Adherens junctions associated protein 1 serves as a predictor of recurrence of squamous cell carcinoma of the esophagus

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Abstract. Esophageal squamous cell carcinoma (ESCC), the most common esophageal cancer in East Asia, is among the six cancers with the highest fatality rates worldwide. Unfortunately, multidisciplinary treatment strategies have not achieved satisfactory outcomes. Therefore, novel insights into the molecular biology of ESCC are required to improve treatment. The gene encoding the transmembrane adherens junctions-associated protein-1 (*AJAP1*) expressed by epithelial cells resides in chromosome 1p36, which is frequently lost or epigenetically silenced in several malignancies. Here, we investigated the expression levels and regulatory mechanism of *AJAP1* transcription. We determined the levels of *AJAP1* mRNA and the genes encoding potentially interacting proteins expressed by ESCC cell lines, as well as the chromosomal copy number of *AJAP1* and the methylation status of its promoter region. *AJAP1* mRNA levels of 78 pairs of surgically resected specimens were determined to evaluate the association of *AJAP1* expression and clinicopathological factors. Nine ESCC cell lines differentially expressed *AJAP1* mRNA, and demethylation of hypermethylated *AJAP1* genomic DNA reactivated *AJAP1* mRNA expression. The copy number of sequences upstream or downstream of the *AJAP1* transcriptional start site was not detectably altered. *AJAP1* mRNA levels correlated inversely with those of ezrin (EZR) and were significantly lower in ESCC tissues compared with adjacent normal tissues. *AJAP1* mRNA levels decreased gradually with increasing tumor stage. Patients with downregulated *AJAP1* transcription were more likely to experience shorter overall and disease-free survival. Multivariate analysis of disease-free survival identified downregulated *AJAP1* transcription as an independent prognostic factor. These results suggest that in ESCC, *AJAP1* acts as a putative tumor suppressor and that *AJAP1* transcription is regulated by promoter hypermethylation. These findings indicate that downregulated *AJAP1* transcription may serve as a novel tumor biomarker to predict recurrence of ESCC after esophagectomy.

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Introduction

Esophageal cancer ranks sixth among all cancers in mortality worldwide because of its extremely aggressive nature (1,2). The predominant histological types of esophageal cancer are adenocarcinoma and squamous cell carcinoma (3). Adenocarcinoma of the distal esophagus predominates in Western countries, whereas esophageal squamous cell carcinoma (ESCC) predominates in Asia (3). The mechanism of carcinogenesis of ESCC differs from that of adenocarcinoma, which has been widely studied in North America and Europe (3). Further, exogenous factors such as smoking, drinking, nitrosamines, and consumption of hot beverages correlate significantly with the development of ESCC but not with adenocarcinoma of the esophagus (4,5). Recent advances in our understanding of the molecular biology of ESCC document the role of genetic alterations in tumorigenesis (6,7). Therefore, a better understanding of the molecular mechanisms of progression and recurrence is of paramount importance, and identification of the genes that mediate ESCC pathogenesis will increase our understanding of the molecular and cellular processes involved and provide new biomarkers that may facilitate diagnosis, risk stratification, and monitoring recurrences of ESCC (8,9).

The transmembrane adherens junctions-associated protein-1 (AJAP1) targets the basolateral membrane of polarized epithelial cells and interacts with E-cadherin-catenin complexes of adherens junctions (10). The locus encoding AJAP1 resides in chromosome 1p36 and is frequently deleted from the genomes of various tumor cells

or is epigenetically silenced, indicating that AJAP1 acts as a tumor suppressor (11-13). Although AJAP1 is involved in cell-cell and cell-extracellular matrix interactions potentially involved in the motility, migration, and invasion of glioblastoma cells (11,14), little evidence is available on its role in oncogenesis. To our knowledge, there are no studies of the expression and regulatory mechanisms of *AJAP1* transcription in gastrointestinal cancers, including ESCC. To address these issues, we analyzed ESCC cell lines and tumor tissues to evaluate *AJAP1* expression and its regulatory mechanisms. Our results indicate that *AJAP1* expression levels provide a potential clinical biomarker of the progression and recurrence of ESCC.

