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p53 Gene Alternation in Squamous Cell Carcinoma of the Esophagus Detected by PCR-Cold SSCP Analysis

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ABSTRACT

Mutations of the p53 tumor suppressor gene play an important role in the development of common human malignancies. Previous reports revealed that the frequencies of p53 alternations in esophageal carcinoma varied from 26% to 87%. The clinical significance of p53 alternations is still disputed. In the present study, we used polymerase chain reaction - "cold" single-strand conformation polymorphism (PCR-"cold" SSCP) - to investigate p53 genetic alternations in 63 surgical specimens of esophageal squamous cell carcinoma (ESC). Our experiments showed that the optimum buffer temperature for "cold" SSCP analysis was 14 ºC while the PCR products were around 200-300 bp in size. Among 63 tumorous samples, p53 alternations was detected in 47 tumors, or an incidence rate of 74.6%. For nontumorous mucosal samples, the incidence of p53 alternations was 55.5% (35/63 samples). Additionally, p53 alternations occurred most frequently at exon 6 (50.8%), followed by exon 7 (33.3%), exon 8 (17.5%) and exon 5 (12.7%). Multiple genetic alternations (≥ 2 exons) between p53 exons 5-8 in the same examined samples were found in 21 (33.3%) of 63 tumors, and in 8 (12.7%) of 63 nontumorous mucosal specimens. Our results further showed that p53 alternations did not correlate with age, depth of tumor invasion, lymph node metastasis, tumor stage, cell differentiation or lymphovascular invasion in ESC ($P > 0.05$). Moreover, the survival rate in patients with p53 alternations was similar to that in patients without p53 alternations ($P > 0.05$). In conclusion, PCR-"cold" SSCP is a rapid and sensitive method for identifying p53 genetic alternations. p53 genetic alternations occur with a relatively high incidence for ESC, but p53 abnormality has no impact on prognosis.

Key Words: esophageal carcinoma; p53 alternations

I. Introduction

The incidence of esophageal carcinoma (ESC) exhibits very striking geographical variation (Parkin *et al*., 1988; Sales and Levin, 1985). In Taiwan, esophageal carcinoma ranks sixth in cancer-caused deaths among male patients (Annual report, 1995). Epidemiological studies have very strongly implicated a variety of risk factors, notably alcohol consumption, tobacco use, deficiencies of certain micronutrients, consumption of food contaminated by mycotoxins, and low consumption of fresh fruit and vegetables. However, none of these factors has been shown to cause esophageal cancer in experimental animals (Craddock, 1993). While efforts have been made instudying multiple therapeutic modality for esophageal carcinoma, the results have been dismal (Wang and Chien, 1983; Wu and Huang, 1979; Wang *et al*., 1992; Fahn *et al*., 1994).

Cancer is the result of an accumulation of genetic alternations that disrupt the control of cell growth and termination differentiation. In recent years, p53 tumor suppressor gene mutation has been found to be the most common genetic alternation in

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human cancers (Hollstein *et al*., 1991). p53 appears to function in a cell cycle control as a negative regulator of cell division in the G1-S phase (Hartwell, 1992; Kastan *et al*., 1991; Livingstone *et al*., 1992; Yin *et al*., 1992). The frequencies of p53 alternations in esophageal carcinoma have been found to vary from 26% to 87% in previous studies (Hollstein *et al*., 1990; Hollstein *et al*., 1991; Casson *et al*., 1991; Bennett *et al*., 1992; Tamura *et al*., 1992; Imazeki *et al*., 1992; Wang *et al*., 1993; Huang *et al*., 1993; Wang *et al*., 1994; Sarbia *et al*., 1994). Except for different investigation modalities, geographic variation may be a major cause of the great difference in the frequencies of p53 alternations (Aguilar *et al*., 1994).

Unlike the ras family of oncogenes, in which mutations occur in two "hot spots", p53 has mutations in multiple sites throughout the open reading frame. Fortunately, most mutations are limited to a region highly conserved among species that spans exons 5 through 8. Thus, it is impractical to directly sequence the polymerase chain reaction (PCR) products for large numbers of samples. Polymerase Chain Reaction Single-Strain Conformation Polymorphism (PCR-SSCP) is a powerful approach for qualitative analysis of the DNA (Yap and McGee, 1992; Mohabeer *et al*., 1991; Yap and McGee, 1992). This method is based on the observation that the electrophoretic mobility of a DNA molecule through a neutral polyacrylamide gel can be altered by the size and shape of the DNA molecule. Under nondenaturating conditions, single stranded DNA has a folded structure that is determined by intermolecular interaction related to its base sequence and is different from the wide-type sequences. As a result, the mutated single-stranded DNA shows a mobility difference in a polyacrylamide gel. Since the introduction of this technique for the detection of polymorphism in the human gene, it has been widely used.

