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World J Gastroentero, 2000; 6(5):750-753
World Journal of Gastroenterology
 ISSN 1007-9327

Infrequent *p*53 gene mutation and expression of the cardia adenocarcinomas from a high-incidence area of Southwest China

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Subject headlines cardia adenocarcinoma/ etiology; protein P53; gene expression; mutation; genes *p*53; polymerase chain reaction; DNA; risk factors

Wang XJ, Yuan SL, Li CP, Iida N, Oda H, Aiso S, Ishikawa T. Infrequent p53 gene mutation and expression of the cardia adenocarcinomas from a high-incidence area of Southwest China. ***World J Gastroentero***, 2000;6(5):750-753

INTRODUCTION

Adenocarcinomas of the cardia are the lesions arising from the proximal stomach or within 3cm of the gastroesophageal junction[1,2]. These cancers tended to be advanced at the time of presentation, usually with poor prognosis[2]. In recent decade, the incidence of adenocarcinoma of gastric cardia and esophagus are increasing steadily, while there has been a decrease in the proportion of the cancers arising from the distal stomach[3-8]. The biological and epidemiological features of the cardia adenocarc inoma were distinct from those arising from the distal stomach[9,10]. The specific etiologic factors involved in the increasing incidence remained unresolved[1,5-10].

Significantly higher levels of DNA aneuloidy[11,12], p53 protein expression[1,12-17] and mutations[1,18] have been reported in gastric cardia tumors, compared with tumors arising in the gastric antrum. p53 expression may be used as a marker in the early detection of cardia adenocarcinoma[2], mutations of p53 gene at specific codons in human cancers may be an indication of specific exposure or genomic susceptibility[14,15,19-22], and may serve as a reporter gene reflecting exposure to specific carcinogen[14,21-23]. The previous studies[1,11-23]. Suggested that the mutation and functional inactivation of p53 gene might play some important roles in the carcinogenesis of cardia adenocarcinomas etiologically.

In this study, the p53 gene mutation spectrum and protein overexpression were investigated in the cardia adenocarcinoma from a high-incidence area of Southwest China, comparison of mutation spectra was made with those in cardia adenocarcinomas from other regions, some clues may be drawn to the etiology and carcinogenesis of cardia adenocarcinomas in the local area.

MATERIALS AND METHODS

Patients and samples

All 20 cases of cardia adenocarcinoma specimens were collected from patients surgically treated in Yanting County Cancer Hospital of Sichuan Province in Southwest China. Of all 20 patients, 17 (85%) were male, 3 (15%) were female, age ranged from 46 to 69 years (mean, 54.9 years). None had received any treatment before the operation; all patients were diagnosed as having cardia adenocarcinoma arising within the gastroesophageal junction[2]. None was associated with Barrett's esophageal epithelium. All tissue specimens were routinely processed, formalin-fixed, and paraffin-embedded. Paraffin sections were stained with hematoxylin and eosin (HE) for histopathological examination, and were used for DNA isolation and detection of p53 protein by immunohistochemi cal staining.

DNA isolation

The paraffin embedded blocks were sectioned at 10μm, attached to glass slides and used for genomic DNA isolation by means of Pinpoint Slide DNA Isolation System (Zymo Research, USA), according to the manuf acturer's instructions. Briefly, a 1mm^2 area of cancer tissue with few other cells except cancer cells was localized on HE staining slides under microscopy in each case; the 10μm slides were de-paraffinized, rehydrated through graded alcohols and air dried. With reference to the HE staining slides of the same case, the PP solution was applied to the localized area of each case. After air dry for 30-45 min, the PP solution embedded tissues were peeled and removed from the glass slides, and added into the tubes

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Received 2000-04-03 **Accepted** 2000-07-31

containing 50μL extraction buffer and 5μL proteinase K solution, then incubated at 55℃ for 4 hours, heated at 95℃ for 10min. The isolated DNA was further purified with DNA binding buffer and mini-spin column and PP wash buffer supplied by the manufacturer.

