OESOPHAGUS

Oesophageal squamous cell carcinoma may develop within a background of accumulating DNA methylation in normal and dysplastic mucosa

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Background: Oesophageal squamous cell carcinoma (OSCC) often arises from preceding dysplastic lesions in the oesophageal epithelium. However, the molecular changes occurring in premalignant lesions are not well understood. An epigenetic change is an example of OSCC that may occur within the epithelium.
Aim: To investigate the methylation status of multiple promoters in cancer-derived DNA, as well as in the background epithelium of OSCC, including dysplastic lesions and non-neoplastic mucosa. The normal epithelium from patients without cancer was also examined. The findings were correlated with the mutational status of *p53*.
Patients and methods: 56 patients with advanced OSCC, 21 patients with intraepithelial neoplasia (IEN), 56 patients with a background of non-neoplastic epithelium, adjacent to the OSCC, and 42 normal control epithelia from healthy volunteers were studied. The promoter methylation status of *SFRP1, SFRP2, DCC, APC, p16^{INK4a}, p14^{ARF}, MINT1, MINT2, MINT31, CACNA1G, COX2, DAPK, hMLH1* and *MGMT* was examined by methylation-specific single polymerase chain reaction or combined bisulphite restriction analysis. The mutation of p53 by direct sequencing was assessed.
Results: DNA methylation was observed in OSCC and in its background epithelium. The frequency of CpG island methylation increased from a baseline level in the background non-neoplastic epithelium, through IEN, to advanced OSCC. However, mutations in *p53* were almost exclusively observed in IEN and OSCC. More extensive DNA methylation was seen in the neoplastic lesions (OSCC or IEN) having a *p53* mutation than in those with wild-type *p53*.
Conclusion: DNA methylation is present at low levels in the non-neoplastic oesophageal epithelium and appears to contribute to the progression of the dysplasia–carcinoma sequence in OSCC carcinogenesis.

Despite the increased incidence of oesophageal adenocarcinoma in the Western World, oesophageal squamous cell carcinoma (OSCC) remains a common type of malignancy worldwide. Tumorigenesis of OSCC is a multistep process and OSCC often develops multifocally within the oesophageal epithelium. Environmental and dietary factors, such as alcohol, tobacco, and high levels of nitrates in the soil and drinking water, have been associated with the aetiology of OSCC.[1 2] Mutations in the *p53* gene are one of the most frequent genetic changes observed in oesophageal cancer and dysplasia.[3–5] A varying *p53* mutational status is often observed in multiple lesions from the same patient with OSCC.[6] Wide areas within the oesophageal mucosa become simultaneously genetically unstable after a prolonged exposure to carcinogens, leading to a pattern of neoplastic transformation described as ''field carcinogenesis''.[7–9]

Epigenetic changes in DNA without concomitant changes in the underlying genetic code are now known to occur often in human cancers.[10–12] Promoter hypermethylation and resulting transcriptional repression of functionally important cancer-related tumour suppressor genes appear to drive tumorigenesis. The promoter CpG island, in normal tissues, is generally protected from aberrant hypermethylation, but this protection may be lost in the early phase of tumorigenesis. We therefore studied the extent of promoter methylation in normal epithelium from patients without cancer and in the oesophageal epithelium, ranging from the background non-neoplastic epithelium to OSCC, and compared the results with the *p53* mutational status.

MATERIALS AND METHODS

Tissue samples

Tumours and the corresponding background non-neoplastic epithelia were obtained from 56 patients with OSCC who underwent curative surgery without prior chemotherapy or radiotherapy between 1996 and 2004 at the Okayama University Medical Hospital, Okayama, Japan. The stage of OSCC was classified according to the TNM classification. Among 56 patients with OSCC, 21 intraepithelial neoplastic (IEN) lesions were identified in the background epithelial specimens with OSCC and sampled for further analysis. IEN lesions were identified and collected using 1.5% Lugol solution sprayed over the resected oesophageal mucosa. The intraepithelial lesion was confirmed histopathologically. IEN was diagnosed when atypical cell proliferation was seen in the upper one third of the epithelium. In situ carcinoma was also included in IEN. Controls of normal oesophageal epithelium were obtained by biopsy using a video endoscope (model Q240, Olympus Optical, Tokyo, Japan) from 42 healthy age-matched volunteers. The mean (standard deviation (SD)) age of the healthy volunteers was 68.0 (13.4) years, 17 (40%) being women. Informed consent was obtained in writing from all patients before recruitment or enrolment into the study. Tissue samples were collected and stored at −80℃.

