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• GASTRIC CANCER •

Somatic mutation analysis of p53 and ST7 tumor suppressor genes in gastric carcinoma by DHPLC

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Abstract

AIM: To verify the effectiveness of denaturing high-performance liquid chromatography (DHPLC) in detecting somatic mutation of p53 gene in gastric carcinoma tissues. The superiority of this method has been proved in the detection of germline mutations, but it was not very affirmative with respect to somatic mutations in tumor specimens. ST7 gene, a candidate tumor suppressor gene identified recently at human chromosome 7q31.1, was also detected because LOH at this site has also been widely reported in stomach cancer.

METHODS: DNA was extracted from 39 cases of surgical gastric carcinoma specimen and their correspondent normal mucosa. Seven fragments spanning the 11 exons were used to detect the mutation of p53 gene and the four exons reported to have mutations in ST7 gene were amplified by PCR and directly analyzed by DHPLC without mixing with wild-type allele.

RESULTS: In the analysis of p53 gene mutation, 9 aberrant DHPLC chromatographies were found in tumor tissues, while their normal-adjacent counterparts running in parallel showed a normal shape. Subsequent sequencing revealed nine sequence variations, 1 polymorphism and 8 mutations including 3 mutations not reported before. The mutation rate of p53 gene (21 %) was consistent with that previously reported. Furthermore, no additional aberrant chromatography was found when wild-type DNA was added into the DNA of other 30 tumor samples that showed normal shapes previously. The positivity of p53 mutations was significantly higher in intestinal-type carcinomas (40 %) than that in diffuse-type (8.33 %) carcinomas of the stomach. No mutation of ST7 gene was found.

CONCLUSION: DHPLC is a very convenient method for the detection of somatic mutations in gastric carcinoma. The amount of wild type alleles supplied by the non-tumorous cells in gastric tumor specimens is enough to form heteroduplex with mutant alleles for DHPLC detection. ST7 gene may not be the target gene of inactivation at 7q31 site in gastric carcinoma.

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INTRODUCTION

Denaturing high performance liquid chromatography (DHPLC) is a relatively new technique with a high degree of sensitivity in the analysis of germline mutations in various inherited diseases[1-3]. It is known that equal amounts of wild-type and mutant DNA are required to form heteroduplexes for ideal DHPLC analysis. The pure mutant DNA could be available easily from germline mutations or tumor cell lines, but it is often not the case in actual tumor specimens because non-tumorous cells may be present in various amounts. Even using other methods, such as LCM, would not guarantee the gain of pure tumor DNA. So, there are few reports with respect to the analysis of somatic mutations in tumors. Fortunately, in more recent reports[4-6], it was proved that DHPLC had the ability to detect the heteroduplexes formed by mixing wild type alleles with homogenous mutant alleles of cell lines over a broad range of mutant allele concentrations, differing from 5 % to 95 %, which suggested that DHPLC may be well suited for the analysis of somatic mutations in tumor tissue samples in which the proportion of mutant and wild-type alleles is variable.

In our investigation about the somatic mutation of 2 genes by DHPLC, PCR amplification of DNA extracted from surgical tumor specimen was directly conducted without mixing with wild-type DNA, only if the existence of both tumor and normal cells was confirmed by pathology in a certain proportion but not strictly in equal amount. One gene we detected was p53 gene, which was reported to have a relatively high frequency of mutation in gastric carcinoma[7-10]. The other one was ST7 gene, which was cloned and mapped to chromosome 7q31.1-7q31.2, a region suspected of containing a tumor suppressor gene involved in a variety of human cancers[11-13]. Strong evidences to support ST7 as the key TSG at this locus have recently been reported by Zenklusen *et al*.[13]. LOH 7q31 in stomach cancer has also been widely reported[14-16], so we detected four exons of ST7 gene that was reported[13,17] to have mutations to clarify the role of ST7 gene in stomach cancer.

MATERIALS AND METHODS

Tissue samples and preparation of DNA

Thirty-nine cases of gastric cancer tissue and corresponding adjacent non-tumorous gastric tissue were obtained by surgical excision from patients at the Oncology Department of the First Affiliated Hospital of China Medical University, including 15

Table 1 Primer sequences used in PCR reactions (shown in the 5' to 3' direction)

Primer name		Forward sequence	Reverse sequence	Size (bp)	Annealing temp. (°C)
P53	Exon2+3	ccaggtgacccagggttg	gcaaggggactgtagatgg	402	62
	Exon4	acctggtcctctgactgctc	gccaggcattgaagtctcat	363	60
	Exon5+6	ccgtgttccagttgctttat	ttaacccctcctcccaga	488	58
	Exon7	tgcttgccacaggtctcc	ccggaaatgtgatgagaggt	301	60
	Exon8+9	ttccttactgcctcttgctt	agaaaacggcattttgagtg	411	57
	Exon10	ctcaggtactgtgtatatac	ctatggctttccaacctagga	218	55
	Exon11	tcatctctcctccctgcttc	ccacaacaaaacaccagtgc	300	60
ST7	Exon3	gtagtgtcactgaacttacgc	gctctctgaaccagaccca	154	55
	Exon4	aggtcttgcttttctctctca	caaaaagccctcccattcag	213	55
	Exon5	tgtcctctactgagtctacc	gtatcctatcaatggcaactg	223	55
	Exon12	gtgtagatgcttccgggttg	taacgagttcctgggggat	187	55

