Risk of *p53* gene mutation in esophageal squamous cell carcinoma and habit of betel quid chewing in Taiwanese

Yih-Gang Goan,[1,2,3] Huang-Chou Chang,[1] Hon-Ki Hsu,[1] Yi-Pin Chou[1] and Jiin-Tsuey Cheng[3,4]

[1]Department of Surgery, Kaohsiung Veterans General Hospital, Taiwan, Republic of China, [2]Department of Surgery, National Yang-Ming University, Taipei, Taiwan, Republic of China, and [3]Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan, Republic of China

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A recent report suggested that BQ (BQ) chewing significantly correlated with the occurrence of esophageal squamous cell carcinoma (ESCC) in Taiwanese. BQ chewing was shown to be associated with *p53* mutation in oral cancers. However, the relationship between BQ chewing and *p53* mutation in ESCC is unclear. Seventy-five primary ESCC patients were enrolled for mutational analysis of the *p53* gene using polymerase chain amplification and direct sequencing of amplified product. Thirty-seven mutations of the *p53* gene were detected in 45.5% (34/75) of tumor specimens. These mutations significantly clustered in exon 5 (21/37) of the *p53* gene. The incidence of *p53* mutations did not associate with clinicopathological characteristics or the habits of cigarette smoking or alcohol consumption. However, BQ chewers exhibited significantly higher incidence of *p53* gene mutations than non-chewers (67.6% vs 32.4%, *P* = 0.007). After controlling the confounding factors of cigarette smoking and alcohol intake, BQ chewing still showed significant association with the incidence of *p53* mutation in ESCCs (RR = 4.23; 95% CI, 1.317–13.60). The A:T to G:C transition (8/37, 21.6%) and G:C to T:A transversion (5/23, 13.5%) were the prevalent spectrum of *p53* gene mutations. All A:T to G:C transitional mutations occurred in patients with the habits of BQ chewing and cigarette smoking. Noticeably, alcohol consumption could enhance this peculiar spectrum of *p53* mutation in ESCC. Accordingly, *p53* might be an important molecular target of BQ carcinogens in the development of ESCC in Taiwanese. (*Cancer Sci* 2005; 96: 758–765)

Esophageal cancer is a serious malignancy. Its overall 5-year survival rate is less than 20%.[1] The incidence of esophageal cancer varies considerably between geographic regions or ethnic groups in a particular area.[2] Results of epidemiological analysis demonstrated that alcohol intake and tobacco use were the major risk factors for ESCC (ESCC) in Western countries.[2,3] However, carcinogens in local diets also have been suggested as playing an important role in the development of ESCC in some high-risk areas. In Taiwan, ESCC is the ninth leading cause of cancer deaths.[4] Besides alcohol and tobacco, betel quid (BQ) has recently been noticed as a major risk factor for developing ESCC in Taiwan. Epidemiological reports from Taiwan, India and Thailand, where BQ chewing is a common addiction, demonstrated that BQ chewing would increase the risk of developing esophageal cancers by 4.7- to 13.3-fold.[5–8] The etiological studies in Taiwan have quantified the risks of esophageal cancer associated with BQ chewing and the consumption of alcohol and tobacco. Those results showed that patients who drank high doses of alcohol (> 40 g/day), smoked tobacco over a long period (> 30 years) and chewed more than 20 BQs per day will significantly increase their risk of occurrence of ESCCs.[5,6] The habit of BQ consumption could act synergistically with tobacco and alcohol use to increase individual susceptibility to esophageal cancer and increase the risk of developing this cancer.[7,9,10]

Previous molecular studies suggested the genetic alteration of *p53* is a significant determining factor in the carcinogenesis of a variety of human cancers.[11–18] The mutational spectrum of the *p53* gene was also thought to be useful information in predicting the type of carcinogen involved in causing a particular type of tumor.[19] Worldwide, alcohol and tobacco are well-known factors contributing to the induction of *p53* gene mutation in ESCC.[20,21] However, dietary carcinogens were also reported as causal factors inducing *p53* mutation in ESCC in some high-risk areas such as China and Southern Brazil.[11,17] Although the prevalence of *p53* mutations in ESCC tissues is well documented in some high-risk regions, this important information regarding *p53* mutation in the etiology of ESCC is still lacking in Taiwan. In addition, BQ chewing has been reported to correlate with the incidence of *p53* gene mutations in human oral cancers.[22] The correlations between the incidences of *p53* gene mutations in ESCC with the habit of BQ chewing have not been rigorously studied in Taiwan.