Abbreviations: COBRA, combined bisulphite restriction analysis; OSCC, oesophageal squamous cell carcinoma; H&E, haematoxylin and eosin; IEN, intraepithelial neoplasia; PAS, periodic acid schiff; PCR, polymerase chain reaction

Haematoxylin and eosin or periodic acid schiff staining

Serial sections were cut from each paraffin wax block, and the sections were counterstained with periodic acid schiff (PAS) with and without diastase digestion, independently. Haematoxylin and eosin (H&E) staining was carried out for histopathological diagnosis.

DNA extraction

From frozen samples, including OSCC, non-neoplastic tissue from patients with OSCC and normal control epithelia from healthy volunteers, DNA was extracted by a standard procedure involving digestion with proteinase K and phenol–chloroform extraction. For the IEN, paraffin-wax-embedded blocks were deparaffinised and 5–10-μm samples were microdissected for unstained serial slides. The dissected samples, which were approximately 50 mm^2, were incubated in 40 μl of lysis buffer (20 mM TRIS-HCl (pH 8.0), 1 mM EDTA, 0.5% Tween 20 and 200 μg/ml proteinase K) at 37℃ for 24 h and then at 95℃ for 15 min to inactivate the proteinase K.

Bisulphite modification and DNA methylation analysis

Extracted DNA was bisulphite modified using an EZ DNA methylation kit (Zymo Research, Orange, California, USA). The methylation status of *MINT1, MINT2, MINT31*, *CACNA1G*, $p16^{INK4a}$, $p14^{ARF}$, *COX2*, *DCC*, *APC1A*, *SFRP1*, *SFRP2* and *DAPK* was evaluated and determined by combined bisulphite restriction analysis (COBRA) as described previously.[12–18] The methylation status of *hMLH1* and *MGMT* was determined by our modified methylation-specific polymerase chain reaction (PCR). Their primer sequences have been described previously.[19]

Mutation analysis of *p53*

We assessed the *p53* mutation from exons 5–9 by direct sequencing of the 56 main oesophageal cancers, 21 IEN, 56 background non-neoplastic epithelia and 42 normal control epithelia from healthy volunteers. PCR was carried out in a 25 μl reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 0.8 mmol/l dNTPs, 1× reaction buffer, 1.5 mmol/l $MgCl_2$ and 0.7 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California, USA). Box 1 describes the primer sequences.

All PCR products were purified using a PCR products presequencing kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), reacted with the Big Dye Terminator FS Ready-Reaction kit (Applied Biosystems) and analysed using an ABI PRISM3100 sequencer (Applied Biosystems). Mutations found were confirmed by independent PCR amplification and by sequencing.

Box 1 Primer sequences

- Exons 5 and 6
  - Forward: 5′-TTTGCTGCCGTGTTCCAGTTG-3′
  - Reverse: 5′-TGGGAGGAGGGGTTAAGGG-3′
- Exon 7
  - Forward: 5′-CTTGGGCCTGTGTTATCTCCT-3′
  - Reverse: 5′-TCAGGAGCCACTTGCCACCCT-3′
- Exons 8 and 9
  - Forward: 5′-CCTTACTGCCTCTTGCTTCTC-3′
  - Reverse: 5′-CTAAGTCTTGGGACCTCTTATCAA-3′.

Statistical analysis

For binary data (methylated *v* non-methylated), specific to patients, locus and tissue type were compared with those of controls (n = 42) and with background non-neoplastic epithelium from patients (n = 56) using Mantel–Haenszel statistics. We compared the matched results for the 56 patients, for non-neoplastic epithelium versus OSCC, using the Wilcoxon signed-rank test. We also compared the non-neoplastic epithelium, IEN and OSCC among the 21 patients with those in all of the three types of sample, using separate Wilcoxon's signed-rank tests for two-way comparisons and using a logistic regression. Differences in the frequencies of DNA methylation were evaluated by Pearson's χ^2 test. The number of methylated genes per patient was analysed using the non-parametric Kruskal–Wallis test and a similar non-parametric test for a trend (procedure ''nptrend'', Stata Corporation). Differences with age were tested using the Student's t test. All reported p values are two-sided and a p value <0.05 was considered significant.

