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DISEASES OF THE ESOPHAGUS

Original article

Aberrant promoter CpG island hypermethylation of the adenomatosis polyposis coli gene can serve as a good prognostic factor by affecting lymph node metastasis in squamous cell carcinoma of the esophagus

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SUMMARY. **There has been no clear evidence demonstrating whether DNA hypermethylation can affect the prognosis of esophageal cancer. We collected tissue from 50 cases of squamous cell carcinoma of the esophagus and tested them for DNA hypermethylation using methylation-specific polymerase chain reaction. CpG island hypermethylations were observed in 10% for p16, 34% for RARβP2, 46% for adenomatosis polyposis coli (APC), 14% for RASSF1A, 84% for FHIT, and 8% for hMLH1. APC promoter hypermethylation was frequently found in patients without lymph node metastasis compared with those with lymph node metastasis (62.5% : 30.8%, $P = 0.025$). The number of metastatic lymph nodes were lower in patients with APC promoter hypermethylation ($0.87 \pm 0.30 : 3.07 \pm 0.72$, $P = 0.008$). Excluding operative mortalities and incomplete resections, 42 patients were analyzed for long-term outcome. During the mean follow-up period of 35 months, 17 developed recurrence and 14 died of cancer. Ten patients died of other causes. In univariable analysis, unmethylation of APC ($P = 0.0015$) and FHIT ($P = 0.0044$), as well as presence of lymph node metastasis ($P = 0.0038$), were risk factors for recurrence. In multivariable analysis, lymph nodes metastasis ($P = 0.050$) and unmethylation of APC promoter ($P = 0.023$) remained as significant risk factors. In conclusion, promoter hypermethylation of the APC gene is related to a lower number of metastatic lymph nodes and to superior prognosis in terms of recurrence, which suggests it might be involved in the process of lymph node metastasis in esophageal cancer.**

KEY WORDS: **APC, esophageal neoplasms, genes, lymph nodes, methylation, neoplasm metastasis.**

INTRODUCTION

Epigenetic changes of DNA have been recently highlighted and DNA methylation study is one of the best studied subjects in the field of cancer epigenetic. Various researches have been performed to identify the clinical significance of DNA methylation patterns in malignant tumors. However, only a limited number of reports have been published for the aberrant promoter hypermethylation in esophageal squamous cell carcinoma. Although the incidence of esophageal cancer is not very high, survival rates remain low despite the recent improvement of treatment strategies. However, none of the molecular markers have been addressed to predict its long-term outcome of esophageal cancer.

We examined whether aberrant promoter hypermethylation of various tumor suppressor genes could be used to predict clinical outcomes of patients with esophageal squamous cell carcinoma after surgical resection, focusing on lymph node metastasis. We also tried to investigate a possible mechanism in which DNA methylation affects lymph node metastasis.

MATERIALS AND METHODS

Sample collection

Among the 161 patients who underwent surgical resection for esophageal carcinoma between October 2000 and May 2005 at Seoul National University Hospital, we selected 50 patients whose histological

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cell type was squamous cell carcinoma and whose frozen specimens were available from our Thoracic Cancer Tissue Bank. Informed consents for tissue collection and gene analyses for research purposes were obtained from individual patients preoperatively according to the policy of the Thoracic Cancer Tissue Bank, Cancer Research Institute, Seoul National University. Recommendations from the Declaration of Helsinki for Biomedical Research involving Human Subjects were also followed. The study protocol, as well as ethical issues, was reviewed by the Seoul National University Hospital IRB (C-0701-004-193).

Forty-seven patients were male and three were female (mean age, 64 years; range, 49–78 years). Esophagectomy and subsequent esophagogastrostomy were performed either at the thoracic or at the cervical level, depending on the location of the tumor. Radical mediastinal and perigastric lymph node dissection were performed in all patients. Two patients had a history of lung cancer, which had been resected curatively, and two patients received preoperative chemotherapy. Patients whose lymph nodes were metastasized by the tumor received postoperative irradiation treatment or systemic chemotherapy. Cancer tissues were obtained from each patient immediately after esophagectomy, quickly frozen in liquid nitrogen, and stored at –80°C until analysis.

