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World J Gastroenterol 2005;11(19):2998-3001
World Journal of Gastroenterology ISSN 1007-9327

• *BRIEF REPORTS* •

Mutations of p53 gene exons 4-8 in human esophageal cancer

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Supported by Ministry of Science and Technology of the People´s Republic of China grants, No. 2003CA04200 and 2004CB720301; National Natural Science Foundation of China grant, No.10490195 and 30271465; Beijing Municipal Science & Technology Commission grant, No. H030230160130 and in part by a grant from Tsinghua University YUYUAN foundation
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Received: 2004-05-29 Accepted: 2004-07-06

Abstract

AIM: To characterize the tumor suppressor gene p53 mutations in exon 4, esophageal cancer and adjacent non-cancerous tissues.

METHODS: We performed p53 (exons 4-8) gene mutation analysis on 24 surgically resected human esophageal cancer specimens by PCR, single-strand conformation polymorphism, and DNA sequencing.

RESULTS: p53 gene mutations were detected in 9 of 22 (40.9%) esophageal cancer specimens and 10 of 17 (58.8%) adjacent non-cancerous tissues. Eight of sixteen (50.0%) point mutations detected were G–A transitions and 9 of 18 (50.0%) p53 gene mutations occurred in exon 4 in esophageal cancer specimens. Only 1 of 11 mutations detected was G-A transition and 4 of 11 (36.4%) p53 gene mutations occurred in exon 4 in adjacent non-cancerous tissues.

CONCLUSION: Mutation of p53 gene in exon 4 may play an important role in development of esophageal cancer. The observation of p53 gene mutation in adjacent non-cancerous tissues suggests that p53 gene mutation may be an early event in esophageal carcinogenesis. Some clinical factors, including age, sex, pre-operation therapy and location of tumors, do not influence p53 gene mutation rates.

Key words: Gene p53; Esophageal cancer

Li LY, Tang JT, Jia LQ, Li PW. Mutations of p53 gene exons 4-8 in human esophageal cancer. *World J Gastroenterol* 2005; 11(19): 2998-3001
http://www.wjgnet.com/1007-9327/11/2998.asp

INTRODUCTION

Esophageal cancer is one of the most fatal cancers both in China and in the rest of the world[1]. The p53 tumor suppressor gene, located in chromosome 17p13, is a well-known transcription factor of cell cycle regulation and mutation of p53 gene is the most common phenomenon in carcinogenesis and tumor progression[2,3].

We investigated p53 (exons 4-8) gene mutations in esophageal cancer and adjacent non-cancerous tissues by PCR and single-strand conformation polymorphism (SSCP) analysis to evaluate p53 mutation correlation and to compare the relationship between p53 (exons 4-8) gene mutations and some clinical factors.

MATERIALS AND METHODS

Specimens

Twenty-two esophageal cancer tissue specimens and 17 non-cancerous tissue specimens, from a high-incidence area of Linxian County in Henan Province, China, were selected for this study. Patients were admitted to hospitals during 1997-1999 (Table 1) and underwent radical surgery for thoracic esophageal carcinoma. The resected specimens were embedded in paraffin and subjected to subsequent molecular analysis after the pathological findings were confirmed as squamous cell carcinoma.

DNA extraction

DNA was extracted using chelating resin for PCR amplification. In brief, three 10-μm-thick paraffin sections were cut, transferred into sterile distilled water containing 20% chelating resin iminodiacetic acid (Sigma, St. Louis, MO), and boiled for 30 min. After centrifugation, the supernatant was transferred to a sterile 500-μL tube and stored at -20 ℃.

