nantly peripheral. To a lesser extent, the hypoxic cell radiosensitizers cause central neurotoxicity and are thus limited in use.

PR-350 (Doranidazole),[8,9] a novel radiosensitizer for hypoxic cells, is a 2-nitroimidazole nucleoside analog and is reportedly less neurotoxic than etanidazole. PR-350 has the ability to sensitize radioresistant tumor cells to the lethal effects of ionizing radiation under extremely hypoxic conditions, and an alternative cell death mechanism following damage by oxygen free radicals, other than necrosis, is now under consideration. This new radiosensitizer PR-350 holds much promise as a way of providing effective anticancer activity in hypoxic tumor cells that are resistant to the usual radiotherapy, and its use in clinical cancer therapy is already in progress. A randomized, controlled trial has been conducted with the aim of clarifying the effects of PR-350 and IOR on locally advanced pancreatic cancer.

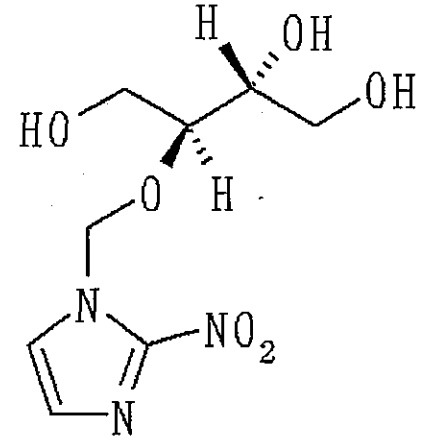

FIGURE 1. Structural formula of PR-350.

PATIENTS AND METHODS

Eligibility

The following patient enrollment criteria were established: (1) Patients must be 20–75 years of age; (2) patients must have a performance status (PS) of 0–2, which projects the survival period to be >3 months; (3) tumors must be unresectable due to invasion to the arterial system and/or peripancreatic nerve plexus; (4) maximal diameters of tumors must be less than that required for radiotherapy; and (5) there is an absence of liver metastasis, other organ metastasis, and peritoneal seeding. Other exclusion criteria were (1) previous radiation therapy or chemotherapy; (2) idiosyncrasy to drugs, including contrast media; (3) presence of serious cardiovascular, pulmonary, renal or hepatic disease; (4) coexistence of an active neoplasm; and (5) any conditions that the physician believed may preclude the trial.

When written informed consent to participate in the trial was obtained from patients who met the above eligibility criteria by preoperative examination, including abdominal CT, ultrasonography, chest x-rays, and routine laboratory tests, patients were registered as potential candidates at the trial central office no later than 1 day before scheduled laparotomy. This was a prospective, randomized, closed-label, controlled study of IOR with or without radiosensitizer PR-350. Patients were randomized and notified by fax. Final eligibility was determined based on operative findings by laparotomy.

Study Design and Treatment

Radiosensitizer PR-350 was synthesized at Pola Chemical Industries, Inc. (Kanagawa, Japan). PR-350 is a 2-nitroimidazole nucleoside analog with a $CH_2OCH(CH_2OH)CH(OH)CH_2OH$ side chain at the N1 position. Its structural formula is illustrated in Figure 1.

After laparotomy, a biopsy specimen from each participant was analyzed to confirm diagnosis. Infusions of PR-350 or placebo were strictly controlled to obtain a suitable concentration for radiotherapy; 2000 mg/m^2 of PR-350 or placebo was infused systematically for ~25 minutes before administration of IOR. Ten to 40 minutes after the completion of PR-350 or placebo administration, the patients were referred to the radiation room and received 25 Gy of IOR. Two weeks following surgery, all patients also received EBRT. The total planned dose of 40 Gy was delivered in 20 fractions, with 2 Gy per fraction and 5 fractions per week, using 10–14-MV photons. The radiation fields included the primary tumor and a margin of 1–3 cm covering the regional lymph nodes; radiation fields were defined using treatment-planning computed tomography 1 or 2 days prior to treatment.

Following completion of EBRT, patients were followed up through outpatient services. Every month, tumor size on restaging CT was compared with initial CT tumor size. The committee for evaluating therapeutic effect measured changes in tumor size, and tumor marker values and laboratory parameters were evaluated every month.

No additional therapy, including chemotherapy and immunotherapy, was allowed for 6 months after IOR treatment.

RESULTS

Patient Characteristics

Between July 1999 and March 2002, 81 patients were fully informed and registered to participate in this clinical study. Thirty-three patients were ineligible due to operational findings of peritoneal seeding, liver metastasis, or extended tumors. Ultimately, 48 patients were enrolled in the trial and administered either PR-350 or placebo. Patient characteristics are given in Table 1. The differences between the 2 treatment groups were not statistically significant.

