Methylation and mutation analysis of p16 gene in gastric cancer

Yi Ding, Xiao-Ping Le, Qin-Xian Zhang, Peng Du

MATERIALS AND METHODS

Specimens
20 specimens of gastric carcinoma and their corresponding adjacent normal-appearing gastric tissue were collected from the First and Second Affiliated Hospital of Medical College of Zhengzhou University and frozen in liquid nitrogen in 30 min. All the specimens were pathologically diagnosed and without radio or chemical therapy before operation.

Analysis of methylation
Tissue DNA was extracted by normal phenol-chloroform method. DNA samples were treated with HpaII and MspI. Primers were synthesized by Shanghai Cell Biology Research Institute of China Scientific Institute and purified with PAGE. The primers of p16 exon 1 (E1): 5'-GAA GAA AGA GGA GGG GCT G-3'; 5'-GCG CTA CCT GAT TCC AAT TC-3'; the primers of exon 2 (E2): 5'-CAC AAG CTT CCT TTC CGT CAT G-3'; 5'-TCT GAG CTT TGG AAG CCT TCA GG -3'. The length of amplified fragments was 336bp and 424bp respectively. The parameter of PCR cycle was: 92 °C 60 s, 60 °C (renaturation temperature of E2 was 58.5 °C) 60 s, 71 °C 90 s. After 24 cycles, the reaction system was thermal retarded at 71 °C for 10 min. 8 µl of PCR products were electrophoresized on 20 g/L agarose gel. After the electrophoresis, the gel was visualized under ultraviolet and photographed.

PCR-SSCP
The primers were the same as mentioned above. The parameter of PCR cycle was: 91.5 °C 60 s, 61.5 °C (E1) or 59.5 °C (E2) 60 s, 70.5 °C 90 s. After 30 cycles, the reaction system was thermal retarded at 70.5 °C for 10 min. PCR products were electrophoresized on 20 g/L agarose gel and stained with ethidium bromide. SSCP was taken on the 80 g/L undenatured polycrylamide gel. After denaturing at 95 °C for 5 min, the samples were ice bathed immediately for 5 to 10 min and electrophorized under constant voltage 160 V for 4-6 h. After electrophoresis the gel was removed and silver stained.

Statistical analysis
Data were analyzed using Fisher’s exact test of probabilities with SPSS 10.0 statistic software.

RESULTS
Methylation analysis of DNA
HpaII is a methylation sensitive restriction endonuclease, when methylation occurs at the second C in the CCGG target sequence, HpaII cannot recognize the target site. However,
Msp I is isoenzyme of Hpa II, and can recognize the target site whether or not methylation occurs at the second C in the CCGG target sequence. The exon 1 and 2 of p16 gene include 2 and 4 5'-CCGG-3' sites. If methylation occurs, Hpa II can not identify the target sequence, the specific patterns would appear after PCR products are electrophoresed (336bp or 424bp) (Figure 1). If no specific bands were amplified by PCR, then no methylation alteration at second C in 5'-CCGG-3' sequence is indicated (Figure 2).

**Homozygous deletion analysis**

After agarose electrophoresis of PCR products, if no amplified products were found at the sites corresponding to 336bp or 424bp, then homozygous deletion of E1 or E2 could be determined. In gastric carcinoma tissues, 4 cases (20%) of E1 deletion and 2 cases (10%) of E2 deletion were found. The 6 cases with homozygous deletion included 1 with well differentiated and 5 with moderately or poorly differentiated gastric carcinoma tissues (Figure 3 and 4).

**PCR-SSCP analysis**

Mobility shift is defined when abnormal bands appear or the position of bands alter. No abnormal alteration was found at E1 of p16 gene (Figure 5). At E2, abnormal single strand of mobility shift exhibited in 2 (10%) cases, in 1 of which (IIIa stage, poorly differentiated adenocarcinoma) mobility shift occurred in both carcinoma and adjacent carcinoma tissues (Figure 6).

