의학박사 학위논문

Molecular Subtypes of Pancreatic Cancer Based on miRNA Expression Profiles Have Independent Prognostic Value

마이크로 RNA 발현 양상에 따른 췌장암의 아형과 예후인자로서의 가치

2016년 8월

서울대학교 대학원

의학과 외과학 전공

권 우 일

Abstract

Molecular Subtypes of Pancreatic Cancer Based on miRNA Expression Profiles Have Independent Prognostic Value

Wooil Kwon

Medicine (Surgery)

The Graduate School

Seoul National University

Background and Aim

Altered microRNAs (miRNA) expression, a typical feature of many cancers, is reportedly associated with prognosis according to several studies. Although numerous studies on miRNAs in pancreatic ductal adenocarcinoma have also attempted to identify prognostic biomarkers, more large-scale clinical studies are needed to establish the clinical significance of the results. Present study aimed to

identify prognosis-related molecular subtypes of primary pancreas cancers using miRNA expression profiling.

Methods

Expression profiles of 1,733 miRNAs were obtained by using microarray analysis of 104 pancreatic tumors of Korean patients. To detect subgroups informative in predicting the patient's prognosis, unsupervised clustering method was applied and then the association of the molecular subgroups with survival time was analyzed. Then, classifiers to predict the subgroup using penalized regression models were identified.

Results

Three PDAC tumor subtypes associated with prognosis based on miRNA expression profiles were determined. These subtypes showed significantly different survival time for patients with the same clinical conditions. This demonstrates that this prognostic molecular subgroup has independent prognostic utility. The molecular subtypes can be predicted with classifiers of 19 miRNAs. Of the 19 signature miRNAs, miR-106b-star, miR-324-3p, and mir-615 were related to a p53 canonical pathway, and miR-324, miR-145-5p, miR-26b-5p, and miR-574-3p were related to a Cox-2 centered pathway.

Conclusions

This study demonstrated that pancreatic cancers may be classified into molecular subtypes based on their miRNA profiles and could be used to predict prognosis. Several classifier miRNAs can discriminate these prognostic molecular subtypes. Further studies are needed for validation.

Keyword: Pancreatic ductal adenocarcinoma, Prognosis, miRNA profile, Molecular subtype

Student Number: 2012-30488

Contents

List of Tables

List of Figures

Introduction

MicroRNAs (miRNAs) are non-coding, 17- to 25-nucleotide-long RNA molecules that regulate gene expression at the post-transcriptional level (1,2). They control gene expression levels by binding to 3□-untranslated regions of target messenger RNA, causing degradation or inhibiting translation (3,4). While miRNAs perform a regulatory role in the development, differentiation, and apoptosis of normal cells, several studies have shown that miRNAs are also related to the pathogenesis of various human diseases including cardiovascular disease, diabetes, mental disorders, viral infections, and cancer (5-9). In particular, dysregulation of miRNAs is known be involved in cancer initiation, progression, and metastasis (9-12). Thus, miRNAs may serve as novel diagnostic, prognostic, and therapeutic markers in clinical oncology.

MicroRNAs have tissue-specific and disease-specific expression patterns. The clinical implications and roles of miRNAs in the pathogenesis of pancreatic cancer also have been of great interest (13). Pancreatic cancer is a malignancy with a dismal outcome, representing the fourth leading cause of cancer-related death in the United States (14), and the fifth in Korea. Despite the highly aggressive nature

of pancreatic cancer, there has been little progress in improving its clinical outcome. The lack of accurate prognostic markers, which are important in establishing an individualized treatment strategy, contributes to the slow progress. Current prognostic factors of pancreatic cancer include clinicopathological features, most of which become available after surgical resection, and the most relevant cancer biomarker, CA19-9, has limited value in prognostic prediction, highlighting the need for additional markers that improve prognosis prediction.

Data have been accumulating on miRNAs related to the prognosis of pancreatic cancer (13,15). However, discrepancies among study results have been observed between the miRNAs and their prognostic value. This could be because of ethnic and regional differences of study populations. Therefore, this study was initiated to investigate molecular features of pancreatic cancer in the Korean population.

The first aim of this investigation was to determine the molecular subtypes of pancreatic cancer that are associated with prognosis by analyzing miRNA expression profiles of pancreatic cancer tissue. Second, this study aimed to develop prognostic models to predict prognostic subgroups by using miRNA profiles from the Korean population. The biological features of the identified prognostic miRNAs were also explored.

Materials and Methods

Study subjects

For this retrospective study, two hundred tissue samples stored between 2009 and 2012 were drawn from the biobank. The biobank is being operated by the department of Hepatobiliary and Pancreas Surgery of Seoul National University Hospital (IRB H-0901-010-267) since 2000. The pancreatic ductal adenocarcinoma (PDAC) samples are stored in the biobank after being processed in the following manner. After obtaining written consent from the patient preoperatively, 5 × 5 mm sections of tissue are sampled from surgical specimen immediately after resection and are stored at -80°C. The initial 200 samples were assessed to ensure high quality of the data. Of these, 96 samples were excluded because of RNA degradation or insufficient RNA content, and 104 samples were valid for the investigation.

The clinicopathologic findings including demographics, operation, adjuvant therapy, TNM stages, and pathologic features were retrospectively collected from the electronic medical records (Table 1). The patients were routinely followed up with

computed tomography (CT) and CA19-9 at 3-, 6-month postoperatively and every 6 months thereafter until 5-year follow up has been completed or until death. Recurrence was defined as newly detected mass or soft tissue density either locally or distantly on CT scan and/or high fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET) scan. The disease free survival (DFS) was defined as the time interval from the operation date to the date of CT or PET on which the recurrence was first detected. Survival data were provided by the Ministry of Public Administration and Security, Korea. The Institutional Review Board of Seoul National University Hospital approved this study (IRB C-1301-095-458).

RNA isolation and gene expression profiling

Total RNA was isolated by using a *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX) as described by the manufacturer. RNA quality was assessed with an Agilent 2100 Expert Bioanalyser using the RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA), and the quantity was determined with an ND-1000 Spectrophotometer (Thermo Fisher Scientific., Wilmington, DE). RNA labeling was

performed with a FlashTag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA) using 1 μg of total isolated RNA as input.

For miRNA gene expression profiling, GeneChip® miRNA 3.0 Array (Affymetrix, Santa Clara, CA), which contains 19,724 assays of 153 species, was used. Of the assays, 1,733 assays target the mature form of human miRNA. The labeled miRNA was hybridized to the array for 16 hours at 48°C and 60 rpm, as described in the protocol. After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450 (Affymetrix) and scanned in a GeneChip Array Scanner 3000 7G (Affymetrix). The probe cell intensity data from the GeneChip® miRNA arrays were analyzed with Expression Console™ software version 1.2, 32-bit version (Affymetrix). This process is summarized in Figure 1.

Statistical analysis

Prognostic molecular subtypes were determined through *k*-means clustering analysis by using 1,733 mature miRNA expression values. The *k*-means clustering aims to group *n* observations into *k* clusters in which each observation belongs to the cluster with the nearest mean, and the algorithm iteratively moves the centers

to minimize the sum of within-cluster variances (16). Gap statistics for estimating number of the clusters (17) were analyzed using clusGap function in R (18).

For construction of the classifier, elastic net regression was used. Elastic net regression is a penalized regression that fits a linear model and selects features simultaneously. This method is useful for a dataset containing a greater number of features than the number of samples, such as microarray data (19,20).

Survival analysis was performed using the log-rank test to determine the survival difference between the clusters. A Cox proportional hazard model was used to analyze the association of miRNAs and clinicopathological variables with survival. The Cox proportional hazard model analysis was conducted using survfit.coxph function from survival library and the Nelson-Aalen-Breslow estimate was adopted for survival curve fitting. To establish the Cox proportional hazard model including significant clinicopathologic variables, stepwise selection procedure was adopted. Stepwise selection is a combination of backward elimination and forward selection. The procedure was conducted using stepAIC function in R.

Chi-square test and Wilcoxon rank test were used to test for the statistical significance of differences in distributions of clinicopathological features between the subgroups.

