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• ORIGINAL RESEARCH •

# Expression, deleton and mutation of *p*16 gene in human gastric cancer

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## Abstract

AIM **To investigate the relationship between the expression of *p*16 gene and the gastric carcinogenesis, depth of invasion and lymph node metastases, and to evaluate the deletion and mutation of exon** 2 **in *p*16 gene in gastric carcinoma.**

METHODS **The expression of P**16 **protein was examined by streptavidin-peroxidase conjugated method** (**S-P**)**; the deletion and mutation of p16 gene were respectively examined by polymerase chain reaction** (**PCR**) **and polymerase chain reaction single strand conformation polymorphism analysis** (**PCR-SSCP**) **in gastric carcinoma.**

RESULTS **Expression of P**16 **protein was detected in** 96.25**%** (77/80) **of the normal gastric mucosa, in** 92.00**%** (45/50) **of the dysplastic gastric mucosa and in** 47.54**%** (58/122) **of the gastric carcinoma. The positive rate of P**16 **protein expression in gastric carcinoma was significantly lower than that in normal gastric mucosa and dysplastic gastric mucosa** (***P***<0.05)**. The positive rate of P**16 **protein expression in mucoid carcinoma** 10.00**%** (1/10) **was significantly lower than that in poorly differentiated carcinoma** 51.22**%** (21/41)**, undifferentiated carcinoma** 57.69**%** (15/26) **and signet ring cell carcinoma** 62.50**%** (10/16) (***P***<0.05)**. The positive rate of p16 protein in 30 cases paired primary and lymph node metastatic gastric carcinoma: There was** 46.67**%** (14/30) **in primary gastric carcinoma,** 16.67**%** (5/30) **in lymph node metastatic gastric carcinoma. The positive rate of lymph node metastatic carcinoma was significantly lower than that of primary carcinoma** (***P***<0.05)**. There was of *p*16 gene mutation in exon** 2**, but** 5 **cases displayed deletion of *p*16 gene in exon** 2 **in the** 25 **primary gastric carcinomas.**

CONCLUSIONS **The expression loss of P**16 **protein related to the gastric carcinogenesis, gastric carcinoma histopathological subtypes and lymph merastasis. The mutation of *p*16 gene in exon** 2 **may not be involved in gastric carcinogenesis. But the deletion of *p*16 gene in exon** 2 **may be involved in gastric carcinogenesis.**

**Subject headings** gastric carcinoma; dysplasis p16/MTS1/CDK4I/CDKN2 gene; mutation deletion; expression; stomach neoplasms; genetics genes

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## INTRODUCTION

Carcinogenesis and progression of human gastric cancer are related to the activation of proto-oncogenes and/or the inactivation of anti-oncogenes and they are the results of genetic alteration accumulated. A recently cloned new tumor suppressor *p*16 gene is located in 9p21, with the full-length of 8.5 kb. It consists of 2 introns and 3 exons, encoding P16 protein-whose molecular mass is 15840 $M_r$, a single strain peptide comprising 148 amino acid, participating in regulating the proliferation of normal cell growth negatively[1,2]. There was a high frequent loss of homozygosis of *p*16 gene in a variety of cancer cell lines such as gliocytoma, melanoma, breast cancer cell lines[2] and in certain primary cancer, for example, leukemia[3], gliomas[4], astrocytomas[5], bladder cancer[6], melanoma[7], oral squamous cell carcinomas[8], squamous cell carcinoma of head and neck neoplasm[9,10]. The frequency of *p*16 gene deletion and mutation is up to 75% in all kinds of human neoplasm, higher than that of the well-known p53 gene. Gastric cancer is common in China[11-30]. In this paper, S-P immunohistochemical staining was used to detect the expression of P16 protein in gastric cancer and precancerous lesions. PCR and PCR-SSCP methods were used to analyse the deletion and mutation of *p*16 gene exon 2. This study aims to evaluate the relationship between P16 protein and the carcinogenesis, progression, histological types as well as biologic behaviors in human gastric cancer, to find a new marker in early diagnosis and to discover the role of deletion and mutation of *p*16 gene in exon 2 in the carcinogenesis and progression of human gastric cancer.

