microRNA expression profile in undifferentiated gastric cancer

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Abstract. Prognosis of patients with undifferentiated gastric cancer is generally poor. The expression of various microRNAs (miRNAs) has not been comprehensively investigated in undifferentiated gastric cancer. Total RNA was extracted from the specimens of 42 undifferentiated gastric cancer tissues and paired normal gastric tissue. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for a set of 72 miRNAs. The expression of each miRNA relative to the internal control RNA was determined using the $2^{-\Delta Ct}$ method. The expression levels of 3 miRNAs (mir-34b, mir-34c and mir-128a) were significantly upregulated and those of 3 miRNAs (mir-128b, mir-129 and mir-148) were downregulated in undifferentiated gastric cancer tissue when compared with those of the paired normal tissues. The probability of survival was significantly lower in patients with high expression levels of mir-20b or 150. There was a correlation between mir-27a and lymph node metastasis. Our investigation provides a list of candidate miRNAs that may be associated with the prognosis in undifferentiated gastric cancer patients. Further study is warranted to identify the target genes of these miRNAs and their function.

Introduction

Gastric cancer is one of the most prevalent forms of human cancers. In Japan, ~50,000 people die of gastric cancer every year. Gastric cancer can be divided into 2 major groups. The intestinal, expanding, or differentiated type; and the diffuse, infiltrative, or undifferentiated type. The former is characterized by expansive growth and liver metastasis; whereas the latter is characterized by infiltrative growth and peritoneal dissemination. The undifferentiated variety includes histologically poorly differentiated adenocarcinoma and signet-ring cell carcinoma. Generally, patients with undifferentiated type tumors have poor prognosis (1,2).

microRNAs (miRNAs) are a subset of small (typically, 21-23 nucleotides in length) non-coding RNA molecules that are believed to regulate the expression of several genes (3). The mature miRNA are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors (3). The precursor is cleaved by cytoplasmic RNase III Dicer into miRNA duplex. One strand of the short-lived duplex undergoes degradation, whereas the other strand serves as mature miRNA (4). Mature miRNAs associate with a cellular complex that is similar to the RNA-induced silencing complex that participates in RNA interference (5). miRNAs have the unique ability to negatively regulate gene expression, thereby resulting in changes in cell development, proliferation and apoptosis (6). These biological properties of miRNAs may provide a potential access to several human diseases including cancers (7). According to the recent findings, it has been reported that miRNAs may play important roles in human cancer by acting as potential oncogenes and antioncogenes (8,9).

In the present study, we examined the expression levels of each of the 72 miRNAs in 42 undifferentiated type gastric carcinoma by using quantitative RT-PCR technique (10-12).

Materials and methods

Tissue samples. Forty-two human gastric tissue samples were obtained from patients who had undergone surgical resection at Nagoya City University Hospital between 1996 and 2005 and diagnosed with poorly differentiated adenocarcinoma or signet-ring cell carcinoma. This study has been approved by the Institutional Review Board and written consent was obtained from each patient. The tumor and its corresponding normal tissue were also obtained. The normal gastric mucosa was obtained from a part of the resected specimen that was the farthest from the tumor. The samples were snap-frozen in liquid nitrogen and stored at -80˚C. Clinicopathological data regarding the samples are shown in Table I.

Total RNA extraction. Total RNA was extracted from the specimens by using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

Targets, primers and probes. Seventy-two miRNAs were selected from the Sanger Center miRNA Registry. All TaqMan miRNA assays were obtained from Applied Biosystems (Foster City, CA).

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Reverse transcriptase reactions. Reverse transcriptase reaction contained RNA samples after adjusting the concentration of each sample to 2 ng/μl; the reaction mixture contained 50 nM stem-loop RT primers, 1X RT buffer, 0.25 mM each of dNTPs, 3.33 U/μl Multiscribe Reverse Transcriptase and 0.25 U/μl RNase inhibitor (Applied Biosystems). The reaction mixture was incubated in an Applied Biosystems 9700 Thermocycler in a 96-well plate for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C and subsequently held at 4˚C. All reverse transcriptase reactions, including the no-template controls and RT minus controls, were run in duplicate.

