

ORIGINAL ARTICLE

췌장암에 대한 예후 생물표지자로서의 MicroRNA-200c

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MicroRNA-200c as a Prognostic Biomarker for Pancreatic Cancer

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Background/Aims: MicroRNA (miRNA) regulates messenger RNA stability and translation. In cancer biology, miRNA affects the growth and metastasis of cancer cells by controlling epithelial-mesenchymal transition (EMT). MiR-200 family (200a/200b/200c/141) and miR-205 are associated with the regulation of EMT. We investigated the prognostic role of EMT-related miRNAs in pancreatic cancer.

Methods: We analyzed miR-200 family and miR-205 expression in tissue samples of 84 patients who underwent radical resection for pancreatic cancer.

Results: Patients were followed from the date of diagnosis until death or censoring. The mean overall survival was 25.0±2.0 months (2-140 months). The R0 resection rate was obtained in 84.5% (n=71) of patients. The relative expressions of miR-200a/200b/200c/141 and miR-205 were 266.9±57.3/18.5±2.2/0.7±0.1/27.2±6.6 folds and 0.1±0.1 compared with human pancreatic ductal epithelial cells, respectively. Overall survival was longer in the low miR-200c expression group than in the high expression group (35 vs. 19 months, p=0.013). Multivariate analysis confirmed that patients with low miR-200c expression survived longer than the high expression group (hazard ratio, 1.771; 95% CI, 1.081-2.900; p=0.023). There was a trend toward longer disease-free survival in low miR-200c group without statistical significance (p=0.061).

Conclusions: The expression of miR-200c may be an important prognosis factor in pancreatic cancer, and it could be a novel therapeutic target of pancreatic cancer. (Korean J Gastroenterol 2015;66:215-220)

Key Words: Pancreatic neoplasms; MicroRNAs; Epithelial-mesenchymal transition; MIRN200 microRNA, human

INTRODUCTION

MicroRNA (miRNA) is a small noncoding RNA molecule consisting of 21-25 nucleotides with a complementary base sequence at the 3'-UTR binding site of target genes, suppressing or accelerating protein synthesis of target genes.^{1,2}

Since it was first identified from *Caenorhabditis elegans* in 1993, more than 700 different types of miRNA have been described in human cells.^{3,4} More than 30% of genes are regulated by miRNA, which is involved in differentiation, growth, and proliferation of cells by regulating messenger RNA translation.^{5,6} Aberrantly upregulated or downregulated ex-

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pression of miRNA is observed in various cancers, suggesting it is important in carcinogenesis.⁷⁻¹² In pancreatic cancer, expression of miRNAs including miRNA-21, miRNA-221/222 and miRNA-181a/b/c is higher than in normal pancreatic tissues.¹³

Epithelial-mesenchymal transition (EMT) is a process by which cell-to-cell adhesion of epithelial cells disappears, and the epithelial cells gain migratory and invasive function like mesenchymal cells. This is closely related to metastasis and prognosis in gastrointestinal cancers (including colorectal cancer, hepatic cancer and pancreatic cancer) since it induces chemotherapy resistance.¹⁴⁻¹⁹ MiRNA-200 family and miRNA-205 regulate EMT by E-cadherin suppressing factors, ZEB1 and ZEB2 (SIP1).²⁰ However, there are a few studies about EMT regulation by miRNA in pancreatic cancer.

Based on this background, the intent of this study is to elucidate the prognostic role of EMT-related miRNAs including miRNA-200 family (miRNA-200a/200b/200c/141) and miRNA-205 in resected pancreatic cancer patients.

SUBJECTS AND METHODS

1. Patients

Study subjects were drawn from among patients who were diagnosed with pancreatic cancer and underwent curative surgery in Seoul National University Bundang Hospital (Seoul, Korea) from 2003 through 2011. Patients who had palliative surgery or expired within 30 days after surgery due to surgical complications were excluded. This left 84 patients in the study. The study was approved by the human subjects committee of Seoul National University Bundang Hospital (IRB No. B-1103-124-302). Informed consent was waived by the board.