## MATERIAL AND METHODS

### *Specimens and treatment*

All specimens were confirmed by pathology. Paraffin-embedded tissue were collected from the department of pathology and fresh resected specimens were from the First Affiliated Hospital of the Nanhua University, among which there were 50 cases of dysplasia of gastric mucosa and 122 cases of gastric cancer (25 cases were resected freshly from September 1995 to December 1996). In the 122 cases of gastric cancer, 29 were well-differentiated adenocarcinoma, 41 were poorly-differentiated adenocarcinoma, 26 were undifferentiated carcinoma, 16 were signet ring cell carcinoma and the other 10 were mucoid carcinoma. There were 81 men

and 41 women, 22 aged below 40 years, 69 aged from 41 to 59 years, and 31 were older than 60 years. The youngest was 15 years and the oldest 79 years ( mean 56 years). Superficial muscles, were invated in 50 cases and deep muscles and the full layer in 72. Sixty-nine cases had lymph node metastasis, 53 had no lymph node metastasis. Thirty cases primary and lymph node metastasis cancer selected randomly were paired and compared. According to Borrmann's classification, 15 were type I, 43 were type II, 47 were type III and 17 were type IV. The 25 cases of fresh resected specimens included cancer, cancer-surroundings and normal mucosa selected far from cancer, were cut into 2 - 4 blocks under sterile conditions. Each block was 2-3 $mm^3$ and stored in -70℃ refrigerator for PCR and PCR-SSCP analysis. The rest tissues were fixed in 100 $mL \cdot L^{-1}$ neutral formalin, resected, dehydrated, cleaned and paraffin-embedded. All paraffin-embedded tissues were cut into sequential slices for 5μm and adhered to the glass which was processed by poly-lys previously.

### *Reagents and instruments*

Rabbit-anti-human P16 protein multiple clonal antibody, streptavidin-peroxidase immunozator kit (S-P kit), and DAB were all bought from Maxim Company, USA. Protase K (Merk, USA), *Sma* I, agar gel, propylene acrylamide, N-N-sulmethyl bipropylene acrylamide, ammonium persulfate, xylene nitrile, bromophenol blue were bought from Shanghai Sangon Company. PCR primer synthesized by Shanghai Sangon, primer sequences of p16 gene exon 2[4].

Sense: 5'-TCT GAC CAT TCT GTT CTC TC-3'

Antisense: 5'-CTC AGC TTT GGA AGC TCT CA-3'

The fragment length of amplification was 384 bp. Primer sequences of β-active served as an internal control.

Sense: 5'-GCG GGG CGC CCC AGG CAC CA-3'

antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

The fragment length of amplification was 548bp.

**Instrument** Ultra low refrigerator (Japan) of -70℃, rotary sector (Germany), microscope (Japan), type 480 DNA amplificatory (PE,USA), type 901 ultraviolet spectrophometer (PE,USA), type DY-IIIB vertical eletrophores and all kinds of centrifuges(Beijing Liuyi).

## METHODS

### *S-P immunohistochemical staining*

Operated as the specification of sp kit, that was: paraffin-embedded tissue slices deparaffined hydrated→endogenous peroxidase blocked→added first antibody→then bridge antibody→added enzyme labeled S-P reagents→DAB colorized →hematoxylin stained→dehydrated→cleaned and paraffin-embedded→observed by microscope.