RESULTS

Clinical and pathological features

Table 1 shows the demographics of the patients with OSCC, with regard to the adjacent background non-neoplastic epithelium, along with those for the patients with IEN and those with normal control oesophageal epithelium (healthy volunteers). The mean age of patients with OSCC was 65.8 years (median age 67 years) and the male:female ratio was 47:9. The mean age of the healthy volunteers was 67.9 years (median age 71 years) and the male:female ratio was 25:17. From a total of 56 oesophageal specimens, 21 with IEN were collected. Patients with OSCC with multiple neoplastic lesions and those with a single neoplastic lesion (main advanced OSCC) were not significantly different in age, sex, pathology of main cancer or stage (data not shown). Figure 1 shows the histopathological features of the advanced OSCC, IEN and background non-neoplastic epithelium. IEN lesions were initially screened as Lugol-negative lesions, and the collected lesions were histopathologically confirmed by H&E and PAS staining. All of the IEN lesions were glycogen negative, as shown by the negative PAS diastase staining.

DNA methylation profiles

Using a panel of 14 promoter loci, the DNA methylation status was assessed using methylation-specific PCR or COBRA in the 56 advanced OSCC, 56 corresponding background non-neoplastic epithelia and 21 IEN lesions from patients with OSCC. The DNA methylation status was also assessed in control oesophageal epithelia from 42 OSCC healthy volunteers without OSCC. Minor promoter hypermethylation was observed in the epithelium of some of the healthy volunteers. Nearly 25% of the controls already possessed methylation in *MGMT* and *SFRP1* (table 2). Compared with the 42 normal control epithelia, the background non-neoplastic epithelium from the 56 patients was significantly more methylated, according to the Mantel–Haenszel test ($\chi_1^2 = 43.5$, $p<0.001$). In a matched comparison of tumour tissue and background non-neoplastic epithelium from the 56 patients, there was significantly higher methylation in the tumour samples ($p<0.001$) and in the 21 patients with OSCC, who also had IEN. All three two-way comparisons showed significantly increased methylation in the tissue with the worse disease (IEN>non-neoplastic epithelium, $p = 0.008$; OSCC>non-neoplastic epithelium, $p<0.001$; and OSCC>IEN, $p = 0.012$). We also compared all three types of sample in these 21 patients using a logistic regression, which showed that there was significant heterogeneity in the methylation status of

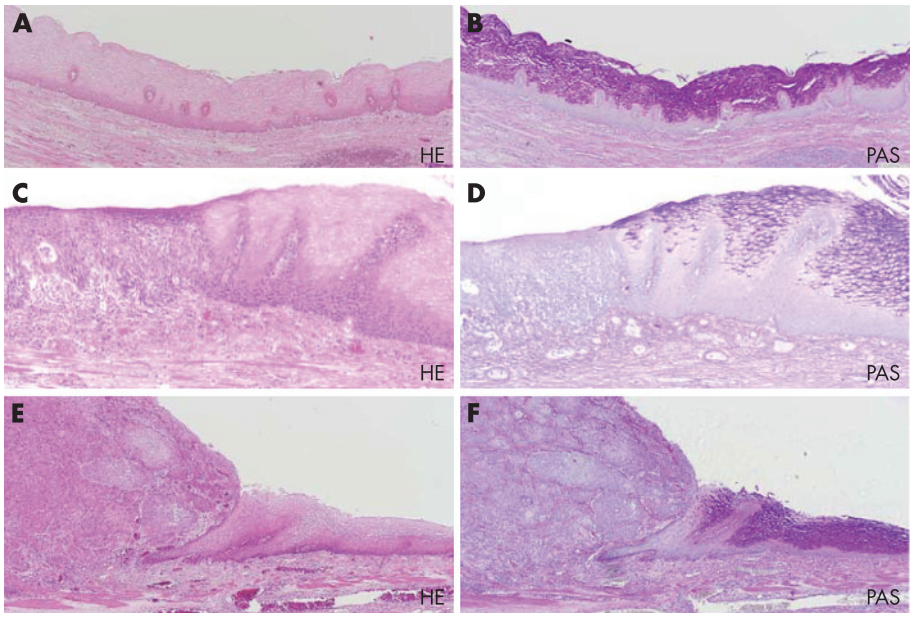

Figure 1 Histopathological features of advanced oesophageal squamous cell carcinoma (OSCC), intraepithelial lesions and background non-neoplastic epithelium. Microscopic results of: (A, B) background non-neoplastic epithelium of OSCC; (C, D) intraepithelial neoplasia (IEN); (E, F) OSCC. Left panels show haematoxylin and eosin (H&E) staining (A, C, E) and right panels show periodic acid schiff (PAS) staining (B, D, F). PAS staining showed the boundary of normal mucosa (right) and IEN (left; ×100). The background non-neoplastic epithelium shows glycogen-positive cells in the upper four fifths region (B), whereas IEN (D) and OSCC (F) contain no glycogen-positive cells.

