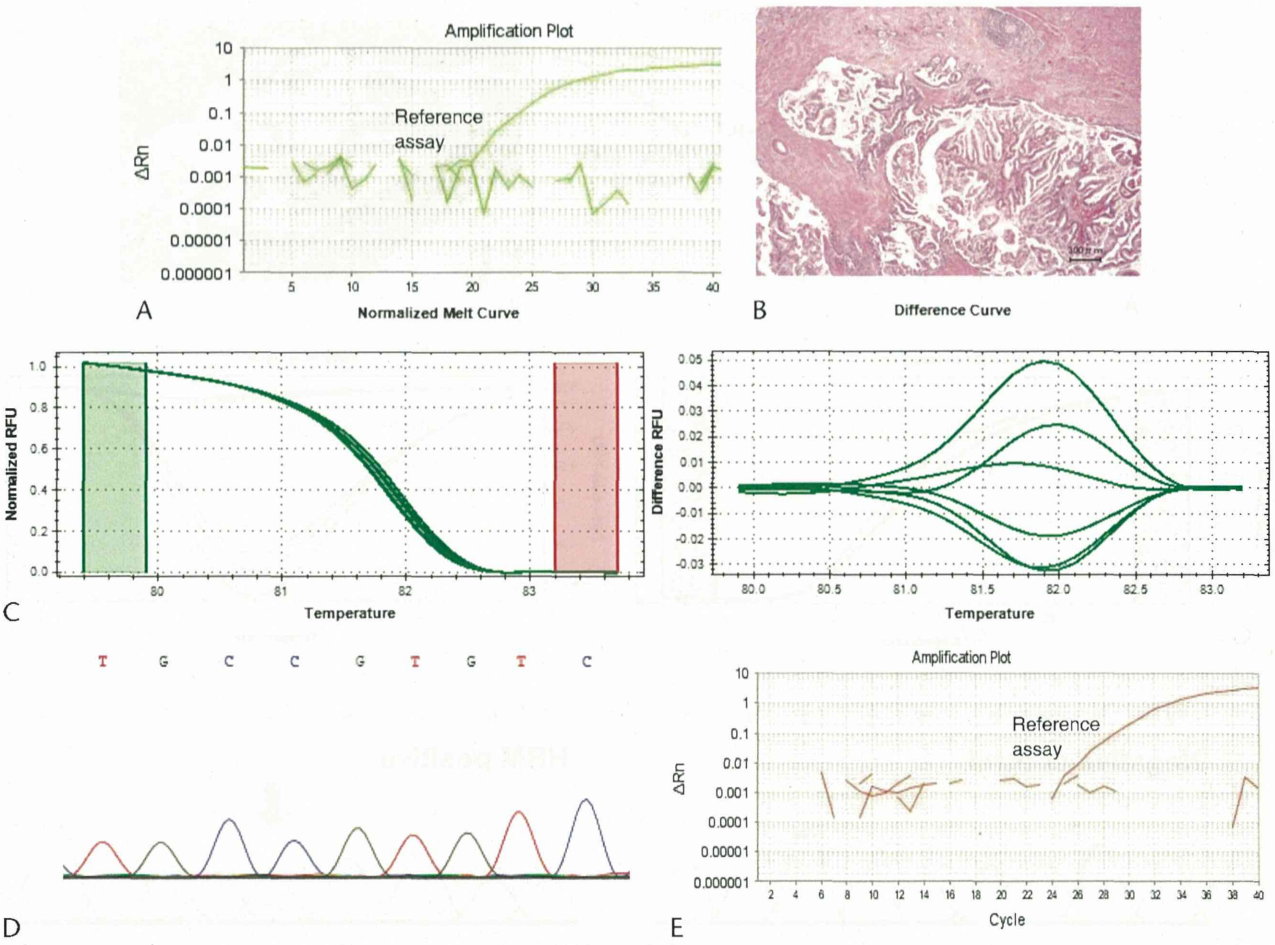

FIGURE 3. Representative case of IPMN with distinct PDAC without *GNAS* codon 201 mutation in DF or resected specimen. Neither *GNAS* R201C nor R201H mutation was detected in DF sample (A). IPMN distinct from PDAC was diagnosed as invasive carcinoma derived from gastric type IPMN (×100) (B). *GNAS* mutation of the IPMN specimen was not detected by any methods including high-resolution melt curve analysis (C), Sanger sequencing (D), and castPCR (E).

were observed in different PanIN lesions microdissected from the same pancreas.[29,38] These findings were inconsistent with the setting of the *KRAS*-mutated mouse model of pancreatic cancer. Taken together, significant overlap between PanIN and IPMN without *GNAS* mutation does really exist; therefore, *GNAS* mutation may become a powerful biologic marker to define certain characteristics of IPMN and PanIN.