### *Genomic DNA extraction[31]*

Frozen tissue of 0.5g was put into liquid nitrogen and powdered immediately, 10× buffer (10$mmol \cdot L^{-1}$ Tris-HCl pH 8.0, 0.1$mol \cdot L^{-1}$ EDTA pH 8.0, 5$g \cdot L^{-1}$ SDS) was added and span in 37℃ water for 1h at the same time, added protase K to the mixture at a final concentration of 100$mg \cdot L^{-1}$ in 50℃ water for 3h and readjusted the protase K as possible reaction. After the mixture lysed completely, 20$mg \cdot L^{-1}$ Rnase reacted in 37℃ water for 1h, saturated phony was put together and bugged slightly for 10 min, centrifuged and extracted up clean liquid transfer to a cleaned plastic tube, saturated phony processed repeatedly 3 times, added 1/10 volume 3$mol \cdot L^{-1}$ NaAc and 2-2.5 times cold ethyl, DNA precipitated by centrifugation, removed ethyl, DNA washed by 700$mL \cdot L^{-1}$ ethyl land centrifuged 3 times, dried, resolved with TE, A260/A280:1.8-1.9, stored at 0℃ for use.

### *PCR amplification*

PCR was performed according to the reference[31] in 50μL reactive volume containing 0.1μg gDNA template, 200$\mu mol \cdot L^{-1}$ each of dCTP, DATP, dGTP, dTTP, 0.25$\mu mol \cdot L^{-1}$ primer, PCR buffer (Tris-HCl 10$mmol \cdot L^{-1}$, pH 8.3, $MgCl_2$ 1.5$\mu mol \cdot L^{-1}$, KCl 50$mmol \cdot L^{-1}$, gelatin 100$mg \cdot L^{-1}$) pre-denatured at 95℃ for 5 min and added 1.5μL of Taq DNA polymerase, 75μL of mineral oil. These samples were subjected to 30 cycles, including: 95℃ 1 min, 60℃ 1 min, 72℃ 1 min, and extended at 72℃ 5 min. Five μL of PCR product and appropriate bromophenol blue was added to the sample point container and electrophoresed at 20$g \cdot L^{-1}$ agarose gel containing 0.5$mg \cdot L^{-1}$ ethidium bromide at tank with 0.5×TBE liquid of electrophoresis, then observed and photographed with ultraviolet radiography.

### *PCR-SSCP analysis [31]*

Five μL digested PCR product mixed with 5μL denatured dissolution (950$mL \cdot L^{-1}$ forman mide, 20$mmol \cdot L^{-1}$ EDTA, 0.05% bromophenol blue, 0.5$g \cdot L^{-1}$ xylene nitrile) denatured at 95℃ 5 min and colded on ice. Solution processed as above was added to the gel containing 80$g \cdot L^{-1}$ polypropylene acrylamide, vertical electrophoresed at 100 V for 4h and gel stained with silver: fixed in 100$mL \cdot L^{-1}$ alcohol for 10 min→oxidized in 100$g \cdot L^{-1}$ nitric acid for 3 min→drip washed for 1 min with double distilled water→stained in 12$mmol \cdot L^{-1}$ silver nitric acid for 20 min→drip washed for 1 min with double distilled water→showed appropriate color in 0.028 mol anhydrous sodium carbonate and 0.19$mL \cdot L^{-1}$ formalin→ended reducing response by 100$mL \cdot L^{-1}$ glacial acetic acid→drip washed with double distilled water→analysis results and photographed. P16 protein expression of confirmed positively cervix carcinoma served as positive control. PBS substituted with first antibody served as negative control.

### *Immunohistochemical determination*

According to Gevadts' standard modefied slightly[32,33], nuclear or plasma stained brown-yellow as positive, (-) indicated no cell stained positive or only plasma stained or the number of nuclear stained positive less than 1 cell, (+) indicated the cells stained weakly or the number of stained cells less than 25%, (++) indicated the cells stained moderately or the stained cells covering about 26%-50%, (+++) indicated cells stained strongly or the number of stained cell more than 50%. The number of nuclear stained positively more than 2 cells per high time sight was considered to be positive. No folding, and no edging-effect fields were chosen to calculate 100 cells per 5 sights and evaluate the average number of positive cells. Positive cells discerned by two

researchers alone and decided on the disagreements together. No products of PCR amplification were loss of homozygosis of *p*16 gene, and abnormal traces found in PCR-SSCP were considered gene mutation.