individual loci among patients and of multiple loci within individual patients.

There was a gradual increase in the number of methylated loci from the normal epithelium (average number: 0.7, 95% CI 0.5 to 0.9), the OSCC-background non-neoplastic epithelium (average number 1.8, 95% CI 1.5 to 2.0), the IEN (average number 3.4, 95% CI 2.7 to 4.0), to the advanced OSCC (average number 3.9, 95% CI 3.5 to 4.4; fig 2). A non-parametric test for a trend was performed on the three groups (background non-neoplastic epithelium, IEN and OSCC) and a significant increase from the background epithelium to OSCC was shown ($p = 0.03$). Most of the individual markers, with the exception of *MINT2*, *SFRP2* and *APC1A,* showed a significant increase in the frequency of methylation, within the range of normal

OSCC, oesophageal squamous cell carcinoma.

Table 1 Pathological features of the samples examined

	OSCC (n = 56)	Intraepithelial neoplasia (n = 21)	Background non-neoplastic epithelium (n = 56)	Normal control epithelium (n = 42)	p Value
Age					
Mean age	65.8	64	65.8	67.9	0.6*
Median age	67	67	67	71	
Sex					
Male	47	18	47	25	0.006†
Female	9	3	9	17	
Lesion					
Single	36	7	36		
Multiple	20	14	20		
Stage					
I	4	1	4		
II	16	7	16		
III	23	6	23		
IV	13	7	13		
Differentiation					
Well	9	5	9		
Moderate	30	10	30		
Poor	17	6	17		
Smoke					
Smoker	43	19	43		
Non-smoker	13	2	13		
Alcohol					
Drinker	45	20	45		
Non-drinker	11	1	11		

OSCC, oesophageal squamous cell carcinoma.
*p Value (two-sided) was calculated using the Student's t test between OSCC and normal epithelium.
†p Value (two-sided) was calculated using Pearson's χ^2 test between OSCC and normal epithelium.

Table 2 Frequency of methylated promoters in each pathological oesophageal lesion

Gene/loci	Number of patients with methylation in designated loci (%)			
	Normal control epithelium (n = 42)	Background non-neoplastic epithelium (n = 56)	Intraepithelial neoplasia (n = 21)	OSCC (n = 56)
MGMT	10 (23.8)	42 (75.0)	17 (81.0)	45 (80.4)
SFRP1	9 (21.4)	25 (44.6)	13 (61.9)	36 (64.3)
$p16^{INK4a}$	0 (0)	18 (32.1)	8 (38.1)	22 (39.3)
DCC	0 (0)	3 (5.4)	8 (38.1)	26 (46.4)
MINT1	0 (0)	1 (1.8)	3 (14.3)	14 (25.0)
MINT2	4 (9.5)	2 (3.6)	1 (4.8)	8 (14.3)
MINT31	1 (2.4)	4 (7.1)	7 (33.3)	18 (32.1)
CACNA1G	0 (0)	6 (10.7)	5 (23.8)	13 (23.2)
DAPK	2 (4.8)	3 (5.4)	5 (23.8)	15 (26.8)
$p14^{ARF}$	0 (0)	2 (3.6)	2 (9.5)	7 (12.5)
SFRP2	2 (4.8)	5 (8.9)	3 (14.3)	11 (19.6)
APC1A	4 (9.5)	10 (17.9)	4 (19.0)	15 (26.7)
COX2	0 (0)	0 (0)	0 (0)	0 (0)
hMLH1	0 (0)	1 (1.8)	0 (0)	6 (10.7)

epithelium, through to OSCC (table 2). The OSCC non-neoplastic epithelium showed more extensive methylation than for the normal (control) epithelium (fig 2). Figure 3 shows the methylation status of each promoter occurring in the sequence from background non-neoplastic epithelium, to IEN and to OSCC, in individual patients. Fifty six patients with OSCC, with or without IEN lesions (IEN lesions were identified in 21 of the patients with OSCC), are shown in the upper panel. OSCCSimilar clusters of promoters are shown to have methylation in the same patients on comparing the IEN lesions and the OSCC. Methylation profiles tend to accumulate along with the development of cancer from background non-neoplastic epithelium through to OSCC, within the same patients.