Toxicity/Tolerability

In regard to any toxicity of PR-350, 1 patient had elevated ALP and patients in the control group experienced nau-

TABLE 1. Patient Characteristics

Characteristics	Control Group	PR-350 Group
Patients (n)	25	22
Gender (n)		
male	20	15
Female	5	7
Age		
Median	61.3	61.1
Range	50–74	45–74
TNM stage		
IVa	22	17
Ivb	3	5

sea and constipation after surgery with IOR. At day 14, abnormal values of RBC, Hb, Ht, Plt, A/G ratio, T-bil, T-chol, UA, Crt, Na, Cl, and Ca were observed, compared with preoperative values. There was no significant difference between the 2 patient groups. All patients, except 1 from the control group, were determined to be negative for toxicity, and the PR-350 compound was considered to be safe.

Efficacy

The efficacy of IOR with PR-350 in the treatment of pancreatic cancer was evaluated using CT examination. Figure 2 illustrates changes in the tumor regression rates of the 2 groups. The committee for evaluating efficacy reported that 9 of 19 patients (47.4%) in the PR-350 group showed effective response, compared with 5 of 23 patients (21.7%) in the control group ($P = 0.1067$, Fisher analysis). At 6 months following radiation administration, the mass reduction rate in the PR-350 group showed significant improvement ($P = 0.0274$).

The tumor marker values in both the PR-350 and control groups suggested therapeutic effects in 15 of 22 patients (68.2%) and 17 of 25 patients (68.0%), respectively, indicating no significant difference between the 2 groups.

Survival

By the time of the last follow-up in July 2003, 17 patients from the PR-350 group and 24 patients from the control group had died of the disease. Survival curves of the 2 trial groups are shown in Figure 3. The median survival period of the PR-350 group was 318.5 days and that of control group was 303.0 days. One-year survival rates of the PR-350 and control groups were 36.4% and 32.0%, respectively. Although the PR-350 group did not show significantly better survival than the control group, 4 of 22 PR-350 patients were alive >2 years after the trial ended, compared with 1 of 25 patients in the control group.

DISCUSSION

In a phase 1 study,[10] we tested the safety and efficacy of the hypoxic cell sensitizer PR-350 in conjunction with IOR in patients with advanced adenocarcinoma of the pancreas. PR-350 was delivered intravenously to the pancreas at a dose of 400–2000 mg/m^2, in conjunction with IOR of 20–40 Gy. In 15 patients treated with PR-350, blood concentrations of PR-350 decreased rapidly, at a mean 50% disappearance time of 3.6–5.0 hours. Eighty-five percent of PR-350 was eliminated without any degradation through the urine. One patient had slight elevation in GOT and GPT values, and another patient experienced a slight decrease in blood pressure with bradycardia. Both patients recovered without any clinical problems, and the adequate dose of PR-350 was determined to be 2000 mg/m^2. Based on this phase 1 study, we designed the double-blind clinical trial with the intent to clarify the effect of PR-350 combined with IOR on patients with locally advanced pancreatic cancer and without liver metastasis.

Although the PR-350 group did not show significantly better survival than the control group in this clinical study, 5 of 22 patients are still alive, and 4 (18.2%) survived for >2 years. This demonstration of extended patient survival is remarkable

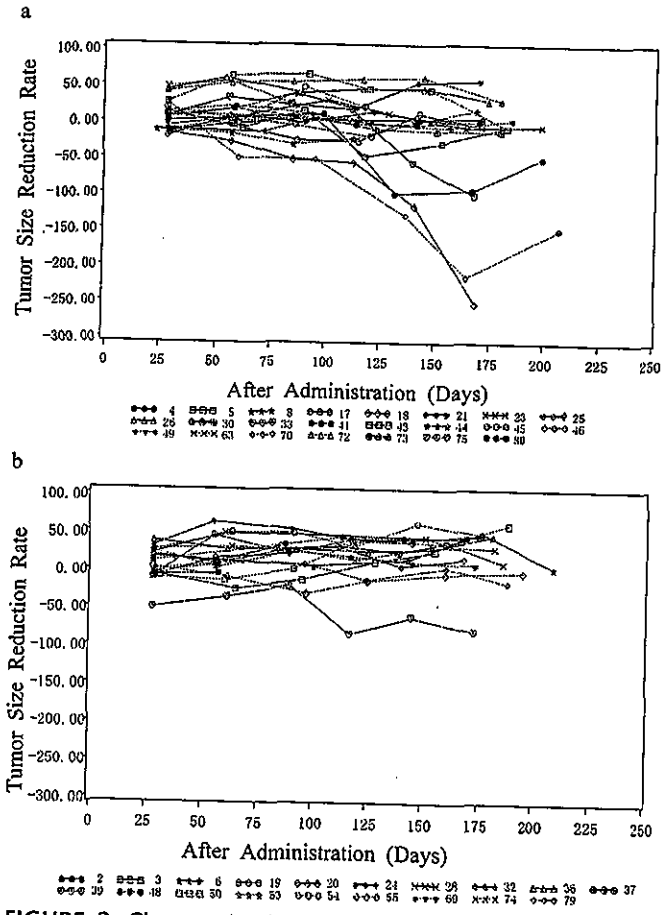

FIGURE 2. Changes in the tumor reduction rates, as determined by CT examination of the control group (A) and PR-350 group (B).

