M, Husgafvel-Pursiainen K, Carpén O. Chromosome 22q alterations and expression of the NF2 gene product, merlin, in gastrointestinal stromal tumors. *Hum Pathol* 2003; **34**: 872-879

14  **Kawaguchi K**, Oda Y, Saito T, Yamamoto H, Tamiya S, Takahira T, Miyajima K, Iwamoto Y, Tsuneyoshi M. Mechanisms of inactivation of the p16INK4a gene in leiomyosarcoma of soft tissue: decreased p16 expression correlates with promoter methylation and poor prognosis. *J Pathol* 2003; **201**: 487-495

15  **Gessner C**, Liebers U, Kuhn H, Stiehl P, Witt C, Schauer J, Wolff G. BAX and p16INK4A are independent positive prognostic markers for advanced tumour stage of nonsmall cell lung cancer. *Eur Respir J* 2002; **19**: 134-140

16  **Hilton DA**, Penney M, Evans B, Sanders H, Love S. Evaluation of molecular markers in low-grade diffuse astrocytomas: loss of p16 and retinoblastoma protein expression is associated with short survival. *Am J Surg Pathol* 2002; **26**: 472-478

17  **Mäkitie AA**, MacMillan C, Ho J, Shi W, Lee A, O'Sullivan B, Payne D, Pintilie M, Cummings B, Waldron J, Warde P, Irish J, Brown D, Gilbert R, Gullane P, Liu FF, Kamel-Reid S. Loss of p16 expression has prognostic significance in human nasopharyngeal carcinoma. *Clin Cancer Res* 2003; **9**: 2177-2184

18  **Haller F**, Gunawan B, von Heydebreck A, Schwager S, Schulten HJ, Wolf-Salgó J, Langer C, Ramadori G, Sültmann H, Füzesi L. Prognostic role of E2F1 and members of the CDK-N2A network in gastrointestinal stromal tumors. *Clin Cancer Res* 2005; **11**: 6589-6597

19  **Romeo S**, Debiec-Rychter M, Van Glabbeke M, Van Paassen H, Comite P, Van Eijk R, Oosting J, Verweij J, Terrier P, Schneider U, Sciot R, Blay JY, Hogendoorn PC. Cell cycle/apoptosis molecule expression correlates with imatinib response in patients with advanced gastrointestinal stromal tumors. *Clin Cancer Res* 2009; **15**: 4191-4198

20  **Huang HY**, Huang WW, Lin CN, Eng HL, Li SH, Li CF, Lu D, Yu SC, Hsiung CY. Immunohistochemical expression of p16INK4A, Ki-67, and Mcm2 proteins in gastrointestinal stromal tumors: prognostic implications and correlations with risk stratification of NIH consensus criteria. *Ann Surg Oncol* 2006; **13**: 1633-1644

21  **Perrone F**, Tamborini E, Dagrada GP, Colombo F, Bonadiman L, Albertini V, Lagonigro MS, Gabanti E, Caramuta S, Greco A, Torre GD, Gronchi A, Pierotti MA, Pilotti S. 9p21 locus analysis in high-risk gastrointestinal stromal tumors characterized for c-kit and platelet-derived growth factor receptor alpha gene alterations. *Cancer* 2005; **104**: 159-169

22  **Schmieder M**, Wolf S, Danner B, Stoehr S, Juchems MS, Wuerl P, Henne-Bruns D, Knippschild U, Hasel C, Kramer K. p16 expression differentiates high-risk gastrointestinal stromal tumor and predicts poor outcome. *Neoplasia* 2008; **10**: 1154-1162

23  **Steigen SE**, Bjerkehagen B, Haugland HK, Nordrum IS, Løberg EM, Isaksen V, Eide TJ, Nielsen TO. Diagnostic and prognostic markers for gastrointestinal stromal tumors in Norway. *Mod Pathol* 2008; **21**: 46-53

24  **Vogiatzi P**, Vindigni C, Roviello F, Renieri A, Giordano A. Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis. *J Cell Physiol* 2007; **211**: 287-295

25  **Kim JW**, Cheng Y, Liu W, Li T, Yegnasubramanian S, Zheng SL, Xu J, Isaacs WB, Chang BL. Genetic and epigenetic inactivation of LPL gene in human prostate cancer. *Int J Cancer* 2009; **124**: 734-738

26  **Shaw RJ**, Liloglou T, Rogers SN, Brown JS, Vaughan ED, Lowe D, Field JK, Risk JM. Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. *Br J Cancer* 2006; **94**: 561-568

27  **Chin L**, Gray JW. Translating insights from the cancer genome into clinical practice. *Nature* 2008; **452**: 553-563

28  **Pfeifer GP**, Besaratinia A. Mutational spectra of human cancer. *Hum Genet* 2009; **125**: 493-506

29  **Ricci R**, Arena V, Castri F, Martini M, Maggiano N, Murazio M, Pacelli F, Potenza AE, Vecchio FM, Larocca LM. Role of p16/INK4a in gastrointestinal stromal tumor progression. *Am J Clin Pathol* 2004; **122**: 35-43

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