A Distinct Expression Pattern of the Long 3′-Untranslated Region Dicer mRNA and Its Implications for Posttranscriptional Regulation in Colorectal Cancer

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OBJECTIVES: Reduced expression of Dicer is associated with global downregulation of microRNAs. Primary Dicer transcripts can be processed at two alternative polyadenylation sites, generating two pools of messenger RNAs (mRNAs) that carry either a long or a short 3′-untranslated regions (3′UTRs), that both encode the same Dicer protein. The short 3′UTR Dicer mRNA is not regulated by miR-103/107. The aim of this study was to investigate the expression of total Dicer mRNA, long 3′UTR Dicer mRNA, and miR-103 in colorectal cancer (CRC).

METHODS: Paired tumor and normal mucosal specimens were obtained from 66 patients with CRC. Real-time reverse transcription PCR of long 3′UTR Dicer mRNA, total Dicer mRNA and miR-103 was carried out using the TaqMan Expression assay and the TaqMan MicroRNA assay.

RESULTS: The median expression level of coding Dicer mRNA in the tumors was significantly lower than that in normal mucosa ($P < 0.001$). There was no significant difference in expression levels of long 3′UTR Dicer mRNA between the tumors and the normal mucosa ($P = 0.90$). The median expression ratio of long 3′UTR Dicer mRNA to total Dicer mRNA in tumors was significantly higher than in normal mucosa ($P < 0.001$). There was no significant difference in expression levels of miR-103 between the tumors and normal mucosa ($P = 0.17$). There was no significant correlation between clinicopathological findings, such as stage, tumor location, and histological grade and expression levels of total Dicer mRNA, long 3′UTR Dicer mRNA, or expression ratio of long 3′UTR Dicer mRNA to total Dicer mRNA.

CONCLUSIONS: These results suggest that both transcriptional and posttranscriptional dysregulation of Dicer expression may be involved in colon carcinogenesis.

INTRODUCTION

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that act as negative regulators of gene expression.[1] Primary miRNAs are transcribed by RNA polymerase II and are processed by RNase III Drosha into long primary miRNAs in the nucleus. The primary miRNAs are transported to the cytoplasm, where they are processed by RNase III Dicer into mature miRNAs, which are able to bind to the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs). The bound miRNAs mediate either the degradation of the mRNA or inhibition of its translation.[2] It has been observed that miRNAs can act as oncogenes (oncomiR) or tumor suppressor genes (tsmiR).[3] In fact, the downregulation of tsmiR expression is a general trait of human cancers.[4] Several lines of evidence have shown that epigenetic silencing and decreased expression of Dicer are implicated in the reduction of tsmiR expression.[5,6]

Colorectal cancer (CRC) is one of the most common causes of cancer-related mortality worldwide. The expression of number of miRNAs is dysregulated in CRC; let-7a and miR-101 are decreased,[7,8] whereas miR-21 and miR-92 are increased.[9,10]

Recent evidence suggests that altered expression of Dicer is associated with dysregulated miRNA expression in various malignancies including breast and ovarian cancer as well as CRC.[11–13] The regulation of Dicer expression remains unclear but several recent studies have demonstrated that Dicer expression is regulated by miR103/107 and let-7 miRNA.[14–16]

Cleavage and polyadenylation are essentially universal steps in the maturation of eukaryotic mRNA transcripts.[17,18] The pre-mRNA is cleaved downstream of the polyadenylation

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signal (typically 5′-AAUAAA-3′) and polyadenylation occurs at the 3′ end of the mRNA. Over half of all mammalian genes including Dicer have one or more polyadenylation sites,[19,20] resulting in mRNA isoforms that encode the same protein but have 3′UTRs of different lengths. The Dicer mRNA (RefSeq NM_030621) 3′UTR is ~4 kb in length and has two poly-adenylation signals, which results in mRNAs of different lengths. One Dicer mRNA is ~6 kb and uses the upstream polyadenylation signal (short 3′UTR Dicer mRNA), whereas another is ~10 kb, and uses the downstream polyadenylation signal (long 3′UTR Dicer mRNA). If more than one poly-adenylation signal occurs in a transcript, the dominant signal is usually located downstream. Polyadenylation site usage can be regulated by physiological conditions such as cell growth and differentiation or by pathological events such as cell transformation.[15,21,22] miRNA-binding sequences are usually located in the 3′UTR of transcripts, so that alternative polyadenylation site usage can affect gene expression through posttranscriptional regulation.[23] In fact, the Dicer mRNA with a short 3′UTR has increased mRNA stability and higher protein expression.[15]

