Snail transcript levels in diagnosis of pancreatic carcinoma with fine-needle aspirate

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ABSTRACT

Expression of the transcription factor Snail that mediates epithelial-mesenchymal transition is correlated with poor prognosis in many tumour types. The aim of this study is to determine, as a proof of principle, whether Snail messenger RNA (mRNA) could be detected in fine-needle aspirate (FNA) biopsies of pancreatic ductal adenocarcinoma (PDAC) and could accurately differentiate malignant from benign pancreatic tissues. We also investigate the expression of Snail mRNA and its clinical significance in PDAC. FNA (22- or 25-gauge needle) samples were obtained from patients from June 1999 to June 2010. FNA samples that were either benign or chronic pancreatitis or confirmed as PDAC were included in this study. Snail mRNA was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The associations of Snail mRNA expression with various clinicopathological parameters was analysed in addition to the relation between its expression and patient survival. Levels of Snail mRNA were increased in tumour samples in comparison to benign and chronic pancreatitis. Transcript copy numbers for Snail were 0.7±0.21 for tumour, 0.16±0.09 for benign (P=0.002) and 0.23±0.12 for chronic pancreatitis (P=0.024). Snail expression was found to be associated significantly with lymph node metastasis (P=0.001), perineural invasion (P=0.038) and elevated preoperative serum carcinoembryonic antigen (CEA) level (P=0.043). Snail mRNA was increased in patients with poor outcome compared with those who remained alive and well. Snail mRNA levels can aid in the pathological evaluation of suspicious cases and may become a valuable asset in obtaining a definitive diagnosis of PDAC. The strong association between Snail expression and lymph node metastasis suggests that Snail mRNA can be used as an adjunct to lymph node positivity to predict survival in pancreatic cancer.

KEY WORDS: Biopsy, fine-needle.
Diagnosis.
Endoscopic ultrasound-guided fine needle aspiration.
Pancreatic neoplasms.
Snail.
Transcription factors.

Introduction

Pancreatic cancer is the fifth leading cause of cancer-related death in industrial Western countries. It is an aggressive disease with <5% survival after five years.[1,2] This is largely attributable to late clinical presentation and limitations in diagnostic methods. More than 80% of patients are diagnosed with pancreatic cancer at a locally advanced or metastatic stage, which excludes a curative surgical resection.[3] It is, therefore, important to identify these tumours in their early stages. Understanding the molecular biology of pancreatic cancer and metastasis may provide insight into its development.

In a patient clinically suspected to have a pancreatic tumour, imaging by conventional computed tomography (CT) scanning has become one of the standard modalities to indicate the size and extent of the tumour. Recently, it has been documented that endoscopic ultrasound (EUS) is a more sensitive modality than imaging by conventional CT for detecting small pancreatic tumours and for detecting invasion of vessels, which may help to assess resectability of pancreatic carcinomas.[4] Imaging modalities alone, however, can neither differentiate benign from malignant lesions nor determine the type of neoplasm.

Recently, EUS-guided fine-needle aspiration (EUS-FNA) has emerged as a very specific and minimally invasive modality for preoperative diagnosis and staging of pancreatic cancer.[5] When performed by experienced endosonographers, the EUS-FNA procedure exhibits complication rates of <1.6%.[6]

Owing to its invasive nature, FNA of the pancreas is not likely to be used routinely for early detection or screening for PDAC. By contrast, these procedures may have benefits in screening high-risk individuals, as well as for the prognosis and predicting the response to treatment in the numerous cases in which the tumour is inoperable. The EUS-FNA sampling method has been shown to provide enough material of sufficient quality to carry out biomarker studies.[7] The presence of molecular biomarkers in EUS-FNA samples is quantifiable and can be standardised.

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Molecular classification of cancer based on expression measurement of many genes and their association to specific phenotypes will have important clinical consequences including improvement of patient care. Specifically, recent studies have shown that molecular classification of PDAC may increase the ability to distinguish normal from malignant tissues, or metastatic from primary tumours, and

also to predict which tumours will have positive or negative outcome.[8,9] Thus, gene expression analysis has potential to improve sensitivity in diagnosing malignant tissues in cytological samples from FNA specimens.