All statistical analyses were executed by using the R program (version 3.0.3; http://www.r-project.org).

Results

Prognostic molecular subgroups of pancreatic cancer based on miRNA expression profiles

The expression profiles of 1,733 miRNAs for 104 pancreatic tumor samples were obtained using microarray analysis. Molecular subtypes of the tumor samples that had similar miRNA expression patterns were determined by using an unsupervised clustering method, that is, *k*-means clustering. *k*-means clustering analysis was performed for k = 2, 3, 4, and 5, where k is the parameter used to determine the number of clusters. Gap statistics, a commonly used measure for estimating number of clusters, was maximized for k=3 and k=5. Because the prediction of patient prognosis was the primary interest, survival analysis was performed for each k value by using the log-rank test. As a result, clusters determined with k = 3 showed the most significant association with overall survival time, with a p-value of 0.021. A classical multidimensional scaling plot that presents relative distances among individuals and the clustering result of three molecular subgroups is shown in Figure 2. Figure 2 also demonstrates that the American Joint

Committee on Cancer (AJCC) stage IIA and IIB distributions in each cluster were not significantly different between clusters. The median survival durations were estimated as 17.2 (n = 20; green in Figure 2), 26.5 (n = 32; red in Figure 2), and 32.4 (n = 52; blue in Figure 2) months for the three clusters, respectively.

Our primary interest was in investigating distinctive features of the molecular subgroup with the poorest prognosis, having an expected survival time of less than 2 years namely, the higher risk group. Additionally, the other two groups' molecular profiles share relatively similar expression patterns, having the shortest distance between their cluster centers. Thus, for simplicity, the higher risk group was compared to a merged cluster of the other two groups (lower risk group), in which median survival duration was longer than 2 years. By merging intermediate and low-risk groups, our classification focuses on differentiating clinically important high-risk group from others. The median survival duration of the lower risk group was 30.5 months, and the survival durations between the two groups (higher and lower risk) were significantly different with a p-value of 0.010 (Figure 3). The clinicopathological characteristics of the two molecular subtypes are summarized in Table 2. There was no significant difference in clinicopathological variables between the subtypes.

The prognostic value of the molecular subgroups of pancreatic cancer

The individual variable analysis of clinicopathological characteristics revealed that residual status (hazard ratio [HR], 1.65; confidence interval [CI], 1.12 2.45; p = 0.04), angiolymphatic invasion (HR, 1.96; CI, 1.22 3.14; p < 0.01), preoperative CA19-9 level (HR, 1.00; CI, 1.00 1.00; p = 0.03), and age (HR, 1.03; CI, 1.00 1.06; p = 0.03) were significantly associated with survival (Table 3).

To investigate the effect of clinical features to survival in the presence of the molecular subgroup variable, stepwise selection procedure for all the clinical variables starting with a Cox proportional hazard regression model with the molecular subgroup as a single covariate. The final selected model contained three clinical features of age, angiolymphatic invasion, and residual status (Table 4). Molecular subgroup was determined to be an independent prognostic factor with the greatest effect on survival duration (HR, 2.30; CI, 1.26 4.19; p = 0.01).

An additional analysis was performed to examine the recurrence predictability of miRNA. The median disease-free survival (DFS) of the higher risk group and lower

risk group was 6.5 and 17.3 months, respectively (p = 0.010). A subpopulation analysis on DFS was performed with the subpopulation of AJCC stage II patients with R status of 0 or 1 who may represent the most commonly confronted candidate for curative intent treatment in the clinic. Of the 104 patients, 93 were identified for this subpopulation. Of the 104 patients, 93 were identified. The DFS difference of prognostic molecular subgroups was also significant, with a median DFS time of 6.9 and 17.8 months for the higher risk group (n = 19) and the lower risk group (n = 74), respectively (p = 0.009) (Table 5, Figure 4a). Further analysis was performed with the subpopulation of AJCC stage II patients with R status of 0 or 1, who had received gemcitabine-based adjuvant chemotherapy. Fifty-one patients were identified as meeting the criteria. Of them, 11 belonged to the higher risk group and 40 to the lower risk group. Again, the lower risk group showed significantly better DFS of 17.2 months over 6.5 months, with a p-value of 0.002 (Table 5, Figure 4b). The same subgroup analysis with AJCC stage II patients but only with R0 revealed similar results as summarized in Table 5.

The same analysis was done on the same subgroup using overall survival (OS) instead of DFS. The result of these sub-analyses are summarized in Table 6. In subgroups of AJCC stage II patients with R0/1 resection and AJCC stage II patients

with R0 resection, the lower risk group revealed significantly better OS compared to the higher risk group (19.0 vs. 32.0 months, p = 0.011; 19.6 vs. 32.0 months, p = 0.037, respectively). The subset of AJCC stage II patients with R0/1 resection who received gemcitabine-based adjuvant chemotherapy the lower risk group also demonstrated better OS (19.0 vs. 32.4 months, p = 0.020). However, although the lower risk group of AJCC stage II patients with R0 resection followed by gemcitabine adjuvant chemotherapy showed better OS with 32.4 months compared to 22.0 months of higher risk group; this did not reach statistical significance (p = 0.081).

A classifier to differentiate the two prognostic molecular groups

Not all of the 1,733 miRNAs have differential expression regarding to the prognostic molecular subgroups, but only small number of the signature miRNAs may determine the subgroups. To determine a classifier to differentiate the two prognostic molecular groups, we applied the elastic net regression method for all 1,733 mature miRNAs and clinical variables including age, gender, residual status,

angiolymphatic invasion, and cancer stage. In the constructed classifier, 19 miRNAs were included, but none of the clinical variables were included (Table 6).

The heatmap of the expression profiles of the 19 miRNAs is demonstrated with the prognostic molecular group in Figure 5. The heatmap shows that miR-574-5p, miR-1244, and miR-4474-5p are up-regulated, whereas the other 16 miRNAs were down-regulated in the higher risk group.

Each of 19 miRNAs was analyzed for its association with overall survival (OS) and DFS to test for the individual prognostic value by using Cox proportional hazard models with three clinical variables as adjusting covariates. Eleven of the 19 miRNAs were independently associated with OS and six were also associated with DFS at the significance level of 0.05 (Table 7). In terms of effect size, miR-574-5p and miR-324-3p had the largest effect sizes of the 19 miRNAs.

Discussion

The possible clinical implications of miRNA expression in oncology are in the areas of diagnosis, therapy, and prognosis. Undoubtedly, all three are equally important in treating cancer patients. Besides the highly aggressive nature of pancreatic cancer, the lack of effective biomarkers contributes to its poor outcome (21). From this point of view, miRNAs represent a new possibility as novel biomarkers that may improve the low survival rate of pancreatic cancer. Currently recognized prognostic factors in resected pancreatic cancer include tumor stage, tumor size, histological differentiation, lymph node status, tumor grade, and resection margin status (22,23). Most of these prognostic factors are obtained postoperatively. Although CA19-9 is a traditionally used prognostic biomarker that can be obtained preoperatively, previous studies have reported little prognostic importance of CA19-9 in pancreatic cancer, having a small proportional HR (24). Therefore, further exploration of markers is needed to fine-tune the process of prognosis prediction for PDAC patients.

MicroRNAs are promising candidates for novel prognostic biomarkers, with the potential for wide clinical utility. miRNAs can be detected from tissue samples

obtained through endoscopic ultrasound-guided cytology or surgical procedures, and they can be also detected from the serum, plasma, or whole blood of patients (24,25). Therefore, prognostic miRNA markers can provide clinical benefits with both preoperative and postoperative usages to predict the patient's prognosis and to establish management strategy.

To date, numerous miRNAs have been reported to be related to the prognosis of pancreatic cancer. miR-142-5p and miR-320c were reported to be predictive of tumor response to gemcitabine (26,27). High expression of miR-200c, miR-142-5p, and miR-204 were reported to be associated with better survival after resection (26,28). In contrast, high expression of miR-155, miR-203, miR-210, miR-222, miR-21, and miR-196a-2 were reported to be associated with poor survival after resection (29-31).