DNA preparation, bisulfite modification, and methylation-specific polymerase chain reaction (PCR)

Genomic DNA samples from frozen esophageal cancer tissue were isolated by the use of the GeneAll® Tissue SV Plus (GeneAll Biotechnology, Seoul, Korea), according to the manufacturer's protocol. One μg of DNA was digested with restriction enzyme (Hind III, Intron Biotechnology, Sungnam, Korea) and bisulfite modification was performed by use of the CpGenome™ Fast DNA Modification Kit (Chemicon, Temecula, CA, USA). Because treatment of genomic DNA with sodium bisulfite converts unmethylated cytosine to uracil whereas it does not convert methylated cytosine, the DNA sequences become different according to the methylation status. PCR primers specific for either methylated or unmethylated DNA sequences were designed. We searched promoter methylation of p16 ^{INK4a}(p16), RARβP2, adenomatosis polyposis coli (APC), FHIT, hMLH1, and RASSF1A genes. Primer sequences of p16 were 5′-GTT TGG AAA GAT ATC GCG GT-3′ (sense) and 5′-CCA CCA ACT CCA TAC TAC TC-3′ (antisense) for the unmethylated reaction and 5′-TGG AAA GAT ATC GCG GTT TT-3′ (sense) and 5′-CCG CCG ACT CCA TAC TAC TC-3′ (antisense) for methylated reaction. The primer sequences of RARβ P2 for the unmethylated reaction were 5′-TTG AGA ATG TGA GTG ATT TGA-3′ (sense) and 5′-AAC CAA TCC AAC CAA AAC AA-3′ (antisense), and for the methylated reaction, they were 5′-TCG AGA ACG CGA GCG ATT CG-3′ (sense) and 5′-GAC CAA TCC AAC CGA AAC GA-3′ (antisense). The primer sequences of APC were 5′-GTG TTT TAT TGT GGA GTG TGG GTT-3′ (sense) and 5′-CCA ATC AAC AAA CTC CCA ACA A-3′ (antisense) for the unmethylated reaction, and 5′-TAT TGC GGA GTG CGG GTC-3′ (sense) and 5′-TCG ACG AAC TCC CGA CGA-3′ (antisense) for the methylated reaction. The primer sequences of FHIT for the unmethylated reaction were 5′-TTG GGG TGT GGG TTT GGG TTT TTA TG-3′ (sense) and 5′-CAT AAA CAA CAC CAA CCC CAC TA-3′ (antisense), and for the methylated reaction, the sequences were 5′-TTG GGG CGC GGG TTT GGG TTT TTA CGC-3′ (sense) and 5′-CGT AAA CGA CGC CGA CCC CAC TA-3′ (antisense). The primer sequences of hMLH1 for the unmethylated reaction were 5′-GGT TAT GGG TAA GTT GTT TTG-3′ (sense) and 5′-CCT AAT CTA TCA CCA CCT CAT C-3′ (antisense), and for the methylated reaction, the sequences were 5′-ACG GGT AAG TCG TTT TGA CG-3′ (sense) and 5′-TAA TCT ATC GCC GCC TCA TC-3′ (antisense). The primer sequences of RASSF1A for the unmethylated reaction were 5′-GGT TTT GTG AGA GTG TGT TTA-3′ (sense) and 5′-CAC TAA CAA ACA CAA ACC AAA C-3′ (antisense); for the methylated reaction, they were 5′-GGG TTT TGC GAG AGC GCG-3′ (sense) and 5′-GCT AAC AAA CGC GAA CCG-3′ (antisense). All PCR amplifications were performed using the Eppendorf thermocycler (Mastercycler®, Eppendorf Corp, Germany) with tube control for accurate annealing temperatures. Every reaction was performed with the hot-start method using the Qiagen HotStart Master Mix Kit (Qiagen Corp, Valencia, CA, USA). The PCR conditions of the four genes were as follows: 95°C for 15 minutes, then 35 cycles of 94°C for 30 seconds, the specific annealing temperature for 45 seconds and 72°C for 1 minute, and a final extension of 5 minutes at 72°C. The specific annealing temperatures of the six genes were 62°C for the methylated p16 gene, 53°C for the unmethylated p16 gene; 57°C for the methylated RARβ P2 gene, 55°C for the unmethylated RARβ P2 gene; 64°C for the methylated APC gene, 58°C for the unmethylated APC gene; 66°C for the methylated FHIT gene, 62°C for the unmethylated FHIT gene; 66°C for the methylated hMLH1 gene, 55°C for the unmethylated hMLH1 gene; and 64°C for the methylated RASSF1A gene, 55°C for the unmethylated RASSF1A gene. CpGenome™ Universal Methylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for methylated alleles of each gene. Water control was added as a negative control. The

