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Gene Cloning of Immunogenic Antigens Overexpressed in Pancreatic Cancer[1]

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The serological analysis of recombinant cDNA expression libraries (SEREX) by utilizing a library derived from a human pancreatic adenocarcinoma cell line and IgG antibodies from an allogeneic patient serum led to the identification of 18 genes: 13 of these were known genes, and 5 were unknown genes. In Northern and RT-PCR analyses, we found that the expression of mRNA of 14 genes was elevated in pancreatic cancer cell lines compared with the levels in normal pancreatic tissues. In addition, the expression of mRNA of hsp105 in colon cancer was greater than that in normal colon tissue. Immunohistochemical analysis using anti-hsp105 antibody revealed that an increased expression of hsp105 is a characteristic feature of pancreatic ductal and colon adenocarcinoma. Furthermore, hsp105 immunoreactivity in some cases of gastric, esophageal, and hepatocellular carcinoma was much stronger than that in normal corresponding tissues. These molecules identified may provide good diagnostic markers for cancer cells.

Key Words: **serological analysis of recombinant cDNA expression libraries (SEREX); pancreatic carcinoma; cancer antigen; heat shock protein105 (hsp105); colon carcinoma.**

Sahin *et al.* (1) introduced a new approach for identification of tumor antigens, and this approach termed serological analysis of recombinant cDNA expression libraries (SEREX), uses diluted sera from cancer patients to detect prokaryotically expressed cDNA libraries prepared from tumor cells. Novel as well as previously defined tumor antigens have been identified using the SEREX method, including MAGE-1 and tyrosinase, both originally identified by expression cloning of epitopes recognized by Cytotoxic T lymphocytes (CTL) (2, 3) CTL with specificity for NY-ESO-1, a cancer-testis antigen initially identified by autologous antibody, have been evidenced in cancer patients (4). Thus, SEREX can define immunogenic tumor antigens that elicit cellular as well as humoral immunity.

In comparison to what is known of other tumors, knowledge of tumor immunity directed against pancreatic cancer is limited. In advanced countries, pancreatic cancer represents the fifth most common cause of cancer-related death, and the incidence appears to be increasing. The prognosis for patients with pancreatic cancer is dismal, and the 5-year survival rate is low, despite advances in diagnostic and treatment modalities (5–7). The development of new treatment modalities and preventive measures are required. In the current study, we applied SEREX analysis on pancreatic cancer and characterized 18 antigens.

MATERIALS AND METHODS

SEREX. The cDNA library of human pancreatic adenocarcinoma cell line, CFPAC-1, in "Uni-ZAP" XR lambda phage vector was obtained commercially (Stratagene, La Jolla, CA). The immunoscreening method used was a modification of previously published studies (1, 8). Sera obtained during routine diagnostic or therapeutic procedures were stored at −80°C until use. To remove antibodies reactive with antigens related to the vector system, sera (1:10 dilution) were absorbed by repeated passage through columns of Sepharose 4B coupled with lysates from *E. coli* Y 1090 and bacteriophage-infected *E. coli* BNN97 (5 Prime 3 Prime, Boulder, CO). Final serum dilutions (1:800) were prepared in 1% bovine serum albumin/TBS. Recombinant phages at a concentration of 1×10^4/15 cm plate were amplified for 6 h at 42°C, covered with nitrocellulose filter, "Hybond"-c extra (Amersham, Buckinghmshire, England), pretreated with isopropyl β-D-thiogalactoside (Wako, Osaka, Japan), and incubated for an ad-

Abbreviations used: SEREX, serological analysis of recombinant cDNA expression libraries; EST, expressed sequence tag; HSP, heat shock protein; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-PCR; CLP, coactosin-like protein; Hsc, heat shock cognate.

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ditional 3 h at 37°C to transfer encoded proteins onto the filter membranes. Membranes were then blocked with 5% (w/v) nonfat dried milk (NFDM)/TBS for 15 h at 4°C. Subsequently, membranes were incubated in a 1:800 dilution of absorbed sera for 15 h at room temperature. After washing in TBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (Southern Biotechnology Associates, Inc, Birmingham, AL) for 1 h at room temperature. After incubation with "ECL" RPN 2106 (Amersham Buckinghamshire, England) for 1 min, the membranes were exposed to autoradiography film to detect antibody-reactive phage plaques. Positive clones were subcloned and retested for serum reactivity, as described above. A total of 3×10^5 recombinants were screened.

Sequence analysis of identified antigens. Positive clones were subjected to *in vivo* excision of pBluescript phagemids using the exassist helper phage/SOLR strain system (Stratagene). Plasmid DNA was purified using "ABI Prism" Miniprep Kits (PE Applied Biosystems, Foster, CA). cDNA inserts were sequenced using an ABI Prism (Perkin–Elmer, Norwalk, CT) automated DNA sequencer. Sequence alignments were determined using BLAST software (GenomeNet, Kyoto).

