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Identification and Cloning of Human G-Protein γ 7, Down-regulated in Pancreatic Cancer

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Differentially expressed genes between normal and cancer tissues of the pancreas were investigated using differential display. Consequently, we identified a fragment cDNA that was expressed in the normal tissue but was rarely expressed in the cancer tissue. This cDNA was screened in cDNA library prepared from the normal pancreatic tissue by rapid amplification of cDNA ends (5′RACE). 859 bp of cDNA was cloned and sequenced, and the inferred amino acid sequence was found to encode a G protein γ subunit with 98% homology to cow G protein γ 7 and complete homology to human G protein γ 7. The decreased expression of the G protein γ 7 was confirmed by Northern blot assay in twelve pancreatic malignancies which included nine duct cell carcinomas, two cystoadenocarcinomas and one blastoma. Reverse transcriptase (RT) -polymerase chain reaction (PCR) assay showed no expression of G protein γ 7 in five of six pancreatic carcinoma cell lines and two pancreatic cancer tissues. Immunohistochemical analysis also displayed positive staining in the normal tissue but no staining in the cancer tissue. The findings demonstrated that the reduced or suppressed expression of human G-protein γ 7 may play an important role in pancreatic carcinogenesis.

Key Words: **differential display; human G-protein γ 7; carcinogenesis; pancreatic cancer.**

Pancreatic cancer is a devastating cancer disease due to its biologically aggressive behavior. Most pancreatic cancers progressed invasion to the peripheral tissues and metastasis to the lymph nodes or distant organs. These characters induce early recurrence and cancer death. Therefore, understanding of the molecular pathogenesis of the pancreatic cancer would be necessary for early diagnosis and better treatment. Recently, various mutations in pancreatic cancer have been reported such as K-ras (1), p53 (2, 3), p16 (3-6), p15 (3, 5) and BRCA2 (7, 8) but little is known so far about the genetic changes that characterize pancreatic cancer. Thus we tried to identify the differentially expressed genes between pancreatic cancer and normal pancreatic tissue. For the purpose, we applied differential display which is a PCR-based method of differential expression cloning, and identified one interesting gene of human G-protein γ 7. G-protein γ is one component of heterotrimeric G-protein which transduces signals across the plasma membrane from a receptor to an effector (9-12). We herein report the identification of human G-protein γ 7 and its status of expression in pancreatic cancers and carcinoma cell lines, and discuss the significance of this gene in pancreatic carcinogenesis.

MATERIALS AND METHODS

Clinical samples and cell lines. Twelve specimens of primary pancreatic cancer were obtained at surgery. The specimens included nine cases of duct cell carcinoma, two cases of cystoadenocarcinoma and one case of blastoma. The tumor and corresponding normal specimens were immediately frozen in liquid nitrogen after resection, and kept at −90 °C until use. The human pancreatic cell lines, PANC-1 and BxPC-3 were provided from Dr. Iguthi, Clinical research institute, National Kyushu cancer center, Fukuoka, Japan. The other human pancreatic cell lines, PK1, PK8, PK9, and MIA Paca2, were provided from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan.

RNA extraction. Total RNA was prepared by a modification of the guanidinium thiocynate method as described previously (13, 14). In addition, 50 μg of total RNA was treated with 1 unit of DNase I (Message Clean Kit, Gene Hunter, Nashville, TN). The treated RNAs were dissolved to 1.0 μg / μl with diethyl pyrocarbonate treated water and then stored at −90 °C until use.

Differential display. Messenger RNA expression in 2 cases of duct cell carcinoma of the pancreas were analyzed by differential display. The cDNAs were synthesized from 2.5 μg of total RNA using anchor primers, gT15X (where X represents G, C, or A) as described previously (15). The anchor primers were end-labeled with γ^{32}P adenosine triphosphates (Amersham, Tokyo, Japan) using T4 polynucleo-

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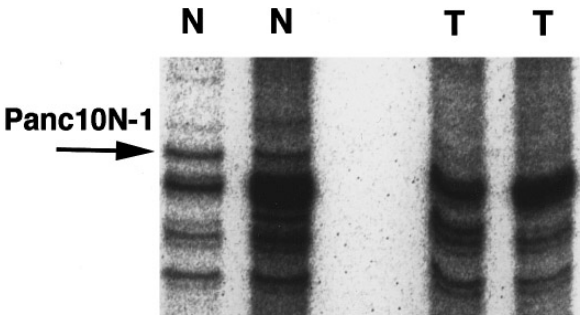

FIG. 1. Differential display of 2 cases of pancreatic carcinomas. Differential display was carried out using GT15C (5′-GTTTTTTTT-TTTTTTTC-3′) and AP-8 (5′-GTTGCGATCC-3′) A differentially expressed gene (PA10N-1) is indicated by an arrow. T means the tumor tissue and N means the normal tissue.