In this study, we determined molecular subgroups of PDAC patients based on miRNA expression profiles and demonstrated that these subgroups can be an independent prognostic factor for survival prediction. The clinicopathological characteristics of the tumors of patients in these molecular subgroups were not significantly different. Furthermore, the molecular subgroup had greater prognostic power than the clinicopathological characteristics within the patient samples of the

same oncologic stage or of similar treatment regimens. Although we defined subgroups associated with OS, DFS was also analyzed as response values to assess the predictability of our molecular groups. Although some of previous studies had poor correlation between the OS and DFS in pancreatic cancer (32), positive correlations between them were reported and DFS was studied as a surrogate outcome variable (32,33). In our sample dataset, correlation between DFS and OS was 0.72 when computed with 64 non-censored cases that have records of different dates for recurrence and death events. Consequently, analysis results show that our molecular subgroups may have also significant association with DFS as well as with OS.

In the multiple regression analysis with covariates of clinicopathological features, the effect of the prognostic molecular subgroup was still significant. Moreover, the HR of the molecular subgroup was greater than any other variables included in the regression model. These analyses emphasize our study result that the prognostic molecular subgroup may have clinical utility as an independent prognostic biomarker for pancreatic cancer.

The prognostic molecular subtypes can be determined by the function of 19 miRNAs' expression levels (Table 6). Six of these 19 miRNAs (miR-574-5p, miR-

1244, miR-145-star, miR-328, miR-26b-star and miR-4321) individually had a significant association with both OS and DFS, and these may be considered as candidates for additional novel prognostic markers. Otherwise, they can be further investigated as therapeutic targets. For example, miR-574-5p, and miR-1244, showed positive HR, thus, down-regulation of miRNAs using anti-miRNA drug may lower the risk (34,35). The exact mechanisms underlying how these miRNAs are associated with an altered prognosis are not fully understood. The investigation of the functional role of the miRNA using the MetaCore™ database (GeneGo, St. Joseph, MI) provided some ideas on possible explanations. Of the 19 miRNAs, miR-106b-star, miR-324-3p, and miR-615 were mapped to the p53 canonical pathway (Figure 6a). It is worth noting that miR-324-3p had the largest effect among the 19 miRNA (Table 6). In addition, miR-324, miR-145-5p, miR-26b-5p, and miR-574-3p were mapped to the Cox-2 pathway (Figure 6b). Their role in pathways linked to oncogenesis may explain the association of these miRNA molecular groups and prognosis. As an individual modulator, miR-106 family were reported as modulators of TGF-β signaling and thus it potentially takes important role in tumorigenesis (36). miR-145 was reported to be associated with glioblastoma in a previous study (37) and the relationship with colon cancer was predicted in a recent study (38).

As the knowledge of cancer-specific miRNA expression profiles and the role of miRNAs in oncogenesis and progression pathways accumulates, more individualized treatment of pancreatic cancer should eventually become available.

Among the 19 miRNAs included in the classifier for the prognostic molecular subgroups, two miRNAs were previously identified to be associated with pancreatic cancer in other studies. miR-145 is often known to suppress tumor and some investigators dictated its potential use in pancreatic cancer diagnosis and treatment, but not yet in prognosis prediction (39,40). In addition, miR-26b was found to have diagnostic value as a panel in whole blood (40). However, none of the 19 miRNAs included in the classifier for the prognostic molecular subgroups have been found in the results of previous studies on prognostic miRNAs. The main differences can be explained by the models used for the analyses. Our classifier model used subgroups sharing patterns of expression profiles as a response variable, whereas other models directly used survival time as a response variable. Additional explanations may come from previously reported sources of inconsistency among studies, such as differences in measurement platforms, lab protocols, and sample sizes. In addition, ethnic difference is an important factor affecting the miRNA expression profiles (41).

This study demonstrated that subgrouping pancreatic cancer according to miRNA expression profiles could provide important prognostic information for pancreatic cancer patients. However, lack of replication of the results is a limitation of this study. Further validation with independent samples remains to be done in order to transfer the study results into the clinical setting.

References

1. Sethi S, Kong D, Land S, Dyson G, Sakr WA, Sarkar FH. Comprehensive molecular oncogenomic profiling and miRNA analysis of prostate cancer. Am J Transl Res 2013; 5: 200-211.
2. Siomi H, Siomi MC. Posttranscriptional regulation of microRNA biogenesis in animals. Mol Cell 2010; 38: 323-332.
3. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009; 136: 642-655.
4. Gu S, Kay MA. How do miRNAs mediate translational repression? Silence 2010; 1: 11.
5. Kim GH. MicroRNA regulation of cardiac conduction and arrhythmias. Transl Res 2013; 161: 381-392.
6. Locke JM, Harries LW. MicroRNA expression profiling of human islets from individuals with and without type 2 diabetes: promises and pitfalls. Biochem Soc Trans 2012; 40: 800-803.
7. Wang W, Kwon EJ, Tsai LH. MicroRNAs in learning, memory, and neurological diseases. Learn Mem 2012; 19: 359-368.

8. Gupta A, Swaminathan G, Martin-Garcia J, Navas-Martin S. MicroRNAs, hepatitis C virus, and HCV/HIV-1 co-infection: new insights in pathogenesis and therapy. Viruses 2012; 4: 2485-2513.

9. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. Adv Exp Med Biol 2013; 774: 1-20.

10. Iorio MV, Croce CM. Causes and consequences of microRNA dysregulation. Cancer J 2012; 18: 215-222.

11. Esquela-Kerscher A, Slack FJ. Oncomirs microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259-269.

12. Xia H, Hui KM. MicroRNAs involved in regulating epithelial-mesenchymal transition and cancer stem cells as molecular targets for cancer therapeutics. Cancer Gene Ther 2012; 19: 723-30.

13. Srivastava SK, Arora S, Singh S, Bhardwaj A, Averett C, Singh AP. MicroRNAs in pancreatic malignancy: progress and promises. Cancer Lett 2014; 347: 167-174.

14. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

15. Papaconstantinou IG, Manta A, Gazouli M, Lyberopoulou A, Lykoudis PM, Polymeneas G, et al. Expression of microRNAs in patients with pancreatic cancer and its prognostic significance. Pancreas 2013; 42: 67-71.

16. MacQeen J. Some methods for classification and analysis of multivariate observations. Proc Fifth Berkeley Symp Math Stat Probab 1967; 1: 281-297.

17. Tibshirani R, Walther G, Hastie T. Estimating the number of clusters in a data set via the gap statistic. Journal of the Royal Statistical Society: Series B (Statistical Methodology). 2001. p. 411 23.

18. Zou H, Hastie T. Regression shrinkage and selection via the elastic net, with applications to microarrays. Technical Report, Department of Statistics, Stanford University, 2003

19. Zou H, Hastie T. Regularization and variable selection via the elastic net. J R Stat Soc Ser B Stat Methodol 2005; 67: 301-320.

20. Jiang B, Gu Y, Chen Y. Identification of novel predictive markers for the prognosis of pancreatic ductal adenocarcinoma. Cancer Invest 2014; 32: 218-225.

21. Brennan MF, Kattan MW, Klimstra D, Conlon K. Prognostic nomogram for patients undergoing resection for adenocarcinoma of the pancreas. Ann Surg 2004; 240: 293-298.

22. Winter JM, Cameron JL, Campbell KA, Arnold MA, Chang DC, Coleman J, et al. 1423 pancreaticoduodenectomies for pancreatic cancer: a single-institution experience. J Gastrointest Surg 2006; 10: 1199-1210; discussion 1210-1211.

23. Fong ZV, Winter JM. Biomarkers in pancreatic cancer: diagnostic, prognostic, and predictive. Cancer J 2012; 18: 530-538.

24. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, Bojesen SE, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. JAMA 2014; 311: 392-404.

25. Brand RE, Adai AT, Centeno BA, Lee LS, Rateb G, Vignesh S, et al. A microRNA-based test improves endoscopic ultrasound-guided cytologic diagnosis of pancreatic cancer. Clin Gastroenterol Hepatol 2014; 12: 1717-1723.

