

## Research Article

## Revisit of Field Cancerization in Squamous Cell Carcinoma of Upper Aerodigestive Tract: Better Risk Assessment with Epigenetic Markers

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### Abstract

We quantified field cancerization of squamous cell carcinoma in the upper aerodigestive tract with epigenetic markers and evaluated their performance for risk assessment. Methylation levels were analyzed by quantitative methylation-specific PCR of biopsied specimens from a training set of 255 patients and a validation set of 224 patients. We also measured traditional risk factors based on demographics, lifestyle, serology, genetic polymorphisms, and endoscopy. The methylation levels of four markers increased stepwise, with the lowest levels in normal esophageal mucosae from healthy subjects without carcinogen exposure, then normal mucosae from healthy subjects with carcinogen exposure, then normal mucosae from cancer patients, and the highest levels were in cancerous mucosae ( $P < 0.05$ ). Cumulative exposure to alcohol increased methylation of homeobox A9 in normal mucosae ( $P < 0.01$ ). Drinkers had higher methylation of ubiquitin carboxyl-terminal esterase L1 and metallothionein 1M ( $P < 0.05$ ), and users of betel quid had higher methylation of homeobox A9 ( $P = 0.01$ ). Smokers had increased methylation of all four markers ( $P < 0.05$ ). Traditional risk factors allowed us to discriminate between patients with and without cancers with 74% sensitivity (95% CI: 67%–81%), 74% specificity (66%–82%), and 80% area under the curve (67%–91%); epigenetic markers in normal esophageal mucosa had values of 74% (69%–79%), 75% (67%–83%), and 83% (79%–87%); and both together had values of 82% (76%–88%), 81% (74%–88%), and 91% (88%–94%). Epigenetic markers done well in the validation set with 80% area under the curve (73%–85%). We concluded that epigenetics could improve the accuracies of risk assessment. *Cancer Prev Res*; 4(12); 1982–92. ©2011 AACR.

### Introduction

Squamous cell carcinoma (SCC) is the major phenotype of esophageal cancer in Asia. Survival of this cancer is

decreased because of field cancerization, in which synchronous and metachronous cancers occur in the upper aerodigestive tract (UADT; ref. 1). Screening and surveillance measures, particularly the use of sophisticated endoscopic technologies such as narrow-band imaging and Lugol staining (2), currently aid physicians in starting early intervention and providing curative treatment.

The populations of patients exposed to well-established risk factors may outnumber the capacity of endoscopists to screen them. For instance, among the adult male population in Taiwan, 46% drink alcohol, 15% chew betel quid, and 57% smoke cigarettes (3), which are all behaviors that result in an increased incidence of SCC (4). Following a negative endoscopy, an efficient risk-assessment method is needed to design surveillance programs for early detection of superficial SCC in the head and neck region and esophagus (2). However, past efforts based on demographic characteristics (5–7), lifestyle risk factors (8, 9), genetic susceptibilities (10–14), serological markers (15–18), and endoscopic findings (19), alone or in combination (15, 20, 21), have not produced models efficient enough to be effective.

Aberrant DNA methylation in histologically normal mucosae has attracted attention as an indicator of past

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**Note:** Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org>).

Presented in Part: Digestive Disease Week Conference, Chicago, USA, 7–10 May 2011 (abstract: Su1129).

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doi: 10.1158/1940-6207.CAPR-11-0096

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exposure to carcinogens and as a marker for future risk prediction (22). Reliable measuring techniques, such as real-time quantitative methylation-specific PCR (qMSP), have been confirmed to be sensitive and accurate in quantifying this early carcinogenic event (23). They offer an opportunity to elucidate whether aberrant DNA methylation can serve as a marker to quantify the field effects associated with SCC of the UADT. If this hypothesis is found to be true, we can compare the performance of risk-assessment methods based on epigenetic markers with those based on traditional risk factors. We also validated the performance of the epigenetic markers with a prospectively recruited independent cohort.

## Materials and Methods

### Subjects and study design

We recruited consecutive Taiwanese patients who received endoscopic screening at the National Taiwan University Hospital between January 2008 and May 2011 due to UADT symptoms, such as globus sensation and dysphagia. This cohort was split into a training set to develop a risk-assessment model for UADT cancer based on epigenetic markers and a validation set to evaluate model performance. Our initial pilot experiment determined the required sample size for hypothesis testing with 123 biopsied samples from 103 patients. The study protocol included thoroughly evaluating traditional risk factors, screening by endoscopy, and quantifying DNA methylation in the biopsied specimens. All participants provided informed consent, and the Ethics Committee of the National Taiwan University Hospital approved the study (no: 200706034R and 200806039R).

### Traditional risk factors

Prior to the endoscopic screening, patients underwent face-to-face interviews, physical examinations, and laboratory tests to identify traditional risk factors for SCCs in the head and neck region (24) and in the esophagus (25) that would be used as baseline comparators for the upcoming epigenetic approach. These included: the demographic characteristics (6, 7) of age, sex, and body mass index (BMI); the lifestyle risk factors (8, 9, 11) of alcohol drinking, betel quid chewing, and cigarette smoking (briefly as "ABC"); polymorphisms in genes encoding enzymes involved in the metabolism of alcohol (10–13), including aldehyde dehydrogenase (*ALDH* 2, alcohol dehydrogenase (*ADH* 1B, and *ADH* 1C); polymorphisms in genes encoding enzymes involved in the metabolism of xenobiotics (14), including glutathione S transferase (*GST* P1, *GST* M1, and *GST* T1); and serological markers (15–18), including increased mean corpuscular volume (MCV), *Helicobacter pylori* infection, and human papillomavirus infection.