Materials and methods

Sample collection. Nine ESCC cell lines (TE1, TE2, TE3, NUEC1, NUEC2, NUEC3, TT, TTn and WSSC) and a control nontumorigenic epithelial cell line (FHs74) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), or were established in our institute. Cells were stored at -80˚C using cell preservative solution (Cell Banker; Mitsubishi Chemical Medience Co., Tokyo, Japan) and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and in an atmosphere containing 5% CO_2 at 37˚C (15,16). Seventy-eight primary ESCC tissues and adjacent normal tissues were acquired from patients who underwent radical esophageal resection at Nagoya University Hospital between December 2001 and October 2013. All tissue samples were diagnosed histologically as ESCC, frozen immediately after resection, and stored at -80˚C. None of the patients underwent preoperative treatment such as chemotherapy and radiation. Specimens were classified histologically using the seventh edition of the UICC staging system for esophageal cancer.

Patients were questioned to determine their levels of alcohol consumption, and excessive alcohol consumption was defined as >210 g/week for ≥3 years (2,17). The mean age of the 78 patients was 64.9±8.2 years (mean ± standard deviation; range, 44-82 years). The male-to-female ratio was 62:16. Nine, 29, 30 and 10 patients were in stages I, II, III and IV, respectively, according to the UICC staging system (seventh edition). The median duration of patient follow-up was 73.7 months (range, 5.3-149 months) or until death. Postoperative follow-up examinations included physical examination, measurement of serum tumor markers every 3 months, and enhanced computed tomography of the chest and abdominal cavity every 6 months. Adjuvant chemotherapy was administered to selected patients according to the patient's condition and the physician's discretion.

The present study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for use of clinical samples and data was required by the Institutional Review Board at Nagoya University, Japan and was obtained from all patients (18).

Analysis of the nucleotide sequences flanking the AJAP1 transcription initiation site. Nucleotide sequence analysis was conducted to determine the presence of CpG islands around the promoter region of *AJAP1*. CpG islands were defined as follows: ≥200-bp region with a GC content >50% and CpG:expected CpG ≥0.6 identified using CpG Island Searcher software (http://cpgislands.usc.edu/) (19-21).

Methylation-specific polymerase chain reaction (MSP-PCR) and 5-aza-2'-deoxycytidine (5-aza-dC) treatment. Genomic DNA samples from 10 cell lines were subjected to bisulfite treatment, and MSP-PCR was conducted to determine the presence or absence of hypermethylation of the *AJAP1* promoter. To evaluate the influence of promoter hypermethylation on *AJAP1* transcription, ESCC cells ($1.5x10^6$) were treated with 10 μM of the DNA methylation inhibitor 5-aza-dC (Sigma-Aldrich) and cultured for 6 days with medium changes on days 1, 3 and 5.

Bisulfite sequence analysis. Genomic DNAs of ESCC cell lines treated with bisulfite were sequenced to verify the accuracy of the MSP-PCR results. After PCR amplification using specific primers (Table I), PCR products were subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA, USA). The DNAs were mixed with 3 ml of a specific primer (M13) and 4 ml of Cycle Sequence mix (ABI PRISM Terminator v1.1 Cycle Sequencing kit; Applied Biosystems, Foster City, CA, USA). Sequence analysis was conducted using an Applied Biosystems ABI310, and sequence electropherograms were generated using ABI Sequence Analysis 3.0 software (Applied Biosystems) (22).

Quantitative real-time reverse transcription-PCR (qRT-PCR). The levels of *AJAP1* mRNA were determined using qRT-PCR. Total RNA (10 μg) isolated from cell lines, 78 primary ESCCs, and adjacent normal tissues were used as templates for cDNA synthesis. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA levels (TaqMan, GAPDH Control Reagents; Applied Biosystems) were quantified to normalize expression levels. qRT-PCR was performed using the SYBR-Green PCR Core Reagents kit (Applied Biosystems) as follows: one cycle at 95˚C for 10 min, 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec. All samples were tested in triplicate, and samples without template were included in each PCR plate as negative controls. Real-time detection of SYBR-Green fluorescence was conducted using an ABI StepOnePlus Real-Time PCR system (Applied Biosystems). The expression level of each sample is shown as the value of the *AJAP1* amplicon divided by that of *GAPDH* (23,24). To identify cell adhesion molecules that may interact with AJAP1, 10 cell lines were analyzed using qRT-PCR to determine the expression levels of the ezrin (*EZR*), focal adhesion kinase (*FAK*), SRC (*SRC*), and dihydropyrimidinase-like 3 (*DPYSL3*) genes (18,25,26). Primers specific for *AJAP1*, *GAPDH*, *EZR*, *FAK*, *SRC*, and *DPYSL3* are listed in Table I.