### *Statistical analysis*

Chiq-square test was used *P* value less than 0.05 was considered to be statistically significant.

## RESULTS

### *P16 protein expression in gastric cancer*

The positive rate of P16 protein expression in 80 cases of normal gastric mucosa was 96% (Figure 1), and in 50 cases of dysplasia mucosa was 90% (Figure 2). In these mucosa P16 protein expression could only be seen in partical adenoepithelial cells. We did not find staining in mucosal epithelial cells, matrix fibrocytes, lymphocytes and smooth myocytes. But in gastric cancer, the ratio was 48% (Figure 3). The positive rate of P16 protein expression in gastric cancer was lower than that in normal and dysplasia mucosa ($P<0.05$). There was no significant difference between the normal gastric mucosa and dysplasia mucosa ($P>0.05$, Table 1).

In the 122 gastric cancer, the positive rate of P16 protein expression was 38%, 51%, 58%, 62% and 10% in well-differentiated adenocarcinoma, poorly-differentiated adenocarcinoma, undifferentiated carcinoma, signet ring cell carcinoma and mucoid carcinoma, respectively. The P16 protein expression in mucoid carcinoma was lower than that in signet ring cell carcinoma, undifferentiated carcinoma and poorly-differentiated adenocarcinoma ($P<0.05$). The positive rate of P16 protein expression was 48% (24/50) in gastric cancer invaded superficial muscle layer and 47% (34/72) in gastric cancer invaded deep muscle and full layer. There was no apparent relevance between P16 protein expression and the depth of invasion ($P>0.05$). In 30 cases of paired primary cancer and lymph node metastasis cancer, the rate of P16 protein expression of the lymph node metastasis cancer was 17%(5/30), significantly lower than that of primary cancer, 47% (14/30), ($P<0.05$).

### *Deletion and mutation of p16 gene exon 2 in gastric cancer*

In 25 fresh resected gastric cancer, there were 7 well-differentiated adenocarcinoma, 13 poorly-differentiated adenocarcinoma, 3 undif ferentiated carcinoma, 1 signet ring cell carcinoma and 1 mucoid carcinoma, cancer-arrounding and normal mucosa were taken at the same time. The PCR amplification showed no product in 1 case of well-differentiated adenocarcinoma, 1 case of poorly-differentiated adenocarcinoma and 1 case of mucoid carcinoma; little product found in 1 case of well-differentiated adenocarcinoma and 1 case of poorly-differentiated adenocarcinoma. There were products of PCR amplification in the rest 20 cases of gastric cancer, tumor adjacent tissue and normal mucosa. All experiments were repeated three times. The result was identical. No product of PCR amplification might indicate the loss of homozygosis of *p*16 gene, little product of PCR amplification was possibly loss of heterozygosis of *p*16 gene or loss of homozygosis of *p*16 gene, but contaminated with normal mucosa (Figure 4). Four of these 5 cases were P16 protein negative expression and 1 case expressed weekly by immunohistochemical staining. No gene mutation was observed in PCR-SSCP analysis after the PCR amplification products cut with *Sam* I (Figure 5, Table 1) (the location of restriction site, and the length of fragment are shown in Figure 6).