Furthermore, the composition of BQ used in Taiwan includes areca (betel nut), slaked lime, *Piper betle* inflorescence and catechu. This differs from the combinations used in other BQ chewing prevalent countries of southern Asia, where tobacco is usually included but fresh *Piper betle* inflorescence and tender areca nut are not.[23] Therefore, the BQ-related mechanism associated with the incidence and spectrum of *p53* mutation in ESCC in Taiwan might differ from other countries and it remains as an interesting area worthy of intensive investigation.

[4]To whom correspondence should be addressed. E-mail: tusya@mail.nsysu.edu.tw
Abbreviations: BQ, betel quid; ESCC, esophageal squamous cell carcinoma; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SSCP, single-strand conformational polymorphism.

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In this study, specimens from a cohort of surgically-treated ESCC patients were collected to characterize their *p53* gene status and evaluate the risk potential of BQ, cigarette smoking and alcohol consumption in the incidence and spectrum of *p53* mutation in ESCC in Taiwanese.

Materials and Methods

Patients and tissue preparation

Between January 1998 and February 2003, 75 primary ESCC tissue samples were collected from 75 consecutive patients who underwent radical esophagectomy at the Department of Thoracic Surgery at Kaohsiung Veterans General Hospital, Taiwan, China. All patients gave informed consent before surgery and none of them received radiation or chemotherapy before surgery. Of the samples taken, snap frozen tissues were available for testing in 40 patients and paraffin-embedded tumor tissues were collected for testing in 35 patients who did not have adequate or available fresh tissue samples for testing. The fresh tumor tissue samples were dissected from the main tumor part of each surgically removed specimen in the operating room, frozen immediately in liquid nitrogen, then stored at −80°C for future analysis. The remaining parts of the specimens were then fixed in formalin solution and embedded in paraffin block for pathological diagnosis. The paraffin-embedded tumor tissues were collected retrospectively from the archives of the Department of Pathology. Total RNA extracted from the frozen tissue samples were used for mutational analysis of the *p53* gene by reverse transcription polymerase chain reaction (RT-PCR). Genomic DNA extracted from the archival paraffin-embedded ESCC specimens were used for the mutational analysis of the *p53* gene by direct amplification and sequencing. The demographic and substance usage information of study subjects was obtained from the records collected by trained interviewers using a pretested semistructured questionnaire. Patients' data were available for age, gender, pathologic TNM stage, and habits of cigarette smoking, alcohol consumption and BQ chewing. The cigarette smokers were defined as regular consumers if their intake was more than 10 cigarettes per day for at least 6 months. Alcohol drinkers were defined as regular consumers if their intake was at least once a week for more than 6 months. Likewise, BQ chewers were defined as regular consumers of areca nut for more than 6 months. The tumor staging was classified according to the TNM system of American Joint Committee on Cancer (AJCC) classification.

Extraction of RNA and RT-PCR amplification

Total RNA was extracted from clinical specimens using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 3 μg of total RNA using 1 μL of random hexamer and the Advantage RT for PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA) in 25 μL of reaction volume. The cDNA encompassing exons 2–11 of *p53* was amplified in two PCR amplifications from 40 frozen specimens that yielded enough high-quality total RNA for the subsequent *p53* mutational analysis. The primer sequences were designed according to the published cDNA sequence of the *p53* gene.[24] For amplification of fragment 1 cDNA encompassing exons 2–4, forward primer 5′-CACGACGGTGACACGCTTCC and reverse primer 5′-CGTGCAACTC-ACAGACTTGG were used; for amplifying fragment 2 cDNA spanning exons 5–11, forward primer 5′-GCTACGGTTTCCGTCTGGGC and reverse primer 5′-TCAGTCTGAGTCAGGCCCTT were used. Three μl of first strand cDNA was mixed with 5 μL of 10× PCR buffer, 1 μL of Taq polymerase (Promega, Madison, WI, USA), 4 μL of 2.5 mM deoxynucleotide triphosphates (dNTP), 1.4 μL each of 10 μM forward and reverse primers. The mixture was first incubated at 94°C for 5 min. Thirty-five cycles of PCR reactions followed, with the conditions of denaturing at 94°C for 45 s; annealing at 57°C (exons 2–4) or 60°C (exons 5–11) for 1 min; and extension at 72°C for 1 min. The complete amplification procedure was carried out by further incubation at 72°C for 10 min.