26. Ohuchida K, Mizumoto K, Kayashima T, Fujita H, Moriyama T, Ohtsuka T, et al. MicroRNA expression as a predictive marker for gemcitabine response after surgical resection of pancreatic cancer. Ann Surg Oncol 2011; 18: 2381-2387.

27. Iwagami Y, Eguchi H, Nagano H, Akita H, Hama N, Wada H, et al. miR-320c regulates gemcitabine-resistance in pancreatic cancer via SMARCC1. Br J Cancer 2013; 109: 502-511.

28. Yu J, Ohuchida K, Mizumoto K, Sato N, Kayashima T, Fujita H, 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.

29. Greither T, Grochola LF, Udelnow A, Lautenschlager C, Wurl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer 2010; 126: 73-80.

30. Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. J Gastrointest Surg 2008; 12: 2171-2176.

31. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007; 297: 1901-1908.

32. Petrelli F, Coinu A, Borgonovo K, Cabiddu M, Barni S. Progression-free survival as surrogate endpoint in advanced pancreatic cancer: meta-analysis of 30 randomized first-line trials. Hepatobiliary Pancreat Dis Int. 2015;14(2):124 31.

33. Milella M, Bria E, Carlini P, Cuppone F, Gelibter A, Nisticò C, Ruggeri, EM. Terzoli FC and DG. Surrogate endpoints for overall survival (OS) in advanced

pancreatic cancer (APC): Analysis of randomized clinical trials (RCTs) exploring gemcitabine (G)-based combinations. Journal of Clinical Oncology, ASCO Annual Meeting Proceedings Vol 25, No 18S (June 20 Supplement). 2007. p. 4575.

34. Broderick J, Zamore PD. MicroRNA theapeutics. Gene Ther. 2011; 18( ): 1104-10.Jacobsen A, Silber J, Harinath G, Huse JT, Schultz N, Sander C. Analysis of microRNA-target interactions across diverse cancer types. Nat Struct Mol Biol [Internet]. 2013;20(11):1325 32.

35. Kota J, Chivukula RR, O' Donnell K a et al.Therapeutic microRNA delivery supresses tumorigenesis in a murineliver cancer model. Cell 2009;137:1005-17.

36. Jacobsen A, Silber J, Harinath G, Huse JT, Schultz N, Sander C, Analysis of microRNA-targ interactions across diverse cancer types. Nat Struct Mol Biol. 2013;20:1325-32.

37. Spermanza MC, Frattini V, Pisati F et al. NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma. Oncotarget. 2012;3(7):723-34.

38. Li L, Hu X, Yang Z, Jia Z, Fang M, 35. Zhang L, et al. Establishing reliable miRNA-cancer association network based on text-mining method. Comput Math Methods Med. 2014;2014:746979.

39. Khan S, Ebeling MC, Zaman MS, Sikander M, Yallapu MM, Chauhan N, et al. MicroRNA-145 targets MUC13 and suppresses growth and invasion of pancreatic cancer. Oncotarget 2014;5:7599-609.

40. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, Bojesen SE, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. JAMA 2014;311:392-404.

41. Ma MZ, Kong X, Weng MZ, Cheng K, Gong W, Quan ZW, et al. Candidate microRNA biomarkers of pancreatic ductal adenocarcinoma: meta-analysis, experimental validation and clinical significance. J Exp Clin Cancer Res 2013; 32: 71.

Tables

Table 1. Demographics and clinicopathological characteristics

	n = 104
Age (mean ± SEM)	64.9 ± 0.9
Sex (M:F)	1:1.04
Operation	
Whipple operation	20 (19.2%)
Pylorus-preserving pancreatoduodenectomy	46 (44.2%)
Distal pancreatectomy	36 (34.6%)
Total pancreatectomy	2 (1.9%)
Adjuvant chemotherapy	93 (89.4%)
Gemcitabine	59 (56.7%)
5-FU	24 (22.1%)
Unknown	10 (9.6%)
Recurrence	75 (72.1%)
Local recur	23 (22.1%)
Distant metastasis	65 (62.5%)
Median follow-up duration (months)	24.5 (range, 2 64)
R status	
R0	83 (79.8%)
R1	15 (14.4%)
R2	6 (5.8%)

Table 1 (cont.)

	n = 104
AJCC 7th staging	
Stage IA	3 (2.9%)
Stage IB	0
Stage IIA	47 (45.2%)
Stage IIB	52 (50.0%)
Stage III	1 (1.0%)
Stage IV	1 (1.0%)
Histological differentiation (*n* = 97)	
Well differentiated	4 (4.1%)
Moderately differentiated	83 (85.6%)
Poorly differentiated	10 (10.3%)
Perineural invasion	91 (87.5%)
Endolymphatic invasion	42 (40.4%)
Endovenous invasion	26 (25.0%)

Table 2. Comparison of clinicopathological features of the two prognostic molecular subtypes

	Lower risk (n = 84)	Higher risk (n = 20)	p-value
Differentiation	78 (92.9%)	19 (95%)	0.960
Well differentiated	3 (3.8%)	1 (5.3%)	
Moderately differentiated	67 (85.9%)	16 (84.2%)	
Poorly differentiated	8 (10.3%)	2 (10.5%)	
Perineural invasion			1.000
Absent	11 (13.1%)	2 (10%)	
Present	73 (86.9%)	18 (90%)	
Venous invasion			1.000
Absent	66 (78.6%)	12 (60%)	
Present	18 (21.4%)	8 (40%)	
Angiolymphatic invasion			0.151
Absent	50 (59.5%)	12 (60%)	
Present	34 (40.5%)	8 (40%)	
Pancreatitis			0.626
Absent	77 (91.7%)	17 (85%)	
Present	7 (8.3%)	3 (15%)	
Residual status			0.088
R0	70 (83.3%)	13 (65%)	
R1	9 (10.7%)	6 (30%)	
R2	5 (6%)	1 (5%)	

Table 2 (cont.)

	Lower risk (n = 84)	Higher risk (n = 20)	p-value
Chemotherapy			0.811
Performed	76 (90.5%)	18 (90%)	
Not performed	8 (9.5%)	3 (15%)	
Chemotherapy regimen			0.546
Unknown	8 (8.5%)	3 (15%)	
5-FU	21 (25%)	3 (15%)	
Gemcitabine	47 (56%)	12 (60%)	
T category			0.609
T1	3 (3.6%)	0 (0%)	
T2	0 (0%)	0 (0%)	
T3	80 (95.2%)	20 (100%)	
T4	1 (1.2%)	0 (0%)	
N category			1.000
N0	40 (48.2%)	10 (50%)	
N1	43 (51.8%)	10 (50%)	
Nx	1 (1.2%)	0 (0%)	
Sex			0.251
Male	44 (52.4%)	7 (35%)	
Female	40 (47.6%)	13 (65%)	
Size (cm)	3.3 ± 1.1	3.1 ± 0.6	0.407
BMI (kg/m^2)	22.8 ± 3.1	22.6 ± 2.5	0.873
Age (years)	65.4 ± 8.9	62.9 ± 11.5	0.276

Table 3. Cox proportional hazard model results of clinicopathological features

Variable	Values	Samples	N	Hazard ratio (95%CI)	*p*-value
Sex	Male	51 (0.49%)	33	1.06 (0.66 - 1.70)	0.81
	Female	53 (0.51%)	37		
Smoking	Non-smoker	96 (0.92%)	65	1.67 (0.66 - 4.18)	0.27
	Smoker	8 (0.08%)	5		
Alcohol	Non-drinker	83 (0.8%)	56	1.20 (0.66 - 2.16)	0.55
	Drinker	21 (0.2%)	14		
Pancreatitis	No pancreatitis	94 (0.9%)	62	1.11 (0.53 - 2.32)	0.78
	Pancreatitis	10 (0.1%)	8		
R status	R0	83 (0.8%)	54	1.65 (1.12 - 2.45)	0.04
	R1	15 (0.14%)	11		
	R2	6 (0.06%)	5		
Chemotherapy	No	11 (0.11%)	9		
	5-FU	24 (0.23%)	12	0.39 (0.16 - 0.93)	0.15
	Gemcitabine	59 (0.57%)	41	0.70 (0.34 - 1.44)	
	Unknown	10 (0.1%)	8	0.77 (0.29 - 1.99)	
T category	T1	3 (0.03%)	2		
	T2	0 (0%)			
	T3	100 (0.96%)	68	1.20 (0.29 - 4.91)	0.56
	T4	1 (0.01%)	0	1.3E-07 (0 - Inf)	

Table 3 (cont.)