PCR amplification

The primers used for PCR amplification of the p53 (exons 4-8) gene were as follows: 5'-TTT TCA CCC ATC TAC AGT CC-3', 5'-CAA GAA GCC CAG ACG GAA AC-3', 5'-CCT GGC CCC TGT CAT CTT CT-3' and 5'-AAG AAA TGC AGG GGG ATA CG-3' for exon 4; 5'-TCT GTC TCC TTC CTC TTC CTA-3', 5'-CAT GTG CTG TGA CTG CTT GT-3', 5'-TGT GCA GCT GTG GGT TGAT TC-3' and 5'-CAG CCC TGT CGT CTC TCC AG-3' for exon 5; 5'-TTG CTC TTA GGT CTG GCC CC-3' and 5'-CAG ACC TCA GGC GGC TCA TA-3' for exon 6; 5'-TAG GTT GGC TCT GAC TGT ACC-3' and 5'-TGA CCT GGA GTC TTC CAG TGT-3' for exon 7; 5'-AGT GGT AAT CTA CTG GGA CGG-3' and 5'-ACC TCG CTT AGT GCT CCC TG-3' for exon 8.

Hot start PCR was performed as follows: 45 cycles of

Table 1 Characteristics of patients with squamous cell esophageal cancer

Patient No.	Age(yr)	Sex	Location of tumor	Therapy of preoperation	Time of operation	Sample	
						Tumor	Adjacent tissue
1	65	M	Upper		1997	+	-
2	48	M	Upper	Radiotherapy	1997	+	+
3	39	M	Lower		1998	+	-
4	61	F	Lower		1998	+	+
5	40	M	Lower		1998	+	+
6	70	F	Mid	Radiotherapy	1998	-	+
7	54	M	Mid		1998	-	+
8	70	F	Lower		1999	+	+
9	50	M	Mid		1999	+	+
10	66	M	Upper		1999	+	+
11	43	M	Mid		1999	+	+
12	60	M	Upper	Radiotherapy	1999	+	+
13	62	M	Upper		1999	+	+
14	60	F	Upper		1999	+	+
15	51	M	Upper		1999	+	+
16	67	M	Upper		1999	+	+
17	53	F	Mid		1999	+	+
18	49	M	Upper		1999	+	-
19	57	F	Lower		1999	+	-
20	61	M	Lower	Chemotherapy	1999	+	+
21	63	M	Lower		1999	+	-
22	65	F	Mid	Radiotherapy	1999	+	-
23	60	M	Mid		1999	+	+
24	54	M	-		1999	+	-

Upper: upper thoracic esophageal cancer, Mid: mid-thoracic esophageal cancer, Lower: lower thoracic esophageal cancer.

denaturation at 95 ℃ for 30 s; annealing at 58 ℃, at 62 ℃, at 60 ℃ and at 60 ℃ for 30 s for exons 4-8; extension at 72 ℃ for 1 min, and final extension at 72 ℃ for 7 min. The amplified products were subjected to electrophoresis in 1.5% agarose gel containing 2 μg/mL ethidium bromide in TBE buffer. After electrophoresis the gels were examined under an ultraviolet light transilluminator.

SSCP analysis

Nonradioactive SSCP was performed as previously reported[25]. Twenty microliters of reaction mixtures containing 52 μL of PCR product (20-200 ng of DNA), 0.2 μL of 1 mol/L methylmercury hydroxide, 3 μL of loading buffer (15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol), and TBE buffer were heated to 90 ℃ for 4 min, then put on ice and electrophoresed in 18% polyacrylamide TBE gel at 300 V, while the temperature was maintained at 20 ℃ for exon 4, at 35 ℃ for exon 5, at 5 ℃ for exon 6, and at 25 ℃ for exons 7 and 8. The gels were stained with 0.5 μg/mL ethidium bromide in TBE buffer for 20 min at room temperature. The bands migrated apart from that of wild type were determined as SSCP positive. The bands possibly mutated by SSCP were extracted from the gels and amplified by 25 cycles of PCR to enrich the mutated alleles.

DNA sequencing

Sequencing was carried out on PCR products of SSCP positive cases. To purify single- or double-stranded PCR products with a range of 100 bp, the PCR products were processed using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. Sequencing was performed by the dideoxy chain termination method using a big dye terminator cycle-sequencing kit (Perkin-Elmer Corporation, Foster City, CA, USA). The same primers were as used for PCR. Cycle sequencing was performed following the protocol, i.e., 30 cycles of denaturation at 95 ℃ for 20 s, annealing at 54 ℃ for 30 s, and extension at 72 ℃ for 3 min. After ethanol precipitation, the samples were analyzed by a genetic analyzer (ABI Prism 310, Perkin-Elmer Corporation). PCR-SSCP analysis and sequencing of the possible positive cases were repeated thrice to rule out contamination and artifacts.

