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RAPID COMMUNICATION

Overexpression of *c-met* in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer

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Abstract

AIM: To investigate *c-met* expression during early pancreatic carcinogenesis.

METHODS: We used 46 bulk tissues and 36 microdissected samples, including normal pancreas, chronic pancreatitis, and pancreatic cancer, for quantitative real-time reverse transcription-polymerase chain reaction.

RESULTS: In bulk tissue analyses, pancreatic cancer tissues expressed significantly higher levels of *c-met* than did chronic pancreatitis and normal pancreas tissues. *c-met* levels did not differ between chronic pancreatitis and normal pancreas tissues. In microdissection-based analyses, *c-met* was expressed at higher levels in microdissected pancreatic cancer cells and pancreatitis-affected epithelial cells than in normal ductal epithelial cells (both, $P < 0.01$). Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* similar to those of pancreatic cancer cells.

CONCLUSION: Overexpression of *c-met* occurs during the early stage of pancreatic carcinogenesis, and a single alteration of *c-met* expression is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells.

Key words: *c-met*; Pancreatic cancer; Chronic pancreatitis; Pancreatic carcinogenesis

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INTRODUCTION

Pancreatic cancer is the fourth and fifth leading cause of cancer death in the United States and Japan, respectively, and has the lowest survival rate of any solid cancer[1-3]. Because the lack of specific symptoms in patients with pancreatic cancer makes early diagnosis difficult, initial diagnosis typically occurs when the tumor has reached an advanced stage[4]. Therefore, we need a better understanding of the early carcinogenesis of pancreatic cancer to facilitate early detection.

The protooncogene *c-met* encodes a member of the family of receptor tyrosine kinases that is a 190-ku glycoprotein comprised of a transmembrane 145-ku β subunit and an extracellular 50-ku α subunit[5,6]. The met receptor binds to and is activated by hepatocyte growth factor/scatter factor (HGF/SF)[7], leading to increased proliferation, altered motility, and enhanced invasion[8]. The wild-type *c-met* gene is amplified or overexpressed in many types of human cancer, including cancers of the breast, stomach, liver, endometrium, nasopharynx, and pancreas[9-14].

Pancreatic ductal cells, which give rise to the most common type of human pancreatic carcinoma, are particularly sensitive to inflammatory and carcinogenic processes[15]. There are reports that pancreatic inflammation may play a key role in early pancreatic carcinogenesis[16]. Rivera *et al* found that *k-ras* mutations that lead to uncontrolled cell growth and may be the principal molecular event in the pathogenesis of pancreatic cancer are present in chronic pancreatitis, providing a genetic basis for the potential progression of chronic pancreatitis to pancreatic cancer[15]. Taken together, the data indicate that the molecular changes associated with chronic pancreatitis

should be studied as a part of a comprehensive strategy to understand pancreatic carcinogenesis. It was reported that *c-met* mRNA expression was increased during development of chronic pancreatitis in dibutyltin-treated mice[17]. Furukawa *et al* also reported that strong immunostaining of c-Met was present in 58% of specimens demonstrating pancreatic hyperplastic epithelia and in 78% of specimens demonstrating ductal adenocarcinoma, respectively[18]. To understand the clinical significance of *c-met* expression in pancreatic carcinogenesis, we need to examine *c-met* expression during early pancreatic carcinogenesis. Therefore, we need accurate measurements of the levels of *c-met* expression in specific cells, such as normal epithelial cells, pancreatitis-affected epithelial cells, and invasive ductal carcinoma (IDC) cells.

The microdissection method, which can isolate specific cells from a frozen section, is used for genetic analysis of specific lesions[19]. Because tumor cells typically represent only 60%-70% of the cells in pancreatic cancer bulk tissues and because the percentage of chronic pancreatitis-affected epithelial cells is very low in bulk chronic pancreatitis tissues[19], cell microdissection is necessary and useful for reliable molecular analyses related to pancreatic carcinogenesis.

In the current study, we examined *c-met* mRNA levels in 46 human pancreatic bulk tissue samples and cells microdissected from 36 samples of pancreas, including normal pancreas, chronic pancreatitis, and pancreatic cancer by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Our goals were to quantify *c-met* expression in chronic pancreatitis, which is an early stage of pancreatic carcinogenesis, and to clarify when overexpression of c-met occurs during pancreatic carcinogenesis.

