RNASEN Regulates Cell Proliferation and Affects Survival in Esophageal Cancer Patients

Nobuyoshi Sugito, Hiroyuki Ishiguro, Yoshiyuki Kuwabara, Masahiro Kimura, Akira Mitsui, Hiroki Kurehara, Takuya Ando, Ryota Mori, Nobuhiro Takashima, Ryo Ogawa, and Yoshitaka Fujii

Abstract

Purpose: MicroRNAs (miRNA) are small noncoding RNAs thought to be involved in physiologic and developmental processes by negatively regulating the expression of target genes. Little is known about the role of miRNAs in normal and cancer cells. It is possible that deregulation of miRNA may contribute to the oncogenesis of some cancers. We studied the expression level of the miRNA processing enzyme (DICER1, DGCR8, and RNASEN) in esophageal squamous cell carcinoma (ESCC).

Experimental Design: The expression levels of DICER1, DGCR8, and RNASEN mRNA in 73 ESCC tissues were compared with that in corresponding normal esophageal epithelium by Taqman real-time reverse-transcription PCR. We also examined RNASEN protein expression in 27 cell lines. The role of RNASEN in cell proliferation in ESCC cells was assessed by small interfering RNA. Paraffin sections of ESCC patients were immunohistochemically investigated.

Results: We found that RNASEN expression levels were enhanced in a fraction of esophageal cancers. Multivariate Cox regression analysis showed that the prognostic effect of RNASEN (P = 0.0036) seems to be independent of disease stage (P = 0.0060). Knockdown of RNASEN in esophageal cancer cell lines resulted in a 46% to 85% reduction in cell number. In an immunohistochemical study, the intensity of RNASEN expression was often increased in the tumor compared with that in normal epithelium.

Conclusions: The relationship between the RNASEN expression and the prognosis of the ESCC patients warrants a further study on the role of miRNA and tumor progression.

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies in Japan, with a 5-year survival rate of 20% to 30% after curative surgery (1, 2). Even in early disease stages, we have experienced many patients developing local tumor recurrence or distant metastases within a short period after surgery.

MicroRNAs (miRNA), which constitute a well-conserved and abundant class of ~22-nucleotide regulatory RNAs, have recently attracted attention. Emerging evidence suggests the potential involvement of altered regulation of miRNAs in the pathogenesis of a limited range of human cancers (3–7). The biological functions of miRNA are not yet fully understood, but it has been suggested that they play a role in the coordination of cell proliferation and cell death during development (8) in addition to their involvement in stress resistance (9). This evidence seems to lend support to the notion that changes in miRNA may be involved in the genesis and/or progression of various human cancers (10).

Double-stranded RNA–specific endonucleases convert precursor forms of miRNA into mature forms through a stepwise process that includes the generation of <70-nucleotide pre-miRNAs with characteristic hairpin structures from the longer nascent transcripts (pre-miRNA) and subsequent processing into the mature form. In humans, DICER1, RNASEN, and DGCR8 are thought to participate in this processing of miRNA, with RNASEN (11, 12) and DGCR8 (13) executing the initial step in the nucleus, after which the resultant pre-miRNAs are exported to the cytoplasm where they are cleaved by DICER1 to generate the final products of <22 nucleotides (14, 15).

In this study, we examined whether the expression of DICER1, DGCR8, and RNASEN, which are essential for miRNA processing, is altered in esophageal cancers. To this end, we examined 73 esophageal cancer cases by means of Taqman real-time reverse-transcription PCR (RT-PCR). We report here that up-regulation of RNASEN in ESCC was associated with shorter postoperative survival.

Materials and Methods

Patients and tumor samples. Esophageal cancers were obtained from 73 patients who underwent surgery at the Nagoya City University Hospital (Nagoya, Japan) between January 1996 and December 2002.
Stages were determined after pathologic evaluation of resected specimens according to the guidelines for clinical and pathologic studies on esophageal carcinoma. All of the cases were classified according to the pathologic tumor-node-metastasis classification (16). The cohort consisted of 59 males and 14 females, with age at diagnosis ranging from 43 to 80 years (median age, 65 years). Five patients had stage 0 disease, 4 had stage I, 14 had stage II, 21 had stage III, and 29 had stage IV. There were 7 patients with poorly differentiated tumors, 33 with moderately differentiated tumors, and 27 with well-differentiated tumors. All of the tumors were confirmed as being ESCC by the Clinical Pathology Department.