Several studies have been made on Dicer expression and its posttranscriptional regulation by miRNAs in different cancers, but little is known about Dicer alternative polyadeny-lation site usage in CRC. To investigate the alternative poly-adenylation site usage by Dicer, we examined the expression of total Dicer mRNA, the long 3′UTR Dicer mRNA, and miR-103 in CRC.

METHODS

Patients. This study was approved by the Institutional Local Genetic Research Ethics Committee of the Hamamatsu University School of Medicine (Hamamatsu, Japan). All of the patients who contributed to this study provided oral and written informed consent. Paired endoscopic biopsy speci-mens of primary tumors and adjacent normal mucosa were obtained from 66 patients with CRC. Biopsy samples were snap frozen in liquid nitrogen and then stored at −80 °C until RNA extraction was performed. CRCs were classified according to the International Union Against Cancer TNM classification.[24] Cancer patients were classified as proximal colon cancer (cecal, ascending, and transverse colon cancers) or distal colon cancer (descending, sigmoid, and rectal cancers) according to the location of the CRC. Table 1 details the patient profiles.

RNA isolation. Total RNA including miRNA and large RNAs (>200 nt) were extracted separately from biopsy materials using the RNeasy Plus Mini Kit and the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were deter-mined using a NanoDrop 1000 (NanoDrop, Wilmington, DE) and the RNA samples were stored at −80 °C.

Reverse transcription and quantitative real-time PCR. Large RNAs were treated with the TURBO DNA-free kit (Life Technologies, Gaithersburg, MD) to remove contaminating DNA according to the manufacturer's instructions. Aliquots of large

Table 1 Clinical characteristic of CRC patients

	CRC ($n=66$)
Age (years), median (range)	71 (41–88)
Sex	
Female:male	22:44
Tumor site	
Proximal colon cancer	31
Distal colon cancer	35
Histological grade	
Well, moderately	58
Poorly and others	8
Lymphatic invasion	
(−)	31
(+)	35
Venous invasion	
(−)	29
(+)	37
Stage	
0	2
I	16
II	24
III	13
IV	11

CRC, colorectal cancer.

RNA (1.6 μg) were reverse transcribed into complementary DNA (cDNA) for reverse transcription PCR using oligo(dT)$_{20}$ and SuperScript III (Life Technologies) in accordance with the manufacturer's instructions. The cDNA was amplified using quantitative real-time PCR. To quantitatively evaluate the expression of total Dicer mRNA (short and long 3′UTR Dicer mRNA) and long 3′UTR Dicer mRNA, commercially available (assay ID Hs 00229023_m1) and custom TaqMan gene expression assays (forward primer: 5′-TTCCCCTGTTTGGTGC TATGTTT-3′, reverse primer: 5′-AGGTAAATTAAGAGGTATTA TAGTTACAGTGCAAAAA-3′, and internal probe: 5′-ATGTA TTATGCTTGAAATTTT-3′) were used, respectively. Custom TaqMan gene expression assays were designed using Primer Express (Life Technologies). Beta-2-microglobulin was used as internal control (part number 4326319E).[25] The reaction mixture comprised 1 μl of template cDNA, 10 μl of TaqMan Universal Master Mix II (Life Technologies), 1 μl of 20 × TaqMan primers, and the probe mixture and in a total volume of 20 μl. Each PCR was performed with precycling heat activation at 95 °C for 10 min, followed by 55 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min in an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystem, Foster City, CA, USA).

Small RNAs were diluted 1,000-fold with nuclease-free water and a 3-μl aliquot of the diluted RNA was reverse trans-cribed by Multiscribed Reverse Transcriptase and miRNA-specific primers (Life Technologies). Quantitative PCR for miR-103 and RNU6B (assays ID 000439 and 001093, respec-tively; Life Technologies) was performed with TaqMan Micro-RNAs Assays using a Applied Biosystems 7500 Fast Real-Time PCR system. RNU6B was used as an internal control.

