Adverse prognosis of epigenetic inactivation in RUNX3 gene at 1p36 in human pancreatic cancer

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Alteration in transforming growth factor-β signalling pathway is one of the main causes of pancreatic cancer. The human runt-related transcription factor 3 gene (RUNX3) is an important component of this pathway. RUNX3 locus 1p36 is commonly deleted in a variety of human cancers, including pancreatic cancer. Therefore, we examined genetic and epigenetic alterations of RUNX3 in human pancreatic cancer. Thirty-two patients with pancreatic cancer were investigated in this study. We examined the methylation status of RUNX3 promoter region, loss of heterozygosity (LOH) at 1p36, and conducted a mutation analysis. The results were compared with clinicopathological data. Promoter hypermethylation was detected in 20 (62.5%) of 32 pancreatic cancer tissues, confirmed by sequence of bisulphite-treated DNA. Loss of heterozygosity was detected in 11 (34.3%) of 32 pancreatic cancers. In comparison with clinicopathological data, hypermethylation showed a relation with a worse prognosis (P = 0.0143). Hypermethylation and LOH appear to be common mechanisms for inactivation of RUNX3 in pancreatic cancer. Therefore, RUNX3 may be an important tumour suppressor gene related to pancreatic cancer.

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Despite its relatively low incidence of approximately 10 cases/100 000 people, pancreatic cancer is still one of the leading causes of cancer-related death in industrialised countries including Japan. The prognosis remains poor, with an overall 5-year survival rate of less than 5% (Jemal et al, 2007). The pathogenesis of pancreatic ductal adenocarcinoma can be described as a step-by-step accumulation of genetic changes, such as K-ras oncogene mutations, p53, p16, and smad4 tumour suppressor gene mutations (Kern et al, 2002), in addition to several epigenetic alterations, which together result in self sufficiency of growth signals, insensitivity to antigrowth signals, evasion of apoptosis, angiogenesis, invasion, and metastasis (Ozawa et al, 2001). Recently, several reports indicated that every silencing mechanism, such as loss of heterozygosity (LOH) and mutations in a gene, or hypermethylation in its promoter region occurred in a tumour suppressor gene resulting in loss of its function in tumorigenesis (Tokumaru et al, 2003).

RUNX3 induced apoptosis in epithelial cells, and the knockout mice of this gene showed hyperplasia in gastric mucosa. In addition, loss of function of RUNX3 caused by DNA hypermethylation, LOH at gene locus, and mutation correlated with the progression of primary gastric cancers (Li et al, 2002). RUNX3 might have the important role of TGF-β and Smad proteins in carcinogenesis. Furthermore, RUNX3 is located on the distal portion of the short arm of human chromosome 1 (1p36), which is commonly deleted in a variety of human cancers, including pancreatic cancer (Nowak et al, 2005; Loukopoulos et al, 2007). Therefore, the genetic and epigenetic alterations in RUNX3 may have an important role in pancreatic cancer.

The aim of our present study was to determine whether the RUNX3 gene alteration might have a role in carcinogenesis in pancreatic cancer. We examined LOH at this gene locus in 1p36 with microdissected DNA, the DNA-methylation status by methylation-specific polymerase chain reaction (MSP) and sequencing, and the mutation of RUNX3 by reverse transcription-polymerase chain reaction (RT-PCR) single-strand conformation polymorphism (RT-PCR-SSCP) in 32 primary pancreatic cancer tissues and corresponding noncancerous tissues. Then, we correlated these results with the clinicopathological data.