Several non-isotopic protocols for SSCP analysis have been reported (Wap and McGee, 1992; Mohabeer *et al*., 1991; Yap and McGee, 1992; Ainsworth *et al*., 1991; Dockhorn-Doworniczak *et al*., 1991; Ballhausen and Kraus, 1993). Most of these protocols require the purchase and use of expensive additional equipment or the use of silver staining techniques (Maekawa *et al*., 1993). In the present study, we have used a so-called "cold" SSCP to investigate the p53 gene alternations in squamous cell carcinoma of the esophagus. This "cold" protocol is a rapid, inexpensive, non-radioactive method for SSCP analysis of standard PCR products (Makino *et al*., 1992). The gel temperature, the most critical parameter influencing SSCP band resolution and reproducibility, is precisely controlled through the use of a thermostatically controlled circulator which accurately maintains a predetermined buffer temperature within the gel unit. Commercially available pre-cast mini-gels ($8.0 \times 8.0 \times 0.1$cm) are used, which are both convenient and inexpensive. Individual SSCP bands can be visualized directly by means of ethidium bromide staining and can be easily cut from the gel for subsequent reamplification or sequencing. In addition to defining the frequency and the status of p53 alternations in our patients, the clinicopathological roles of the p53 alternations are also discussed.

II. Materials and Methods

1. Patients and Tumor Samples

Fresh tumorous and non-cancerous esophageal mucosal samples were collected from 63 consecutive patients with histologically confirmed esophageal squamous cell carcinoma (ESC) who underwent subtotal esophagectomy at Veterans General Hospital-Taipei between December 1993 and November 1996. Patients who received preoperative irradiation or chemotherapy were excluded from the study. Of these 63 patients, 60 were males and 3 were females. The mean age (± SD) was 64.1 years (± 10.4 years). The clinicopathological factors of these patients were estimated according to the TNM classification proposed by the International Union Against Cancer (UICC) in 1987 (IUAC, 1987). All the patients have been regularly followed up with systemic examinations of blood biochemistry studies, chest radiography, abdominal and cervical sonograms, and whole body bone radiostopic scanning every 3 to 6 months. A computer tomogram (CT) scan of the chest or abdomen was also done, if necessary. The mean follow-up period (± SD) has been 38.0 months (± 4.6 months). All tissue samples for examination were taken at surgery and placed in liquid nitrogen as soon as possible.

Postoperative chemoradiotherapy was administered for 24 patients with advanced stage (> stage IIb) lesions. The adjuvant therapy was given 1 month after surgery. The dose of irradiation was 5,000-6,000 rads (1,000 rads/week), and the combination regimens of chemotherapy consisted of 5-fluorouracil (5-FU) (600 mg/m^2/day), cisplatin (20 mg/m^2/day), and leucovorin (120 mg/m^2/day) for 4 days. Chemotherapy was concomitantly administered in the first week of irradiation.

2. Preparation of PCR Products

Genomic DNA was extracted from archival fresh-frozen specimens of squamous cell carcinoma

and nontumorous mucosae of the esophagus. The cocktail mixture for PCR amplification consisted of H_2O 69.6 μl, 10 × PCR buffer 9.6 μl, 4 dNTP mixture (2.5 mM) 9.6 μl, primer 1 (10 μM) 1.8 μl, primer 2 (10 μM) 1.8 μl, and Taq polymerase 0.96 μl. As shown in Table 1, the working primers included p53 exon 5 to exon 8. PCR amplifications were performed in 10 μl volumes with 500 ng of genomic DNA and 15 μl of the cocktail mixture for a total of 30 cycles. In the present study, the size of the PCR products was in a range from 196 bp to 287 bp. No specific purification of PCR products was performed prior to SSCP analysis. However, to obtain unambiguous results, it was important that a single, clear band of PCR product (double-stranded DNA) was found on gel electrophoresis after PCR.

