Alteration of epithelial-to-mesenchymal transition gene expression in periostin-treated cholangiocarcinoma

***Suchada Siritunyong*[1,2] *Jumaporn Sonongbua*[1,2] *Peti Thuwajit*[2] *and Chanitra Thuwajit*[2]*

[1]*Graduate Program in Immunology,* [2]*Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700*

**Corresponding author: cthuwajit@yahoo.com; chanitra.thu@mahidol.ac.th*

ABSTRACT

Periostin (PN) is increased in cholangiocarcinoma (CCA) tissues and relates to patient poor prognosis. Epithelial-to-mesenchymal transition (EMT) is a process characterized by changes of carcinoma to mesenchymal cell and essential for cancer progression. This study aims to investigate the expression of EMT-related genes in PN-induced CCA cell migration. Nine EMT genes including two matrix metalloproteinase genes (*MMP-10*, *MMP-13*), two mesenchymal genes (*ASMA, VIM*), two epithelial genes (*CK19, CDH1*), and three EMT-related transcription factors (*SNAIL1, SLUG, TWIST2*) were explored in terms of their expression levels in cells with and without PN treatment by real time PCR. The results showed up-regulations (>1.2 fold) of *MMP-10, SNAIL1, TWIST2, ASMA,* and *VIM*, and a significant up-regulation of *MMP-13* whereas *CK19* was significantly down-regulated (≤0.8 fold) in PN-treated CCA cells. No alterations of *SLUG and CDH1* were observed. The different results in PN- and transforming growth factor-β-treated CCA cells reveal specific EMT gene expression pattern depending on the stimulant. Understanding of EMT genes in PN-induced CCA cells might help in inhibiting cancer progression.

Keywords: epithelial-to-mesenchymal transition, periostin, cholangiocarcinoma

INTRODUCTION

Our group has recently revealed that periostin (PN), a secreted protein, is solely produced from cholangiocarcinoma (CCA)-associated fibroblasts and has significant association with patient short survival time (Utispan *et al*., 2010). PN induces proliferation, migration, and invasion of CCA cells *in vitro* (Utispan *et al*., 2010, Uitspan *et al*., 2012).

Epithelial-mesenchymal transition (EMT) is a phenomenon that cancer cells become mesenchymal-like cells, which facilitates cancer progression (Thiery, 2002). The characteristic of EMT is the increase in expression of mesenchymal markers and the reduction of epithelial markers mediated via certain EMT-related transcription factors.

Several lines of evidence exhibited the different patterns of EMT-related genes depending on both cancer cell types and stimulants (Yan and Shao, 2006; Kim *et al*., 2011; Techasen *et al*., 2012). However, there have been no reports about EMT gene expression pattern in PN-treated CCA cells.

This study aims to measure the expression levels of EMT-related genes including *MMPs*, *ASMA, VIM*, *CK19, CDH1*, *SNAIL1, SLUG,* and *TWIST2* in PN-treated CCA cells. The ultimate goal is to understand PN-mediated EMT in CCA and to propose an alternative way to inhibit fibroblast-driven disease progression.

MATERIALS AND METHODS

Cholangiocarcinoma cell culture

Human CCA cell line, KKU-M213 was cultured in Ham-F12 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, antibiotic and anti-fungal agent at 37°C in humidified 5% CO_2 incubator.

Migration induction assay (Wound healing assay)

KKU-M213 cells were seeded into 6-well culture plate until reaching 90% confluence. Wound was made by a sterile pipette tip. The scratched cells were treated with medium with or without 100 ng/ml recombinant PN. After 24 h treatment, cells were photographed for determination of the migration efficiency which is calculated by the following formula:

$$\% \text{ wound healing} = \frac{\text{wound space at 0 h - wound space at 24 h}}{\text{wound space at 0 h}} \times 100$$

EMT gene expression measurement: real time RT-PCR

Total RNA extraction and cDNA synthesis

Total RNA were extracted from three millions cells of KKU-M213 by PerfectPure RNA (5 PRIME) following the manufacturer's instructions. The amount and quality of total RNA were measured by spectrophotometer. Five micrograms of total RNA were converted to cDNA by M-MLV reverse transcriptase using SuperScript™III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions. The cDNA was kept at -20°C until use.

Real time PCR

Expression levels of EMT-related genes were determined by SYBR-Green-based real time PCR in Light Cycler®480 II machine (Roche Applied Sciences). The condition of real time PCR for *MMP-10, MMP-13, SNAIL1, SLUG, ASMA, VIM, CDH1,* and *CK19* was 95°C for 10 min followed by 95°C for 15 s and 60°C (58°C for *TWIST2*) for 45 s for 50 cycles. *ACTB* was used as an internal control. The expression level of each gene in PN-treated CCA cells was calculated by $2^{-\Delta\Delta_{CP}}$.