Analysis of the *p53* mutation

Exons 5–9 of the *p53* gene were amplified and sequenced. Most of the changes found in *p53* were missense mutations. A total of 35 of 56 OSCC tumour samples (62.5%, 38 mutations) and 12 of 21 samples of IEN (57%, 14 mutations) harboured *p53* mutations (fig 4). However, only 2 of 56 background non-neoplastic mucosa showed *p53* mutations (3.5%, 2 mutations), and no mutation was found in the normal mucosa (fig 4). The *p53* mutational profiles in the IEN were different from those of the corresponding main advanced OSCC, within the same patient, there being one exception (patient 17; table 3).

Comparison of DNA methylation and *p53* mutation

The methylation status of the 14 promoter loci and the mutational status of *p53* were compared for the 21 patients with IEN and the 56 patients with advanced OSCC (table 4). In both groups of patients, higher levels of methylation were observed in the lesions with *p53* mutations (IEN; $p<0.006$, OSCC; $p<0.001$). No specific methylation marker or pattern was related to the *p53* mutation.

DISCUSSION

This study is the first to show that the non-neoplastic oesophageal epithelium in patients with OSCC already accumulates a low level of DNA methylation in the gene promoters.

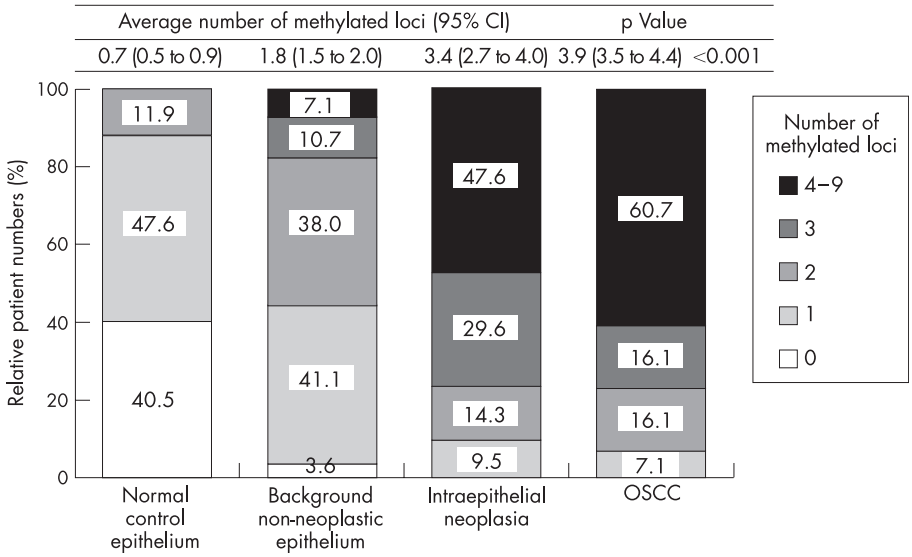

Figure 2 Histopathological status of oesophageal lesions and DNA methylation profiles. A gradual increase in the number of methylated loci was observed from the normal control epithelium through the background non-neoplastic epithelium of OSCC and intraepithelial neoplasia to advanced OSCC. p Value was calculated by the non-parametric Kruskal–Wallis test.

Table 3 Comparison of *p53* mutation and methylation frequency between intraepithelial neoplasia of the oesophagus and advanced oesophageal squamous cell carcinoma

