

Clinicopathological and prognostic significance of the microRNA processing enzyme DICER1 mRNA expression in colorectal cancer patients

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Abstract. The cytoplasmic ribonuclease DICER1 is one of the key enzymes in microRNA (miRNA) processing, essential for the production of mature miRNA. The effect of DICER1 expression in tumor cells on the prognosis of patients with several cancers has been examined with controversial results in various cancer types. In particular, the clinical significance of DICER1 expression in colorectal cancer (CRC) patients has yet to be determined. The aim of this study was to evaluate the correlation between the DICER1 mRNA levels and the clinicopathological characteristics and prognostic significance in CRC patients. Tumor and normal adjacent tumor tissues from 260 patients with CRC (Dukes' stage A: 40 cases, Dukes' B: 68 cases, Dukes' C: 88 cases and Dukes' D: 64 cases) were examined. The DICER1 mRNA levels were measured using the TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR) method. The expression levels of DICER1 mRNAs showed a significant decrease in CRC tissues as compared to normal ones ($P=0.039$). A statistically significant association was observed between DICER1 mRNA expression and tumor size, depth of invasion, lymph node metastasis, lymphatic invasion and Dukes' stage. In Kaplan-Meier survival curve analysis, overall survival (OS) and disease-free survival (DFS) rates of patients with a low DICER1 mRNA expression were significantly worse compared to patients with a high DICER1 mRNA expression (OS $P<0.001$; DFS $P<0.001$). In the Cox multivariate analysis, DICER1 mRNA expression in CRC tissues was identified as an independent prognostic factor for OS [hazard ratio (HR), 0.30; 95% confidence interval (CI), 0.13-0.64; $P=0.001$] and DFS (HR, 0.23; 95% CI, 0.10-0.48; $P=0.001$). This study

demonstrated that a reduced DICER1 mRNA expression is associated with poor prognosis in CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer mortality worldwide (1). Despite the fact that recent advances in chemotherapeutic regimens and combination with radiotherapy have improved survival of advanced-stage CRC patients, an increased risk of recurrence and metastasis, and thus high mortality rates, are associated with advanced-stages of the disease (2). Therefore, identification of novel prognostic biomarkers to improve patient outcome and to assess individual prognosis is required.

MicroRNAs (miRNAs) are a class of small, mature non-coding 21-25 nucleotides that participate in the regulation of cell differentiation, cell cycle progression and apoptosis (3-5). miRNAs target protein-coding mRNAs at the post-transcriptional level by direct cleavage of the mRNA or by inhibition of protein synthesis (6). It has been hypothesized that miRNAs are highly involved in cancer development (4). The synthesis and maturation of miRNAs requires a set of proteins known collectively as miRNA-processing machinery (7). miRNAs are transcribed by polymerase II as long primary transcripts (pri-miRNAs). Pri-miRNAs are spliced in the nucleus by the enzyme Drosha to form 70-100 nucleotide hairpin precursors of miRNA (pre-miRNA) (8). The pre-miRNAs are exported to the cytoplasm by Exportin-5, where they are further processed by the RNase III endonuclease enzyme DICER 1, resulting in short double-stranded miRNA (miRNA duplex) of 19-24 nucleotides (9,10).

Cytoplasmic ribonuclease type III DICER1 is a key enzyme involved in the miRNA processing pathway that regulates RNA-based gene silencing by the cleavage of miRNA precursor (11-16). Mounting evidence suggests that DICER1 expression levels are associated with clinical outcomes in lung, ovarian, breast and prostate cancers (17-21). Therefore, it is of interest to determine whether or not DICER1 may be used as a prognostic marker to predict an individual patient's risk. Using the immunohistochemical method (IHC), only a few clinical studies have investigated the usefulness of DICER1 protein levels as a prognostic factor in CRC patients (22,23). However, the prognostic values of DICER1 protein levels in these studies

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were contradictory. Furthermore, the prognostic significance of DICER1 mRNA level in CRC patients has yet to be elucidated.