Copy number analysis. *AJAP1* copy numbers of 10 cell lines were determined using TaqMan Copy Number Assays (Applied Biosystems). Twenty nanograms of genomic DNA was amplified using specific primer pairs according to the manufacturer's instructions. Two assays were employed

Table I. Primers and annealing temperature.

Gene	Experiment	Type	Sequence (5'-3')	Product size (bp)	Annealing temperature (˚C)
AJAP1	qRT-PCR	Forward	GTTAGCACAACGGAGCCTTC	105	60
		Reverse	GATGATCTGATGGACAGCCA		
	MSP	Forward	GGTCGCGAGTTTCGCGTTTC	184	64
		Reverse	CCGATCTCCGACTCTCGATC		
	U-MSP	Forward	GTGTTGATTGGTGGTGGAGT	152	64
		Reverse	TCCCAACACACAACTCTTAC		
	Bisulfite sequencing	Forward	GTTTTTAGGATTTAGGTGAG	316	60
		Reverse	CTACTAACTCCTAAAACTAC		
SRC	qRT-PCR	Forward	CTGACCGCATGGACCGT	107	58
		Reverse	AAGCCAACCTGTCACTTGGTA		
EZR	qRT-PCR	Forward	GATAGTCGTGTTTTCGGGGA	91	60
		Reverse	CTCTGCATCCATGGTGGTAA		
FAK	qRT-PCR	Forward	GCCAAAAGGATTTCTAAACCAG	110	64
		Reverse	CCTGGTCCACTTGATCAGCTA		
DPYSL3	qRT-PCR	Forward	AGAAGAAGGAGGGAGGGAGC	110	60
		Reverse	CTCCCTTGATAAGGAGACGG		
GAPDH	qRT-PCR	Forward	GAAGGTGAAGGTCGGAGTC	226	60
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

AJAP1, adherens junctions associated protein 1; SRC, cellular SRC; EZR, ezrin; FAK, focal adhesion kinase; DPYSL3, dihydropyrimidinase-like 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; MSP, methylation specific PCR; U-MSP, un-methylation specific PCR.

as follows: upstream (assay ID Hs04540488_cn, chromosome 1, map position 4798221 in *AJAP1* intron 2) and downstream (assay ID Hs01575789_cn, chromosome 1, map position 4834502 in the intron 4 to exon 5 of *AJAP1* gene). Data were analyzed using CopyCaller software (Invitrogen Life Technologies, Carlsbad, CA, USA). Copy number loss was defined as the copy number value equal to 1 determined in the regions upstream, downstream, or both of the *AJAP1* loci.

Statistical analysis. Correlations between the levels of *AJAP1* mRNA with those of *EZR*, *FAK*, *SRC*, and *DPYSL3* were analyzed using the Spearman's rank correlation test. Differences in the levels of *AJAP1* mRNA between ESCC and adjacent normal tissues were analyzed using the Mann-Whitney test. The χ^2 test was used to analyze the significance of the association between the expression levels of *AJAP1* and clinicopathological parameters. Overall and disease-free survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. We performed multivariate regression analysis using the Cox proportional hazards model to identify prognostic factors, and variables with P-values <0.05 were entered into the final model. Statistical analyses were performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA). P<0.05 was considered statistically significant.

Results

Expression, methylation, and copy number analysis of cell lines. *AJAP1* harbors a CpG island (length 3305 bases, 70.3% GC, and Observed CpG:Expected CpG=0.883) flanking the transcription initiation site (Fig. 1A). *AJAP1* mRNA expression levels differed among the nine ESCC cell lines, and five expressed levels <50% of that of the control FHs74 cells (Fig. 1B). MSP-PCR detected methylation of the DNAs of NUEC2, TE1, TE2, and TTn cells, which expressed reduced levels of *AJAP1* mRNA. When we compared the levels of *AJAP1* mRNA in ESCC cell lines before and after demethylation, reactivation of *AJAP1* transcription was detected in cells with complete methylation of *AJAP1* before treatment with 5-aza-dC. Moreover, there was no detectable loss of copy number in ESCC cell lines and FHs74 cells (Fig. 1B).