Transcription factors that orchestrate epithelial-mesenchymal transition (EMT) have been correlated with increasing histological grade and poor prognosis for several types of carcinoma. For example, increased expression of Snail, Slug, or Twist correlate with higher histological grade and decreased relapse-free survival in patients with breast carcinoma, pancreatic ductal adenocarcinoma, oesophageal adenocarcinoma and colorectal carcinoma patients.[10–13] Expression of EMT markers is associated with higher tumour stage and shorter disease-free survival in oesophageal and colorectal carcinomas.[12,13] High levels of Twist have been reported as a poor prognostic factor in carcinomas of the female gynecological tract.[14] Twist is also up-regulated in prostatic adenocarcinoma of high Gleason grade and correlates with disease stage and histological grade of urothelial carcinoma.[15] Thus, much preliminary data suggest that these transcription factors may be useful as tumour markers.

Justin *et al*.[16] found that Twist and Slug were identified in both the nucleus and cytoplasm of benign pancreatic ductal epithelium, chronic pancreatitis and PDAC. Compared with normal ductal epithelium, nuclear levels of Twist are decreased in PDAC. None of the Twist and Slug showed significant differences in staining indices among the diagnostic groups. There were no correlations between Twist and Slug expression and histological grade. Birgit *et al*.[17] found that 70% human pancreatic cancer tissues showed expression of Snail, and Snail did not express in normal tissue and chronic pancreatitis tissue.

In the present study, we quantitatively measure the expression of Snail messenger RNA (mRNA) in pancreatic cancer, chronic pancreatitis and normal tissue on FNA samples by quantitative real-time polymerase chain reaction (qRT-PCR). We investigate relations between expression of the Snail and clinicopathological parameters to assess its potential value as a prognostic indicator in pancreatic adenocarcinoma. We also determine whether Snail could be a diagnostic marker to distinguish pancreatic adenocarcinoma from chronic pancreatitis on FNA samples.

Materials and methods

Human tissues

The study was approved by the Affiliated Hospital of Weifang Medical College Committee. After obtaining informed consent, EUS-FNA (22- or 25-gauge needle) samples were obtained from 202 patients from June 1999 to June 2010, who were initially evaluated for suspicious pancreatic lesions that were mostly detected by CT scan, and in some cases by abdominal ultrasound. FNA samples that were either benign (n=10) or chronic pancreatitis (n=16) or confirmed as PDAC (n=84) were included in this study. The remaining 49 cases, which were not included in this study, were diagnosed as intraductal papillary mucinous neoplasm (n=13), neuroendocrine tumours (n=17) and other non-pancreatic malignancies (n=9). In four cases the pathological diagnosis was undetermined. The cytopathologist assessed the adequacy of these FNA samples with standard techniques at the time of the biopsy. The samples were kept at room temperature for 20–30 min and then stored frozen at –80°C.

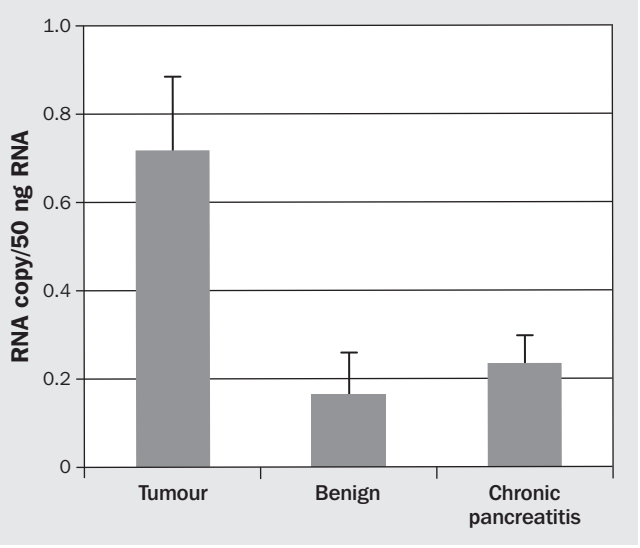

Fig. 1. Levels of transcripts of Snail in tumour samples in comparison to benign and chronic pancreatitis (expressed as transcript copy number per 50 μg messenger RNA and standardised with β-actin).

Quantitative PCR

RNA was isolated from tissue samples (FNA samples) using a standard RNAzol procedures. For reverse transcription-PCR, complementary DNA (cDNA) was synthesised in a 20-μL reaction mixture with 1 μg RNA, as described previously (AB Gene Reverse Transcription System; ABGene, Surrey, UK). The qRT-PCR system used the Amplofluor Uniprimer system (Intergen, Oxford, UK) and Thermo-Start (ABgene, Epsom, Surrey, UK). Specific primer pairs for Snail (forward: 3′-TCTTTCCTCGTCAGGAAGc-5′, reverse 3′-ACTGAACCTGACCGTACACTGCTGGAAGGTAAACTCTG-5′) was designed by the authors by using Beacon Designer software and were manufactured by Shenggong, Shanghai, China.