A mixture consisting of 10 μl of PCR product (roughly 500 ng of DNA), 16 μl loading buffer (containing 10% bromphenol blue and 10% xylene cyanol, 0.1 mM EDTA in formamide) was prepared to yield a total volume of 26 μl. This mixture was heated to 95 ºC for 10 minutes and then plunged into ice prior to loading the 26 μl onto the gel for SSCP analysis.

3. Polyacrylamide Gel Analysis With Cold SSCP

A 6% gradient polyacrylamide TBE gel (29:1 acrylamide to bis-acrylamide cross-linking) was used with the matching gel electrophoresis unit (Novex, San Diego, CA). The buffer chamber was filled with 1.5 × TBE buffer (90mM Tris, 92 mM boric acid, 2.5 mM EDTA) for this gel. A thermostatically controlled refrigerated circulator (B402-D, TKS, Taiwan) was used to maintain a constant temperature of 14 ºC. The circulator was equipped with a Y-tube on the fluid output to allow it to supply the inner and the outer buffer chambers of the gel apparatus simultaneously. These tubes had an internal diameter of 4.8 mm, and each supplied 1350-2000 ml/min. This rapid rate of flow allowed the circulator to easily maintain a constant temperature within the gel unit.

A pre-run of 6% glycerol gel at 100 volts was done. Then, 26 μl of mixture samples prepared as described above was loaded onto the gel. The gel was run at 200 volts (Model 1000/500, Bio-Rad, CA) until the blue marker reached the bottom of the gel. The running time was approximately 2 hours. The temperature of the buffer chamber nearest the gel was monitored with thermister during the gel run.

After completion of the gel run, the gels were stained with a 0.5 μg/ml solution of ethidium bromide in 1 X TBE buffer for 15 minutes. Ethidium bromide-stained bands were visualized using a 340 nm UV viewing box and photographed.

4. Statistical Analysis

The frequency distribution was tested using the Chi-square (χ^2) test with Fisher's exact test. The cumulative survival rates were calculated using the Kaplan-Meier method, and the statistical difference in survival rates was determined by the log rank. The statistical significance was assumed if P-value < 0.05.

III. Results

1. Clinicopathological Findings

All clinicopathological findings are listed in Table 2. Among 63 patients, 33 (52.4%) were older than 65 years. For the tumor length, 47 (74.6%) patients had tumors larger than 5 cm, and 16 (25.4%) were less than 5 cm. The tumor locations were distributed as follows : cervical, 5 (7.9%); upper thoracic (Tu), 7 (11.1%); middle thoracic (Tm), 32 (50.8%); lower thoracic (Tl), 19 (30.2%), and none in the abdomen. Histologically, tumor grades consisted of 8 (12.7%) with good differentiation, 45 (71.4%) with moderate differentiation, and 10 (15.9%) with poor differentiation. Microscopic evidence of lymphovascular invasion was detected in 15 cases (23.8%). The depth of tumor invasion included T1, 9 cases (14.3%); T2, 9

Table 1. The Working Primers of p53 Exons 5-8 for Cold PCR-SSCP Analysis of p53 Gene Alternation

Primes		Sequences		Molecular Weight	Fragment Length (bp)	Annealing Temperature
5R	5'	ACC CTG GGC AAC CAG CCC TG	3'	6128.0	287	59.8 ºC
5L	5'	TTT ATC TGT TCA CTT GTG CCC	3'	6409.2		50.3 ºC
6R	5'	CTC CCA GAG ACC CCA GTT GC	3'	6103.0	236	57.8 ºC
6L	5'	TCA GAT AGC GAT GTG AGC AG	3'	6286.1		60.4 ºC
7R	5'	CAG TGT GCA GGG TGG CAA GT	3'	6318.1	196	55.8 ºC
7L	5'	GCC ACA GGT CTC CCC AAG GC	3'	6128.0		59.8 ºC
8R	5'	CCA CCG CTT CTT GTC CTG CT	3'	6051.0	213	55.8 ºC
8L	5'	GAC CTG ATT TCC TTA CTG CC	3'	6099.0		60.4 ºC

Table 2. The Correlations Between Clinicopathologic Findings and p53 Genetic Alternations (N = 63)