Bisulfite sequence analysis. Sequence analysis revealed that all CpG sites in TTn DNA (complete methylation) were CG (cytosine and guanine) and that the corresponding positions in NUEC1 DNA (absence of methylation) were TG (thymine and guanine) (Fig. 2). These results confirm the MSP-PCR results.

Analysis of the levels of AJAP1 mRNA and those representing potentially interacting molecules. We evaluated the expression levels of genes encoding other cell adhesion molecules that

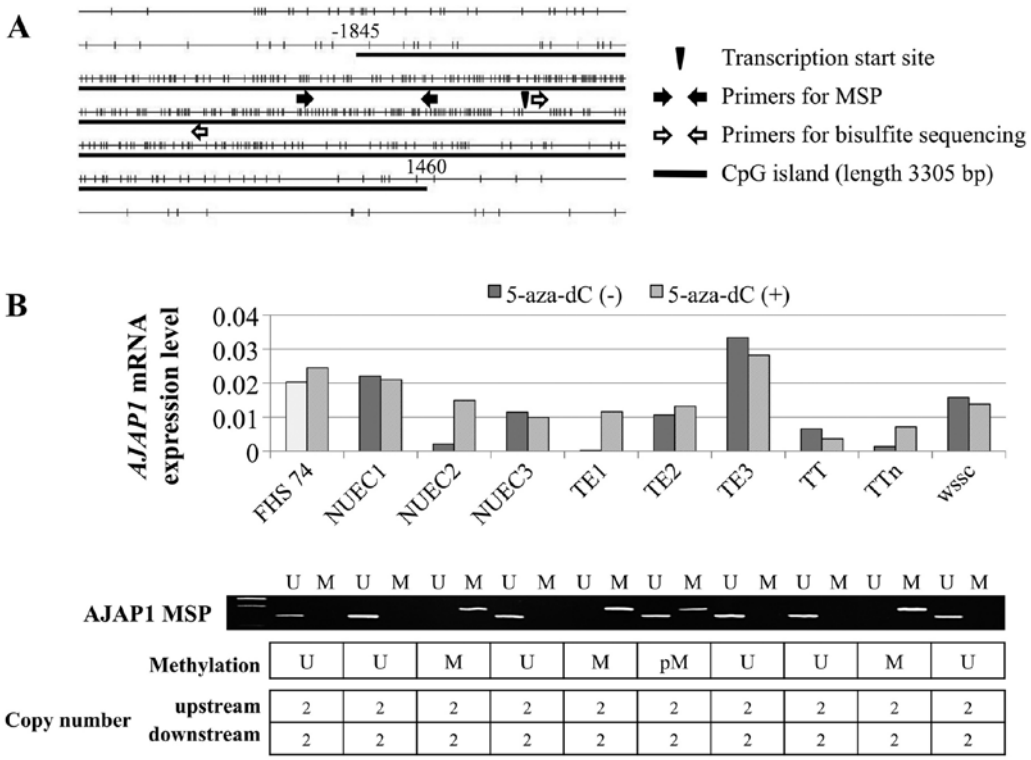

Figure 1. (A) The CpG island (indicated by the underline) is centered on the *AJAP1* transcription initiation site and extends upstream into the promoter region. (B) *AJAP1* mRNA levels in control FHs74 cells and nine ESCC cell lines before and after 5-aza-dC treatment. The methylation and copy number of *AJAP1* in the 10 cell lines are shown below the graph. M, methylated; pM, partially methylated; U, unmethylated.