Pieces of tumor tissue were carefully selected for maximum tumor content. We confirmed the section of tumor content by H&E staining. We used a criterion of tumor content at least 80% and above. All samples were snap frozen in liquid nitrogen followed by storage at −80°C until use. Total RNA was extracted from esophageal cancer tissues and from normal esophageal mucosa taken from a site as distant as possible from the tumor using Absolutely RNA RT-PCR Miniprep kits (Stratagene, La Jolla, CA). Concentration of total RNA was adjusted to 200 ng/mL using a spectrophotometer. Reverse transcription was done using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI PRISM 7500 (Applied Biosystems). PCR conditions were 50°C for 15 minutes using 10 μg total RNA, random hexamer primers (Roche Applied Science, Alameda, CA), and SuperScript II enzyme (Invitrogen, Carlsbad, CA).

Real-time RT-PCR using Taqman probes. Real-time quantitative PCR amplification of the cDNA template corresponding to 20 ng total RNA was done using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI PRISM 7500 (Applied Biosystems). PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. DICER1-, RNASEN-, and DGCR8-specific Taqman probes were synthesized from sequences in exons 15, 11, and 2 (Assays-on-Demand Gene Expression system, DICER1 assay ID, Hs00229023_m1; RNASEN assay ID, Hs00203008_m1; DGCR8 assay ID, Hs00256020_s1; Applied Biosystems). Expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (Assays-on-Demand Gene Expression system, assay ID Hs99999905_m1; Applied Biosystems).

Cell culture. Human esophageal carcinoma cell lines: TE series, a gift from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan); KYSE series, obtained from Japanese Collection Research Bioresources (Tokyo, Japan) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen-Life Technologies, Inc., Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin. Normal human esophageal mucosa cell line (Hert1A), 293T, HCT116, and SW480 were purchased from the American Type Culture Collection (Manassas, VA). Cell lines were cultured essentially according to the vendor’s instructions.

Small interfering RNA transfection and cell proliferation assay. All small interfering RNA (siRNA; Dharmacon, Lafayette, CO), control (siCONTROL Non-Targeting Pool), and RNASEN (siGENOME SMART-pool Reagent) transfections were done with Cell Line Nucleofector kits (Amaxa, Cologne, Germany) according to the manufacturer’s instructions. Cells were analyzed at 72 hours after siRNA treatment. Cell proliferation assay was done in duplicate, and cells were counted in 20 random fields.

Western blot analysis. Equal amounts of total cell lysate solubilized in Laemmli sample buffer were subjected to SDS-PAGE and then transferred to Immobilon-P filters (Millipore Corp., Bedford, MA). The filters were first incubated with primary antibody, rabbit polyclonal anti-Drosha (RNASEN; Abcam, Cambridge, United Kingdom), and mouse monoclonal anti-α-tubulin (Sigma, St. Louis, MO) followed by

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**Table 1.** Relationship between expression levels of DICER1, DGCR8, RNASEN and various clinicopathologic characteristics

<table>
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<th>Characteristics</th>
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*Two-sided Fisher’s exact test.
horseradish peroxidase–conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Cell Signaling Technology, Beverly, MA; anti-goat IgG, MBL, Nagoya, Japan). For visualization, an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom) was used.

**Immunohistochemistry.** ESCC sections were obtained from Nagoya City University Hospital. For antigen unmasking, deparaffinized sections were boiled in citrate buffer [10 mmol/L sodium citrate buffer (pH 6.0)] before incubation with primary antibodies. RNASEN and Ki67 protein levels were examined using rabbit polyclonal antibodies and mouse monoclonal antibodies (Abcam). Antibody staining was done using the DAKO EnVision system (DAKO EnVision labeled polymer, peroxidase).