PCR and asymmetric PCR amplification

To amplify the DNA segments of exons 5-8 of p53, 100ng-200ng aliquots of template DNA were added in a total volume of 25μL- PCR reaction mixture containing 1×PCR buffer, 0.2mM of each dNTP (PE Applied Biosy stems), 1μm of each primer and 2.0 units of AmpliTaq Gold DNA polymerase (Perkin Elmer). Initial denaturation at 94 was performed for 9min, followed by 50 cycles of 30 sec at 94℃, 30 sec at 52℃ or 58℃ (depending on the primers), 45 sec at 72℃ and a final extension of 7min at 72℃. The primers used in this study covered the portions of exons 5 - 8 of the human p53 gene as described before[24].

Twenty microliters of PCR products were run on a 2.0% agarose gel for examining the specificity of PCR reaction and DNA recovery, the specific bands were cut, recovered by Sprec-DNA Recovery Filter Tubes (Takara Biomedical, Japan), and purified according to the manufacturer's instruction. The recovered DNA fragments were used as templates for asymmetric PCR amplification. The two single-stranded DNA fragment of each exon were amplified with asymmetric PCR by using only one primer, respectively, except of the doubled primer, the conditions of asymmetric PCR were the same as those of the first PCR amplification.

DNA sequencing

Asymmetric PCR products were purified by alcohol precipitation, one fourth of purified product was used for DNA sequencing. DNA sequencing was performed by dideoxynucleotide method using the Thermo Sequenase cycle sequencing kit (Amersham, Life Science) and γ^{32}p-ATP (Amersham, Pharmacia Biotech). The sequence ladder was resolved in 7.0M urea and 6% polyacrylamide gel. After electrophoresis, the gel was dried in the Bio-Rad gel dryer before exposure to X-ray film for 6-12 hours at -70℃. When base changes were identified repeated sequencing of the same or complementary DNA strand was performed for confirmation[19].

Immunohistochemical analysis of p53 proteins

P53 protein was detected immunohistochemically with the avidin-biotin-peroxidase method[25]. Briefly, tissue sections were de-paraffinized and re-hydrated through graded alcohols. Antigen retrieval was performed by microwave oven heating in 0.1mM citrate buffer (pH 6). Then, endogenous peroxidase activity was blocked with 3% H_2O_2, and after treatment with normal serum, the sections were incubated with CM1 polyclonal rabbit p53 antibody (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:500, overnight at 4℃, with biotylated second antibody 20min, and with avidin-biotin-peroxidase complex 20min at room temperature. Subsequently, the sections were subjected to color reaction with 0.02% 3, 3 diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 in PBS (pH 7.4), and were counterstained with hematoxylin lightly. In each staining run, a known p53 positive sample of colon cancer was added as a positive control, and a section of the same colon cancer tissue incubated in PBS instead of CM1 was included as a negative control.

Approximate percentage of p53 positive tumor cell nuclei in carcinoma were as sessed and scored as follows: negative (-), <10% (+), 10% - 50% (++), >50% (+++); The intensity of staining in p53 positive cell nuclei was compared with the negative control and scored as follows: negative (-), weak (+), moderate (++), strong (+++)[25].

RESULTS

p53 mutations in cardia adenocarcinoma

In all 20 cases of cardia adenocarcinomas, only 2 mutations (10%, 2/20) of p53 gene were detected in 2 cases. There were ATC-ACC, T to C transition in one occurred at codon 195 in exon 6 and CGC-AGC, C to A transversion in another at codon 283 in exon 8. Two were missense mutations. A representative mutation is shown in Figure 1.

p53 overexpression in cardia adenocarcinomas

All immunostainings for p53 protein were confined to the cell nuclei (Figure 2), the positive cell number varied from 10% to more than 50%; The intensity of staining ranged from moderate to strong. Seven out of 20 (35%, 7/20) were detected with p53 protein overexpression.