Factors	No. of patients	p53 Alternations		P-values*
	(%)	Positive (%)	Negative (%)	
Age (years)				
< 65	33 (52.4)	26 (78.8)	7 (21.2)	0.305
≥ 65	30 (47.6)	21 (70)	9 (30)	
T-Status				
T1	9 (14.3)	5 (55.6)	4 (44.4)	0.416
T2	9 (14.3)	8 (88.9)	1 (11.1)	
T3	35 (55.6)	26 (74.3)	9 (25.7)	
T4	10 (15.9)	8 (80)	2 (20)	
N-Status				
N0	22 (34.9)	16 (72.7)	6 (27.3)	0.802
N1	41 (65.1)	31 (75.6)	10 (24.4)	
M-Status				
M0	44 (69.8)	33 (75)	11 (25)	0.912
M1	19 (30.2)	14 (73.7)	5 (26.3)	
Tumor Stages				
I	6 (9.5)	4 (66.7)	2 (33.3)	0.939
II	19 (30.2)	15 (78.9)	4 (21.1)	
III	19 (30.2)	14 (73.7)	5 (26.3)	
IV	19 (30.2)	14 (73.7)	5 (26.3)	
Cell Differentiation				
Well	8 (12.7)	5 (62.5)	3 (37.5)	0.296
Moderate	45 (71.4)	36 (80)	9 (20)	
Poor	10 (15.9)	6 (60)	4 (40)	
Lymphovascular invasion				
Negative	48 (76.2)	38 (79.2)	10 (20.8)	0.137
Positive	15 (23.8)	9 (60)	6 (40)	

*Chi-square (χ^2) test with Fisher's exact test was used to test the frequency distribution of p53 alternations. Statistical significance was assumed if P-value < 0.05.

cases (14.3%); T3, 35 cases (55.6%); and T4, 10 cases (15.9%). Forty-one patients (65.1%) were found to have lymph node metastasis, and 19 patients (30.2%) were associated with distant lymph node metastasis or nodal M1 lesion. Thus, the distribution of tumor stages was 6 in stage I (9.5%), 19 in stage II (30.2%), 19 in stage III (30.2%), and 19 in stage IV (30.2%).

2. p53 Genetic Alternations

An electrophoretic mobility shift between the tumor or nontumor esophageal specimens and their paired normal tissues (internal control) is characteristic of a genetic alternation. Figure 1 shows examples of mutants showing altered mobility in cold PCR-SSCP of p53 exon 7. In total, p53 alternations were observed in 47 (74.6%) of 63 tumors, and in 35 (55.5%) of 63 nontumorous mucosae. As shown in Table 3, p53 genetic alternations in tumorous specimens occurred most frequently at exon 6 (50.8%), followed by exon 7 (33.3%), exon 8 (17.5%) and exon 5 (12.7%). Similar to the tumorous results, the most frequent p53 genetic alternation in nontumorous mucosal specimens was located at exon 6 (41.3%), followed by exon 7 (19.0%), exon 8 (11.1%) and exon 5 (3.2%). Multiple genetic alternations (≥ 2 exons) between p53 5-8 exons in the same examined sample occurred in 21 (33.3%) of 63 tumors and in 8 (12.7%) of 63 nontumor mucosal specimens.

3. Correlation Between p53 Alternations and Clinical Data and Survival

In the present series, tumorous p53 alternations did not have a positive correlation with age (P = 0.305), depth of tumor invasion (P = 0.416), lymph node metastasis (P = 0.802), tumor stages (P = 0.939), cell differentiation (P = 0.296) or microscopic evidence of lymphovascular invasion (P = 0.137), respectively (Table 2). In addition, there was no difference in the survival rate between patients with and without p53 genetic alternations in ESC (P > 0.1) (Fig.2). The overall survival rate was 66% at one year, 48% at 3 years, and 28% at 5 years in the present series.

IV. Discussion

Much attention has recently been given to the role of the p53 gene in tumorigenesis, where it is believed to function as a tumor suppressor gene (Hollstein *et al.*, 1991; Hartwell, 1992; Kastan *et al.*, 1991; Livingstone *et al.*, 1992; Yin *et al.*, 1992). The p53 gene, located at 17p13, encodes a 53-kilodalton nuclear phosphoprotein. The majority of p53 mutations occur in the core domain, which contains the sequence-specific DNA binding activity of the p53 protein (residues 102-292), and they result in loss of DNA binding (Ostrowski *et al.*, 1994). The point mutations of p53 gene alternations can be clearly evaluated by SSCP analysis. The "cold" SSCP technique can rapidly and sensitively identify mutant SSCP bands under constant, optimized temperature conditions. This protocol is convenient and can usually be completed in less than 2.5 hours. However, precise and constant temperature control during electrophoresis is critical for consistent detection of SSCP bands. The optimum buffer temperature for SSCP analysis is defined as the temperature at which the maximum number of actual mutations is revealed by discernible mobility shifts. The optimum buffer temperature may vary greatly with individual products. The majority usually fall within the range of 10~20 ºC. Initial SSCP runs may be conducted over a series of temperatures with 2~5 ºC increments within this range in order to determinine the ideal temperature. In the present study, we found the optimum temperature for "cold" SSCP analysis was 14 ºC while the PCR products were around 200~300 bp in size.