2. RNA extraction from pancreatic cancer tissue

RNA from formalin-fixed, paraffin embedded pancreatic cancer tissue was extracted with a nucleic acid extraction kit (Ambion, Austin, TX, USA) and an RNeasyasylic Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.²¹⁻²⁴ The relative expression of miRNAs of pancreatic cancer tissues was measured with immortalized human pancreatic ductal epithelial (HPDE) cells as the expression reference. HPDE cells were cultured in 5% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) and mixture of low glucose

(DMEM; Gibco-BRL) containing 750 ng/mL puromycin and medium M3 base (Incell Co., San Antonio, TX, USA), mixed in 3:1 ratio. After the cultured cell line was isolated by Trypsin-EDTA (Gibco-BRL) and collected with centrifugation (300×g, 5 min), RNA extraction was performed by manufacturing protocol.

3. Quantitative real-time PCR

Isolated and quantified 5 µg of RNA was incubated in reverse transcription premix (Maxime RT premix; Intron, Seoul, Korea) at 42°C for 50 minutes and at 95°C for 10 minutes to synthesize cDNA. With use of miScript (SYBR) Green PCR kit (Qiagen, Valencia, CA, USA), synthesized cDNA was mixed with 3 ng of cDNA, 10 µL of SYBR Green master mix, 1 µL of universal primer and 1 µL of each miRNA primer (Table 1) and titrated with diethylpyrocarbonate treated water, resulting in a total volume of 20 µL. After incubation at 95°C for 15 minutes, sequential incubation at 94°C for 15 seconds, at 55°C for 30 seconds and at 70°C for 30 seconds was repeated 45 times. The PCR results were analyzed by quantifying miRNA according to Ct values with 7500 Real-time PCR System (Applied Biosystems, Foster, CA, USA). U6 was used as an internal control for quantification.

4. Statistics

The average values of continuous variables, standard error of the means and median values were calculated and categorical variables were measured. To evaluate the effects of miRNA on recurrence or metastasis, patients were dicotomized by the mean value of miRNA and Student's t-test was performed. The relationship between miRNA expression and survival time after the diagnosis was evaluated with Pearson's correlation analysis. Survival period and disease-free survival period between the operation and recurrence were analyzed by Kaplan-Meier curves with log rank

Table 1. The MicroRNA (miRNA) Primer Sequences for Quantitative Real-time PCR Analysis

miRNA	Primer sequences
miRNA-200a	CGTAACACTGTCTGGTAACGATGT
miRNA-200b	GGTAATACTGCCTGGTAATGATGA
miRNA-200c	CTGCCGGGTAATGATGGA
miRNA-141	GCTAACACTGTCTGGTAAAGATGG
miRNA-205	CTTCATTCCACCGAGTCTG
U6	GGCAGCACATATACTAAATGGAA

test. We evaluated the prognostic factors affecting survival time by using univariate and multivariate analysis with Cox proportional hazards model. The prognostic factors of which p-values were less than 0.1 in univariate analysis were included in multivariate analysis. Null hypotheses of no difference were rejected if p-values were less than 0.05, or, equivalently, if the 95% CIs of risk point estimates excluded 1. Most statistical analyses were carried out by IBM SPSS statistics version 20.0 (IBM Co., Armonk, NY, USA).

RESULTS

1. Baseline characteristics of enrolled patients

There were 52 male and 32 female patients (Table 2). The mean age was 62.6 ± 0.9 years. Pathologically, 79 patients (94.1%) had adenocarcinoma. Other cancers included one adenosquamous carcinoma, one mucoepidermoid carcinoma, two undifferentiated carcinomas, and one anaplastic carcinoma. R0 resection was done in 71 patients (84.5%). The pathologic stage of pancreatic cancer was mostly stage II, consisting of 37 patients with stage 2A (44.1%) and 45 pa-

tients with stage 2B (53.6%). Seventy patients (83.3%) experienced recurrence during the follow-up period (25 in locoregional and 45 in distant metastases). The mean time of recurrence was 16.0 ± 2.2 months and the average overall survival period was 25.0 ± 2.3 months (2-140 months).

2. Expression of miRNA-200 family and miRNA-205 in pancreatic cancer

Relative expressions of miRNA-200a/200b/200c/141 and miRNA-205 in pancreatic cancer tissue were 266.9 ± 57.3 folds in miRNA-200a, 18.5 ± 2.2 folds in miRNA-200b, 0.7 ± 0.1 folds in miRNA-200c, 27.2 ± 6.6 folds in miRNA-141 and 0.1 ± 0.1 folds in miRNA-205 (Table 3). There were no significant differences in miRNA expression in patients in terms of recurrence or recurrence patterns (data not shown).