Variable	Values	Samples	N	Hazard ratio (95%CI)	*p*-value
N category	N0	50 (0.48%)	29	1.48 (0.91 - 2.38)	0.11
	N1	53 (0.51%)	40		
Differentiation	Well	4 (0.04%)	3	1.20 (0.37 - 3.84)	0.17
	Moderate	83 (0.8%)	56	2.57 (0.67 - 9.82)	
	Poor	10 (0.1%)	8	1.2E-07 (0 - Inf)	
Perineural invasion	Absent	13 (0.13%)	9	1.31 (0.70 - 2.46)	0.64
	Present	91 (0.88%)	61		
Angiolymphatic invasion	Absent	62 (0.6%)	37	1.96 (1.22 - 3.14)	<0.01
	Present	42 (0.4%)	33		
Venous invasion	Absent	78 (0.75%)	52	1.40 (0.81 - 2.40)	0.22
	Present	26 (0.25%)	18		
AJCC stage	IA	3 (0.03%)	2		
	IIA	46 (0.44%)	27	0.97 (0.23 - 4.09)	0.14
	IIB	52 (0.5%)	39	1.36 (0.33 - 5.65)	
	III	1 (0.01%)	0		
	IV	1 (0.01%)	1	NA	
Preoperative CEA				1.01 (0.98-1.04)	0.54
Preoperative CA19-9				1.00 (1.00- 1.00)	0.03
BMI				1.01 (0.94-1.09)	0.79
Age				1.03 (1.00-1.06)	0.03

Table 4. Cox proportional hazard model with molecular group determined by miRNA expression profiles along with three clinicopathological prognostic factors that were significantly associated with overall survival time

	Hazard ratio	95% CI	*p*-value
Molecular group	2.293	1.255–4.192	0.007
Age	1.048	1.018–1.078	0.001
Angiolymphatic invasion	2.088	1.278–3.413	0.003
Residual status	1.841	1.224–2.769	0.003

Table 5. The disease-free survival (DFS) analysis and subgroup analysis of AJCC stage II patients between higher risk group and lower risk group

	Median DFS duration (months)		
	Higher risk group	Lower risk group	*p*-value
Overall	6.5 (*n* = 20)	17.3 (*n* = 84)	0.010
Stage II; R0 & R1	6.9 (*n* = 19)	17.8 (*n* = 74)	0.009
Stage II; R0	9.4 (*n* = 13)	18.6 (*n* = 66)	0.040
Stage II; Gemcitabine; R0 & R1	6.5 (*n* = 11)	17.2 (*n* = 40)	0.002
Stage II; Gemcitabine; R0	6.5 (*n* = 7)	17.8 (*n* = 37)	0.009

Table 6. The overall survival (OS) analysis and subgroup analysis of AJCC stage II patients between higher risk group and lower risk group

	Median OS duration (months)		
	Higher risk group	Lower risk group	*p*-value
Overall	17.2 (*n* = 20)	30.5 (*n* = 84)	0.010
Stage II; R0 & R1	19.0 (*n* = 19)	32.0 (*n* = 74)	0.011
Stage II; R0	19.6 (*n* = 13)	32.0 (*n* = 66)	0.037
Stage II; Gemcitabine; R0 & R1	19.0 (*n* = 11)	32.4 (*n* = 40)	0.020
Stage II; Gemcitabine; R0	22.0 (*n* = 7)	32.4 (*n* = 37)	0.081

Table 7. Nineteen miRNA included in the classifier to predict the prognostic molecular group that was determined through the elastic net regression model analysis

Classifiers[†]	Parameter estimate	Cox model for OS (n=104)		Cox model for DFS (n=93)	
		Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
miR-106b-star	-3.891	0.262 (0.052,1.306)	0.102	0.304 (0.059,1.571)	0.156
miR-324-3p	-3.087	0.101 (0.013,0.780)	**0.028**	0.143 (0.020,1.049)	0.056
miR-943	-1.315	0.432 (0.205,0.908)	**0.027**	0.567 (0.270,1.192)	0.134
miR-1292	-1.244	0.801 (0.375,1.714)	0.568	0.754 (0.361,1.576)	0.453
miR-4474-5p	1.095	1.972 (0.980,3.969)	0.057	1.484 (0.738,2.982)	0.268
miR-935	-0.818	0.780 (0.520,1.170)	0.231	0.925 (0.612,1.397)	0.709
miR-668	-0.684	0.794 (0.433,1.455)	0.455	1.032 (0.562,1.896)	0.918
miR-574-5p	0.573	10.609 (1.483,75.908)	**0.019**	7.709 (1.212,49.015)	**0.030**
miR-1244	0.579	4.396 (1.512,12.779)	**0.007**	5.067 (1.790,14.343)	**0.002**
miR-4763-5p	-0.467	0.760 (0.434,1.331)	0.337	0.882 (0.511,1.523)	0.653
miR-145-star	-0.418	0.437 (0.277,0.691)	**<0.001**	0.582 (0.375,0.904)	**0.016**
miR-328	-0.338	0.310 (0.154,0.624)	**0.001**	0.491 (0.254,0.948)	**0.034**
miR-26b-star	-0.324	0.530 (0.342,0.82)	**0.004**	0.606 (0.382,0.960)	**0.033**

Table 7 (cont.)

Classifiers†	Parameter estimate	Cox model for OS (n=104)		Cox model for DFS (n=93)	
		Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
miR-615-5p	-0.301	1.132 (0.616,2.078)	0.69	1.179 (0.649,2.141)	0.589
miR-1914-star	-0.247	0.427 (0.237,0.771)	**0.005**	0.563 (0.316,1.004)	0.052
miR-564	-0.233	0.390 (0.199,0.762)	**0.006**	0.586 (0.297,1.153)	0.121
miR-3194-5p	-0.154	0.380 (0.187,0.773)	**0.008**	0.555 (0.282,1.091)	0.088
miR-4746-3p	-0.078	0.525 (0.26,1.059)	0.072	0.568 (0.287,1.124)	0.104
miR-4321	-0.002	0.449 (0.234,0.861)	**0.016**	0.536 (0.298,0.967)	**0.038**

Regression parameter estimates obtained from the regression model show that miR-4474-5p, miR-574-5p, and miR-1244 are up-regulated in high risk group. Hazard ratios for overall survival (OS) duration and disease-free survival (DFS) duration obtained from Cox proportional hazard regression models for individual miRNAs with adjusting covariates of age, angiolymphatic invasion, and residual status.

† miRNAs having *p*-values less than 0.05 in the Cox proportional hazard model analysis are printed in bold.

Figures

Figure 1. The schematic flow of RNA isolation and gene expression profiling method is illustrated.

