

Impact of Snail and E-cadherin expression in pancreatic neuroendocrine tumors

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Abstract. Pancreatic neuroendocrine tumors (PNETs) are a rare type of malignancy with a prognosis that is relatively good, compared with that of pancreatic adenocarcinoma. However, a number of patients with PNETs have distant metastasis and a less favorable prognosis. Epithelial-mesenchymal transition (EMT) is essential for tumor progression and metastasis. Snail and E-cadherin serve key roles in the process of EMT in numerous tumor types, including gastric and pancreatic adenocarcinoma. However, the role of EMT in PNETs remains unclear. The aim of the present study was to investigate the significance of Snail and E-cadherin expression in PNETs. Tissue samples were obtained from 40 resected PNETs. The expression of Snail and E-cadherin was evaluated in the primary tumors using immunohistochemical staining. The association between protein expression and patient clinicopathological features was then analyzed. High and low Snail expression levels were observed in 11 (27.5%) and 29 (72.5%) patients, respectively. Preserved and reduced E-cadherin expression was observed in 19 (47.5%) and 21 (52.5%) patients, respectively. Patients with low Snail expression and preserved E-cadherin expression had a significantly lower risk of vascular invasion, lymphatic invasion, lymph node metastasis and liver metastasis and a lower WHO classification, as compared with the group that included patients with high Snail and reduced E-cadherin, high Snail and preserved E-cadherin, and low Snail and reduced E-cadherin expression. In addition, the patients with low Snail expression levels and preserved E-cadherin expression had more a favorable prognosis compared with the other group. The present study indicates that EMT serves an important role in tumor

progression in PNETs. Immunohistochemical evaluation of Snail and E-cadherin is useful for predicting the risk of vessel invasion and metastasis in PNETs.

Introduction

Pancreatic neuroendocrine tumors (PNETs) are a rare type of malignancy with an annual incidence of 1/100,000 people, and account for ~1-2% of all pancreatic tumors (1). PNETs are divided into functional tumors, which cause specific hormonal syndromes such as hypoglycemic attack, and non-functional tumors (2). The prognosis of PNETs is relatively favorable compared with that for pancreatic adenocarcinoma; however, certain patients with PNETs exhibit distant metastasis and have a less favorable prognosis (3). In 2010, the World Health Organization (WHO) categorized PNETs into G1 and G2 PNETs and neuroendocrine carcinoma (NEC), based on mitotic counts and the Ki-67 index (4). These classifications are useful for predicting prognosis and postoperative recurrence (5,6). However, certain patients with low-risk pathologic features may unexpectedly experience distant metastasis and postsurgical recurrence (5). Little is currently known about the underlying molecular mechanisms by which distant metastasis is promoted in patients with PNETs.

Invasion into the surrounding normal tissue is a critical step for primary tumors to metastasize to distant areas (7). Recent studies have reported that the epithelial-mesenchymal transition (EMT) serves an important role in tumor progression and metastasis (8). Through EMT, cancer cells acquire the ability to migrate and infiltrate through the extracellular matrix, which leads to distant metastasis (7). At the molecular level, the transcriptional reprogramming of EMT is triggered by the transcription factors Snail, Slug and Twist, leading to subsequent suppression of specific adhesion molecules, including E-cadherin (9). E-cadherin is expressed in epithelial cells and performs an essential role in cell-cell contact (10). The expression of E-cadherin is decreased during EMT in embryonic development, tissue fibrosis and cancer (10). Snail was initially identified in *Drosophila* as the zinc-finger transcriptional repressor that binds to the E-boxes of the E-cadherin promoter (11,12). Snail is able to effectively induce EMT via suppressing the transcription of E-cadherin during tumor progression (13). The loss of E-cadherin expression

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promotes Wnt signaling, and is associated with high levels of Snail in the nucleus (14). It was reported that high expression levels of Snail and low expression levels of E-cadherin are inversely correlated with the prognosis of patients with breast cancer (14). With the aim of gaining insight into the underlying molecular alterations in metastatic PNETs, the present study focused on EMT by evaluating Snail and E-cadherin expression. The significance of Snail and E-cadherin expression patterns in distant metastasis and prognosis was investigated in patients who had received surgery for PNETs.

Materials and methods

Patients and tumor samples. Formalin-fixed paraffin-embedded blocks were obtained, containing tissue samples from 40 patients with histologically proven PNETs and who underwent surgical resection at Kagoshima University Hospital (Kagoshima, Japan) between January 1995 and December 2015. All resected tissue specimens were histologically examined using hematoxylin and eosin staining according to the tumor-node-metastasis classification and WHO classification 2010 systems (4,15). The present study was approved by the Institutional Ethics Review Board of Kagoshima University Hospital, and written informed consent was obtained from all patients.