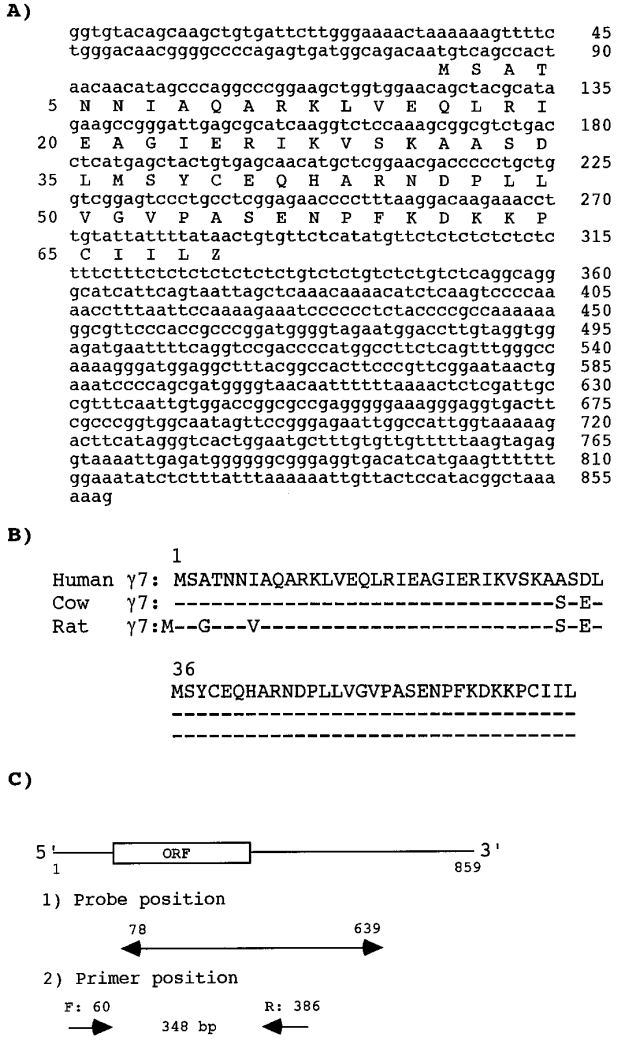

FIG. 2. A, Nucleotide sequence of the PA10N-1 obtained by 5′RACE. Open reading frame is shown at the nucleotides 79-285. Complete amino acid sequence of human G-protein γ 7 inferred from the nucleotide sequence. Sequence alignment of human G-protein γ 7 compared with other mammalian G-protein γ 7 is shown in B. Probe position for Northern blot assay and primer position are shown in C.

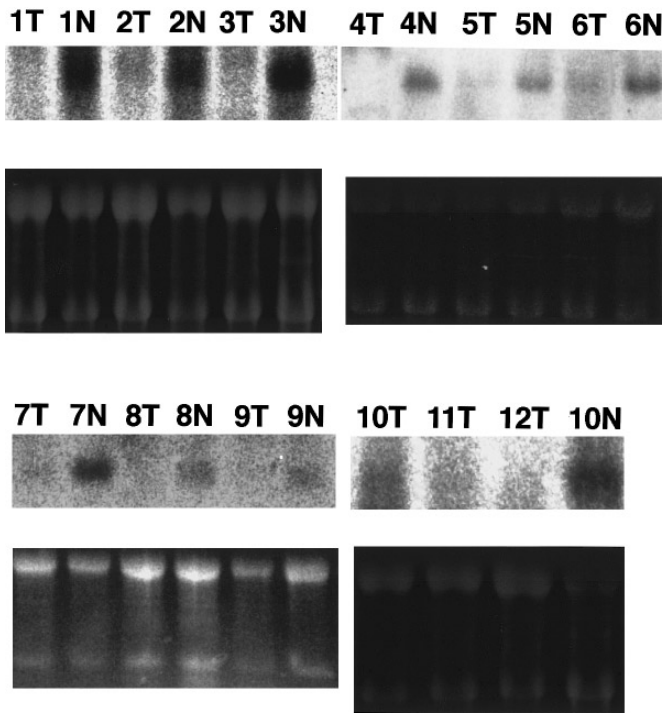

FIG. 3. Northern blot assay for G-protein γ 7 in twelve cases of pancreatic carcinoma. Ethidium bromide staining demonstrated a similar loading of undegraded RNA in each lane. Note the suppressed expression of G-protein γ 7 in tumor tissues. T means the tumor tissue and N means the normal tissue.

