Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas

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Summary It has been reported that the rho genes, which consist of a ras-related small GTPase protein family, regulate cytoskeletal structures and have the potential to transform cultured cells. To investigate the biological relevance of the rho genes in pancreatic carcinogenesis, we examined expressions of the rhoA, B and C genes by polymerase chain reaction after reverse transcription (RT-PCR) in 33 cases of ductal adenocarcinoma of the pancreas. In addition, mutations of the K-ras, rhoA, B and C genes were studied in the same series of tumour tissues to correlate with rho gene expressions. The expression levels of the rhoC gene were significantly higher in tumours than in non-malignant portions (P < 0.001). Metastatic lesions overexpressed the rhoC gene compared with primary tumours (P < 0.05). Carcinoma tissues with perineural invasion and lymph node metastasis exhibited significantly higher expressions of the rhoC gene than tumours without these manifestations (P < 0.001 and P < 0.05 respectively). Overexpression of the rhoC gene significantly correlated with poorer prognosis of patients with pancreatic adenocarcinoma (P < 0.05). In contrast, the expression levels of the rhoA and B genes showed no significant relationship with clinicopathological findings. Mutation was not found either in the rhoA, B or C gene sequences examined. K-ras gene mutation, detected in 27 out of 33 (81.8%) cases, did not affect the expression levels in any of the rho genes. These suggest that elevated expression of the rhoC gene may be involved in the progression of pancreatic carcinoma independent of K-ras gene activation.

Keywords: pancreatic carcinoma; rho; K-ras; gene expression; mutation

Ductal adenocarcinoma of the pancreas is characterized by an extremely poor prognosis with an overall 5-year survival rate of only 3% (Warshaw and Castillo, 1992). There has been progress in molecular genetic analysis of pancreatic carcinogenesis. Inactivations of tumour-suppressor genes such as p53 and allelic loss of chromosome 18q are reported (Barton et al, 1991; Hohne et al, 1992; Scarpa et al, 1993; Suwa et al, 1994; Seymour et al, 1995). Point mutation at codon 12 of the K-ras oncogene is frequently observed and is considered to be a crucial step in pancreatic carcinogenesis (Almoguer et al, 1988; Smit et al, 1988; Hruban et al, 1993). However, the molecular genetic changes that contribute to aggressive characteristics of pancreatic carcinoma still remain to be elucidated.

To date, a number of small GTP-binding proteins have been identified and are thought to be involved in signal transduction pathways that control a diverse set of essential cellular functions such as cell growth, cell differentiation, cytoskeletal organization, intracellular vesicle transport and secretion (Hall, 1990). The RAS family members are critical components of GTP-binding proteins and mutation at either codon 12, 13 or 61 makes RAS proteins in their active GTP-bound state, resulting in oncogenic potential (Boguski and McCormick, 1993). Rho proteins constitute one of the RAS-related subfamilies and are involved in cytoskeletal organization and cell motility by coordinated assembly of focal adhesion and stress fibres (Ridley and Hall, 1992). In addition, Rho proteins are regulators of gene expression by activating the Jun nuclear kinase and the serum response factor, and are necessary for cell cycle progression (for reviews Olson, 1996). The Rho family consists of at least ten proteins, and three rho isoforms have been identified in the human genome. They are designated as the rhoA, rhoB and rhoC genes respectively (Yeramian et al, 1987; Chardin et al, 1988). Cells transformed by oncogenic RAS reveal changes in their morphology through cytoskeletal actin structures (Bar-Sagi and Feramisco, 1986). Malignant transformation of NIH3T3 cells is induced by transfection of the Aplysia rho gene, which has 95, 94 and 92% homology to the human rhoA, B and C genes respectively. Activated RhoB augments focus formation of NIH3T3 cells transformed by oncogenic Ras (Prendergast et al, 1995). These suggest that Rho proteins may play a role in Ras signal transduction and cell transformation.