Polymerase chain reaction. Real-time PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7500 real-time PCR System. The 10 μl PCR included 0.67 μl of the RT product, 1X TaqMan® Universal PCR master mix, 0.2 μM TaqMan® probe, 1.5 μM forward primer and 0.7 μM reverse primer. The reactions were incubated in a 96-well plate at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression of each miRNA relative to the internal control RNA was determined using the $2^{-\Delta Ct}$ method (13).

Statistical analysis. The data were $\log_{10}$ and expressed as the mean ± SD. We used student's t-test and Z test to assess the differences between the variables. Kaplan-Meier method was used to calculate the survival rates and log-rank test was used to test the significance in the difference in the patient survival. Cox regression analysis was used to test the relationship between the variables and patient survival; Cox proportional hazards model was used to assess the contribution of the factors to the patient survival. Data analyses were performed using the Stat View for Windows version 5.01 (SAS Institute, Inc., Cary, NC). $P<0.05$ was considered as statistically significant.

Table I. Clinicopathological characteristics.

Age (years)	24-91 (mean, 64)
Gender	
Male	23
Female	19
Histological type	
Poorly differentiated	36
Signet-ring cell	6
Serosal invasion	
Absent	26
Present	16
Lymph node invasion	
Absent	16
Present	26
n1	12
n2	11
n3, 4	3
Lymphatic permeation	
Absent	4
Present	37
Unknown	1
Vascular permeation	
Absent	9
Present	32
Unknown	1

Results

miRNA expression profiling in undifferentiated type gastric cancer. During the preliminary experiments, we tested let-7a and U6B as internal controls by using the Z test. Expression of both let-7a and U6B varied among individuals. The expression levels of let-7a varied less than that of U6B between the tumor and normal gastric tissues (Fig. 1A and B). Therefore, we used let-7a as the internal control.

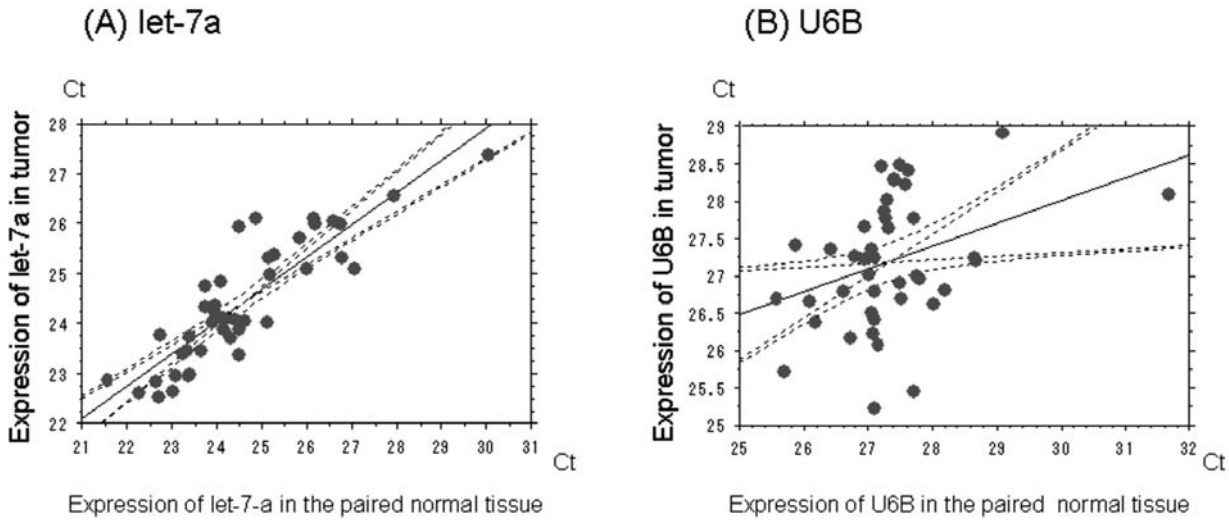

Figure 1. (A) Relationship between the expression levels of let-7a in the tumor and those in the corresponding normal tissues on the scatter chart. (B) Relationship between the expression levels of U6B in the tumor and those in the corresponding normal tissues on the scatter chart.