In one of our preliminary studies, we performed SSCP analysis at 8 ºC and found a relatively low rate of p53 genetic alternation (35%, 14/40 cases) in ESC.

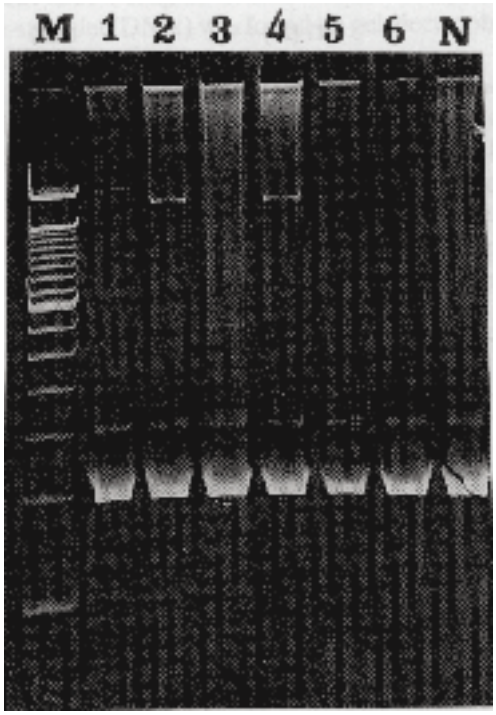

Fig. 1. Examples of p53 exon 7 fragments run on gradient polyacrylamide TBE gel at 200 volts for 2 hours and stained with ethidium bromide. The left lane (M-lane) was the φ X 174 Hae III-digest molecular weight marker. Lanes 1-5 were the tumorous samples with p53 mutant bands, and lane 6 was the tumorous sample without a p53 mutant band. The right land (N-lane) was used as a negative control.

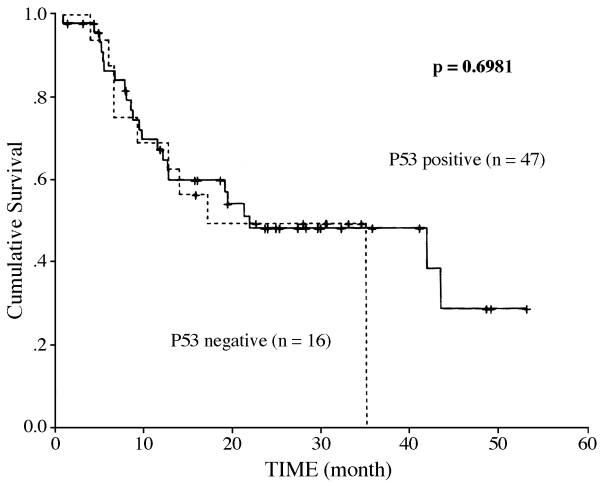

Fig. 2. The cumulative survival rates for patients with and without p53 genetic alternations in esophageal carcinoma. No survival difference was found between these two groups of patients (P > 0.1).

Table 3. The Frequencies of p53 Genetic Alternations Detected by Means of PCR-"Cold" SSCP in Esophageal Squamous Cell Carcinoma (N = 63)

	p53 Exons 5-8								p53 Genetic Alternations		
	Exon 5		Exon 6		Exon 7		Exon 8		Negative	Single exons (+)	Multiple exons (+)
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)			
No. of Cases	55	8	31	32	42	21	52	11	16	26	21
(%)	87.3	12.7	49.2	50.8	66.7	33.3	82.5	17.5	25.4	41.3	33.3
											(74.6)

Additionally, the p53 alternation rates for each of exons 5-8 at 8 ºC were 12.5% (5/40 tumors), 10% (4/40 tumors), 5% (2/40 tumors) and 15% (6/40 tumors), respectively. When the controlled temperature was increased from 8 ºC to 14 ºC, the overall rate of p53 genetic alternation increased to 74.6%. Changes in the rate were particularly obvious at exons 6 (50.8%, at 14 ºC) and 7 (33.3%, at 14 ºC).