In the present study, we investigated quantitative and qualitative alterations of rhoA, B and C gene expressions to clarify their biological relevance to pancreatic carcinogenesis and their relationship with K-ras gene mutation.

MATERIALS AND METHODS

Patients and tissue preparation

Thirty-three cases of ductal adenocarcinoma of the pancreas were obtained by surgical resection at Kyoto University Hospital between 1990 and 1995 under written informed consent. Clinical staging was determined according to the classification of the WHO (Gibson and Sobin, 1978). In 17 out of 33 cases a non-tumorous portion of the pancreas was also acquired. In five cases only metastatic lesions without their corresponding primary pancreatic tumours were obtained because of incomplete radicality. They comprised three liver metastatic lesions and two cases of peritoneal dissemination. After inappropriate tissues were removed, tissue specimens of non-malignant lesions were immediately stored at
–80°C. Tissues of tumours were embedded in OCT tissue compound (Miles, Elkhart, IN, USA), and cryostat sections were cut at 5 μm thickness. The tumour portion was identified under a microscope after haematoxylin and eosin (H & E) staining.

**RNA extraction and RT-PCR**

Total RNA of tumour tissues was extracted from sections adjacent to the H & E-stained sections using Trizol (Life Technologies, MD, USA) according to the manufacturer’s protocol. RNA from a non-malignant portion of the pancreas surrounding the tumours was also extracted.

Gene expressions were determined by polymerase chain reaction after reverse transcription (RT-PCR) according to the method described previously (Arao et al., 1994). The PCR primers for rho gene amplification are listed in Table 1. As the rho genes have strong homology with each other and only cDNA sequences are available we tried several sets of primers. We chose primer sets that gave only one PCR product in polyacrylamide gel electrophoresis with an appropriate internal restriction site and gave no products from genomic DNA (data not shown). The condition of PCR was as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 1 min in a thermal cycler (Perkin–Elmer Cetus). Gene expression was presented by the relative yield of the PCR product from target sequences to that from the β2-microglobulin gene. Mean values from three independent experiments were taken as results.

**PCR-SSCP analysis and direct sequencing**

PCR-SSCP analysis was performed to determine gene mutations according to the method of Orita et al. (1989) with minor modifications. For the SSCP analysis of the K-ras gene, exon 1 was focused because all the point mutations were confined in exon 1 (Suwa et al., 1994). In the SSCP analysis of the rhoA, B and C genes, the fragments including codon 14, which is equivalent to codon 12 of the oncogenic mutation in the K-ras gene (Moscow et al., 1994) were investigated. The fragments analysed were codons from 1 to 69 for the rhoA, 4 to 83 for the rhoB and 3 to 81 for the rhoC genes. In brief, PCR fragments were generated from 10 ng of complementary DNA in a 10-μl mixture containing 1.25 mM dATP, dTTP, dGTP; 1.5 mM magnesium chloride; 20 pmol of each primer; 10 mM Tris-HCl (pH 8.8); 50 mM potassium; 0.45 units Taq polymerase (Gibco BRL); and 0.1 μl of [α-32P]dCTP (3000 Ci mmol−1). PCR was carried out for 35 cycles (1 min at 94°C, 2 min at 55°C and 1 min at 72°C) in a Thermal cycler (Perkin–Elmer Cetus). An aliquot (2.5 μl) from 10 μl of amplified products was diluted with 20 μl of stop solution (0.1% sodium dodecyl sulphate (SDS), 10 mM EDTA). The mixture was added to loading solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. Samples were heat denatured at 95°C for 3 min and then loaded on 6% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at room temperature with a constant power of 35 W for 3 h with a cooling fan. The exposure of autoradiography was carried out overnight.

Direct sequencing of the K-ras gene was performed as described previously (Suwa et al., 1994) using a Circumvent DNA Sequencing kit (New England Biolab, ML, USA) according to the manufacturer’s protocol.

