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Correlation of miR-494 expression with tumor progression and patient survival in pancreatic cancer

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ABSTRACT. MicroRNA-494 (miR-494) expression is aberrant in various types of human cancer. However, the prognostic value of miR-494 in pancreatic cancer remains unclear. The level of miR-494 expression was determined in 99 pairs of primary pancreatic cancer and their corresponding, adjacent non-tumor tissues by using quantitative reverse transcriptase polymerase chain reaction. We also analyzed the associations between miR-494 expression and clinicopathological features. The survival correlations were analyzed by using the Kaplan-Meier method and Cox proportional hazards model. The level of miR-494 expression was significantly downregulated in pancreatic cancer tissues (mean relative expression level ± SD, 0.48 ± 0.11) as compared to matched adjacent normal tissues (1.80 ± 0.28, $P < 0.05$). We found

significant correlations between the miR-494 expression levels and TNM stage (P = 0.009), lymphatic invasion (P = 0.036), vascular invasion (P = 0.011), distant metastasis (P = 0.007), and tumor grade (P = 0.031). Pancreatic cancer patients with a low miR-494 expression level had a shorter overall survival than those with a high miR-494 expression level (P < 0.05). Reduced miR-494 expression in pancreatic cancer tissues is correlated with tumor progression and might be an independent, poor prognostic factor for patients with pancreatic cancer.

Key words: microRNA; miR-494; Survival; Prognosis; Pancreatic cancer

INTRODUCTION

Pancreatic cancer is the sixth leading cause of death due to malignant disease in China and the fourth leading cause of cancer-related death in the USA (Guo et al., 2005; Siegel et al., 2014). Pancreatic carcinogenesis is a multistep process involving multiple genetic and epigenetic alterations (Macgregor-Das et al., 2013). Hence, a better understanding of the molecular mechanisms involved in pancreatic carcinogenesis will be helpful for identification of new and effective prognostic biomarkers.

MicroRNAs (miRNAs) are small non-coding regulatory RNAs that suppress gene expression through partial complementary elements in the 3'-untranslated regions of their target messenger RNAs (mRNAs)(Bartel 2004). miRNAs have been shown to be involved in various critically important physiological processes such as cell proliferation, cell division, cell differentiation, cell apoptosis, tumorigenesis, hematopoiesis, and the nervous system patterning (Ambros, 2004; Harfe, 2005). A particular type of miRNA-miR-494-is downregulated in different types of cancer tissues. Moreover, it is a tumor suppressor and induces cell cycle arrest, cell senescence, and apoptosis, but suppresses cell proliferation (Diakos et al., 2010; Ohdaira et al., 2012; Yamanaka et al., 2012). Li et al. found that downregulation of miR-494 via loss of SMAD4 increased FOXM1 and β-catenin signaling in pancreatic cancer cells. However, the association between the expression of miR-494 and the clinicopathological characteristics of pancreatic cancer remain unclear. Thus, the aim of this study was to evaluate the clinical significance of miR-494 in pancreatic cancer.

MATERIAL AND METHODS

Patients and tumor tissues

The study protocol was approved by the Ethics Committee of Shandong Provincial Hospital affiliated to ShanDong University, and all patients provided written informed consent for the use of their tissues. A total of 99 pairs of human pancreatic cancer tissues and matched normal adjacent pancreatic tissues were obtained during surgery between July 2006 and August 2012 at the Department of Hepatobiliary Surgery, Shandong Provincial Hospital, which is affiliated to ShanDong University. The diagnosis was based on pathological evidence. The specimens were immediately snap-frozen and stored at -80°C for future miR-494 extraction. None of the patients received chemotherapy or radiotherapy before the surgical excision.

Quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from pancreatic cancer tissues and matched normal adjacent tissues by homogenizing the tissue in Trizol (Invitrogen, USA) according to the manufacturer's instructions. Primers for miR-494 and endogenous control U6 snRNA were obtained from Applied Biosystems (Foster City, USA). The concentration and purity of RNA were determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies, USA). cDNA was generated using the PrimeScript RT reagent kit (Takara Co. Ltd., Dalian, China) in 20 µL of final reaction volume containing 0.5 µg of RNA, 0.5 µL Prime-Script RT enzyme mix, 4 µL 5X PrimeScript buffer, and 1 µL RT primer. The reaction mixture was incubated at 42°C for 60 min and at 85°C for 5 min. Quantitative real-time polymerase chain reaction was performed to evaluate the miR-494 expression using SYBR Premix Ex Taq (Takara Co. Ltd.) and measured in a LightCycler 480 System (Roche, Basel, Switzerland). The amplification was conducted in the following steps: denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Relative quantification of miRNA expression was performed using the $2^{-\Delta\Delta C}{}_{T}$ value. The raw data were presented as the relative quantity of target miRNA, normalized with respect to U6 snRNA and relative to a calibrator sample.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between the two groups were estimated using the Student *t*-test and the Chi-square test. Overall survival (OS) was measured up to the date of death from any cause or, for living patients, the date of last contact. OS was estimated using the Kaplan-Meier method, and the differences in survival were compared using the log-rank test. A Cox proportional hazards model was used for multivariate analysis. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Expression of miR-494 in pancreatic cancer

The level of miR-494 expression was significantly downregulated in pancreatic cancer tissues (mean relative expression level ± SD, 0.48 ± 0.11) when compared with matched adjacent normal tissues (1.80 ± 0.28, $P < 0.05$, Figure 1). The relative miR-494 expression level was classified as high or low in relation to the median value.

Association between miR-494 expression and clinicopathological variables of pancreatic cancer patients

The relationships between miR-494 expression levels and clinicopathological characteristics in individuals with pancreatic cancer are summarized in Table 1. We found significant correlations between miR-494 expression levels and the TNM stage ($P = 0.009$), lymphatic invasion ($P = 0.036$), vascular invasion ($P = 0.011$), distant metastasis ($P = 0.007$), and tumor grade ($P = 0.031$). However, we did not find a significant association of the miR-494 expression levels with sex ($P = 0.62$), age ($P = 0.21$), tumor size ($P = 0.11$), and tumor location ($P = 0.45$).

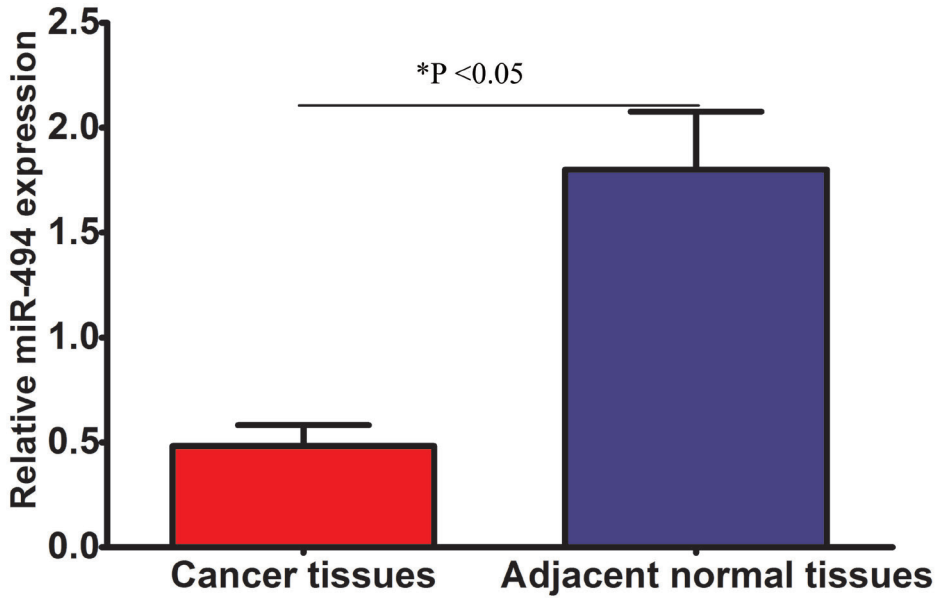

Figure 1. The level of miR-494 expression in pancreatic cancer tissues as compared to matched adjacent normal tissues (P < 0.05).