Genomic DNA extraction and amplification

A commercial kit (DNeasy tissue kit; Qiagen, Santa Clarita, CA, USA) was used to extract genomic DNA from 35 paraffin-embedded ESCC tumor sections. The histologically confirmed ESCC area was dissected to ensure at least 80% of tumor cells in the preparation. Mutational analysis of exons 4–8 encoding the DNA binding domain of *p53* was performed. The coding region of each exon was individually amplified from extracted genomic DNA using the primer pairs as described previously.[25] The sequences of PCR primers used were: exon 4 forward primer, 5′-ATCTACAGTCCCCCTTGCCG; exon 4 reverse primer, 5′-GCAACTGACCGTGCAAGTCA; exon 5 forward primer, 5′-GCTGCCGTGTTCCAGTTGCT; exon 5 reverse primer, CCAGCCCTGTCGTCTCTCCA; exon 6 forward primer, 5′-GGCCTCTGATTCC-TCACTGA; exon 6 reverse primer, 5′-GCCACTGACAACCACCCTTA; exon 7 forward primer, 5′-TGCCACAGGTCTCCCCAAGG; exon 7 reverse primer, 5′-AGTGTGCAGGGTGGC-AAGTG; and exon 8 forward primer, 5′-CCTTACTGCCTCTTGCTTCT; exon 8 reverse primer, 5′-ATAACTGCACCCTTGGTCTC. The conditions of PCR amplifications were the same as those described above for cDNA amplification, except the annealing temperature was 55°C.

Direct sequencing

The PCR products were purified individually using Wizard SV gel and a PCR clean-up system (Promega), then sequenced at the sequencing laboratory of National Sun Yat-Sen University (Kaohsiung, Taiwan, China). Primers for PCR amplifications were used as the primers for sequencing both orientations of the corresponding PCR products. Mutations were analyzed using the Gene Blast system on the National Center for Biotechnology Information website. All detected mutations were doubly confirmed either with cDNA synthesized from another batch of RNA isolation, or another batch of genomic DNA extraction.

Statistical analysis

Fisher's exact test and Pearson's χ^2 test were used to test the associations between dichotomous categorical variables. Multiple logistic regression analysis was used to estimate the hazardous ratio of cigarette smoking, alcohol drinking and BQ chewing associating with the incidence of *p53* mutations. All statistical manipulations were performed with SPSS-10.0

for Windows software (SPSS, Chicago, IL, USA). All tests were analyzed two-sided, and a probability value less than 0.05 was considered to be statistically significant in this series.

Results

A total of 75 ESCC patients who underwent esophagectomies were enrolled in this study. The clinical characteristics of 71 male and 4 female patients are listed in Table 1. The average age of these patients at the time of esophagectomy was 60.7 years (ranging from 33 to 81 years). Advanced stage IIB, III and IV cancers were diagnosed in 61.3% (46/75) patients. The majority of ESCC patients were cigarette smokers (84%, $P < 0.001$) and alcohol drinkers (68.0%, $P = 0.004$) (Table 1).

Of the patients in this study, the percentage of BQ chewers and non-chewers was approximately equal (50.7% vs 49.3%, $P = 0.908$). Ten patients were non-smokers, non-drinkers and non-chewers. In the present series, ESCC patients with the habit of BQ chewing were significantly younger than patients who did not have the habit (mean 54.3 vs 67.1 years; $P < 0.001$). Patients with the habit of alcohol drinking were also significantly younger than non-drinkers (58.5 vs 64.8 years; $P = 0.033$). However, no significant age difference existed in patients with or without the habit of smoking (60.3 vs 62.3 years; $P = 0.608$) (Table 2).