Reverse transcription-PCR (RT-PCR). The following eight human pancreatic cancer cell lines were used: (a) CFPAC-1, Capan-2, HPAF-II, BxPC-3, and MIA-PaCa-2 from ATCC, and (b) SUIT-2 (9) and (c) PaCa-2 and Panc-1 from Dr. Kyohgo Itoh of Kurume University, Kurume, Japan. Various cancer and normal tissues were provided from Departments of Surgery II, Kumamoto University School of Medicine. Total RNA was isolated from various tissues and cell lines using the TRIZOL Reagent (GIBCO BRL, Rockville, MD). The total RNA of some of the human normal tissues were purchased from Clontech (Palo Alto, CA). Integrity of RNA was checked by electrophoresis in formalin/Mops gels. Two micrograms of each total RNA sample were subjected to cDNA synthesis using random hexamer primer and Superscript reverse transcriptase (GIBCO/BRL, Rockville, MD). As a control for genomic DNA contamination, all cDNA synthesis reactions were set up in duplicate with the additional sample lacking reverse transcriptase. Gene-specific PCR primers were designed to amplify fragments of 500–1100 bp and used in RT-PCRs consisting of initial denaturation at 95°C for 5 min, and 30–35 amplification cycles at an annealing temperature of 55°C. KM-PA-1/apg-2 PCR primer sequences were: sense, 5′-CTGTGTTGTTTC-GGTTCCTTG-3′ and antisense, 5′-ATTGGCTCCTCATTTTCCTCA-3′. KM-PA-3/β-actin: sense, 5′-CCT-CGCCTTTGCCGATCC-3′ and antisense, 5′-GGATCTTCATGA-GGTAGTCAGTC-3′. KM-PA-4/CLP: sense, 5′-GCCATGAGCAAGAGGTCCAAG-T-3′ and antisense, 5′-ACCTTTGCCCTTCTCTGTGTCA-3′. KM-PA-5/HALPHA44: sense, 5′-TCTGTGAAACTGGTGCTGGAAA-3′ and antisense, 5′-GCTCGTGGTATG-CCTTTTCTGC-3′. KM-PA-6/unknown: sense, 5′-ACCTCCCCTTATTTCTACAAC-3′ and antisense, 5′-TTTTTATTGAACTGCCTATTT-3′. KM-PA-8/cytokeratin18: sense, 5′-CTGAGTCCTGTCCTTTCT-3′ and antisense, 5′-CCATCTGTAGGGCGTAGC-3′. KM-PA-9/polyA binding protein: sense, 5′-CCGAGATGAACCCCAGTGC-3′ and antisense, 5′-GGAGAAACATACAAAACCA-3′. KM-PA-10/VLCAD: sense, 5′-TCC-ATCCGAACCTCTGCTGTGC-3′ and antisense, 5′-ATCCCCTTCTTGTGTTTTATCA-3′. KM-PA-13/unknown: sense, 5′-AGAAACTGGTAAAAGAGAAAA-3′ and antisense, 5′-ATGAATACACAGCAGCAAGAA-3′. KM-PA-14/CGI55 protein: sense, 5′-GGCAGCAGAGAACAAGAAAAA-3′ and antisense, 5′-CACATCAGGAGCAGAA-GCACT-3′. KM-PA-16/unknown: sense, 5′-CTTTAGCAGCCTTACAAC-3′ and antisense, 5′-CCTCAAGTGATTCTCCTG-3′. KM-PA-17/dbpA: sense, 5′-GCCCCC-AGTATCGCCCTCAG-3′ and antisense, 5′-CCTGCTTTTTGCTTTATTTC-3′. KM-PA-18/hsp105: sense, 5′-TCAGTCCCCTCCTTCTTTACAG-3′ and antisense, 5′-AGATGC-CGTAGAGATGGTGAAA-3′. PCR products were visualized by ethidium bromide staining after separation over a 1.5% agarose gel. After normalization by β-actin mRNA as a control, the expression of mRNA in pancreatic cancer cell lines and in various normal and cancer tissues was compared.

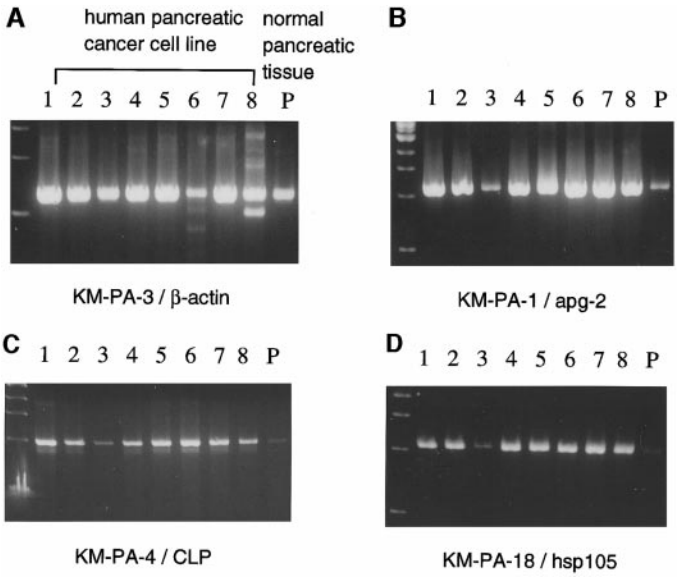

FIG. 1. mRNA expression of 4 genes encoding for SEREX-defined pancreatic cancer antigens in 8 pancreatic cancer cell lines and in normal pancreatic tissues, determined by RT-PCR, using gene-specific primers. Pancreatic cancer cell lines investigated are: Lane 1, CFPAC-1; lane 2, Capan-2; lane 3, HPAF-II; lane 4, BxPC-3; lane 5, SUIT-2; lane 6, MIA-PaCa-2; lane 7, PaCa-2; lane 8, Panc-1; and for lane P, normal pancreatic tissues were investigated. Antigens investigated were A, KM-PA-3/β-actin, 30 cycles; B, KM-PA-1/apg-2, 30 cycles; C, KM-PA-4/CLP, 35 cycles; and D, KM-PA-18/hsp105, 35 cycles.