A standard reference curve was established for each marker using serial 10-fold dilutions of the recombinant

plasmid DNA containing the target sequence or cDNA synthesized from synthetic target RNA. Each sample was run in triplicate and a negative control without template was also run in each reaction plate.

Statistical analysis. Data analysis was performed by PASW statistics (version 18.0, IBM Corporation, Somers, NY). Differences in target gene expression between tumor and adjacent normal mucosa were analyzed by the Wilcoxon signed-rank test. The impact of clinicopathological factors, such as tumor stage, grade, and location on the expression of target gene in cancer tissues, was analyzed by the Mann–Whitney U-test. Correlations between the expression levels of total Dicer mRNA in cancer tissues and long 3′UTR Dicer mRNA, as well as between total Dicer mRNA and miR-103 in cancer tissues, were determined from Spearman's rank correlation. All statistical tests were two sided, and P values <0.05 were considered to be statistically significant.

RESULTS

Dicer mRNA expression in CRC and adjacent normal mucosa. The median expression level of total Dicer mRNA in CRC tumors was 8.6 (range; 0–220), which was significantly lower than that in normal mucosa (29 (2.1–380): $P<0.001$, Figure 1a). There was no significant difference in expression levels of long 3′UTR Dicer mRNA between tumors and normal mucosa (3.9 (0–45) vs. 4.1 (0–27): $P=0.90$, Figure 1b). The median expression ratio of a long 3′UTR Dicer mRNA to total Dicer mRNA in tumors was significantly higher than that in normal mucosa (0.44 (0.047–1.3) vs. 0.15 (0.038–0.59): $P<0.001$, Figure 1c).

Association between Dicer mRNA expression in CRC and clinicopathological features. There was no significant difference in expression levels of total Dicer mRNA between stage 0–II CRC and stage III–IV CRC (7.7 (2–130) vs. 9.7 (0–220): $P=0.54$, Table 2). Similarly, there was no significant difference in expression levels of long 3′UTR Dicer mRNA or in the median expression ratio of long 3′UTR Dicer mRNA to total Dicer mRNA between stage 0–II CRC and stage III–IV CRC (3.9 (0.6–45) vs. 4.0 (0–19): $P=0.95$, 0.50 (0.047–1.3) vs. 0.39 (0.086–0.81): $P=0.53$, respectively, Table 2). The expression level of total Dicer mRNA, long 3′UTR Dicer mRNA, or expression ratio of long 3′UTR Dicer mRNA to total Dicer mRNA was not associated with tumor location, histological grade, lymphatic invasion, and venous invasion (all $P>0.05$, data not shown).

miR-103 expression. There was no significant difference in expression levels of miR-103 between tumors and normal mucosa (3.9 (0–45) vs. 4.1 (0–27): $P=0.90$, Figure 1d). The expression levels of miR-103 are not associated with stage, tumor location, lymphatic invasion, and venous invasion (all $P>0.05$, data not shown). Neither long 3′UTR Dicer mRNA expression nor coding Dicer mRNA expression was correlated with miR-103 expression (both $P>0.05$, Table 3).