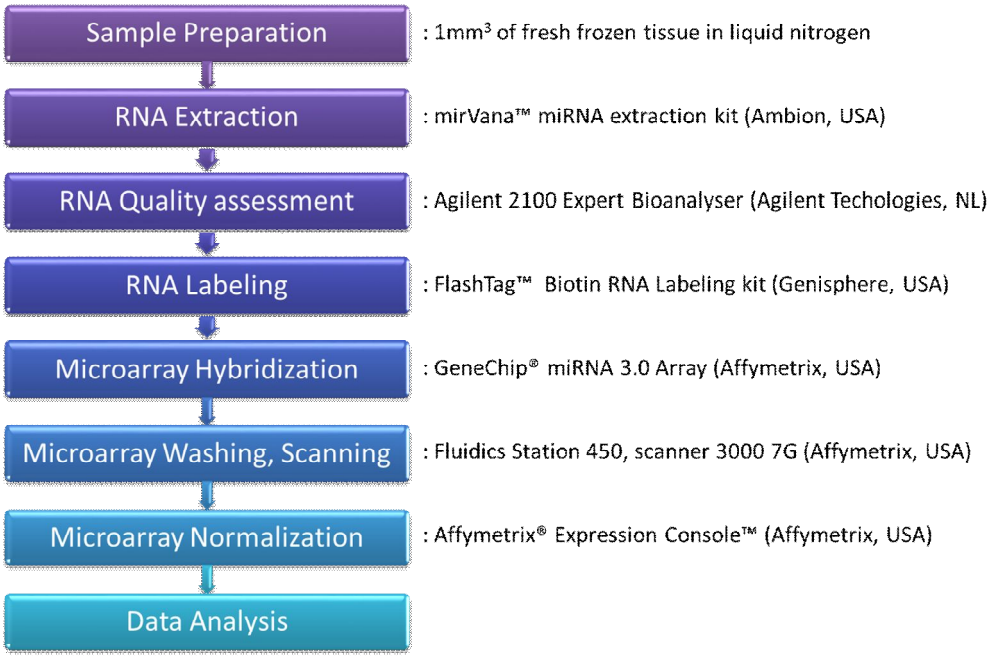


Figure 2. Multidimensional scaling plot of the three groups determined by k-means clustering analysis of miRNA expression profiles with k = 3. Median survival times of the three molecular subgroups were 17.2, 26.5, and 32.4 months, respectively. To visualize the relationship of the molecular groups and cancer stages, AJCC stages of each individual are marked with distinctive point types.

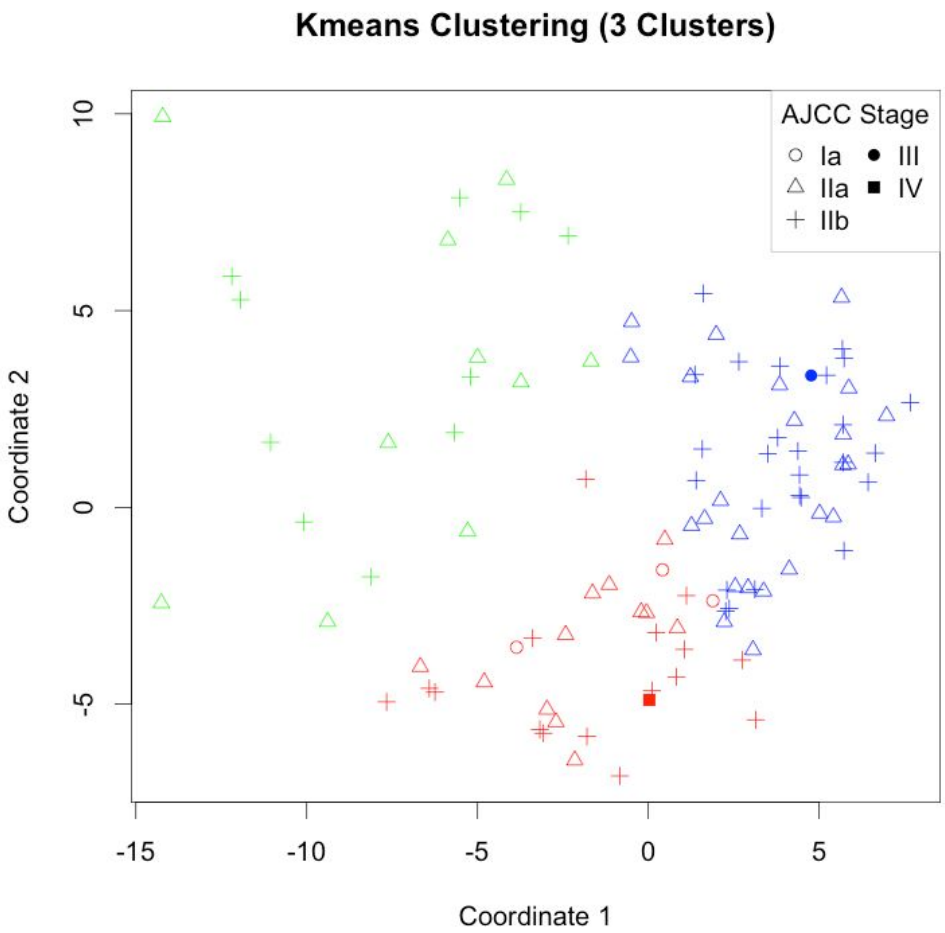


Figure 3. Kaplan-Meier curves for the two groups (higher risk and lower risk) after merging two similar groups with a median survival time of more than 2 years. Median survival times were 30.5 and 17.2 months for the lower risk group and the higher risk group, respectively.

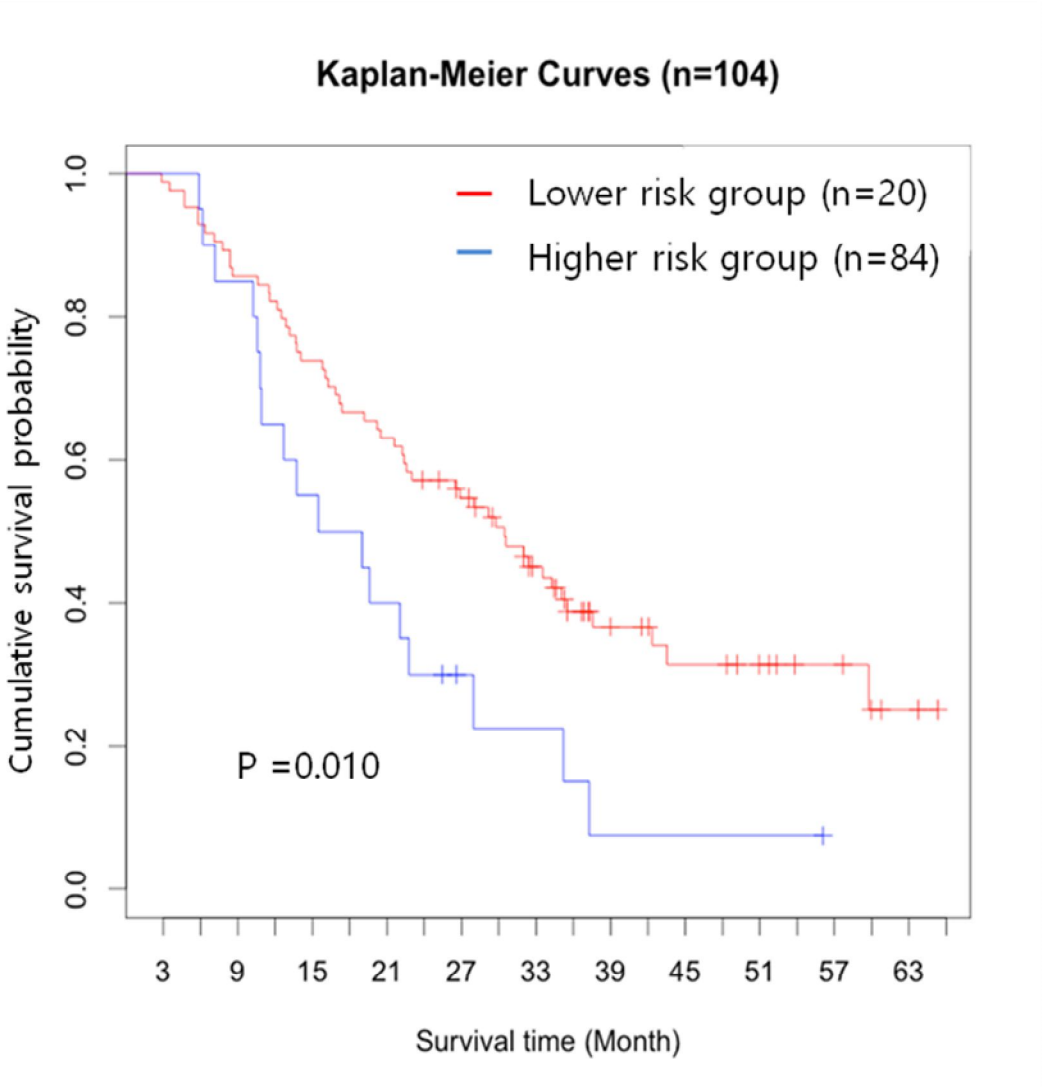
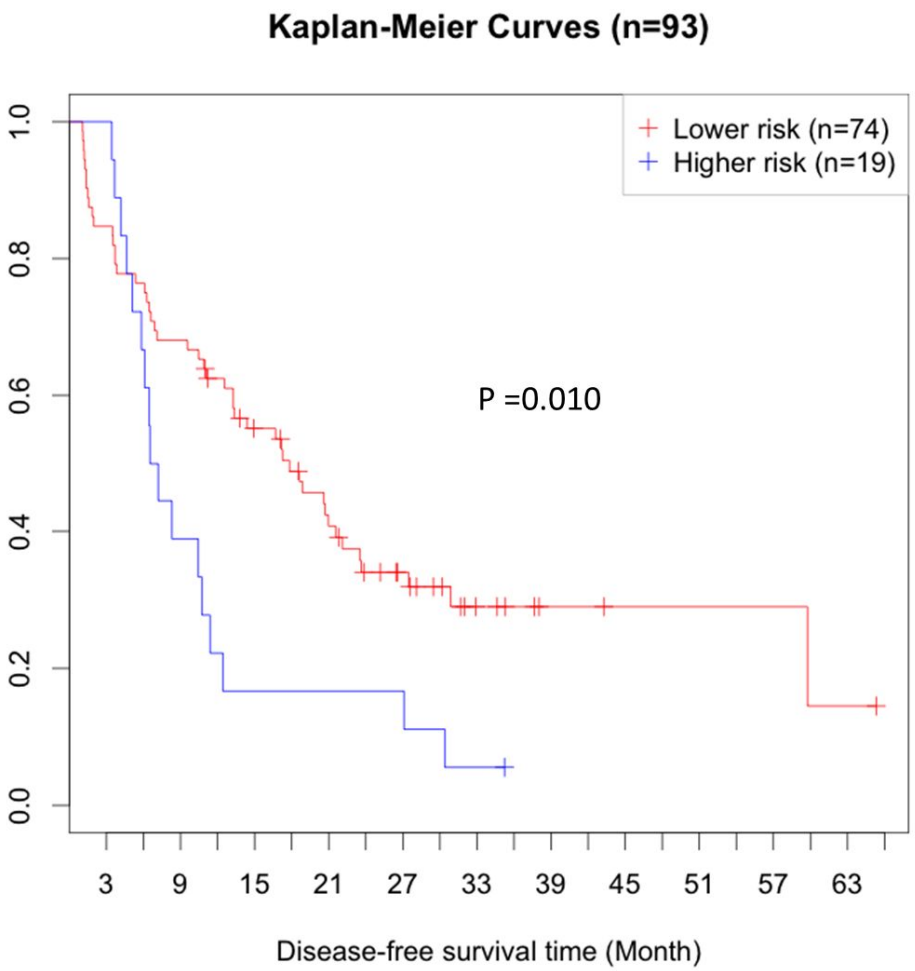