Using the iCycler IQ system (Bio-Rad), which incorporates a gradient thermocycler and a 96-channel optical unit, the pancreatic tissue cDNA was simultaneously assayed in duplicate reactions with a standard hot-start qRT-PCR master mix. Conditions were as follows: enzyme activation at 95°C for 12 min for 1 cycle, followed by 60 cycles of denaturing at 95°C for 15 sec, annealing at 55°C for 40 sec, and extension at 72°C for 25 sec. With use of purified plasmids as internal standards, the levels of each tight junction molecule cDNA (copies per 50 ng RNA) in the pancreatic samples were calculated. Q-PCR for β-actin was also performed on the same samples to correct for any residual differences in the initial level of RNA in the specimens (in addition to spectrophotometry). The products of Q-PCR were verified on agarose gels (not shown).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 11.0 (SPSS, Chicago, IL, USA) by using a two-sample Student's t-test and the nonparametric Mann-Whitney

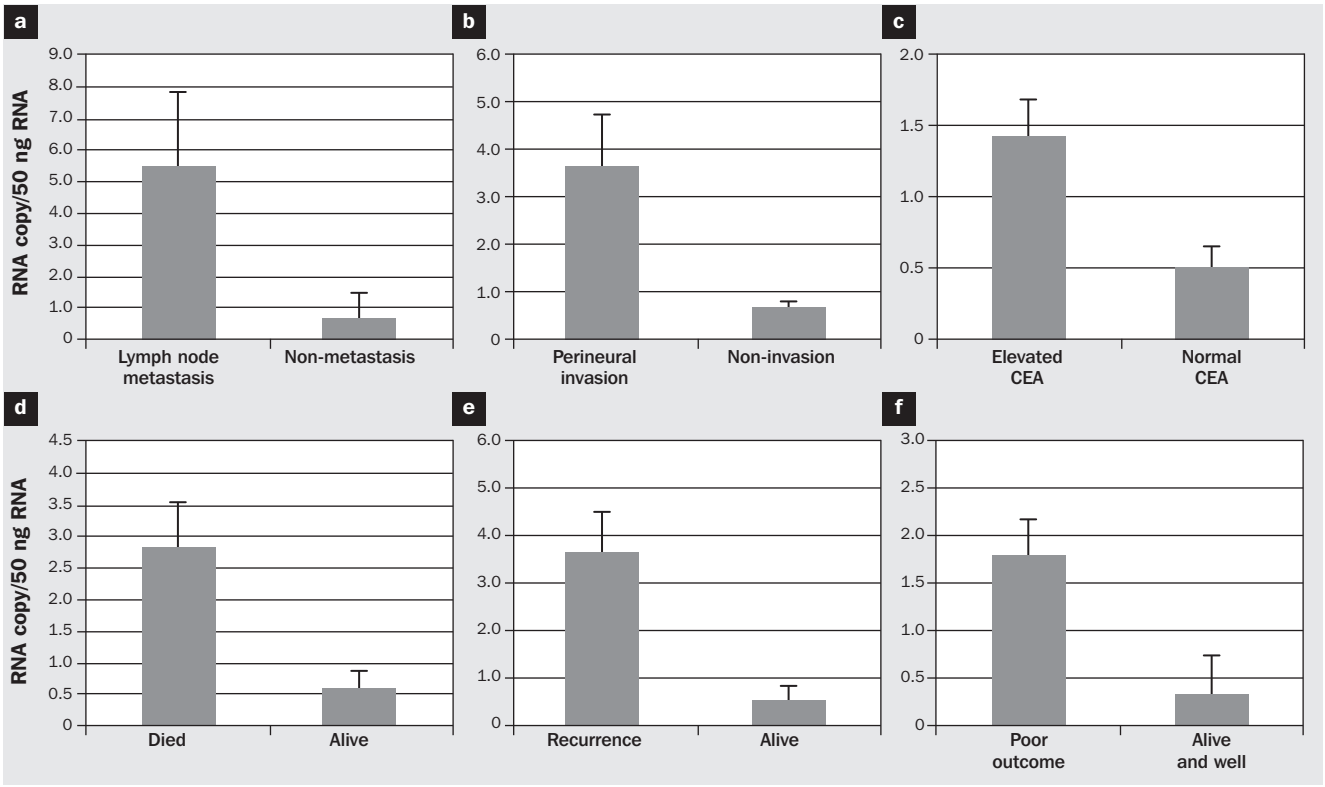

Fig. 2. Comparison of transcript levels of Snail and clinicopathological characteristics. Levels of transcripts of Snail expression in **a)** lymph node metastasis, **b)** perineural invasion and **c)** elevated preoperative serum carcinoembryonic antigen level. **d)** Comparison of transcript levels of Snail and death. **e)** Comparison of transcript levels of Snail and recurrences. **f)** Comparison of transcript levels of Snail in poor-outcome patients compared with those who remained disease-free.

confidence interval and test, where appropriate. $P<0.05$ was considered statistically significant.

Results

Snail shows increased expression in tumour tissues

Levels of transcripts of Snail were increased in tumour samples in comparison to benign and chronic pancreatitis (expressed as transcript copy number per 50 ng mRNA and standardised with β-actin; Fig. 1). Transcript copy numbers for Snail were 0.74±0.21 for tumour, 0.16±0.09 for benign ($P=0.002$) and 0.23±0.12 for chronic pancreatitis ($P=0.024$).

Snail expression and clinicopathological characteristics

Our patient follow-up of 89.4 months allowed us to examine the qRT-PCR data with regard to patient outcome. Snail expression was found to be associated significantly with lymph node metastasis (Fig. 2a, $P=0.001$), perineural invasion (Fig. 2b, $P=0.038$) and elevated preoperative serum carcinoembryonic antigen (CEA) level (Fig. 2c, $P=0.043$). Higher expression of Snail was found in patients who had died of pancreatic cancer (Fig. 2d, 2.8±0.4, $P=0.048$). Furthermore, Snail was significantly increased in those with recurrences (Fig. 2e; recurrence 3.8±0.4; alive and well 0.62±0.09; $P=0$ .03). When all poor outcomes are considered together, it can be observed that Snail increased in patients with poor outcome (metastatic disease, recurrence and death from pancreatic cancer) compared with those who remained alive and well after 89.4 months (Fig. 2f).