**Immunofluorescence.** Het1A, TE10, TE12, TE13, and TE15 cells were grown on Lab-Tek chamber slides (Nalge Nunc International K.K., Rochester, NY). Cells were washed once with PBS, fixed with 1% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 on ice for 20 minutes. Cells and ESCC deparaffinized sections
were then incubated for 1 hour at 37°C in 1% bovine serum albumin and 4% fat in TBS with rabbit anti-Drosha (RNASEN) antibody (1:50 dilution) and mouse anti-Ki67 antibody (1:50 dilution) obtained from Abcam. Secondary antibodies conjugated with Alexa Fluor 488 (rabbit polyclonal) and Alexa Fluor 568 (mouse monoclonal; Molecular Probes, Eugene, OR) were used at a dilution of 1:300. Immunofluorescence microscopy and image acquisition were followed by confocal microscopy done using a laser scanning confocal imaging system (LSM5 PASCAL, Carl Zeiss, Jena, Germany).

Statistical analysis. The following biostatistical analyses were done with StatView software (Abacus Concepts, Berkeley, CA). Student’s t test was used to determine the best cutoff value for separating two characteristic groups in terms of gene expression levels. The associations between various clinicopathologic characteristics and expression levels of DICER1, RNASEN, and DGCR8 were examined by means of Fisher’s exact test. Kaplan-Meier estimates of overall survival time were compared using the log-rank test. Cox regression analysis of factors potentially related to survival was used to identify independent factors that might have a significant joint effect on survival. All tests were two tailed, and the significance level was set at \( P < 0.05 \).

Results

Relationships between DICER1, DGCR8, and RNASEN expression levels and clinicopathologic findings. We did realtime RT-PCR analysis using Taqman probes (Taqman Gene Expression Assays) to examine 73 ESCC cases for DICER1, DGCR8, and RNASEN expression. A histogram of expression of DICER1 and RNASEN showed a frequency distribution with two prominent peaks at log2 values (Supplementary Data). Patients could be divided most clearly and consistently into two groups with low and high expression. DICER1 distribution threshold was set at 0.9 of the log2 ratio, and RNASEN distribution threshold was set at 1.1 of the log2 ratio. In contrast to DICER1 and RNASEN, the distribution of DGCR8 seemed to follow a normal distribution. Accordingly, the median expression level (i.e., 1.9 of the log2 value) was chosen as the threshold value to be used for further analysis.

Our investigation focused on whether expression levels of DICER1, DGCR8, and RNASEN had any relationship with clinicopathologic characteristics of ESCC. We found that there was a statistically significant association between RNASEN expression levels and lymph node metastasis (Table 1). Cases with low RNASEN expression showed significantly greater prevalence of N0 than those with high RNASEN expression (\( P = 0.02 \); Table 1).

High RNASEN expressions correlate with poor prognosis in ESCC. We next examined whether expression levels of DICER1, DGCR8, and RNASEN were associated with patient survival after surgery. Kaplan-Meier survival curves showed that the probability of survival was significantly lower for patients with high levels of RNASEN expression (\( P = 0.0003 \), log-rank test; Fig. 1), whereas high expression of DGCR8 tended to be associated with poor prognosis (\( P = 0.10 \), log-rank test; Fig. 1). The effect of various factors on prognosis was studied by univariate Cox regression analysis (Table 2). It was shown that, in addition to disease stage (\( P = 0.0006 \), high RNASEN expression was a significant predictive factor for poor prognosis (\( P = 0.0008 \)), whereas DICER1 and DGCR8 expression levels did not show significant associations with survival (\( P = 0.53 \), \( P = 0.11 \); Table 2).

The interrelationships between possible prognostic factors and survival were further analyzed by means of Cox proportional hazards modeling using disease stage, lymph node metastasis, vein invasion, lymphatic invasion, and histology as well as expression levels of DICER1, DGCR8, and RNASEN as variables. Overexpression of RNASEN, in addition to disease stage (\( P = 0.018 \)), was identified as a significant and independent prognostic factor (\( P = 0.0069 \)) for surgically
treated ESCC patients. The hazard ratio for earlier death was 4.56 (95% confidence interval, 1.52-13.75) for high versus low expression levels of RNASEN (Table 2). These findings suggest that expression levels of RNASEN have a significant effect on postoperative survival of ESCC patients.

**RNASEN expression in ESCC cell lines and tumor samples.** We examined RNASEN expression in 27 cell lines at the mRNA (Fig. 2A) and protein (Fig. 2B) levels. All cell lines expressed RNASEN protein at varying degrees. RNASEN expression in many esophageal cancer cell lines was found to be higher when compared with the normal human esophageal mucosa cell line Het1A; expression of RNASEN in the colon cancer cell lines HCT116 and SW480 was low. A modest correlation was found between RNASEN protein levels and mRNA expression quantified by Taqman real-time RT-PCR.