Table 1. Relationship between miR-494 and clinicopathological characteristics in 99 patients with pancreatic cancer.

Parameters	Number of cases	miR-494 expression		P value
		Low	High	
Gender				
Male	51	27	24	0.62
Female	48	17	31	
Age				
<60 years	49	16	33	0.21
≥60 years	50	28	22	
Tumor Size				
<2 cm	67	25	42	0.11
≥2 cm	32	19	13	
Location				
Head	58	28	30	0.45
Other	41	16	25	
Lymphatic invasion				
Positive	48	31	17	0.036
Negative	51	13	38	
Vascular invasion				
Positive	46	30	16	0.011
Negative	53	14	39	
Distant metastasis				
Positive	42	32	10	0.007
Negative	57	12	45	
TNM stage				
I/II	54	37	17	0.009
III/IV	45	7	38	
Histologic grade				
High/moderate	56	33	23	0.031
Poor	43	11	32	

Association between miR-494 expression and prognosis of pancreatic cancer patients

To evaluate the prognostic value of miR-494 expression in pancreatic cancer, survival curves were constructed by using the Kaplan-Meier method and compared by using the log-rank test. Pancreatic cancer patients with low miR-494 expression level had a shorter overall survival than those with high miR-494 expression level (Figure 2). The 5-year overall survival rate in the low-expression groups was 12.0% compared with 49.2% in the high-expression group (log-rank test, P = 0.036).

To determine whether the expression of miR-494 was an independent risk factor for poor prognosis, both clinicopathological factors and the level of miR-494 expression were evaluated by performing multivariate Cox regression analysis. We found that the miR-494 expression level (hazard ratio [HR] = 2.85, 95% confidence interval [CI]: 1.54-8.45, P = 0.019) was an independent factor for predicting the overall survival of pancreatic cancer patients (Table 2).

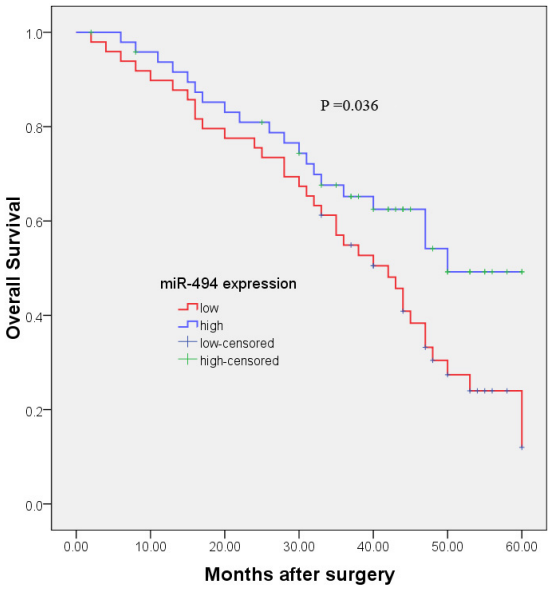

Figure 2. Comparison between the overall survival in pancreatic cancer patients with high and low miR-494 expression levels (P = 0.036).

Table 2. Multivariate analysis of factors associated with overall survival in pancreatic cancer patients.