Northern blot analysis. RNA from three pancreatic cancer cell lines, CFPAC-1, Panc-1, and PaCa-2 and normal pancreatic tissues were used for Northern blots. Human Colon Tumor Multi-Sample Total RNA Northern Blot was purchased from Biochain Institute, Inc. (Hayward, CA). Integrity of RNA was checked by electrophoresis in formalin/Mops gels. Gels with 20μg of RNA per lane were blotted onto nylon membranes, prehybridized, and subsequently incubated with the specific ^{32}P-labeled each cDNA probe overnight at 42°C in hybridization solution. The membranes were then washed at progressively higher stringency. Autoradiography was conducted at −70°C for 1–2 days by using Kodak X-Omat-AR film and intensifying screen. Thereafter the filters were stripped and rehybridized with 18S ribosomal RNA to prove RNA integrity and assess loading of equal amounts of RNA.

Immunohistochemical examination. Immunohistochemical examinations were made using the avidin-biotin complex immunoperoxidase technique with vectastain ABC-PO (rabbit IgG, mouse IgG) kit (Vector Laboratories, Inc. Burlingame, CA). The primary antibodies used in this study, mouse monoclonal anti-human β-actin (Sigma, St. Louis, MO), mouse monoclonal anti-human cytokeratin18, rabbit polyclonal anti-human apg-2, and rabbit polyclonal anti-human hsp105 (Santa Cruz, Santa Cruz, CA) were purchased. Four-micrometer-thick sections of formalin-fixed and paraffin-embedded tissue samples were deparaffinated and immersed in 1% H_2O_2 (diluted in methanol) for 30 min to inactive endogenous peroxidase. After further rinsing with phosphate-buffered saline (PBS), the sections were incubated with normal horse or goat serum for 30 min at room temperature to block nonspecific binding and then incubated with primary antibody at a dilution of (1:3400; anti-β-actin, 1:80; anti-apg-2 and anti-hsp105) for 14 h at 4°C. After wash-

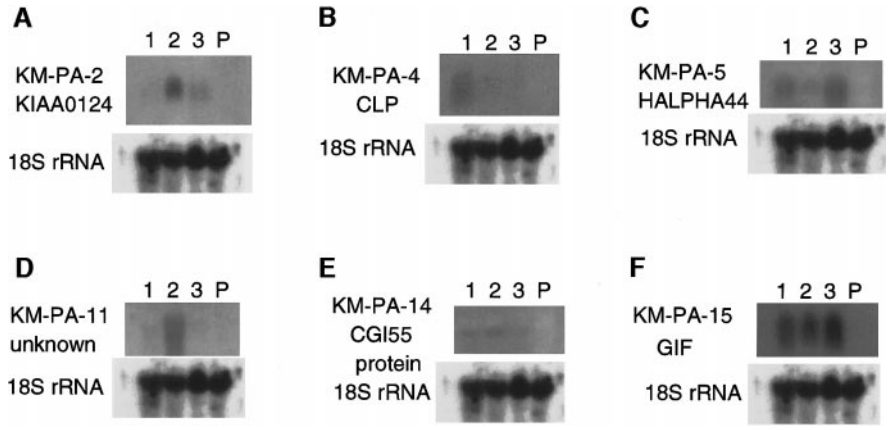

FIG. 2. Expression of 6 genes in 3 pancreatic cancer cell lines and in normal pancreas tissue, determined by Northern blot analysis. Pancreatic cancer cell lines investigated were: Lane 1, CFPAC-1; lane 2, Panc-1; lane 3, PaCa-2; and for lane P, normal pancreatic tissue. Antigens investigated were: A, KM-PA-2/KIAA0124; B, KM-PA-4/CLP; C, KM-PA-5/HALPHA44; D, KM-PA-11/unknown; E, KM-PA-14/CGI55 protein; F, KM-PA-15/GIF. Hybridization of 18S ribosomal RNA is shown in lower panel, as the control.

ing with PBS, the sections were further incubated with biotinylated anti-mouse or -rabbit IgG for 1 h at room temperature and washed in PBS. After the addition of streptavidin-biotin-conjugated peroxidase and incubation for 1 h at room temperature, the sections were washed in PBS, and the location of the proteins was visualized by incubating the sections with diaminobenzidine. Sections were counterstained with methyl green.

RESULTS

Identification of immunoreactive cDNA clones. A cDNA expression library consisting of 2.0×10^6 primary clones was prepared from CFPAC-1, a human pancreatic adenocarcinoma cell line, and 3×10^5 independent clones were immunoscreened with an absorbed allogeneic serum from a 43-year-old Japanese woman who had undergone pancreatoduodenectomy, there was a recurrence in the liver, and chemotherapy or radiation therapy were not proscribed. Sixty-three cDNA clones were identified by their reactivity to the serum, and their sequences were analyzed. Comparisons of the sequences showed that these clones represented cDNAs from 18 distinct genes, designated as KM-PA-1 through KM-PA-18. Of these KM-PA-1 was represented by 46 overlapping clones, and others by one clone each (Table 1). A homology search through BLAST software revealed that 13 of the 18 genes corresponded to previously known ones, one was expressed sequence tag (EST), and the other 4 were unknown.