Statistical analysis

Results among p53 exon 4-8 mutations and clinical factors were analyzed using χ^2 test and Fisher's exact test. $P<0.05$ was considered statistically significant.

RESULTS

p53 gene mutations in esophageal carcinomas

We found p53 gene mutations in 9 of 22 specimens of esophageal carcinomas (40.9%). Sixteen of eighteen mutations were caused by single-nucleotide substitutions, among them, nine were missense mutations leading to amino acid substitution, and the other five were silent mutations without any amino acid change. Two of eighteen mutations were caused by deletions (Table 2). Eight of sixteen point mutations detected were G-A transitions at various codons. Nine of eighteen (50.0%) p53 gene mutations occurred in exon 4 at codons 102, 107, 111, 131, 135.

p53 gene mutations in adjacent non-cancerous tissues

We found p53 gene mutations in 10 of 17 specimens of adjacent non-cancerous tissues (58.8%). All mutations were

caused by single-nucleotide substitutions, among them, seven were missense mutations leading to amino acid substitution, and the other four were silent mutations without any amino acid change. Only 1 of 11 mutations detected was G-A transitions at codon 145. No deletion was found (Table 3). Four of eleven (36.4%) p53 gene mutations occurred in exon 4 at codons 58, 107, 111, and 141.

Table 2 p53 gene mutations in tumor tissue from esophageal cancer patients

No.	Exon	Codon	Nucleotide	Amino acid
1	None			
2	4	111	CTG-CTA	Leu-Leu
	4	131	GGG-GAG	Gly-Glu
3	None			
4	5	179	CCA-CTA	Pro-Leu
5	None			
6	-	-	-	-
7	-	-	-	-
8	4	107	TAC-TAT	Tyr-Tyr
9	None			
10	None			
11	4	102	ACC-ACT	Thr-Thr
	4	135	CAT-TAT	His-Tyr
	5		16 bp deletion	
12	None			
13	None			
14	4	131	GGG-GAG	Gly-Glu
	6	196	CGA-TGA	Arg-stop
15	None			
16	None			
17	None			
18	7i	242	CTG-CCG	Leu-Pro
19	None			
20	4	111	CTG-CTA	Leu-Leu
	4	131	GGG-GAG	Gly-Glu
	5	152	CCG-CCA	Pro-Pro
	6	208	GAC-AAC	Asp-Asn
	7i	250	CCC-TCC	Pro-Ser
21	6	208	GAC-AAC	Asp-Asn
	7i	248	CGG-TGG	Arg-Trp
22	None			
23	None			
24	4		6 bp deletion	

Table 3 p53 mutation in adjacent non-cancer tissue from esophageal cancer patients

No.	Exon	Codon	Nucleotide	Amino acid
1	-			
2	7i	256	ACA-GCA	Thr-Ala
3	-			
4	5	144	CAG-CGG	Gln-Arg
5	None			
6	5	145	CTG-CTA	Leu-Leu
7	4	111	CTG-CAG	Leu-Gln
8	4	141	TGC-TGT	Gys-Cys
	7i	256	ACA-GCA	Thr-Ala
9	None			
10	None			
11	4	107	TAC-TAT	Tyr-Tyr
12	None			
13	4	58	TTG-TGG	Leu-Trp
14	None			
15	None			
16	None			
17	5	144	CAG-CGG	Gln-Arg
18	-			
19	-			
20	None			
21	-			
22	-			
23	7i	267	CCT-TCT	Pro-Ser
24	8	282	CGG-CGC	Arg-Arg

p53 gene mutations and their correlation with clinical factors

40.9% and 58.8% of p53 gene mutations occurred in esophageal carcinoma specimens and adjacent non-cancerous tissue specimens. A comparison of p53 gene mutations in esophageal carcinoma specimens and adjacent non-cancerous tissue specimens with other clinical factors, including age, sex, pre-operation therapy and location of tumors, showed no clear correlation between two kinds of specimens ($P>0.05$).