Table 2 p53 mutations in sporadic gastric carcinomas

Specimen No.	Pathology	Exon	Codon	Mutation	Effect	DHPLC (temp)	DHPLC gradient (B% in 4.5 min)
H3	Poorly diff.	6	188	CTG>CCG	Leu188Pro	63	60-66
54	Tubular	7	246	Del 24bp[a]	8 amino acid deletion	57,59,61	54-63
57	Tubular	6	215	AGT>GGT	Ser215Gly	62	57-66
64	Tubular	5	167	Ins 3bp[a]	Gln insertion	60	57-66
77	Tubular	7	235	Del 1bp(C)[a]	Framshift (246stop)	61	53-62
79	Papillary	8	301	CCA>CTA	Pro301Leu	60	54-63
86	Poorly diff.	5	161	GCC>GGC	Ala161Gly	61	53-62
133	Papillary	5	135	TGC>TGG	Cys135Trp	62	57-66
36		Intron 7		C>T, T>G		57	54-63

[a]Mutation that hasn't been reported previously.

cases of intestinal type (4 cases of papillary type and 11 cases of tubular type) and 24 cases of diffuse type (18 cases of poorly differentiated type, 4 cases of mucinous type and 2 cases of signet-ring cell type). Their average age was 58.5 years(from 42 to 79 years), male/female was 27/12. A portion of tissue was frozen and stored at -80 ℃ for DNA extraction, and the remaining tissue was fixed in 10 % buffered formalin for histological examination. DNA was isolated by a standard proteinase K digestion and phenol-chloroform extraction procedure.

PCR conditions

Primer pairs for the amplification are listed in Table 1. Oligonucleotide primers for exons of p53 were published before[18]. The underlying sequence was based on the NCBI database. The average fragment length ranged from 150 to 500 bp. Fragment amplification was accomplished with the UNO II PCR system (Biometra, Germany). PCR reactions were performed in a volume of 20-50 μl with 35 cycles consisting of a denaturation step at 94 ℃ for 45 s, primer annealing for 30 s and an elongation step at 72 ℃ for 1 min. The final step was extended at 72 ℃ for 5 min.

DHPLC analysis and DNA sequencing

Prior to DHPLC analysis, heteroduplex formation of the PCR products was carried out by heating for 5 min at 94 ℃ followed by cooling to 25 ℃ at a rate of 0.03 ℃ S^{-1}. DHPLC was performed using the transgenomic WAVE DNA fragment analysis system (transgenomic, Ohama, USA). Four μl of the PCR products was loaded on the DNASep column and DNA was eluted at a flow rate of 0.9 ml/min within a linear acetonitrile gradient consisiting of buffer A (0.1 M triethylammonium acetate, TEAA) and buffer B (0.1 M TEAA, 25 % acetonitrile). Elution of DNA from column was detected by absorbing at 260 nm. The optimal melting temperature for each fragment was selected by using WaveMaker 4.1 software (transgenomic) or by a software described before which is available freely at http:// insertion.Stanford.edu/melt.html. PCR amplification of the DNA extracted from surgical tumor specimen was directly conducted without mixing with wild-type DNA. The original PCR products of any tumor sample showing an aberrant DHPLC elution profile and its corresponding normal tissue sample were purified using a PCR fragment purification kit (Takara, Dalian), then sequenced directly. Sequence analysis was conducted with the same primers as those used in the original PCR using an ABI 377 automated DNA sequencer. The PCR products of those tumor samples that showed normal DHPLC chromatography were mixed with wile-type at the ratio of 2:1 prior to the reannealing step, and run again.

PCR reaction and DHPLC analysis of all the samples with positive results were repeated at least 2 times and a double direction sequencing was used.

RESULTS

p53 mutations

The results of DHPLC analysis are summarized in Table 2. Mutations of the p53 gene were investigated in 39 surgical specimens of primary gastric cancer by DHPLC. Altogether, 9 aberrant DHPLC chromatographies were found and all of them were from tumor tissues, while their normal-adjacent counterparts running in parallel showed a normal shape. Subsequent sequencing revealed nine sequence variations of 8 mutations and 1 polymorphism. The 8 mutations were localized at exons 5, 6, 7 and 8, including 5 missense mutations, 1 frameshift deletion of 1 bp, 1 in frame insertion of 3 bp and 1 in frame deletion of 24 bp (one example is shown in Figure 1). The latter 3 base pairs changes were not reported previously. Through the European Bioinformatics Institute (EBI) available

at ftp://ftp.ebi.ac.uk/pub/databases/p53/, one polymorphism was localized in intron 7. The positivity for p53 mutations was significantly higher in intestinal-type carcinoma (40 %, 6/15) than that in diffuse-type (8.33 %, 2/24) carcinoma of the stomach ($P<0.01$, χ^2 test).

