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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

DOK2 as a Marker of Poor Prognosis of Patients with Gastric Adenocarcinoma After Curative Resection

Hiromichi Miyagaki, MD, Makoto Yamasaki, MD, PhD, Tsuyoshi Takahashi, MD, PhD, Yukinori Kurokawa, MD, PhD, Hiroshi Miyata, MD, PhD, Kiyokazu Nakajima, MD, PhD, FACS, Shuji Takiguchi, MD, PhD, Yoshiyuki Fujiwara, MD, PhD, Masaki Mori, MD, PhD, FACS, and Yuichiro Doki, MD, PhD

Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

ABSTRACT

Background. DOK2 is known as the substrate of chmeric p210bcr/abl oncoprotein characterizing chronic myelogenous leukemia with Philadelphia chromosome. Reduced DOK2 expression was recently reported in lung adenocarcinoma, suggesting that this protein acts as a tumor suppressor in solid tumors. The purpose of this study was to determine the significance of DOK2 in gastric cancer.

Methods. The study subjects were 118 patients who underwent curative surgery for gastric cancer, as well as 7 gastric cancer cell lines. The tissues and cell lines were analyzed for DOK2 gene and protein expressions by histopathology and immunohistochemistry, and also using a microsatellite marker for loss of heterozygosity. Correlation of survival with clinicopathological parameters was investigated by univariate and multivariate analyses.

Results. DOK2 expression was confirmed in the normal gastric mucosa. Considerable differences in the gene expression were noted among the gastric cell lines. Positive DOK2 expression was noted in the noncancerous regions of all pathological specimens, whereas 59 (50.0%) specimens of 118 patients were negatively stained in the tumor. Loss of heterozygosity was observed in 54.5% of DOK2(−) cases. DOK2(−) patients were more likely to develop recurrence than DOK2(+) and showed poorer 5-year overall survival (59.1%) than DOK2(+) (76.4%, $P = .0403$). Multivariate analysis identified pT (hazard ratio [HR] = 2.748, 95% confidence interval [95% CI] = 1.061–8.927, $P = .0361$), pN (HR = 2.486, 95% CI = 1.264–4.932, $P = .0086$), and DOK2(−) (HR = 2.343, 95% CI = 1.211–4.727, $P = .0112$) as significant and independent determinants of poor survival.

Conclusions. Our data suggest the potential usefulness of DOK2 as a marker of poor prognosis in patients with gastric cancer after curative resection.

Gastric cancer is a global health problem with estimated new stomach cancer cases of 989,600 and cancer-related deaths of 738,000 in 2008 alone, with the highest incidence in Eastern Asia, Eastern Europe, and South America.[1] Advances in the diagnosis and treatment have offered excellent long-term survival for patients with early diagnosis of gastric cancer; however, the prognosis of those with advanced cancer remains poor and heterogeneous. Assessment of prognosis through clinicopathological features remains inadequate, especially with advanced cancer even when using the staging system of tumor-node-metastasis (TNM) classification, because of the considerable variability and heterogeneity within the same stage.[2,3] Therefore, it is necessary to identify novel biological markers that allow a more accurate identification of high-risk population for recurrent disease and help in the design of appropriate treatment strategies for individual population.

DOK1–3 are adaptor proteins that function in feedback loops to modulate tyrosine kinase signaling including epidermal growth factor receptor, platelet-derived growth factor receptor, c-Kit, Tie2, and Her2/*Neu*.[4–10] The interaction between DOK1–3 and these tyrosine kinases in hematopoietic cells is well described. DOK1 and DOK2 are crucial regulators in chronic myelogenous leukemia (CML) with Philadelphia chromosome.[8] Furthermore,

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M. Yamasaki, MD, PhD
e-mail: myamasaki@gesurg.med.osaka-u.ac.jp

Dok-1/Dok-2 double-knockout mutants spontaneously developed transplantable CML-like myeloproliferative disease, and thus both are considered to be involved in leukemogenesis.[11,12]

Little is known about DOK1–3 expression in nonhematopoietic cells and solid tumors. Recently, Berger et al.[13] reported the presence of low expression of DOK2 in lung adenocarcinoma, suggesting that this protein acts as a tumor suppressor. The purpose of the present study was to determine the expression of DOK2 in gastric cancer and gastric cancer cell lines. The results indicated the clinical significance of DOK2 in the evaluation of prognosis of patients with gastric cancer.

MATERIALS AND METHODS

Patients

From October 2001 to March 2004, 118 consecutive patients diagnosed histopathologically with gastric adenocarcinoma underwent surgery at Osaka University Hospital. In all patients, the gastric adenocarcinoma was newly diagnosed, and none had received chemotherapy or radiotherapy before surgery; the tumor was resected curatively and pathologically diagnosed as pStage I–III. After surgery, the patients were surveyed every 3 months by clinical examination, and serum tumor markers [carcinoembryonic antigen (CEA), CA19-9], every 6 months by computed tomography (CT) scan and abdominal ultrasonography, and annually by endoscopy until tumor relapse was evident. Patients with tumor relapse received chemotherapy as long as their systemic condition permitted. Adjuvant chemotherapy was provided for 24 patients; 1 patient received TS-1 (tegafur, gimeracil, oteracil potassium) with paclitaxel, and the other 23 patients received oral fluoropyrimidines TS-1, 5-FU (fluorouracil), UFT (tegafur, uracil) or 5′DFUR (doxifluridine) for at least 6 months. The median follow-up period after surgery was 63.8 months (range, 5.5–132.4 months). Various clinicopathological parameters, such as age, gender, histological classification (according to the World Health Organization [WHO]), depth of invasion, nodal metastasis, pathological-tumor-metastasis (pTNM) stage (according to TNM Classification System of Malignant Tumors, 7th ed. [UICC]), and lymphovascular invasion status were evaluated by reviewing the medical and pathologic reports, and hematoxylin and eosin (H&E)-stained tumor tissue sections.[2] There were 6 frozen samples (N1–N6) chosen at random from 118 patients for reverse transcription polymerase chain reaction (RT-PCR) analysis described below. This study adhered to the guidelines established by the Declaration of Helsinki, and all patients provided informed written consent.

Gastric Cancer Cell Line

The gastric cancer cell lines MKN74, MKN45, MKN45P, MKN7, NUGC3, RERFGC1B, and AGS were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% (v/v) FBS (GIBCO BRL, Grand Island, NY) and penicillin/streptomycin (1000 units/mL; GIBCO BRL).

Laser Microdissection

Six frozen samples (N1–N6) were sectioned with a cryostat (Leica Microsystems, Wetzlar, Germany) at 6 μm thickness. The sliced samples were immediately fixed with a mixture of 100% ethanol and acetic anhydride (18:1). After staining with H&E, only normal gastric mucosal cells were differentially dissected from stromal cells and lymphocyte using a laser microdissection (LMD) system (Leica LMD System, Leica Microsystems).

Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA from frozen normal gastric mucosa dissected by LMD was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and total RNA from gastric cancer cell line was extracted using Trizol reagent (Gibco BRL, Grand Island, NY) following the instructions provided by the manufacturer. Total RNA was reverse transcribed to cDNA in a 20 μl volume using Reverse Transcription System (A3500 Promega, Madison, WI). The reaction condition was based on the information provided by the manufacturer.

RT-PCR was carried out in a reaction mixture containing 2 μl of cDNA, 12.5 μl ampliTaq GOLD (Applied Biosystems, Foster City, CA), and 10.5 μl water. The cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 68°C (58°C for B2M) for 30 s, 72°C for 2 min, and a final extension at 72°C for 7 min. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Peripheral mononuclear blood cells (PMBC) were prepared as a positive control with standard Ficoll-Hypaque density gradient separation techniques. The details of DOK2 gene specific primers were reported by Favre et al.[14] To verify the quality and integrity of synthesized cDNA, the *beta-2 microglobulin* (*B2M*) gene was used as an internal control: 5′-TGTCTTTCAGCAAGGACTGG-3′ (sense) and 5′-CCTCCATGATGCTGCTTACA-3′ (antisense) (NCBI-Acc. AF072097, nucl. pos. 1021–1168).

Immunohistochemical Staining

DOK2 protein contents were examined by immunohistochemical staining of formalin-fixed and paraffin-embedded gastric cancer tissue and normal gastric mucosa sections (3.5 μm). One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry. Briefly, after deparaffinization in xylene and dehydration in graded ethanol solutions, tissue sections were heated at 121°C for 20 min in ethylenediaminetetraacetic acid (EDTA)-tris buffer, pH 9.0, for antigen retrieval. Then, endogenous peroxidase activity was blocked by incubation with 30 ml/l hydrogen peroxide for 20 min. After overnight incubation with mouse monoclonal primary antibody DOK2 (sc-17830 Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:200) at 4°C, staining was performed by the labeled streptavidin-biotin (LSAB) method. Negative controls of immunohistochemical reactions included omission of the primary antibody. Lymphocytes were used as positive control. DOK2 staining in each gastric cancer sample was judged positive when the cancer cells in the section were immunoreactive to DOK2. All slides were assessed independently by 2 pathologists and then by consensus in case of disagreement. Both pathologists were blinded to the clinicopathological data.