PCR products were analyzed on a 2.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination.

Immunohistochemical staining

We made a tissue microarray from paraffin blocks of each specimen using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Immunohistochemical staining was performed for APC (Abcam, Cambridge, UK) using the Ultra LP Detection kit from Labvision (Fremont, CA, USA), for β-catenin (BD Transduction laboratories, Lexington, KY, USA) using the Vector kit (Vector laboratories, Burlingame, CA, USA), and for E-cadherin (Novocastra, Newcastle, UK) using the LSAB kit (DAKO, Carpinteria, CA, USA), according to the manufacturer's protocol. Briefly, sections were deparaffinized, rehydrated, and then heated in a microwave for 15 minutes within a citric acid buffer (0.01 M, pH 6.0) for antigen retrieval. After blocking endogenous peroxidase activity with 3% hydrogen peroxide, slides were immersed in the blocking solution, followed by incubation with the primary antibody for 1 hour at room temperature. For APC, primary antibody enhancer and horseradish peroxidase polymer (Labvision Ultra LP Detection kit) were sequentially applied. For β-catenin, biotinylated secondary antibody and avidin-biotinylated peroxidase (Vector kit) were used, and for E-cadherin, biotinylated secondary antibody and peroxidase-labeled streptavidin were sequentially applied. The sections were visualized using 3,3′-diaminobenzidine and counterstained with Mayer's hematoxylin. For negative controls, normal rabbit IgG was used instead of the primary antibody.

Immunohistochemical reactivity for APC was classified into three groups, 0 (no reactivity), 1+ (weak reactivity), and 2+ (moderate to strong reactivity). For β-catenin, we followed the evaluation method previously described.[1] The intensity of membranous staining of β-catenin was graded as 0 (negative, complete loss of membrane expression), 1+ (weak), 2+ (moderate) or 3+ (strong), and the proportion of tumor cells showing membranous expression was graded as 0 (<5% of tumor cells), 1 (5–25%), 2 (26–50%), 3 (51–75%), or 4 (>75%). A final score of each sample was achieved by multiplying the intensity and the proportion value; we then defined final scores of 6–12 as preserved membranous expression, and 0–5 as reduced expression. Cytoplasmic expression of β-catenin was classified as follows: 0, negative (no cytoplasmic staining); 1+, <5% of tumor cells with cytoplasmic staining; 2+, 5–25%; 3+, >25%. We defined the score 0 and 1 as preserved cytoplasmic staining and 2–3 as accumulated expression. Nuclear staining for β-catenin was scored as follows: negative (no nuclear staining), 1+ (1–5% of tumor cells with nuclear staining), 2+ (5–10%), 3+ (>10%). With respect to E-cadherin staining, if more than 50% of tumor cells showed distinct membranous staining, the tumor was defined as having preserved expression (score 2+). If 5–50% of tumor cells showed membranous staining, the tumor was deemed as reduced expression (score 1+). Membranous staining in less than 5% of tumor cells was considered as negative (score 0).