Lifestyle risk factors were recorded according to frequency (alcohol where one time indicates at least 15.75 gm of ethanol: 0, never; 1, once per week; 2, once to twice per week; 3, 3–4 times per week; and 4, 5 or more times per week; betel quid: 0, never; 1, less than 1 piece daily; 2, 1–10

pieces daily; 3, 11 to 20 pieces daily; and 4, more than 20 pieces daily; cigarettes: 0, never; 1, less than 0.5 pack daily; 2, 0.5–1 pack daily; 3, 1–2 packs daily; and 4, more than 2 packs daily) and duration (0, never; 1, less than 1 year; 2, 1–10 years; 3, 11–20 years; and 4, more than 20 years). The cumulative lifetime exposure was calculated by multiplying the frequency and duration, and then categorized into 3 levels: level 1, never (0); level 2, light to moderate (1–11); and level 3, heavy ( $\geq 12$ ). Polymorphic genotypes were determined from peripheral blood leucocytes with PCR-RFLP (the primers are provided in the Supplementary Table S1). Diagnosis of *H. pylori* infection was made histologically or serologically (Diagnostic Products Co.; ref. 26) as appropriate. Human papillomavirus infection was serologically diagnosed by ELISA with commercially available kits (Wuhan EIAab Science Co., Ltd.; ref. 27). The cutoff value for human papillomavirus IgG was set at 600 U/mL based on 3 SDs above the mean optical density obtained from serum samples of negative controls.

### Endoscopic examination and tissue sampling

After evaluating traditional risk factors, patients underwent endoscopic examinations by white-light, narrow-band imaging, and Lugol staining in a single session, which is a method shown to be accurate in the detection of cancers of the UADT (28, 29). We evaluated endoscopic findings of numerous irregular-shaped multifocal unstained areas over the background esophageal mucosae, namely, Lugol voiding lesions (LVL) after spraying of Lugol solution (19), as one of the traditional risk factors. A biopsy was done on any suspicious lesions along the UADT before chemotherapy or radiation therapy and also on normal-appearing mucosae in the esophagus. Normal-appearing mucosa was defined as an area that did not seem brownish ("brownish" indicated microvascular proliferation) under narrow-band imaging and, after being sprayed with Lugol's solution, was stained ("unstained" indicated glycogen depletion). For patients with either head and neck cancer or esophageal cancer, biopsies for normal-appearing mucosae were routinely done 3 cm above the squamocolumnar junction in the esophagus; in cases with neoplastic lesions close to this area, the biopsy sites were moved to at least 5 cm away from the neoplastic margin. In addition to histologic confirmation, a set of specimens was stored at  $-80^{\circ}\text{C}$  until genomic DNA was extracted.

### Internally validating epigenetic markers and quantifying DNA methylation levels

Genomic DNA was sent to the National Cancer Center Research Institute in Japan. The DNA methylation of the epigenetic markers was quantified with the qMSP. The laboratory work was similar to the details previously reported that used an independent test set of 60 tumor samples derived from Japanese individuals (30). In brief, 14 genes whose promoter CpG islands were methylated in esophageal SCCs were isolated by a genome-wide screening of genes reexpressed after esophageal SCC cell lines were treated with the demethylating agent 5-aza-2'-deoxycytidine. Most of the

14 genes were also methylated at low levels in adjacent esophageal mucosae. We analyzed 4 genes correlated with smoking duration in this study: homeobox A9 (*HOXA9*), neurofilament heavy polypeptide (*NEFH*, 200 kDa), ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), and metallothionein 1M (*MT1M*).

Laboratory analyses were done by laboratory technicians who were blinded to the clinical status of the samples analyzed. As in our previous report (30), DNA was digested by *Bam*HI, denatured, and treated by 15 cycles denaturing for 30 seconds at 95°C and incubating for 15 minutes at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mmol/L hydroquinone. The samples were desalted and then desulfonated in 0.3 N NaOH. The qMSP was done with primer sets specific to either the methylated or unmethylated sequence (M or U set) with the sodium bisulfite-treated DNA and an iCycler Thermal Cycler (Bio-Rad Laboratories). We compared the amplification of sample DNA to that of standard DNA that was prepared from a plasmid with a cloned PCR product and that had known copy numbers of the cloned DNA. The amount of methylated and unmethylated DNA in the sample was quantified.

### Statistical analysis

Data are expressed as average  $\pm$  SD (median; range) for continuous variables and as a percentage for categorical variables. The measurement of DNA methylation levels in the biopsied specimens was the starting point for the following statistical analyses: (i) comparing methylation levels according to stage of carcinogenic sequence and level of carcinogen exposure; (ii) building logistic-regression models to determine the likelihood of UADT cancers based on epigenetic markers, traditional risk factors, and both together; and (iii) comparing the predictive value of epigenetic markers on a validation set of patients not used in the initial model building phase.

First, the ability of each epigenetic marker to serve as an indicator for the field effects was confirmed by seeking a stepwise relationship among the methylation levels that followed the carcinogenic sequence related to the chronic irritation of ABC, which are the 3 principal determinants of SCC. We simulated the carcinogenic sequence by categorizing the biopsied specimens into 4 groups: group 1, normal esophageal mucosae of healthy subjects with no exposure to ABC; group 2, normal esophageal mucosae of healthy subjects with at least one of the above exposures; group 3, normal esophageal mucosae of cancer patients; and group 4, cancerous mucosae. Methylation levels were expected to increase in a stepwise manner in linear regression analyses as the group number increased. Scatter plots were drawn to describe the results. Pairwise comparisons between groups were also made using the nonparametric Mann-Whitney *U* test. We also correlated methylation levels in the normal-appearing esophageal mucosae (i.e., groups 1, 2, and 3) with cumulative lifetime exposures to ABC. Tests for linear trends and pairwise comparisons between subjects with different exposure levels were done in a similar manner. In cancerous tissues (i.e., group 4), the

association between methylation levels and clinicopathologic characteristics was evaluated.