tide kinase (New England Biolabs, Beverly, MA). A PCR amplification of differential display was performed with 25 cycles for 1 min at 95 °C, 1min at 40 °C, 1min at 72°C in a thermal cycler ASTEC PC 800 (ASTEC, Fukuoka, Japan). The amplified cDNA was electrophoresed on 6 % polyacrylamide gels. The gels were dried and analyzed using the Bio Image Analyzer (Bas1000; Fuji, Kanagawa, Japan). Interesting bands were cut off and cloned by the TA cloning method (Invitorogen, San Diego, CA). These clones were sequenced by ABI sequencer and analyzed by the GenBank using BLAST, FAST and EST homology search programs.

5′ Rapid amplification of cDNA ends (5′ RACE) and cloning. For searching the full-length of the partial cDNAs, 5′RACE was performed by using Marathon Ready cDNA library (CLONTECH Laboratories, Inc, Palo Alto, CA) prepared from the normal pancreatic tissue. We modified the manufacture's instruction (#PT1156-1). The first PCR was performed with the adapter primer 1 (AP1) and gene specific primer 1 (GSP1) end-labeled with γ^{32}P adenosine triphosphates using T4 polynucleotide kinase. The first PCR product was electrophopresed on a 1% agarose gel. The gel was dried and analyzed using the Bio Image Analyzer. Amplified DNA fragments were cut off, then dissolved into 20 μl of distilled water and thereafter were heated at 94 °C for 5 minutes. This mixture was used for the template of the second PCR. The second PCR was performed with nested adapter primer 2 (AP2) and nested gene specific primer 2(GSP2). The first and the second PCR conditions were same, consisiting of 30 cycles with 94 °C for 1 min, 59 °C for 1 min, 72 °C for 4 min. The final amplified DNA fragments were cloned, sequenced and analyzed by the same methods as described in the explanation of differential display.

Northern blot assay in clinical samples. 30 μg of total RNA was electrophoresed using 1% agarose gel. The RNA was blotted onto a

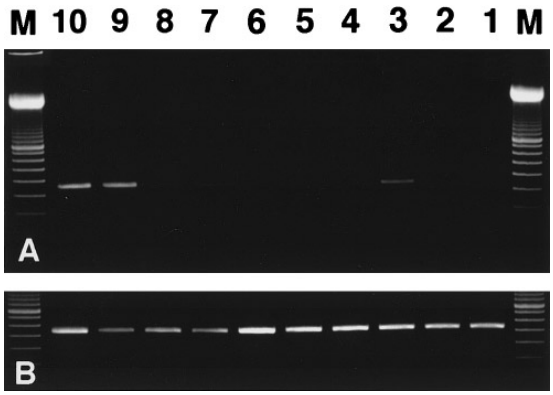

FIG. 4. The expression of G-protein γ 7 in six pancreatic carcinoma cell lines, two pancreatic cancer tissues and two normal pancreatic tissues. All pancreatic cell lines except MIA Paca 2 and two pancreatic cancer samples do not express the amplified DNA. MIA Paca 2 shows weakly amplified DNA which is lesser signal than two normal pancreatic tissues. M : Marker, Lane 1: PANC-1, Lane 2: BxPC-3, Lane 3: MIAPaCa2, Lane 4: PK1, Lane 5: PK8, Lane 6: PK9, Lane 7 and 8: Pancreatic cancer tissues, Lane 9 and 10: Normal pancreatic tissues.

nylon membrane, Hybond N+ (Amersham, Tokyo, Japan) and fixed to the membrane using Stratalinker UV cross-linker (Stratagene, La Jolla, CA). The cDNA probe was purified from agarose gels using the QIAEX II gel extraction kit (Qiagen, Chatsworth, CA) and labeled with α^{32}P dCTP (Amersham) by random primed labeling. Hybridization was performed overnight at 42 °C and the blots were washed with 1 × SSPE/ 0.25% SDS for at least 10 min. The blots were analyzed using the Bio Image Analyzer (Bas1000; Fuji)

Reverse transcriptase polymerase chain reaction (RT-PCR) in pancreatic carcinoma cell lines. The cDNAs for RT-PCR analysis were synthesized from 8.0 μg of total RNA extracted from the pancreatic carcinoma cell lines in a 30 μl reaction mixture as described previously (16, 17). The mixture was incubated at 37°C for 60 min, heated to 80°C 10 min, and chilled on ice.