In this study, we investigated the association between the clinicopathological characteristics and prognostic value of DICER1 mRNA in 260 CRC patients.

Patients and methods

Patients and tissue samples. A total of 260 CRC patients were studied between September, 2000 and April 1, 2006 at the Teikyo University Hospital (Tokyo, Japan). The median follow-up period was 45 months (range, 24-70). The samples were obtained from patients who did not receive any chemotherapy or radiotherapy prior to surgery. Immediately following surgical resection, primary CRC and normal adjacent tumor tissues (normal tissue) were mounted using Tissue-Tek O.C.T Compound (Sakura Finetech Co., Ltd., Tokyo, Japan) and frozen in liquid nitrogen. The tissues were then stored at -80°C until laser-capture micro-dissection (LCM). The study protocol conformed to the guidelines of the ethics committee, and was approved by the review board of the Teikyo University, while written informed consent was obtained from the patients.

Follow-up of patients. Post-operative follow-up was performed along the guidelines published by the Japanese Society for Cancer of the Colon and Rectum. Confirmation of recurrence in the patients was required to evaluate imaging or pathological diagnosis. Physical examination and tumor marker (CEA and CA19-9) testing was conducted every 3 months for 3 years and then every 6 months for 5 years. Computed tomography (CT) or magnetic resonance imaging (MRI) scans were repeated every 3 months for 3 years and then every 6 months for up to 5 years, following surgery. Colon evaluation, including colonoscopy or colon radiography was performed every 2 years or annually for 3 years.

LCM and RNA isolation. Frozen sections ($10\ \mu\text{m}$) of CRC and normal tissues were prepared using a Leica CM 1900 cryostat (Leica, Wetzlar, Germany) at -25°C . The sections were placed on membrane-coated glass slides (Leica), fixed in 75% alcohol for 30 sec and stained with 0.5% violet-free methyl green (Sigma-Aldrich, St. Louis, MO, USA). After staining, the sections were air-dried and micro-dissected using a Leica AS LMD system (Leica). LCM caps were stored at -80°C until RNA isolation.

Total RNAs were extracted using a miRNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) and were treated with DNase I, according to the manufacturer's instructions (Qiagen). Total RNA was reverse-transcribed to complementary DNA (cDNA), using the SuperScript II reverse transcriptase system with random hexamer primers, according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA).

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) for DICER1 mRNA. The relative expression levels of DICER1 and GAPDH mRNA (internal control) were determined by quantitative real-time PCR amplification (qRT-PCR) using a LightCycler 480 (Roche Diagnostics Corp., Indianapolis, IN, USA). The amplifications of these genes were performed using the LightCycler

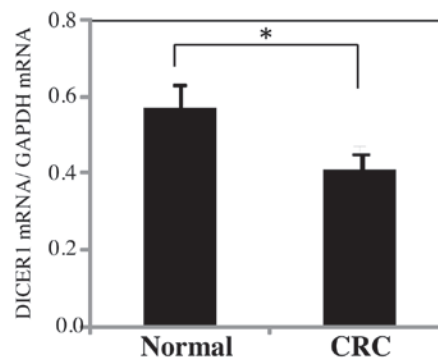


Figure 1. Comparison of DICER1 mRNA levels between colorectal cancer (CRC) and normal tissues. Primary CRC and normal adjacent tumor tissues (normal tissues) were obtained from 260 CRC patients. DICER1 mRNA levels were detected using TaqMan PCR assay and normalized to GAPDH mRNA. Data are shown as the mean \pm standard error. * $P=0.039$.

480 Probe Master (Roche Diagnostics Corp.) and TaqMan Gene Expression Assays for DICER1 (Hs00229023_m1) and GAPDH (Hs02758991_g1) (Applied Biosystems, Inc., Carlsbad, CA, USA). The PCR conditions of these genes are 95°C for 5 min, followed by 40 cycles 95°C for 1 sec, 60°C for 20 sec. All the samples were performed in triplicates. The expression levels of DICER1 mRNA were normalized to GAPDH mRNA expression.