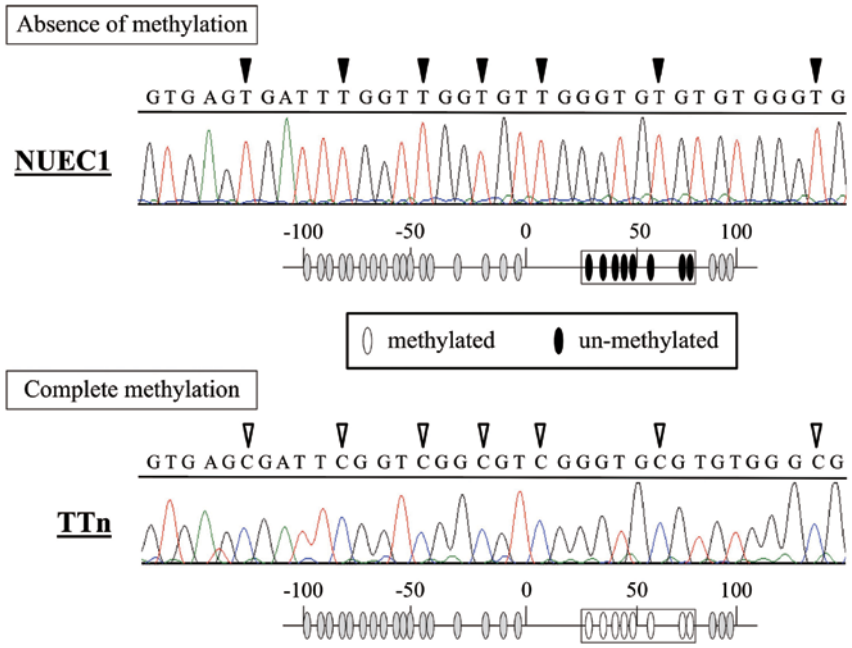

Figure 2. Representative results of bisulfite sequence analysis. All CpG sites in NUEC1 cells were converted to TG, and those of TTn were CG.

may functionally interact with *AJAP1*. The relative expression levels of *EZR*, *FAK*, *SRC*, *DPYSL3*, and *AJAP1* mRNAs in the ESCC and FHs74 cell lines are shown in Fig. 3A. The *AJAP1* mRNA levels correlated inversely with those of *EZR* (correlation coefficient -0.661, P=0.038), and there was no significant correlation with the levels of *FAK*, *SRC* and *DPYSL3* mRNAs (Fig. 3B).

Clinical significance of AJAP1 mRNA levels. In resected samples, *AJAP1* mRNA levels were lower in ESCC tissues compared with those of adjacent normal tissues in 67 (86%) of 78 patients. *AJAP1* mRNA levels gradually decreased according to the UICC stage (Fig. 4A). The *AJAP1* mRNA levels of 45 patients with ESCC were less than half of those of adjacent normal tissues, and these patients were