MATERIALS AND METHODS

Patients, sample collection, microdissection, and DNA preparation

Thirty-two primary pancreatic cancer tissues and corresponding noncancerous tissues were collected at Nagoya University Hospital from pancreatic cancer patients during pancreatico-duodenectomy,
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30 s, after the initial denaturation step (94°C for 5 min). Polymerase chain reaction products were purified directly using the QIA quick Gel Extraction Kit (QIAGEN, Hilden, Germany). Purified DNA fragments were subcloned into TA cloning vector (Invitrogen™, Carlsbad, CA, USA). Six cloning samples were picked out from one methylated tumour tissue. Each cloning DNA was mixed with 3 μl of specific primer (M13, 4 μl of Cycle Sequence Mix (ABI PRISM Terminator v.1.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA). Samples were subjected to the following cycling conditions: 95°C for 30 s; 25 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 4 min followed by purification by ethanol precipitation. Sequence analysis was carried out using an Applied Biosystems ABI310, and sequence electropherograms were generated by ABI Sequence Analysis 3.0.

RESULTS

Polymerase chain reaction amplification using random-primed cDNA of 32 primary pancreatic cancer tissues was performed using oligonucleotide primers in the presence of [α-32P]dCTP, followed by electrophoretic separation on 6% nondenaturing polyacrylamide gels both in the presence of 5% glycerol at room temperature and in its absence at 4°C. RUNX3 ORF (1248-bp) is divided into four overlapped fragments and each fragment was amplified. The primer pairs used for RUNX3 mutation were S1 (sense, 5'-GGCGCTGTTATGCGTATTCC-3'), AS1 (antisense, 5'-CTCAGGGAATGTCTTCTGT-3'), amplifying a 370-bp fragment; S2 (sense, 5'-GTGACTGTGATGGCAGGCAA-3') and AS2 (antisense, 5'-GGTTCCAGGTGCCCTGGATT-3'), amplifying a 398-bp fragment; S3 (sense, 5'-ACAAGCCATCTTGAGCCAAA-3') and AS3 (antisense, 5'-GAGAACTGGTAGGAGCCAGA-3'), amplifying a 368-bp fragment; S4 (sense, 5'-CTACCACGTCTACTAGGGGA-3') and AS4 (antisense, 5'-CCCACGTCTCTGTGAAAGG-3'), amplifying a 326-bp fragment. The PCR amplification consisted of 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, after the initial denaturation step (94°C for 5 min) in F1–R1 and in the presence of 10% dimethylsulfoxide (F2–R2, F3–R3, F4–R4).

Statistical analysis

The correlation between the methylation status of RUNX3 mRNA and clinicopathological data was analysed by Fisher’s exact test or χ² test for independence. Overall survival rates were calculated using the Kaplan–Meier method, and difference in survival curves was analysed using the log-rank test. Independent prognostic factors were identified by multivariate analysis using the Cox proportional hazards regression model. Data are expressed as mean ± s.d. Statistical significance was considered as P < 0.05.

RESULTS

Microsatellite analysis of RUNX3

We first examined DNA samples obtained by microdissection from the 32 primary pancreatic cancer tissues and corresponding noncancerous tissues for LOH using two microsatellite markers, D1S234 and D1S247, which are close to the RUNX3 locus. D1S234 is telomeric to the locus. Allelic imbalance in one or two markers was observed in 11 (34.3%) of the 32 cases (Figure 1). We judged the 11 cases as having an LOH at RUNX3 ORF (1248-bp) is divided into four overlapped fragments and each fragment was amplified. The primer pairs used for RUNX3 mutation were S1 (sense, 5'-GGCGCTGTTATGCGTATTCC-3'), AS1 (antisense, 5'-CTCAGGGAATGTCTTCTGT-3'), amplifying a 370-bp fragment; S2 (sense, 5'-GTGACTGTGATGGCAGGCAA-3') and AS2 (antisense, 5'-GGTTCCAGGTGCCCTGGATT-3'), amplifying a 398-bp fragment; S3 (sense, 5'-ACAAGCCATCTTGAGCCAAA-3') and AS3 (antisense, 5'-GAGAACTGGTAGGAGCCAGA-3'), amplifying a 368-bp fragment; S4 (sense, 5'-CTACCACGTCTACTAGGGGA-3') and AS4 (antisense, 5'-CCCACGTCTCTGTGAAAGG-3'), amplifying a 326-bp fragment. The PCR amplification consisted of 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, after the initial denaturation step (94°C for 5 min) in F1–R1 and in the presence of 10% dimethylsulfoxide (F2–R2, F3–R3, F4–R4).