MATERIALS AND METHODS

Pancreatic tissues

Tissue samples were obtained at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) as described previously[20]. In brief, tissue samples were removed as soon as possible after resection and divided into at least three bulk tissue specimens. The first sample was embedded in OCT compound (Sakura Findek, Tokyo, Japan), snap-frozen for microdissection, and stored at -80℃ until use. The second sample was fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin and eosin (H&E) staining. The third sample was snap-frozen for bulk tissue analysis and stored at -80℃ until use. Tissues adjacent to the specimens were examined histologically, and the diagnosis was confirmed by pathologists. Thirteen pancreatic cancer tissue specimens were obtained from tumoral lesions of resected pancreas with primary pancreatic cancer. Twelve normal pancreatic and 11 pancreatitis-affected pancreatic tissue specimens were taken from peripheral tissues away from the tumor or pancreas resected due to mass-forming pancreatitis. Written informed consent was obtained from all patients, and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

Table 1 *c-met* and *β-actin* primer sequences and product size

Primer	Forward	Reverse	Product size
	Sequence 5′-3′	Sequence 5′-3′	
c-met	tgatgatgaggtggacaca	ctatggcaaggagcaaaga	149
β-actin	aaatctggcaccacaccttc	ggggtgttgaaggtctcaaa	139

Pancreatic cancer cell lines

Fourteen pancreatic cancer cell lines, ASPC-1, BxPC-3, KP-1N, KP-2, Panc-1, Suit-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2, NOR-P1 (established in our laboratory), Capan-1, Capan-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, Virginia), and four primary cultured pancreatic fibroblasts derived from resected pancreatic tumors were used. Cells were maintained as described previously[21].

RNA Isolation

Total RNA was extracted from bulk tissues with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) per the manufacturer's protocol. Total RNA was extracted from cells isolated by microdissection with the standard acid guanidinium thiocyanate-phenol-chloroform protocol[22] with or without glycogen (Funakoshi, Tokyo, Japan).

Quantitative analysis of c-met mRNA expression by real-time RT-PCR

Quantitative real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) with a LightCycler Quick System 350S (Roche Applied Science, Mannheim, Germany) as described previously[23]. In brief, the reaction mixture was first incubated at 50℃ for 15 min to allow for reverse transcription. PCR was initiated with one cycle of 95℃ for 10 min to activate modified Taq polymerase followed by 45 cycles of 94℃ for 15 s, 55℃ for 20 s, and 72℃ for 10 s, and one cycle of 95℃ for 0 s, 65℃ for 15 s, and + 0.1℃/s to 95℃ for melting analysis. Each sample was run twice. In addition, any sample showing more than 10% deviation in the values was tested a third time. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of *c-met* mRNA expression was calculated from a standard curve constructed with total RNA from the Capan-1 pancreatic cancer cell line. The range of threshold cycles was from 20-35 cycles for *c-met* primers[24] and from 5-30 cycles for *β-actin* primers[25] (Table 1). Expression of *c-met* mRNA was normalized to that of *β-actin* mRNA.

Microdissection-based quantitative analysis of c-met mRNA

Frozen tissues were cut into 8-μm-thick sections. One section was stained with H&E for histologic examination. IDC cells from 13 sections, pancreatitis-affected epithelial cells from 12 sections, and normal ductal epithelial cells from 11 sections were selectively isolated with a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. After microdis-

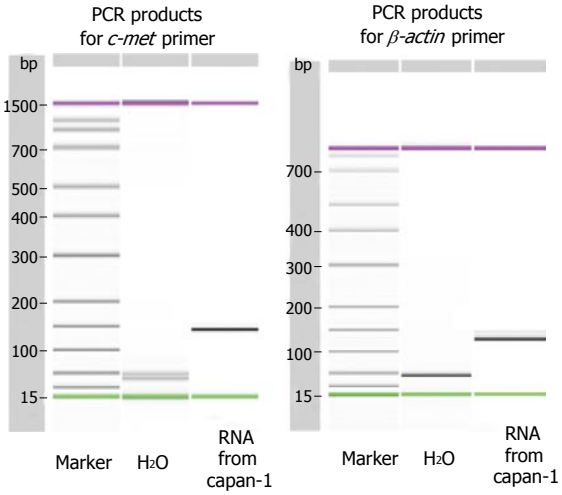

Figure 1 PCR products were analyzed with an Agilent Bioanalyzer 2001. A single 149-bp band was observed for *c-met* primer pairs (left panel). A single 138-bp band was observed for *β-actin* primer pairs (right panel). Each primer pair used in the present study produced a single melting peak on real-time RT-PCR and a single prominent band of the expected size on microchip electrophoresis.