Antibodies. The rabbit anti-human polyclonal antibody directed against Snail (catalogue no. ab85936; Abcam, Cambridge, UK) was diluted at 1:500. The mouse anti-human monoclonal antibody against E-cadherin (catalogue no. M361229-2; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was diluted at 1:500.

Immunohistochemical staining. All specimens were fixed in 10% formalin at room temperature and processed routinely. Sections (3 μ m-thick) were cut from the paraffin blocks of primary tumors. Following deparaffinization in xylene and rehydration in graded solutions of ethanol, endogenous peroxidase activity was blocked by immersing the slides in absolute methanol solution containing 3% hydrogen peroxide for 30 min at room temperature. The sections were then treated with 1% goat serum albumin for Snail (catalogue no. S-1000) or 1% horse serum albumin for E-cadherin (catalogue no. S-2000) (both Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature to block nonspecific reactions. Heat-induced antigen retrieval via autoclave pretreatment (120°C for 5 min) in citrate buffer solution (pH 6.0) was performed. The sections were rinsed with PBS and incubated with the Snail antibody (dilution, 1:500) for 180 min and the E-cadherin antibody (dilution, 1:500) for 60 min at room temperature. Following incubation, the specimens were visualized using an Vectastain Elite ABC IgG Rabbit (catalogue no. PK-6101) or Mouse (catalogue no. PK-6102) kits (both Vector Laboratories, Inc.) and the Liquid DAB+ Substrate Chromogen system (catalogue no. K3468; Dako; Agilent Technologies, Inc.), according to the manufacturer's protocol. The slides were counterstained with hematoxylin prior to mounting. All reactions were performed with positive controls (mouse heart tissue for Snail and normal pancreatic tissue for E-cadherin). For the negative control, the primary antibody was replaced with PBS. No significant staining was observed in the negative-control sections.

Table I. Clinicopathological parameters of pancreatic neuroendocrine tumors (n=40).

Characteristics	n (%)
Age, years	
>60	17 (42.5)
≤60	23 (57.5)
Gender	
Male	17 (42.5)
Female	23 (57.5)
Tumor size	
>20 mm	13 (32.5)
≤20 mm	27 (67.5)
Lymph node metastasis	
No	33 (82.5)
Yes	7 (17.5)
Liver metastasis	
No	31 (77.5)
Yes	9 (22.5)
WHO classification	
G1	22 (55.0)
G2	15 (37.5)
NEC	3 (7.5)
Vascular invasion	
No	24 (60.0)
Yes	16 (40.0)
Lymphatic invasion	
No	28 (70.0)
Yes	12 (30.0)
Functionality	
Functioning	23 (57.5)
Insulinoma	15 (37.5)
Glucagonoma	4 (10.0)
Gastrinoma	4 (10.0)
Nonfunctioning	17 (42.5)

WHO, World Health Organization; NEC, neuroendocrine carcinoma.

Evaluation of immunohistochemistry. All tissue sections were simultaneously assessed by two investigators who were blinded to the patient clinicopathological data. Each slice was observed under an Olympus CX31 optical microscope (Olympus Corporation, Tokyo, Japan) (magnification, x400). Stained cells were assessed and quantified in five randomly selected fields. The immunohistochemical staining of Snail was classified as high expression if ≥10% of neoplastic cells exhibited nuclear staining, or as low expression if <10% of neoplastic cells were stained. The expression of E-cadherin was compared between tumor cells and normal islet cells located adjacent to the tumor. Tumor cells that exhibited equivocal staining to normal islet cells were considered to have preserved expression of E-cadherin, whereas those that exhibited less intense staining patterns compared with the normal islet cells, or those that did