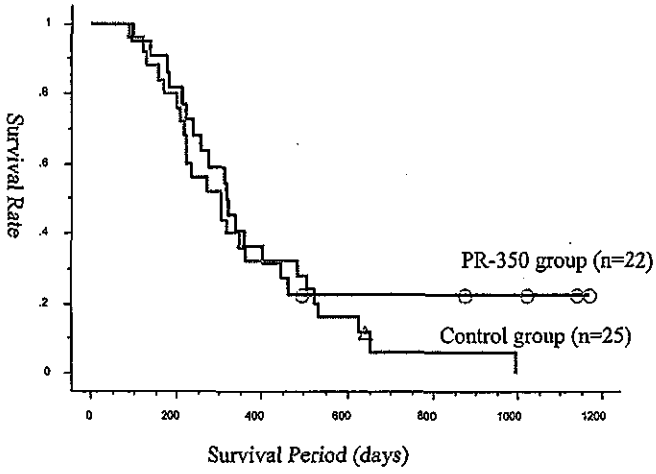

FIGURE 3. Kaplan-Meier analysis of the survival curves of the PR-350 and control groups.

in comparison with other reports.[11,12] Li et al[13] conducted a randomized, controlled study on the efficacy of gemcitabine (GEM)-concurrent chemoradiotherapy (CCRT) and 5-FU–CCRT in treating locally advanced cancer. GEM-CCRT appeared to be significantly more effective than 5-FU–CCRT; however, only 1 of 18 patients (5.6%) survived for >2 years. The median survival for 5-FU–CCRT patients was 201 days, which is a considerably shorter period than the 303-day median survival seen in the control group from our study.

It should be emphasized that organ metastasis, including that of the liver, which is frequently seen in pancreatic cancer, is often the cause of death. Since occult liver metastasis that is not detectable during surgery and radiochemotherapy grows rapidly in a couple of months, it is reasonable that the survival curve of the PR-350 group matched that of the control group in the early period of the trial just following radiotherapy. Recent improvements in the best supportive care and use of a novel chemotherapy agent of GEM have helped patients with pancreatic cancer and metastatic liver tumor(s) survive ~1 year. The survival curves after 1 year between the 2 groups in this trial vary widely, which suggests that radiotherapy with PR-350 contributes to local control of primary regions. Chemotherapy with PR-350 seems to be a promising strategy to treat patients without distant metastasis by controlling primary tumor into dormant status.

CONCLUSION

Recent progress in molecular biology and genomic engineering has made possible the evaluation of tumor characteristics. Tumor sensitivity to anticancer agents can now be evaluated before development of a treatment plan and selection of drugs, which brings us closer to patient-tailored therapy. Tumor sensitivity to radiotherapy also varies across pancreatic tumors, as shown in the study of chemotherapy. It is important to clarify the mechanism of tumor resistance to radiotherapy to develop a useful and harmless strategy for individual patients. In addition, future studies are needed to clarify the mechanism of the efficacy of radiotherapy accompanied by radiosensitizer PR-350, so that a more effective strategy for pancreatic cancer may be established.

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Advancements in Pancreatic Cancer Research in Japan and Unfolding Prospective

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In commemorating the 35th anniversary of the founding of the Japan Pancreas Society (JPS), we are highlighting the significant contributions of our Japanese investigators in the advancement of the fields of epidemiology, pancreatic carcinogenesis, early diagnosis, and surgical and medical management of pancreatic cancer. In addition, future research and clinical direction in the areas of gene therapy, immunotherapy, and other new horizons in pancreatic carcinogenesis are emphasized. Each key expert presents his or her accomplishments in respective fields as they relate to current knowledge of the biology, diagnosis, and treatment of pancreatic cancer.