**Statistical analysis**

The results of RT-PCR were statistically analysed using the Mann–Whitney U-test. Spearman’s correlation coefficient was used to determine a relationship between rhoA, B and C expressions. Post-operative survival was defined as the period from the first operation for pancreatic carcinoma to the time of death and was analysed by the log-rank test. Patients who died of post-operative complications were excluded from the analysis.

**RESULTS**

**Expression of the rhoA, B and C genes in relation to clinicopathological findings**

Representative profiles of RT-PCR products are shown in Figure 1. The expression of the rho genes, K-ras mutation status and clinicopathological findings are summarized in Table 2. Ductal adenocarcinoma of the pancreas revealed significantly higher levels of rhoC expression than did non-tumorous portions (mean ± s.d. =
Table 2 Expressions of rho A, B, C genes and mutation of K-ras gene in pancreatic adenocarcinoma

| Case | Age/Sex | Histology* | Stageb | rho gene expression | K-ras gene mutationc | Case | Age/Sex | Histology | Stage | rho gene expression | K-ras gene mutation |
|------|---------|------------|--------|---------------------|---------------------|------|---------|-----------|--------|---------------------|---------------------|
| 1    | 62/M    | W          | III    | 1.30 1.34 0.87      | CGT                 | 19   | 68/F    | M         | IV     | 0.88 0.32 0.32      | GTT                 |
| 2    | 70/M    | W          | III    | 1.87 2.5 1.89       | GTT                 | 20   | 51/M    | M         | IV     | 1.76 1.14 1.21      | WT                  |
| 3    | 71/F    | W          | I      | 1.57 1.22 0.74      | GAT                 | 21   | 60/M    | M         | IV     | 1.24 0.79 0.53      | WT                  |
| 4    | 54/F    | W          | I      | 1.07 0.57 0.58      | WT                  | 22   | 41/F    | M         | II     | 1.27 0.36 0.55      | WT                  |
| 5    | 66/M    | W          | III    | 1.78 1.13 1.10      | GAT                 | 23   | 60/F    | M         | IV     | 0.47 0.72 1.02      | GTT                 |
| 6    | 75/M    | M          | III    | 1.72 0.36 0.81      | GTT                 | 24   | 74/M    | M         | III    | 1.11 0.72 0.64      | GTT                 |
| 7    | 63/F    | M          | IV     | 1.09 0.50 0.62      | CGT                 | 25   | 71/F    | M         | III    | 1.39 0.72 0.67      | GTT                 |
| 8    | 63/M    | M          | I      | 1.25 0.67 0.49      | GTT                 | 26   | 61/M    | M         | IV     | 0.95 0.19 0.67      | GTT                 |
| 9    | 73/M    | M          | IV     | 1.72 0.74 1.00      | GAT                 | 27   | 73/M    | P         | III    | 1.17 0.58 0.62      | GTT                 |
| 10   | 79/M    | M          | III    | 1.15 0.72 0.57      | GAT                 | 28   | 66/M    | P         | IV     | 0.99 0.68 0.26      | WT                  |
| 11   | 73/F    | M          | I      | 1.86 1.44 0.52      | GAT                 | 29   | 72/M    | Meta IV  |        | 0.92 0.62 1.07      | GTT                 |
| 12   | 55/M    | M          | IV     | 1.05 0.32 0.38      | GTT                 | 30   | 80/M    | Meta IV  |        | 2.21 2.45 0.89      | GTT                 |
| 13   | 52/M    | M          | III    | 2.32 1.30 1.75      | WT                  | 31   | 72/M    | Meta IV  |        | 1.58 0.83 1.06      | GTT                 |
| 14   | 55/F    | M          | III    | 1.48 0.23 0.60      | GAT                 | 32   | 69/M    | Meta IV  |        | 1.11 0.36 0.87      | GTT                 |
| 15   | 63/F    | M          | IV     | 1.1 0.58 0.80       | CGT                 | 33   | 46/F    | Meta IV  |        | 1.48 1.12 1.22      | GTT                 |
| 16   | 57/M    | M          | IV     | 0.69 0.26 0.60      | GAT                 | 17   | 69/F    | M         | III    | 1.56 1.03 1.25      | GTT                 |
| 18   | 54/M    | M          | III    | 1.41 0.47 0.99      | CGT                 | 19   | 68/F    | M         | IV     | 0.88 0.32 0.32      | GTT                 |