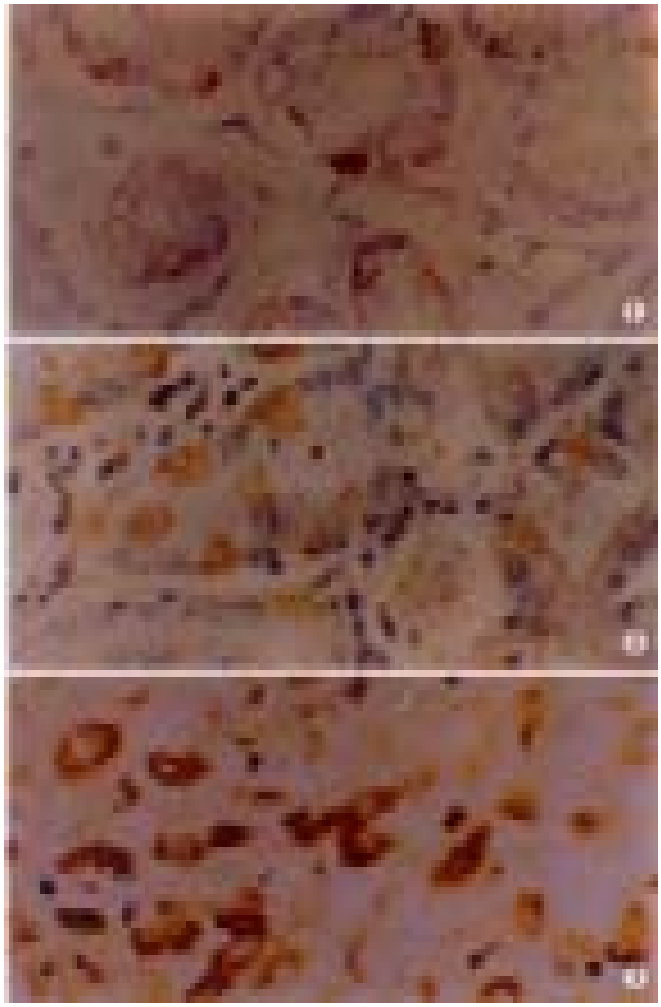

**Figure 1** *p*16 protein expression in normal gastric mucosa. ×400
**Figure 2** *p*16 protein expression in dysplastic gastric mucosa. ×400
**Figure 3** *p*16 protein expression in gastric carcinoma. Immunohistochemic stain. Arrow shows the undifferentiated carcinoma positive cell. ×400

**Table 1** P16 protein expression, p16 gene mutation and deletion gastric cancer

| Hisological types | *n* | P16 protein | | | | *p*16 gene | | |
|---|---|---|---|---|---|---|---|---|
| | | - | + | ++ | +++ | Positive rate (%) | Mutation | Deletion |
| A. Normal gastric mucosa | 80 | 3 | 41 | 20 | 16 | 96 | 0/25 | 0/25 |
| B. Dysplasia gastric mucosa | 50 | 5 | 12 | 19 | 14 | 92 | 0/25 | 0/25 |
| C. Gastric cancer | 122 | 64 | 13 | 20 | 25 | 48 | 0/25 | 5/25 |

A,B *vs* C, $P<0.05$. Mutation and deletion of p16 gene: B is tumor adjacent tissue.