Second, the predictive value of our epigenetic markers was assessed by developing logistic-regression models that used the methylation levels in normal-appearing esophageal mucosae to predict the probability of a patient having head and neck cancer, esophageal cancer, and UADT cancer based on the training samples. Following univariate logistic-regression analyses, an epigenetic model was built using backward elimination of predictors from the full 4-predictor model that excluded those with  $P \geq 0.05$ . We excluded strong linear dependencies among the predictors by examining tolerance and the variance inflation factor. We also excluded the statistical significance of interactions and nonlinear terms.

We developed logistic-regression models using the traditional risk factors to compare with the epigenetic model. Continuous predictors included age, BMI, levels of cumulative lifetime exposures to ABC, and MCV. For the categorical predictors, the baseline comparators were: female, unexposed to ABC, without *H. pylori* infection, without human papillomavirus infection, and carrying these genotypes: *ALDH2*\*1/2\*1 (fast metabolizers), *ADH1B*\*1/\*2 or \*2/\*2 (fast), *ADH1C*\*1/\*2 or \*2/\*2 (slow), *GSTP1* *ile/val* or *val/val* (fast), *GSTM1* *present* (active), and *GSTT1* *present* (active). Univariate logistic-regression analyzed each predictor and those predictors with  $P < 0.05$  were included in a multivariate regression model. We also assessed interaction terms, in particular, between the polymorphic genotypes and exposures to ABC. A traditional model for the probability of a patient having UADT cancer was built using backward elimination based on Akaike's information criterion.

To further assess if our epigenetic markers improved on or added value to the traditional methods, we added the epigenetic markers individually to the final traditional model and determined the independence and magnitude of the contribution of the epigenetic markers after adjusting for the effects of traditional risk factors. A combined model was constructed based on Akaike's information criterion. The significance of each predictor is presented as an OR and a 95% CI. A 2-tailed  $P < 0.05$  indicated statistical significance. All statistical analyses were done using the SAS software system, version 9.2 (SAS Institute Inc.).

The performance of the above predictive models was compared by graphically displaying their relative performances on a receiver operating characteristic (ROC) curve that plotted the sensitivity against the false-positive rate (i.e., 1-specificity) over a range of cutoff values. The area under the curve (AUC) measured the performance of the test in correctly distinguishing subjects with and without cancer and the corresponding 95% CIs were calculated using 1,000 bootstrap sample sets (drawn with replacement) of the same size of the original dataset (31). The model calibration was assessed using the Hosmer-Lemeshow test for goodness of fit, which evaluated whether the predicted probabilities agreed with the observed probabilities in deciles. The bootstrap method evaluated statistical differences between the

AUCs of epigenetic, traditional, and combined models over the same training set. We determined the optimal cutoff value for each model by selecting the point closest to theoretically perfect performance in discrimination and calculated the corresponding sensitivity and specificity pair.

Finally, to validate the epigenetic model, we recruited an independent validation set of a sample size similar to the training set at the National Taiwan University Hospital and quantified their DNA methylation levels in normal esophageal mucosae in a similar manner. We fitted an epigenetic model using the training set, saved the details of the fitted model such as the parameter estimates and SEs, and tested its performance on the validation set. The derived AUC was compared with that based on the training set using the  $z$  test.

### Sample size estimation

The sample size of this prospective study was planned previously based on our previous study (30). That study found that methylation levels in the normal esophageal mucosae of cancer patients (i.e., group 3) were  $2.7\% \pm 2.5\%$  (average  $\pm$  SD; median: 2.4%; range: 0%–16.3%) for *HOXA9*,  $7.2\% \pm 5.1\%$  (6.2%; 0.4%–20.3%) for *NEFH*,  $1.8\% \pm 3.6\%$  (0.9%; 0%–22.4%) for *UCHL1*, and  $1.9\% \pm 1.7\%$  (1.3%; 0%–6.8%) for *MT1M*. However, the methylation levels of healthy controls (i.e., groups 1 and 2) were not available. To test the hypothesis that methylation levels in the normal esophageal mucosae could differentiate cancer patients from noncancer subjects, we used the initial 10 healthy controls in the pilot experiment to estimate the required sample size; their average methylation levels were 1.2% for *HOXA9*, 0.2% for *NEFH*, 0.2% for *UCHL1*, and 0.6% for *MT1M*. To detect a significant difference between the case (i.e., group 3) and control (i.e., groups 1 and 2) at  $\alpha = 0.05$ ,  $\beta = 0.2$ , and a single-tail hypothesis, it was estimated that a total of 64 samples for each group would suffice using the Student  $t$  test. To compare the model based on epigenetic markers with the one based on traditional risk factors, we set a minimally acceptable level of accuracy at 80%: that is, a level that is commonly considered a prerequisite for population-based screening. Knowing that the average accuracy of traditional methods was approximately 70% (5, 20), an overall sample size of 231 was required to detect this difference at  $\alpha = 0.05$  and  $\beta = 0.2$  using the  $\chi^2$  test. The sample size estimation was done using the nQuery Advisor 7.0.

## Results

### Patients' characteristics

Between January 2008 and April 2009, 272 consecutive patients were screened. Seventeen (6.3%) subjects were excluded, including 8 having a history of bleeding tendency, 2 with failed endoscopic examination, and 7 refusing endoscopic biopsy. The remaining 255 patients were recruited to the study as the training set. The demographic and clinicopathologic characteristics between cases and controls are shown in the Table 1. Histories of ABC were confirmed in 190 (74.5%) subjects; among them, 66 (34.7%) were double positives and 84 (42.2%) were triple positives. Of

the 146 (57.3%) patients diagnosed with SCC of the UADT, 49 were diagnosed with head and neck cancer (including 4 with oral cavity cancer, 2 with laryngeal cancer, and 43 with hypopharyngeal cancer), 91 with esophageal cancer, and 6 patients with synchronous head and neck and esophageal cancers. Of the 109 subjects who had undergone the similar diagnostic procedures but had negative results, 46 (42.2%) had no history of ABC while 63 (57.8%) had used at least one of these 3 risk factors.