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Hypermethylation of RUNX3 promoter region in pancreatic cancer

To investigate whether the gene silencing was due to hypermethylation of RUNX3, MSP was performed in the 32 primary pancreatic
cancer tissues and corresponding noncancerous tissues. Promoter hypermethylation was detected in 20 (62.5%) of the 32 primary pancreatic cancer tissues and in only two of the corresponding noncancerous tissues (Figure 1). To confirm the methylation of the RUNX3 promoter region, genomic bisulphite-treated DNA of primary pancreatic cancer tissues, which showed methylation by MSP, were sequenced. Every case showed at least one methylated CpG island of the sequenced fragments. A representative case is shown in Figure 2.

Mutational analysis of RUNX3 in pancreatic cancer tissues

To investigate the mutation status of this gene, RT-PCR-SSCP analysis was performed. We could not see any aberrant bands (Figure 3). No mutations or polymorphisms were detected in the 32 pancreatic cancer tissues. As we used the bulk frozen samples, normal cells such as fibrosis cells were contaminated in the tumour tissues, making it difficult to identify aberrant bands.

Statistical analysis of clinicopathological data and our findings

Subsequently, we analysed the correlation between the clinicopathological data and results of our findings. Table 2 shows the correlation between the clinicopathological data and methylation status. Interestingly, RUNX3 hypermethylation was significantly correlated with a worse prognosis \( (P = 0.0143) \) (Figure 4). No other correlation with any clinicopathological parameter was found.

To evaluate the value of RUNX3 methylation as an independent prognostic determinant, multivariate analysis was performed with prognostic factors that had been found to be significant by univariate analyses. The analysis identified lymph node metastasis, invasion of retroperitoneal tissue, and hypermethylation of RUNX3 gene as the variables for independently predicting overall survival (Table 3).

DISCUSSION

Transforming growth factor-\( \beta \) plays a key role in regulating the growth and differentiation of many cell types. In TGF-\( \beta \)1-null
animals, proliferation of the gastric epithelium is stimulated and hyperplasia occurs (Crawford et al., 1998). TGF-β is known to be a potent inhibitor of pancreatic acinar and duct cell proliferation in vitro (Bisgaard and Thorgerisson, 1991; Logsdon et al., 1992).

RUNX3 is a runt domain transcription factor involved in this signalling pathway. RUNX3 protein binds with the Smad2 and Smad3 proteins. Recently, it has been reported that RUNX3 was one of the tumour suppressor genes in gastric cancer and testicular yolk sac tumour. Runx3-null mice reportedly develop hyperplasia of the gastric mucosa through activation of cellular proliferation and suppression of apoptosis in epithelial cells (Li et al., 2002).

Interestingly, 1p36, where RUNX3 exists, is a region commonly deleted in a wide variety of human carcinomas, including pancreatic cancer. To date, there are many reports regarding the TGF-β signalling pathway in pancreatic cancer (e.g. TGF-β receptor II, Smad2 and Smad4), but only a few deal with this gene's alterations in pancreatic cancer (Li et al., 2004; Wada et al., 2004). Moreover, there are no reports regarding primary pancreatic cancer. Our study further supports a role for RUNX3 in pancreatic cancer.

The 1p36 region is believed to harbour tumour suppressor genes, because previous studies identified frequent allelic imbalance at 1p36 in various types of human cancers (Schwab et al., 1996). RIZ1 and p73 genes are located on 1p36, and LOH was detected at each gene locus in pancreatic cancer (Sakurada et al., 2001; Sphyris et al., 2004). It is thought that these are one of the tumour suppressor genes in pancreatic cancer, and we think that RUNX3 may also be a candidate.