Of the 13 known genes, KM-PA-1 is identical to human apg-2 (10), which belongs to the hsp110 family. It is listed on the SEREX database as NY-CO-32 or -40 and NGO-St-81, as related for screening of colon and gastric cancers. KM-PA-4 is identical to coactosin-like protein (CLP), of which gene is mapped to each end of the Smith–Magenis syndrome (SMS) common deletion region (11). Coactosin is an actin-binding protein from *Dictyostelium discoideum*. KM-PA-8/cytokeratin 18 is a cellular skeletal protein which is normally coexpressed with cytokeratin 8 and is found in most simple ductal and glandular epithelia. The majority of adenocarcinomas and basal cell carcinomas express cytokeratin 18, but most squamous cell carcinomas do not (12, 13). KM-PA-12/HLA-Cw heavy chain (MHC Class I) is listed on the SEREX database as LONY-BR-26 and related to screening for breast cancer. KM-PA-15/human glycosylation-inhibiting factor (GIF) is an immunosuppressive cytokine involved in the regulation of IgE synthesis. Secretion of bioactive GIF appears to be restricted in suppressor T cells (14). KM-PA-17/DNA binding protein A (dbpA) is a family member of the nucleic-acid-binding proteins containing a cold-shock domain, and is regarded as a transcriptional regulator (15). KM-PA-18, identical to human heat shock protein 105 (hsp105) (16), is listed on the SEREX database as NY-CO-25, as related to screening for colon cancer. Of the four unknown genes, KM-PA-16 has some homology with unknown genes listed on the SEREX database and identified in screening of gastric cancers.

Determination of gene expression of SEREX-defined pancreatic cancer antigens in pancreatic cancer cell lines and in normal pancreatic tissues. We compared the expression of genes encoding for SEREX-defined cancer antigens in normal pancreatic tissue with that

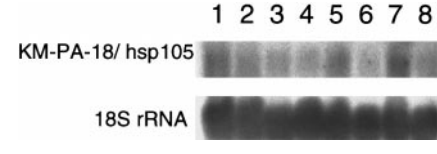

FIG. 3. Northern blot analysis of hsp105 in 4 cases of normal colon and colon cancer tissues. Lanes 1, 3, 5, and 7, colon cancer; lanes 2, 4, 6, and 8, normal colon. Hybridization of 18S ribosomal RNA is shown in lower panel, as the control.

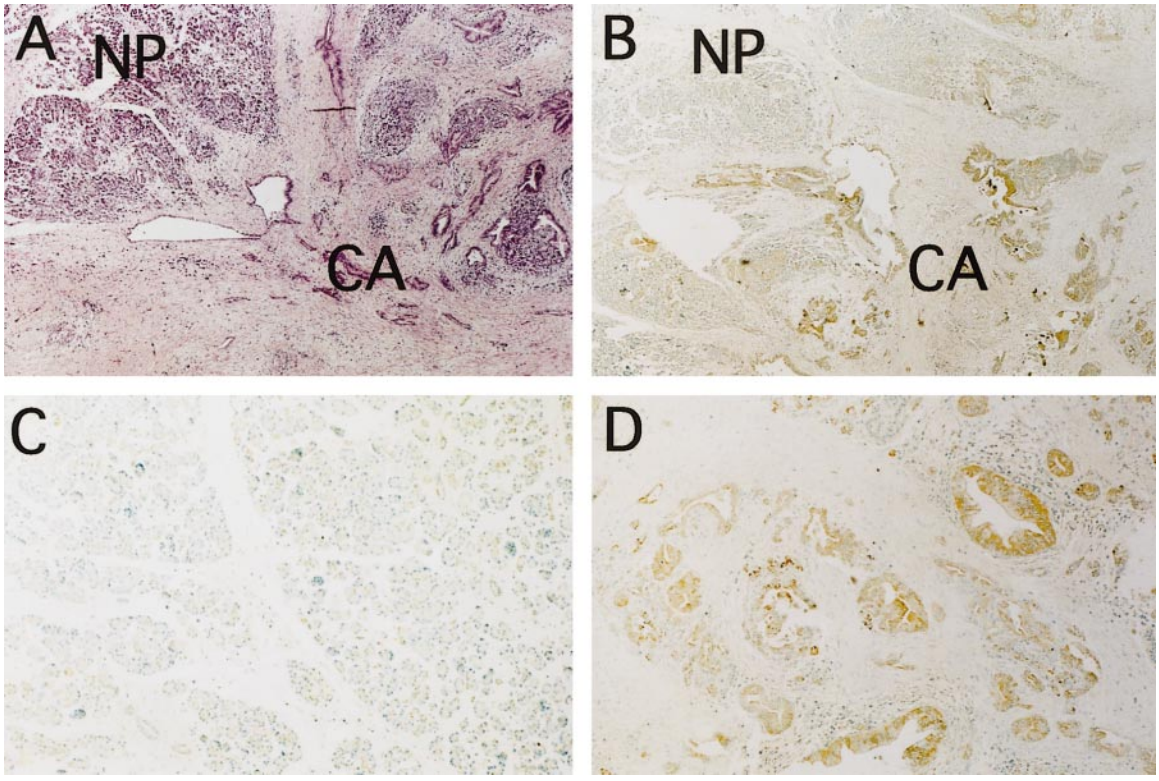

FIG. 4. Immunohistochemical analysis of hsp105 in pancreatic ductal adenocarcinoma and in the normal pancreas. (A) Hematoxylin and eosin (H&E) staining of a section, including carcinoma, stroma, and inflammatory cells in pancreatic ductal adenocarcinoma and acinar, islet and duct cells in the normal pancreas. CA, carcinoma lesion; NP, normal pancreas. Adjacent sections were used for the following staining procedures: (B) Strong hsp105 immunostaining (brown staining) was observed in the carcinoma cells. In contrast, no significant hsp105 labeling was observed in cells in the adjacent normal pancreas. (C) Observation under higher magnification revealed faint immunoreactivity of hsp105 in the cytoplasm of acinar, islet, and ductal cells in adjacent normal pancreatic lesion, albeit the intensity being much lower than that in the carcinoma cells. (D) Hsp105 immunostaining observed in carcinoma lesion, under higher magnification. As seen in B, hsp105 immunoreactivity in carcinoma cells was localized mainly in the cytoplasm. Objective magnifications, ×18 (A, B) and ×45 (C, D). Sections were counterstained with methyl green (B–D). Scale bars: (A, B) 500μm, (C, D) 200μm.