Statistical analysis. Data were shown as the mean \pm standard error. The correlations were analyzed using the Student's t-test, the Chi-square test and analysis of variance (ANOVA). The cut-off value of DICER1 mRNA was determined by receiver operating characteristic (ROC) curves, which used the JMP 9.0 software (SAS Inst. Inc., Cary, NC, USA). Overall survival (OS) and disease-free survival (DFS) curves were analyzed using the Kaplan-Meier method and the differences were examined using log-rank tests. Cox proportional-hazards regression analysis was used to estimate univariate and multivariate hazard ratios for OS and DFS. P values were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using the JMP 9.0 software (SAS Inst. Inc.).

Results

Expression of DICER1 mRNA in CRC and normal tissues. A comparison of DICER1 mRNA expression levels of the primary CRC and normal adjacent tumor tissues (normal tissues) were compared (Fig. 1). The samples were collected from 260 CRC patients. DICER1 mRNA levels were normalized by GAPDH mRNA levels. In this study, DICER1 mRNA levels of CRC tissues showed a significant decrease as compared to normal tissues ($P=0.039$).

Correlation between the expression of clinicopathological factors and DICER1 mRNA in tumor tissues. This study comprised 260 CRC patients (153 men and 107 women), with a mean age of 67 years (range, 27-88). To evaluate the correlation between the DICER1 mRNA levels and the clinicopathological characteristics, patients were divided

Table I. Clinicopathological data and DICER1 mRNA expression in 260 CRC patients.

Variables	Total no. of patients	DICER1 mRNA expression, no. of patients (%)		P-value
		High	Low	
Gender				0.909
Male	153	64 (41.83)	89 (58.17)	
Female	107	44 (41.12)	63 (58.88)	
Tumor size (cm)				0.001 ^a
<5	147	74 (50.34)	73 (49.66)	
≥5	113	34 (30.09)	79 (69.91)	
Depth of invasion				0.002 ^a
≤pT2	24	17 (70.83)	7 (29.17)	
≥pT3	236	91 (38.56)	145 (61.44)	
Localization				0.860
Colon	150	63 (42.00)	87 (58.00)	
Rectum	110	45 (40.91)	65 (59.09)	
Histological type				0.319
Well	174	76 (43.68)	98 (56.32)	
Unwell	86	32 (37.21)	54 (62.79)	
Lymph node metastasis				0.008 ^a
Negative	124	62 (50.00)	62 (50.00)	
Positive	136	46 (33.82)	90 (66.18)	
Lymphatic invasion				0.014 ^a
Negative	150	72 (48.00)	78 (52.00)	
Positive	110	36 (32.73)	74 (67.27)	
Venous invasion				0.804
Negative	106	45 (42.45)	61 (57.55)	
Positive	154	63 (40.91)	91 (59.09)	
Liver metastasis				0.178
Negative	222	96 (43.24)	126 (56.76)	
Positive	38	12 (31.58)	26 (68.42)	
Peritoneum dissemination				0.116
Negative	243	104 (42.80)	139 (57.20)	
Positive	17	4 (23.53)	13 (76.47)	
Dukes' stage				0.015 ^a
A	40	25 (62.50)	15 (37.50)	
B	68	30 (44.12)	38 (55.88)	
C	88	32 (36.36)	56 (63.64)	
D	64	21 (32.81)	43 (67.19)	

^aP=0.039. CRC, colorectal cancer.

into the high- and low-level groups. The cut-off level for DICER1 mRNA was set at 0.275 based on analysis of the ROC curve. As shown in Table I, a statistically significant association was observed between DICER1 mRNA expression and tumor size, depth of invasion, lymph node metastasis, lymphatic invasion and Dukes' stage.