A total of 37 mutations were found in 34 of 75 (45.3%) tested specimens. Mutational analysis within exons 2–11 of *p53* was performed in 40 of 75 (53%) ESCC tumors, which provided enough high-quality RNA for testing, and 20 (50%) of these cancers displayed mutation within exons 5–8. No mutation was detected outside exons 5–8 in 40 frozen ESCC specimens. Only exons 4–8 were analyzed for *p53* gene mutations in 35 patients using genomic DNA extracted from paraffin-embedded ESCC tumors, and 14 (40%) of these tissues displayed mutation within exons 5–8. Both methods provided a comparable mutation rate of the *p53* gene in our present work ($P = 0.385$). No significant correlation between the incidence of *p53* mutations and gender, pTNM stages, depth of tumor invasion, regional lymph-node involvement, smoking status or alcohol consumption could be found in our series. Surprisingly, BQ chewers had a significantly higher incidence of *p53* mutations than non-chewers ($P < 0.001$) (Table 1).

Due to the small number of female patients ($n = 4$) and all of them being non-smokers, non-drinkers and non-BQ-chewers, they were excluded from further statistical analysis. In logistic regression analysis, no significant associations between the incidence of *p53* mutation and well-known lifestyle risk factors such as cigarette smoking (relative risk [RR]: 1.615; 95% confidence interval [CI]: 0.355–7.399) and alcohol consumption (RR: 1.015; 95% CI: 0.366–2.817) could be detected in the present study. However, ESCC patients who had the habit of BQ chewing had a significantly higher risk of *p53* mutation (RR: 3.066; 95% CI: 1.159–8.114). As almost all ESCC patients harboring *p53* mutations had a cigarette

Table 1. Clinicopathologic parameters of esophageal squamous cell carcinoma (ESCC) patients and the correlation with *p53* gene mutation in ESCC samples

Characteristic	Patients	*P*	P53 gene mutation (*N* = 75)		*P*
	Total *N* = 75 (%)		Positive *N* = 34 (%)	Negative *N* = 41 (%)	
Gender					*P = 0.121*
Male	71 (94.7)		34 (100)	37 (90.2)	
Female	4 (5.3)		0	4 (9.8)	
Stage					*P = 0.959*
I	6 (8.0)		2 (5.9)	4 (9.8)	
IIA	23 (30.7)		10 (29.4)	13 (31.7)	
IIB	17 (22.7)		8 (23.5)	9 (22.0)	
III	27 (36.0)		13 (38.2)	14 (34.1)	
IV	2 (2.7)		1 (2.9)	1 (2.4)	
Depth of invasion					*P = 0.927*
T1	8 (10.7)		3 (8.8)	5 (12.2)	
T2	28 (37.3)		13 (38.2)	15 (36.6)	
T3	37 (49.3)		17 (50.0)	20 (48.8)	
T4	2 (2.7)		1 (2.9)	1 (2.4)	
Node involvement					*P = 0.585*
Absence	29 (38.7)		12 (35.3)	17 (41.5)	
Presence	46 (61.3)		22 (64.7)	24 (58.5)	
Cigarette		*P < 0.001*			*P = 0.123*
Smoker	63 (84.0)		31 (91.2)	32 (78.0)	
Non-smoker	12 (16.0)		3 (8.8)	9 (22.0)	
Alcohol		*P = 0.004*			*P = 0.512*
Drinker	50 (66.7)		24 (70.6)	26 (63.4)	
Non-drinker	25 (33.3)		10 (29.4)	15 (36.6)	
Betel quid chewing		*P = 0.908*			*P = 0.007*
Chewer	38 (50.7)		23 (67.6)	15 (36.6)	
Non-chewer	37 (49.3)		11 (32.4)	26 (63.4)	

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Table 2. Clinical characteristics of patients with esophageal squamous cell carcinoma and their lifestyle habits (cigarette smoking, alcohol drinking and betel quid [BQ] chewing)

Characteristic	Cigarettes		*P*	Alcohol		*P*	BQ		*P*
	smoker	non-smoker		drinker	non-drinker		chewer	non-chewer	
Gender (*n*)			*P* < 0.001*			*P* = 0.01*			*P* = 0.054*
Male	63	8		50	21		38	33	
Female	0	4		0	4		0	4	
Age			*NS*			*P* = 0.033			*P < 0.001*
Mean (years)	60.3	62.3		58.5	64.8		54.3	67.1	
Stage (*n*)			*NS*			*NS*			*NS*
I	5	1		3	3		2	4	
IIA	17	6		14	9		10	13	
IIB	15	2		10	7		8	9	
III	24	3		21	6		13	14	
IV	2	0		2	0		1	1	

*Fisher's exact test; *NS*, not significant.