DISCUSSION

The data presented in this study show that: (i) total Dicer mRNA expression in CRC is lower than in adjacent normal mucosa, (ii) long 3′UTR Dicer mRNA expression in CRC is similar to adjacent normal mucosa, and the ratio of long 3′UTR Dicer mRNA to total Dicer mRNA in cancer is higher than in
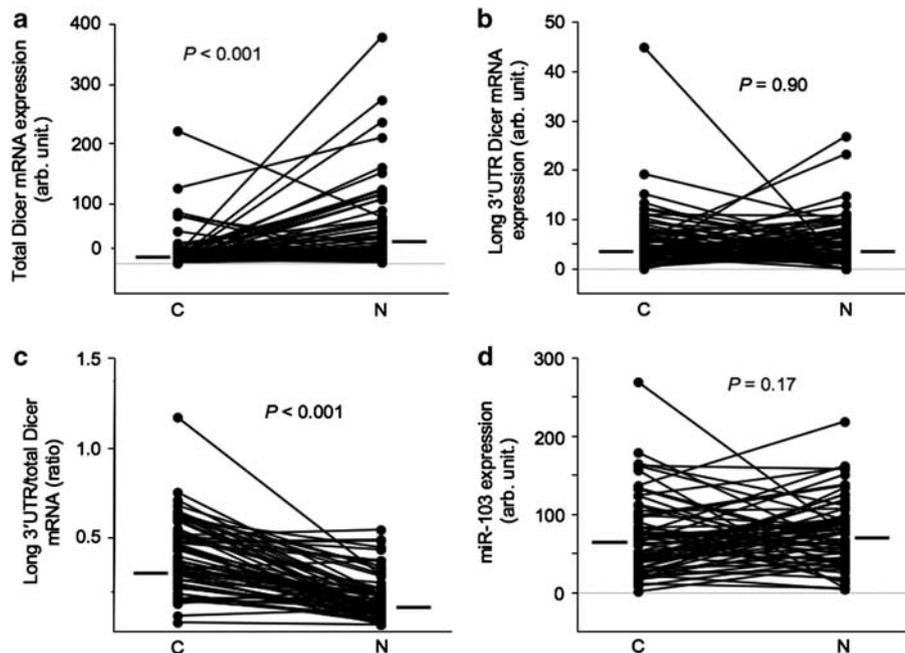

Figure 1 Comparison of gene expression in paired samples of primary colorectal cancer (CRC) (C) and adjacent normal (N) mucosa. (**a**) Total Dicer messenger RNA (mRNA) expression in CRC and normal mucosa. (**b**) Long 3′-untranslated region (3′UTR) Dicer mRNA expression in CRC and normal mucosa. (**c**) Ratios of long Dicer 3′UTR mRNA to total Dicer mRNA in CRC and normal mucosa. (**d**) miR-103 expression in CRC and normal mucosa.

Table 2 Expression of Dicer mRNAs and miR-103 in CRC according to stage

	Stage 0–II	Stage III–IV	*P*
Total Dicer mRNA (arb. units), median (range)	7.7 (2–130)	9.7 (0–220)	0.54
Long 3′UTR Dicer mRNA (arb. units)	3.9 (0.6–45)	4.0 (0–19)	0.95
Long 3′UTR/total Dicer mRNA (ratio)	0.50 (0.047–1.3)	0.39 (0.086–0.81)	0.53
MiR-103 (arb. units)	53 (9.1–270)	67 (1.5–180)	0.53

CRC, colorectal cancer; mRNA, messenger RNA; 3′UTR, 3′-untranslated region.
P value was analyzed by Mann–Whitney test; $P < 0.05$ was considered statistically significant.

Table 3 Correlation between expression of miR-103 and Dicer mRNAs in CRC

	miR-103 (r)	*P*
Total Dicer mRNA (arb. units)	0.12	0.33
Long 3′UTR Dicer mRNA (arb. units)	0.062	0.62

CRC, colorectal cancer; mRNA, messenger RNA; 3′UTR, 3′-untranslated region.
Spearman's rank correlation analysis was applied to determine the correlation; $P < 0.05$ was considered statistically significant.

adjacent normal mucosa, (iii) miR-103 in cancer is expressed similarly to adjacent normal mucosa, (iv) neither total Dicer mRNA expression nor long 3′UTR Dicer mRNA in cancer is correlated with miR-103 expression in cancer and clinico-pathological features, such as TNM stage, tumor location, histological grade, and vessel invasion.

Expression of Dicer varies among tumor types and may be correlated with cancer progression. Upregulation of Dicer was found in prostate, esophageal, and oral cancer,[26–28] whereas Dicer downregulation was found in neuroblastoma, breast, ovarian, and advanced lung cancer.[5,29–31] Opinions are divergent on Dicer mRNA expression in CRC.[13,32,33] Ciosea *et al*. reported that Dicer mRNA expression decreased in CRC,[32] whereas Papachristou *et al*. found that Dicer mRNA levels did not display any significant differences between normal epithelium and CRC.[33] Furthermore, Stratmann *et al*. found a significant increase of Dicer mRNA expression in the primary tumor from rectal cancer patients compared with normal mucosa but not in CRC patients.[13] We have demonstrated that Dicer mRNA is downregulated in cancer compared with that in normal mucosa in agreement with the work by Ciosea *et al*. Therefore, it is possible that dysregulation of Dicer mRNA expression is induced by genomic instability at the Dicer loci at 14q, which are frequently observed to have loss of heterozygosity in CRC.[34,35] Further studies are needed to clarify the genetic abnormality at the Dicer loci.