The PCR amplification was carried out with 28 cycles with 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min. To ensure that the RNA was of sufficient purity for RT-PCR, a PCR assay with primers specific for the gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out in each case, except that 22 cycles were performed under the previously described oligonucleotide primers and PCR condition (16, 17).

Immunohistchemical staining. All samples were fixed in buffered formalin and embedded in paraffin. Serial sections of 5 μm thickness were cut; one section was stained with hematoxylin-eosin for morphological investigation and the other section was immunostained by the streptavidin-biotin-peroxidase method (LSAB Kit, Dako, Kyoto, Japan) (18). The procedure was based on the manufacturer's instructions. A negative control was made by exposing the section to the rabbit serum instead of the primary antibody.

RESULTS

The Differentially Expressed Gene

PCR products by the differential display were electrophoresed and each lane displayed about 30 bands. Most bands showed the same pattern between the tumor and the normal samples in all 2 cases. However, one band appeared in all normal specimens but either disappeared or substantially decreased in all tumor tissue specimens (Fig. 1). The cDNA re-amplified from this band showed about 180 bp size DNA fragment. The re-amplified cDNA was cloned by the TA cloning method. Five clones were analyzed; three clones were the same clone and named Panc 10N-1 while the other two were different clones. The sequence of Panc10N-1 was homology to one gene entered in EST program.

Identification of Human G-Protein γ 7

The Panc 10N was screened by modified 5′RACE technique. GSP1 for Panc 10N-1 was 5′CCCTCGGCGCCGGTCCACAATTGAAAC3′ and GSP2 was 5′CCCATCGCTGGGGGATTTCAGTTATTC3′. We could ob-

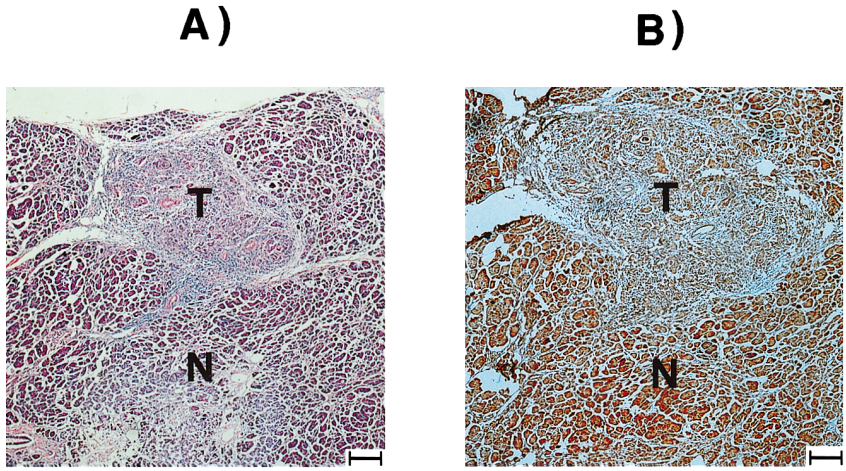

FIG. 5. Representative case of pancreatic cancer. A, Cancer (T) and normal (N) tissue of the pancreas. B, Immunohistochemical staining for G-protein γ 7 in the same area. Note the positive staining in the normal acinar and duct cells, and the negative staining in the cancer tissue. Scale bar: 120 μm.

tain finally a 859 bp cDNA which contained an open reading frame (Fig. 2). The nucleotide sequence of the cDNA showed high homology to bovine G-protein γ 7. The amino acid sequence was 98 % homology to bovine G-protein γ 7 and equal to human G-protein γ 7 which was previously reported by Ray (19). Thus we concluded that the Panc 10N-1 was the cDNA encoding the human G-protein γ 7. The nucleotide sequence data reported in this paper are available in the DDBJ, EMBL and GeneBank nucleotide sequence database with the following accession number AB010414.

Northern Blot Assay for Clinical Samples and RT-PCR for Pancreatic Carcinoma Cell Lines

To confirm the decreased expression of human G-protein γ 7 mRNA in pancreatic cancer, Northern blot assay was performed in twelve clinical samples (Fig. 3). Human G-protein γ 7 mRNA was displayed as a 1.5-kb band and its expression was decreased in cancer tissues of all cases. On the contrary, normal pancreatic tissues showed expression of human G-protein γ 7 mRNA. The probe position for G-protein γ 7 mRNA was shown in Fig. 2. RT-PCR disclosed that only one cell line, MIA Paca2 and two normal pancreatic tissues showed an amplified DNA fragment, but the other five cell lines and two pancreatic cancer tissues (duct cell carcinomas) showed no amplification (Fig. 4). Oligonucleotide primer pairs for G-protein γ 7 mRNA were as follows: up (5′- CCCCAGAGTGATGGCAGACAAT -3′) and down (5′-TTTGGGGACTTGAGATGTTTTG-3′) as shown in Fig. 2.