Table 3. Logistic regression analysis of cigarette smoking, alcohol drinking and betel quid (BQ) chewing with incidence of *p53* mutations in male patients with esophageal squamous cell carcinoma

Potential risk factor	Relative risk	95% CI	*P*
BQ chewing	3.066	1.159–8.114	0.024
Cigarette smoking	1.615	0.355–7.399	0.535
Alcohol drinking	1.015	0.366–2.817	0.977
Cigarette smoking†			
BQ chewing	3.014	1.118–8.129	0.029
Alcohol drinking†			
BQ chewing	4.286	1.338–13.731	0.014
Alcohol drinking†			
Cigarette smoking†			
BQ chewing	4.233	1.317–13.603	0.015
BQ chewing†			
Alcohol drinking†			
Cigarette smoking	1.555	0.304–7.956	0.596
Cigarette smoking†			
BQ chewing†			
Alcohol drinking	0.430	0.115–1.610	0.210

†Controlled factors in logistic regression analysis; CI, confidence interval.

smoking habit, it was difficult to evaluate the independent interacting effect of cigarette smoking, alcohol drinking and BQ chewing on the incidence of *p53* mutations. However, after controlling the confounding influences of smoking and alcohol drinking, the effect of BQ on the incidence of *p53* mutation remained statistically significant (RR: 4.233; 95% CI: 1.317–13.603). Noticeably, alcohol drinking would enhance the risk potential of BQ chewing on the incidence of *p53* mutation (RR: 4.286; 95% CI: 1.338–13.731; Table 3).

The identified mutation spectrums of our ESCC patients are summarized in Table 4. Only one ESCC patient who displayed mutation in the *p53* gene was a non-smoker, non-drinker and non-chewer in our present series. The exons 5–8 of the *p53* gene were the frequent sites of mutations observed in ESCC tumor tissues in the present series. No mutation was detected outside exons 5–8 in any ESCC tumors. All the detected mutations in this series were point mutations and most of them were missense mutations except for four silent mutations. There were 32 tumors that exhibited a single mutation, one cancer had two distinct mutations, and one cancer had three mutations in the *p53* gene. More than one sample harbored mutation in codons 131 (4 specimens), 150 (2 specimens), 151 (2 specimens) and 193 (2 specimens).

Among these 37 point mutations, 56.7% (21/37) were transversion mutations and 43.2% (16/37) were transition mutations. The A:T to G:C transitions (21.6%) and G:C to T:A transversion mutations (16.2%) were the two common types of nucleotide substitutions in our present study (Table 5). The A:T to G:C and A:T to T:A mutations were predominantly observed in patients who were BQ chewers and cigarette smokers. Interestingly, alcohol consumption seems to have the potential to enhance the incidence of A:T to G:C transition in patients with the habits of BQ chewing and tobacco usage. The majority of G:C to T:A transversions (5/6) were detected in patients with a smoking habit (Table 6).

Discussion

The *p53* gene is a multifunctional tumor suppressor gene. It is often referred to as 'the guardian' of the human genome.[26] It was also suggested to be the prime molecular target of various carcinogens.[12,20,27] In the screening of *p53* gene mutations, PCR-SSCP is the commonly adopted method. Exons 5–8, the DNA binding region of the *p53* gene, were the usual sites detected by this method. Consequently, mutations outside these target regions will be missed using PCR-SSCP.[13] Alternatively, RT-PCR with subsequent cDNA sequencing also has been used for detecting *p53* gene mutations in human tumors[13,28] and was suggested as a method with comparable results as the SSCP analysis in detecting *p53* mutation.[13] In our present series, 20 of 40 (50%) tumors using RT-PCR with subsequent cDNA sequencing and 14 of 35 (40%) tissues using direct sequencing of amplified PCR products of genomic DNA displayed mutations within exons 5–8 of the *p53* gene. The incidences of *p53* gene mutation detected by both methods also were not significantly different in our present series.