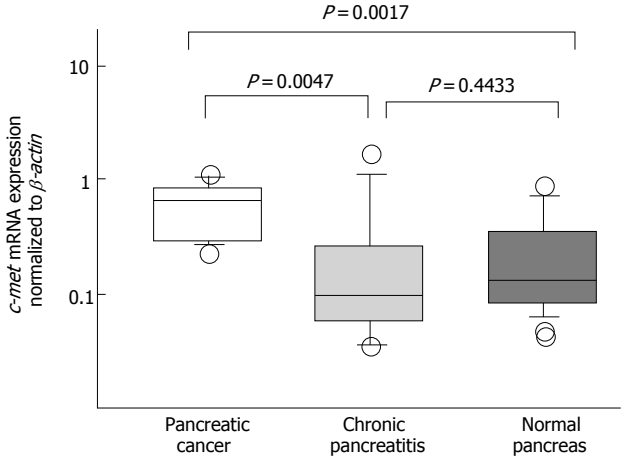

Figure 2 We performed quantitative real-time RT-PCR to quantify *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). *c-met* was overexpressed in pancreatic cancer tissues in comparison to expression in normal pancreatic (P = 0.0017) and chronic pancreatitis tissues (P = 0.0047). Levels of *c-met* did not differ between normal pancreatic and chronic pancreatitis tissues (P = 0.4433).

section, total RNA was extracted from the selected cells and subjected to real-time RT-PCR for quantitative measurement of *c-met* as described previously[19].

PCR products sized by the Agilent 2100 Bioanalyzer

Microchip electrophoresis was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). A DNA 1000 Assay Kit was purchased from Agilent Technologies. As shown in Figure 1, each PCR product was analyzed with DNA 1000 Lab Chips (Agilent Technologies) per the manufacturer's protocol[26].

Statistical analysis

Data were analyzed with the Kruskal-Wallis test for comparison of three groups and Mann-Whitney U test for comparison of two groups because normal distribution was not obtained after logarithmic transformation. Statistical significance was defined as $P < 0.05$. Because we performed multiple comparisons of our real-time RT-PCR data, we conservatively used the Bonferroni correction, and therefore, the adjusted significance level was $P < 0.017$.

RESULTS

Quantitative analyses of c-met expression in bulk pancreatic tissues

In the bulk tissue analyses, we measured *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). As shown in Figure 2, the level of *c-met* expression in normal pancreatic tissues was similar to that in chronic pancreatitis tissues. The median values were 0.130 for normal pancreatic tissues and 0.107 for chronic pancreatitis tissues (P = 0.44). The median value of *c-met* expression in pancreatic cancer tissues was 0.678, which was approximately 5-fold greater than that in normal pancreatic tissues (P = 0.0017) and 6-fold greater than that in chronic pancreatitis tissues (P = 0.0047). All data from bulk tissue analyses indicated that *c-met* was overexpressed in pancreatic cancer, but not chronic pancreatitis tissues.

Quantitative Analysis of c-met expression in 14 pancreatic cancer cell lines and 4 primary pancreatic fibroblast cultures

To confirm expression of *c-met* mRNA in pancreatic cancer cell lines, total RNA was isolated from 14 pancreatic cancer cell lines. As shown in Figure 3, *c-met* was expressed in all 14 pancreatic cancer cell lines with median value of 0.742. The 4 primary cultures of normal pancreatic fibroblast1, 2, 3 and 4 (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) expressed low levels of *c-met* mRNA with a median value of 0.023. Pancreatic cancer and chronic pancreatitis tissues usually contain abundant stromal cells, such as fibroblasts. Most of the bulk pancreatic cancer tissues in the present study also contained many desmoplastic changes and the chronic pancreatitis tissues contained stromal components. Therefore, the level of *c-met* expression detected in bulk tissue analyses may not represent the true levels of expression by specific cells due to dilution of these specific cells by contaminating cells such as stromal fibroblasts.

Quantitative analysis of c-met in microdissected pancreatic cancer cells, pancreatitis-related epithelial cells, and normal pancreatic epithelial cells

In general, bulk pancreatic tissue is complex, containing ductal epithelial cells, acinar cells, fibroblasts, islet cells, and mesenchymal cells. Tumor cells comprise only 60%-70% of the cells in bulk tissue specimens of pancreatic cancer[19]. Therefore, data from bulk tissue analyses may not accurately reflect *c-met* levels in specific cells, such as pancreatic cancer cells, pancreatitis-affected epithelial cells, and normal ductal epithelial cells. It has been reported that *c-met* is expressed in acini and pancreatic islets in normal human pancreas[27]. To avoid the influence of contami-

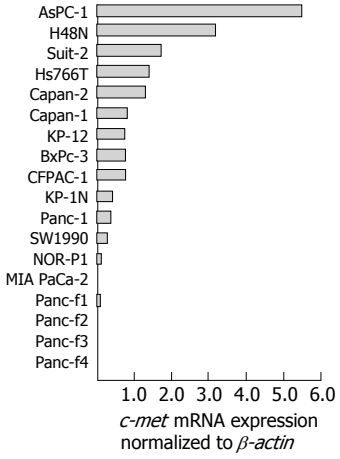

Figure 3 *c-met* expression in pancreatic cancer cell lines and primary pancreatic fibroblasts. The median value of *c-met* expression from pancreatic cancer cell lines was 0.742. In contrast, the median value of *c-met* expression in 4 primary cultures of pancreatic fibroblasts (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) was 0.023.