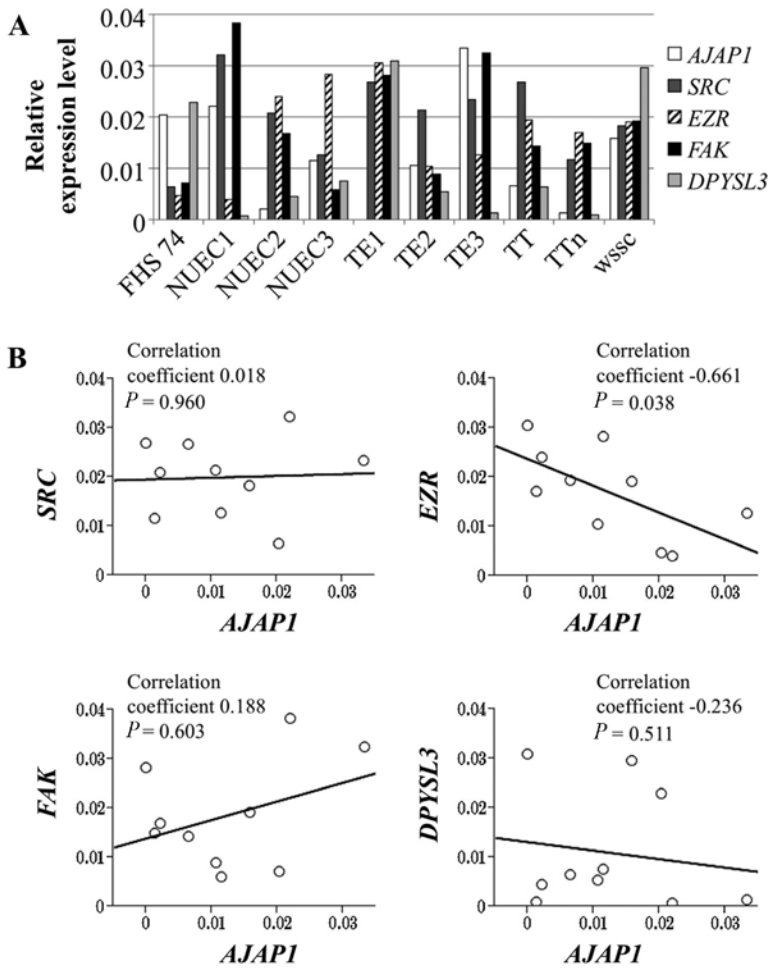

Figure 3. (A) The graph indicates the relative levels of *AJAP1*, *SRC*, *EZR*, *FAK*, and *DPYSL3* mRNAs in control FHs74 cells and nine ESCC cell lines. (B) Relation of mRNA levels between *AJAP1* and those of *SRC*, *EZR*, *FAK* and *DPYSL3*.

designated as the 'downregulated AJAP1 transcription' group in the following analyses. Downregulation of *AJAP1* transcription associated significantly with male individuals but not with clinicopathological factors (Table II). Patients with downregulated *AJAP1* transcription were more likely to experience shorter overall survival compared with that of other patients (5-year survival rates were 40 and 66%, respectively; P=0.046) (Fig. 4B). Moreover, the disease-free survival of patients with downregulated *AJAP1* transcription was significantly shorter compared with those of other patients (3-year survival rates were 37 and 64%, respectively; P=0.009) (Fig. 4C). Multivariate analysis of disease-free survival identified downregulated *AJAP1* transcription as an independent prognostic factor (hazard ratio 2.04, 95% confidence interval (CI), 1.11-3.90; P=0.022) (Table III).

Discussion

Despite numerous and intensive recent studies devoted to improving the treatment of esophageal cancer, clinical outcomes remain unsatisfactory as indicated dramatically by 5-year survival rates of 49.3 and 2.8% for localized and metastatic disease, respectively (1,27). To develop novel treatment options for ESCC, molecular biological approaches were applied to identify specific molecular diagnostic markers and therapeutic targets (28). We decided to study *AJAP1* for this purpose, because it encodes a transmembrane protein of adherens junctions in epithelial cells that plays pivotal roles in cell growth and migration and is involved in the pathogenesis of glioblastoma (11,13).

Here, we determined the levels of *AJAP1* expression in patients with ESCC to determine the underlying regulatory mechanism. We detected reduced levels of *AJAP1* mRNA in 78 and 86% of ESCC cell lines and resected ESCC tissues, respectively, and the loss of *AJAP1* expression correlated with methylation of the *AJAP1* promoter region without loss of copy number. Moreover, *AJAP1* transcription was restored when ESCC cell lines were treated with 5-aza-dC. Downregulation of *AJAP1* was associated with worse patient outcomes, particularly postoperative recurrence. The present study shows that the levels of *AJAP1* mRNA were frequently decreased in ESCC cell lines and tissues, indicating that AJAP1 plays a role in the pathogenesis of ESCC.

In the ESCC cell lines, differentially expressed *AJAP1* mRNA, and *AJAP1* promoter hypermethylation were detected only in cells with significantly decreased *AJAP1* mRNA levels. Further, *AJAP1* mRNA levels were increased in cells treated with a DNA methylation inhibitor, indicating that promoter hypermethylation is a pivotal regulatory mechanism of *AJAP1* transcription, which is consistent with studies of patients with