in pancreatic cancer cell lines, but not with that in pancreatic cancer tissue because pancreatic cancer tissues contained not only carcinoma cells, but also many stroma and inflammatory cells. To examine the expression of individual SEREX-defined 18 genes in 8 pancreatic cancer cell lines and in normal pancreatic tissues, we designed gene-specific PCR primers. The utility of these primers for RT-PCR was tested to show expected PCR products in 13 primer pairs. Thereby expression of 13 genes in 8 pancreatic cancer cell lines and in normal pancreas tissue was evaluated using these 13 pairs of gene-specific primers. As shown in Fig. 1A, KM-PA-3/β-actin mRNA was equally expressed in pancreatic cancer cell lines and normal pancreatic tissues, and the mRNA of KM-PA-14/CGI 55 protein and KM-PA-17/DNA binding protein A was also equally expressed (Table 2). However, we found that the expression of mRNA of KM-PA-1/apg-2, KM-PA-4/CLP, and KM-PA-18/hsp105 was specifically increased in pancreatic cancer cell lines compared with the levels in normal pancreas tissues (Figs. 1B, 1C, and 1D). The expression levels of mRNA for KM-PA-5/HALPHA4, KM-PA-6/unknown, KM-PA-8/cytokeratin 18, KM-PA-9/poly A binding protein, KM-PA-10/VLCAD, KM-PA-13/unknown, and KM-PA-16/unknown were also increased in pancreatic cancer cell lines (Table 2).

Furthermore, expression of 9 genes in 3 pancreatic cancer cell lines and in normal pancreas tissue was evaluated by Northern blot analysis. As shown in Fig. 2, we found that the expression of mRNA of KM-PA-2/KIAA0124, KM-PA-4/CLP, KM-PA-5/HALPHA4, KM-PA-11/unknown, KM-PA-14/CGI 55 protein, KM-PA-15/GIF was detected only in pancreatic cancer cell lines, but not in normal pancreatic tissue. The expression of mRNA of KM-PA-6/unknown, KM-PA-16/unknown and KM-PA-18/hsp105 were also detected only in pancreatic cancer cell lines (data not shown). Furthermore, the results of Northern blot analysis of hsp105 in 4 independent specimens of normal colon and colon cancer tissues showed that the expression levels of hsp105 mRNA in 3 cases of colon cancer were greater than those in normal colon tissues (Fig. 3). Analyses of expression in multiple normal tissues of

TABLE 1

Genes Isolated by SEREX of a Pancreatic Ductal Adenocarcinoma Cell Line

Gene designation	Gene/sequence identity	SEREX database search[a]
KM-PA-1	apg-2 (heat shock protein 110 family)	NGO-St-81, NY-CO-40, NY-CO-32
KM-PA-2	EST (KIAA0124)	—
KM-PA-3	β-actin	—
KM-PA-4	coactosin-like protein (CLP)	—
KM-PA-5	HALPHA44 (alpha-tubulin)	—
KM-PA-6	unknown	—
KM-PA-7	CDC-like kinase (CLK3)	—
KM-PA-8	cytokeratin 18	—
KM-PA-9	polyA binding protein	—
KM-PA-10	very-long-chain-acyl-CoA-dehydrogenase (VLCAD)	—
KM-PA-11	unknown	—
KM-PA-12	HLA-Cw heavy chain (MHC Class I)	LONY-BR-26
KM-PA-13	unknown	—
KM-PA-14	CGI 55 protein	—
KM-PA-15	glycosylation-inhibiting factor (GIF)	Mz19-16a, Hom-HD1-21
KM-PA-16	unknown	NGO-St-95, NGO-St-103
KM-PA-17	DNA binding protein A (dbpA)	—
KM-PA-18	heat shock protein 105 (KIAA0201)	NY-CO-25

[a] Dash means no strong homology.

mRNA for KM-PA-2/KIAA0124 and KM-PA-18/hsp105 (KIAA0201) detected by Northern blot analysis are available from web site (http://www.kazusa.or.jp/). The mRNA of KM-PA-2/KIAA0124 is especially abundantly detected in skeletal muscle, testis, and colon. As for KM-PA-18/hsp105 (KIAA0201), the mRNA level is higher in testis, brain, and lung. The expression of mRNA for KM-PA-4/CLP was detected predominantly in placenta, lung, liver, and kidney (10).

Immunohistochemical analysis of β-actin, cytokeratin18, apg-2, and hsp105 expressed in pancreatic ductal adenocarcinoma. Immunohistochemical analysis of tumors excised from 4 patients with pancreatic ductal adenocarcinoma was made using commercially available antibodies specific to 4 antigens defined in this study and all 4 tumors gave the similar results as follows. The examination with anti-β-actin antibody showed strong immunoreactivity both in carcinoma and in adjacent normal pancreas tissues. Expression of cytokeratin18 protein was stronger in normal pancreas cells than in malignant cells. Expression of apg-2 was as strong in malignant cells as in normal pancreatic cells (data not shown). On the contrary, analysis made using anti-hsp105 antibody revealed immunoreactivity exclusively in carcinoma cells but not in stroma or inflammatory cells in carcinoma lesion nor in acinar, islet, and ductal cells in the adjacent normal pancreas (Figs. 4A and 4B) as observed under a lower magnifi-