Data collection and statistical analysis

We collected clinical variables including gender, age, pathological TNM stage, number of positive lymph nodes, development of recurrence, and survival. Among the 50 patients, six patients underwent incomplete resection (five microscopic and one gross residual tumor) and two patients died early postoperatively. Excluding those eight patients, 42 patients were followed regularly at the clinic. The medical records were collected until the closing date of August 31, 2007. The mean follow-up period was 35 ± 21 months (range, 4–81 months). Careful history taking, physical examinations, and chest X-rays were performed every 3 months, and chest computed tomography scans and esophageal contrast studies were obtained on an annual basis. If there were any symptoms or observations suggesting recurrence, additional evaluations were performed.

Statistical differences between groups were examined by the use of χ^2-test and Fisher's exact test with continuity corrections. As the distribution of patients' number in each category was not appropriate for analysis, we merged it as follows: for the age, older (≥60 years) and younger (<60 years); for the T stage, early (≤T2) and late T stage (>T2); for the immunohistochemical staining of APC, negative (score 0) and positive (score 1+ and 2+); for immunohistochemical staining of E-cadherin, negative (score 0) and positive (score 1+ and 2+). Overall survival and recurrence patterns were investigated by using the Kaplan–Meier method, and the difference between groups determined by risk factors were tested by using the log–rank test. Cox regression analysis was used to explore the influence of independent prognostic factors in a multivariable model. The factors were chosen by a stepwise forward method, with criteria for variable inclusion and exclusion of 0.10. A 5% significance level was considered for statistical significance.

RESULTS

Promoter methylation and clinical features

Promoter CpG island hypermethylations were observed in 10% (5/50) for p16, 34% (17/50) for

Table 1 Association between DNA methylation and clinicopathological features of esophageal squamous cell carcinoma

	p16		RARβP2		APC		FHIT		hMLH1		RASSF1A	
Feature	U	M	U	M	U	M	U	M	U	M	U	M
Male	42	5	31	16	26	21	8	35	43	4	42	5
Female	3	0	2	1	1	2	0	3	3	0	1	2
	$P = 1.000$		$P = 1.000$		$P = 0.588$		$P = 1.000$		$P = 1.000$		$P = 0.048$	
Age ≤60	12	0	8	4	8	4	3	9	12	0	11	1
Age >60	33	5	25	13	19	19	5	33	34	4	32	6
	$P = 0.319$		$P = 1.000$		$P = 0.313$		$P = 0.379$		$P = 0.560$		$P = 1.000$	
T1, T2	14	1	11	4	8	7	4	11	13	2	12	3
T3, T4	31	4	22	13	19	16	4	31	33	2	31	4
	$P = 1.000$		$P = 0.474$		$P = 0.951$		$P = 0.220$		$P = 0.574$		$P = 0.415$	
N0	21	3	15	9	9	15	4	20	21	3	20	4
N1	24	2	18	8	18	8	4	22	25	1	23	3
	$P = 0.661$		$P = 0.616$		$P = 0.025$		$P = 1.000$		$P = 0.340$		$P = 0.697$	

U, unmethylated; M, methylated.

RARβP2, 46% (23/50) for APC, 14% (7/50) for RASSF1A, 84% (42/50) for FHIT, and 8% (4/50) for hMLH1 from the cancer tissue.

Pathological TNM stages included stage I in eight patients, IIA in 15 patients, stage IIB in four patients, stage III in 22 patients, and stage IV in one patient. The numbers of harvested lymph nodes were 34.8 per patient (2–118). We analyzed for an association between T or N stage and individual gene methylation. APC promoter hypermethylation was frequently found in patients without lymph node metastasis compared with those with lymph node metastasis (62.5%; 15/24 vs 30.8%; 8/26, $P = 0.025$). Other genes, however, did not show significant association with T or N stage (Table 1).

We analyzed the number of metastatic lymph nodes according to the methylation status of the APC gene. The number of metastatic lymph nodes was lower in patients with APC promoter hypermethylation (0.87 ± 0.30 vs 3.07 ± 0.72 nodes, $P = 0.008$, Figure 1).