Microsatellite Analysis

A total of 30 frozen paired normal and tumor tissue samples were randomly selected from 118 patients described previously. The DNA was extracted using QIAmp DNA Mini Kit (Qiagen). The microsatellite marker D8S560, which is located at 164 kbp downstream of DOK2 gene, was selected for loss of heterozygosity (LOH) analysis. The specific PCR primer was designed as follows: 5′-GGCATTTCAGAGGACC-3′ (sense) and 5′-TGCAAA GATGGGCTCAG-3′ (anti-sense). The cycling conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. Equal amounts of PCR products were electrophoresed on 15% SDS-polyacrylamide gel and visualized by cyber green staining. In each case, the results were analyzed by visual inspection. In addition, cases presenting equivocal results and cases delimiting critical regions of loss were analyzed by comparison of allele intensities in matched normal/tumor DNA using scanning densitometry with a computerized ChemiDoc XRS Plus (Bio-Rad Laboratories, Hercules, CA). Quantification was performed using the Quantity One program. The relative ratio of both tumor and normal alleles was determined, normalized, and then compared. LOH was assigned when the intensity ratio of the two tumor sample alleles differed by at least 30% from that observed on normal DNA.[15]

Quantitative Real-Time PCR

Total RNA was extracted from the same 30 frozen paired normal and tumor samples used for microsatellite marker analysis, by the Trizol reagent (GIBCO). Real-time monitoring of PCRs was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of DOK2 and B2M. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 15 s, annealing at 66°C for 18 s, and elongation at 72°C for 30 s. The products were then subjected to a temperature gradient from 55 to 95°C at 0.1°C/s, with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against B2M mRNA expression.

Statistical Analysis

Correlations between DOK2 expression and various clinicopathological parameters were evaluated by the χ^2 test and Fisher exact probability test. Prognostic variables were assessed by log-rank test, and overall survival (OS) and relapse-free survival (RFS) were analyzed by the Kaplan and Meier method. Cox proportional hazards regression model was used to evaluate the independent prognostic factors. These analyses were carried out using JMP version 8.0.1 (SAS Institute, Cary, NC) for Windows. A P value of less than .05 denoted the presence of statistical significance.

RESULTS

DOK2 Gene Expression in Normal Gastric Mucosa and Gastric Cancer Cell Lines by RT-PCR

DOK1 mRNA expression was observed in all normal gastric mucosa and gastric cancer cell lines, while DOK3 mRNA expression was hardly observed in both. DOK2 mRNA expression was observed in all normal gastric mucosa samples (N1–N6) (Supplementary Fig. 1a). In gastric cancer cell lines, the expression was strong in MKN7 and AGS, and weak in MKN74. On the other hand, MKN45, MKN45P, NUGC3, and RERFGC1B were negative for DOK2 (Supplementary Fig. 1b). There were considerable differences in DOK2 expression level between gastric cell lines. Thus, we focused on DOK2 and assessed the correlation between DOK2 expression and clinicopathological outcome.

DOK2 Protein Expression in Gastric Cancer by Immunohistochemistry

Samples from the entire group of 118 patients (Table 1) that contained both cancerous and noncancerous tissues were evaluated for DOK2 protein expression by immunohistochemistry. Abundant DOK2 protein expression was detected in the mucosa of all normal gastric tissues (100%; Fig. 1a, b). The cytoplasm of the normal gastric parietal cells stained strongly for DOK2, while that of chief cells showed moderate reactivity.

In cancer cells, 59 of 118 patients (50.0%) showed positive DOK2 expression (Fig. 1c, d), mainly in the cytoplasm of tumor cells while the remaining 59 (50.0%) were negative (Fig. 1a, b). The pattern of DOK2 expression in cancer cells was almost similar to that in normal gastric chief cells. Positive staining was almost homogeneous at single cancer nest and among different areas (surface, central, and deepest areas) of the cancer lesion. There was a close agreement between the two pathologists on the pathological assessment with interobserver variation of less than 5%.

TABLE 1 Correlation between DOK2 expression and various clinicopathological parameters

Parameters	DOK2 expression		*P* value
	Positive	Negative	
All cases	59	59	
Age (<66/≥66)	24/35	34/25	.0971
Gender (female/male)	21/38	15/44	.3175
Differentiation (poorly/well & mod)	40/19	21/38	.0008
pT (T3–4a/T1–2)	39/20	39/20	1.0000
pN (N2–3/N0–1)	20/39	17/42	.6918
pStage (I/II/III)	18/17/24	16/20/23	.8259
Lymphatic infiltration (positive/negative)	49/10	46/13	.6428
Venous invasion (positive/negative)	22/37	26/33	.5742
Relapse cases ($n = 35$)	12	23	.0430
Lymph node ($n = 10$)	3	7	.3220
Peritoneum ($n = 10$)	5	5	1.0000
Hematogenous ($n = 18$)	4	14	.0105
Liver ($n = 14$)	2	12	.0083

Hematogenous included bone, lung, brain, and liver metastases as the first site of metastasis

Well & Mod well and moderately differentiated carcinomas (including papillary adenocarcinomas), *Poorly* poorly differentiated carcinomas (including signet ring cell carcinomas and mucinous adenocarcinoma), *pT pN pStage* (pathological classification) according TNM Classification of Malignant Tumors (UICC) 7th ed

DOK2 mRNA Expression Evaluated by Quantitative Real-Time PCR

To assess the relevance of DOK2 protein level to DOK2 mRNA expression, we conducted quantitative real-time PCR in 30 frozen paired normal and tumor samples from the 118 cases. As shown in Fig. 2, DOK2 mRNA expression was significantly lower in DOK2(−) cases than DOK2(+) (fold change 2.41, $P < .001$), which was consistent with protein expression evaluated by immunohistochemistry.

Relationship Between Loss of Heterozygosity and Low DOK2 Expression

To assess the relevance of the reduced DOK2 expression to genomic status, microsatellite analysis was performed in 30 patients with gastric cancer. Allelic loss of DOK2 was observed in 6 of 30 cases, while the results in 7 cases were noninformative. DOK2 protein expression level correlated significantly with allelic loss of DOK2. The loss was not detected in any of the patients with positive DOK2 expression, but was detected in 6 of 11 cases (54.5%) negative for DOK2 expression (Table 2). Correlation of the results of microsatellite analysis and histopathological grade showed that the six cases with allelic loss comprised five cases with differentiated tumors and one with poorly differentiated tumor (Table 2). The 17 heterozygous cases consisted of 6 (35.3%) with differentiated tumors and 11 (64.7%) with poorly differentiated tumors. In DOK(−) cases with heterozygosity, three of five cases (60.0%) had poorly differentiated tumors, which was similar to DOK(+) cases ($P = 1.000$).

Correlation Between DOK2 Expression and Clinicopathological Parameters

Table 1 lists the correlations between DOK2 expression and various clinicopathological parameters. Tumors of the differentiated type (including papillary and well and moderately differentiated adenocarcinomas), based on histopathological grade, were significantly more likely to be negative for DOK2 expression than those positive for DOK2 [DOK2(−): 66.7%, DOK2(+): 33.3%, $P = .0008$]. There were no significant correlations with other parameters, including age, gender, pT, pN, pStage, lymphatic infiltration, and venous invasion (Table 1).