Figure 4. (a) Kaplan-Meier curves for disease free survival time of the molecular subgroups among subpopulation show that the molecular subgroups have prognostic values even in R0 or R1 resected stage II pancreatic cancer patients. (b) The molecular subgroups still retain their prognostic values when the subpopulation is further limited to patients treated with gemcitabine.

(a)



(b)

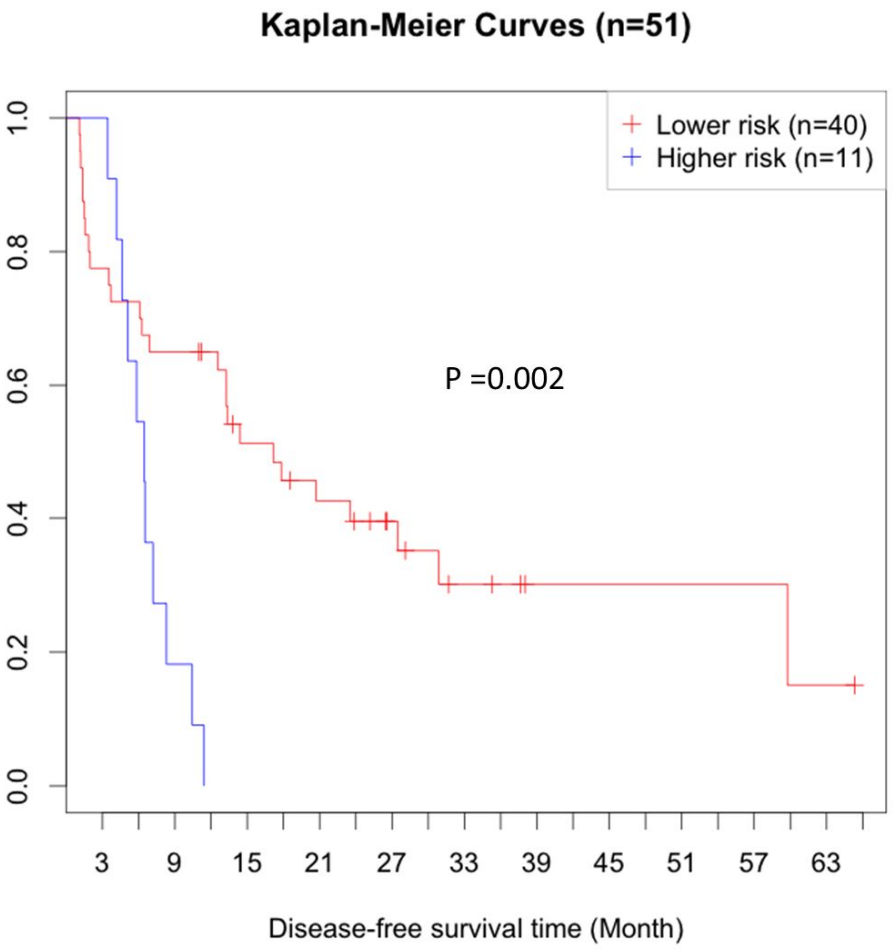

Kaplan-Meier Curves (n=51)
Lower risk (n=40)
Higher risk (n=11)
P =0.002
1.0
0.8
0.6
0.4
0.2
0.0
3
9
15
21
27
33
39
45
51
57
63
Disease-free survival time (Month)

Figure 5. Heatmap of the 19 miRNAs included in the classifier model for the two molecular subtypes and original *k*-means clustering groups. Color bar on the top indicates the prognostic molecular subgroups and is determined by the clustering analysis where green indicates the higher risk group.