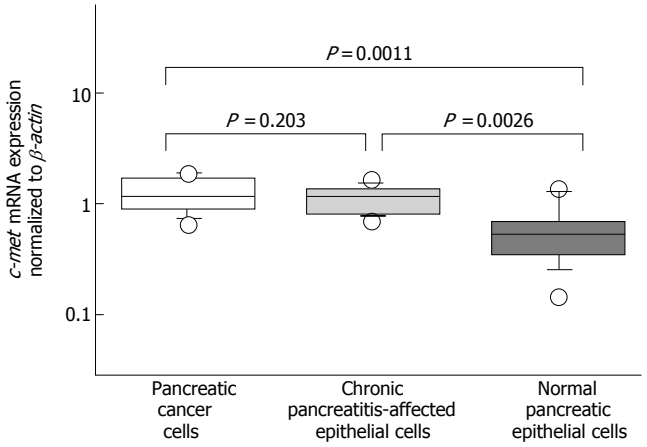

Figure 4 Quantitative analysis of *c-met* mRNA levels in microdissected pancreatic cancer cells (n = 13), pancreatitis-affected epithelial cells (n = 11), and normal pancreatic ductal epithelial cells (n = 12). *c-met* levels in pancreatic cancer cells (median, 1.208) were 2.21-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.0011). *c-met* levels in pancreatitis-affected epithelial cells (median, 1.211) were 2.22-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.005). *c-met* levels in microdissected normal ductal epithelial cells were the lowest with a median value of 0.546. Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* that approached those in pancreatic cancer cells.

nating non-ductal cells, we used a laser-microdissection method to select specific cells for analysis. For microdissection analyses, we isolated pancreatic cancer cells from 13 sections, normal pancreatic ductal epithelial cells from 12 sections, and pancreatitis-affected epithelial cells from 11 sections. As shown in Figure 4, *c-met* expression in pancreatic cancer cells (median, 1.208) was 2.21-fold higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.0011). *c-met* level in normal pancreatic ductal epithelial cells was the lowest. Interestingly, the *c-met* level in pancreatitis-affected epithelial cells (median, 1.211) was significantly higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.005). These data suggested that *c-met* was overexpressed in chronic pancreatitis-affected epithelial cells at levels close to those in pancreatic cancer cells.

DISCUSSION

We performed quantitative real-time RT-PCR to measure *c-met* expression in pancreatitis-affected epithelial cells, which may progress to pancreatic cancer[28-30], to clarify the significance of *c-met* expression in an early stage of pancreatic carcinogenesis. Bulk tissue analyses revealed that *c-met* was overexpressed in pancreatic cancer, but *c-met* expression did not differ between chronic pancreatitis and normal pancreatic tissues. In microdissection-based analyses, we found that pancreatitis-affected epithelial cells expressed high levels of *c-met* that approached those of pancreatic cancer cells. Although it may be difficult to make comparison because microdissection data are based on c-met mRNA levels in single cell, our findings appear to be inconsistent with those of previous immunohistochemical studies in which the positive rates of c-Met were 58%, 80%, and 78% of specimens demonstrating hyperplastic epithelia, severely dysplastic epithelia, and pancreatic ductal adenocarcinoma, respectively[18]. However, Furukawa *et al*[18] also reported that pancreatic cancer patients with diffuse c-Met immunostaining survived longer than c-Met-negative patients. Welm *et al* reported that overexpression of c-*Met* alone did not result in development of tumors, although c-Met acted cooperatively with other genetic alterations, such as overexpression of *MYC*, to induce mammary tumorigenesis[31]. In addition, there have been several reports that the cumulative risk of pancreatic cancer among patients with chronic pancreatitis is only 1% to 4%[28-30]. Taken together, these data suggest that overexpression of *c-met* occurs in the early stage of pancreatic carcinogenesis but is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells. However, these data also suggest that pancreatitis-affected epithelial cells expressing high levels of *c-met* in conjunction with other genetic or epigenetic changes may have the potential to progress to pancreatic cancer. Therefore, *c-met* may be a useful marker for identifying persons with high-risk lesions that may progress to pancreatic cancer.

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