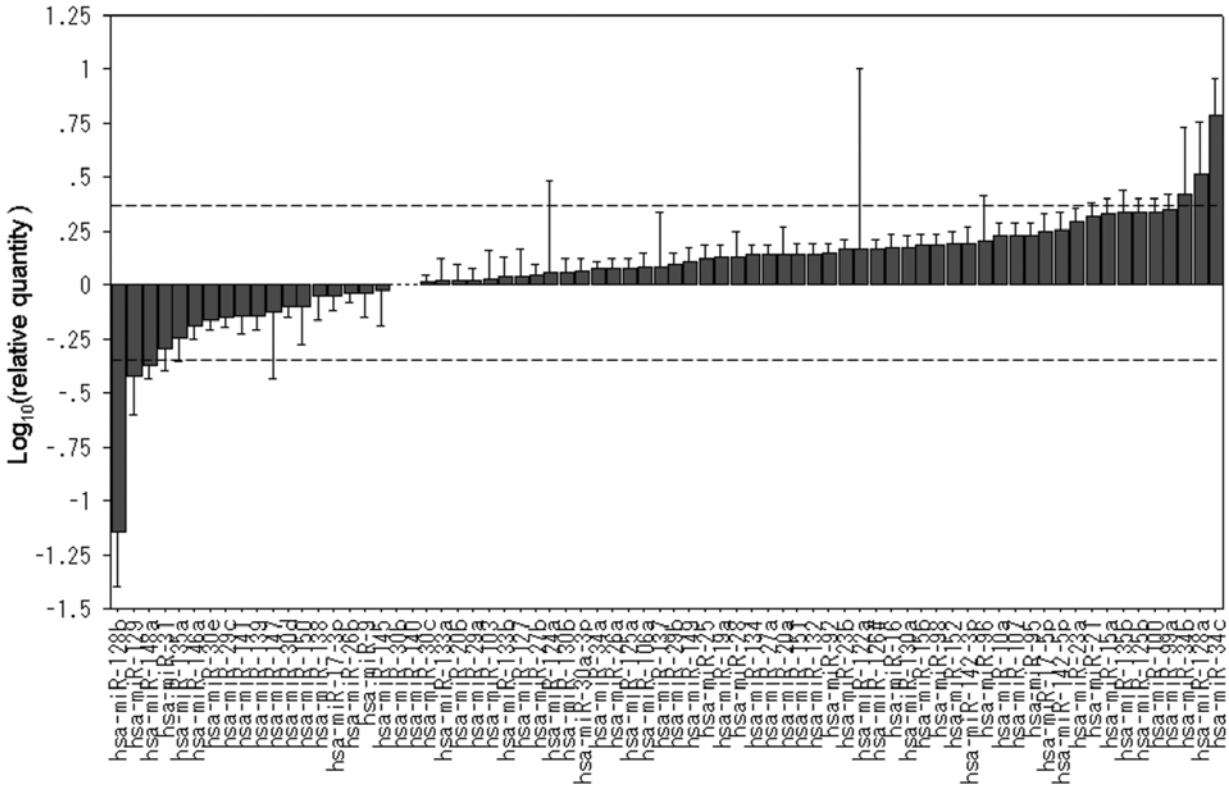

Figure 2. Fold change in the expression of each of the 72 miRNAs in undifferentiated type gastric cancer in comparison with those of the corresponding normal tissue. Two broken lines represent 2-fold change lines.