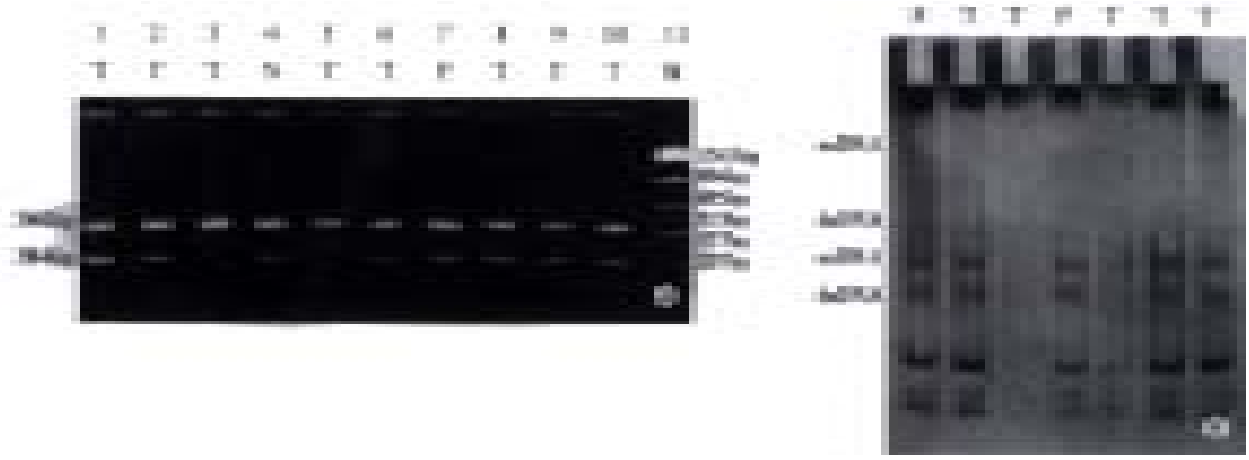

**Figure 4** PCR amplification product in exon 2 of p16 gene.
Lines 1-4,5-6,8-10 (T: gastric cancer); Line 7 (P: tumor adjacent tissue); Line 4 (N: normal gastric mucosa); Line 11 (M: marker) Little PCR product in line 3 and no PCR product in line 5.

**Figure 5** The exon 2 of p16 gene analyzed by SSCP. segment a 135bp, segment b 249bp.
Lines 2,3,5,6,7 (T: gastric cancer); Line 4 (P: tumor adjacent tissue); Line 1 (N: normal gastric mucosa).
No electrophoresis band on line 3, weak electrophoresis band on line 5, and no abnormal electrophoresis band in all lines.

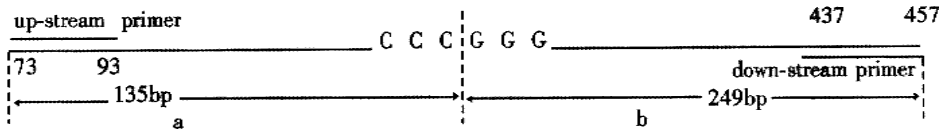


**Figure 6** The length of PCR amplification of p16 gene exon 2 and the *Sam* I (CCCGGG) restriction site.

## DISCUSSION

The *p*16 gene is a tumor suppressor gene that participates in the negative regulation of the cell growth and proliferation, and a hot spot in the molecular biological research of neoplasm since its discovery in 1993. The product of p16 gene-P16 protein is the inhibitor of CDK4. Its function is to default the activity of CDK4 by binding with CDK4 against Cyclin $D_1$ competitively, then inhibits the phosphation of Rb protein, transcription factor such as E2F when combined with the dephosphated Rb protein can not be released and activated, thus cells arrest in $G_0/G_1$ phase, resulting in cell dividing and proliferation suppressed. If p16 gene was abnormal, its function of negative regulation of cell growth would be lost. CDK4 combines to cyclin $D_1$ and PRb phosphates, lots of transcription factors would be released. The cell from $G_1$ phase enters into S phase rapidly, cell proliferates excessively and results in carcinogenesis and progression[1,2,34-36]. A lot of investigations show that there were P16 protein deletion and *p*16 gene abnormality in various primary cancers and cancer cell lines. The alteration forms of *p*16 gene were deletion, mutation[2,9,37-43], rearrangement, insertion[44-48], translocation[49-51]and hyper methylation of CpG islands presented in promotor sequence[52-58]. Such alterations consequently change the gene activity, cause abnormality, structure change of the product of *p*16 gene expression and the loss of its physiological functions. Recently, it has been reported that the exoteric *p*16 gene was transfected into the cancer cell which *p*16 gene deletes. The cancer cell restored *p*16 gene expression and cell growth was remarkably inhibited[59-67]. It has also been documented that the CpG island methylated of *p*16 gene cancer cell line was treated with 5-aza-2'-deoxycytidin. Cancer cell restored *p*16 gene expression and showed growth inhibition[68-71]. All these indicated that *p*16 gene and its product played important roles in the carcinogenesis.