Endoscopic DNA tests using DF for the detection of *KRAS*, *GNAS*, and *TP53* mutations in pancreatic neoplasms have been reported.[21–23] Duodenal fluid is a more ideal specimen for the screening of these gene mutations in the whole pancreas than specimens obtained by endoscopic ultrasonography–fine needle aspiration (EUS-FNA) or pancreatic juice specimens obtained during endoscopic retrograde pancreatography (ERP) for the following reasons. One is that DF may contain neoplastic cells derived from the entire pancreatic ductal system, whereas cystic fluid obtained by EUS-FNA contains only neoplastic cells from a limited area. The other reason is that DF can be more easily and safely collected by endoscopy than pancreatic juice obtained by ERP or cystic fluid aspirated by EUS-FNA because ERP and EUS-FNA are associated with the risks for pancreatitis, bleeding, and perforation.

The high consistency of *GNAS* status between genomic DNA extracted from tissue specimens and that extracted from DF suggests that *GNAS* mutation assessment using DF can be clinically applied to discriminate IPMN from other pancreatic cysts. The majority of patients who are proven to have IPMN by *GNAS* mutation detection in DF will be successfully managed according to the guidelines. On the other hand, patients with pancreatic cysts other than IPMN such as mucinous cystic neoplasm, serous cystic neoplasm, and nonneoplastic cyst may be included in those that exhibit *GNAS* wild-type by DF DNA test because *GNAS* mutation is specific to IPMN.[20] We routinely perform EUS, computed tomography, and magnetic resonance imaging for patients with pancreatic cysts at the time of initial assessment, and differential diagnosis of pancreatic cysts can be reliably made in most patients especially based on EUS findings of cyst appearance and multilocularity,[39] and thus, patients diagnosed as having IPMN with *GNAS* wild-type according to DF DNA test may have the indication of pancreatic juice cytology for the early detection of concomitant PDAC in addition to the routine radiographic surveillance of IPMN. Although pancreatic juice cytology during ERP is currently considered to be the only way to detect noninvasive adenocarcinoma concomitant with IPMN as described in our previous article,[15] assessment of secreted pancreatic cancer–associated proteins in DF may have a value similar to that of pancreatic juice cytology for the detection of distinct PDAC. This can be explained by the fact that we

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previously showed that quantification of carcinoembryonic antigen or S100P in DF could discriminate PDAC from benign pancreatic diseases,[25] and this test can be repeatedly performed without worrying about the adverse events. Therefore, combining *GNAS* status assessment with quantification of pancreatic cancer–associated proteins using DF would increase the likelihood of diagnosing concomitant PDAC at earlier stages.

Distinct PDAC was often found in the remnant pancreas more than 10 years after resection of IPMN.[4] Although any patients with IPMN without distinct PDAC did not exhibit the metachronous development of distinct PDAC in their remnant pancreas in this study cohort, the median surveillance period of these patients was only 46 months. Longer follow-up period of these patients is needed to reveal the true prevalence of the development of distinct PDAC after resection of IPMN.

In conclusion, the mutational analyses of resected IPMN in the present study indicate that distinct PDAC frequently develops in the pancreas with gastric type IPMN without *GNAS* mutations. *GNAS* mutation assessment of DF is a safe and easy test for predicting the presence of *GNAS* mutation in IPMN, whereas the detection of gastric subtype using a noninvasive test remains to be determined.

ACKNOWLEDGMENT

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Micro RNA-373 is Down-regulated in Pancreatic Cancer and Inhibits Cancer Cell Invasion

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ABSTRACT

Background. Micro RNAs (miRNAs) are small noncoding RNAs that have gained attention as key molecules in the malignant characteristics of cancers, and several recent investigations also have identified some miRNAs as potential key regulators to inhibit the malignant characteristics of tumors. MiRNA-373 (miR-373) has recently been reported to induce E-cadherin, which is a key regulator of epithelial-mesenchymal transition (EMT). However, the role of miR-373 in the characteristics of cancer cells is not still well known.

Methods. We investigated the expression levels of miR-373 in pancreatic cancer cell lines and its effect on the invasiveness of pancreatic cancer by using in vitro and in vivo models. We also analyzed the expression of miR-373 using formalin-fixed paraffin-embedded ($n = 152$) and microdissected frozen ($n = 57$) samples from pancreatic tissues.

Results. The levels of miR-373 expression were low in pancreatic cancer cell lines. In formalin-fixed paraffin-embedded and microdissected frozen samples, miR-373 expression was significantly down-regulated in pancreatic cancer compared with that in healthy pancreas ($P < 0.001$ and $P = 0.005$, respectively). We also found that reexpression of miR-373 repressed transforming growth factor-β–induced EMT, leading to inhibition of invasiveness of cancer cells. Furthermore, reexpression of miR-373 significantly inhibited peritoneal dissemination in vivo ($P < 0.001$).

Conclusions. MiR-373 is down-regulated in pancreatic cancer, and its reexpression represses the invasiveness of pancreatic cancer cells.

Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries and has the lowest patient survival rate of any solid cancers.[1–3] The cancer death rates of most malignancies have decreased in recent years because of improvements in early detection and treatment; however, the overall 5-year survival of patients with pancreatic cancer has increased only slightly, from 3 to 5 %. This is due to early and aggressive local invasion, metastasis, and dissemination of the pancreatic cancer cells.[1,4] Identifying novel molecules to repress the invasiveness of pancreatic cancer may be helpful to improve the prognosis of patients with pancreatic cancer.

Micro RNAs (miRNAs) have recently gained attention as key molecules in carcinogenesis.[5] MiRNAs are small noncoding RNA gene products of approximately 22 nucleotides that down-regulate gene expression by binding to the 3′ untranslated regions of specific target messenger RNAs (mRNAs), leading to mRNA degradation or inhibition of translation.[6–8] MiRNAs are predicted to regulate as many as 30 % of human transcripts.[9] Some miRNAs are already reported to play a key role as tumor suppressors or oncogenes in human cancer, and we have also reported that miRNA expression is related to cell proliferation, invasiveness, and chemoresistance of pancreatic cancer.[10–17]

Several recent investigations have identified some miRNAs as key regulators of epithelial-mesenchymal transition (EMT).[18–20] EMT is characterized by loss of E-cadherin expression and is related to the invasive

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behavior of pancreatic tumors and other malignancies. Regulating EMT is considered to be important in the improvement of patient prognosis.[21–23] Members of the miR-200 family and miR-205 were down-regulated in cells that had undergone EMT in response to transforming growth factor (TGF)-β, and reexpression of miR-200 family genes prevented TGF-β–induced EMT.[18] These findings suggest that miRNAs may become new therapeutic targets to control EMT in malignant tumors. MiRNA-373 (miR-373) was recently reported to directly induce E-cadherin, which was closely related to the promotion of tumor invasion.[24] However, the role of miR-373 in pancreatic cancer progression is unknown.

In this study, we investigated miR-373 expression in pancreatic cancer and the effect of miR-373 reexpression on pancreatic cancer cells. We found that miR-373 reexpression repressed EMT and inhibited the invasiveness of pancreatic cancer cells. We also found that miR-373 reexpression significantly inhibited peritoneal dissemination in vivo.

METHODS

Cell Culture

Twelve pancreatic cancer cell lines, including NOR-P1, which was established in our laboratory, and three colon cancer cell lines were used. All cell lines were maintained as described previously.[25,26] The details are described in the supplement materials.

RNA Isolation from Microdissected and Formalin-Fixed Paraffin-Embedded Samples

Laser microdissection was performed as described previously.[27–29] After microdissection, total RNA was extracted from selected cells as described previously.[30] Total RNA was extracted by using an RNeasy formalin-fixed paraffin-embedded Kit (Qiagen, Tokyo, Japan) with DNase I treatment according to the manufacturer's instructions.

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction for Analysis of miRNA Expression

Cultured and microdissected cells were analyzed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) by using SuperTaq Polymerase (Ambion) and a mirVana RT-PCR miRNA Detection Kit (Ambion) according to the manufacturer's instructions. All reactions were performed in triplicate. The details are described in the supplement materials.

TABLE 1 Primers

Variable		Primer
E-cadherin	Fw	TCAGCGTGTGTGACTGTGAA
	Re	AGGCTGTGCCTTCCTACAGA
Vimentin	Fw	GCCATCAACCGAGTTGAAG
	Re	CGATGTAGTTGGCGAAGCG
18 s	Fw	GTAACCCGTTGAACCCCATT
	Re	CCATCCAATCGGTAGTAGCG
CD44	Fw	GGGATTGGTTTTCATGGTTG
	Re	CGTACCAGCCATTTGTGTTG

Fw forward, *Re* reverse

TABLE 2 Antibodies

Antibody	Supplier	Concentration	Test
Rabbit anti-E-cadherin (#4605)	Cell Signaling	1:1,000	IF
			WB
Mouse anti-vimentin (V6630)	SIGMA	1:40	IF
		1:200	WB
Goat anti-β-actin (sc-1616)	Santa Cruz	1:1,000	WB
Goat anti-rabbit Alexa 488	MolProbes	1:200	IF
Goat anti-mouse Alexa 546	MolProbes	1:200	IF

IF immunofluorescence, *WB* Western blot

Transfections

Transfections were performed by electroporation with a Nucleofector system (Amaxa Biosystems, Koln, Germany). All studies were done in triplicate. The details are described in the supplement materials.

Cell Proliferation Assay and Invasion Assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide as described previously.[31] The invasiveness of cancer cells was evaluated by counting the number of cells invading a Matrigel-coated transwell as reported previously.[26] The details are described in the supplement materials.