Excluding patients of operative mortality and those who underwent incomplete resections, 42 patients were analyzed for long-term outcome. During the follow-up period, 17 patients (40.5%) developed recurrence and 14 died. Additional 10 patients died without evidence of recurrence. Clinical variables such as gender, age, TNM stages, and gene methylation patterns were tested as to whether they affect clinical outcomes. For survival analysis, none of the methylation status of the six genes affected survival. When we analyzed recurrence, unmethylation of APC ($P = 0.0015$) and FHIT ($P = 0.0044$), as well as the presence of lymph node metastasis ($P = 0.0038$), were risk factors in univariable analysis (Figure 2). In the Cox regression model, we found that lymph nodes metastasis ($P = 0.050$) and unmethylation of APC promoter ($P = 0.023$) remained as significant risk factors (Table 2).

APC promoter methylation and immunohistochemical staining

To evaluate downstream proteins of the Wnt/β-catenin signal pathway, we compared promoter methylation of APC with the immunohistochemical stain results of APC, β-catenin, and E-cadherin. However, there was no correlation between APC promoter hypermethylation and the APC immunohistochemical stain ($P = 0.539$). We were not able to find any significant correlation between APC methylation and β-catenin protein expression either (Figure 3). We then included the Wnt/β-catenin pathway molecules along with the clinical variables for the analysis of recurrence. In univariable analysis, none of the

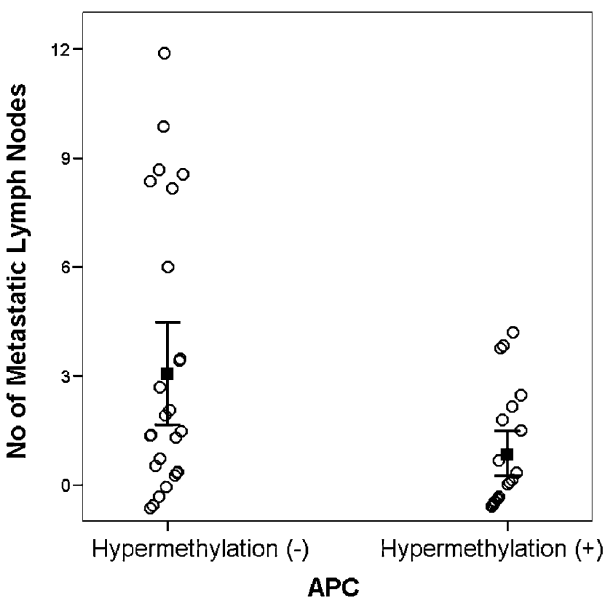

Fig. 1 Comparison of number of lymph nodes metastasized by the tumor according to the adenomatosis polyposis coli (APC) hypermethylation.

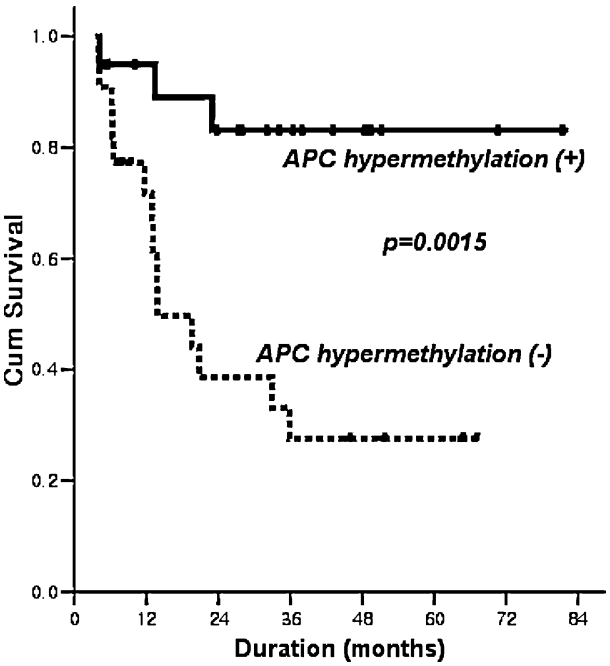

Fig. 2 Probability of having recurrence in patients with resected esophageal squamous cell carcinoma in relation to adenomatosis polyposis coli (APC) promoter hypermethylation. A statistically significant difference in recurrence rate was observed between APC promoter methylation-positive and negative groups ($P = 0.0034$).

immunohistochemical stain results showed significance as a risk factor for recurrence. However, in the Cox regression model, we found that lymph node metastasis ($P = 0.004$), unmethylation of APC promoter ($P = 0.028$), increased expression of APC ($P = 0.027$), and decreased expression of E-cadherin ($P = 0.020$) remained as significant risk factors for the development of recurrence (Table 3).