This investigation showed that the positive rate of P16 protein expression in gastric cancer was remarkably lower than that in dysplasia and that in normal gastric mucosa ($P<0.05$). The result indicated that gastric carcinogenesis was probably related to the loss of P16 protein expression. But there was no significant difference between the normal mucosa and the dysplasia mucosa of the stomach ($P>0.05$). The positive rate of P16 protein expression in gastric cancer was not identical with other reports[72£¬73]. The cause was not clear. It was possibly related to the different standards of determination, reagents and some uncertain factors. However, the quantity of P16 protein expression increased from normal mucosa to precancerous lesions and gastric cancer ($P<0.05$). Following pathological lesions, P16 protein expression increased. This change may inhibit cell proliferation. The positive rate of P16 protein expression in mucoid carcinoma was significantly lower than in poorly-differentiated adenocarcinoma, undifferentiated carcinoma and signet cell carcinoma ($P<0.05$). The result suggested that the alteration of *p*16 gene was different in various histological types gastric cancer. The discrepancy of P16 protein expression exists in various histological types of lung and esophageal cancer[33,74]. There was difference of P16 protein expression and deletion of *p*16 gene in various differentiation types of gliomas. But the deletion of *p*16 gene concurred with the expression of P16 protein[4]. In 30 cases of primary gastric cancer paired with lymph node metastasis cancer, the positive expression rate of P16 protein in metastasis cancer was lower than in primary gastric cancer

($P<0.05$), which was in agreement with the reported results[75]. The result convincingly suggested that the P16 protein deletion might be related to gastric cancer metastasis and indicate P16 protein expression heterogentity in gastric cancer[76]. What was more intriguing that 2 neighboring lymph nodes metastasis cancer migrated from primary cancer had positive expression P16 protein. Expression of P16 protein is not only related to neoplasms metastasis but also related to prognosis and progression. Expression of P16 protein is low, clinical prognosis is bad[77-81]. We also investigated the relevance between various factors such as age, sex, the depth of invasion and Borrman classification and P16 protein expression in gastric cancer. There was no significant difference ($P>0.05$). The positive expression of P16 protein could merely be observed in partial adeno-epithelial cells of normal and dysplasia gastric mucosa, and weakly positive expression or undetectable in gastric mucosa epithelium cells, interstitial lymphocytes, fibroblasts and smooth muscle cells, which is contrary to some published files[82]. Nevertheless, others confer that the undetectability of P16 protein expression in neoplasm interstice[32], normal lung tissue[83] and normal urinoepithelial cells[82] might attribute to a paucity of P16 molecule in $G_0/G_1$[84] phase cells or short halftime of P16 protein[85].

Among some human neoplasms, *p*16 gene alterations always resided in exon 2[5]. There was no product of PCR amplification in 3 of 25 cases possibly due to the loss of homozygosis. Little product of PCR in 2 of 25 cases amplification might be the loss of heterozygosis or loss of homozygosis but normal mucosa contaminated. In the 5 cases, the expression of P16 protein was negative in 4 cases and weekly positive in one. The results manifested that 4 cases might be the loss of homozygosis and 1 case might be the loss of heterozygosis among the 5 cases of gastric cancer and the deletion of *p*16 gene is possibly related to the carcinogenesis and progression of gastric carcinoma. The rate of deletion in this study was slightly lower than that reported by others[74]. It was likely that only exon 2 was examined or inadequate for specimens or other unknown factors. Nevertheless, PCR amplification products were found in the rest 20 cases of gastric cancer, normal gastric mucosa and cancer-surrounding mucosa. No abnormal PAGE band and mutation of *p*16 gene was found by SSCP analysis digestion product of PCR amplification. We suggested that *p*16 gene was not involved in the carcinogenesis of gastric cancer, which coincided with other authors[86,87]. We also found that the frequency of *p*16 gene deletion was lower than that of deletion P16 protein expression. P16 protein was undetectable in normal and dysplasia gastric mucosa epithelial cells but in partial adenoepithelium. Some other uncertain mechanisms might exist in the regulation of *p*16 gene and the expression level of P16 protein[88-90], which require further studies.

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