The overall mutation frequency of the *p53* gene was 45.3% in our present series, which is lower than that of some

Table 4. Characteristics of *p53* mutations in esophageal squamous cell carcinoma

ID	Smoking	Drinking	Betel	*P53* mutation			
				Exon/codon	Nucleotide mutation	Base change	Amino acid change
1	+	+	–	5/128	CCT→CTT	C→T	Pro→Leu
2	+	+	+	5/131	AAC→AGT	AC→GT	Asn→Ser
3	+	–	+	5/131	AAC→AGC	A→G	Asn→Ser
4	+	+	+	5/131	AAC→AGC	A→G	Asn→Ser
5	–	+	+	5/131	AAC→AAG	C→G	Asn→Lys
				5/151	CCC→GCC	C→G	Pro→Ala
				8/275	TGT→TGC	T→C	Cys→Cys
6	+	–	+	5/133	ATG→AAG	T→A	Met→Lys
7	+	+	+	5/142	CCT→CCC	T→C	Pro→Pro
8	+	+	+	5/150	ACA→AAC	CA→AC	Thr→Asn
9	+	+	+	5/150	ACA→ACT	A→T	Thr→Thr
10	+	+	–	5/151	CCC→CAC	C→A	Pro→His
11	+	–	–	5/151	CCC→ACC	C→A	Pro→Thr
				5/153	CCC→GCC	C→G	Pro→Ala
12	+	–	–	5/154	GGC→GTC	G→T	Gly→Val
13	+	+	+	5/159	GCC→TCC	G→T	Ala→Ser
14	–	–	–	5/161	GCC→GGC	C→G	Ala→Gly
15	+	+	+	5/175	CGC→CAC	G→A	Arg→His
16	+	+	+	5/179	CAT→CTT	A→T	His→Leu
17	+	+	+	5/184	AGA→AAA	A→T	Arg→Lys
18	+	+	+	5/185	AGC→AGA	C→A	Ser→Arg
19	+	–	–	5/186	GAT→AAT	G→A	Asp→Asn
20	+	+	+	6/193	CAT→CGT	A→G	His→Arg
21	+	+	+	6/193	CAT→CCT	A→C	His→Pro
22	+	–	+	6/203	CAT→GAT	C→G	Asp→Asp
23	+	+	+	6/210	AAC→AGC	A→G	Asn→Ser
24	+	+	+	7/237	ATG→GTG	A→G	Met→Val
25	+	+	–	7/238	TGT→TTT	G→T	Cys→phe
26	+	+	+	7/239	AAC→GAC	A→G	Asn→Ser
27	+	+	+	7/241	TCC→TTC	C→T	Ser→Phe
28	+	+	–	7/242	TGC→TAC	G→A	Cys→Tyr
29	+	+	+	7/249	AGG→AGT	G→T	Arg→Ser
30	+	–	+	7/256	ACA→GCA	A→G	Thr→Ala
31	+	–	–	7/259	GAC→TAC	G→T	Asp→Tyr
32	+	–	–	8/266	GGA→GAA	G→A	Gly→Glu
33	–	+	+	8/273	CGT→CTT	G→T	Arg→Leu
34	+	+	–	8/294	GAG→GCG	A→C	Glu→Ala

Table 5. Prevalence of base pair alterations in *p53* mutations in patients with esophageal squamous cell carcinoma

Base pair alteration	Incidence (*n* = 37)
Transition mutation	43.2%
A:T→G:C	8
T:A→C:G	2
C:G→T:A	2
G:C→A:T	4
Transversion mutation	56.7%
G:C→T:A	6
C:G→G:C	5
C:G→A:T	4
A:T→T:A	3
A:T→C:G	2
T:A→A:T	1

well-documented high-risk areas of ESCC such as northern Europe,[18] the mainland of China[29] and northern Iran.[30] However, it is comparable with the results of Thailand[15,16] and India[31] where BQ chewing is also a major addiction. These different incidences of *p53* gene mutation indicate that different risk factors might be involved in the development of ESCC in these areas. In our present work, the majority of ESCC patients were cigarette smokers and drinkers of alcohol. BQ chewers and non-chewers were distributed equally in our patients. These observations are consistent with the results of epidemiological studies suggesting that consumption of tobacco and alcohol are cumulative risk factors for ESCC[6,32] and BQ chewing adds a limited risk to smoking and drinking habits for contracting esophageal cancer.[6] However, when considering the effect on the *p53* mutation profile, BQ chewing provides an obviously significant risk for the occurrence of *p53* mutation in ESCCs (RR = 4.233; 95% CI, 1.317–13.603) in our work. Even after controlling the confounding influences of smoking and alcohol, BQ chewing remained significant in its effect on the incidence of *p53* mutation in ESCCs. These results indicate that carcinogens of BQ might associate with the occurrence of *p53* mutation in ESCC. Consequently, the correlation with the incidence of *p53* mutation would be an important BQ-related risk of