Correlation Between DOK2 Expression and Clinical Outcome

Disease relapse was diagnosed after surgery in 35 of 118 patients (29.7%), and the median time to relapse was 14.8 months. The relapse rate in DOK2(−) patients was

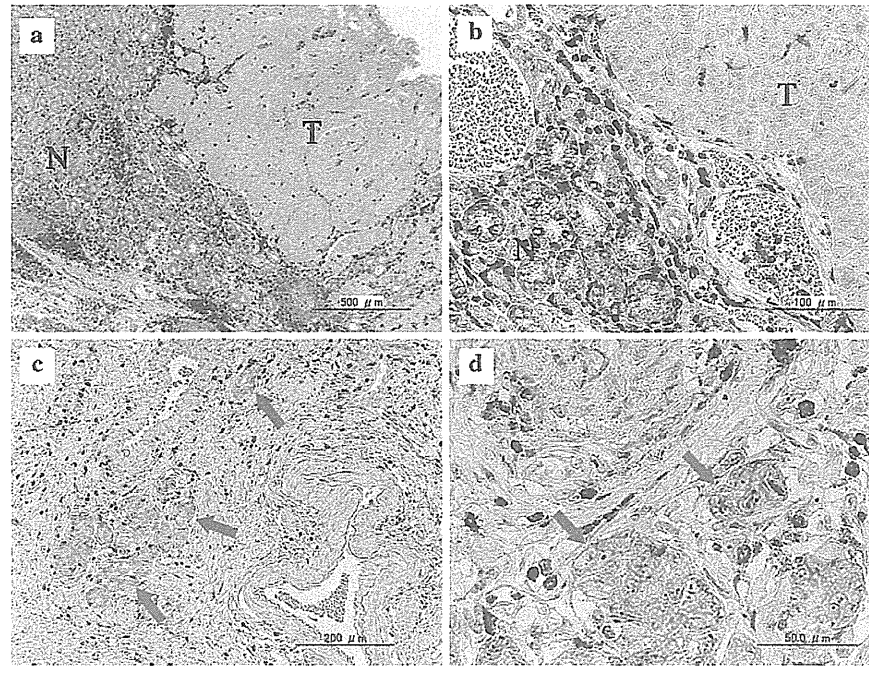

FIG. 1 DOK2 expression by immunohistochemistry. **a** Low magnification of representative DOK2-negative gastric adenocarcinoma (T) diagnosed as moderately differentiated adenocarcinoma, and adjacent normal gastric mucosa (N) positive for DOK2 (original magnification ×40). **b** High-magnification view of **a** (original magnification ×200×). **c** Representative DOK2-positive gastric adenocarcinoma (*arrows*) diagnosed as poorly differentiated adenocarcinoma (original magnification ×100) **d** High-magnification view of **c** showing cytoplasmic staining of tumor cells (*arrows*) (original magnification ×400)

significantly higher compared with those with DOK2(+) tumors ($P = .0430$). Significantly poorer OS and RFS were noted in patients with DOK2(−) tumors than those with DOK2(+) [5-year OS: DOK2(−) 59.1%, DOK2(+) 76.4%, $P = .0403$, 5-year RFS: DOK2(−) 58.1%, DOK2(+) 73.0%, $P = .0334$] (Fig. 3a, b).

Stratification analysis by histopathological type showed similar tendency in all types, including differentiated type, and poorly differentiated type (including poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas) [differentiated type; 5-year OS: DOK2(−) 57.6%, DOK2(+) 76.8%, $P = .2857$, poorly differentiated type; 5-year OS: 61.9%: 76.2%, respectively, $P = .0897$, data not shown].

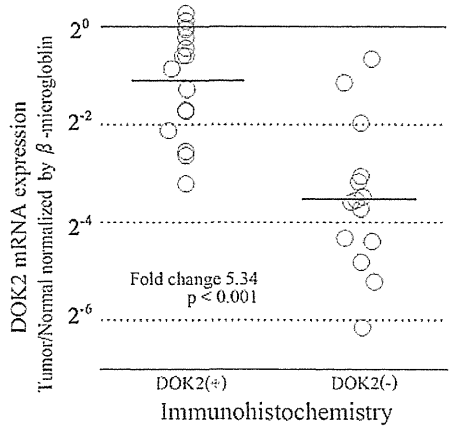

FIG. 2 DOK2 mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase-polymerase chain reaction on 30 paired clinical samples (normalized by *beta-2 microglobulin*). The mean DOK2 mRNA expression in DOK2(−) cases evaluated by immunohistochemistry was significantly lower compared with that of DOK(+) cases ($P < .001$; *t* test)

Univariate analysis showed significant relationship between OS and pT (hazard ratio [HR] = 3.354, 95% confidence interval [95% CI] = 1.509–8.900, $P = .0020$), pN (HR = 2.720, 95% CI = 1.437–5.131, $P = .0024$), lymphatic infiltration (HR = 3.167, 95% CI = 1.143–13.133, $P = .0239$), and negative DOK2 expression (HR = 1.960, 95% CI = 1.032–3.873, $P = .0395$), but not with age, gender, histology, venous invasion, or adjuvant chemotherapy (Table 3).

Multivariate analysis using the aforementioned 4 significant parameters identified pN (HR = 2.486, 95% CI = 1.264–4.932, $P = .0086$) as the poorest prognostic factor, followed by negative DOK2 expression (HR = 2.343, 95% CI = 1.211–4.727, $P = .0112$) and pT (HR = 2.748, 95% CI = 1.061–8.927, $P = .0361$) (Table 3).

DISCUSSION

The present study showed that while DOK2 is always expressed in the normal epithelium, its expression in gastric cancer cell varies considerably among patients, and the expression was downregulated in approximately half of the patients. This result suggested that loss of DOK2 expression might possibly play a role in the development of gastric cancer. LOH analysis also showed the involvement of reduced DOK2 expression in allelic loss of 8p21.3. High

TABLE 2 Results of microsatellite marker analysis (D8S560)

	LOH	Heterozygosity	Noninformative	*P* value
DOK2 expression				.005
Positive ($n = 16$)	0	12	4	
Negative ($n = 14$)	6	5	3	
Histological grade				.069
Well & Mod ($n = 15$)	5	6	4	
Poorly ($n = 15$)	1	11	3	

P value was calculated by Fisher exact test excluding noninformative cases

LOH loss of heterozygosity, *Well & Mod* well and moderately differentiated carcinomas (including papillary adenocarcinomas), *Poorly* poorly differentiated carcinomas (including signet ring cell carcinomas and mucinous adenocarcinoma)

frequency of the allelic loses has been reported in gastric cancer and many other cancers.[13,15–25] Various tumor suppressor genes are considered to reside on this locus, and the simultaneous loss of these tumor suppressors may trigger tumorigenesis, lending support to the tumor suppressive role of this gene.[13,26–29] The results of this study point to the importance of loss of DOK2 expression in histological grade of gastric cancer. Interestingly, most of the DOK2(−) cancers with allelic loss were of the differentiated type, while the DOK2(−) cancers without allelic loss did not correlate with histological grade. This result indicates that the pathologic differentiation of gastric cancer involves other genes in this locus but not DOK2 expression. How allelic loss determine the histopathological grade is not well understood at present, and further studies are needed to clarify the relationship.

Furthermore, clinicopathological analysis in this study revealed that patients with DOK2(−) tumors were at a significantly high risk for relapse and showed poorer overall survival than patients with DOK2(+) tumors. The prediction of recurrence and metastasis after curative resection could allow us to determine the need for intensive follow-up and adjuvant therapy. In gastric cancer therapy, it is important to prevent metachronous metastasis after curative surgical resection. In this study, 35 of 118 patients had a relapse, similar to the number of relapses reported in a previous study.[30] Recent studies described the beneficial effects of certain adjuvant chemotherapies for the treatment of recurrence in certain stages.[31,32] Staging according to TNM classification has been successful to some extent but is still insufficient. Thus, the use of predictive markers of tumor recurrence and metastasis that are independent of traditional TNM classification is clinically important. The present study indicated that DOK2 is not only an independent prognostic factor but also a candidate predictor of metachronous metastasis. The results showed no difference in OS between patients with and without DOK2 expression in the first 2 years after surgery [2-year OS: DOK2(−) 82.6%, DOK2(+) 87.85%, $P = .4717$], although differences in RFS were present between the 2 groups [2-year RFS: DOK2(−) 65.5%, DOK2(+) 84.4%, $P = .0152$]. In the present study, the liver was the first site for metastasis, accounting for 52% in DOK2(−) patients, which was much higher than 16.7% in DOK2(+) patients (Table 1). Prognosis after liver metastasis might occur in general after a longer period than after metastases at other locations, most likely because of the ease of detection and availability of effective therapeutic options such as surgical resection or radiofrequency ablation.[33–39] The present study also showed that prognosis after relapse was better for patients with liver metastasis than peritoneal metastasis (15–16 vs.