The Dicer mRNA has two polyadenylation sites, but the regulation of which site is used remains unknown. Recent researches suggest that polyadenylation site choice can be influenced by physiological conditions including cell growth, differentiation, developmental stage, and pathological events such as cancer.[21,22,36] Mayr and Bartel found that cancer cell produce more mRNAs with short 3′UTR, including for Dicer, which indicates that the proximal polyadenylation site is used more frequently.[15] The results of the current study are contrary to those of Mayr and Bartel and one reason may be the low transcriptional activity of Dicer in CRC. In a recent investigation into the 3′end-processing of mRNAs, short 3′UTR

isoforms were found to be relatively more abundant when genes were highly expressed but conversely long 3′UTR isoforms were more abundant when genes were expressed at low levels.[37] Elongated transcripts have also been observed in a mouse model of B-cell leukemia/lymphoma.[22] Our findings are consistent with this observation that expression of long 3′UTR Dicer mRNA is higher relative to short 3′UTR Dicer mRNA in cancer than in normal mucosa and is associated with the downregulation of total Dicer mRNA expression in cancer.

The expression of many miRNAs, including miR103/107, is affected to a lesser extent by Dicer expression because it only regulates a subset of miRNAs.[16,38,39] As a result, the high level of miR-103/107 expression in breast cancer induces a reduction in the level of Dicer mRNA and results in the global downregulation of all miRNAs. There is a significant association between miR-103/107 expression and Dicer protein expression, and clinical relapse.[16] miR-103/107 binds to the 3′UTR of Dicer mRNA, and inhibits protein expression by a posttranscriptional mechanism. Interestingly, the long 3′UTR Dicer mRNA has miR-103/107-binding sites in its 3′UTR but these are not present in the short 3′UTR Dicer mRNA. Therefore, the short 3′UTR Dicer mRNA is not directly regulated by miR-103/107. In the present study, miR-103 levels in CRC were not significantly different from normal mucosa. miR-103 levels in cancer cells might be sufficient to produce a reduction in the long 3′UTR Dicer mRNA, which results in the posttranscriptional downregulation of total Dicer mRNA, however, there might be other mechanisms to make a reduction in the long 3′UTR Dicer mRNA.

Contrary to previous observations, Dicer mRNA expression was not associated with stage, tumor location, and histological grade in the current study. In terms of stage, the downregulation of Dicer mRNA might be an early event in colon carcinogenesis. In addition, long 3′UTR Dicer mRNA expression was not correlated with the same clinicopathological features. It is possible that this discrepancy is a result of the small population size of the study, which could be addressed with a larger number of cases in subsequent studies.

We can conclude that the dysregulation of Dicer expression is involved in colon carcinogenesis through transcriptional and posttranscriptional gene regulation in CRC.

CONFLICT OF INTEREST

Kuriyama, Tetsunari Takai, Ken-ichi Yoshida, Takanori Yamada, and Mitsushige Sugimoto; critically reviewed manuscript: Satoshi Osawa, Ken Sugimoto, Hiroaki Miyajima, and Shigeru Kanaoka.

Potential competing interests: Shigeru Kanaoka and Yasushi Hamaya are entitled to a share of royalties received by the University on licensing intellectual property to Olympus. The terms of these arrangements are being managed by the University in accordance with its conflict of interest policies.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✎ Expression of Dicer varies among tumor types and may be correlated with cancer progression.

✎ Long 3′UTR Dicer mRNA is regulated posttranscriptionally by miR-103/107, but short 3′UTR Dicer mRNA is not posttranscriptionally regulated.

WHAT IS NEW HERE

✎ Dicer mRNA expression in CRC is lower than that in normal mucosa.

✎ The ratio of long 3′UTR Dicer mRNA expression to short 3′UTR Dicer mRNA expression in cancer is relatively high; Dicer mRNA is more sensitive to posttranscriptional regulation in CRC than in normal mucosa.

✎ Dysregulation of Dicer expression may be involved in colon carcinogenesis at transcriptional and posttranscriptional levels.

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