Quantitative Assessment of mRNA Levels by One-Step qRT-PCR

One-step qRT-PCR was performed by using a Quanti-Tect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) with a Chromo4 Real-Time PCR Detection System (Bio-Rad) as described previously.[28] We designed specific primers as described in Table 1.

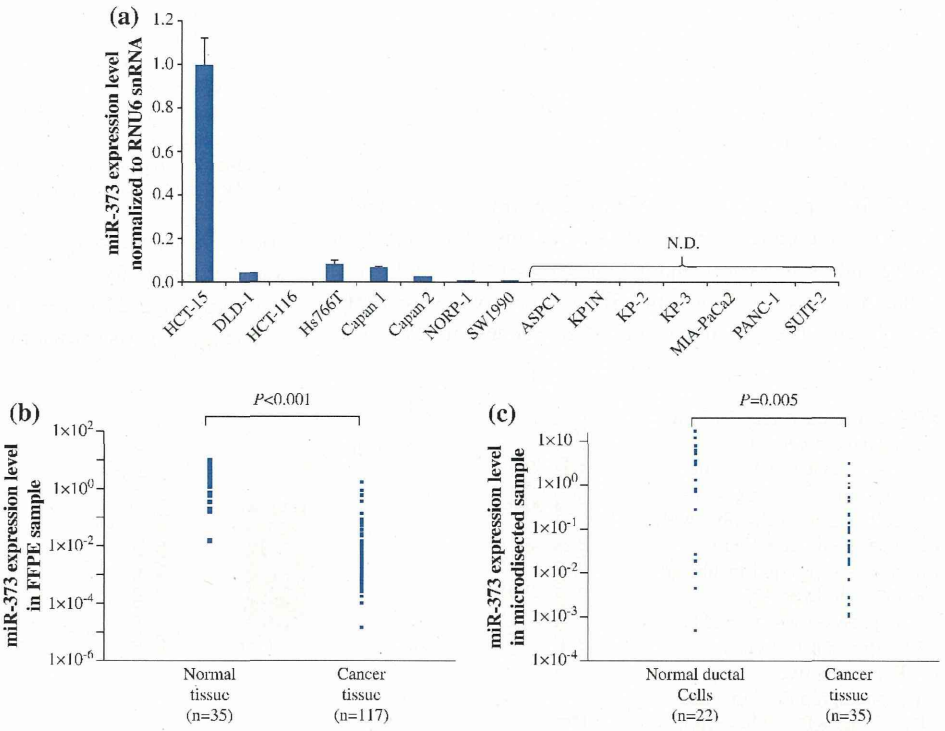


FIG. 1 MiR-373 expression in pancreatic cancer cell lines and tissue samples. **a** MiR-373 expression in 12 pancreatic cancer cell lines and in 3 colon cancer cell lines. The expression of miR-373 was normalized to the level of U6 snRNA. Values are expressed relative to the expression in HCT-15 cells, which was given the arbitrary value of 1.00. The level of miR-373 expression was significantly higher in the HCT-15 cell line compared with other cell lines (more than 20 times compared with Hs766T). Only five pancreatic cancer cell lines expressed miR-373, with the Hs766T cell line showing the highest level. *ND* not detected. **b** Relative expression of miR-373 in formalin-fixed paraffin-embedded (FFPE) samples and **c** in microdissected cells

Western Blot Analysis

Cultured pancreatic cancer cells were lysed in PRO-PREP protein extraction solution (iNtRON Biotech, Kyunggi, South Korea) according to the manufacturer's instructions. The details and antibody used in this study are described in the supplement materials and Table 2.

Immunofluorescence

Immunofluorescence was performed on cells seeded on glass-bottomed dishes (Matsunami, Osaka, Japan). The details are described in the supplement materials.

Statistical Analysis

All calculations were performed with JMP 7.0.1 software (SAS Institute, Cary, NC). Differences in expression levels were analyzed with Student's *t* test. For qRT-PCR data, each sample was analyzed twice or in triplicate. Any sample showing a deviation in value of more than 10 % was tested a third time. Data were analyzed by the Mann–Whitney *U*-test when normal distribution was not obtained. All differences were considered to be statistically significant if the *P* value was <0.05.

RESULTS

MiR-373 Expression in Pancreatic Cancer Cell Lines and Tissue Samples

We investigated the levels of miR-373 expression in cultures of 12 different pancreatic cancer cell lines and in 3 colon cancer cell lines, including HCT-15 cells, which were previously reported to express high levels of endogenous miR-373.[32] As shown in Fig. 1a, miR-373 was expressed at a significantly higher level in HCT-15 cells compared with the other cell lines. Five pancreatic cancer cell lines expressed miR-373, but seven did not. Of the pancreatic cell lines examined here, Hs766T cells expressed the highest level of miR-373, but its expression level was very low compared with that in HCT-15 cells.