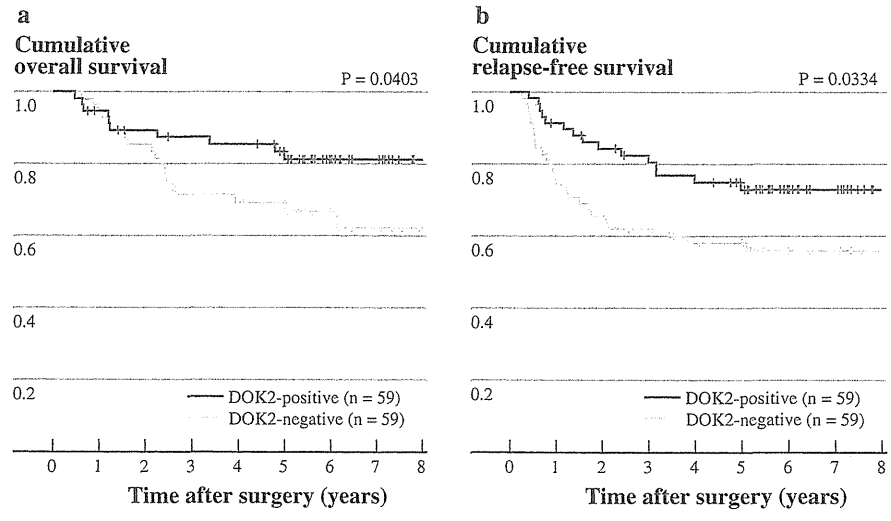

FIG. 3 Kaplan–Meier curves for overall and relapse-free survival according to DOK2 expression. Overall survival curve (**a**) and relapse-free survival curve (**b**) according to DOK2 expression for all patients. Differences between the two groups were evaluated by log-rank test. *Ordinate* survival rate, *abscissa* time after surgery (years)

TABLE 3 Results of univariate and multivariate survival analyses of overall survival by Cox proportional hazards model

Parameter	*n*	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
DOK2 expression (negative/positive)	59/59	1.960	1.032–3.873	.0395	2.343	1.211–4.727	.0112
Age (<66/≥66)	58/60	1.584	0.842–3.031	.1535			
Gender (female/male)	36/82	0.511	0.219–1.059	.0721			
Differentiation (poorly/well & mod)	57/61	0.857	0.454–1.612	.6306			
pT (T3–4a/T1–2)	78/40	3.354	1.509–8.900	.0020	2.748	1.061–8.927	.0361
pN (N2–3/N0–1)	37/81	2.720	1.437–5.131	.0024	2.486	1.264–4.932	.0086
Lymphatic infiltration (positive/negative)	96/22	3.167	1.143–13.133	.0239	1.063	0.266–5.346	.9343
Venous invasion (positive/negative)	48/70	1.643	0.873–3.101	.1226			
Adjuvant chemotherapy (yes/no)	24/94	0.882	0.357–1.884	.7613			

6 months). These short-term outcomes might result from the association between DOK2 expression and predisposition to relapse pattern.

In conclusion, the present study demonstrated the expression of DOK2 in normal gastric mucosa and 50% of gastric cancer samples. Our data pointed to the potential usefulness of DOK2 as a marker for prediction of prognosis of patients with gastric cancer after curative resection. Further prospective studies are necessary to clarify the clinical significance of such prediction. Moreover, the potential mechanism of poor prognosis in patients with low DOK2 expression should be evaluated. The present findings could open the door for exploration of efficacious treatment strategies and the development of new therapeutic modalities for gastric cancer.

DISCLOSURES Hiromichi Miyagaki and all authors of the manuscript: "Evaluation of DOK2 protein expression in gastric adenocarcinoma" declare no conflict of interest and no financial ties to disclose.

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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

Let-7 Expression Is a Significant Determinant of Response to Chemotherapy through the Regulation of IL-6/STAT3 Pathway in Esophageal Squamous Cell Carcinoma

Keijiro Sugimura, Hiroshi Miyata, Koji Tanaka, Rie Hamano, Tsuyoshi Takahashi, Yukinori Kurokawa, Makoto Yamasaki, Kiyokazu Nakajima, Shuji Takiguchi, Masaki Mori, and Yuichiro Doki

Abstract

Purpose: Cisplatin-based chemotherapy is widely used for esophageal cancer, sometimes in combination with surgery/radiotherapy, but poor response to chemotherapy is not uncommon. The aim of this study was to examine whether miRNA expression is useful to predict the response to chemotherapy in patients with esophageal cancer.

Experimental Design: Using pretreatment biopsy samples from 98 patients with esophageal cancer who received preoperative chemotherapy, we measured the expression level of several miRNAs whose expression was altered in cisplatin-resistant esophageal cancer cell lines compared with those parent cell lines and examined the relationship between the miRNA expression and response to chemotherapy. *In vitro* assays were conducted to clarify the mechanism of miRNA-induced changes in chemosensitivity.

Results: The expression levels of 15 miRNAs were altered in cisplatin-resistant cells. Of these, low expression of let-7b and let-7c in before-treatment biopsies from 74 patients of the training set correlated significantly with poor response to chemotherapy, both clinically and histopathologically. Low expression of let-7c also correlated with poor prognosis ($P = 0.032$). The relationship between let-7b and let-7c expression and response to chemotherapy was confirmed in the other 24 patients of the validation set. In *in vitro* assay, transfection of let-7c restored sensitivity to cisplatin and increased rate of apoptosis after exposure to cisplatin. Let-7c directly repressed cisplatin-activated interleukin (IL)-6/STAT3 prosurvival pathway.

Conclusions: Let-7 expression in esophageal cancer can be potentially used to predict the response to cisplatin-based chemotherapy. Let-7 modulates the chemosensitivity to cisplatin through the regulation of IL-6/STAT3 pathway in esophageal cancer. *Clin Cancer Res; 18(18); 5144–53. ©2012 AACR.*

Introduction

Despite recent advances in surgical techniques and perioperative management, the prognosis of patients who undergo surgery alone for esophageal cancer remains poor (1). Neoadjuvant chemotherapy or chemoradiotherapy followed by surgery has emerged as a promising strategy for advanced esophageal cancer and in fact, good responders to such preoperative therapy show better survival (2, 3). However, the reported response rate to cisplatin-based chemotherapy, which is widely used for esophageal cancer, is only modest, ranging from 25% to 48% (4–7) and nonresponders likely receive no survival benefit (8). The ability to predict the response to chemotherapy before treatment should limit the application of chemotherapy to selected patients who are likely to show some benefits, and allow tailoring such therapy to the individual patient with esophageal cancer.

miRNAs are noncoding RNAs of approximately 22 nucleotides in size and act by repressing the translation of target mRNA by binding to the 3′-untranslated region of those mRNAs (9). miRNAs exist stably in various tissues and play pivotal roles in differentiation and development (10). In addition, aberrant expression of miRNAs is reported in various types of cancers. In esophageal cancer, miR-21 and miR-93 are reported to be upregulated, whereas miR-375, miR-27b, miR-203, miR-205, and let-7c are downregulated (11, 12). Recent studies also showed the involvement of several miRNAs in resistance to anticancer treatment including chemotherapy and radiotherapy. Giovannetti and colleagues (13) reported that overexpression of miR-21was associated with poor outcome in gemcitabine-treated patients with pancreatic cancer. In our previous study using residual tumor after chemotherapy, we showed the involvement

Authors' Affiliation: Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Yamada oka, Suita, Osaka, Japan

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Corresponding Author: Hiroshi Miyata, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2, Yamada oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-3251; Fax: 81-6-6879-3259; E-mail: hmiyata@gesurg.med.osaka-u.ac.jp

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Translational Relevance

Chemotherapy is one of the essential treatments in esophageal cancer. It is also important to predict the response to chemotherapy before treatment to avoid unnecessary treatment. In this study, we investigated whether we could predict the response to cisplatin-based chemotherapy for esophageal cancer by analyzing the miRNA expression using biopsy samples before treatment. Of the several miRNAs associated with resistance to cisplatin, let-7b and let-7c expression is potentially useful to predict the response to chemotherapy. We also found that let-7 modulates the chemosensitivity to cisplatin through the regulation of interleukin (IL)-6/STAT3 pathway in esophageal cancer. This result should help doctors and scientists dealing with chemotherapy for gastrointestinal cancers including esophageal cancer.

of upregulated miR-200c expression in chemoresistance in esophageal cancer and that this effect is mediated through activation of the Akt signaling pathway (14).

In the present study, we examined whether we could predict the response to chemotherapy before treatment in patients with esophageal cancer, by using endoscopic biopsies. The results showed that low expression of let-7 measured before treatment is associated with low sensitivity to cisplatin-based chemotherapy in esophageal cancer. The molecular mechanism of the involvement of let-7 expression in chemosensitivity was also investigated.

Materials and Methods

Patients, treatment, and samples

Biopsy samples were obtained under esophagoscopy from 98 patients with histopathologically confirmed primary thoracic esophageal squamous cell carcinoma who subsequently underwent surgical resection between 2000 and 2011 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan. Informed consent was obtained from each patient before participation in this study. These 98 patients were divided at random into 2 independent groups; 74 in the training set and the remaining 24 patients in the validation set. Biopsy samples of the patients were obtained before preoperative chemotherapy. The samples were confirmed to contain cancerous tissue. All patients received neoadjuvant chemotherapy, which consisted of 2 courses of 5-fluorouracil (5-FU), cisplatin, and Adriamycin, using the following protocol: Cisplatin was administered at 70 mg/m^2, Adriamycin was administered at 35 mg/m^2 intravenously on day 1, and 5-FU was administered continuously from days 1 to 7 at 700 mg/m^2/d. Two courses of chemotherapy were provided after an interval of 4 weeks (8). The median follow-up period was 22.4 months. Thirty (30.6%) patients died during the follow-up period. Patients were divided into 2 groups: the first 74 patients were categorized as the training set whereas the second group of 24 patients was categorized as the validation set (Supplementary Table S1).