Recently, it has been found that there are several mechanisms are involved in inactivation of the p53 tumor suppressor gene product in cancers. The first and most common in human cancers is occurs through mutation. The second occurs via the viral encoded oncogene products. The third way occurs via amplification of the mdm-2 gene. A fourth mechanism occurs due to the abnormal location of the p53 protein in the cytoplasm. Because p53 is a transcription factor, localizing this protein in the cell cytoplasm will not permit it to function as a tumor suppressor gene (Ostrowski *et al*., 1991). In addition to a high frequency of p53 alternation in ESC, our results also showed a rather high incidence (55.5%) of p53 genetic alternation in the nontumorous mucosal of the esophagus. Similarly, we found a relatively high incidence (43.7%) of aneuploid DNA contents in our previous DNA flow cytometric analysis of 119 nontumor mucosal specimens (Wang *et al*., 1997). This evidence may hint that p53 or other genetic mutations may precede invasion in esophageal carcinogenesis, and that multifocal esophageal neoplasms may arise from independent clones of transformed cells (Bennett *et al*., 1992).

The prognostic value of p53 genetic alternations has been evaluated recently with controversial results. Some groups have proposed p53 alternations as a significant prognostic indicator in ESC (Wang *et al*., 1994; Maesawa *et al*., 1994; Monges *et al*., 1996; Muro *et al*., 1996; Ohashi *et al*., 1997). However, several other series have suggested that p53 dysfunction has no impact on the prognosis for ESC (Cho *et al*., 1994; Coggi *et al*., 1997; Vijeyasingam *et al*., 1994; Hardwick *et al*., 1997). In the current study, no association was found between p53 genetic alternations and clinicopathologic variables, including TNM factors, tumor stage, cell differentiation, and lymphovascular invasion. Moreover, our results also support the idea that p53 alternations do not correlate with the survival rate. Therefore, p53 protein dysfunction cannot be used as a prognostic indicator to guide patient management.

In conclusion, PCR with "cold" SSCP analysis is a rapid and sensitive method for identifying p53 genetic alternations for ESC. The proper temperature for "cold" SSCP analysis seems to be 14 ºC . Although p53 genetic alternations may occur with a rather high rate of incidence, expression of p53 abnormalities is probably not a useful biomarker for prognostic prediction, at least for patients with resectable tumors.

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利用低溫PCR-SSCP方法分析食道類上皮癌的p53基因突變

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摘 要

p53基因突變在人類惡性腫瘤的發生上扮演一個十分重要的角色，文獻的報告：食道癌發生p53基因不正常的機率有26%~87%的不同情況。此外，p53基因不正常在臨床上的意義也存在許多的爭議。在本研究，我們利用PCR-cold SSCP的方法來分析63例食道癌切除標本上，腫瘤及非腫瘤食道黏膜發生p53基因不正常的情形，而本實驗所作的毯old□SSCP分析中，我們的早期結果發現：在食道癌的DNA PCR的產物之大小為200~300 bp時，其最適當的"冷"緩衝液的溫度為14劍。在14 劍下利用SSCP方法分析食道癌p53基因突變，我們的結果顯示： 在63個腫瘤中有47個(74.6%)腫瘤標本上有p53基因不正常的現象，而在非腫瘤的食道黏膜亦可高達55.5%（35/63食道黏膜標本）；而在p53基因的Exons 5-8中，以Exon 6發生不正常的機會為最高(50.8%)，其次依序為Exon 7 (33.3%)、 Exon 8 (17.5%)及Exon 5 (12.7%)。在同一檢體中發現有21個腫瘤同時有二個或以上的Exons發生不正常的現象，佔33.3%；在非腫瘤黏膜則有8例，佔12.7%。在與臨床病理的關係中，p 5 3 基因的不正常和病人的年齡、腫瘤大小或侵犯的深度、腫瘤期、細胞分化程度及腫瘤內淋巴或血管侵犯現象，在統計上都沒有關係(P > 0.05)。此外，在腫瘤上有無p53基因的突變和病人手術等治療後的活存預後並無關係(P > 0.05)。

總之，PCR-毯old紱SCP是一個快速且敏感的方法，可用來偵測腫瘤的p53基因不正常。台灣地區食道癌發生p53基因不正常機率相當的高，但p53基因不正常無法用為預測食道癌病人的活存預後。