Variable	Hazard ratio	95%CI	P value
Gender	0.87	0.35-1.68	0.45
Age	1.28	0.65-1.54	0.33
Tumor Size	1.23	0.75-2.92	0.24
Location	1.48	0.54-2.62	0.74
Lymphatic invasion	2.92	1.37-10.39	0.016
Vascular invasion	2.18	1.03-16.86	0.008
Distant metastasis	3.03	2.15-18.92	0.012
TNM stage	2.98	1.88-14.12	0.007
Histologic grade	1.21	0.78-8.27	0.06
miR-494 expression	2.85	1.54-8.45	0.019

DISCUSSION

Although pancreatic cancer has been considerably studied over the past several decades, new and effective treatment regimens are lacking, and chemotherapy and radiation therapy remain ineffective (Neoptolemos et al., 2004). Therefore, surgery is the only curative treatment option; however, only 15-20% pancreatic cancer patients are eligible for surgery at the time of presentation. Further, of those who undergo successful surgical resection, the 5-year survival rate is only 15-23% (Simianu et al., 2010). To develop novel treatment strategies for treatment of pancreatic cancer patients, a better understanding of the molecular pathogenesis of pancreatic cancer is required. In addition, accurate prediction of the prognosis for individual pancreatic cancer patients is of great importance, and molecular biomarkers that could serve as prognostic factors would be useful in determining an individualized treatment plan for a pancreatic cancer patient (Hurwitz et al., 1992). However, the biomarkers used in these group of patients today are not satisfactory (Winter et al., 2013), and additional markers need to be tested to fine-tune this process.

Recent studies have indicated that mutations or misexpression in miRNAs are correlated with various human cancers and can act as oncogenes or tumor-suppressor genes by controlling the expression of protein-coding miRNAs (Esquela-Kerscher et al., 2006). Varied expression of miRNAs in normal tissues compared with cancerous tissues and the significant correlation between specific expression and prognosis imply that miRNAs are determinants of certain clinical outcomes (Lu et al., 2005; Volinia et al., 2006). miR-494 has been found to be downregulated in different types of cancer tissues and is a tumor suppressor, as it induced cell cycle arrest, cell senescence, and apoptosis and suppresses cell proliferation (Diakos et al. 2010; Ohdaira et al. 2012; Yamanaka et al. 2012). Ohdaira et al. found that miR-494 suppressed cell proliferation and induced senescence in A549 lung cancer cells (Ohdaira et al. 2012). Furthermore, Romano et al. (2012) found that miR-494 was regulated by ERK1/2 and modulated by tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small-cell lung cancer through BIM downregulation. The findings by Kim et al. (2011) indicated that miR-494 was a negative regulator of *KIT* in gastrointestinal stromal tumors (GISTs), and overexpressing miR-494 in GISTs might be a promising approach to GIST treatment. Previously, Li et al. found that downregulation of miR-494 via the loss of SMAD4 increased FOXM1 and β-catenin signaling in pancreatic cancer cells. However, the association between miR-494 expression and the clinicopathological characters of pancreatic cancer remain unclear. In the present study, we found that the level of miR-494 expression was significantly downregulated in pancreatic cancer tissues, and that significant correlations existed between miR-494 expression levels and TNM stage, lymphatic invasion, vascular invasion, distant metastasis, and tumor grade. These results indicated that the miR-494 deregulation was involved in invasion/metastasis of pancreatic cancer. Therefore, the level of miR-494 expression might be correlated with a poor prognosis of patients with pancreatic cancer. To evaluate the prognostic value of miR-494 expression in pancreatic cancer, survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Using these analyses, we found that pancreatic cancer patients with low miR-494 expression levels had shorter overall survival than those with high miR-494 expression levels. To determine whether miR-494 is an independent risk factor for poor prognosis, both clinicopathological factors and the level of miR-494 expression were evaluated by performing multivariate Cox regression analysis. Results showed that miR-494 expression level (HR = 2.85, 95%CI: 1.54-8.45, P = 0.019) was an independent factor in predicting the overall survival of pancreatic cancer patients. To the best of our knowledge, this is the first study investigating the clinicopathological and prognostic values of miR-494 in pancreatic cancer.

In conclusion, reduced miR-494 expression level in pancreatic cancer tissues was correlated with tumor progression and might be an independent poor prognostic factor for patients with pancreatic cancer. In the future, a larger sample population is needed to confirm the prognostic value of miR-494 expression in pancreatic cancer.

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