TABLE 2

mRNA Expression Detected by RT-PCR in Pancreatic Cancer Cell Lines and in Normal Pancreatic Tissues[a]

Pancreatic cancer cell line	Gene[b]												
	PA-1	PA-3	PA-4	PA-5	PA-6	PA-8	PA-9	PA-10	PA-13	PA-14	PA-16	PA-17	PA-18
CFPAC-1	+++	+++	++	+++	+	++	++	++	++	+++	+	+++	++
Capan-2	+++	+++	++	++	+	++	++	++	+	+++	−	+++	++
HPAF-II	+	+++	+	+	+	+++	+	+	−	+++	−	++	+
BxPC-3	+++	+++	++	++	++	+++	++	+	++	+++	++	+++	++
SUIT-2	+++	+++	++	++	+	−	+++	++	+	+++	+	+++	++
MIA-PaCa-2	+++	++	++	+	+	+	++	++	+	+++	++	+++	++
PaCa-2	+++	+++	++	+++	++	++	+++	+	+	+++	++	+++	++
Panc-1	+++	+++	++	+	+	+++	+++	++	++	+++	++	+++	++
Pancreas[c]	+	+++	+	+	−	+	+	+	+	+++	+	+++	+

[a] The level of expression was determined by the intensity of ethidium bromide stained RT-PCR products: +++, strong amplification; ++, moderate amplification; +, weak amplification; −, no amplification.

[b] The SEREX-defined genes are listed in Table I and "KM-" is deleted from gene designation.

[c] Normal pancreatic tissues.

cation. Observation at a higher magnification revealed faint immunoreactivity of hsp105 in the cytoplasm of acinar, islet, and ductal cells in the adjacent normal pancreatic lesion, although the intensity was much lower than that seen in the carcinoma cells (Figs. 4C and 4D). Hsp105 immunoreactivity was localized mainly in cytoplasm of the carcinoma cells (Fig. 4D).

Immunohistochemical analysis of hsp105 expressed in various carcinomas. To investigate whether an increased expression of hsp105 as proteins is a characteristic feature of pancreatic ductal adenocarcinoma or not, we then examined expression in each of 4 cases of colon, gastric, esophageal, and hepatocellular carcinomas. Hsp105 immunoreactivity in all 4 cases of colon carcinoma and 2 cases of gastric, esophageal, and hepatocellular carcinomas showed much stronger than those in normal colon, gastric and esophageal mucosae, and in the normal liver. To our surprise, in all 4 cases of colon carcinoma, hsp105 immunostaining showed the very strong cytoplasmic labeling observed in carcinoma cells. In contrast, no significant hsp105 labeling was observed in epithelial cells of adjacent normal colonic mucosa, as observed in a lower magnification field (Figs. 5A and 5B). While observation in a higher magnification field revealed faint immunoreactivity of hsp105 in epithelial cells of the adjacent non-cancerous colonic mucosa, albeit the intensity being much lower than that in the malignant cells (Figs. 5C and 5D). Hsp105 immunoreactivity was localized mainly in the cytoplasm but faint staining was also evident within the nucleus (Fig. 5D).

Immunohistochemical analysis of hsp105 expressed in various normal tissues. RT-PCR analysis revealed hsp105 transcripts in some normal tissues such as brain, lung, heart, stomach, thymus and testis (data not shown). Northern blot analysis also showed higher expression of mRNA of hsp105 in brain, lung, and testis (http://www.kazusa.or.jp/). We then examined the expression of hsp105 as proteins in various normal tissues. In immunohistochemical analysis with anti-hsp105 polyclonal antibody, hsp105 protein was evidently detected in several tissues including epithelium of esophagus and mucosa of stomach (gastric gland), visceral muscle (smooth muscle) of gastrointestinal system, lung, liver, heart (cardiac muscle) and testis. However, the intensity of staining signal in these tissues is much lower than that observed in carcinoma. In addition, the expression of hsp105 protein in brain, small intestine, colon, pancreas, spleen and thymus was weak (Fig. 6).

DISCUSSION

In the current study, we applied SEREX analysis on pancreatic cancer, and characterized 18 antigens. In these antigens, there was no cancer-testis (CT) antigen, or mutational antigen (data not shown). Some of them were overexpressed gene products in cancer cells and they are expressed in various normal tissues. Especially, hsp105 showed strong expression in pancreatic and colon adenocarcinoma.

Even cancer antigens expressed in normal tissues proved to be good candidates for cancer immunotherapy. Genes encoding tumor epitopes that are capable of inducing CTLs against adenocarcinomas and squamous cell carcinomas, for example, SART-1, SART-3, or CypB, are ubiquitously expressed at the mRNA level (17–19). As for melanoma, many epitopes are non-mutated self-peptides derived from melanosomal proteins, MART-1, gp100, or tyrosinase. Some epitopes of these antigens are highly immunogenic common epitopes in many melanoma patients. Immunization with these normal self-peptides may possibly result in autoimmune problems against melanocytes, such as vitiligo, poliosis, uveitis, dysacusis, or meningismus. However, dramatic tumor regression with only occasional vitiligo was observed in some patients prescribed immunotherapy with melanoma epitopes together with administration of Interleukin-2 (20). HER-2/neu is another cancer antigen overexpressed in breast and gastrointestinal tract cancer cells, and immunotherapy with HER-2/neu provided some achievement. Since the tumor antigen may also be present in normal tissues, there has been some concern about adverse immune reactions toward normal tissues elicited by cancer immunotherapy. However, the expression of excess amount of antigen in tumor tissues does provide an increased therapeutic ratio (21, 22). These observations suggest the possibility of some antigens defined in this study by SEREX, highly expressed in several kinds of adenocarcinoma cells, as a candidate antigen for cancer immunotherapy. However we must be careful about induction of harmful autoimmune diseases by immunotherapy using these antigens, because they were expressed in normal tissues of vital organs. To evaluate the clinical usefulness of these antigens for cancer immunotherapy, we are now examining its capacity to elicit T cell immunity directed against tumors in both human and mouse.