DISCUSSION

In this study, we found that aberrant promoter hypermethylation frequently occurs in esophageal squamous cell carcinoma, as in other malignancies. Among the six genes we tested, FHIT, APC, and RARβP2 were more frequently hypermethylated with prevalence of 84, 46, and 34%, respectively, whereas that of p16, RASSF1A, and hMLH1 was low. There are limited published reports concerning DNA methylation of esophageal squamous cell carcinoma; hence, not enough information is available. APC promoter methylation was reported as one of the most frequent epigenetic alteration in esophageal adenocarcinoma ranging from 68 to 92% of tumors.[2,3] However, in esophageal squamous cell carcinoma, the frequency of APC methylation was reported to be as low as 50%.[4] Our series, where a larger number of samples had been used, showed similar results of 46%. According to the literature, the frequency of RARβP2 methylation in squamous cell carcinoma of the esophagus varies from 25–55%,[5,6] which is similar to our result of 34%. It is of interest that the frequency of p16 methylation was only 10% in our series, whereas others reported as high as 64–80%.[7,8] In a different paper, however, it was reported as only 18%.[9] For hMLH1, the frequency of methylation is more controversial. The reported frequency varies from as low as 20%[9,10] to as high as 62–67%.[11,12] Our data showed only 8%. The situation is similar for FHIT. The reported frequency of methylation for FHIT varies from 14 to 70%.[6,9,13–16] Our result demonstrated 84%, which is the highest frequency reported. For RASSF1A, the reported frequency varies from 24 to 51%[6,17,18] and is a little higher than our own data of 14%. We do not have a clear explanation for such a large difference. Possible explanations may be ethnic differences, different patient selection, or a different set of PCR primer sequence used. These discrepancies could also be attributed to an intrinsic problem of specificity related to PCR.

As the aberrant promoter hypermethylation of tumor suppressor genes will result in decreased expression of tumor suppressor protein, it is logical to postulate that promoter methylation will offer a

Table 2 Risk factors for freedom from recurrence: univariable and multivariable analysis of the clinical factors and gene hypermethylation

Factors (risk)	Log rank *P*-value	Cox regression	
		HRP	*P*-value
Gender (male)	0.5993	–	–
Age (≥60)	0.7904	–	–
T stage (>2)	0.4739	–	–
N stage (N1)	0.0038	3.196 (1.001–10.207)	0.050
p16 (methylated)	0.7039	–	–
RARβP2 (methylated)	0.2089	–	–
APC (methylated)	0.0015	0.226 (0.063–0.814)	0.023
FHIT (methylated)	0.0044	–	–
hMLH1 (methylated)	0.2528	–	–
RASSF1A (methylated)	0.3815	–	–

APC, adenomatosis polyposis coli; HR, hazard ratio.