Clinical and histopathological evaluation of response to chemotherapy

The clinical response to chemotherapy was evaluated according to the World Health Organization Response Criteria for Measurable Diseases (15). Complete response (CR) represented total regression of the tumor. Partial response (PR) represented more than 50% reduction in primary tumor size on computed tomography (CT). Progressive disease (PD) represented more than 25% increase in the primary tumor or appearance of new lesion. Stable disease (SD) represented cases that did not meet the criteria of PR or PD. For evaluation, both the CR and PR were grouped together into the responders whereas the SD and PD were grouped as non-responders. The clinical response was assessed retrospectively by 2 investigators (K. Sugimura and H. Miyata) in a blinded fashion. The histopathologic response was also categorized according to the criteria of the Japanese Society for Esophageal Diseases (16). The percentage of viable residual tumor cells within the entire cancerous tissue was defined as follows: grade III, no viable residual tumor cells; grade II, less than two-third residual tumor cells; grade I, more than two-third residual tumor cells; and grade 0, no significant response to chemotherapy. The histopathologic response was assessed retrospectively by 2 investigators (K. Sugimura and K. Tanaka) in an independent manner and any disagreements were resolved by consensus.

Cell culture

Human esophageal squamous cell lines, TE1/TE5/TE8/TE9/TE10/TE11/TE13, were obtained from the Riken Bioresource Center Cell Bank. All cells were cultured in RPMI-1640 media (Life Technologies), containing 10% FBS (Sigma-Aldrich Co.) and 1% penicillin/streptomycin (Life Technologies), in a humidified atmosphere under 5% CO_2 at 37°C.

Establishment of cisplatin-resistant cell lines

Cisplatin-resistant cell lines (TE8-R and TE10-R) were cultured through gradual increase in cisplatin concentration [*cis*-diamminedichloroplatinum (II), Wako], as described previously (14). The cultured cells were exposed cisplatin at an initial concentration of 2 μmol/L. Three days later, the cells were cultured in cisplatin-free medium until confluence. Next, cisplatin concentration was increased by 2- to 3-fold. This cycle was repeated until cisplatin concentration reached 35 μmol/L.

Isolation of RNA

Total RNA was isolated from cells or tissues using TRIzol reagent (Life Technologies) according to the protocol provided by the manufacturer. Briefly, 100 mg of tissue samples was homogenized with 1 mL of TRIzol reagent using a power homogenizer. After homogenization, the samples were mixed with 0.2 mL of chloroform. The samples were

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shaken vigorously for 15 seconds and then centrifuged at 12,000 × *g* for 15 minutes at 4°C. The supernatant in the tube was mixed with 0.5 mL of 100% isopropanol and then incubated at room temperature for 10 minutes. After centrifugation at 12,000 × *g* for 10 minutes at 4°C, the supernatant was removed and washed with 1 mL of 75% ethanol. After centrifugation at 7,500 × *g* for 5 minutes at 4°C, the supernatant was removed and the pellet was dried for 5 minutes. The RNA pellet was resuspended in RNase-free water and adjusted into appropriate concentration.

Reverse transcription PCR and TaqMan miRNA assay

TaqMan miRNA Assay (Applied Biosystems) was used to measure miRNA levels. This assay detects only the mature form of the specific miRNAs. First, 10 ng of RNA was reverse transcribed and the resulting cDNA was amplified using the following specific TaqMan microRNA assays. Assay IDs were hsa-miR-135a ID 000460, hsa-miR-96 ID 000186, hsa-miR-141 ID 000463, hsa-miR-101 ID 2253, hsa-miR-146a ID 000468, hsa-miR-489 ID 0002358, hsa-miR-545 ID 0002267, hsa-miR-99a ID000435, hsa-let-7b ID 002619, hsa-miR-204 ID 000508, hsa-let-7c ID 000379, hsa-miR-202 ID 002363, hsa-miR-10a ID 000387, hsa-miR-136 ID 000592, hsa-miR-145 ID 002278, and RNU48 ID:001006. The PCRs were carried out in the 7500HT Sequence Detection System (Applied Biosystems), as recommended by the manufacturer. Amplification data were normalized to RNU48 expression. Quantification of relative expression was conducted using the $2^{-\Delta\Delta C_t}$ method (17).

Interleukin-6 quantitative reverse transcription PCR

For reverse transcriptase reaction, the Reverse Transcription System (Promega) was used according to the protocol provided by the manufacturer. Real-time quantitative reverse transcription PCR (qRT-PCR) was carried out using designed oligonucleotide primers and Light Cycler (Roche Diagnostics), and the amount of interleukin (IL)-6 mRNA expression was calculated. The expression of IL-6 was normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The designed PCR primers were as follows: IL-6: forward primer, 5′-CCTTCCAAAGATGGCTGAAA-3′, reverse primer, 5′-ATCTGAGGTGCCCATGCTAC-3′; GAPDH: forward primer, 5′-CAACTACATGGTTTACATGTTC-3′, reverse primer, 5′-AAATGAGCCCCAGCCTTC-3′.

miRNA microarray

The miRNA expression profiling was conducted with 1,000 ng of RNA extracted from 2 esophageal cell lines (TE8 and TE10) and the corresponding cisplatin-resistant cell lines (TE8-R and TE10-R) using the TaqMan Array Human MicroRNA Panel (version 1, Applied Biosystems). This qRT-PCR array contains the 365 target microRNAs as well as the endogenous controls. Normalization was conducted with RNU48. The expression of each miRNA in cisplatin-resistance cell line was compared with that in the control parent cell line, and the ratio of miRNA expression in cisplatin-resistance cell line to control cell line was calculated for all 365 miRNAs.

miRNA transfection

TE11 and TE13 cells were transfected with 30 nmol/L pre-miR miRNA precursor molecules of has-let-7c (#PM10436, Applied Biosystems) using SiPORT NeoFX (Ambion) in 6-well plates or 6-cm dishes according to the instructions supplied by the manufacturer. Pre-miR negative control (Applied Biosystems) was also used as a control.

MTT assay

Cell viability was determined by MTT (Sigma-Aldrich) assay. Let-7c or negative control miRNA–transfected cells were seeded into 96-well plates in culture medium. After 24 hours, the medium was changed with a medium containing the following concentration of cisplatin (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, or 400 μmol/L). After incubation for 6 hours, the medium was changed into normal medium. Seventy-two hours after culture, the cells were stained with 20 μL MTT (5 mg/mL) at 37°C for 4 hours and subsequently solubilized in 100 μL of 0.004N HCl-isopropanpol. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories).

Apoptosis assay

Apoptosis was assessed by the flow cytometric detection of phosphatidyl serine externalization using Annexin V and propidium iodide staining (Bio Vision). TE13 cells, after transfection with pre-let-7c and pre-miR negative controls, were treated with 40 μmol/L cisplatin for 6 hours. The cells were harvested and processed for Annexin V staining using the procedure described by the supplier. Briefly, cells were trypsinized gently and resuspended with 500 μL of 1× binding buffer and then treated with 5 μL of Annexin V-FITC and 5 μL of phosphatidylinositol (PI). After incubation for 5 minutes on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (BD Bioscience).

ELISA assay

After 24-hours culture, the cells were exposed to 5 μmol/L CDDP (mentioned above) or medium only. The supernatants were collected (24, 48, or 72 hours) and centrifuged. IL-6 protein level was measured using ELISA kits (#D6050, R&D Systems) according to the protocol provided by the manufacturer.

Western blotting

Cells were washed with ice-cold PBS and harvested from the culture dish. The cells were lysed in RIPA buffer (25 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 500 KIE/mL aprotinin) containing phosphatase inhibitor. The extracts were centrifuged and the supernatant fractions were collected for Western blot analysis. The following antibodies were used

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in this study: at 1:2,000 for anti-human p-STAT3 (Tyr705) antibody (#9145, Cell Signaling), 1:2,000 for anti-human STAT3 antibody (#9132, Cell Signaling), 1:2,000 for anti-human p-Akt antibody (#9271, Cell Signaling), 1:2,000 for anti-human Akt antibody (#4691, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4370, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4695, Cell Signaling), 1:10,000 for anti-human β-actin (#A2066, Sigma-Aldrich), and 1:2,000 for all secondary antibodies. Immune complexes were detected using the Detection Kit (GE HealthCare).