### Quantifying field effects with epigenetic markers

A total of 397 biopsied specimens were obtained in the training cohort and DNA methylation levels were successfully quantified in 372 (93.7%) of them. The methylation levels of all 4 epigenetic markers increased in a stepwise manner ( $P < 0.001$ ) based on the exposure to alcohol, betel quid, or cigarettes (Fig. 1). The normal esophageal mucosae of healthy subjects not exposed to alcohol, betel quid, or cigarette (group 1) had methylation levels of  $2.8\% \pm 3.2\%$  (average  $\pm$  SD; median: 1.8%; range: 0.3%–15.4%) for *HOXA9*,  $0.4\% \pm 0.7\%$  (0.2%; 0%–4.1%) for *NEFH*,  $0.3\% \pm 0.5\%$  (0.2%; 0%–2.4%) for *UCHL1*, and  $0.9\% \pm 1.7\%$  (0.4%; 0.1%–10.7%) for *MT1M*. The normal esophageal mucosae of healthy subjects with at least one of the above exposures (group 2) had methylation levels of  $4.8\% \pm 5.7\%$  (2.7%; 0.2%–29.3%) for *HOXA9*,  $0.6\% \pm 1.1\%$  (0.3%; 0%–7.4%) for *NEFH*,  $1.2\% \pm 3.3\%$  (0.3%; 0%–23.7%) for *UCHL1*, and  $1.0\% \pm 0.7\%$  (0.9%; 0%–3.1%) for *MT1M*. The normal esophageal mucosae of cancer patients (group 3) had methylation levels of  $9.2\% \pm 9.9\%$  (5.8%; 0%–65.8%) for *HOXA9*,  $0.7\% \pm 1.0\%$  (0.4%; 0%–7.3%) for *NEFH*,  $2.0\% \pm 5.0\%$  (0.4%; 0%–42.8%) for *UCHL1*, and  $1.4\% \pm 2.2\%$  (0.9%; 0.1%–18.0%) for *MT1M*.

Methylation levels in the normal esophageal mucosae correlated to cumulative lifetime exposures to ABC (Supplementary Figs. S1–7). Notably, a dose-dependent relationship was noted between the methylation levels of *HOXA9* and alcohol consumption ( $P < 0.01$ ). The methylation levels of *UCHL1* and *MT1M* were higher in drinkers ( $P < 0.05$ ). Although we did not identify a linear relationship between the methylation levels and dosage of betel quid use or cigarette smoking, betel quid users had significantly higher *HOXA9* methylation levels than nonusers ( $P = 0.01$ ) and smokers had consistently higher methylation levels for the 4 markers ( $P < 0.05$ ). Regarding the concurrent use of ABC, the methylation levels of 4 marker genes were higher in individuals who had at least one risk factor ( $P \leq 0.01$ ). The methylation levels of *HOXA9* increased in a stepwise manner with the number of exposures ( $P < 0.01$ ), suggesting an additive effect.

When the data was stratified by the presence of numerous LVLs, which are a well-recognized indicator of SCC risk, higher methylation levels were noted in the cases with numerous LVLs (Supplementary Fig. S8), and the levels of the *HOXA9* and *UCHL1* genes reached statistical significance ( $P < 0.01$ ). Stratifying the data by infection with *H. pylori* or human papillomavirus did not yield significant differences in the methylation levels of noncancerous mucosae.

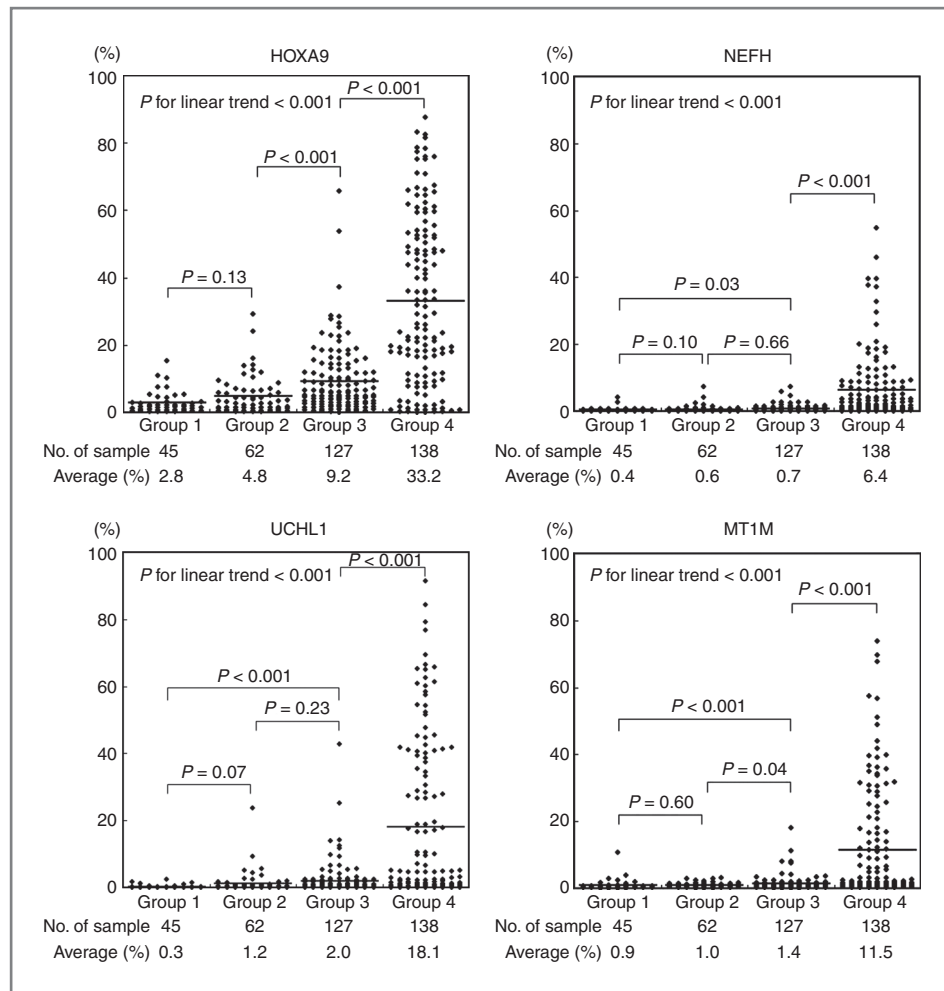
**Table 1.** Patient characteristics of the training set