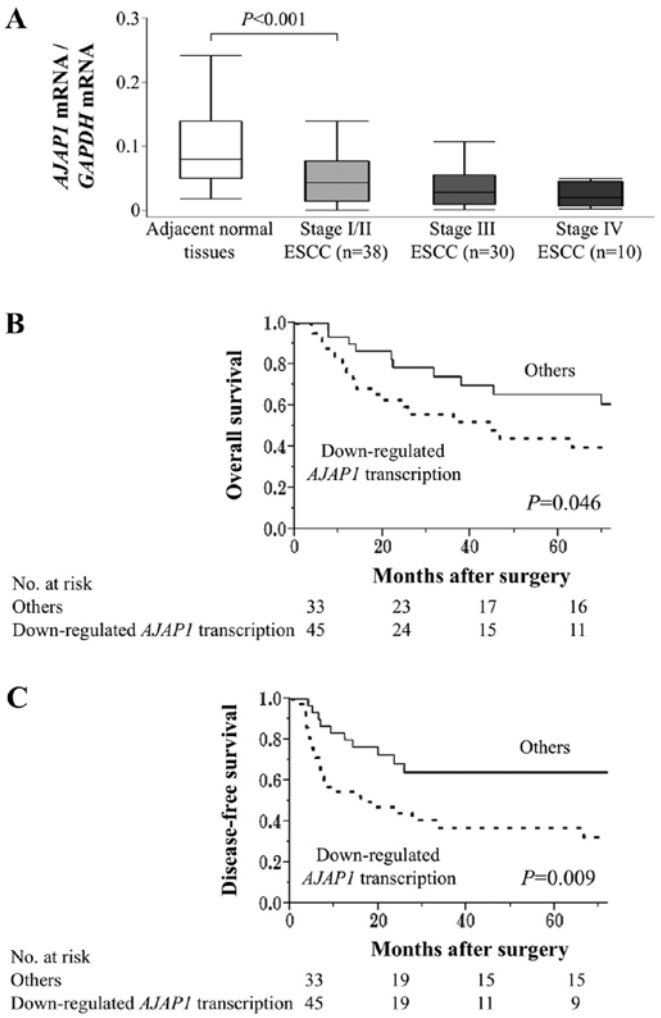

Figure 4. (A) A stepwise decrease in *AJAP1* mRNA levels in ESCC tissues was observed with increasing UICC stage. (B) Patients with downregulated *AJAP1* transcription experienced significantly shorter overall survival compared with other patients. (C) Disease-free survival was significantly shorter in patients with downregulated *AJAP1* transcription compared with other patients.

glioma (11,14,29,30). In contrast, we identified some ESCC cells with reduced expression of *AJAP1* mRNA without DNA methylation, leading us to assume that other mechanisms regulate *AJAP1* transcription, such as loss of heterozygosity (LOH), because *AJAP1* resides within chromosome 1p36, a known hotspot of chromosomal alterations (13,31,32). However, we did not detect a loss of *AJAP1* copy number in ESCC cell lines. Because copy number analysis addressed a limited region of the *AJAP1* locus, further investigations, including detection of LOH and epigenetic modifications other than DNA methylation are required.

In the present study, we determined the relative levels of mRNAs encoding selected cell adhesion molecules to identify novel proteins that may interact with AJAP1 in ESCC cells. Thus, cell adhesion molecules act coordinately to mediate the migration and invasion of tumor cells (33). We found that the levels of *AJAP1* mRNA correlated significantly with those of *EZR* in ESCC and FHs74 cell lines. EZR is a member of the ezrin-radixin-moesin family and acts as a cross-linker between the plasma membrane

Table II. Association between the expression of *AJAP1* mRNA and clinicopathological parameters of 78 patients with squamous cell carcinoma of the esophagus.

Clinicopathological parameters	Downregulated *AJAP1* transcription (n)	Other (n)	P-value
Age (years)			0.874
<65	24	17	
≥65	21	16	
Gender			0.016[a]
Male	40	22	
Female	5	11	
Preoperative symptoms			0.705
Absent	8	7	
Present	37	26	
Brinkman index			0.167
<1,000	23	22	
≥1,000	22	11	
Excessive alcohol consumption			0.453
Absent	9	9	
Present	36	24	
CEA (ng/ml)			0.225
≤5	42	28	
>5	3	5	
SCC (ng/ml)			0.240
≤1.5	27	24	
>1.5	18	9	
Tumor size (cm)			0.724
<5.0	20	16	
≥5.0	25	17	
UICC T factor			0.838
T1-2	16	11	
T3-4	29	22	
Differentiation			0.961
Moderate to well	38	28	
Poor	7	5	
Lymphatic involvement			0.421
Absent	10	10	
Present	35	23	
Vessel invasion			0.898
Absent	28	21	
Present	17	12	
Intraepithelial spread			0.875
Absent	12	20	
Present	13	20	
Lymph node metastasis			0.522
Absent	24	20	
Present	21	13	

χ^2 test. [a]Statistically significant (P<0.05). CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control.