Previously, Wada et al. (2004) reported that nine of 12 pancreatic cancer cell lines exhibited no expression of RUNX3 by both

### Table 1: Clinicopathological features and results of RUNX3 alterations in pancreatic cancer tissues

| Case | Gender | Location | Stage | Pathology | Hypermethylation | LOH* |
|------|--------|----------|-------|-----------|------------------|------|
| 1    | M      | Ph       | III   | Tub. poor | —                | —    |
| 2    | F      | Ph       | IVa   | Tub. mod  | —                | —    |
| 3    | M      | Ph       | IVb   | Tub. mod  | —                | M    |
| 4    | F      | Ph       | III   | Tub. mod  | —                | —    |
| 5    | F      | Phb      | III   | Anap. duc | —                | M    |
| 6    | F      | Ph       | IVb   | Tub. well | M                | M    |
| 7    | M      | Ph       | III   | Tub. mod  | —                | —    |
| 8    | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 9    | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 10   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 11   | M      | Ph       | IVb   | Tub. mod  | —                | M    |
| 12   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 13   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 14   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 15   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 16   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 17   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 18   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 19   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 20   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 21   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 22   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 23   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 24   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 25   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 26   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 27   | F      | Ph       | IVb   | Tub. mod  | —                | —    |
| 28   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 29   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 30   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 31   | M      | Ph       | IVb   | Tub. mod  | —                | —    |
| 32   | F      | Ph       | IVb   | Tub. mod  | —                | —    |

**Hypermethylation**

- N: normal tissue
- T: tumour tissue

**LOH**

- D1S234: LOH detected
- D1S247: LOH detected

| LOH* | Cases |
|------|-------|
| LOH*: cases in which LOH was detected in at least one locus. |
| 11/32 (34.3%) |

**LOH+**: cases in which LOH was detected in at least one locus. **NI**: not informative. **–**: unmethylated; open circle = LOH detected; closed circle = retention of heterozygosity.

**Figure 4**: Survival stratified by methylation status in primary pancreatic cancer. RUNX3 hypermethylation was significantly correlated with a worse prognosis ($P=0.0143$). RUNX3 = human runt-related transcription factor 3 gene.
northern blot analysis and RT-PCR. All of the nine cell lines showed methylation of the promoter CpG island of the gene. Moreover, hemizygous deletion of RUNX3, as detected by fluorescence in situ hybridisation, was found in most of the cell lines that lacked RUNX3 expression. Our results using primary pancreatic cancer tissue were compatible with their findings. Li et al (2004) reported that RUNX3 expression was low-to-absent in normal pancreatic tissues, but increased in a third of cancer tissues by RT-PCR and immunohistochemistry. RUNX3 expression was present only in islets of the normal pancreas. They

### Table 2
Clinicopathological features and results of RUNX3 hypermethylation in pancreatic cancer tissues

| Variable          | No. of cases | Hypermethylation | P*  |
|-------------------|--------------|------------------|-----|
|                   |              | +                | –  |
| **Age**           |              |                  |    |
| <60               | 10           | 5                | 5  | 0.325 |
| ≥60               | 22           | 15               | 7  |
| **Gender**        |              |                  |    |
| M                 | 18           | 13               | 5  | 0.198 |
| F                 | 14           | 7                | 7  |
| **Tumour size**   |              |                  |    |
| TS1               | 5            | 2                | 3  | >0.9999 |
| ≥TS2              | 27           | 18               | 9  |
| **S**             |              |                  |    |
| –                 | 18           | 11               | 7  | 0.854 |
| +                 | 14           | 9                | 5  |
| **RP**            |              |                  |    |
| –                 | 10           | 6                | 4  | 0.844 |
| +                 | 22           | 14               | 8  |
| **CH**            |              |                  |    |
| –                 | 13           | 9                | 4  | 0.515 |
| +                 | 19           | 11               | 8  |
| **DU**            |              |                  |    |
| –                 | 21           | 13               | 8  | 0.923 |
| +                 | 11           | 7                | 4  |
| **PV**            |              |                  |    |
| –                 | 12           | 8                | 4  | 0.706 |
| +                 | 20           | 12               | 8  |
| **A**             |              |                  |    |
| –                 | 27           | 16               | 11 | 0.379 |
| +                 | 5            | 4                | 1  |
| **PL**            |              |                  |    |
| –                 | 27           | 16               | 11 | 0.379 |
| +                 | 5            | 4                | 1  |
| **DPM**           |              |                  |    |
| –                 | 26           | 15               | 11 | 0.242 |
| +                 | 6            | 5                | 1  |
| **N**             |              |                  |    |
| 0                 | 14           | 8                | 6  | 0.581 |
| 1                 | 18           | 12               | 6  |
| **Differentiation** |            |                  |    |
| Mod               | 21           | 12               | 9  | 0.241 |
| Poor              | 6            | 5                | 1  |