Immunohistochemical Staining

G-protein γ 7 stained strongly in the normal pancreatic tissue. The acinar and duct cells showed diffuse staining in the cytoplasm. In contrast, the staining intensity decreased in the carcinoma tissue (Fig. 5). The immunohistochemical results were similar to the findings of both Northern blot assay and the RT-PCR. The primary antibody for G-protein γ 7 was obtained from SANTA CRUZ BIOTECHNOLOGY, Inc (California 95060 U.S.A) and used at a dilution of 1:100.

DISCUSSION

Differential display is a rapid tool to isolate the differentially expressed genes between two or more populations (20). Most clones obtained by this method are located on the 3′ end of unknown mRNAs, then investigators need to sequence the 5′ end of these clones. We got two possible differentially expressed cDNAs which showed homology to the genes entered on the EST homology search programs(data not shown). Then we applied modified 5′RACE method to identify the amino acid coding resion of the two cDNAs. 5′RACE technique was difficult because PCR of the 5′RACE is progressed with only adapter primer and nonspecific genes are occasionally amplified. To select the gene specific PCR product, we end-labeled the gene specific primer for the first PCR with γ^{32}P adenosine triphosphates. Marathon ready cDNA libraries are useful to perform the nested PCR because two adapter primers are located on bilateral edges of inserted cDNA. This modified technique enabled us to obtain the specific 5′RACE products of the two cDNAs. We thus consider that this procedure consisting of differential display, homology search by EST programs and modified 5′RACE was a useful tool for cloning.

Differentially expressed genes between pancreatic carcinoma and normal pancreatic tissue were previously reported, such as angiogenin (21), sst2 somatostatin receptor gene (22, 23), KAI-1 (24), V-ATPase protein (25), urokinase plasminogen activator and its receptor (26), SAP-1 (27) and neogenin (28). In this study, we identified a new carcinoma related gene, human G-protein γ 7, which is down-regulated in pancreatic cancer. Heterotrimeric G-protein is composed of α, β, and γ subunits. Signal transducing elements of the G-protein are not only the α subunit which binds and hydrolyzes GTP, but the $\beta\gamma$ subunit also plays a major role in signal transmission (29-31). G-protein γ subunits determine the functional specificity of the $\beta\gamma$ subunits complex because the known β subunits are highly similar at the amino acid level, but the γ subunits are much more divergent (10, 32, 33). Additionally, the γ subunits localize and stabilize the heterotrimeric G-protein to the cellular membrane, because the γ subunits have CAAX box which is the site of C-terminal prenilation (34-37). Membrane localization of the heterotrimeric G-protein is important for signal transducing such as rhodopsin-transducin (38) and $\beta\gamma$ adenylyl cyclase interaction (29). As reported previously, G -protein γ 7 was expressed in a variety of tissues and may regulate widely distributed signal transduction pathway (19, 39).

To our knowledge, there is no report studying the expression of γ 7 gene in human cancer tissues. Our present study demonstrated that the expression of γ 7 mRNA and protein is down-regulated not only in the variety of primary pancreatic malignancies such as duct cell carcinoma, cystoadenocarcinoma, and blastoma but also in pancreatic cancer cell lines. The findings suggest that the γ 7 coupled G-proteins are associated with pancreatic carcinogenesis irrespective of histological subtypes. Association between cancer and heterotrimeric G-proteins is expected by previous reports. Mutations that constitutively activate the several α subunits by inhibiting their intrinsic GTPase activity are occurred in human endocrine tumors, such as pituitary GH secreting tumors and thyroid hyperfunctioning adenomas, where it induces a constitutive activation of the adenylyl cyclase-cAMP pathway (40). cAMP and cholera toxin which elevates intracellular

cAMP inhibit the cell growth of several carcinoma cell lines (41-44). The G protein $\beta\gamma$ subunits clearly control signals involved in cell growth, but there is no evidence for mutations of these molecules in human tumors (40). Genetic changes of ras mediated signals occasionally occur in early phase of pancreatic carcinogenesis (1), but the changes of heterotrimeric G-proteins mediated signals are unknown at present. We will further investigate the role of γ 7 coupled G-proteins for understanding the pancreatic carcinogenesis.

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