One of the most significant accomplishments in pancreatic cancer research in Japan was the establishment of the National Pancreatic Cancer Registry by the JPS nearly a quarter of a century ago. This monumental project, involving 350 participating institutions, resulted in a unique and encompassing database created by providing standardized criteria and terminology, facilitating comparison of clinical and pathologic data, and analyzing the treatment outcomes of over 23,000 cases in the Registry. The wealth of information generated from the success of this Registry led to the first English edition of the *Classification of Pancreatic Carcinoma*, published initially in 1996, and its more recent second edition, published last year. The Japan Pancreas Society also published the fifth Japanese edition of the *General Rules for the Study of Pancreatic Cancer* in 2002.[1–3]

The National Pancreatic Cancer Registry permitted comparison of the survival curves according to stage in the classification of pancreatic cancer between the JPS and the Union International Contre le Cancer (UICC). These comparisons revealed that stratification in the JPS classification system is much better than in the UICC system and may indicate that the JPS system is more reliable in predicting outcomes. It is also noteworthy that the prognosis of pancreatic cancer is defined by the histology and extent of disease and that preoperative histologic diagnosis and diagnostic imaging are fundamental in the management of this malignancy. In this Japanese Registry, there were 822 identified cases with tumors less than 2 cm in diameter (TS1 pancreatic cancer). However, small-size pancreatic cancer does not necessarily mean early-stage pancreatic cancer. Thirty-seven percent of small pancreatic cancer cases already demonstrate lymph node metastases, and 8% have N3 metastasis. Nevertheless, the Japanese investigators have encountered stage 1 disease with an excellent (58%) 5-year survival rate. Advances in diagnostic imaging, intraoperative radiation therapy, chemotherapy and immunotherapy, and knowledge of pancreatic endocrine neoplasia and intraductal papillary mucinous tumors are also reviewed in this issue.

Most pancreatic cancer originates in the ductal epithelial cell of the exocrine pancreas. However, based on embryologic and functional evidence, there is extensive interaction between the exocrine and endocrine pancreas during carcinogenesis, an event considered to be a metabolic disease. Although most of our clinical effort and activities currently focus on the diagnosis and treatment of the disease, research evidence has indicated that the development of pancreatic cancer progresses over several years.[4] This stage is called intraepithelial neoplasia (PanIN) or carcinoma in situ.[5,6] The best approach to improving survival for pancreatic cancer patients involves the detection of early-stage tumors, preferably in the PanIN state.

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The PanIN classification formed the foundation for a series of recent molecular analyses that helped define the prognosis model for the phases of pancreatic neoplasia with genetic mutations or alterations, such as K-*ras* gene mutation, abnormal expression of p53, inactivation of DPC4, or inactivation of p16. These have become the biomarkers that can be detected in tissue, serum, and pancreatic juice and are currently being evaluated for their specificity and sensitivity in the early diagnosis of pancreatic cancer as well as for their role in the progression of pancreatic adenocarcinoma. The new horizons and frontiers in pancreatic carcinogenesis and genomic research are also being investigated by our Japanese basic and clinical investigators with the hope that the knowledge gained in these areas will provide us with more useful tools in our goal toward the prevention and better treatment of pancreatic cancer.

Pancreatic cancer remains the malignancy with the worst prognosis among solid tumor carcinomas: the worldwide 5-year patient survival rate is less than 5%. This special issue of *Pancreas* successfully focuses on the key contributions from Japan and provides a wealth of Japanese experiences in the management of this malignancy. It also identifies new frontiers for future investigations that may help in the prevention and appropriate future management of pancreatic cancer.

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Oncolytic Virotherapy as a Novel Strategy for Pancreatic Cancer

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Abstract: We have developed a novel gene therapy that targets genetic alterations in pancreatic cancer using oncolytic replication–selective adenoviruses in tumor cells. E1B-55kDa-deleted adenovirus (AxE1AdB) can selectively replicate in *TP53*-deficient human cancer cells but not cells with functional *TP53*. Consecutive injection with AxE1AdB markedly inhibited the growth of human pancreatic tumors in severe combined immunodeficiency disease mice. Furthermore, AxE1AdB displayed the ability to enhance gene expression as a virus vector. It is reported that uracil phosphoribosyl transferase (UPRT) overcomes 5-FU resistance. The therapeutic advantage of a replication-selective adenovirus that expresses UPRT (AxE1AdB-UPRT) was thus evaluated in an intraperitoneum-disseminated tumor model. Combined treatment with 5-FU and AxE1AdB-UPRT dramatically reduced the disseminated tumor burden without causing toxicity in normal tissues. We also clarified the process of AxE1AdB-inhibited tumor angiogenesis through the preserved E1A region: an adenoviral E1A protein binds to pRB, forcing the quiescent cell into the S phase. We constructed a double-mutant, replication-selective adenovirus (AxdAdB-3) containing a mutation in the RB-binding motif of the E1A region and a deletion of large E1B-55kDa. AxdAdB-3 swiftly induced cancer cell death in vitro and showed a potent antitumor effect in vivo. These results strongly suggest that AxdAdB-3 possesses a wider therapeutic potential than previously believed, given that most pancreatic cancers have abnormalities in both the TP53 and RB pathways.