					Number of methylation		*p53* mutation spectrum	
Patient number	Sex	Age	Differentiation	Stage	IEN	OSCC	IEN	OSCC
1	M	51	Moderate	IV	6	6	C580T	G422A
2	M	67	Poor	III	4	4	C449T	C535T
3	M	53	Poor	III	7	7	G437A/A659G	G818T
4	M	62	Poor	IV	4	6	Deletion	A659G
5	M	70	Moderate	IV	2	4	w	T400G
6	M	76	Poor	III	3	4	w	C637T
7	M	54	Well	III	3	6	w	A659G
8	F	73	Well	II	2	5	w	G824A
9	M	70	Well	III	4	5	w	G743A
10	F	69	Well	II	4	3	A659G	w
11	M	74	Moderate	II	3	3	A671G	w
12	M	61	Moderate	II	4	4	A659G	w
13	M	70	Moderate	II	5	5	A659G	w
14	M	69	Poor	I	3	2	T755C	w
15	F	54	Moderate	III	2	2	T421C/A659G	w
16	M	68	Well	II	3	2	A659G	w
17	M	46	Moderate	IV	6	5	A659G	A659G
18	M	63	Moderate	IV	2	3	w	w
19	M	60	Moderate	II	4	3	w	w
20	M	66	Moderate	IV	2	5	w	w
21	M	69	Well	IV	3	4	w	w

OSCC, oesophageal squamous cell carcinoma; F, female; IEN, intraepithelial neoplasia; M, male; w, wild-type *p53*.

The number of gene promoters with DNA methylation increased gradually from the normal healthy epithelium, through OSCC-background non-neoplastic epithelium and IEN identified in the epithelium of OSCC, to the main advanced form of OSCC.

Hiyama *et al*[1] and Morita *et al*[2] have reported that smoking or alcohol intake increases the risk of OSCC. Among 56 patients with OSCC in the present study, 43 (77%) had a history of smoking, whereas 45 (80%) had consumed alcohol (table 1). However, neither smoking nor alcohol was significantly associated with the methylation status of the examined promoters (data not shown).

Mutation in the *p53* gene has been shown to play an important role in oesophageal carcinogenesis. We therefore assessed *p53* mutation in exons 5–9. We identified 38 mutations in 35 OSCC samples (three patients having double mutations), 14 mutations in 12 IEN (two patients having double mutations) and 2 mutations in 2 corresponding background non-neoplastic mucosa. Most of the mutations identified were located in ''hot spots'' as shown in the IARC TP53 MUTATION database.[20] The two background non-malignant mucosal specimens with a *p53* mutation may have come from micro-malignant areas, which could not be easily identified by the routine microscopy used. Interestingly, these two areas also had frequent promoter methylation (data not shown). No significant difference was seen in *p53* mutational status for sex or age (data not shown).

In this study, *p53* mutations were almost exclusively observed in the IEN and OSCC samples and rarely in the background epithelium. Conversely, an average of two of 14 promoter loci examined had acquired methylation in the corresponding non-neoplastic mucosa and 0.7 loci showed methylation in age-matched normal oesophageal epithelium from healthy volunteers (fig 2). However, there was no significant association between the number of methylated

Table 4 Methylation frequency in multiple promoter versus *p53* mutational status in the intraepithelial neoplasia of the oesophagus or advanced oesophageal squamous cell carcinoma

	Intraepithelial neoplasia (n = 21)			OSCC (n = 56)		
Gene/loci	*p53* wild (n = 9)	*p53* mutant (n = 12)	p Value*	*p53* wild (n = 21)	*p53* mutant (n = 35)	p Value*
MGMT	7 (77.8)	10 (83.3)	0.7	16 (76.2)	29 (82.9)	0.5
SFRP1	3 (33.3)	10 (83.3)	0.01	11 (52.4)	24 (68.6)	0.09
p16INK4a	2 (22.2)	6 (50.0)	0.2	6 (28.6)	16 (45.7)	0.3
DCC	3 (33.3)	5 (41.7)	0.7	6 (28.6)	19 (54.3)	0.1
MINT1	2 (22.2)	1 (8.3)	0.4	3 (14.3)	11 (31.4)	0.2
MINT2	0	1 (8.3)	0.4	2 (9.5)	6 (17.1)	0.5
MINT31	2 (22.2)	5 (41.7)	0.1	6 (28.6)	12 (34.3)	0.8
CACNA1G	2 (22.2)	3 (25.0)	0.9	2 (9.5)	12 (34.3)	0.05
DAPK	0	5 (41.7)	0.02	4 (19.0)	11 (31.4)	0.4
p14ARF	1 (11.1)	1 (8.3)	0.8	1 (4.8)	6 (17.1)	0.2
SFRP2	2 (22.2)	1 (8.3)	0.4	1 (4.8)	10 (28.6)	0.04
APC1A	1 (11.1)	3 (25.0)	0.4	4 (19.0)	11 (31.4)	0.4
COX2	0	0	NA	0	0	NA
hMLH1	0	0	NA	0	5 (13.9)	0.08
Average number of methylated loci (95% CI)	2.4 (1.6 to 3.3)	4.1 (3.3 to 4.9)	0.006†	2.9 (2.1 to 3.5)	4.9 (4.1 to 5.2)	<0.001†