Parameters	Controls (n = 109)	Cases (n = 146)	Total (n = 255)
Demographic characteristics			
Age, mean $\pm$ SD, range (y)	59.8 $\pm$ 14.3, 25–90	59.8 $\pm$ 10.7, 32–89	59.5 $\pm$ 12.4, 25–90
Male sex (%)	78 (71.6)	136 (93.2)	214 (83.9)
BMI, mean $\pm$ SD, range (kg/m <sup>2</sup> )	23.0 $\pm$ 3.5, 17.0–33.1	22.1 $\pm$ 3.4, 15.6–34.3	22.5 $\pm$ 3.5, 15.6–34.3
Lifestyle risk factors (%)	57 (52.3)	133 (91.1)	190 (74.5)
Levels of alcohol drinking			
Never	70 (64.2)	24 (16.4)	94 (36.9)
Light to moderate	26 (23.9)	36 (24.7)	62 (24.3)
Heavy	13 (11.9)	86 (58.9)	99 (38.8)
Levels of betel quid chewing			
Never	92 (84.4)	65 (44.5)	157 (61.6)
Light to moderate	7 (6.4)	30 (20.6)	37 (14.5)
Heavy	10 (9.2)	51 (34.9)	61 (23.9)
Levels of cigarette smoking			
Never	68 (62.4)	24 (16.5)	92 (36.1)
Light to moderate	20 (18.3)	56 (38.3)	76 (29.8)
Heavy	21 (19.3)	66 (45.2)	87 (34.1)
Genetic polymorphisms (%)			
Encoding enzymes in the metabolism of alcohol			
ALDH2 genotype	41 (37.6)	128 (87.7)	169 (66.3)
ALDH2*1/2*1, fast	15 (36.6)	36 (28.1)	51 (30.2)
ALDH2*1/2*2 or 2*2/2*2, slow	26 (63.4)	92 (71.9)	118 (69.8)
ADH1B genotype	39 (35.8)	128 (87.7)	167 (65.5)
ADH1B*1/*1, slow	4 (10.3)	37 (28.9)	41 (24.6)
ADH1B*1/*2 or *2/*2, fast	35 (89.7)	91 (71.1)	126 (75.4)
ADH1C genotype	38 (34.9)	126 (86.3)	164 (57.3)
ADH1C*1/*1, fast	34 (89.5)	101 (80.2)	135 (82.3)
ADH1C*1/*2 or *2/*2, slow	4 (10.5)	25 (19.8)	29 (17.7)
Encoding enzymes in the metabolism of xenobiotics			
GSTP1 genotype	36 (33.0)	121 (82.9)	157 (61.6)
ile/ile, slow	31 (86.1)	90 (74.4)	121 (77.1)
ile/val or val/val, fast	5 (13.9)	31 (25.6)	36 (22.9)
GSTM1 genotype	40 (36.7)	128 (87.7)	168 (65.9)
present, active	19 (47.5)	54 (42.2)	73 (43.5)
null, inactive	21 (52.5)	74 (57.8)	95 (56.5)
GSTT1 genotype	41 (37.6)	128 (87.7)	169 (66.3)
present, active	20 (48.8)	62 (48.4)	82 (48.5)
null, inactive	21 (51.2)	66 (51.6)	87 (51.5)
Serological markers			
MCV, mean $\pm$ SD, range (fL)	89.3 $\pm$ 8.3, 63.5–104.1	93.0 $\pm$ 6.9, 67.1–111.5	92.0 $\pm$ 7.7, 63.5–111.5
<i>H. pylori</i> infections (%)	40/103 (38.8)	70/141 (49.7)	110/244 (45.1)
Human papillomavirus infections (%)	7/41 (17.1)	30/128 (23.4)	37/169 (21.9)
Endoscopic findings (%)			
Negative study	109	0	109 (42.7)
Head and neck SCC	0	49 (33.6)	49 (19.2)
Esophageal SCC	0	91 (62.3)	91 (35.7)
Synchronous head and neck and esophageal SCC	0	6 (4.1)	6 (2.4)
Presence of numerous LVLs in the background mucosae	6 (10.5)	68 (48.6)	74 (29.0)

The cancerous mucosae (group 4) had methylation levels of 33.2%  $\pm$  24.7% (28.8%; 0%–87.6%) for *HOXA9*, 6.4%  $\pm$  10.0% (2.3%; 0%–54.9%) for *NEFH*, 18.1%  $\pm$  23.7%

(3.9%; 0%–91.4%) for *UCHL1*, and 11.5%  $\pm$  16.7% (2.0%; 0.1%–73.9%) for *MT1M*, which was a broad distribution with higher average values than each of the 3 normal groups

Figure 1. Methylation levels of the epigenetic markers along the carcinogenic sequence in the training set. Group 1, normal esophageal mucosae of healthy subjects without exposure to alcohol, betel quid, or cigarette; group 2, normal esophageal mucosae of healthy subjects with at least one of the above exposures; group 3, normal esophageal mucosae of cancer patients; group 4, cancerous mucosae.