In addition, in 2 cases with p53 gene mutations, one was p53 protein-positive (96 - 236, codon 195: ATC-ACC); one was negative for p53 protein (96-237, codon 283:CGC-AGC). All paracancerous normal tissues were p53-negative. In 5 cancers with lymph node metastasis, 2 were p53-positive of both cancer and lymph node, and 3 were p53-negative in both tissues. The positive cases with p53 gene mutations and/or p53 protein overexpression are summarized in Table 1.

Table 1 The positive cases with *p*53 gene mutation and/or p53 protein overexpression in cardia adenocarcinomas

Patient No.	Exon	Codon	Nucleotide change	Amino acid change	p53protein expression
96-114	-	-	-	-	+++
96-123	-	-	-	-	+++
96-166	-	-	-	-	++
96-185	-	-	-	-	++
96-236	6	195	ATC-ACC	Ile-Thr	+++
96-237	8	283	CGC-AGC	Arg-Ser	-
96-270	-	-	-	-	++
96-432	-	-	-	-	++

The negative cases without *p*53 gene mutations and protein expression were not listed.

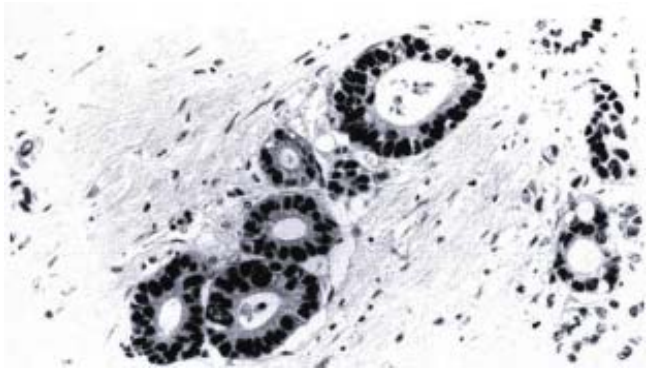

Figure 1 Results of sequence analysis of the p53 gene in the cardia adenocarcinomas from a high incidence area of Southwest China, case 96-236 showed a ATC-to ACC mutation at codon 195 of exon 6.

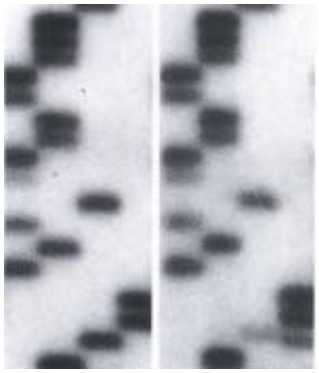

Figure 2 Immunoreactivity of p53 protein of cardia a denocarcinoma, p53 protein was located in the nuclei of cancer cells, most cancer cells were positive for p53 protein. ABC ×20

DISCUSSION

The present study investigated the *p*53 gene mutation and protein expression in a series of 20 cases of cardia adenocarcinomas from a high incidence area of Southwest China, the rates of p53 mutation and protein expression were lower than those reported previously.

There were less reports of p53 mutation and protein expression in cardia aden ocarcinomas than those in other cancers (http:/www.irac.fr/p53/ homepage. htm)[26], such as, hepatocellular carcinoma[27-29], esophageal cancer and gastric cancer[14,20-22]. Li *et al*[30] detected p53 mutations in 3 out of 4 cardia adenocarcinomas (3/4), 6 mutations were identified in exons 5-8 of p53 gene in 6 out of 14 cardia adenocarcinomas (6/14, 42.9%) from Linxian, China, 3 were missense mutations, and 3 were non-sense mutations[18]. Recently, a high p53 mutation rate (63%, 26/41) in cardia adenocarcinomas from North Ireland was reported[1]. As for the p53 protein expression, the positive rates reported in cardia adenocarcinoma were 59% (24/41)[1], 44% (11/25)[2], 56% (20/ 36)[12], and 76.5% (13/17)[13], respectively. These results suggested that p53 mutation and expression played an important role in carcinogenesis of cardia adenocarcinoma, and *p*53 gene mutations reflected exposures to specific carcinogens in the environment[14,19,21-23].