Table II. Expression profiles of miRNA in gastric carcinoma (poorly differentiated adenocarcinoma or signet-ring cell carcinoma) vs. the corresponding adjacent normal tissues and chromosomal loci and predicted targets for these miRNAs.

	Chromosomal locus	Predicted target genes
Upregulated		
mir-34b	11q23.1	*DLL1*, *NOTCH1*, *STRAP*, *TMEM22*, *TH*, *VEZATIN*, *FLOT2*, *NAV1*, *ELMOD1* and *CNTNP1*
mir-34c	11q23.1	*DLL1*, *NOTCH1*, *PLEKHF-1*, *BBS1*, *VEZATIN*, *NAV1*, *RAB43*, *CNTN2*, *VAMP2* and *ASB1*
mir-128a	2q21.3	*PPP4C*, *PXMP2*, *PLK2*, *ADCY3*, *HLX1*, *PHF6*, *DVL2*, *DCP2*, *GPAM* and *ZNF385*
Downregulated		
mir-128b	3p22.3	*PPP4C*, *PXMP2*, *PLK2*, *ADCY3*, *HLX1*, *PHF6*, *DVL2*, *DCP2*, *GPAM* and *ZNF385*
mir-129	7q32.1	*DEFB119*, *PORI*, *NSUN5*, *SOX4*, *EIF2C3*, *EFNB2*, *TMEM23*, *STAT5B*, *CIB2* and *CTNND1*
mir-148a	7p15.2	*DYNLL2*, *SNS*, *LBR*, *INOC1*, *LAMA4*, *SFRS2IP*, *MITF*, *RPS6KA5*, *ATP6AP2* and *MEOX2*

Expression level of each miRNA is shown in Fig. 2. The expression levels of 52 miRNAs were higher in the tumor than in the normal gastric tissue; on the other hand, those of 20 miRNAs were lower in the tumor than in the normal gastric tissue. The expression levels of 3 miRNAs (mir-34b, mir-34c and mir-128a) were >2-fold higher in the tumor than in the normal gastric tissue; the expression levels of 3 other miRNAs (mir-128b, mir-129 and mir-148a) were <1/2 of those observed in the normal gastric tissue (Table II).

Association between expression levels of miRNAs and lymph node metastasis. Next, we investigated the correlation between expression levels of miRNAs and lymph node metastasis, which is the most significant prognostic factor in gastric cancer in our examples. There was a significant correlation between the mir-27a expression and lymph node metastasis (Fig. 3); however, there was no significant correlation between the expression levels of other miRNAs and lymph node status in gastric cancer.

Association between expression levels of miRNAs and postoperative survival. Next, we examined whether the expression level of each miRNA was associated with postoperative patient survival. Among the 72 miRNAs, the Kaplan-Meier survival curves demonstrated that the probability of survival was significantly lower in patients

Table III. Univariate and multivariate analyses of the expression levels of miRNAs and various clinical characteristics.

	Univariate analysis		
	HR (95% CI)	Unfavorable/Favorable	P-value
Age (years)	1.65 (0.52-5.19)	>64/≤64	0.3939
Gender	2.43 (0.77-7.72)	Female/Male	0.1317
Histology	3.00 (0.39-23.33)	Poor/Signet	0.2946
Serosal invasion	1.21 (0.39-3.77)	Positive/Negative	0.7420
Lymph node metastasis	9.90 (1.28-76.92)	Positive/Negative	0.0284
Lymphatic permeation	2.38 (0.51-11.08)	Negative/Positive	0.2706
Vascular permeation	3.95 (0.50-31.25)	Positive/Negative	0.1939
mir-20b	3.42 (1.03-11.36)	High/Low	0.0448
mir-150	6.76 (0.87-52.63)	High/Low	0.0680
	Multivariate analysis		
	HR (95% CI)	Unfavorable/Favorable	P-value
Lymph node metastasis	9.90 (1.26-76.92)	Positive/Negative	0.0290
mir-20b	2.01 (0.59-6.85)	High/Low	0.2614
mir-150	6.10 (0.76-50.00)	High/Low	0.0897

Table IV. Chromosomal loci and predicted targets for mir-20b, mir-150 and mir-27b.