DISCUSSION

The wild-type p53 protein has an inhibitory effect on cell proliferation and transformation and this effect has been believed to be mediated by its ability to arrest cells in G_1 phase of the cell cycle[2]. Recent studies also reported that p53 gene mutation was correlated with an increased risk of developing human papilloma virus-associated cancers[4]. In addition to its role in cell regulation, p53 has been implicated in DNA synthesis and repair[5], maintenance of genomic stability[6], cell differentiation and apoptosis[7]. Point mutational damage to p53 could significantly alter regulatory tumor suppressor activity of this gene[8].

In the worldwide, esophageal cancer is the fourth most common malignancy, after gastric, colorectal and hepatocelluar malignancies[9]. It has been reported that squamous cell carcinoma accounts for 70% of esophageal cancers[10]. During the last 30 years, an increased incidence of 350%, particularly of adenocarcinoma has been described in population of USA, Western Europe and Sweden[11-13]. Because surgical resection alone could rarely result in long-term survival, efforts are focused now on combined modality therapy in an attempt to improve its local control and to eliminate micrometastatic diseases present at the time of resection.

We found p53 tumor-suppressor gene mutations in 9 of 22 (40.9%) esophageal carcinoma specimens examined and 10 of 17 (58.8%) adjacent non-cancerous tissue specimens examined from Linxian County, China. Gao *et al*[14], detected p53 gene mutations in normal epithelia (33.3%), basal cell hyperplasia (13.0%, BCH), and dysphasia (36.4%, Dys). Shi *et al*[15], showed that p53 gene mutations were detected in 70% of 43 surgically resected esophageal cancer specimens and in p53 positive immunostaining non-cancerous lesions adjacent to cancer containing BCH (47%) and Dys (67%). Fujiki *et al*[16], reported that the p53 gene mutation rates were 28.6% in esophageal cancer specimens, 20% in Dys, and 50% in BCHs. The observation of p53 gene mutation in adjacent non-cancerous tissues suggests that such mutations may occur even before morphological changes can be observed. The different p53 gene mutation rates were also reported from other esophageal carcinoma high-incidence areas, ranging 30-75%[17-20]. The mutation frequency was significantly different. The difference may be due to different exons examined or due to different tumor samples, which contained inflammatory and stromal cells. A mutation might be hidden by the wild-type sequence of the gene in normal cells and was not detectable by screening procedures or there were differences in areas where specimens were collected and in examination methods.

Most point mutations of p53 gene have been known to

occur at codons 97-292[21]. Greenblatt *et al*[22], reported that p53 played an important role in transcription of target genes in a sequence-specific manner. Our study showed that, except one mutation in codon 58, 28 of 29 identified point mutations were involved in Linxian County, a highly conserved region. It is worth noting that 8 of 16 point mutations detected were G-A transitions and 2 of 18 mutations were caused by deletions in esophageal carcinoma tissues, but only 1 of 11 mutations detected was G-A transition and no deletion was found in adjacent non-cancerous tissues. G-A transitions may be a result of carcinogen-induced alkylations of DNA. The formation of O^6-methylguanine may cause a mispair for thymine in DNA replication. In the subsequent round of DNA replication, an adenine would replace the original guanine and result in G-A transitions[23].

It has been reported that mutations have rarely been found outside exons 5-8 of the p53 gene[24]. Shi *et al*[15], reported the exon distributions of p53 mutations in Henan Province, China. The exon 4 mutation was not found both in esophageal carcinoma and in adjacent non-cancerous tissue specimens. But in our study, 9 of 18 (50.0%) p53 gene mutations occurred in exon 4 in esophageal carcinoma tissues and 4 of 11 (36.4%) p53 gene mutations occurred in exon 4 in adjacent non-cancerous tissues. The results may be due to the differences the methods we used contributed to the higher detection rate of genetic alteration.

Our findings indicate that the mutation of p53 gene in exon 4 may play an important role in the development of esophageal cancer. The observation of p53 gene mutation in adjacent non-cancerous tissues suggests that p53 gene mutation may be an early event in esophageal carcinogenesis.

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Science Editor Wang XL and Li WZ **Language Editor** Elsevier HK