Key Words: gene therapy, replication-selective adenovirus, TP53, RB, uracil phosphoribosyl transferase

(*Pancreas* 2004;28:326–329)

Although gene therapies have proven effective against pancreatic tumors in in vivo experiments,[1–4] sufficient therapeutic effects have not been established in clinical trials. Currently, adenovirus vectors that lack genes essential to viral replication are the most favorable gene therapy tools in the clinical treatment of cancer. The major difficulties of this approach involve transfecting adenovirus into every cancer cell of a tumor and effectively inducing gene expression. These limitations result in an incomplete antitumor effect and subsequent regrowth of tumors. Increasing the titer of the adenovirus is an alternative method that increases gene expression in solid tumors, including those of pancreatic cancer. At the same time, however, this strategy brings with it the adverse effects of adenovirus gene therapy.

In an attempt to increase the antitumor effect and efficiency of gene expression and delivery, various groups have experimented with replication-competent viruses. Adenovirus E1 gene products, in addition to transactivating other early gene promoters, prepare the cellular environment for optimal viral replication by associating with a number of key cell cycle proteins. Replication-selective viruses may overcome the limitations of gene transfer of conventional adenoviral vectors. Viral replication in a small fraction of tumor cells leads to amplification and extension of the antitumor effect of gene expression. Cell death is due exclusively to viral replication and cell lysis.

In this paper, we introduce a novel therapeutic strategy for pancreatic cancer involving an oncolytic replication–selective adenovirus. It is demonstrated that oncolytic replication–selective adenoviruses are also useful as vectors of antitumor genes because they significantly increase gene expression in tumors only.

REPLICATION-SELECTIVE ADENOVIRUS TARGETING TP53 ABNORMALITY

A malignant tumor develops in a multistep process that results from the mutation of several specific genes involved in the control of cell growth and programmed cell death. The *TP53* gene is mutated in more than half of human tumors,[5] which indicates that it plays a key role as a tumor suppressor. Because TP53 is functionally inactivated in many human tumors, including pancreatic tumors,[6] and because the prognosis of patients with LOH on 17p is worse when pancreatic cancer is present,[7] transduction of the wild-type *TP53* gene into can-

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cer cells using a virus vector is an attractive therapeutic strategy for pancreatic cancer. However, one of the limitations of this strategy and other cancer gene therapies is the low efficiency at which therapeutic genes can be delivered in vivo, especially into solid tumors.

To replicate, small DNA tumor viruses encode proteins to shut down the *TP53* gene. In an adenovirus, the E1B-encoded proteins E1B-19kDa and E1B-55kDa are responsible for turning off *TP53*. The former acts downstream of TP53 to prevent apoptosis, whereas the latter physically associates with TP53 to prevent TP53-mediated transactivation.[8] The E1B-55kDa protein binds to TP53 and prevents it from stimulating the promoters of growth arrest genes such as P21 and GADD45.[9,10]

The widely used adenovirus vector with a deletion of E1 (essential to replication) is unable to replicate in infected cells. Several research groups have demonstrated that when the E1A-deleted adenovirus vector is linked to a plasmid expressing the E1A gene, it is able to replicate by transcomplementation of the E1A gene product. An adenovirus vector containing the E1A-expressing plasmid can amplify adenovirus vector–mediated transduced genes such as luciferase,[11] thymidine kinase,[12] and lacZ.[13] If the E1B-55kDa–deleted adenovirus were used as a helper virus, it would also be able to amplify the effects of an adenovirus vector carrying a therapeutic gene. To develop further strategies of gene therapy for cancer, we constructed the E1B-55kDa–deficient adenovirus (AxE1AdB) and examined the effects of a combination of this mutant adenovirus and other adenovirus vectors on pancreatic cancer cell lines.[14] AxE1AdB produced a stronger oncolytic effect against pancreatic cancer cell lines when compared with *TP53* gene therapy using a replication-incompetent adenovirus vector. Co-infection with AxE1AdB and the E1-deficient adenovirus expressing the reporter *lacZ* gene resulted in the replication of both viruses and a marked increase in reporter gene expression in pancreatic cancer cells without TP53 function. Pancreatic cancer cells infected with both the E1B-55kDa–deficient adenovirus and the adenovirus vector for human interleukin-2 (AxCAhIL-2) produced 110 times more IL-2 than cells infected with AxCAhIL-2 alone. Moreover, injection of the pancreatic cells with AxE1AdB and AxCAhIL-2 resulted in a complete regression of the established tumors (Fig. 1).