	Chromosomal locus	Predicted target genes
mir-20b	Xq26.2	*CC2D1A*, *PTPN4*, *ZDHHC1*, *APP*, *BAMBI*, *VSX1*, *FASTK*, *MAP3K9*, *PLEKHM1* and *WEE1*
mir-150	19q13.33	*TNFRSF4*, *IGFBP2*, *CCDC22*, *MYB*, *CENTD3*, *ELOVL3*, *ELK1*, *COL1A1*, *ETF1* and *VAMP2*
mir-27a	19p13.12	*PDE3B*, *PLK2*, *ST14*, *ADCY3*, *CLPP*, *SLC25A25*, *PRR3*, *YPER3*, *TANFKBH* and *GPAM*

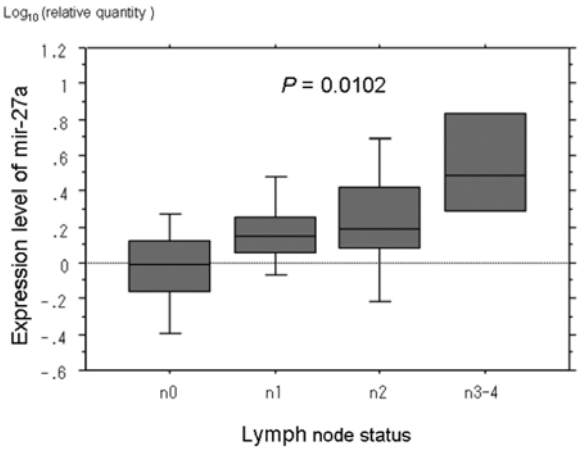

Figure 3. Correlation between the expression of mir-27a and the stage of lymph node metastasis.

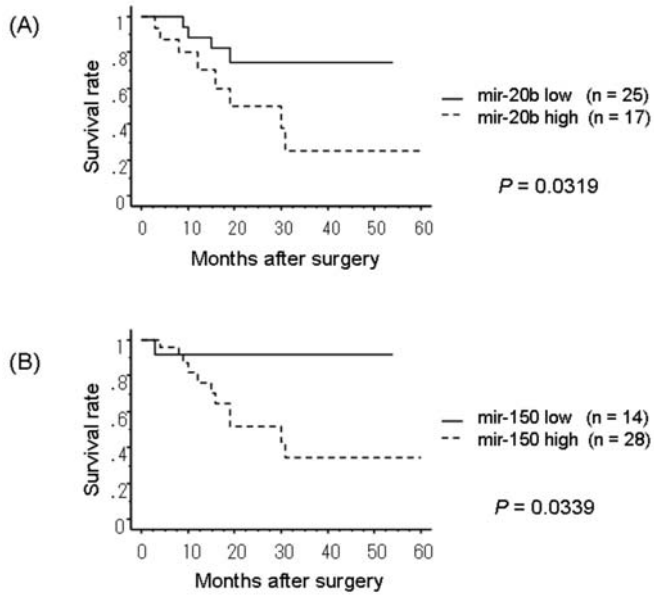

Figure 4. (A) The overall survival rate of undifferentiated type gastric cancer patients and the expression levels of mir-20b. Expression levels of mir-20b were high/low. (B) The overall survival rate of undifferentiated type gastric cancer patients and the expression levels of mir-150. Expression levels of mir-150 were high/low.

with high expression levels of mir-20b or mir-150 (Fig. 4). Expression levels of other miRNAs including those of 6 miRNAs listed in Table II (high and low expression levels in the tumor) and that of mir-27a (which correlated with lymph

node metastasis) did not correlate with the prognosis. The prognostic impact of various factors was studied by univariate Cox regression analysis (Table III). According to the results, in addition to lymph node metastasis (P=0.0300), high expression level of mir-20b was a significant predictor of poor prognosis (P=0.0448); however, that of mir-150 did not show a significant association with postoperative patient survival (P=0.0680) in gastric carcinoma.