3. Survival and prognosis by expression of miRNA-200 family and miRNA-205 in pancreatic cancer

The correlation between expressions of each miRNA and survival were evaluated. The miR-200c expression was inversely correlated with survival period ($r = -0.22$, $p = 0.043$), but the others were not associated (data not shown).

The subjects were dichotomized by the mean value of miRNA-200c; high miRNA-200c group (> 0.65 , $n = 29$) and low miRNA-200c group (< 0.65 , $n = 55$). The mean values of miRNA-200c were 1.5 ± 0.2 in high miRNA-200c group and 0.3 ± 0.03 in low miRNA-200c group. Overall survival was significantly longer in low miRNA-200c group (35 vs. 19 months, $p = 0.013$) (Fig. 1A). By Cox proportional hazard model, survival was shorter in the high miRNA-200c group (hazard ratio, 1.771; 95% CI, 1.081-2.900; $p = 0.023$) (Table 4).

When analyzing the correlation between miRNA-200a/200b/200c/141 or miRNA-205 expression and disease free survival (DFS), there was a trend toward longer DFS in low

Table 2. Baseline Characteristics of 84 Resected Pancreatic Cancer Patients

Characteristic	Data
Age (yr)	64 (44-83)
Sex, male	52 (61.9)
Pathology, adenocarcinoma	79 (94.1)
R0 resection	71 (84.5)
pT stage	
T1/T2	0 (0)/2 (2.4)
T3/T4	81 (96.4)/1 (1.2)
pN stage	
N0/N1	36 (42.9)/48 (57.1)
AJCC stage	
IB	1 (1.2)
IIA/IIB	37 (44.1)/45 (53.6)
III	1 (1.2)
Invasion	
Angiolymphatic	39 (46.4)
Venous	17 (20.2)
Perineural	59 (70.2)
Adjuvant chemotherapy	43 (51.2)
Gemcitabine based	17 (20.2)
Adjuvant radiation therapy	45 (53.6)
Recurrence	70 (83.3)
Local recurrence	25 (29.8)
Distant metastasis	45 (53.6)

Values are presented as median (range) or n (%).
AJCC, American Joint Committee on Cancer.

Table 3. MicroRNA (miRNA) Expression Levels in Pancreatic Cancer Tissue Comparing with Immortalized Pancreatic Duct Epithelial Cells

miRNA	HPDE cells	Mean \pm SEM
miRNA-200a	1	266.9 \pm 57.3
miRNA-200b	1	18.5 \pm 2.2
miRNA-200c	1	0.7 \pm 0.1
miRNA-141	1	27.2 \pm 6.6
miRNA-205	1	0.1 \pm 0.1

HPDE, human pancreatic ductal epithelial; SEM, standard error of the mean.