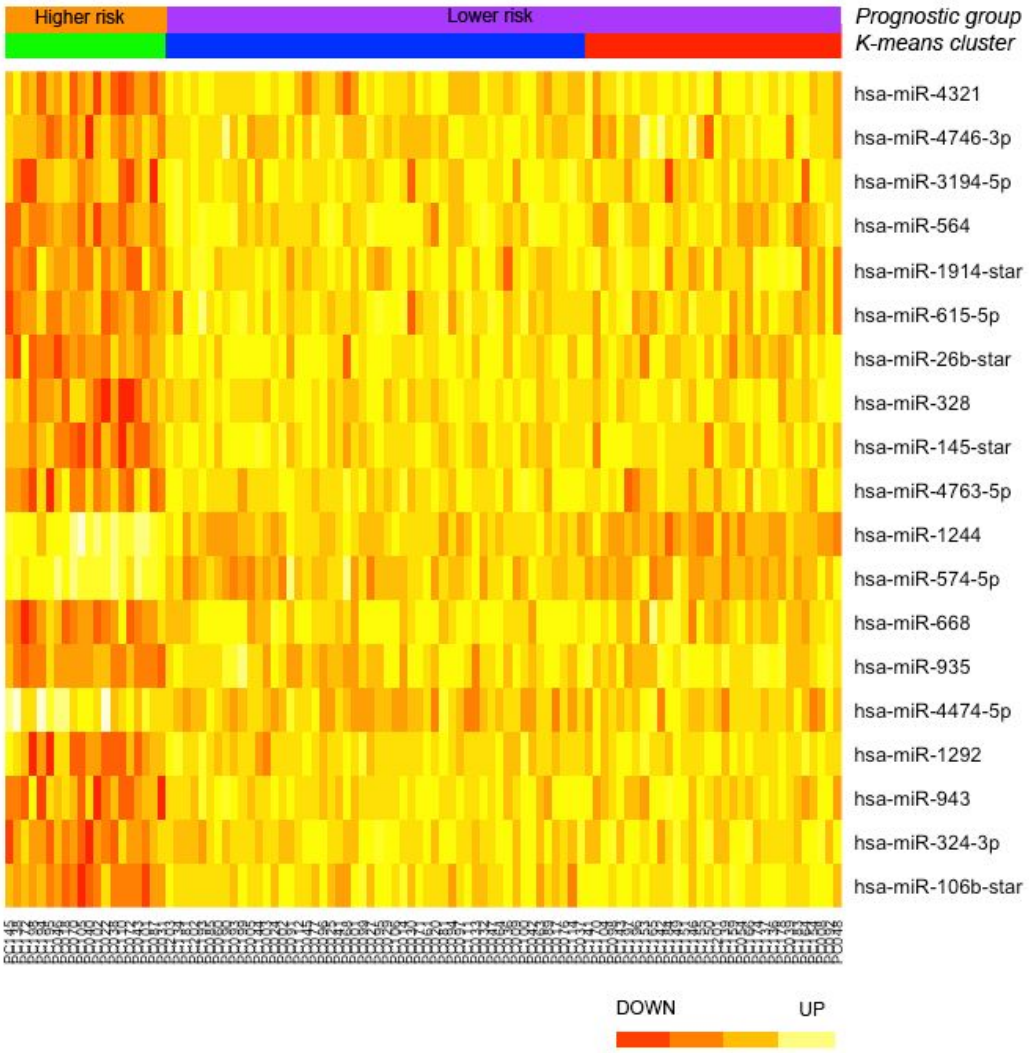
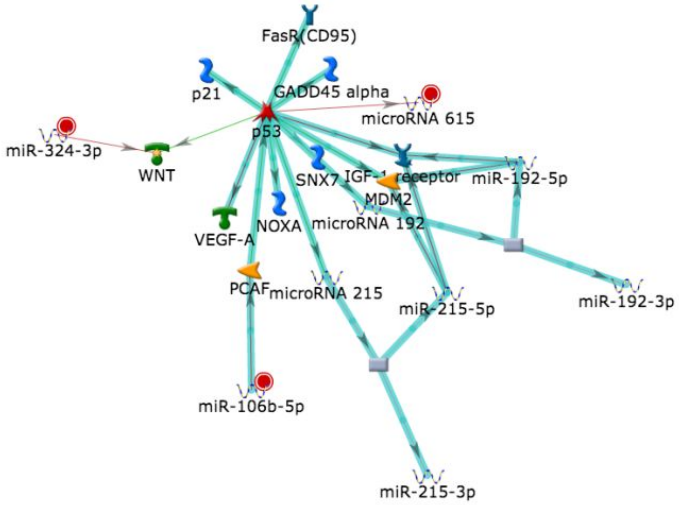
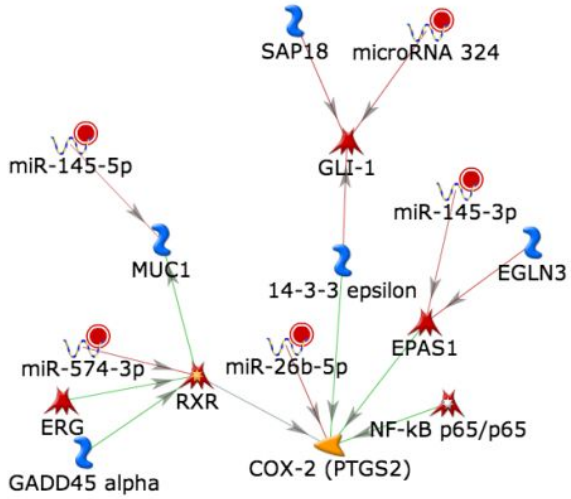


Figure 6. MetaCore™ network analysis results are illustrated. (a) In the first subnetwork, miR-324-3p, miR-106b-5p, and miR-615 are mapped to the p53 canonical pathway. (b) In the second subnetwork, miR-324, miR-145-5p, miR-26b-5p, and miR-574-3p are mapped to a Cox-2 centered pathway.

(a)



(b)



국문초록

마이크로 RNA 발현 양상에 따른 췌장암의 아형과 예후인자로서의 가치

서울대학교 대학원

의학과 외과학 전공

권 우 일

<배경 및 목적> 다양한 암종에서 마이크로 RNA (miRNA) 발현 양상이 변한다는 것은 잘 알려져 있으며, 여러 연구에서 변화된 miRNA 발현 양상의 일부는 예후와 관련이 있다는 것이 보고되어 있다. 췌장암에서도 miRNA에 대한 연구들이 여럿 진행되었고, 이를 통해 예후를 반영할 수 있는 miRNA를 찾기 위한 많은 노력이 있었다. 그러나 연구자마다 결과가 상이하고 대규모의 연구가 아니어서 임상에 적용할 만큼 명확한 결과를 얻지 못했다. 또한 대부분의 결과가 서양에서 이루어진 연구라서 동양, 특히 국내의 결과가 부족한 상황이다. 따라서 본 연구에서는 한국인에서 발생한 췌장암의 miRNA 발현 양상을 살펴보고, miRNA 발현 양상에 따라서 췌장암을 아형으로 분류하여 예후인자로서의 가치가 있는지 알아보도록 했다. 더불어 이런 아형

을 결정짓는 miRNA를 확인해보도록 하였다.

<대상 및 방법> 2009년에서 2011년 사이에 서울대병원에서 췌장암으로 수술한 환자에게서 채취하여 인체유래물 저장소에 저장되어 있던 췌장암 조직 200개 중 실험에 적합한 104개를 선별하여 연구를 진행하였다. 마이크로어레이를 통해 이 104개의 췌장암 조직에서 1,733개의 miRNA 발현 양상을 분석하였다. 예후를 예측하기 위한 아형으로 분류하기 위해 miRNA 발현 양상으로 *k*-means clustering analysis를 시행하고 이 아형에 따른 생존분석을 시행하였다. 분자 수준의 아형을 포함하여 다른 임상병리학적 인자에 대하여 예후인자 분석을 시행하여 예후인자로서의 가치를 확인하였고, 이 췌장암 아형을 구분 짓는 miRNA를 elastic net regression을 통하여 확인하였다.

<결과> *k*-means clustering analysis을 통해 예후가 가장 상이한 3개의 췌장암 아형으로 나누었다. 그 중 예후가 가장 나쁜 고위험군과 비교적 좋은 저위험군 2개의 아형으로 재분류 하였다. 이 두군 간에 임상병리학적인 차이는 없었으며, 이 아형들은 다변량분석에서 독립적인 예후 예측인자이었다. Elastic net regression을 통하여 19개의 miRNA가 이 두 군을 구분함을 확인하였다. 이 중 miR-106b-star, miR-324-3p, miR-615sms p53 canonical pathway와 연관이 있는 것이었으며, miR-324, miR-145-5p, miR-26b-5p, miR-574-3p는 Cox-2 centered pathway와 연관이 있었다.

<결론> 이 연구는 비슷한 임상병리학적 특징을 갖는 췌장암이라도 miRNA 발현 양

상에 기반하여 다른 예후를 갖는 아형으로 분자학적 수준에서 췌장암을 분류할 수 있음을 시사한다. 또한 이러한 아형을 구분 짓는 miRNA를 확인할 수 있었으나 이를 검증하기 위해서는 더 큰 규모의 더 많은 연구들이 필요하겠다.

주요어: 췌장암, 예후, 마이크로 RNA 발현양상, 분자학적 아형

학번: 2012-30488