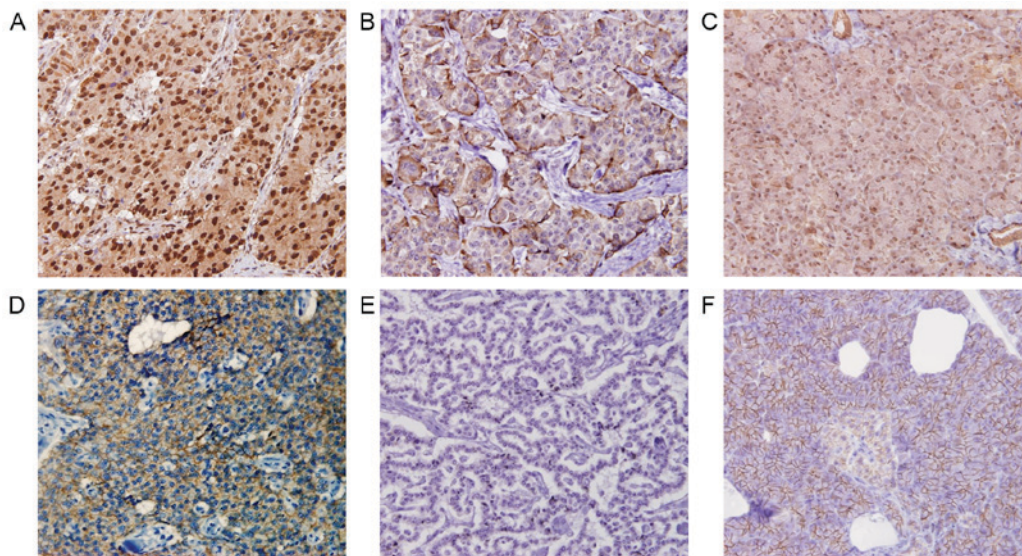


Figure 1. Representative images of (A-C) immunohistochemical Snail and (D-F) E-cadherin expression in pancreatic neuroendocrine tumors and normal pancreatic tissues. (A) High levels of nuclear staining of Snail in pancreatic neuroendocrine tumor. (B) Low levels of nuclear staining of Snail in pancreatic neuroendocrine tumor. (C) Snail expression in normal pancreatic tissue. (D) Preserved levels of membrane staining of E-cadherin in pancreatic neuroendocrine tumor. (E) Reduced levels of membrane staining of E-cadherin in pancreatic neuroendocrine tumor. (F) E-cadherin expression in normal pancreatic tissue. All images were captured at magnification, x400.

not stain at all, were considered to have reduced expression of E-cadherin.

Statistical analysis. Associations between different categorical variables were assessed using Fisher's exact test and the χ^2 test. The Kaplan-Meier method was used for survival analysis, and variations in survival rates were estimated using the log-rank test. All statistical analysis was performed using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA). All tests were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. Table I lists the clinicopathological features of the patients enrolled in the present study. The study group comprised 17 men and 23 women with an age range between 18 and 79 years (mean, 56.1 years). Lymph node metastasis was observed in 7 patients (17.5%). Distant metastasis was observed in 9 patients (22.5%), and the site of metastasis was the liver. Postoperative recurrence or an increase in the residual tumor size was observed in 10 (25.0%) patients.

Expression of Snail and E-cadherin in PNETs. Fig. 1 depicts representative images of Snail and E-cadherin expression. Snail expression was identified in the nucleus of neoplastic cells. The proportion of immunoreactive cells varied, ranging from 0 to $>50\%$ of neoplastic cells. The expression of E-cadherin in neoplastic cells was heterogeneous, ranging from 0 to $\sim 100\%$ of neoplastic cells. High and low Snail expression levels were observed in 11 (27.5%) and 29 (72.5%) patients, respectively. Preserved and reduced E-cadherin expression was observed in 19 (47.5%) and 21 (52.5%) patients, respectively.

Association between Snail and E-cadherin expression and clinicopathological features. Table II demonstrates the association between Snail/E-cadherin expression and various clinicopathological factors. High Snail expression levels were significantly associated with high incidences of lymphatic and vascular invasion and liver metastasis ($P=0.0078$, $P<0.0001$ and $P=0.0067$, respectively; Table II). Reduced E-cadherin expression levels were also significantly associated with high incidences of lymphatic and vascular invasion and liver metastasis ($P=0.0015$, $P=0.0270$ and $P=0.0214$, respectively; Table II). No significant difference was observed in Snail and E-cadherin expression according to gender, tumor size and tumor functionality. Furthermore, on the basis of the expression profiles of Snail and E-cadherin, all patients were divided into two groups as follows: Low Snail and preserved E-cadherin expression, and the 'other group' (patients with high Snail and reduced E-cadherin, high Snail and preserved E-cadherin, and low Snail and reduced E-cadherin expression) (Table III). Patients in the 'other group' experienced a markedly increased lymph node metastasis, liver metastasis, G2 and NEC in the WHO classification, vascular invasion and lymphatic invasion, as compared with in the low Snail and preserved E-cadherin expression group. Patients with low Snail and preserved E-cadherin expression were not observed to exhibit lymph node metastasis or liver metastasis during the present study. In total, 1/22 patients with a G1 classified tumor experience liver metastasis; these particular tumor tissues exhibited high Snail and reduced E-cadherin expression levels.