*Histology: W, well; M, moderately; P, poorly differentiated tubular adenocarcinoma; Meta, metastatic adenocarcinoma; stage according to the classification of WHO; all the K-ras mutations involved codon 12. WT, wild type.

Figure 2 Relationship between the expression levels of the rhoC gene in ductal adenocarcinoma of the pancreas and clinicopathological findings. (A) Expressions in tumour tissues (T) and in non-tumorous portions (N); (B) expressions in primary tumours (P) and in metastatic lesions (M); (C) tumours with perineural invasion and those without perineural invasion; (D) tumours with lymph node metastasis and without metastasis. *P < 0.001; **P < 0.05

0.82 ± 0.36 and 0.49 ± 0.20 respectively) (Figure 2A). There were no significant differences in the expression levels of the rhoA or B genes between carcinoma tissues and non-malignant portions (data not shown). Metastatic lesions showed significantly higher rhoC mRNA levels (1.02 ± 0.14) than primary pancreatic carcinoma tissues (0.78 ± 0.38, Figure 2B). These levels were not different between liver metastasis and peritoneal dissemination. The expression of the rhoC gene in tumours with perineural invasion was 1.04 ± 0.34 and that in primary tumours with lymph node metastasis exhibited 0.86 ± 0.38. These expression levels were significantly higher than those without perineural invasion (0.61 ± 0.18) and those without metastasis (0.57 ± 0.09) respectively (Figures 2c and 2d).
There were no significant associations between rho gene expressions and other clinicopathological findings such as tumour size, location, age or sex (data not shown). Correlations between rho A, B and C expressions are shown in Figure 3. Gene expressions between rhoA and rhoB showed moderately positive correlation (correlation coefficient $r = 0.68; P < 0.0001$). Weakly positive correlation was observed between rhoB and rhoC expressions ($r = 0.50; P < 0.005$), and between rhoA and rhoC expressions ($r = 0.50; P < 0.005$).
As the mean value of rhoC expression in carcinoma tissues was 0.82, cases were divided into two groups at this level – high expression and low expression. Patients in the high expression group revealed significantly poorer prognosis than the patients in the low expression group (P < 0.05, Fig. 4). Although case 5 was a well-differentiated adenocarcinoma, it was one of the high-expression group with perineural invasion (Figure 5a) and lymph node metastasis (data not shown) and revealed poor prognosis (survival period was 186 days). In contrast, case 28 was a poorly differentiated carcinoma but was one of the low-expression group without perineural invasion (Figure 5b) and the patient survived for 462 days after surgical resection. No mutation was found in the fragments of the rhoA, B or C genes examined either by PCR-SSCP or by direct sequencing (data not shown).

K-ras gene mutation

Mutation of the K-ras gene was found in 27 out of 33 (81.8%) pancreatic carcinoma tissues both by PCR-SSCP and by direct sequencing. There were no mutations in any of the non-tumorous portions examined. Sequence analysis revealed that all the K-ras mutations detected were at codon 12. Mutational patterns were from GGT to GAT in 13, GTT in ten, and CGT in four cases (Table 2). The expression levels of the rhoC gene in tumour tissues with K-ras mutation were not significantly different from those without (0.81 ± 0.55 and 0.83 ± 0.32 respectively). The presence of K-ras gene mutation had no relationship with clinicopathological findings (data not shown).