**DISCUSSION**

**Methylation of p16 gene**

In the process of multistage canceration, abnormality of gene expression may be controlled by genetic mechanism and epigenetic mechanism. Epigenetic mechanism is indicated by methylation alteration at 5 mC which cause gene expression abnormality without change in the DNA sequence and product of gene expression, and is a key mechanism causing genomic instability and canceration. Hypermethylation at CpG induces abnormality of DNA conformation stability which may influence the binding of specific protein and DNA regulating sequence, and cause gene silence. Inability to transcribe the tumor suppressor genes resulted in dysfunction of the genes and induced the development of carcinoma[10, 11].

Regional hypermethylation plays an important role in the alteration of gene expression in human carcinoma and in the progression of carcinoma. In the present paper, methylation at CpG in exons 1 and 2 of p16 gene in gastric carcinoma tissues was detected by treatment of methylation sensitive restriction endonuclease combined with PCR technique. The results showed that abnormal methylation was present in 5 and 9 of 20 cancer tissues, respectively, but no abnormality was found in corresponding adjacent normal gastric mucosa, suggesting.
an association between methylation of p16 gene and gastric carcinogenesis. Homozygous deletion of p16 gene occurred, to some extent, in many kinds of human carcinoma cells. However, gene mutation rarely occurred and the frequency of homozygous deletion was low in primary carcinoma. It is interesting that in some human carcinoma without site mutation or homozygous deletion, for example, in the pulmonary cancer cell line in oat cell type, the frequency of remethylation at p16 CpG island is 78 % resulting in the loss of p16 gene transcription activity. The same phenomena exists in mammary, prostate, gastric and colon carcinomas, especially in the colon carcinoma with the frequency of methylation being high as 92 %. In the cells of colon carcinoma without homozygous deletion, methylation occur at both alleles of p16 gene, and is related to its entire deactivation[11]. Based on the fact that the alteration of methylation of p16 gene and other genes occur in many kinds of carcinomas lacking of mutation and deletion, methylation might be a key mechanism of deactivation of tumor suppressor genes in primary carcinoma. Expression of p16 gene in gastric carcinoma is decreased significantly[22-23]. However, the frequency of mutation and deletion of p16 gene is low, suggesting that abnormal methylation might be a key mechanism in alteration of the gene expression in gastric carcinoma.

The results in the present study showed that abnormal methylation mainly appeared in poorly differentiated gastric carcinoma. Two cases with methylation in both exons were poorly differentiated and progressive gastric carcinoma. Hypermethylation of exon 2 mainly exhibited in the cases of late stage of gastric carcinoma, suggesting that hypermethylation of exon 2 is related to the differentiated degree and the clinical progression of gastric carcinoma, and thus might be a late event. Kampster et al[20], reported in their study on methylation of p16 gene in esophageal carcinoma that alteration of methylation at exon 2 was obviously related to clinical stage and progression of carcinoma, and a correlation existed between hypermethylation of exon 1 and no gene expression. Yi et al[21] reported that methylation of p16 gene in colorectal cancer was obviously related to the Duke’s stage. Methylation of p16 gene was increased gradually with the progression of carcinoma, and could induce detectable alteration and consequence to late stage which may be related to stage of gastric carcinoma.

Deletion and mutation of p16 gene
Deletion and mutation of p16 gene are also important mechanisms responsible for the dysfunction of tumor suppressor genes. Abnormality in 9p21-22 of chromosome has been reported in many kinds of carcinoma cells, and p16 gene is an important gene located in this region. By analysis of the sites adjacent to p16 gene, simultaneous mini-deletions (<200bp) of p16 allele were found in many carcinomas, and homozygous deletion of p16 gene has been testified in many kinds of primary carcinoma[23-24]. Lu et al[25] detected that the deletion of E1 of p16 gene in 16.4 % of gastric carcinoma tissues, Wu et al[23], reported a rate of 10 % (6/60). Different deletion rates of p16 gene in gastric carcinoma were reported by the other investigators[26-27]. In the present study, deletion of E1 and E2 was detected in 20 % and 10 %, respectively, of 20 cases gastric carcinoma cases, but amplified products appeared in corresponding normal gastric mucosa tissues. Mutation of p16 gene mainly includes nonsense, missense, and frame shift mutation. The frequency of mutation is significantly lower than deletion with 70-90 % being present on E2[25]. Mutation of p16 gene in gastric carcinoma is rare, but the frequency is much higher than the natural mutation (10-6-10-4) of general genes, thus, it is conceivable that mutation of p16 gene might be involved in the development and progression of gastric carcinoma. In the present study, mutation of p16 gene E2 was detected in 2 cases of gastric carcinoma tissues, and no E1 mutation was found. Both the gastric carcinoma cases with mutation were progressive gastric carcinoma. One of them exhibited mobility shift in both carcinoma and adjacent carcinoma tissues, and belonged to IIIa stage and poorly differentiated adenocarcinoma, suggesting that mutation of p16 gene might be a late event in the process of gastric carcinoma. It has been reported that the mutation site of p16 gene is the same as that of p53 gene, i.e. at CpG. It is believed that mutation is induced by nucleotide methylation[21]. It is suggested that mutation of p16 gene might be the consequence of the DNA genomic instability, and gradually causes the canceration.