Conversely, in our series, only 2 cases of *p*53 gene mutation (10%) and 7 cases of p53 protein expression (35%) were detected in 20 cardia adenocarcinomas from a high incidence area of China. It had been proposed that the comparison of *p*53 gene mutation spectra in tumors of different origin might reveal similarities or differences concerning the endogenous and exogenous molecular processes contributing to tumor development[24], and might reflect site-specific difference (cardia or antrum) or regional exposure to particular environmental agents[31]. The results in this study suggested that *p*53 gene mutation might play some role in some subset of cardia adenocarcino mas, but, it is not the major causation in carcinogenesis of gastric cardia in local area, or the risk factors in the local environment are different from those in other regions.

To the best of our knowledge[1,18,26,30], the two mutations detected in the present study were rare in cardia adenocarc inomas. Mutation of *p*53 gene at codon 195 from ATC to ACC was reported in ovarian cancer[32], brain tumors[33], lung cancers[34,35], and adenocarcinoma of Barrett's esophagus[36], etc.[26]; Mutation at codon 283 was of polymorphism in previous studies: It was from CGC to TGC in hepatocellular carcinoma[37] and colorectal cancers[38], CGC to CTC in ovarian tumor[39], CGC to GGC in lung cancer[40,41], etc[26]. But, in this series, the mutation at codon 283 was from CGC to AGC, C:G-A:T transversion, no such a mutation was reported previously. It was further suggest ed that the environmental risk factors and their mechanism of actions in local area were different from those in other regions[1,18,30].

The disconcordance between p53 protein expression and *p*53 gene mutation was noted in many other studies[1,25,35], one case with *p*53 gene mutation was negative for p53 protein immunohistochemical staining in this study, since the frameshifts or non-sense mutations in the coding sequences of the gene might result in truncated or unstable forms, even non-expression[25]. No mutations in exons 5-8 were detected in other 6 cases with positive p53 prote in, the possibilities to account for this apparent disconcordance might be: Firstly, the missense mutation lay in the region of the gene not screened in this study. Although *p*53 gene mutations occurred frequently in the so-called 'hot-spot' region of exons 5-8[35], a review of 50 studies that carried out sequencing of the entire coding region of *p*53 gene reported that 13% up to 30% of missense mutations were located outside exons 5-8[23]; Another possibility was that the mechanism other than mutations resulted in inactivation and stabilization of p53 protein. Binding to viral oncop roteins, e.g. HPV E6, and cellular oncoproteins, e.g. mdm2, had been shown to result in p53 stabilization and in the inactivation

of wild-type function[41,42]. Our previous studies indicated that there was a high prevalence of HPV16 of esophageal cancer patients and health population in this high incidence area[43,44]. In these instances, p53 overexpression represented the fun ctional, but not the structural, inactivation of *p*53 gene[1].

We conducted a preliminary investigation of *p*53 gene mutation and p53 protein expression in 20 cases of cardia adenocarcinomas from a high incidence area in Southwest China, to elucidate the etiology and carcinogenesis of cardia adenocarcinoma in the local area. The low *p*53 gene mutation and expression rates, and different mutation spectrum suggested that *p*53 gene mutation and functional inactivation might play some roles in the subset of cardia adenocarcinomas in this high incidence area. Besides *p*53 gene abnormalities, there might be some other environmental risk factors and other genetic abnormalities contributing to the high incidence of cardia adenocarcinomas in this area, which deserves further investigation.

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Edited by Wu XN
Proofread by Zhu LH and Ma JY