*p Value was calculated by Pearson's χ^2 test.
†p Value was calculated by the non-parametric Kruskal–Wallis test.
OSCC, oesophageal squamous cell carcinoma;

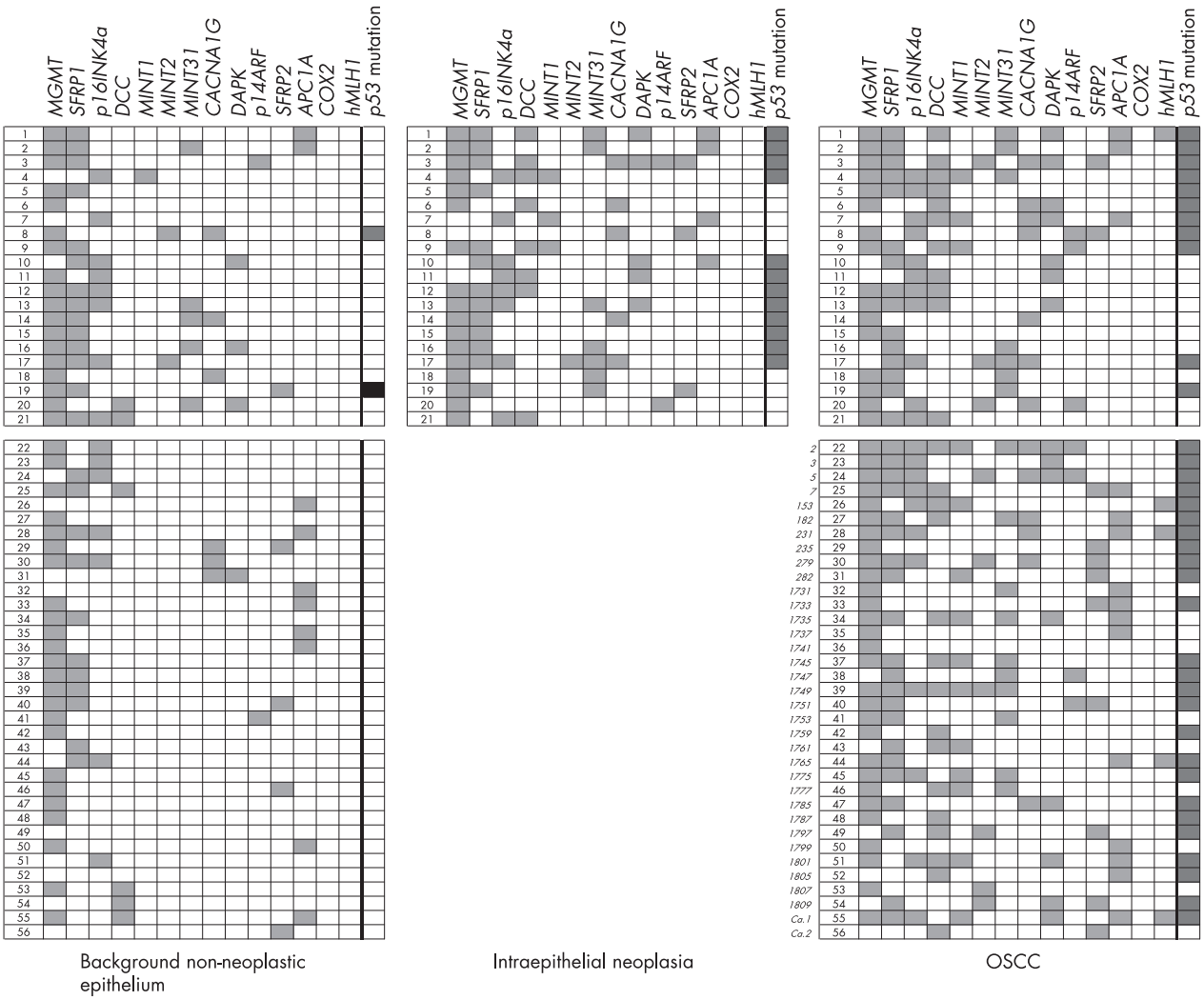

Figure 3 The methylation status of individual promoter methylation in background non-neoplastic epithelium, intraepithelial neoplasia and in oesophageal squamous cell cancer tissue from 56 patients with oesophageal squamous cell cancer (OSCC). Promoter methylation and *p53* mutational status in 21 patients with OSCC accompanying intraepithelial neoplasia (IEN) are shown in the upper panel. Promoter methylation and *p53* mutational status in 25 patients with OSCC, not accompanying IEN, are shown in the lower panel. The number of methylated loci and the frequency of *p53*-mutated patients were similar in IEN and OSCC. Methylated loci (closed boxes in the first 14 columns); non-methylated loci (open boxes); *p53* mutant (closed boxes in the last column); *p53* wild (open boxes).

genes or loci examined and the sex or age either in the OSCC or in the normal mucosa from healthy volunteers (data not shown). Interestingly, within 21 samples of IEN or 56 OSCC samples, a significantly higher number of loci showed methylation in samples with *p53* mutation, compared with the samples without the mutation. These results suggest that the oesophageal epithelium, which has accumulated methylation, becomes highly susceptible to mutations in the *p53* gene. The promoter methylation of *MGMT* has been shown to be associated with the G:C to A:T mutations in *p53* in colorectal and other cancers.[21–24] By contrast, *MGMT* methylation has not been shown to be associated with a *p53* mutation in gastric carcinoma or OSCC.[25 26] In our study, no association was found between aberrant *MGMT* methylation and the frequency of *p53* mutations (table 4) or the presence of G:C to A:T transitions in *p53* (data not shown).