It is reported that uracil phosphoribosyl transferase (UPRT) overcomes 5-FU resistance by catalyzing the synthesis of 5-fluorouridine monophosphate (FUMP) from uracil and phosphoribosylpyrophosphate (PRPP). The antitumor effect of 5-FU is enhanced by augmenting 5-fluorodeoxyuridine monophosphate (FdUMP) (converted from FUMP), which inhibits thymidylate synthetase (TS). We studied the effectiveness of gene therapy using adenovirus-mediated UPRT in overcoming the 5-FU resistance seen in pancreatic cancer.[15] Transduction of the UPRT gene resulted in an increase of FdUMP and subsequent sensitivity of various pancreatic cancer cells to 5-FU. Although in vivo gene transduction of UPRT followed by administration of 5-FU resulted in regression of intraperitoneal pancreatic tumors, the high dose of adenovirus needed to obtain a complete reduction of the tumors produced adverse effects, including severe diarrhea with dehydration. FdUMP, which is converted from 5-FU in normal mucosal cells, inhibits cell growth, thereby causing gastrointestinal toxicity. We manufactured a replication-competent adenovirus expressing UPRT (AxE1AdB-UPRT). As expected, selective replication and amplification of the UPRT gene did occur in cells with abnormal *TP53* genes. In contrast, human fibroblast cells with normal *TP53* genes appeared to be resistant to the replication of AxE1AdB-UPRT. The restricted replication-competent adenovirus augmented the antitumor effect without producing adverse effects, in contrast to a replication-incompetent adenovirus.

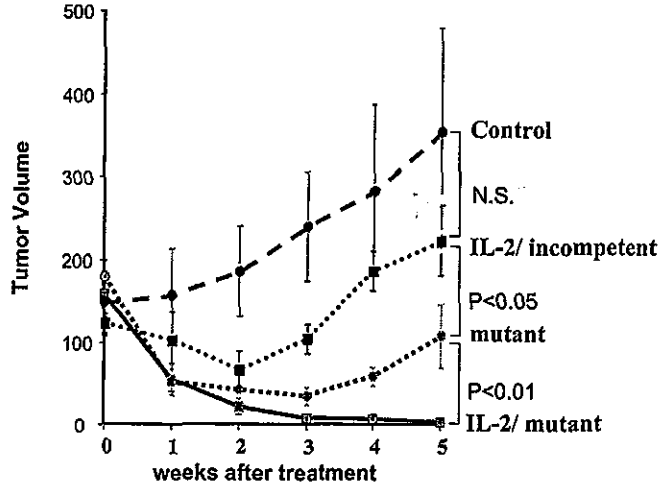

FIGURE 1. Tumor growth in SCID mice was treated with AxE1AdB and AxCAhIL2. Treatment with AxE1AdB plus AxCAhIL2 caused significant tumor regression compared with AxCAhIL2 alone or AxE1AdB alone.

This replication-selective adenovirus is useful in laparoscopic examination and aids in the diagnosis of tumor staging. We confirmed its efficacy using a mouse model of intraperitoneally disseminated pancreatic cancer. Injection of this virus and the GFP-expressing adenovirus vector made it possible to detect peritoneal dissemination and metastasis to lymph nodes, which were stained with green color and observable under fluorescence, as shown in Figure 2.

ANTIANGIOGENESIS EFFECT OF E1B-55kDa–DELETED ADENOVIRUS

E1A proteins, which are divided into 2 subtypes, bind to various cellular proteins such as CREB-binding protein (CBP)[16] and p300,[17] and have a significant role in the inhibition of angiogenesis. Aggressive tumors, including those of

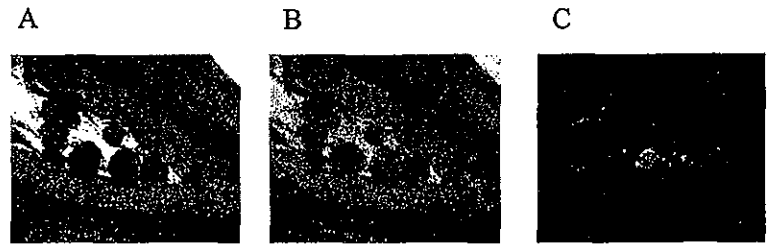

FIGURE 2. Detection of the abdominal dissemination of tumors. Disseminated pancreatic tumors (A) were stained with green color under fluorescence (C). B: Image was taken under light and fluorescence.

pancreatic cancer, often have an insufficient blood supply, partly because tumor cells grow faster than endothelial cells and partly because the newly formed vascular supply is disorganized. When tumor cells are exposed to hypoxia, hypoxia-inducible factor-1α (HIF-1α) is stabilized and activated to promote the transcription of several genes, such as vascular endothelial growth factor (VEGF).[18] In the presence of E1A hypoxia-induced VEGF, however, the binding of E1A to p300/CBP inhibits mRNA synthesis.