**Growth suppression in ESCC cell lines by reduction of RNASEN.** The identification of strong expression of RNASEN in ESCC, in association with shortened survival, prompted us to explore the possible biological significance of RNASEN in ESCC development. We introduced RNASEN siRNA into ESCC cell lines (TE10, TE12, and TE13, which expressed high levels of RNASEN) by electroporation. Introduction of RNASEN siRNA reduced RNASEN protein levels by >90% in ESCC cell lines, whereas transfection of the control (nontargeting siRNA) did not (Fig. 3A). Knockdown of RNASEN in esophageal cancer cell lines resulted in 76.5% (TE10), 46.4% (TE12), and 84.8% (TE13) reductions in the number of cells counted in 20 random fields at 72 hours after transfection.

**Protein levels of RNASEN in ESCC lines and human tissue samples.** In normal esophageal epithelium, immunohistochemistry showed that RNASEN is abundant in the nuclei of basal and lower spinous layer cells. In esophageal cancer samples, RNASEN was strongly expressed in the nuclei and cytoplasm, and particularly, strong staining was observed in the peripheral part of the tumor (Fig. 4A). RNASEN staining was mainly seen in the nuclei of the cell lines (Fig. 4B). Staining for Ki67, a proliferation marker, seemed to be positive in cells with positive RNASEN expression (Fig. 4C and D).

**Discussion**

RNase III proteins, which are grouped into three classes based on domain organization, play key roles in miRNA biogenesis (13). RNASEN (class II protein) contains two RNase III domains and one double-stranded RNA–binding domain. RNASEN cleaves pri-miRNAs to release hairpin-shaped pre-miRNAs, which are subsequently cut by DICER1 (class III) to generate mature miRNAs. Whereas DICER1 and other simple RNase III proteins (class I) have been studied intensively, class II enzyme RNASEN proteins have not been characterized...
in detail. The size of the pri-miRNA processing complex (650 kDa) suggests that RNASEN may interact with other proteins to form a functional complex. In this complex, RNASEN interacts with DGCR8, which contains two double-stranded RNA-binding domains (13). RNASEN is located at 5p13.3 whose amplification has been reported in ESCC specimens and cell lines by comparative genomic hybridization (17–21), which suggests that RNASEN may be a key gene in the development of esophageal cancer.

In the present study, we showed that the higher expression levels of RNASEN detected in a significant fraction of ESCC are associated with shorter postoperative survival. To the best of our knowledge, ours is the first report of changes in RNASEN expression in ESCC. It should be noted that, among the
variables used in the multivariate Cox regression analysis, RNASEN seems to have a prognostic effect ($P = 0.0069$) independent of the disease stage ($P = 0.018$). Interestingly, expression of RNASEN, but not DGCR8 or DICER1, is correlated with poor prognosis in esophageal cancer.

RNASEN protein was strongly stained in tumor samples; particularly, strong staining was observed in the tumor periphery, suggesting a possibility of the involvement of RNASEN in tumor invasion to surrounding tissues. Furthermore, it seems that strong staining was observed in the cytoplasm of ESCC, a finding that might indicate aberrant localization of RNASEN. Growth suppression in ESCC cell lines was observed when RNASEN expression was reduced by siRNA. Staining for Ki67, a proliferation marker, correlated with the RNASEN staining. These findings suggest that RNASEN may be involved in tumor growth and development. Our experiment using RNASEN siRNA supports the notion that the high RNASEN expression observed in our study might have a functional role in the development of esophageal cancers rather than being a mere surrogate marker.

In esophageal cancer patients, prognostic markers, including cyclinD1 (22–24), Ecadherin (22, 24), MDM2 (22, 24), fascin (25), and HGF (26), have been reported. We previously showed that expression of survivin (27), pituitary tumor transforming gene 1 (28), peroxisome proliferator-activated receptor $\gamma$ (29), DNA fragmentation factor 45 (30), and excision repair cross complementing 3 (31) may be prognostic markers for ESCC. RNASEN may now be added to this list. Although the precise molecular mechanism of RNASEN up-regulation requires clarification, our data indicate that RNASEN may be a good candidate molecular prognostic marker. A further study on the role of miRNA on ESCC progression is warranted.

Acknowledgments

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References

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