Prognostic impact of Snail and E-cadherin expression. The 5-year and 10-year survival rates for the patient cohort were determined to be 91.7 and 59.5%, respectively. Fig. 2 presents the overall survival (OS) and progression-free survival (PFS) rates following surgery in the aforementioned two groups, based on the expression levels of Snail and E-cadherin. The

Table II. Association between the expression of Snail/E-cadherin and clinicopathological factors in pancreatic neuroendocrine tumors.

Characteristics	Total, n	Snail expression, n		P-value	E-cadherin expression, n		P-value
		Low	High		Reduced	Preserved	
Total	40	29	11		21	19	
Age, years (mean \pm SD)		56.0 \pm 3.20	56.4 \pm 4.46	NS	59.4 \pm 3.62	52.5 \pm 3.63	NS
Gender				NS			NS
Male	17	11	6		9	8	
Female	23	18	5		12	11	
Tumor size				NS			NS
>20 mm	13	9	4		9	4	
\leq 20 mm	27	20	7		12	15	
Lymph node metastasis							NS
No	33	26	7		15	18	
Yes	7	3	4	NS	6	1	
Liver metastasis				0.0067			0.0214
No	31	26	5		13	18	
Yes	9	3	6		8	1	
WHO classification				NS			NS
G1	22	18	4		9	13	
G2 + NEC	18	11	7		12	6	
Vascular invasion				<0.0001			0.0270
No	24	23	1		9	15	
Yes	16	6	10		12	4	
Lymphatic invasion				0.0078			0.0015
No	28	24	4		10	18	
Yes	12	5	7		11	1	
Functionality				NS			NS
Functioning	23	18	5		12	11	
Nonfunctioning	17	11	6		9	8	

NS, not significant; WHO, World Health Organization; NEC, neuroendocrine carcinoma.

‘other group’ exhibited a poorer OS rate, as compared with the low Snail and preserved E-cadherin expression group, although these differences were not statistically significant (Fig. 2A). By contrast, a significant difference was observed between the two groups with regard to the rate of PFS ($P=0.0099$; Fig. 2B).

Discussion

The present study demonstrated that the analysis of Snail and E-cadherin co-expression may be useful for predicting vascular invasion, lymphatic invasion and liver metastasis in patients with PNETs. The PNET tissue samples in the present study were divided into the following two groups: Tumors with low Snail and preserved E-cadherin expression, and the other tumors which consisted of any tumor tissues with high Snail and reduced E-cadherin, high Snail and preserved E-cadherin, and low Snail and reduced E-cadherin expression. Tumors in the low Snail and preserved E-cadherin expression group and in the ‘other group’ were considered to have

epithelial and mesenchymal phenotypes, respectively. PNETs with a mesenchymal phenotype exhibited a significantly higher frequency of vascular and lymphatic invasion, lymph node metastasis and liver metastasis. Even in G2 tumors and NEC, those with low Snail and preserved E-cadherin expression did not have lymph node and liver metastasis. In PNETs, liver metastasis is the most frequent mode of recurrence and a significant adverse prognostic factor (16,17). Our study indicated that EMT serves an important role in lymphatic invasion, vascular invasion, lymph node metastasis and liver metastasis in PNETs. Immunohistochemical evaluations of the EMT markers Snail and E-cadherin were useful for predicting metastasis.

It has been reported that EMT performs a fundamental role in tumor invasion and metastasis in various types of cancer. In pancreatic adenocarcinoma, numerous previous studies have revealed that Snail, zinc finger E-box-binding homeobox (ZEB) 1, ZEB2 and nectins are associated with EMT and with poor prognosis (18-20). However, the role of EMT in

Table III. Association between the expression of Snail and E-cadherin and clinicopathological factors in pancreatic neuroendocrine tumors.

Characteristic	Snail and E-cadherin expression, n			P-value
	Total	Snail low +E-cadherin preserved	Other group ^a	
Total	40	16	24	
Age, years (mean \pm SD)		51.8 \pm 4.23	59.0 \pm 3.22	NS
Gender				NS
Male	17	6	11	
Female	23	10	13	
Tumor size				NS
>20 mm	13	4	9	
\leq 20 mm	27	12	15	
Lymph node metastasis				0.0295
No	33	16	17	
Yes	7	0	7	
Liver metastasis				0.0060
No	31	16	15	
Yes	9	0	9	
WHO classification				0.0349
G1	22	10	12	
G2 + NEC	18	6	12	
Vascular invasion				0.0007
No	24	15	9	
Yes	16	1	15	
Lymphatic invasion				0.0009
No	28	16	12	
Yes	12	0	12	
Functionality				NS
Functioning	23	10	13	
Nonfunctioning	17	6	11	

^a‘Other group’ included patients with high Snail and reduced E-cadherin, high Snail and preserved E-cadherin, and low Snail and reduced E-cadherin expression. NS, not significant; WHO, World Health Organization; NEC, neuroendocrine carcinoma.