We speculated that the replication-selective adenovirus has an inhibitory effect on tumor angiogenesis through E1A-mediated inhibition of the p300 function. To analyze this antiangiogenesis property, we infected pancreatic cancer cells with several mutant constructs and demonstrated that the oncolytic replication–selective adenovirus (AxE1AdB) inhibits the production of VEGF in vitro and neovascularization in vivo.[19] VEGF and HIF-1α expression in various cancer cells in hypoxia was confirmed. Under hypoxic conditions, there was less HIF-1α protein in cancer cells infected with AxE1AdB than in cells infected with the mutant E1A-type adenovirus. In vivo, the cancer cells infected with AxE1AdB were significantly inhibited in contrast to the angiogenesis in flank skin and microvessel count by immunohistochemical staining of mutant E1A CD31. These results suggest that the E1A region preserved in the adenovirus AxE1AdB inhibited tumor angiogenesis not only by binding with p300 but also by participating in the degradation process of HIF-1α protein under hypoxic conditions. Since several groups have developed new oncolytic replication–selective adenoviruses, the findings from this study on E1A have important and immediate implications for future projects in developing and refining gene therapy for cancer.

E1A-MUTATED ADENOVIRUS

The E2F family of transcription factors is required for transcription of several genes involved in DNA and deoxynucleotide synthesis by inducing the G1-S transition of cell cycle.[20] The binding of 2 proteins, RB and a related protein p107, to E2F inhibits its ability to activate transcription. The RB protein was initially identified as the product of the prototype tumor suppressor gene *RB*.[21] The underphosphorylated form of RB may act as a growth suppressor by blocking exit from the G0 or G1 phase. Phosphorylation of RB inhibits its growth suppression function, allowing the cell to enter the S phase. The mechanisms of action of the *RB* gene have been clarified through studies of the E1A oncogene of human adenovirus type 5. The binding of proteins, such as adenovirus E1A or SV40 large T-antigen gene products, to RB negates the requirement of RB phosphorylation and allows quiescent cells to enter the cell cycle.

Recently, another adenovirus with mutation in the E1A region was reported to be an alternative mode of cancer therapy. It is hypothesized that an adenovirus with a deletion in the RB-binding (CR2) region of E1A or with mutations in the p300-binding region (CR1) of E1A selectively replicates in cancer cells with defects in the RB pathway (eg, *RB* mutation, *P16* loss, cyclin D amplification).

We expected that an E1A mutant adenovirus unable to bind pRB would be selectively replicated in tumor cells with dysregulated cell cycles but would not be replicated in normal cells, which have tightly regulated cell cycles. We constructed a double-mutant, replication-selective adenovirus (AxdAdB-3) that contained a mutation in the RB-binding motif of the E1A region and the same E1B-55kDa deletion as AxE1AdB.[22] The effect of AxdAdB-3 on pancreatic cancers was evaluated both in vitro and in vivo. AxdAdB-3 induced cancer cell death efficiently in vitro and had a more potent antitumor effect in vivo (Fig. 3). These results strongly suggest that AxdAdB-3 has great therapeutic potential since most pancreatic cancers have abnormalities in both the TP53 and/or RB pathways and may be a promising new tool in gene therapy.

DISCUSSION

Cancer therapy employing viral replication has been previously reported. Patients with cervical cancer have been treated with direct injection of wild-type adenovirus,[23] which then replicated and destroyed cancer cells. However, wild-type viruses are able to replicate in both tumor cells and normal cells, and systemic manifestation of viral disease was observed in some immunocompromised patients. The E1B-55kDa–deleted adenovirus ONYX-015 (ONYX Pharmaceuticals, Richmond, CA) can selectively replicate in tumor cells[24] without requiring any additional procedures. ONYX-015 is able to replicate in and lyse TP53-deficient human tumor cells but not cells with functional TP53. Moreover, the released viruses infect neighboring cells, and their subsequent proliferation re-

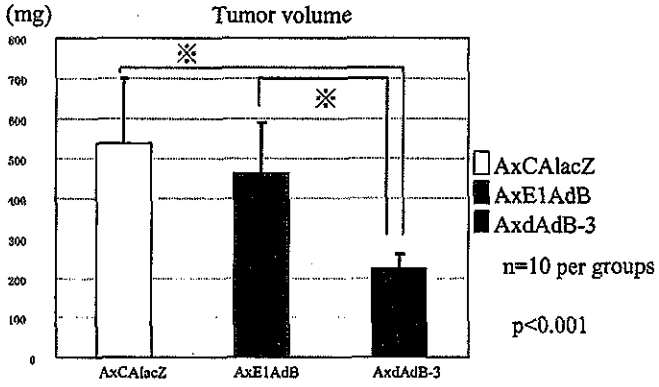

FIGURE 3. Therapeutic effect of oncolytic replication–selective adenovirus AxdAdB3. Tumor weight in animals treated with AxdAdB3 was significantly reduced when compared with the control (AxCAlacZ) and AxE1AdB groups.

sults in a destructive cycle. This is characteristic of mutant adenoviruses, distinguishing their use in cancer therapy from other gene therapy approaches, which are limited due to the nonreplicating character of conventional virus vectors. Phase I clinical testing of ONYX-015 began in April 1996 in patients with head and neck cancer[25] and has continued in patients with pancreatic cancer[26] and liver metastasis of colorectal cancer.[27]

There are several theoretical advantages to using oncolytic replication–selective adenoviruses over replication-defective adenoviruses in cancer gene therapy. Our studies have clarified that replication-competent adenoviruses are not only strong weapons in and of themselves but that they are also useful carriers of genes that possess antitumor activity because they are virus vectors specific to tumors without normal TP53 function or intact RB pathways. Whether these experimental results are universally valid requires confirmation in future clinical trials.