We also compared the mutational status of the *p53* and the pattern of DNA methylation in the IEN and the OSCC in the same patient (table 3 and fig 3). Interestingly, the frequency of methylation in the same individual was very similar. Moreover, almost the same promoter (loci) showed methylation both in the IEN and in the OSCC. The pattern of methylation in individual loci, shown in fig 3, indicates a progressive accumulation of methylations from non-neoplastic epithelium to IEN to OSCC in individual patients. Thus, probably a field of methylation precedes and has some causative relationship with the development of intraepithelial lesions and cancer. By contrast, the specific mutations in *p53* almost always differed between IEN and tumour lesions from the same patients. These results suggest that the oesophageal epithelium, affected by environmental carcinogens, accumulates methylation in increasing numbers of gene promoters over time and that these epigenetic changes eventually contribute to OSCC carcinogenesis. Also, epigenetic changes in a wide epithelial field appear to increase the probability of acquiring changes in genes such as *p53*, which may occur independently in multiple sites and may also contribute to carcinogenesis.

In summary, this study provides novel evidence for the field carcinogenesis in OSCC that is closely linked to the progressive methylation of multiple promoters and *p53* mutations.

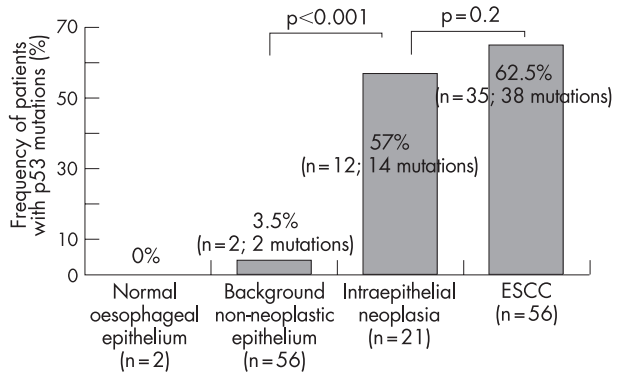

Figure 4 Mutational analysis of *p53*. The frequency of *p53* mutation among patients with normal oesophageal epithelium, background non-neoplastic epithelium, intraepithelial neoplasia and, oesophageal squamous cell carcinoma (OSCC). Exons 5–9 of *p53* were amplified and sequenced. A total of 35 of 56 main patients with OSCC (62.5%, 38 mutations) and 12 of 21 patients with intraepithelial neoplasia (57%, 14 mutations) showed *p53* mutations. However, only 2 of 56 background non-neoplastic epithelia and none of the normal control epithelia showed *p53* mutations. p Value was calculated by Pearson's χ^2 test.

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