Statistical analysis

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we used the cross-validation method. Data were expressed as mean ± SD. Clinicopathologic parameters were compared using the χ^2 test and continuous variables were compared using Student t test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. $P < 0.05$ denoted the presence of a statistically significant difference. Statistical analysis was conducted using the JMP Ver. 8.0 software.

Results

Altered expression of 15 miRNAs in cisplatin-resistant cells

PCR-based microarray analysis was conducted to compare the expression of miRNAs in cisplatin resistance cells and control cells using 2 pairs of cell lines; TE8/TE8-R and TE10/TE10-R. The miRNA microarray analysis in TE8/TE8-R and TE10/TE10-R cisplatin-resistant cells showed altered expression (by more than 1.7-fold) in 128 (35.0%) and 177 (48.5%) miRNAs among 365 miRNAs, respectively, compared with control cells. Among the miRNAs with altered expression in cisplatin-resistant cells, 15 miRNAs showed overlap in the 2 cell lines. Among these 15 miRNAs, miR135a, miR-96, miR-141, miR-101, miR-146a, miR-489, and miR-545 were upregulated, whereas miR-99a, let-7b, miR-204, let-7c, miR-202, miR-10a, miR-136, and miR-145 were downregulated in cisplatin-resistant cells, compared with control cells (Table 1). Accordingly, we selected these 15 miRNAs as candidates for the response to chemotherapy in esophageal cancer.

Table 1. Fold change in the expression of 15 microRNAs in cisplatin-resistant cells compared with parental cells

microRNA	TE8R/TE8 fold change	TE10R/TE10 fold change
Upregulation		
miR-135a	6.08	12.07
miR-96	3.40	3.85
miR-141	2.41	25.37
miR-101	1.75	2.21
miR-146a	1.97	1,556.1
miR-489	1.78	5.30
miR-545	1.84	3.09
Downregulation		
miR-99a	0.49	0.12
let-7b	0.37	0.39
miR-204	0.35	0.29
let-7c	0.26	0.11
miR-202	0.02	0.01
miR-10a	0.57	0.06
miR-145	0.52	0.03
miR-136	0.54	0.002

Low expression of let-7c is associated with poor response to chemotherapy and poor prognosis

To determine whether the 15 miRNAs are implicated in the response to chemotherapy, we carried out qRT-PCR using pretreatment biopsy samples in 74 patients in training set group with esophageal cancer who underwent preoperative chemotherapy followed by surgery (Table 2). With regard to the clinical response in 74 patients of the training set, CR and PR was achieved in 3 and 30 patients, respectively, whereas SD and PD was observed in 35 and 6 patients, respectively. Thus, 33 (44.6%) patients were categorized as responder whereas the remaining 41 (55.4%) patients were categorized as nonresponders. Expression of the 15 miRNAs was confirmed in the biopsy samples. We also divided the 74 patients of the training set into 2 groups on the basis of the median value of the expression level of each miRNA; the high expression group ($n = 37$) and the low expression group ($n = 37$). Among 15 selected miRNAs, high expression levels of let-7b and let-7c correlated significantly with the clinical response to chemotherapy in esophageal cancer ($P = 0.019$, $P = 0.005$ respectively). However, the expression of the other microRNAs did not correlate with chemosensitivity. Next, we examined whether the expression of let-7b and let-7c is associated with the histopathologic response. With regard to the histopathologic response in 74 patients of the training set, complete tumor regression (grade III) and major tumor regression (grade II) was observed in 3 and 9 patients, respectively, whereas minor tumor regression (grade I) and almost no tumor regression (grade 0) was observed in 54 and 8 patients, respectively. Similar to the clinical response, high expression of let-7b and let-7c correlated significantly with better histopathologic response (Fig. 1A and B). Thus, the expression of let-7b and let-7c in pretreatment biopsy samples determined the response to chemotherapy in patients with esophageal cancer.

Next, we examined whether the expression of let-7b and let-7c is associated with the prognosis of patients who underwent preoperative chemotherapy followed by surgery for esophageal cancer. High expression of let-7c correlated significantly with longer survival in patients who received preoperative chemotherapy (Fig. 1D). High expression of

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Table 2. Relationship between the expression of 15 microRNAs and clinical response

miRNA	Responders ($n = 33$) high/low	Nonresponders ($n = 41$) high/low	P
miR-135a	23/10	14/27	0.640
miR-96	19/14	18/23	0.350
miR-141	19/14	18/23	0.350
miR-101	19/14	18/23	0.350
miR-146a	20/13	17/24	0.160
miR-489	18/15	19/22	0.640
miR-545	19/14	18/23	0.350
miR-99a	15/18	22/19	0.640
let-7b	22/11	15/26	0.019
miR-204	15/18	22/19	0.640
let-7c	23/10	14/27	0.005
miR-202	16/17	21/20	1.000
miR-10a	21/12	16/25	0.061
miR-145	20/13	17/24	0.160
miR-136	16/17	21/20	1.000

NOTE: Data are number of patients.

let-7b also tended to correlate with longer survival, but this tendency did not reach statistical significance (Fig. 1C). We could not find significant relationship between let-7c expression and any clinicopathologic parameter in patients who received preoperative chemotherapy followed by surgery.

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we examined the relationship between let-7c expression and chemosensitivity using biopsy samples of the second group of 24 patients in validation set group. The results confirmed that high expression of let-7c also correlated significantly with the clinical response in esophageal cancer.

Induction of let-7c expression restores chemosensitivity and increases apoptosis after genotoxic chemotherapy

In the next series of studies, we established the relationship between let-7c expression and chemosensitivity using esophageal squamous cell carcinoma cell lines. First, we determined let-7c expression in each esophageal cancer cell line and found relatively low expression of let-7c in TE11 and TE13 cells compared with other esophageal cancer cell lines (Supplementary Fig. S1a). To evaluate the biologic effect of let-7c, pre-let-7c was transfected into TE11 and TE13 cells, and let-7c expression was confirmed in the let-7c–transfected cells (Supplementary Fig. S1b). The MTT assay showed that let-7c–transfected cells were significantly more sensitive to cisplatin than control cells. Furthermore, the IC_{50} of let-7c–transfected

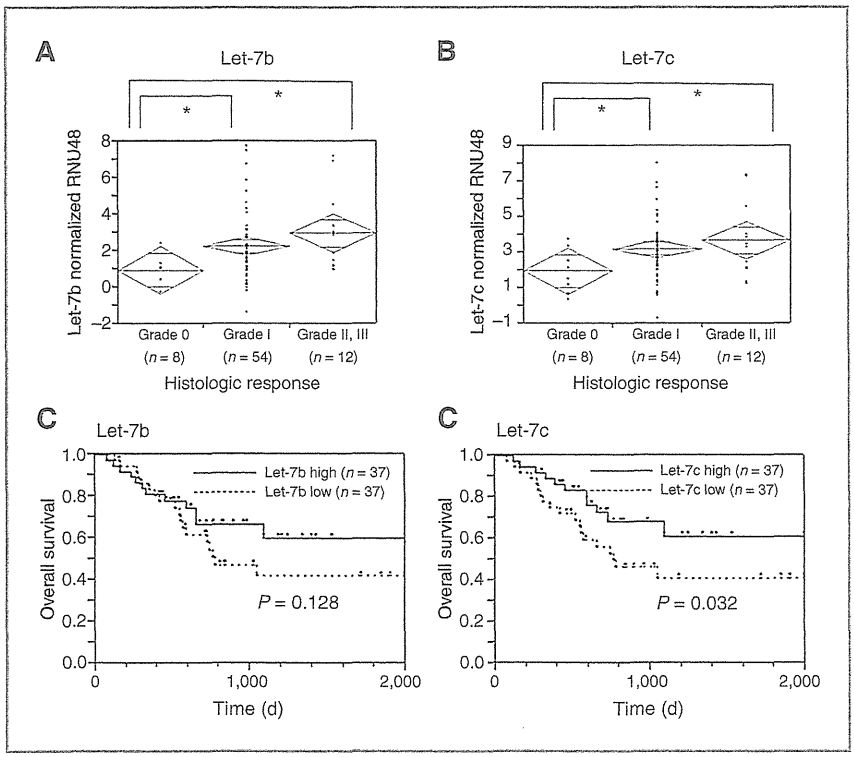

Figure 1. Association of let-7b and let-7c expression with histologic response and overall survival of patients treated with preoperative chemotherapy. A and B, the expression of let-7b and let-7c was higher in patients with histologic response of grade II–III/I than in those with grade 0 (let-7b: $P = 0.014/0.02$; let-7c: $P = 0.032/0.025$). C and D, overall survival curves of 74 patients with esophageal cancer according to let-7b and let-7c expression. High expression of let-7c correlated significantly with longer survival ($P = 0.032$). High expression of let-7b showed similar effect ($P = 0.128$).