( $P < 0.001$ ). Cancers of the head and neck and of the esophagus had similar methylation levels (Supplementary Fig. S9). No association was found between methylation levels and clinicopathologic characteristics, including TNM staging, tumor differentiation, and multiplicity (data not shown).

#### Comparing performance between head and neck cancer and esophageal cancer

Methylation levels in the normal esophageal mucosae were significantly associated with the risk of SCC for the 4 epigenetic markers (Table 2). To predict head and neck cancer, AUCs for each of the epigenetic markers were 83% for *HOXA9* (bootstrap 95% CI: 77%–88%), 69% for *NEFH* (60%–74%), 67% for *UCHL1* (59%–74%), and 74% for *MT1M* (67%–81%). To predict esophageal cancer, similarly, the AUCs were 78% for *HOXA9* (73%–83%), 71% for *NEFH* (65%–77%), 73% for *UCHL1* (67%–78%), and 71% for *MT1M* (65%–77%). Overall, to predict UADT cancer, the AUCs for each of the markers were 80% for *HOXA9* (75%–84%), 69% for *NEFH* (64%–75%), 71% for *UCHL1* (66%–76%), and 72% for *MT1M* (66%–77%). Notably, *HOXA9* showed the best discriminative ability and an

additional dichotomous analysis showed that OR was 10.189 (5.430–19.118) when the cutoff point of the *HOXA9* methylation level was set at 9.2% (the average value of cancer group).

An optimal epigenetic model to predict UADT cancer was constructed with 2 predictors of *HOXA9* and *NEFH* methylation levels (AUC: 83%; bootstrap 95% CI: 79%–86%). The model had similar performances for head and neck cancer and for esophageal cancer (Supplementary Fig. S10). The sensitivity, specificity, and AUC were 76% (65%–87%), 75% (67%–83%), and 85% (80%–90%), respectively, in predicting head and neck cancer, and 72% (63%–81%), 73% (65%–81%), and 82% (77%–87%), respectively, in predicting esophageal cancer.

Thus, we confirmed that our 4 epigenetic markers were robust as a quantitative measure for field cancerization along the UADT.

#### Comparing performance between traditional risk factors and epigenetic markers

The traditional risk factors were compared between cases and controls using univariate logistic-regression analyses (Table 2). Both groups were similar in age. The patients with

**Table 2.** Logistic-regression models fitted on the training set to screen for SCC at the upper aerodigestive tract based on traditional risk factors or epigenetic markers

Risk factors	Univariate analyses			Multivariate analyses with model selection		
	Crude OR	95% CI	P	Adjusted OR	95% CI	P
Models based on the traditional risk factors						
Age	1.009	0.990–1.028	0.357			
Male sex	5.405	2.515–11.618	<0.001			
BMI	0.935	0.875–0.998	0.042			
Lifestyle risk factors						
Levels of alcohol drinking	1.199	1.148–1.252	<0.001			
Levels of betel quid chewing	1.131	1.080–1.185	<0.001			
Levels of cigarette smoking	1.176	1.122–1.233	<0.001			
Any one of the above	10.533	5.766–19.239	<0.001	4.172	1.515–11.488	0.006
Genetic polymorphisms						
<i>ALDH2</i> * 1/2*2 or 2*2/2*2, slow	1.563	0.841–2.904	0.158			
<i>ADH1B</i> * 1/*1, slow	1.904	0.881–4.115	0.101			
<i>ADH1C</i> * 1/*1, fast	0.437	0.165–1.161	0.097			
<i>GSTP1</i> ile/ile, slow	1.923	0.767–4.817	0.163			
<i>GSTM1</i> null, inactive	0.822	0.434–1.559	0.549			
<i>GSTT1</i> null, inactive	0.943	0.502–1.773	0.856			
MCV	1.061	1.011–1.114	0.015	1.060	1.000–1.124	0.049
<i>H. pylori</i> infection	1.553	0.927–2.601	0.094			
Human papillomavirus infection	1.487	0.598–3.696	0.391			
Presence of numerous LVLs in the background mucosae	14.965	6.176–36.263	<0.001	9.384	3.058–28.795	<0.001
Models based on the epigenetic markers						
<i>HOXA9</i> methylation level (%)	1.153	1.101–1.209	<0.001	1.141	1.086–1.198	<0.001
<i>NEFH</i> methylation level (%)	1.670	1.280–2.179	<0.001	1.339	1.078–1.664	0.008
<i>UCLH1</i> methylation level (%)	1.199	1.069–1.345	0.002			
<i>MT1M</i> methylation level (%)	1.474	1.153–1.884	0.002			

SCC tended to be male, lower in BMI, and more likely to have the ABC habits. Serologically, their MCVs were higher, but their rate of *H. pylori* and human papillomavirus infections was similar to that of the controls. Endoscopy found that the cancer patients were more likely to have numerous LVLs in the background mucosae. A significant interaction was found between carriers of the genetic polymorphisms of an inactive *ALDH2*\*2 allele and levels of alcohol consumption (OR: 1.212, 95% CI: 1.092–1.344,  $P < 0.001$ ), as observed in the Hardy–Weinberg equilibrium, indicating that this genotype modified alcohol-related cancer risk.