ST7 mutations

All of the DHPLC chromatographies from 39 tumor tissues showed a normal single peak shape, just the same as with those from their corresponding normal adjacent tissues.

DISCUSSION

Some prescreening methods, such as single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE), have been widely used for mutation analysis[19-35], but they were characterized by their lower sensitivity and more labor intensity[1,36,37]. A relatively new technique, DHPLC, is believed to be a superior method for its economic, automatic, time-saving features and higher sensitivities ranging from 95 % to 100 % for germline mutation detection[2,3,38-40]. However, with respect to somatic mutation in actual tumor specimens, one potential drawback that should be considered was that heteroduplexes formed by normal and mutant alleles were necessary for DHPLC detection. Such heteroduplexes were usually got by mixing tumor DNA with an normal equal amount of wild-type DNA when germline mutation was detected. Excitingly, some more recent reports[4-6] showed that heteroduplexes could still be detected by DHPLC when they changed the concentration of homogenous mutant alleles of cell lines from 5 % to 95 %. Their results indicated that DHPLC might also be well suitable for the analysis of somatic mutations in tumor tissue samples in which the proportion of mutants and wild-type alleles was variable. It has been demonstrated that many gastric cancers contained abundant non-neoplastic stromal cells[9]. So when somatic mutations in gastric cancer tissues were detected, it might not only be unnecessary but also laborious to mix normal wild-type alleles with tumor alleles. Further more it might yield a pseudo-negative result if the tumor cell concentration was relatively low in specimens.

In our study, the PCR products, which were amplified from the extracted DNA of surgical tumor specimens without mixing with extra wild type alleles, were directly analyzed by DHPLC. In the analysis of p53 gene mutations, 9 aberrant DHPLC chromatographies were found and all of them were from tumor tissues, while their normal-adjacent counterparts running in parallel showed a normal shape. Subsequent sequencing revealed nine sequence variations of 8 mutations and 1 polymorphism. The mutation rate (21 %, 8/39) was similar to the previously reported frequency of 20 % to 50 %[7-10]. Furthermore, no additional aberrant chromatography was found when wild-type DNA was added into the DNA of other 30 tumor samples that showed a normal shape previously. These results indicate that the amount of wild type alleles supplied by non-tumorous cells in actual gastric tumor specimens is enough to form heteroduplex with mutant alleles for the detection by DHPLC. So, DHPLC is a very convenient method for the detection of somatic mutations in gastric carcinomas.

In contrast to previous studies, p53 mutations did not follow a random distribution among different subtypes. The positivity for p53 mutations was significantly higher in intestinal-type carcinomas (40 %) than in diffuse-type carcinomas (8.33 %). This phenomenon was also observed in other reports[9,41]. Two hot spots for p53 gene mutations in gastric cancer at codon 251 and codon 173[42] were not observed in our study. Interestingly, we found three new mutations that have not been reported in the database of p53 mutations. Considering that all the patients in our study were from the northeast area of China, the above differences might be due to the different etiologies of gastric cancer in different geographical areas.

Evidence of LOH 7q31 has been found in many kinds of malignant tumors and also in gastric carcinomas[14-16], indicating that a putative tumor suppressor gene at this locus may be involved in the pathogenesis of many neoplasms. Strong evidences to support ST7 as the key TSG at this locus were recently reported by Zenklusen *et al*.[13]. Using a prostate cancer-derived cell line they showed that ST7 could suppress *in vivo* tumorigenicity. In addition, they described protein-truncating mutations of ST7 in three out of seven breast cancer derived cell lines and in four out of 10 primary colon carcinomas. But in our investigation on 39 cases of gastric carcinoma, no mutation was found. This could be due to the fact that in the previous study all the tumors were pre-screened for LOH at 7q31, thus increasing the likelihood of detecting a mutation. Other mechanisms of inactivation such as promoter hypermethylation, homozygous deletion, or genomic rearrangement that were not explored in our study were the common mechanisms of ST7 inactivation in stomach cancer. Our results were coincident with a few other recent reports[17,43-45] supporting the absence of ST7 alterations. In these studies, the researchers also failed to detect any further coding mutations in all exons of ST7 in a wide-range of carcinomas and cell lines, including that of ovarian, colon, breast, esophagus, head and neck, pancreatic and prostate. Our results extend the spectrum of malignant tumor types examined for ST7 somatic alterations, and suggest that one of the other tumor suppressor genes, or an undiscovered gene at 7q31 is the target involved in carcinogenesis of gastric carcinoma at this locus.

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