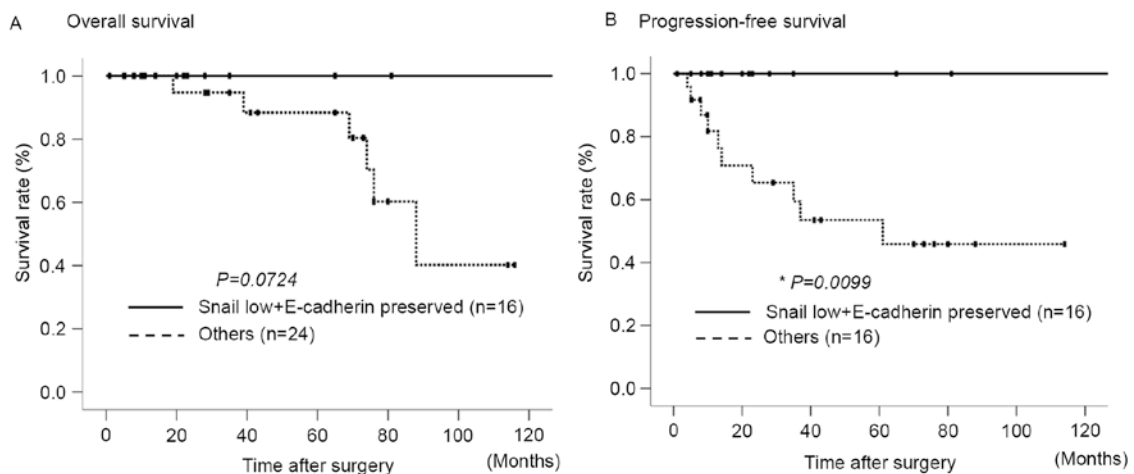


Figure 2. Kaplan-Meier survival curves for patients with pancreatic neuroendocrine tumors who underwent surgical resection. All patients were divided into two groups: The group with low Snail and preserved E-cadherin expression, and the ‘other group’ that included patients with high Snail and reduced E-cadherin, high Snail and preserved E-cadherin, and low Snail and reduced E-cadherin expression. (A) No significant difference in the overall survival rate was observed between the two groups. (B) A significant difference in the progression-free survival rate was observed between the two groups.

PNETs is still unclear. Fendrich *et al* (21) immunohistochemically evaluated the expression patterns of the EMT markers E-cadherin, Snail and Twist in human PNETs, and observed a loss of E-cadherin and the overexpression of Snail and Twist in the majority of malignant PNETs. In addition, the formation of islet cell tumors in the RIP-Tag2 transgenic mouse model was prevented by inhibiting Snail expression using polyethylene glycol. This result indicated that EMT serves a key role in the tumorigenesis of PNETs. Galvan *et al* (22) also used immunohistochemistry to assess the expression of EMT markers in 91 cases of gastroenteropancreatic (GEP) NET, including 22 cases of PNET. It was reported that alteration of the E-cadherin/ β -catenin complex, specifically the high Snail expression and cytoplasmic E-cadherin pattern, reduced the survival rates of patients with GEP-NETs. Taken together, the current study and previous reports on PNETs have demonstrated that tumor cells that have acquired an invasive phenotype through EMT may easily detach from the primary tumor, invade surrounding tissues and enter microvessels, therefore spreading into the circulation (21,22).

In the present study, the expression of Snail and E-cadherin was not significantly associated with the rate of OS. This is partly as the recurrence of PNETs, which most commonly involves liver metastasis, is usually treatable by transcatheter arterial chemoembolization or transcatheter arterial infusion, and then effectively controlled (23-25). The current study had various limitations; the study design was retrospective, and the patient cohort was small. However, the expression patterns of Snail and E-cadherin were identified to be potent predictors of lymph node and liver metastasis. To the best of our knowledge, this is the first study to demonstrate that EMT may perform an important role in the spread of tumor cells into the lymphatic flow and circulation, and in the subsequent establishment of metastasis in PNETs. Additional studies, with larger patient cohorts are required to verify the results of the present study.

In conclusion, the current study demonstrated that EMT may serve an important role in vessel invasion and metastasis in PNETs, and that the immunohistochemical evaluation of Snail and E-cadherin expression is useful for predicting the invasive and metastatic phenotype of PNETs.

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