Next, we measured miR-373 expression levels in formalin-fixed paraffin-embedded samples derived from 117 cases of invasive ductal carcinoma and from 35 cases of healthy pancreatic tissues. The miR-373 expression level was significantly lower in both healthy and cancer tissues compared with that in HCT-15 cells. Also, the miR-373 expression levels were significantly lower in pancreatic cancer tissues than in healthy pancreatic tissues (Fig. 1b; $P < 0.001$). We also measured miR-373 expression levels in 35 invasive ductal carcinoma cells and in 22 healthy pancreatic ductal epithelial cells by using microdissected samples and found that the expression levels were significantly lower in

invasive ductal carcinoma cells compared with healthy ductal epithelial cells (Fig. 1c; $P = 0.005$).

MiR-373 Reexpression Inhibited the Invasiveness of Pancreatic Cancer Cells

We investigated the role of miR-373 in the invasiveness of pancreatic cancer cells, which is an important aspect of malignant progression and metastasis. SUIT-2 and KP-2 cells, which showed no miR-373 expression, were transfected with the precursor miR-373, seeded in Matrigel-coated inner wells at a density of 2×10^5 cells per well, and cultured for 48–72 h. MiR-373 was significantly overexpressed 24 h after transfection with precursor miR-373 (Fig. 2a). As shown in Fig. 2b, the number of invading

FIG. 3 Morphologic changes of pancreatic caner cell lines after miR-373 precursor transfection. **a** MiR-373 precursor transfection resulted in morphologic change from a fibroblastoid to an epithelial-like appearance. **b** Alteration of E-cadherin, vimentin, and CD44 expression in SUIT-2 and KP-2 cells at each time point from 24 h after transfection of miR-373. **c** Immunoblotting **d** up-regulation of E-cadherin *(green)* and down-regulation of vimentin *(red)* in SUIT-2 and KP-2 cells. *Bar* = 100 μm. *DAPI* 4′,6-diamidino-2-phenylindole ▶