Correlation between DICER1 mRNA levels and OS and DFS. The prognostic significances of DICER1 mRNA levels

was evaluated for the OS in all 260 patients and DFS in the 196 patients who underwent curative surgery. The average follow-up period for OS was 39.4±25.8 months and that of DFS was 38.8±21.5 months. In each analysis, patients were divided into the high and low DICER1 mRNA expression groups, as described above.

Fig. 2 shows the Kaplan-Meier OS curve of the CRC patients based on the status of DICER1 mRNA levels. The OS of patients in the low DICER1 group showed significantly

Table II. Univariate and multivariate analysis of prognostic factors for OS.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
Tumor size	0.65	1.92 (1.22-3.04)	0.005 ^a	-0.23	0.79 (0.43-1.47)	0.467
Depth of invasion	2.23	9.34 (2.07-164.75)	0.008 ^a	0.13	1.10 (0.19-20.85)	0.907
Lymph node metastasis	1.25	3.50 (2.12-6.05)	<0.001 ^a	0.03	1.03 (0.51-2.13)	0.945
Lymphatic invasion	0.99	2.70 (1.71-4.33)	<0.001 ^a	0.13	1.14 (0.61-2.16)	0.683
Venous invasion	1.00	2.71 (1.64-4.69)	<0.001 ^a	0.27	1.31 (0.66-2.76)	0.444
Histological type	0.65	1.92 (1.22-3.04)	0.005 ^a	0.52	1.67 (0.89-3.15)	0.108
Liver metastasis	1.61	4.98 (2.94-8.23)	<0.001 ^a	-0.23	0.79 (0.31-1.96)	0.618
Peritoneum dissemination	1.94	6.95 (3.60-12.46)	<0.001 ^a	0.11	1.11 (0.38-3.03)	0.840
Serum CEA	1.17	3.23 (1.93-5.60)	<0.001 ^a	0.15	1.16 (0.57-2.39)	0.686
Serum CA19-9	0.63	1.87 (1.07-3.20)	0.027 ^a	0.07	1.07 (0.52-2.14)	0.855
Dukes' stage	1.47	4.33 (3.11-6.21)	<0.001 ^a	1.18	3.27 (1.75-6.22)	0.001 ^a
DICER1 mRNA	-1.93	0.15 (0.02-0.46)	0.002 ^a	-1.19	0.30 (0.13-0.64)	0.001 ^a

^aP=0.039. OS, overall survival; CI, confidence interval.

worse survival rates as compared to the high DICER1 group (P<0.001). Fig. 3 shows the Kaplan-Meier DFS curves of the CRC patients, based on the status of DICER1 mRNA levels. The DFS of patients in the low DICER1 group also showed significantly worse survival rates as compared to the high DICER1 group (P<0.001). These findings suggest that a low expression of DICER1 mRNA is associated with worse OS and DFS in CRC patients.

Univariate and multivariate Cox analyses for OS. Table II shows the results of the univariate and multivariate Cox proportional hazard regression analyses for OS in the CRC patients. Multivariate analysis was performed for factors exhibiting statistical significance in the univariate analysis. In the univariate analysis, tumor size, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, histological type, liver metastasis, peritoneal dissemination, serum CEA, serum CA19-9, Dukes' stage and DICER1 mRNA level, while in the multivariate analysis, Dukes' stage and DICER1 mRNA showed statistical significance for OS. Table III shows the results of univariate and multivariate

Cox analyses for DFS in CRC patients who underwent curative surgery (n=196). In the univariate analysis, depth of invasion, venous invasion, serum CEA, Dukes' stage and DICER1 mRNA, while in the multivariate analysis, venous invasion, Dukes' stage and DICER1 mRNA showed statistical significance for DFS.

These results suggest that DICER1 mRNA levels of tumor tissues have an independent prognostic value for OS and DFS in CRC patients.

Discussion

In the present study, we aimed to examine the association of clinicopathological variables and the prognostic value of DICER1 mRNA in 260 CRC patients. Our findings demonstrate that the expression of DICER1 mRNA of CRC tissues showed a significant correlation between the tumor size, depth of invasion, lymph node metastasis, lymphatic invasion and Dukes' stage. Furthermore, the low expression of DICER1 mRNA in CRC tissue showed a markedly poor prognosis for OS and DFS in CRC patients.