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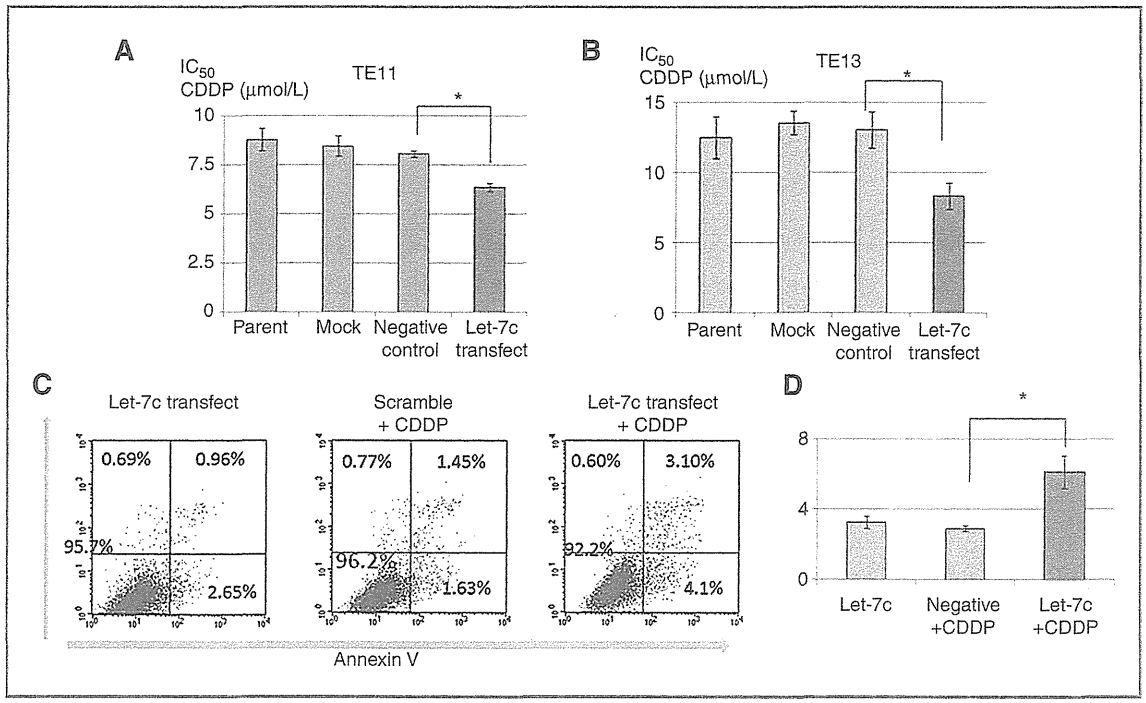

Figure 2. Overexpression of let-7c increases chemosensitivity in esophageal cells. A and B, the IC_{50} levels of cisplatin in TE11 and TE13 esophageal cells were significantly lower in let-7c transfected cells than in negative control transfected cells. Data are mean ± SD. *, $P < 0.01$. C, apoptotic cells were detected by flow cytometry using Annexin V and PI staining. Apoptotic cells were regarded as Annexin-V–positive cells. D, transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control. Data are mean ± SD of 3 experiments. *, $P < 0.01$.

cells was significantly smaller than that of the negative control (Fig. 2A and B).

We also examined the effect of let-7c transfection on apoptosis. For this purpose, we used flow cytometry to determine the percentages of Annexin-V–positive cells among let-7c–transfected cells and control cells treated with cisplatin. Transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control (2.9% vs. 6.1% at 24 hours, $P = 0.049$, Fig. 2C and D). Thus, induced expression of let-7c restored chemosensitivity and increased apoptosis after genotoxic chemotherapy in esophageal cancer cells.

Cisplatin activates IL-6/STAT3 prosurvival signaling pathway

What is the mechanism of let-7c–mediated chemosensitivity of esophageal cells? To answer this question, we hypothesized that let-7c expression regulated apoptosis in cisplatin-treated cells through downregulation of IL-6–mediated signaling pathway. This was based on Target scan and miRBase Targets database, which showed that IL-6 is potential target of let-7c, and also on previous finding of IL-6 as a putative let-7 target (18). In addition, a recent study has shown that IL-6 is released by genotoxic chemotherapy to protect cancer cell from cell death (19). First, we showed that cisplatin activated IL-6 mRNA in esophageal cancer cells (Fig. 3A). Next, we assayed IL-6 levels by ELISA. Cisplatin significantly increased the amount of IL-6 in the conditioned media (Fig. 3B). Furthermore, phosphorylated STAT3, which is downstream of IL-6, was induced by cisplatin in esophageal cancer cells (Fig. 3C and D). These results suggest that cisplatin activates the IL-6/STAT3 signaling pathway in an autocrine manner in esophageal cancer cells.

Next, we investigated whether activation of IL-6/STAT3 pathway protects cisplatin-exposed cancer cells from apoptosis. For this purpose, we examined cell viability and apoptosis in cisplatin-treated IL-6 knockdown cells and control cells. MTT assay showed that knockdown of IL-6 in esophageal cancer cells significantly reduced cell viability (Fig. 3E), and Annexin V assay showed that knockdown of IL-6 in esophageal cancer cells significantly increased the rate of apoptosis (Fig. 3F and G). These results indicate that cisplatin activates IL-6/STAT3 pathway in cancer cells, paradoxically providing protection of cancer cells against cell death.

Let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy

We examined whether let-7 represses the activation of IL-6/STAT3 signaling pathway after cisplatin chemotherapy. Expression of IL-6 mRNA was significantly reduced after

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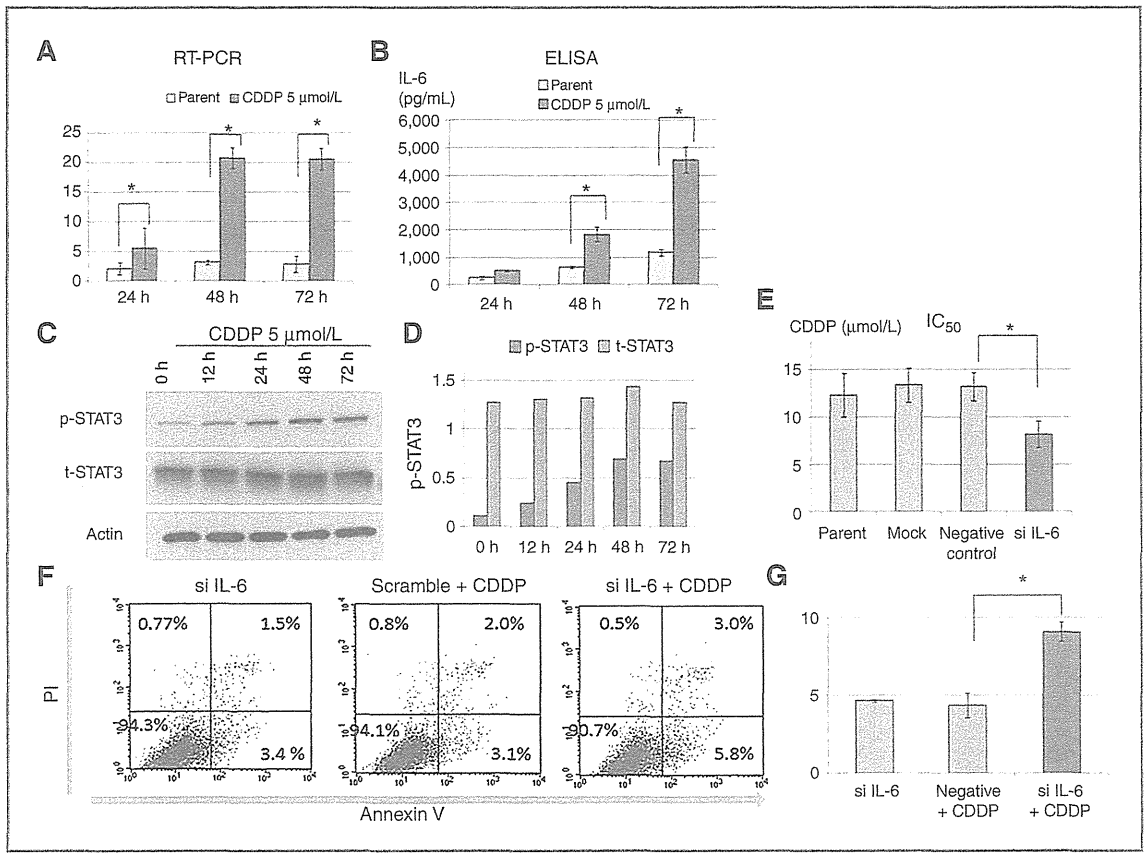