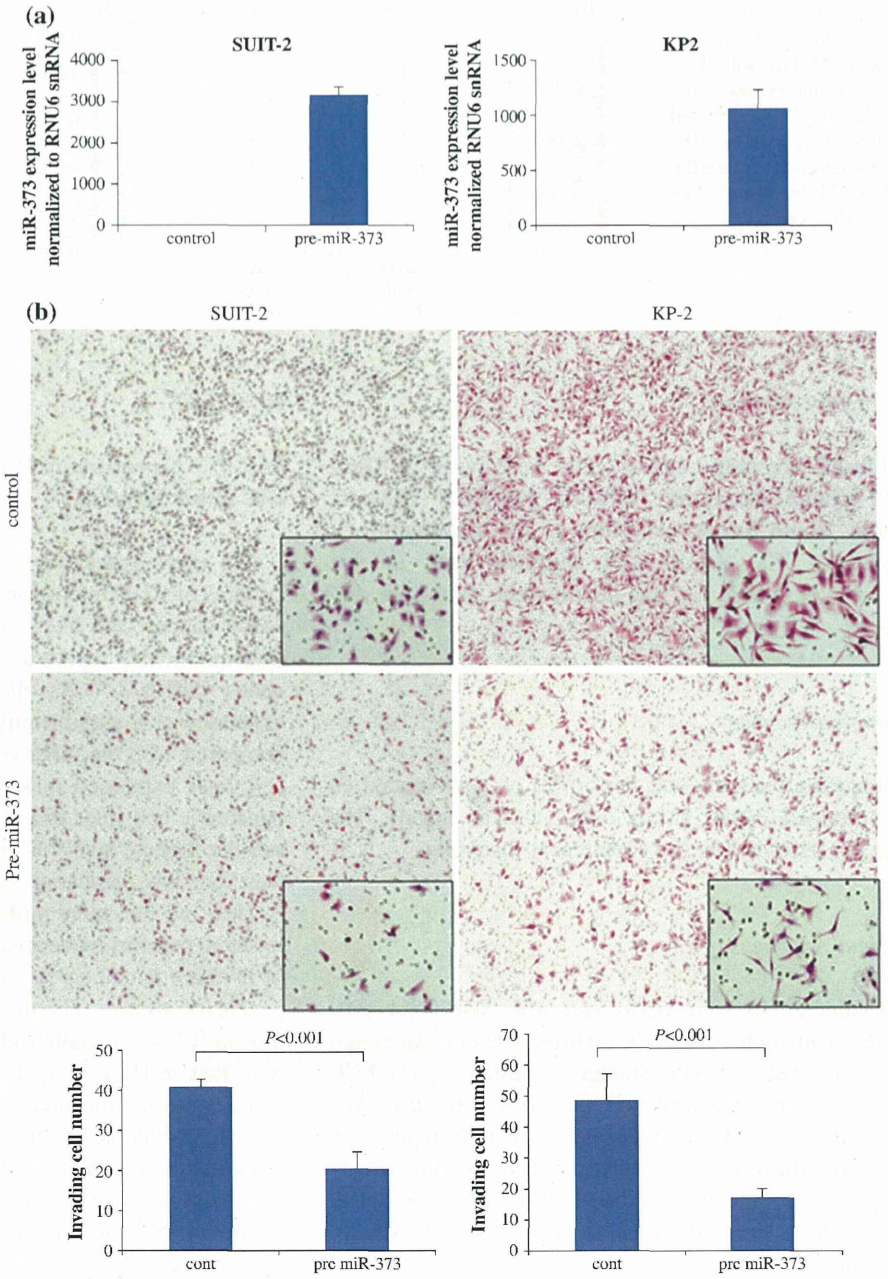
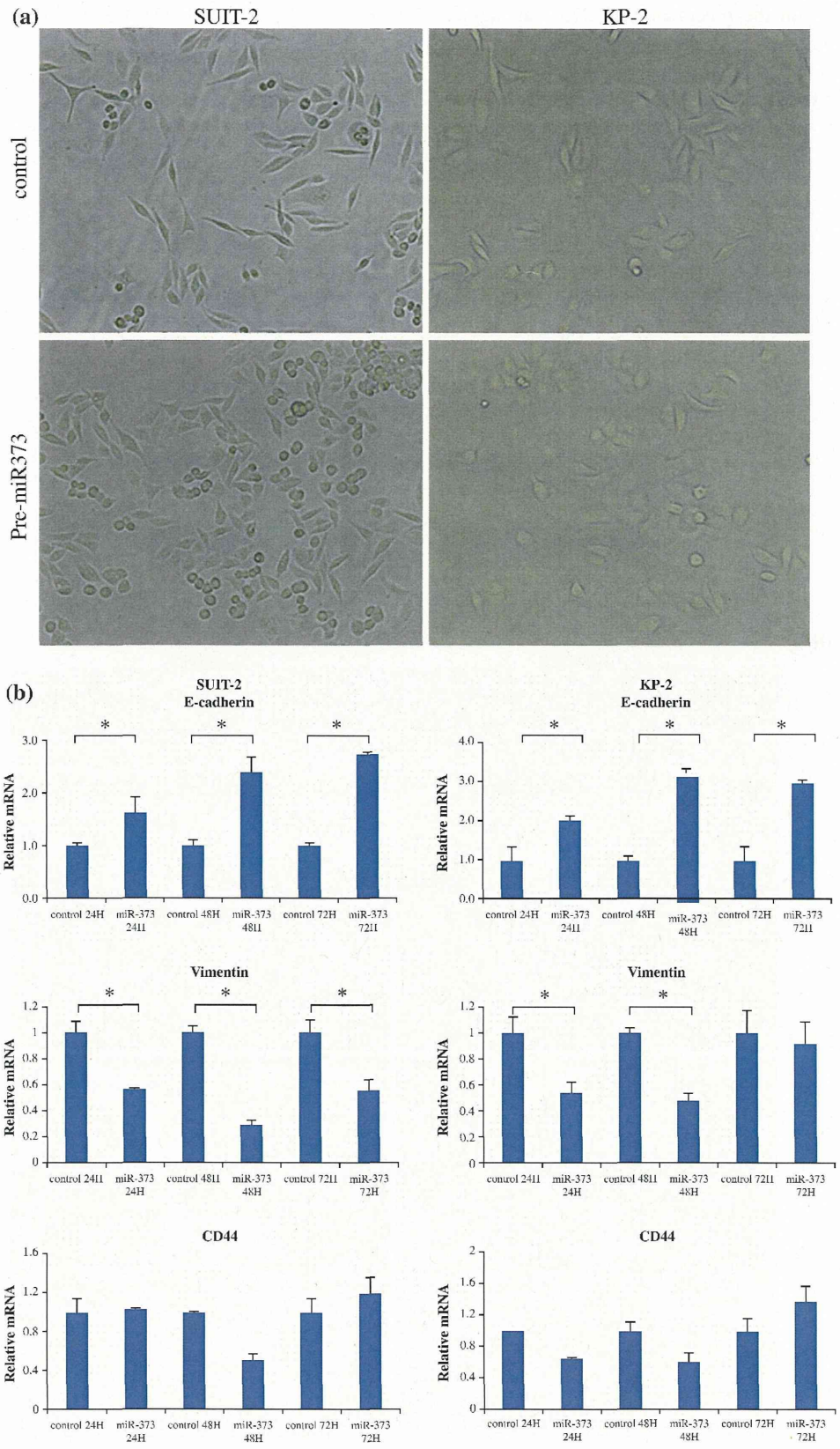


FIG. 2 Effect of miR-373 on the invasive potential of pancreatic cancer cells. After transfection, cells were seeded in a cell migration chamber with the inner well coated with Matrigel (20 μg) and incubated for 48–72 h. MiR-373 overexpression was verified 24 h after transfection (**a**) Representative microphotographs of invaded SUIT-2 and KP-2 cells (original magnification, 40 × ; *right lower inset,* 200 ×) treated with miR-373 precursor or control precursor (**b**) The miR-373 precursor suppressed the invasion of SUIT-2 and KP-2 cells