Discussion

Successful management and treatment of PDAC remains one of the key challenges in clinical oncology. Although early-stage pancreatic carcinoma can be treated surgically, most cases present at an advanced stage, when surgical resection is not possible because of vascular dissemination of tumour and its spread to regional lymph nodes. In addition, the differential diagnosis of pancreatic carcinoma and pseudotumoural masses caused by chronic pancreatitis is often difficult because of their similar imaging features and clinical presentations. Several protein and nucleic acid markers identified in blood have been shown to have diagnostic potential, but they lack specificity and sensitivity.[18–23]

In this study, we examined the feasibility of the use of qRT-PCR to detect Snail mRNA in FNA biopsies of pancreatic tissue. Levels of transcripts of Snail were increased in tumour samples in comparison to benign and chronic pancreatitis, and the differential levels of Snail mRNA production in the frozen tissue samples clearly separated pancreatic cancer, normal tissues and chronic pancreatitis tissues for the FNA sets of samples. This finding suggests that the quality of cells sampled with EUS-FNA is sufficient for successful qRT-PCR analysis of pancreatic tissues. It also confirms that the observed differences in mRNA production are specific to the disease state. qRT-PCR analysis of Snail mRNA could be used in clinical practice as an ancillary method to improve the diagnostic potential of the FNA procedure. In addition, a combination of these diagnostic procedures could improve the negative predictive value of pancreatic FNAs.

We further studied the levels of Snail mRNA and clinical significance in pancreatic adenocarcinoma. The results showed that Snail mRNA was positively associated with lymph node metastasis, perineural invasion, elevated preoperative serum CEA level and recurrence. Higher expression of Snail mRNA was found in patients who had died of pancreatic cancer.

In conclusion, these results demonstrate that Snail mRNA levels can aid in the pathological evaluation of suspicious cases and may become a valuable asset in obtaining a definitive diagnosis of PDAC. Additional studies are needed to analyse retrospectively archived formalin-fixed paraffin wax-embedded samples and prospectively evaluate the utility of the use of Snail mRNA in conjunction with the EUS-FNA procedure to complement the current standard histological and cytological diagnosis of pancreatic diseases. Nevertheless, the strong association between Snail expression and lymph node metastasis suggests that Snail mRNA can be used as an adjunct to lymph node positivity to predict survival in pancreatic cancer. □

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