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Table 6. Correlation of *p53* mutational spectrum with cigarette smoking, alcohol drinking and betel quid chewing in 71 male esophageal squamous cell carcinoma patients

Smoking	Drinking	Betel	†*P53* mutation +/–	G→T	G→A	C→A	C→T	C→G	A→G	A→T	A→C	T→C	T→A	Total number of mutations
+	+	+	17/15	2	1	2	1	0	6	3	1	1	0	17
+	–	+	4/0	0	0	0	0	1	2	0	0	0	1	4
+	+	–	5/11	1	1	1	1	0	0	0	1	0	0	5
+	–	–	5/6	2	2	1	0	1	0	0	0	0	0	6‡
–	+	+	2/0	1	0	0	0	2	0	0	0	1	0	4§
–	–	–	1/9	0	0	0	0	1	0	0	0	0	0	1
Total			34/41	6	4	4	2	5	8	3	2	2	1	37

†*p53* mutation +/–: number of ESCC patients with/without *p53* gene mutation; ‡two mutations detected in one patient; §both patients had two mutations.

developing ESCC in Taiwanese. Noticeably, our data also revealed that alcohol consumption could enhance the effect of BQ chewing on *p53* mutation in ESCC (RR = 4.286; 95% CI, 1.338–13.731). This finding was concordant with the results obtained from the studies of oral squamous cell carcinomas,[22,33] but in contrast to those analyzing results of head and neck tumors.[34] These discrepant results might also indicate that BQ carcinogens may exert differential interacting effects on tumors at distinct anatomic sites.

More than 18 585 somatic mutations of the *p53* gene have been found in human cancers worldwide.[35,36] Of these, approximately 560 mutations were identified in the coding region of the *p53* gene in human esophageal cancers.[20] Transitions or transversions at A:T base pairs and transitions of C:G to T:A or G:C to A:T are the predominant types of mutation spectrum of the *p53* gene in squamous cell carcinomas of the esophagus.[37] In our study, approximately 56.8% (21/37) of mutations occurred in exon 5 and almost all detected mutations (33/37) were of the missense type. No mutation outside exons 5–8 was detected in our series either by cDNA sequencing or direct sequencing of genomic DNA. Alterations at the A:T base pair ($n = 16$), C:G to G:C transversion ($n = 5$), and G:C to T:A or A:T mutations ($n = 10$) were the prevalent types of *p53* mutation in our present work.

Transversions of G:C to T:A and G:C to A:T transitions occurred in 27% of detected mutations in our patients. Almost all of G:C to T:A and G:C to A:T mutations were detected in patients with a history of cigarette smoking with or without the habits of alcohol consumption or BQ chewing. This finding is consistent with the frequent detection of G:C to T:A transversions and G:C to A:T transitions in cancers exposed to carcinogens such as benzo(a)pyrene, the representative chemical of polycyclic aromatic hydrocarbons (PAH) and nitrosamine in tobacco smoke.[32] The PAH in cigarettes could be activated as nucleophilic species, which form quanine adducts in DNA that eventually resolved predominantly G:C to T:A transversions. The nitrosamine in cigarette smoke was reported to be the culprit for G:C to A:T mutations, due to its capability of inducing nucleotide methylation or deamination.[38]