Table III. Univariate and multivariate analysis of prognostic factors for DFS.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
Tumor size	0.27	1.31 (1.31-2.31)	0.369	–	–	–
Depth of invasion	1.26	3.51 (1.09-6.48)	0.034 ^a	-0.44	0.66 (0.16-4.39)	0.604
Lymphatic invasion	0.52	1.69 (0.94-2.97)	0.077	–	–	–
Venous invasion	0.70	2.00 (1.13-3.67)	0.018 ^a	0.54	1.73 (0.95-3.21)	0.072
Histological type	0.40	1.49 (0.81-2.66)	0.195	–	–	–
Serum CEA	0.63	1.87 (1.05-3.30)	0.035 ^a	0.39	1.48 (0.82-2.62)	0.189
Serum CA19-9	0.60	1.82 (0.91-3.46)	0.089	–	–	–
Dukes' stage	0.87	2.39 (1.54-3.91)	<0.001 ^a	0.73	2.07 (1.28-3.53)	0.003 ^a
DICER1 mRNA	-1.54	0.21 (0.09-0.43)	<0.001 ^a	-1.46	0.23 (0.10-0.48)	0.001 ^a

^aP=0.039. DFS, disease-free survival; CI, confidence interval.

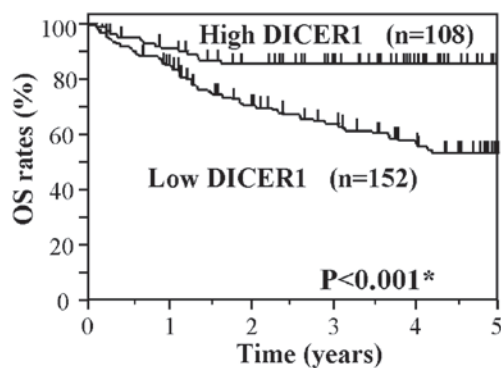


Figure 2. Overall survival (OS) curves based on DICER1 mRNA expression. The OS for 260 CRC patients was analyzed using the Kaplan-Meier survival curve. CRC patients were divided into the low and high DICER1 mRNA group. The OS of patients in the low-DICER1 mRNA groups showed markedly worse survival rates as compared to the high-DICER1 mRNA group. Log-rank test, P<0.001.

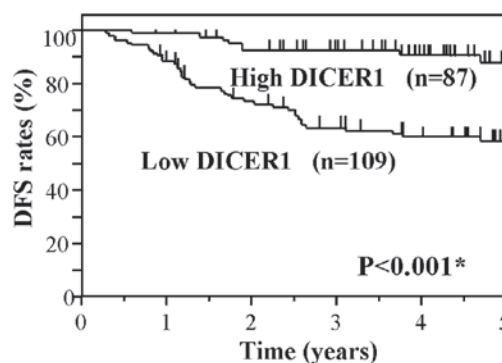


Figure 3. Disease-free survival (DFS) curves based on DICER1 mRNA expression. The DFS for 196 CRC patients who underwent curative surgery was analyzed using the Kaplan-Meier survival curve. CRC patients were divided into the low and high-DICER1 mRNA groups. The DFS of patients in the low-DICER1 mRNA group showed markedly worse survival rates as compared to the high-DICER1 mRNA group. Log-rank test, P<0.001.

DICER1 is a key enzyme responsible for the cleavage of miRNA precursors that is necessary for the production of mature miRNAs (11-16). DICER1 is capable of splicing the hairpin-like structure RNA and double-stranded RNA into mature miRNA or siRNA. MiRNAs are highly involved in several developmental and biological cell processes including timing of cell development, haematopoiesis, organogenesis, apoptosis, cell differentiation and proliferation (12,25).