*pAnalysed by Fisher’s exact test or χ² test for independence. *Tumour size according to the Classification of Pancreatic Carcinoma; A = arterial invasion; CH = choledocal invasion; DPM = dissected peripancreatic tissue margin; DU = duodenal invasion; F = female; PL = peripancreatic nerve plexus invasion; M = male; mod = moderately differentiated adenocarcinoma; N = lymph node metastasis; poor = poorly differentiated adenocarcinoma; pTNM = pathological TNM; PV = portal vein invasion; RP = retroperitoneal invasion; S = serosal invasion. *Classified according to the classification of The General Rules for the Clinical and Pathological Study of Primary Pancreatic Cancer. April 2002, Pancreatic Cancer Study Group of Japan.

### Table 3
Multivariate analysis of patients with pancreatic cancer

| Variable          | Odds ratio | 95% CI     | P     |
|-------------------|------------|------------|-------|
| Tumour size (≥2.0 cm) | 1.995      | 0.639–6.226 | 0.2342 |
| Lymph node metastasis | 2.388      | 1.026–5.561 | 0.0435* |
| Invasion of retroperitoneal tissue (d.p.m.) | 5.486      | 1.409–21.358 | 0.014* |
| Invasion of plexus nerve (P) | 1.759      | 0.591–5.239 | 0.3103 |
| Hypermethylation  | 3.157      | 1.226–8.130 | 0.0172* |

*Statistical significance. CI = confidence interval.
also found that all metastases of pancreatic cancer tissues were devoid of or displayed only very faint RUNX3 expression by immunostaining.

Some groups have advocated islet cells as the cells of origin of pancreatic ductal adenocarcinoma (Pour et al., 2003). This would mean that the islet cells in pancreatic tissue are the tissue-specific stem cells in which cancer cells begin from the alteration in the oncogenes or tumour suppressor genes. RUNX3 is expressed in the tissue-specific stem cells, and only in islet cells in normal tissue. When cancer tissue has grown from the tissue-specific stem cells, the cancer cells express the RUNX3 protein. Some cancer tissues do not express RUNX3. In those cancer cells, RUNX3 gene is methylated. In cases with metastatic lesions, more aggressive tumour cells from the original lesion exist, such as RUNX3-methylated cells. Hence, the metastatic pancreatic cancer cells do not express RUNX3 gene.

Thus, it may be hypothesised that there is indeed loss of RUNX3 expression by promoter hypermethylation or LOH in some primary tumours compared with normal islets, and almost a complete loss in metastatic tumours. Our finding that the survival in methylated cases in RUNX3 gene was significantly worse than that in unmethylated patients is compatible with this hypothesis, although pointing to a tumour suppressor role for RUNX3 in pancreatic cancer.

Nine of 11 LOH detected cases had hypermethylation of the RUNX3 promoter region. These findings imply that silencing of RUNX3 occurred biallelically. Complete silencing of this gene leads to the progression of cancer, and then relates to the worse prognosis.

In conclusion, we have clearly demonstrated for the first time that RUNX3 is frequently methylated in primary pancreatic cancer tissues, frequent hemizygous deletion occurs at its locus in 1p36, and RUNX3-inactivated cases showed worse survival. We propose that inactivation of RUNX3 plays an important role in alteration of the TGF-β signalling pathway and in the tumorigenesis of pancreatic cancer.

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