REFERENCES
1. Yskoob JF, Fan XG, Hu GL, Zhang Z. DNA methylation and carcinogenesis in digestive neoplasms. World J Gastroenterol 1996; 4:174-177
2. Pollock PM, Pearson JV, Haylor ND. Compilation of somatic mutation of the CDKN2 gene in human cancers: non random distribution of base substitutions. Genes Chromosomes Cancers 1996; 15:77-88
3. Hayashi K, Metzger R, Salonga D, Danenberg K, Leichman LP, Fink U, Sendler A, Kelsan D, Schwartz GJ, Grossman S, Lenz HJ. Danenberg PV. High frequency of simultaneous loss of p16 and p16 beta gene expression in squamous cell carcinoma of the esophagus but not in adenocarcinoma of the esophagus or stomach. Oncogene 1997; 15:1481-1488
4. Song ZY, Xu RZ, Qian KD, Tang XQ, Zhao XY, Lin M. Abnormal expression of p16/CDKN2 gene in human gastric carcinoma. Xiu Xian Zaohua Bae 1997; 5:139-140
5. Igaki H, Sasaki H, Tachimori Y, Kato H, Watanabe H, Kimura T, Harada Y, Sugimura T, Terada M. Mutation frequency of the p16/CDK2 gene in primary cancer in the upper digestive tract. Cancer Res 1995; 55:3421-3423
6. Ocampo A, Hussian SP, Hagiwara K, Spilare EA, Ruscik MR, Demetrick DJ, Serrano M, Hannon GJ, Shiokaki M, Zariwala M. Mutations in the p16Nk4/MTS1/CDK4 in primary cancer in the upper digestive tract. Cancer Res 1995; 55:1448-1451
7. Mori T, Miura K, Aoki T, Nishihiro T, Morii S, Nakamura Y. Frequent somatic mutation of the MTS1/CDK4 (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous carcinoma. Cancer Res 1996; 54:3366-3379
8. Gonzalez-Zulueta M, Brender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, Jones PA. Methylation of the 5’ CpG island of the p16/CDK2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res 1995; 55:4531-4535
9. Zhang J, Lai MD, Chen J. Methylation status of p16 gene in colorectal carcinoma and normal colon mucosa. World J Gastroenterol 1999; 5:451-454
10. Issa JP, Ottaviano YL, Celeno P, Hamilton SR, Davidson NE, Baylin SB. Methylation of oestrogen receptor Cpg island links aging and neoplasia in human colon. Nat Genet 1994; 7:536-540
11. Zing JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 1997; 18:989-982
12. Wang B, Shi LC, Zhang WB, Xiao CM, Wu JF, Dong YM. Expression of tumor suppressor gene p16 in gastric cancer and precancerous lesions. Shijie Huaren Xiaohua Zazhi 2001; 9:39-42
13. Zhou Y, Gao SS, Li YX, Fang ZM, Zhao X, Qi YJ, Wei JP, Zou JX, Liu G, Jiao LH, Bai YM, Wang LD. Tumor suppressor gene p16 and RB expression in gastric cardia precancerous lesions from subjects at a high incidence area in northern China. World J Gastroenterol 2002; 8:423-425
14. Huang JS, Su Q, Chen ZC, He XT, Long ZF, Ling H, Zhang L. Expression, deletion and mutation of p16 gene in human gastric cancer. World J Gastroenterol 2001; 7:515-521
15. Wei T, Wei MX, Yang SM. Expression of cyclin D1 P16 and RB protein in gastric cancer. Shijie Huaren Xiaohua Zazhi 2000; 8:234-235
16 Zhu ZY, Tian X, Wang X, Yang XL. Mutation of p16 and APC gene in gastric cancer. Shijie Huaren Xiaohua Zazhi 2000; 8: 1418-1419