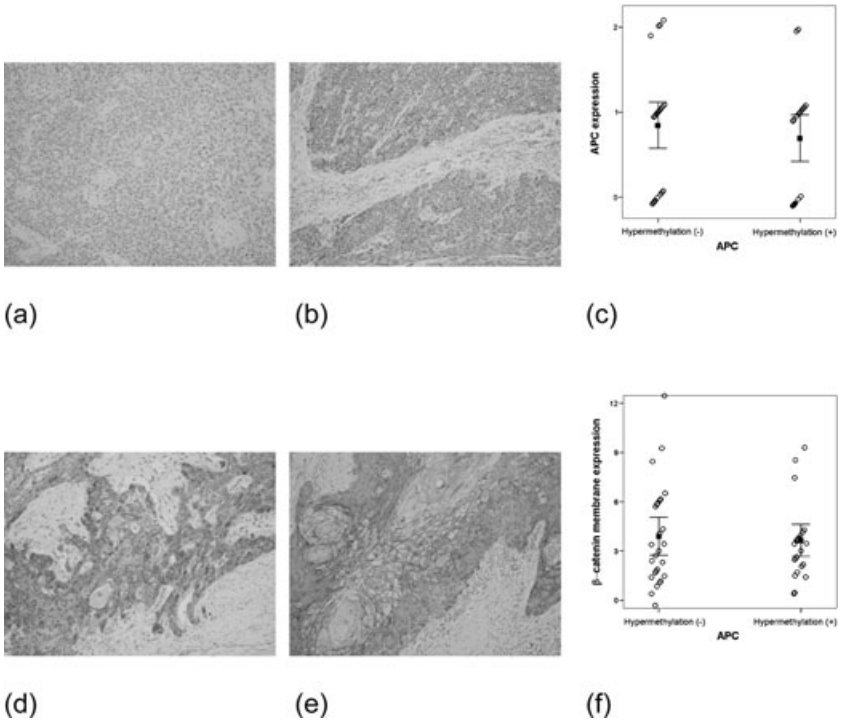

Fig. 3 Immunohistochemical staining of adenomatosis polyposis coli (APC) and β-catenin proteins. Note (a) a negative and (b) a positive staining for APC; (d) a reduced and (e) a preserved staining of β-catenin on the cell membrane (×200). There was no correlation between APC promoter hypermethyaltion and expression of either (c) APC or (f)membranous β-catenin.

poor prognosis. In esophageal adenocarcinoma, for example, Brock and associates suggested that positive methylation status for multiple genes in esophageal adenocarcinoma was a predictor of poor survival.[2] However, in our reports, the aberrant promoter hypermethyation of APC gene, which is a well-known tumor suppressor gene, showed strong correlation with improved outcome in terms of recurrence. We also found that the APC promoter hypermethylation was closely correlated with N staging. When we further analyzed the number of metastasized lymph nodes, APC hypermethyation was related to a fewer number of metastasized lymph nodes. Based on these observations, we postulated that the hypermethylation of APC may influence a protective effect on the malignant cells from being metastasized either into lymph nodes or into the systemic circulation.

Invasion and metastases, the most life-threatening properties of malignant tumors, consist of sequential changes in host–tumor interaction. The suppression of cell–cell adhesiveness may trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumor.[19] Reduced cell–cell adhesiveness is thus considered indispensable for both the early and the late carcinogenesis steps. In such cell–cell adhesion, E-cadherin–catenin adhesion complex has been considered as the prime mediators of calcium-dependant cell–cell adhesion.[20] More evidence is now appearing to suggest that an inverse correlation is found between the expression of the E-cadherin–

Table 3 Risk factors for freedom from recurrence: univariable and multivariable analysis of the clinical factors and the Wnt/β-catenin pathway molecules

Factors (risk)	Log rank *P*-value	Cox regression	
		HRP	*P*-value
Gender (male)	0.5993	–	–
Age (≥60)	0.7904	–	–
T stage (>2)	0.4739	–	–
N stage (N1)	0.0038	6.850 (1.843–25.463)	0.004
APC methylation (methylated)	0.0015	0.215 (0.055–0.846)	0.028
APC staining (positive)	0.3916	4.034 (1.174–13.859)	0.027
β-catenin staining (preserved)	0.6467	–	–
E-cadherin staining (positive)	0.7431	0.047 (0.004–0.617)	0.020

APC, adenomatosis polyposis coli; HR, hazard ratio.

catenin complex and the invasive behavior of tumor cells.