Involvement of miRNA-base regulatory mechanisms is important in several diseases, including cancer (25-27). A general deregulation of miRNAs has been described as a key feature of several cancer types. Therefore, alternation of DICER1 expression may affect the development and progression of cancer via the loss of miRNA-mediated gene regulation.

DICER1 expression in cancer and normal tissues has shown inconsistent results in various cancer types. In this

study, DICER1 mRNA levels of CRC showed a significant decrease compared to normal tissues. Similar to our findings, a decreased DICER1 expression has been shown in CRC, lung, gastric and ovarian cancers (17,20,28,29). In their study, Papachristou *et al* (30) reported that the mRNA levels of DICER1 did not exhibit significant differences in normal and CRC tissues. Conversely, the overexpression of DICER1 has been reported in prostate adenocarcinoma, ovarian cancer and acute myeloid leukemia (19,31,32). The correlation between the DICER1 levels in CRC tissues and their clinicopathological factors are also noteworthy. We have demonstrated significant correlations between the DICER1 mRNA and tumor size, depth of invasion, lymph node metastasis, lymphatic invasion and Dukes' stage. Using the IHC method, Faggad *et al* (23) reported that the DICER1 protein expression in CRC tissues showed a significant correlation with tumor grade, lymph node metastasis, localization of tumor and tumor stage, thereby partially supporting our findings.

We also evaluated the prognostic value of DICER1 mRNA in CRC tissues. The effect of expression on prognosis has been studied in several cancers with controversial outcomes in various cancer types. In patients with ovarian, lung and breast cancers, decreased DICER1 levels in the tumor tissues showed a poor prognosis (17,18,21). Similarly, in patients with myeloma, nasopharyngeal carcinoma and chronic lymphocytic leukemia, decreased DICER1 levels were also a marker of poor prognosis (33-35). Conversely, the overexpression of DICER1 expression has been reported as a prognostic factor in prostate adenocarcinoma (19). Regarding CRC, the results of prognostic values of DICER1 in tumor tissues are also controversial.

Recently, Faber *et al* (24) reported that the overexpression of DICER1 predicts poor survival in CRC patients with pT2 or pT3 stages and without metastatic disease (pN0 and pM0). By contrast, Faggad *et al* (23) reported that the downregulation of DICER1 is a prognostic factor in CRC patients with WHO stage I, II, III and IV. These studies examined the DICER1 protein levels of tissue microarrays (TMA) using IHC staining. In this study, we selected the DICER1 mRNA detection using the Taqman RT-PCR method, with the aim of obtaining high sensitivity and objective analysis, since a small sample size of TMA may limit the value of TMA due to tumor heterogeneity.

Furthermore, Grelier *et al* (21) compared the prognostic value of DICER1 of breast cancer in the two measuring methods: real time RT-PCR and IHC. They reported that DICER1 mRNA levels were predictive for metastatic-free survival. However, the protein expression was not informative for survival. Of note, our data showed that low expression levels of DICER1 mRNA in CRC tissues significantly correlate with poorer OS and DFS, thereby supporting the findings of Faggad *et al* (36). The expression level of DICER1 directly influences the biosynthesis of miRNA. In ovarian cancer, a link between reduced DICER1 expression and a global down-regulation of miRNA are reported.

The aggressive tumors are thought to have decreased total microRNA levels, contributing to their poor differentiation. The downregulation of miRNA may have an impact on the development of tumor cells leading to poor prognosis of patients. However, the reason for the discrepancy with the survival results reported by Faber *et al* (24) is to be studied in future large-scale studies of each CRC stage. To the best

of our knowledge, this is the first study to demonstrate the independent prognostic value of a decreased expression of DICER1 mRNA in CRC.

In conclusion, our study has demonstrated that reduced DICER1 mRNA expression of tumor tissues shows a prognostic significance in CRC patients. Our finding of DICER1 mRNA expression being an independent marker capable of predicting high-risk patients is potentially useful in the individualized management and monitoring of CRC patients. In the future, these molecules may serve as a novel target with beneficial therapeutic applications.

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