Figure 3. Cisplatin activates prosurvival IL-6/STAT3 signaling pathway. A, cisplatin significantly increased the expression of IL-6 mRNA in esophageal cancer cells at 24-, 48-, and 72-hour exposure. B, cisplatin significantly increased the expression of IL-6 protein in supernatants of conditioned medium at 24-, 48-, and 72-hour exposure. C, Western blot analysis of phosphorylated STAT3 and total STAT3 after cisplatin exposure. Exposure to cisplatin induced phosphorylated STAT3 in esophageal cancer cells. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. D, semiquantitative analyses of expression of pSTAT3 and t-STAT3 in (C) by using densitometer. E, the IC_{50} level of cisplatin in siIL-6–transfected cells is significantly lower than in negative control transfected cells. F and G, transfection of siIL-6 significantly increased the proportion of apoptotic cells after cisplatin, compared with the negative control. Data in (A), (B), (E), and (G) are mean ± SD of 3 experiments. *, $P < 0.01$.

cisplatin treatment in let-7c transfected cells compared with control cells. The level of secreted IL-6 in the conditioned medium after cisplatin treatment was also significantly reduced in let-7c–transfected cells compared with control cells (Fig. 4A). Furthermore, phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells after cisplatin treatment, although the induced expression of let-7c had no apparent effect on the expression of Akt and extracellular signal–regulated kinase (Erk), which are downstream of IL-6 (Fig. 4B and C). Taken together, these results indicate that let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy in esophageal cancer cells.

Finally, we examined the relationship between let-7c and IL-6 expression in clinical samples obtained from 40 patients with esophageal cancer. Let-7c expression of cancer tissue is significantly lower than that of noncancerous tissue (Fig. 4D). In contrast, IL-6 expression was significantly higher in cancer tissue than in noncancerous tissue (Fig. 4E). Moreover, IL-6 expression correlated inversely with let-7c expression in noncancerous tissue and esophageal cancer tissue (Fig. 4F).

Discussion

In multimodal therapy for esophageal cancer, chemotherapy is often combined with radiation and/or surgery. If prediction of the response to chemotherapy before surgery is possible, one can offer another treatment option for patients who show resistance to chemotherapy. In the present study, we investigated whether we could predict the response to cisplatin-based chemotherapy by analyzing the miRNA expression in esophageal cancer using biopsy samples before treatment. The results showed that low expression of let-7b and let7c is associated with low chemosensitivity in patients with esophageal cancer. The

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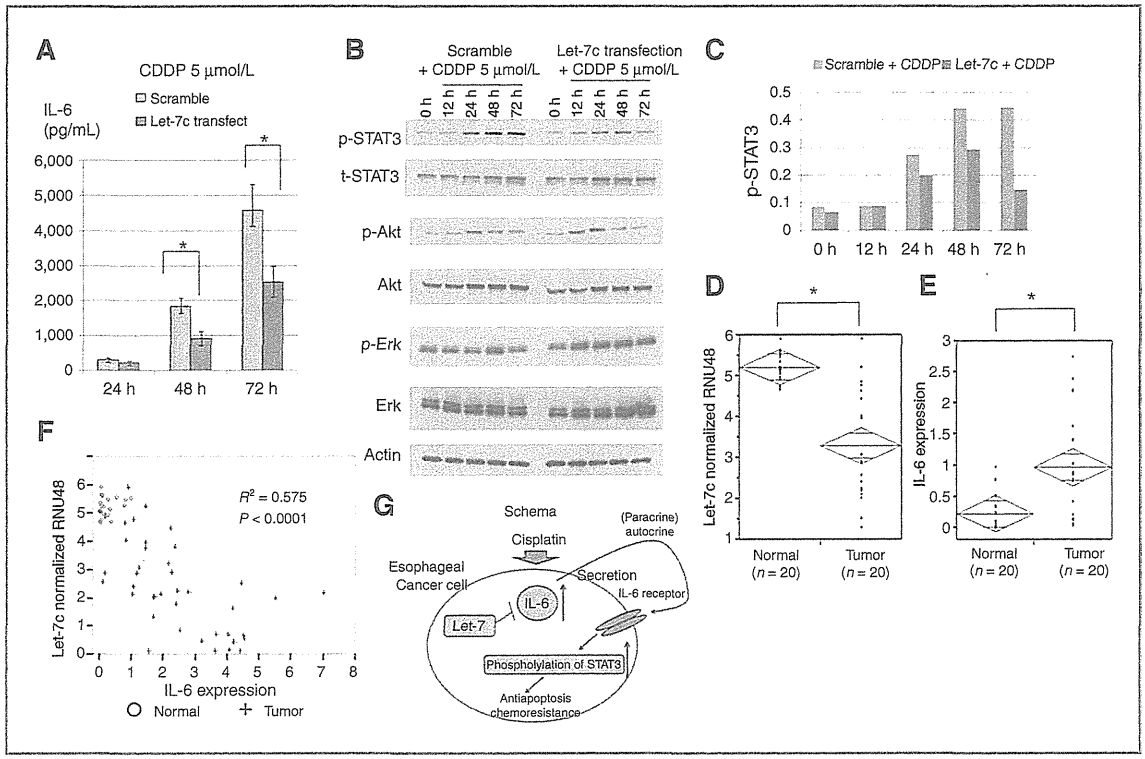

Figure 4. Overexpression of let-7c represses IL-6/STAT3 prosurvival pathway after cisplatin exposure. A, cisplatin significantly reduced IL-6 production in conditioned medium of let-7c–transfected cells compared with control cells at 48 and 72 hours. Data are mean ± SD of 3 experiments. *, $P < 0.01$. B, Western blot analysis of differential expression of proteins downstream of IL-6 after cisplatin exposure. Phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells. Representative data of 3 experiments with similar results. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. p-Akt and p-Erk means phosphorylated Akt and Erk, respectively. C, semiquantitative analyses of expression of pSTAT3 in (B) by using densitometer. D, let-7c expression in esophageal cancer tissue is significantly lower than that of noncancerous tissue. E, IL-6 mRNA expression in cancer tissue is significantly higher than that of noncancerous tissue, determined by real-time RT-PCR. F, IL-6 mRNA expression correlated inversely with let-7c expression in noncancerous tissue ($n = 20$) and esophageal cancerous tissue ($n = 40$). G, schematic overview of relationship between let-7 and IL-6/STAT3 pathway in chemoresistance. IL-6 expression is upregulated after cisplatin exposure in esophageal cancer cells. In autocrine manner (although paracrine manner may also exist), increased expression of IL-6 upregulates phosphorylation of p-STAT3, resulting in antiapoptosis and chemoresistance. Let-7 restores sensitivity to cisplatin through repressing IL-6/p-STAT prosurvival pathway by inhibiting directly IL-6 expression.

results also showed that the effect of let-7 expression on chemosensitivity of esophageal cancer is mediated through let-7–induced repression of the IL-6/STAT3 pathway, which is prosurvival pathway activated through exposure to genotoxic agents such as cisplatin.

A few studies have reported the clinical use of miRNA expression for prediction of response to chemotherapy. Yang and colleagues (20) conducted miRNA microarray in 69 patients with epithelial ovarian cancer who had received cisplatin-based chemotherapy and reported significantly reduced let-7i expression in chemotherapy-resistant patients. They confirmed the clinical relevance of let-7i as a biomarker to predict chemotherapy response in a validation set of another 72 patients. However, the underlying mechanism of the involvement of let-7i expression in chemosensitivity of ovarian cancer was not clarified in their study. Another study by Nakajima and colleagues (21), which evaluated the expression of several miRNAs in 46 patients with recurrent or residual colon cancer, showed that upregulation of miR-181b and let-7g was significantly associated with poor response to 5-FU–based antimetabolite S-1. However, their finding of the correlation between high expression of let-7 and poor response to chemotherapy is different from our results.

The involvement of let-7 family in chemosensitivity has been examined in several *in vitro* studies. In pancreatic cancer cells, the expression of let-7b,c,d,e was significantly reduced in gemcitabine-resistant cancer cells, and upregulation of let-7 expression resulted in the reversal of epithelial–mesenchymal transition in gemcitabine-resistant cancer cells (22). In hepatocellular carcinoma cells, let-7 inhibited Bcl-xL expression, which is an antiapoptotic member of the Bcl-2 family and known to induce apoptosis in cooperation with anticancer drugs that target Mcl-1, antiapoptotic Bcl-2 protein (23). In oral cancer cells, let-7d negatively regulated EMT expression by targeting Twist and Snail and played an