17 Yang SM, Yang LS, Li L, Deng LY, Wang CY, Yuan XB, Shen XD. Methylation of MTS1/ P16 gene and expression of P16 protein in gastric cancer. Shijie Huaren Xiaohua Zazhi 2000; 8: 1427-1429

18 Zhao Y, Zhang XY, Shi XJ, Hu PZ, Zhang CS, Ma FC. Expression of P16, P53 and proliferating cell nuclear antigen in gastric cancer. Shijie Huaren Xiaohua Zazhi 1999; 7: 246-248

19 Li GX, Li GQ, Zhao CZ, Xu GL. Relationship between telomerase hTRT and the expression of tumor suppressor gene p53 and p16. Shijie Huaren Xiaohua Zazhi 2002; 10: 591-593

20 Jiang YX, Zhao MY, Geng M, Chao YC, Wang XY. Expression of P16, cerb-2 protein in gastric tumor. Shijie Huaren Xiaohua Zazhi 2002; 10: 1050-1051

21 Yang ZL, Li YG, Huang YF, Wang QW. Expression of cyclin D1, CDK-4, P16 and Rb in gastric cancer. Shijie Huaren Xiaohua Zazhi 2000; 8: 362-363

22 Wang GT. Progression in the study on gastric precancerous lesions and its reversion. Shijie Huaren Xiaohua Zazhi 2000; 8: 1-4

23 Kempster S, Phillips WA, Baindur-Hudson S, Thomas RJ, Dow C, Rockman SP. Methylation of exon 2 of p16 is associated with late stage oesophageal cancer. Cancer Lett 2000; 150: 57-62

24 Yi J, Wang ZW, Cang H, Chen YY, Zhao R, Yu BM, Tang XM. P16 gene methylation in colorectal cancers associated with Duke’s staging. World J Gastroenterol 2001; 7: 722-725

25 Hui AM, Shi YZ, Li X, Takayama T, Makuuchi M. Loss of p16 (INK4) protein, alone and together with loss of retinoblastoma protein, correlate with hepatocellular carcinoma progression. Cancer Lett 2000; 154: 93-99

26 Lin SC, Chang KW, Chang CS, Liu TY, TzengYS, Yang FS, Wong YK. Alterations of p16/ MTS1 gene in oral squamous cell carcinomas from Taiwanese. J Oral Pathol Med 2000; 29: 159-166

27 Liggett WH Jr, Sidoransky D. Role of the p16 tumor suppressor gene in cancer. J Clinical Oncol 1998; 16: 1197-1206

28 Lu YY, Gao CF, Cui JQ. Deletion and down-regulation of mts1/p16 gene in human gastric cancer. Zhonghua Zhongliu Zazhi 1996; 18: 189-191

29 Wu MS, Shun CT, Sheu JC, Wang HP, Wang JT, Lee WJ, Chen CJ, Wang TH, Lin JT. Overexpression of mutant p53 and c-erbB-2 proteins and mutations of the p15 and p16 genes in human gastric carcinoma: with respect to histological subtypes and stages. J Gastroenterol Hepatol 1998; 13: 305-310

30 Jiang HX, Liu ZM, Zhuang YQ, Yang DH, Jiang YQ, Li JQ. Homologous deletion of p16 gene in human gastric carcinoma. Huaren Xiaohua Zazhi 1998; 6: 934-935

31 Lee YY, Kang SH, Seo JY, Jung CW, Lee KU, Choe KJ, Kim NK, Koeffler HP, Bang YJ. Alterations of p16/INK4A and p15/INK4B genes in gastric carcinomas. Cancer 1997; 15: 1889-1896

32 Tang SH, Luo HS. Aberration of p16 gene and p18 gene in gastric carcinoma. Shijie Huaren Xiaohua Zazhi 2001; 9: 91-93

Edited by Xia HHX