The interrelationship between the possible prognostic factors and patient survival was further analyzed by means of the Cox proportional hazards model using lymph node metastasis and expression levels of mir-20b and mir-150 as variables. It was observed that the increased expression levels of mir-20b or mir-150 were not significant independent prognostic factors in undifferentiated gastric cancer patients. As expected, lymph node metastasis was an independent prognostic factor (not shown).

Discussion

There have been several clinicopathological investigations regarding the comparison between the differentiated with undifferentiated type of gastric carcinoma (2,14-16). It was reported that the 5-year survival rate was better in patients with differentiated type of gastric carcinoma when compared with those with undifferentiated type (1,2). Therefore, it is extremely important to improve the prognosis of undifferentiated type of gastric carcinoma.

Recently, miRNA-mediated regulation of cell growth and apoptosis has been reported (17,18). Additionally, it has been reported that miRNAs have the potential to behave as oncogenes or antioncogenes (19). By examining the expression levels of miRNA, it was reported certain miRNA are specifically involved in cancer (10,11,18,20). The relationship between miRNAs and gastric carcinoma was reported by Kim *et al* (21) and Li *et al* (22).

In the present study, it was shown that the higher expression level of mir-20b and mir-150 in undifferentiated gastric cancer is associated with shorter postoperative patient survival. It should be noted that among the variables used for multivariate Cox regression analysis (i.e., lymph node metastasis, mir-20b and mir-150), mir-20b and mir-150 did not appear to have a significant prognostic impact that was independent of lymph node metastasis.

It was reported that mir-20 was upregulated in megakaryocytopoiesis (23). mir-20b is located on the chromosome Xq26-a location whose amplification was found in the pleural and asocitic fluid of gastric cancer patients and not found in gastric cancer tissue (24). It was reported that mir-150 was significantly deregulated in chronic lymphocytic leukemia patients (25). The locus of mir-150 is chromosome 19q13 and amplification of this site has been reported as well in patients with gastric carcinomas (26). Thus, it is possible that the amplification of these 2 loci is mediated by the increased expression of mir-20b or mir-150. The potential binding partners of these 2 miRNAs are listed in Table IV (miRBAse, Pictar and TagetScan were used). Among them, there are several genes that are of interest, including *Wee1* (27), *BAMBI1* (28), *TNFRSF4* (29) and *IGFBP2* (30) and that have been reported to be involved in cell cycle control and in apoptosis. Future studies could be aimed at assessment of the mechanisms underlying the effects of mi-20b or mir-150 on these functions.

Table II lists 6 genes that are either highly upregulated (>2-fold) or highly downregulated (<1/2) in the tumor when compared with that observed in the normal gastric tissue. The potential binding partners of these 6 miRNAs are also listed in the table. Of all the genes, the functions of only a few are known; of these *NOTCH1* (31,32), *STRAP* (33) and *PLK2* (34) have been suggested to be involved in apoptosis.

In the present study, mir-27a was shown to be associated with lymph node metastasis (Fig. 3). Those with more extensive lymph node involvement tended to have a higher expression level of mir-27a in the tumor. The potential binding partners of mir-27a are listed in Table IV. mir-27a is located on chromosome 19p13; amplification of this locus has been reported in gastric carcinoma (35). mir-27a was associated with lymph node metastasis; however, it was not associated with the prognosis of patients with undifferentiated gastric carcinoma. This is probably due to the lack of any correlation between the stage of lymph node metastasis and prognosis.

The present study will serve as the basis for further studies regarding the function of miRNAs in carcinoma, particularly in the undifferentiated type of gastric carcinoma. The association that we observed between the expression levels of certain miRNAs and the prognosis in the present study must be considered with caution because several miRNAs were studied simultaneously and it should be confirmed with another set of specimens and additional functional studies.

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