Model performance was compared in terms of ROC curves (Supplementary Fig. S11). Overall exposure to ABC had a higher sensitivity (93%, 95% CI: 90%–96%) but a lower specificity (45%, 95% CI: 35%–54%), while the presence of endoscopic LVLs had a lower sensitivity (50%, 95% CI: 44%–56%) but a higher specificity (92%, 95% CI: 88%–98%). The AUCs increased as the model was based on MCV; then that based on overall exposure to ABC, endoscopic LVLs, and MCV (the traditional model; goodness-of-fit test,  $P = 0.77$ ); then that based on methylation levels of *HOXA9* and *NEFH* (the epigenetic model; good-

ness-of-fit test,  $P = 0.18$ ); and then that based on the overall exposure to ABC, endoscopic LVLs, and methylation levels of *HOXA9* and *NEFH* (the combined model shown in Table 3; goodness-of-fit test,  $P = 0.70$ ), which was the most accurate. The sensitivity and specificity pairs of the optimal cutpoints were 56% (95% CI: 48%–64%) and 57% (48%–66%) for the MCV-based model, 74% (67%–81%) and 74% (66%–82%) for the traditional model, 74% (69%–79%) and 75% (67%–83%) for the epigenetic model, and 82% (76%–88%) and 81% (74%–88%) for the combined model. The performance of epigenetic model (AUC: 83%, bootstrap 95% CI: 79%–87%) was similar to that of the traditional model (AUC: 80%, 95% CI: 67%–91%;  $P = 0.51$  for the comparison). After adding the epigenetic markers, the combined model (AUC: 91%, 95% CI: 88%–94%) was more accurate than the traditional model ( $P < 0.001$ ).

#### Comparing performance between training and validation sets

Between May 2009 and May 2011, a total of 224 patients who underwent endoscopic screening were recruited as the validation set. They included 74 with negative endoscopies,

**Table 3.** Logistic-regression models fitted on the training set to screen for SCC at the upper aerodigestive tract based on the combination of traditional risk factors and epigenetic markers

Risk factors	Multivariate analyses with model selection		
	Adjusted OR	95% CI	P
Models based on both traditional risk factors and epigenetic markers			
Exposure to at least one of ABC	4.557	1.141–18.208	0.032
Presence of numerous LVLs in the background mucosae	8.428	2.458–28.903	<0.001
<i>HOXA9</i> methylation level (%)	1.118	1.039–1.204	0.003
<i>NEFH</i> methylation level (%)	1.350	1.042–1.749	0.023

86 with head and neck cancer (6 with oral cavity cancer, 6 with oropharyngeal cancer, 5 with laryngeal cancer, and 69 with hypopharyngeal cancer), 59 with esophageal cancer, and 5 patients with synchronous head and neck and esophageal cancers. Their distribution of age ( $58.2 \pm 12.5$  y), gender (86.6% men), BMI ( $22.2 \pm 3.7$  kg/m<sup>2</sup>), and exposure to ABC (80.4%) was similar to that of the original training cohort. Methylation levels of the 4 markers in the normal esophageal mucosae of the control group were  $2.9\% \pm 2.9\%$  (average  $\pm$  SD; median: 1.8%; range: 0%–12.4%) for *HOXA9*,  $0.4\% \pm 0.7\%$  (0.1%; 0%–6%) for *NEFH*,  $0.5\% \pm 0.7\%$  (0.1%; 0%–3.2%) for *UCHL1*, and  $0.2\% \pm 0.5\%$  (0.1%; 0%–3.4%) for *MT1M*. The normal esophageal mucosae of the cancer group had methylation levels of  $13.5\% \pm 16.8\%$  (5.9%; 0.1%–84.6%) for *HOXA9*,  $1.1\% \pm 4.2\%$  (0.2%; 0%–47.6%) for *NEFH*,  $3.8\% \pm 11.7\%$  (0.6%; 0%–82.0%) for *UCHL1*, and  $2.4\% \pm 10.2\%$  (0.2%; 0%–92.3%) for *MT1M*. When the epigenetic model fit in the training set was applied to the validation set, the AUC was 80% (bootstrap 95% CI: 73%–85%; goodness-of-fit test,  $P = 0.16$ ); the difference in discrimination between the training and validation sets was not statistically significant ( $P = 0.43$ ; Fig. 2). The *HOXA9* remained the best discriminative marker with 78% AUC (95% CI: 72%–84%) in predicting UADT cancer.

## Discussion

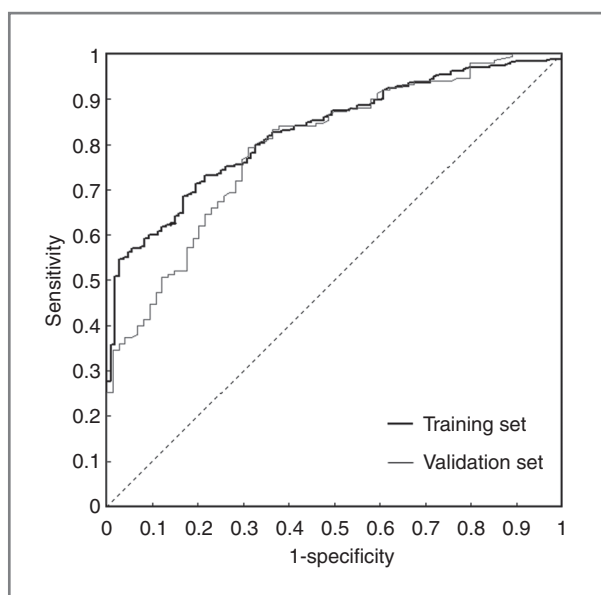
Our present study quantified use of epigenetics for cancerization with small diagnostic biopsies from normal esophageal mucosae. We confirmed that this approach is equivalent to the traditional risk factor method, which typically requires a comprehensive set of clinical data. The biological significance of the epigenetic approach is supported by studies investigating the pathogenesis of SCC, which find that *HOXA9* protein expression is deregulated in SCC (32), that oncogenesis is triggered by silencing *NEFH* to activate the Akt/ $\beta$ -catenin pathway (33), that the prognosis of SCC is worsened by hypermethylating *UCHL1* (34) that interacts with *p53* through the deubiquitination pathway (35), and that overexpressing *MT* is associated with metastatic and proliferative activity (36). None of these 4 genes had previously been correlated with carcinogen exposure or with cancer risk prediction.