To explain our result, we hypothesized that APC methylation can perform as a protector of malignant cell metastasis by affecting the E-cadherin–catenin cell–cell adhesion complex through the Wnt/β-catenin pathway. It is well known that APC acts as a scaffold to promote phosphorylation, ubiquitination, and degredation of β-catenin, thus regulating the level of free β-catenin in the cell. For example, mutation of APC results in truncated APC protein, which can still complex with, but not degrade, β-catenin.[21] The result of APC mutation is therefore an increase in cellular free β-catenin, which will hold cells together by binding to E-cadherins, both at the adherens junctions and the actin cytoskeleton.[22] On the other hand, the increased level of cellular free β-catenin may enter the nucleus and directly bind to the transcription factors Lef and Tcf, leading to the activation of gene expression, which subsequently triggers a cascade of tumor formation.[23–25] Besides point mutation or deletion, aberrant hypermethylation was found to act as one of the mechanisms of APC gene inactivation in colorectal cancer.[26] Our postulation is that when APC promoter hypermethylation occurs, APC protein will decrease, and subsequently, the level of cellular free β-catenin will increase. The increased level of β-catenin will result in increased E-cadherin–catenin complex, which confers a protective effect of tumor cells from metastasis. There is additional literature that supports our hypothesis. By isolating and analyzing highly invasive clones in oral cancer cells, Kudo and colleagues demonstrated that invasion and metastasis of oral squamous cell cancer cells require methylation of E-cadherin and/or degradation of membranous β-catenin.[27] So far, none of the literature has reported a correlation between APC methylation and lymph node metastasis in either esophageal cancer or other type of cancers.

It is noteworthy that the finding of aberrant promoter hypermethylation of any gene demands further investigation but does not imply functional significance until the promoter methylation can be associated with the loss of expression. To prove our hypothesis, we investigated downstream proteins of Wnt/β-catenin pathway by performing immunohistochemical staining for APC, β-catenin, and E-cadherin. Unfortunately, however, we could not clearly demonstrate a statistically significant correlation between APC methylation status and its downstream protein, APC and β-catenin. Although we failed to associate APC methylation with loss of protein expression, we believe it can be attributed to using clinical samples that are heterogenous in the aspect of molecular events. Because DNA methylation is not a single mechanism for gene silencing in carcinogenesis, the research using clinical samples can be frequently subjected to bias. For example, in our previous reports of methylation study for lung cancer, the promoter hypermethylation did not always coincide with decreased expression of downstream proteins.[28,29] Even though there was no significance in univariable analysis, we were able to demonstrate the expression levels of APC and E-cadherin as well as methylation status of APC promoter, which were related to the recurrence in multivariable analysis. It is indeed interesting because the odd ratios of each factor coincide well with our hypothesis (Table 3).

Our observation suggested that APC promoter hypermethylation might affect the metastasis of esophageal squamous cell carcinoma by means of the Wnt/β-catenin signal pathway. Because we had not calculated an appropriate sample size at the time of study design, the power of testing of Cox proportional hazard model may not be limited. To further convince our result, we performed post-hoc testing for APC methylation using PASS statistical package (NCSS, Kaysville UT, USA). The power of post-hoc testing for APC methylation was 0.77243 in Table 2, and 0.74929 in Table 3. These high values of post-hoc testing power suggested that the alternative hypothesis, "APC methylation affects recurrence," could be supported in high probability. However, because the sample size of our study was small and had not been determined prospectively, and because the follow-up period was relatively short, our study has a limitation for generalization. Accordingly, future investigations are mandatory to clarify the mechanism of APC gene methylation for preventing lymph node metastasis. To achieve such a goal, future endeavors, such as larger series studies including far advanced stage patients and testing metastasized lymph nodes, are necessary. For more concrete evidence, we need *in vitro* studies where one can demonstrate the reversal of downregulation of APC protein and increase of cellular β-catenin when hypermethylated APC is reactivated by demethylation.

Although we were not able to demonstrate a clear mechanism, we demonstrated that APC promoter hypermethylation was associated with fewer occurrences of lymph node metastasis and, as a consequence, resulted in better clinical outcome in respect to recurrence-free status. Our observation may be used to identify high-risk patients who may possess occult lymph node metastasis, and subsequently be used to identify patients who need more aggressive lymph node dissection or who can receive benefits from postoperative adjuvant systemic chemotherapy. Additionally, our results suggested that the development of a new therapeutic modality targeting on the Wnt/β-catenin pathway may be promising in the prevention of cancer cell metastasis.

Acknowledgments

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