Table III. Prognostic factors for disease-free survival of 78 patients with squamous cell carcinoma of the esophagus.

		Univariate			Multivariate		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥65)	37	1.70	0.87-3.35	0.119			
Gender (male)	62	2.69	1.06-9.06	0.035	1.86	0.70-6.45	0.227
Preoperative symptoms	63	1.29	0.60-3.20	0.541			
Brinkman index (≥1,000)	33	1.61	0.83-3.12	0.153			
Excessive alcohol consumption	60	0.99	0.47-2.32	0.975			
CEA (>5 ng/ml)	8	1.38	0.47-3.25	0.521			
SCC (>1.5 ng/ml)	27	0.80	0.37-1.61	0.539			
Tumor size (≥5.0 cm)	42	1.20	0.62-2.31	0.591			
UICC T factor (T3-4)	51	1.60	0.79-3.48	0.197			
Tumor differentiation (poor)	12	1.75	0.74-3.67	0.186			
Lymphatic involvement	58	5.18	1.85-21.5	<0.001	4.37	1.38-19.4	0.011[a]
Vessel invasion	29	1.89	0.97-3.66	0.062	1.39	0.70-2.76	0.346
Intraepithelial spread	33	1.02	0.52-1.97	0.951			
Lymph node metastasis	34	1.92	0.95-4.18	0.069	1.01	0.43-2.08	0.973
Downregulated *AJAP1* transcription	45	2.53	1.26-5.51	0.009	2.19	1.07-4.90	0.032[a]

[a]Statistically significant in multivariate analysis (P<0.05). CI, confidence interval; CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model.

and the actin cytoskeleton (34). Inactive EZR is located in the cytoplasm, and its C-terminal domain, an F-actin-binding site, is masked by the EZR N-terminal domain or those of other ERM proteins (35). Moreover, EZR is a key signaling molecule that is involved in a wide variety of cellular processes such as cell adhesion, survival, and motility as well as signal transduction (36,37). Moreover, EZR contributes to tumorigenesis, development, invasion, and metastasis, likely through regulation of adhesion molecules and signaling to other cell membrane channels in tumors, including ESCC (38-40). Further, the AKT/EZR/NF-κB signaling pathway regulates the epidermal growth factor-induced epithelial-mesenchymal transition (EMT) in squamous cell carcinoma of the tongue (41). Our present results indicate a possible interaction between AJAP1 and EZR that may provide the first step required to understand the role of AJAP1 in oncogenesis. Further investigation of the correlation between the expression of AJAP1 and EMT-associated molecules are required.

We show here that *AJAP1* mRNA levels gradually decreased as a function of UICC tumor stage, highlighting the diagnostic implications of analyzing *AJAP1* expression in esophageal tissues. Downregulation of *AJAP1* mRNA in ESCC tissues associated significantly with worse prognosis after curative esophagectomy, particularly with disease-free survival. This finding emphasizes that *AJAP1* expression is a potential biomarker for patients with ESCC who are susceptible to recurrence.

Taken together, our analyses of *AJAP1* promise to improve clinical management of ESCC as follows: i) The expression levels of *AJAP1* in biopsy tissue obtained using endoscopic surveillance may identify patients requiring intensive systemic treatment or neoadjuvant therapy; ii) the expression levels of *AJAP1* in surgical specimens may predict recurrence and subsequent adverse prognosis, leading to the design of appropriate therapeutic strategies; and iii) demethylating agents targeting *AJAP1* may serve as therapeutics. However, this study is limited by its lack of direct functional analysis of AJAP1, and we are unable to conclude that AJAP1 acts a suppressor of ESCC. Further, the mechanisms that regulate *AJAP1* expression, other than promoter hypermethylation, remain to be determined. Further studies are therefore necessary to identify the molecular mechanisms underlying the phenotypes of ESCC cells.

In summary, our data suggest that *AJAP1* expression is frequently suppressed in ESCC and that hypermethylation of the *AJAP1* promoter region is a pivotal regulatory mechanism of *AJAP1* expression in ESCC. Downregulation of *AJAP1* in ESCC tissues may represent a promising biomarker for predicting ESCC recurrence.

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