Our study provided abundant information regarding the effects of traditional risk factors. As expected, ABCs were strong determinants that led to a lower BMI and a higher MCV, indicating poor nutritional status (7). However, judgment-based merely on the overall exposure to ABC was insufficient because the false-positive rate (55%) was high. Both of the infectious pathogens *H. pylori* and human papillomavirus were not associated with SCC risk (37, 38). Although several risk alleles have been previously identified (10–13), they served as effect modifiers rather than direct causes. Although the value of endoscopic LVLs has been confirmed, the usefulness of Lugol staining as a first-line screening tool is limited by a lower sensitivity (50%), in addition to significant side effects. A substantial proportion of subjects with cancer risk would be lost from surveillance if the risk assessment was solely based on the presence of endoscopic LVLs. However, the above limitations of traditional risk factors might support the credibility of our findings since our findings are similar to other literature reports (summarized in the Supplementary Table S2; refs. 15, 19, 20).

Taking into consideration both measured and unmeasured risk factors/genetic susceptibilities, molecular information derived from histologically normal mucosae has been extensively investigated, such as the field effects of oral leukoplakia, Barrett's esophagus (39), and colon neoplasm (40, 41). Mutations of *p53* or *p53* oncoprotein are relatively late events in carcinogenesis (42, 43), so, despite their biological plausibility as cancer predictors, they are insufficient for risk assessments for SCC in the UADT. Because aberrant DNA methylation usually precedes neoplastic transformation, qualitative studies have suggested that the presence of methylated genes may increase in a sequence from normal mucosa to precursor lesions to SCC (44–46). Our study further found that this phenomenon is quantitative for individual gene markers.

Research on clinical applications found that a candidate gene approach measuring the methylation of *AHRR*, *p16INK4a*, *MT1G*, and *CLDN3* had unsatisfactory sensitivity of 50% and specificity value of 68% when screening for SCC in China with esophageal-balloon cytology (47). In contrast, the performance of our 4-gene panel may effectively identify subjects who will benefit from endoscopic surveillance. Our panel's superiority may be due to our





**Figure 2.** ROC curve shows the prediction of upper aerodigestive tract cancer with epigenetic markers in the training and validation sets. The area under the ROC curve was 83% (bootstrap 95% CI: 79%–87%) in the training set and 80% (bootstrap 95% CI: 73%–85%) in the validation set (training vs. validation ROC,  $P = 0.43$ ). The dashed line represents a test of no discrimination.

meticulous search for candidate genes by oligonucleotide microarray, which decreased bias in choosing targets (30), and to using qMSP to extract full information without truncating the methylation value (23). Imaging-enhanced endoscopies minimized the risk of contamination during sample collection (28, 29). The similar methylation patterns between the 2 ethnically different populations from Japan and Taiwan further strengthens the generalizability of our approach. Our approach is accurate, inexpensive with the cost estimated at one USD per specimen marker, and high throughput. This can allow surveillance with sophisticated technologies to focus on high-risk patients, which is potentially cost effective.

Intriguingly, the *HOXA9* gene, which is essential for embryogenesis and organogenesis, was identified as the most informative marker. This finding underscored the significance of *HOX* genes in regulating cell proliferation and differentiation, which is a balance that shifts in tumorigenesis (48). Also noteworthy are the contributions of overall exposure to ABC and of endoscopic LVLs, which remained significant in the combined model that yielded the highest AUC of 91%. This result may partially reflect the design of our screening procedure on the basis of alcohol

and cigarette use (30) rather than on betel quid chewing. Betel quid chewing is a well-recognized class-1 carcinogen, so residual confounding cannot be ruled out. Recruiting more patients with heavy exposure to betel quid would be worthwhile because markers tailored to this substance could be identified by methylated DNA immunoprecipitation microarray (49).

Our risk-assessment models predicted the risk of actually finding SCC at the endoscopy when the biopsies were taken. The results may need to be verified by longitudinal data following the development of aberrant methylation in those with ongoing exposure to carcinogens. This would allow us to elucidate how many individuals with positive methylation markers would eventually go on to develop SCC (50). Individuals with normal mucosae, low methylation levels, and exposure to carcinogens may also benefit from risk stratification by modifying lifestyle risk factors to reduce cancer risk. In addition, although similar etiologic factors are involved in the development of head and neck cancer and esophageal cancer, they are not created equally. Our patients with head and neck cancer were mainly composed of those with hypopharyngeal cancer, so our results may not be so readily applied to those with oral cavity cancer. Therefore, this topic warrants further investigation.

In conclusion, we successfully quantified the field for cancerization using epigenetic markers. Our work provides a better model for risk assessment and may potentially be generalized across the high-risk regions to allocate the limited endoscopic resources for the surveillance and early detection of UADT cancers.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

The authors thank Luan-Yin Chang, MD, PhD, Department of Pediatrics, College of Medicine, National Taiwan University, for helping to determine the cutoff point to positively determine human papillomavirus infection.

#### Grant Support

This study was supported by research grants from the National Science Council (NSC96-2314-B-002-092-MY3) and the Taipei Institute of Pathology.

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Received February 22, 2011; revised July 15, 2011; accepted August 31, 2011; published OnlineFirst September 27, 2011.

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# Cancer Prevention Research

## Revisit of Field Cancerization in Squamous Cell Carcinoma of Upper Aerodigestive Tract: Better Risk Assessment with Epigenetic Markers

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*Cancer Prev Res* 2011;4:1982-1992. Published OnlineFirst September 27, 2011.

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