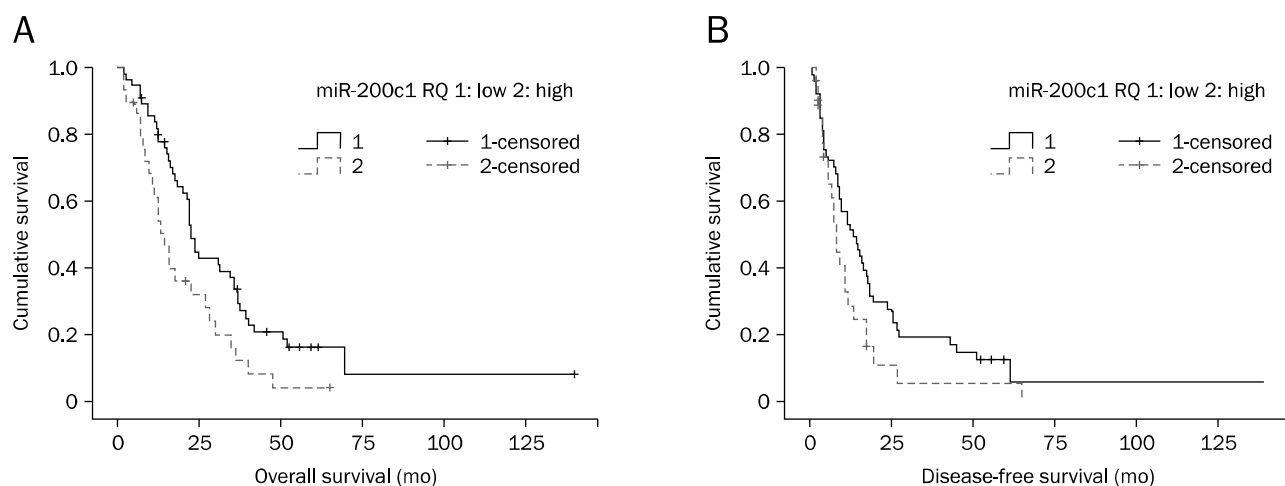


Fig. 1. Overall survival ($p=0.013$) (A) and disease-free survival ($p=0.061$) (B) according to the level of microRNA (miRNA)-200c.

Table 4. Prognostic Factors of Pancreatic Cancer

	HR	95% CI	p-value
Univariate analysis			
Age (> 60 yr)	1.081	0.652-1.793	0.763
Sex (male)	1.286	0.794-2.085	0.307
Margin invasion (positive)	1.249	0.615-2.536	0.539
pN (positive)	0.955	0.593-1.539	0.850
Angiolymphatic invasion (positive)	1.314	0.819-2.108	0.258
Venous invasion (positive)	0.992	0.551-1.788	0.979
Perineural invasion (positive)	0.953	0.571-1.591	0.854
Post operative chemotherapy	0.866	0.540-1.390	0.552
Post operative gemcitabine	0.524	0.279-0.982	0.044
Post operative radiation therapy	1.036	0.645-1.664	0.883
High miRNA-200c (> 0.65)	1.849	1.131-3.025	0.014
Multivariate analysis			
Post operative gemcitabine	0.551	0.293-1.036	0.064
High miRNA-200c (> 0.65)	1.771	1.081-2.900	0.023

HR, hazard ratio.

miRNA-200c group, however, it did not reach a statistical significance (24 vs. 12 months, $p=0.061$, Fig. 1B).

DISCUSSION

EMT is a process that is closely associated with metastasis and prognosis in gastrointestinal cancer including pancreatic cancer.^{14,15} MiRNA-200 family and miRNA-205 are reported to regulate EMT and affected the prognosis in several cancers. However, there are only a few studies about these in pancreatic cancer. Based on this, we evaluated the role of miRNA-200 family and miRNA-205 as an EMT-related prognostic marker in curatively resective pancreatic cancer.

In this study, the results of quantitative RT-PCR revealed

higher miRNA-200a/200b/141 and lower miRNA-200c/205 expression in pancreatic cancer than in HPDE cells. Moreover, the high miRNA-200c group, where the mean value was over 1.5 fold of the reference, suffered a high recurrence rate and poor prognosis. In the present study, the expression of miRNA-200c and miRNA-205 were suppressed in pancreatic cancers as in a previous study,²⁵ and miRNA-200c was an independent prognostic factor. However, the overall survival was longer in the patient group with low miRNA-200c expression, unlike previous studies. These conflicting results suggest that roles of miRNA differ by various interactions between miRNA and the surrounding environment. MiRNA and associated transcription factors function in a heterogeneous fashion in regulating genes in the context of tumor development, growth, metastasis and apoptosis. High levels of the miRNA-200 family are related to recurrence and poor prognosis in patients with ovarian cancer,²⁶⁻²⁸ although in other cancers that is not the case.^{29,30} Similarly, miRNA-200c inhibits the metastatic ability of colon cancer cells by targeting ZEB1.³¹ However, another study observed that serum miRNA-200c levels were significantly higher in stage IV than in low stage colorectal cancer and high levels of miRNA-200c was associated with grave prognosis including lymph node and distant metastasis.³² Further study about the complex gene regulatory mechanism by miRNA-200c and related transcription factors is warranted.

This study is limited in that the results cannot reflect all pancreatic cancer patients because we included only patients who had received surgical treatment and who were mostly stage II. Retrospective data collection produces errors

as medical records are not intended for research and can contain errors. As disease stage and lymph node metastasis are prognostic of pancreatic cancer, this may increase statistical error and reduce significance. Furthermore, the small study sample size might have been the reason that influencing factors affecting survival in the univariate analysis lost statistical significance in the multivariate analysis.