Table 1 List of primers used in this study (F = forward, R = reverse)

Gene	Primer	Sequence(5'->3')	Tm (^{O}C)	Size (bp)
SNAIL1	F	TCTGAGGCCAAGGATCTCCAGGC	59.70	243
NM_005985	R	CAGGTTGGAGCGGTCAGCGAA	59.30	
SLUG	F	AATATGTGAGCCTGGGCGCCCT	60.30	163
NM_003068	R	GCTCTGTTGCAGTGAGGGCAAGAA	59.60	
TWIST2	F	GCAAGAAGTCGAGCGAAGAT	52.71	221
NM_057179	R	CAGCTTGAGCGTCTGGATCT	54.05	
ASMA	F	AGGAAGCAGCTCTATGCTAACAAT	59.30	379
NM_001613	R	AACACATAGGTAACGAGTCAGAGC	61.00	
VIM	F	CAGGTGGGACCAGCTAACCAA	59.60	152
NM_003380	R	TGCCAGACGCATTGTCA	58.20	
CDH1	F	GCCTGGGACTCCACCTACA	54.60	147
NM_004360	R	TCTGAGGCCAGGAGAGGAG	53.70	
CK19	F	AGCTAGAGGTGAAGATCCGCGAC	64.20	155
NM_002276	R	GGCATTGTCGATCTGCAGGACAA	62.40	
MMP-10	F	TGGCCCTCTCTTCCATCATA	57.30	95
NM_002425	R	CTGATGGCCCAGAACTCATT	57.30	
MMP-13	F	GCAGCTGTTCACTTTGAGGA	57.30	136
NM_002427	R	CACCAATTCCTGGGAAGTCT	57.30	
ACTB	F	CACACTGTGCCCATCTACGA	53.90	162
NM_001101	R	CTCCTTAATGTCACGCACGA	52.30	

RESULTS AND DISCUSSION

PN-induced cell migration and MMP expressions in CCA cells

PN (100 ng/ml) could significantly induce CCA cell migration (P=0.01) (Fig. 1A) together with the increased expression levels of *MMP-10* (P=0.104) and *MMP-13* (P <0.01) (Fig. 1B). This may indicate the potential of PN to induce CCA cell migration and then the production of MMPs facilitates cells to invade.

PN-induced alterations EMT-related gene expressions

The down-regulation of *CK19* was the hallmark of EMT. The results confirmed that PN induced EMT of CCA cells leads to a significant down-regulation of *CK19* (P=0.038) (Fig. 2C). The up-regulations of *ASMA* and *VIM* were observed in PN-treated CCA cells, which were similar to TGF-β treatment (Fig. 2B). Both PN and TGF-β activated *SNAIL1* EMT-related transcription factor while only PN induced *TWIST2* (Fig. 2A). Though *CDH1* was not down-regulated as expected, the aberrant translocalization from the cytoplasm to the nucleus will be further investigated. Our study confirms that the decreased *CK19* and the increased *ASMA*, *VIM* and *SNAIL1* are specific

EMT expression alteration pattern of CCA cells activated by TGF-β, whereas the alteration patterns of *CK19*, *ASMA*, *VIM*, *SNAIL1* and *TWIST2* are for PN-treated CCA cells.

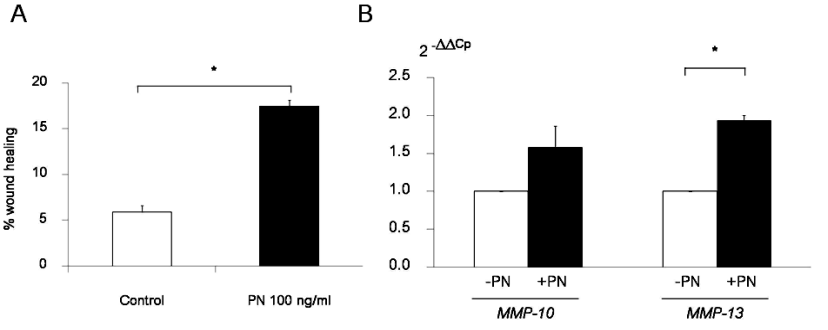

Figure 1 PN-induced cell migration and *MMPs* expression. (A) Wound healing assay. Bars represent mean ± SD of two independent measurements. (B) PN-induced *MMP-10* and *MMP-13* expressions in PN-treated CCA cells measured by real time PCR. *ACTB* was used as an internal control. Bars represent mean ± SD of triplicate experiments. *P-value <0.05.

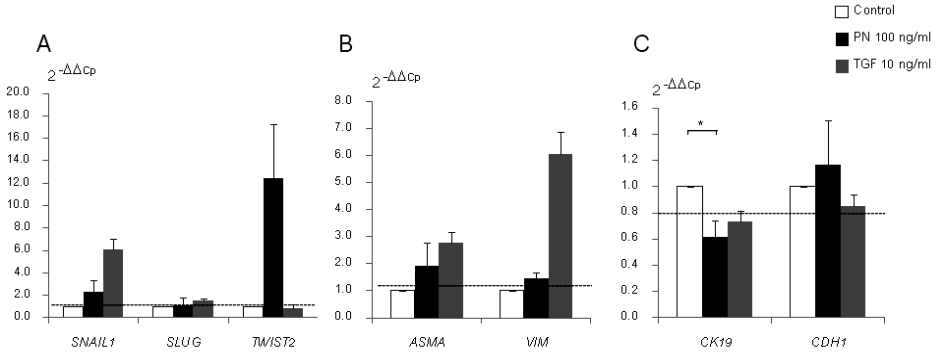

Figure 2 PN-induced EMT-related gene expression. Relative gene expression of >1.2 compared to control was set as up-regulation whereas ≤0.8 was set as down-regulation. Bars represent mean ± SD of three independent experiments. *P-value <0.05.

CONCLUSION

The alterations of EMT gene expressions in PN-treated CCA cells help cells to migrate (increased *ASMA, VIM* and decreased *CK19*) and invade (increased *MMPs*) via either *SNAIL1* or *TWIST2* regulator. This may in part explain PN-induced CCA progression.

ACKNOWLEDGEMENTS

KKU-M213 is from Assoc. Prof. Banchob Sripa, Khon Kaen University and

TGF-β is from Associate Professor Tuangporn Suthiphongchai, Mahidol University. This work is a part of thesis entitled "The expression pattern of epithelial-mesenchymal transition markers in periostin-induced cholangiocarci noma".

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