In our 18 antigens, there were two kinds of heat shock proteins with high molecular weight. One was apg-2, and the other was hsp105. Both of them belong to hsp110/105 family, and hsp105 was 68% identical in sequence to apg-2. The expression of apg-2 transcripts was found to be ubiquitous in various mouse tissues, the most abundant in the testis and ovary and plays a role in case of non-stress conditions (10). Hsp105 consists of hsp105α and hsp105β, hsp105α is a constitutively expressed 105 kDa hsp which is induced by a variety of stresses, whereas hsp105β is a 90-kDa hsp which is specifically induced by heat shock at 42°C. Hsp105β is a truncated form of hsp105α, and produced

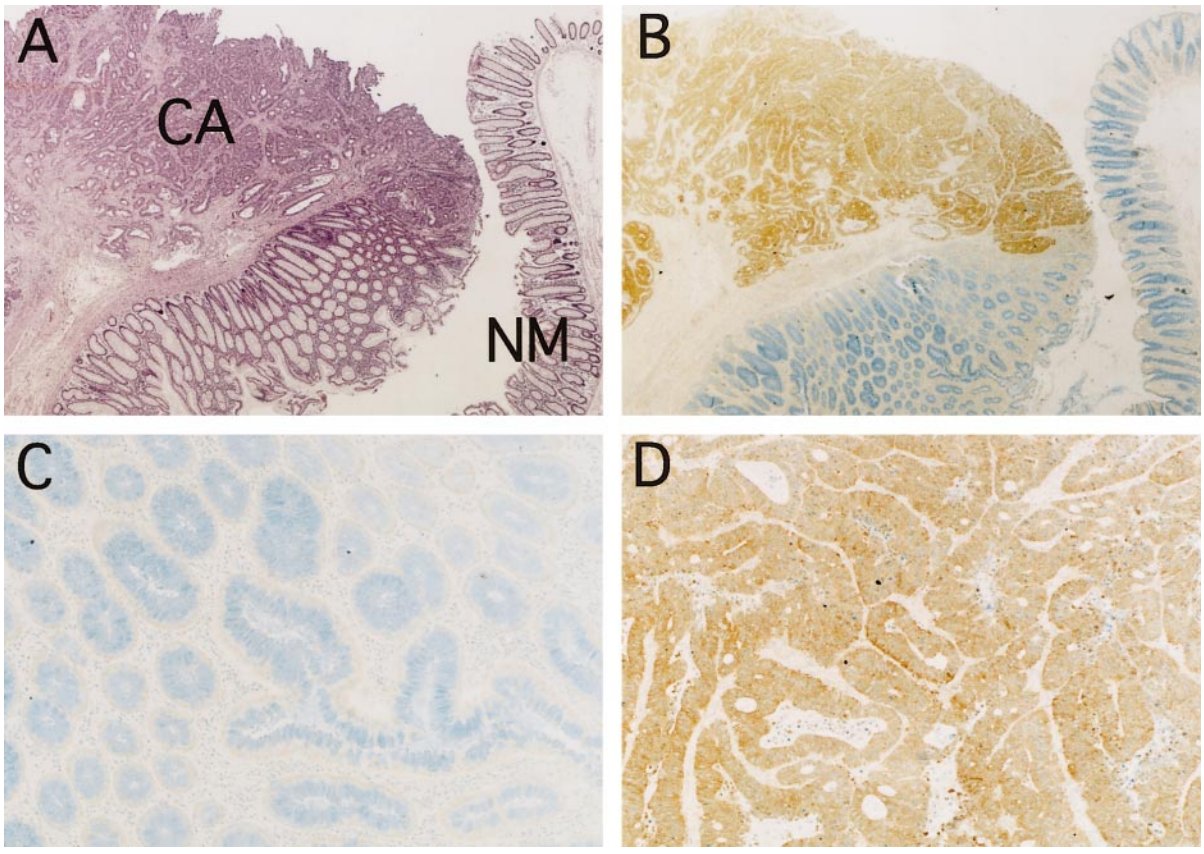

FIG. 5. Immunohistochemical analysis of hsp105 in colon carcinoma and in adjacent normal colonic mucosa. (A) H&E staining of a section, including carcinoma and epithelial cells in the adjacent normal colonic mucosa. CA, carcinoma lesion; NM, normal mucosa. Adjacent sections were used for the following staining procedures. (B) Strong hsp105 immunostaining colored brown was evident in the carcinoma cells. In contrast, no significant hsp105 labeling was observed in epithelial cells in the normal colonic mucosa. (C) Observation under higher magnification revealed that epithelial cells in adjacent noncancerous colonic mucosa showed faint immunoreactivity of hsp105, albeit the intensity being much lower than in carcinoma cells. (D) Hsp105 immunostaining observed in carcinoma lesion under higher magnification. As seen in B, hsp105 immunoreactivity in carcinoma cells was localized mainly in the cytoplasm, with faint nuclear immunoreactivity. Objective magnifications: ×9 (A and B), and ×90 (C and D). Sections were counterstained with methyl green (B–D). Scale bars: (A, B) 1 mm, (C, D) 100 μm.

by alternative splicing of hsp105α gene (15). Hsp105 is expressed in most adult mouse tissues, but level is the highest in brain (23). Transient increases in levels of hsp105 during mouse embryogenesis suggests that this protein has an important role in development of the mouse embryo (24). Hsp105 was found to exist as complexes associated with hsc70/hsp70 in cultured mouse cells, under both nonstressed and stressed conditions (25). However, the function of apg-2 and hsp105 remains to be proved.