DISCUSSION

In the present study we examined the expression levels of the rhoA, B and C genes, and mutation status of the K-ras gene and the three rho gene isoforms in human ductal adenocarcinoma of the pancreas. Although the rho genes have been suspected to be involved in cell transformation, there are few reports about rho gene expressions in human tumour tissues. Recently, in vitro assay revealed that activated RhoA protein is necessary for the motility of keratinocytes induced by hepatocyte growth factor (Takaishi et al, 1994) and that the invasive activity of rat MM1 cells across the mesothelial cell monolayer is inhibited by Clostridium botulinum exo-enzyme C3 that specifically inactivates Rho proteins (Imamura et al, 1996). Serum-dependent invasive activity of hepatoma cells is regulated by activated RhoA protein (Yoshioke et al, 1995). In the present study, rhoC gene expression was significantly higher in tumour portions than in non-tumour portions of the pancreas. Furthermore, tumours with lymph node metastasis and those with perineural invasion exhibited significantly higher expression of the rhoC gene than those without these manifestations, irrespective of histological grading or differentiation. Levels of the rhoC mRNA were also significantly higher in metastatic lesions than in primary pancreatic carcinomas. These suggest that overexpression of the rhoC gene occurs during pancreatic carcinogenesis and that its overexpression may be associated with the invasive characteristics of pancreatic cancer. As matched pairs of primary and metastatic lesions could not be compared in the present study, it remains to be elucidated whether overexpression of the rhoC gene is directly associated with metastatic process or not. In the current study, rho gene expressions were only moderately associated each other. RhoA and RhoC proteins are predominantly associated with the submembranous actin network and RhoB is found in association with multivesicular bodies (Robertson et al, 1995). Although rhoA and rhoC expression levels are not different in human breast cancer cell lines and normal mammary epithelial cells, rhoB expressions show a dramatic variation and are implicated in cell proliferation (de Cremoux et al, 1994). RhoA has weakly transforming activity in NIH3T3 cells (Avraham and Weinberg, 1989). The rhoB gene is an immediate–early response gene for epidermal growth factor and the v-src oncogene (Jahner and Hunter, 1991). Recently, it has been suggested that RhoC regulates microfilament organization in the apical pole of intestinal epithelial cells (Nusrat et al, 1995). Target proteins for each Rho protein could be different (Watanabe et al, 1996) and the function in carcinogenesis, if any, may be different in individual Rho proteins.

The incidence of K-ras gene mutation in ductal adenocarcinoma of the pancreas in the present study is similar to other previous reports (Almoguera et al, 1988; Smit et al, 1988; Hruban et al, 1993). It is reported that oncogenic Ras may cause some aspects of the malignant phenotype by deregulating the Rho family protein function (Khosravi-Far and Der, 1994). In the present study, the expression levels of the rho genes have not been affected by the mutational status of the K-ras gene. The activities of Ras and Rho family proteins may be coordinately regulated by dual-function proteins such as mCDC25 and p190 through GTP/GDP cycles (Khosravi-Far and Der, 1994). Thus, it remains possible that the Ras mutation status influences the Rho function by upregulating the level of the active GTP-bound form irrespective of Rho protein amount. In the current study mutational change was not detected in any of the rho genes. Mutational activation of the rhoA gene is not observed in lung, breast, colon and ovarian tumours (Moscow et al, 1994). Thus, rho genes are unlikely to be activated by mutation during pancreatic carcinogenesis. However, sequencing of entire coding regions have to be determined to confirm whether mutation exists or not in the rho genes. In conclusion, the expression level of the rho genes in ductal adenocarcinoma of the pancreas is not affected by K-ras gene mutation and rhoC gene overexpression may play a role in tumour invasion resulting in poorer prognosis.

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ABBREVIATIONS

PCR, polymerase chain reaction; RT-PCR, PCR after reverse transcription; SSCP, single-strand conformation polymorphism

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