Mutations at the A:T base pair was suggested resulting from exposure to DNA-reactive agents such as acetaldehyde, a metabolite of ethanol.[32] Alterations at the A:T base pair were found in 43.2% (16/37) of all detected mutations in this series, which was higher than that reported in the complied International Agency for Research on Cancer (IARC) data base (21.5%) but comparable with those results reported from the high-risk areas of Western Europe, such as Normandy and northern Italy (47%), where alcohol consumption is considered to be a major risk factor for the development of esophageal cancer. Alcohol is well accepted as the major risk factor for A:T base pair mutations of the *p53* gene in esophageal cancer in these regions.[32] However, in our present work, approximately 94% (15/16) of ESCC patients detected with A:T base pair alterations in tumor tissues were BQ chewers and only 81% (13/16) of them had the habit of alcohol drinking. In addition, even though the overall incidence of A:T base pair alteration was comparable with the results from high-risk areas of Western Europe, more than 57% (8/14) of A:T base pair mutations were A:T to G:C transitions, which was significantly higher than the result (16%) of Western countries, such as France and Germany.[18,21,39] Furthermore, recent results of *p53* mutation profiles from an ESCC high-incidence area of India showed that all of their A:T base pair alterations were detected in ESCC specimens obtained from patients without a history of alcohol consumption, and A:T to G:C transition was the only type of alteration at the A:T base pair.[40] Accordingly, carcinogens other than alcohol might be involved in the occurrence of A:T base pair alteration in ESCC in Taiwanese.

All A:T to G:C transitions found in our study were detected in the ESCC specimens obtained from patients who were BQ chewers and cigarette smokers, with or without alcohol drinking. This is consistent with the results of oral cancer studies, which also showed that all A to G mutations were detected in patients with the habits of BQ chewing and tobacco use.[22] These results imply that BQ chewing might play a special role in alterations at the A:T base pair, especially for the peculiar type of A to G mutation evident in cases of ESCC in Taiwan. The mechanisms behind the BQ-related ESCC in Taiwanese also might be different from other countries where BQ chewing is prevalent, such as India, due to the peculiar combination of BQ contents that contain areca (betel nut), slaked lime, catechu, as well as *Piper betle* leaves. The areca nut contains abundant alkaloids such as arecoline, arecaidine, arecolidine, guvacoline and guacine.[41] These alkaloids could undergo nitrosation to produce a variety of BQ-specific nitrosamines that interact with DNA to form adducts. In combination with the highly

susceptible character of the esophagus, these tumor-initiating nitrosamines could exert their carcinogenic effects on esophageal cells and transform them into neoplasms.[42–44] The mutation of A:T to G:C transition usually resulted from the attack of reactive oxygen species (ROS) induced by environmental mutagens either by deaminating adenine to form hypoxanthine or directly incorporating into DNA.[45–47] In BQ, the slaked lime could generate mutagenic ROS such as nitric oxide, nitrogen oxide, hydroxyl radicals or H_2O_2 by causing chronic inflammation in the submucosal area.[48] The nitrogen oxide could deaminate adenine to form hypoxanthine, leading to the formation of A to G mutation. The calcium hydroxide content of lime could also form ROS that might cause oxidative damage in cellular DNA.[48] The safrole, which is abundant in *Piper betle* inflorescence, could also cause cytogenetic insult through covalently binding to adenine residue to form a stable safrole–DNA adduct in the esophageal mucosa that could contribute to the A to G mutation.[49,50] Despite the fact that G:C to A:T and G:C to T:A mutations of the *p53* gene were the commonly detected mutation spectrum in oral cancers associated with BQ chewing and cigarette smoking in Taiwanese,[22] alterations at the A:T base pair were the prevalent types of mutations encountered in ESCC in our series, which is consistent with the results from India.[48] The dissimilar results between ESCC and oral cancer could be explained by the hypothesis that BQ might induce oral cancers by direct contact, while a systematic mechanism might also be involved in the development of ESCC. This finding also might indicate that a complex carcinogenic mixture of BQ can have a different mutagenic impact on different anatomic sites in the digestive system.

In conclusion, to our best knowledge, this is the first reported mutation profile of the *p53* gene in ESCC in Taiwanese. Of course, our data are preliminary in that we have analyzed a relatively small number of patients. However, our findings remain impressive. Patients diagnosed with ESCC who were BQ chewers were significantly younger than those without the habit. BQ chewing was significantly associated with the incidence of *p53* mutation in ESCC in Taiwanese. Alcohol intake could strengthen the effect of BQ chewing on *p53* gene mutation. A prevalent mutation spectrum of A to G transition was noticed in Taiwanese ESCC tumors. The carcinogens of BQ might be important risk factors contributing to this peculiar mutational spectrum. The awareness of the risk of BQ on *p53* mutation in ESCC might be helpful in the future exploration of the exact molecular mechanism of BQ carcinogens in the carcinogenesis of esophageal cancers.

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