(a)
SUIT-2
KP-2
control
Pre-miR373
(b)
SUIT-2
E-cadherin
KP-2
E-cadherin
Relative mRNA
control 24H
miR-373 24H
control 48H
miR-373 48H
control 72H
miR-373 72H
Vimentin
CD44

cells transfected with the precursor miR-373 was significantly smaller compared with the number of invading cells transfected with the control precursor in both SUIT-2 and KP-2 cells ($P < 0.001$ and < 0.001, respectively). However, there were no differences in proliferation between

FIG. 4 **a** TGF-β1–induced EMT in pancreatic cancer cells. **b** MiR-373 repressed TGF-β1–induced EMT in pancreatic cancer cells; mRNA *(upper panel)* and immunoblotting *(lower panel)*. **c** Phase contrast microscopy *(left panel)* and immunofluorescence staining *(right panel)* of SUIT-2 cells. E-cadherin *(green)*, vimentin *(red)*, and 4′,6-diamidino-2-phenylindole (DAPI; *blue*). *Bar* = 50 μm ▶

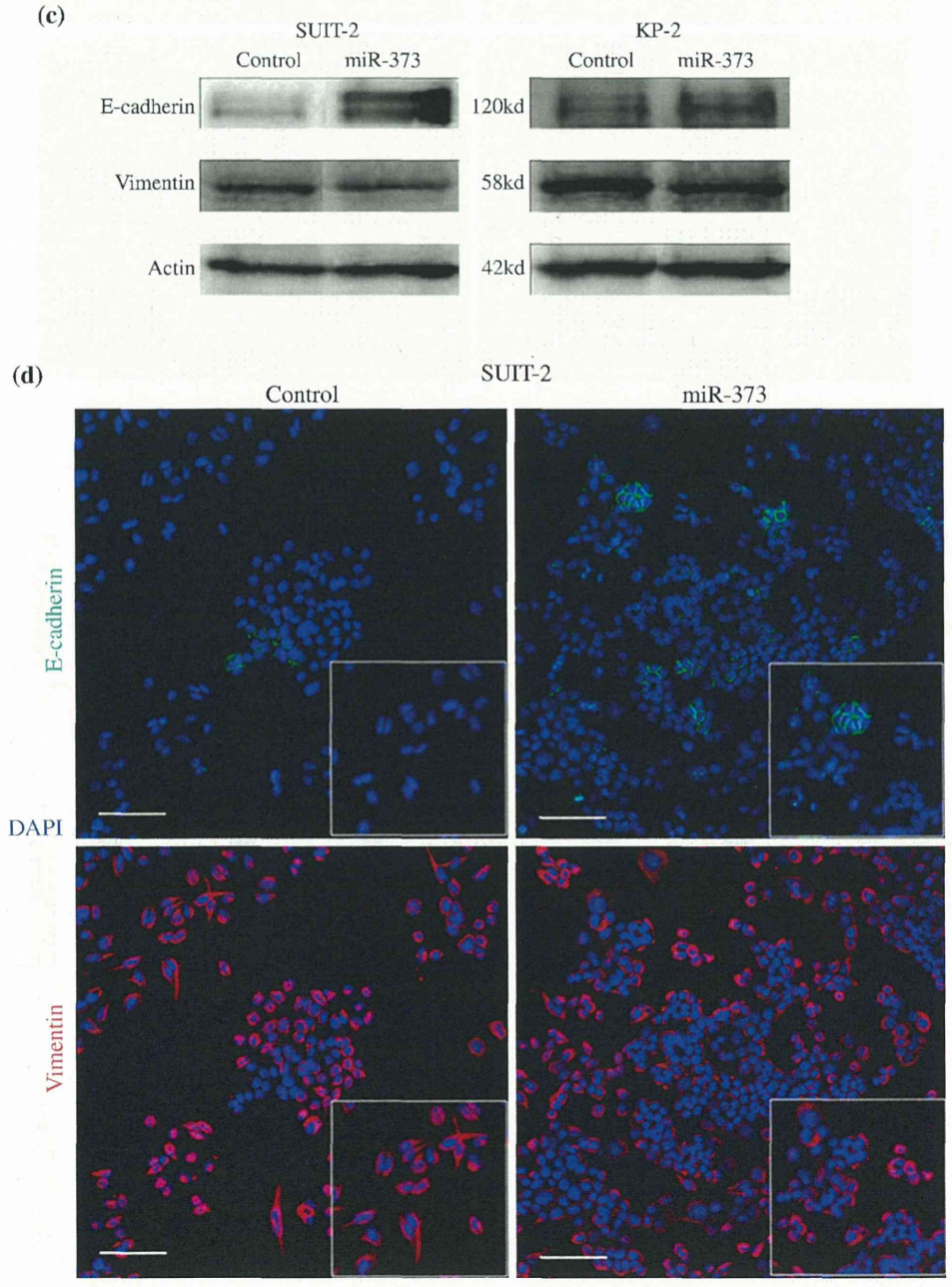
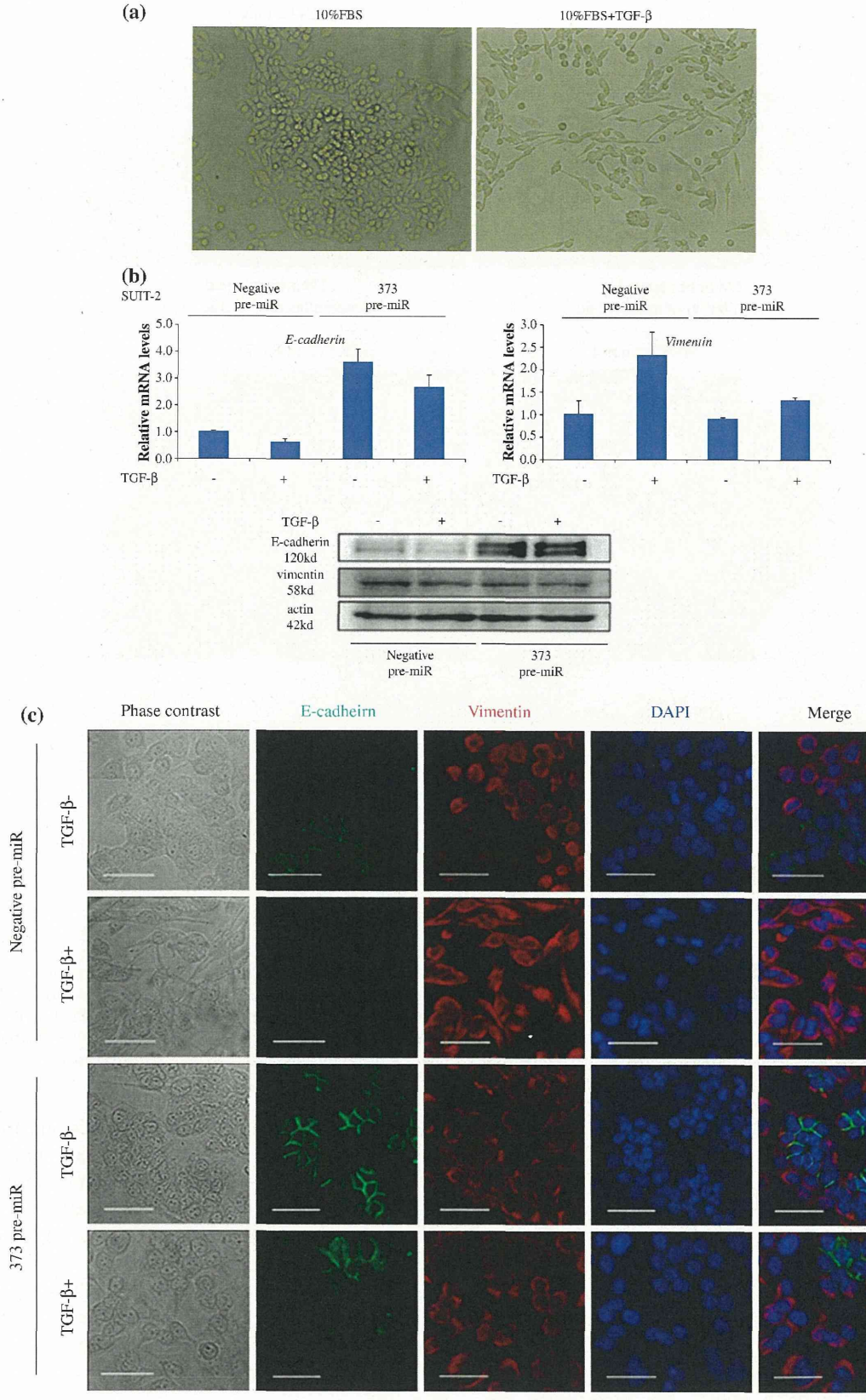


FIG. 3 continued


(a)
10%FBS
10%FBS+TGF-β
(b)
SUIT-2
Negative pre-miR
373 pre-miR
E-cadherin
Relative mRNA levels
5.0
4.0
3.0
2.0
1.0
0.0
TGF-β - + - +
Negative pre-miR
373 pre-miR
Vimentin
Relative mRNA levels
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TGF-β - + - +
TGF-β - + - +
E-cadherin 120kd
vimentin 58kd
actin 42kd
Negative pre-miR
373 pre-miR
(c)
Phase contrast
E-cadheirn
Vimentin
DAPI
Merge
Negative pre-miR
TGF-β-
TGF-β+
373 pre-miR
TGF-β-
TGF-β+