The expression of miRNA was investigated by using extracted RNA from specimens in this study. Further study about measuring miRNA expression by in situ hybridization analysis of cancer tissue is necessary.

In conclusion, this study found that miRNA-200a/200b/141 was increased and miRNA-200c/205 was decreased in pancreatic cancer. Increased miRNA-200c expression is associated with poor prognosis. Further studies on mechanisms of miRNA-200c in the progression and metastasis of pancreatic cancer will be required.

REFERENCES

- Lai EC. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002;30:363-364.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215-233.
- Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 1993;75:843-854.
- Ambros V. microRNAs: tiny regulators with great potential. *Cell* 2001;107:823-826.
- Lu M, Zhang Q, Deng M, et al. An analysis of human microRNA and disease associations. *PLoS One* 2008;3:e3420.
- Wienholds E, Plasterk RH. MicroRNA function in animal development. *FEBS Lett* 2005;579:5911-5922.
- Krützfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 2005;438:685-689.
- Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834-838.
- Scherr M, Venturini L, Battmer K, et al. Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. *Nucleic Acids Res* 2007;35:e149.
- He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network—another piece in the tumour-suppression puzzle. *Nat Rev Cancer* 2007;7:819-822.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-866.
- Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 2006;6:259-269.
- Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 2007;297:1901-1908.
- Wells A, Yates C, Shepard CR. E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* 2008;25:621-628.
- Thompson EW, Williams ED. EMT and MET in carcinoma—clinical observations, regulatory pathways and new models. *Clin Exp Metastasis* 2008;25:591-592.
- Kajiya H, Shibata K, Terauchi M, et al. Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* 2007;31:277-283.
- Yang AD, Fan F, Camp ER, et al. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res* 2006;12:4147-4153.
- Hiscox S, Jiang WG, Obermeier K, et al. Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation. *Int J Cancer* 2006;118:290-301.
- Hiscox S, Morgan L, Barrow D, Dutkowskij C, Wakeling A, Nicholson RI. Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). *Clin Exp Metastasis* 2004;21:201-212.
- Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008;10:593-601.
- Abrahamsen HN, Steiniche T, Nexø E, Hamilton-Dutoit SJ, Sørensen BS. Towards quantitative mRNA analysis in paraffin-embedded tissues using real-time reverse transcriptase-polymerase chain reaction: a methodological study on lymph nodes from melanoma patients. *J Mol Diagn* 2003;5:34-41.
- Godfrey TE, Kim SH, Chavira M, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. *J Mol Diagn* 2000;2:84-91.
- Doleshal M, Magotra AA, Choudhury B, Cannon BD, Labourier E, Szafranska AE. Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. *J Mol Diagn* 2008;10:203-211.
- Zhang X, Chen J, Radcliffe T, Lebrun DP, Tron VA, Feilottter H. An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. *J Mol Diagn* 2008;10:513-519.
- Yu J, Ohuchida K, Mizumoto K, et al. MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. *Mol Cancer* 2010;9:169.
- Iorio MV, Visone R, Di Leva G, et al. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67:8699-8707.
- Nam EJ, Yoon H, Kim SW, et al. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* 2008;14:2690-2695.
- Helleman J, Jansen MP, Burger C, van der Burg ME, Berns EM. Integrated genomics of chemotherapy resistant ovarian cancer: a role for extracellular matrix, TGFbeta and regulating microRNAs. *Int J Biochem Cell Biol* 2010;42:25-30.

29. Eitan R, Kushnir M, Lithwick-Yanai G, et al. Tumor microRNA expression patterns associated with resistance to platinum based chemotherapy and survival in ovarian cancer patients. *Gynecol Oncol* 2009;114:253-259.
30. Leskelä S, Leandro-García LJ, Mendiola M, et al. The miR-200 family controls beta-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients. *Endocr Relat Cancer* 2010;18:85-95.
31. Chen ML, Liang LS, Wang XK. miR-200c inhibits invasion and migration in human colon cancer cells SW480/620 by targeting ZEB1. *Clin Exp Metastasis* 2012;29:457-469.
32. Toiyama Y, Hur K, Tanaka K, et al. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. *Ann Surg* 2014;259:735-743.