On the other hand, several hsps, such as hsp70, hsp90, and gp96 can bind endogenous peptides including tumor antigenic peptides processed by ubiquitin-proteasome pathway and can be used for cancer immunotherapy. Immunotherapy of mice with preexisting cancers with hsp70 or gp96 preparations derived from autologous cancer cells resulted in activation of tumor-reactive CTL and retarded progression of the primary cancer, a reduced metastatic load and prolongation of the life-span. Immunization with endogenous hsp-peptide complexes might elicit pathological autoimmune responses. However, in several hundreds of mice that have been immunized with tumor-derived GP96, no adverse consequences indicative of autoimmune phenomena have been detected (26). These observations may also suggest the possible usefulness of apg-2 and hsp105 for cancer immunotherapy.

We found that in spite of little expression of hsp105 protein in normal colon and pancreas, when they transformed to be carcinoma, the expression of hsp105 proteins surprisingly increased. This result shows the possibility of hsp105 as a tumor marker of colon and pancreatic adenocarcinoma. To evaluate this clinical usefulness of hsp105, it may be reasonable to set up enzyme-linked immunosorbent assay (ELISA)-detection of hsp105 or anti-hsp105 antibodies in sera of cancer patients and normal donors. It is also important to investigate a relationship between carcinogenesis and increased expression of hsp105.

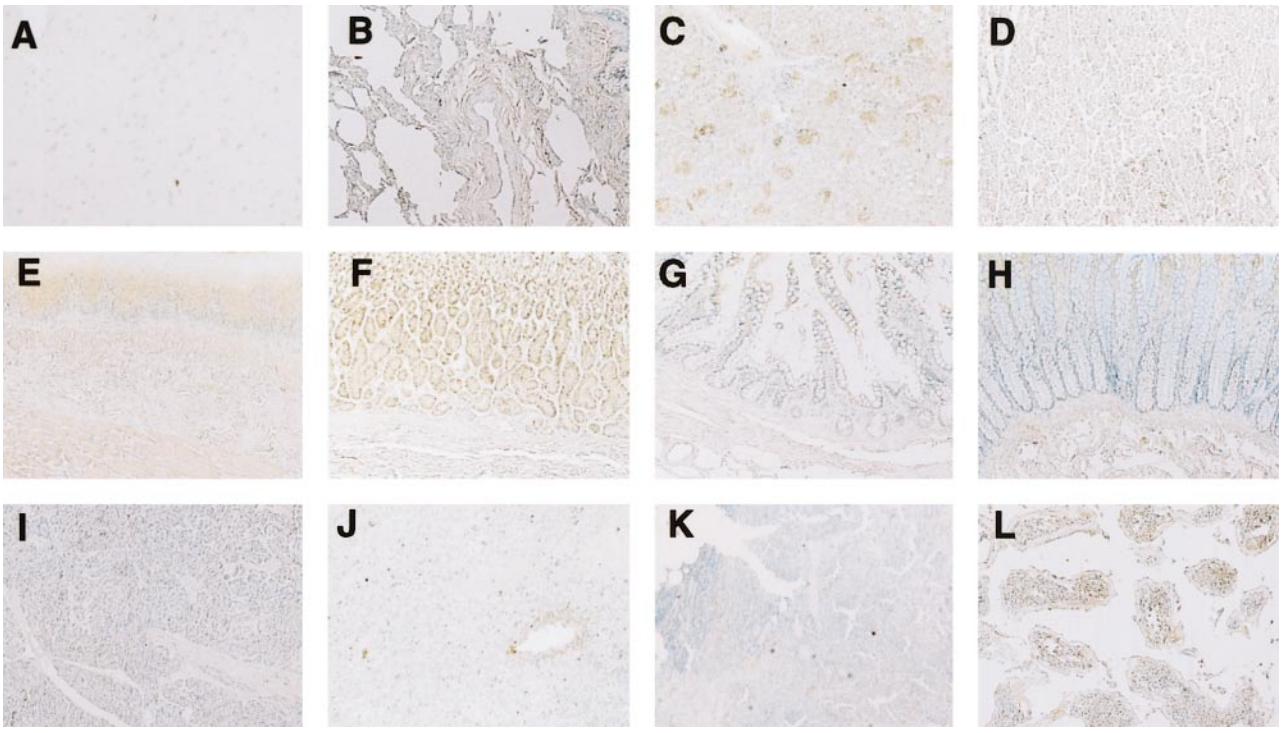

FIG. 6. Immunohistochemical analysis of hsp105 expressed in various normal tissues. A, brain; B, lung; C, liver; D, heart; E, esophagus; F, stomach; G, small intestine; H, colon; I, pancreas; J, spleen; K, thymus; L, testis. Hsp105 protein was evidently detected (brown staining) in several tissues including epithelium of esophagus and mucosa of stomach (gastric gland), visceral muscle (smooth muscle) of gastrointestinal system, lung, liver, heart (cardiac muscle), and testis. However, the intensity of staining signal in these tissues is much lower than that observed in carcinoma. In addition, the expression of hsp105 protein in brain, small intestine, colon, pancreas, spleen, and thymus was weak.

Taken together, these results suggest that some antigens defined in this study by SEREX, may be a pertinent cancer antigen for future cancer-diagnosis and related immunotherapy.

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