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Chromosome 12, frequently deleted in human pancreatic cancer, may encode a tumor-suppressor gene that suppresses angiogenesis

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Several lines of evidence have suggested that the long arm of chromosome 12 may carry a tumor-suppressor gene(s) that plays a role in pancreatic ductal carcinogenesis. We have previously found a significant association between loss of heterozygosity of the 12q arm and a poor prognosis in pancreatic cancer patients. In this study, we introduced a normal copy of chromosome 12 into some pancreatic ductal carcinoma cells. Both anchorage-dependent and -independent proliferations as well as invasiveness were similar throughout the hybrid clones when compared with their corresponding parental cells. In sharp contrast, significant suppression of tumorigenesis was observed after inoculation of the hybrid clones into nude mice. Measurements made up to 1 month later showed that there was a significant delay in the growth of tumors into which the introduced normal copy of chromosome 12 had been restored. More significantly, using our dorsal skin chamber and an intravital microscopy system experiment in SCID mice, we demonstrated and visualized directly that implantation of the hybrids failed to promote the angiogenic phenotype encountered in the parental cells. Gene expression profiling using the complementary DNA microarray system identified a set of 24 genes differentially expressed between the hybrids and parental cells. An additional set of 18 genes was also identified that were differentially expressed between the hybrid clone that lost its growth-suppression activity and one that retained such activity. Another set of 25 genes mapped on 12q was detected that showed high expression levels in the hybrid clones retaining growth-suppressive activity. In summary, this study provides the first functional evidence of the existence of an additional tumor-suppressor gene(s) on chromosome 12, whose absence is responsible for the pathogenesis in pancreatic ductal carcinogenesis.
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Ductal adenocarcinoma is the most frequent malignancy arising in the pancreas. Although the incidence of this disease is only 3.6% of the cancer cases in Japan, the number of cancer deaths caused by this disease accounts for up to 6.4% of the total (http://www.ncc.go.jp/en/statistics/2001/index.html). The mean 5-year survival rate of this disease is poor; it is below 5% in Japan (http://www.mc.pref.osaka.jp/ocr_e/ocr/index.html#survival) and worldwide.[1] This poor prognosis is partly due to the lack of symptoms arising only at the late stage; nearly 80% of pancreatic cancer patients already harbor metastases at the time of diagnosis. Detection of small, resectable cancers would improve the outcome of

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this deadly disease;[2] but the optimal approach to early detection of pancreatic cancer has not yet been established. Thus, acquisitions of efficient approaches for accurate detection at the earliest stages as well as development of efficient methods for treatment are among the tasks with the highest priority in conquering pancreatic cancer.

Tumor-suppressor genes (TSGs) and their products are attractive candidates as molecular targets for early genetic diagnosis because their functional losses should be followed by switching toward a malignant phenotype. Moreover, there is the possibility of inventing valuable techniques for clinical management of this disease by supplementation of the lost functions of TSGs. Despite the continuous progress in molecular biology, the genetic events involved in the initiation and progression of pancreatic ductal adenocarcinoma remain largely unclear. Cytogenetic, allelotype, and somatic cell hybrid studies in human cancers have suggested that chromosome 12 may carry a TSG(s) that plays a role in the carcinogenises of prostate,[3,4] stomach,[5–7] male germ cells,[8] and pancreas.[9–11] Furthermore, we previously demonstrated that 12q-loss of heterozygosity (LOH) is significantly associated with a poor prognosis in patients with pancreatic cancer.[12] *DUSP6* on 12q was found to be inactivated in pancreatic cancer,[13] and introduction of this gene-induced apoptosis.[14] However, no structural abnormality was observed in this gene, and its localization was outside the smallest region of overlap (SRO). Hence, there is a possibility of the localization of an unknown TSG(s) on 12q that is associated with a poor prognosis in pancreatic cancer patients. To address this possibility and to isolate and characterize the TSG(s) on 12q, we first tried to demonstrate the factor on chromosome 12 that harbored tumor-suppressor activity by means of introduction of a normal copy of chromosome 12.

Materials and methods

Cell Lines

The pancreatic cancer cell lines used were PCI-35 and MIAPaCa2; the former is a generous gift from Dr Hiroshi Ishikura at Hokkaido University, and the latter was purchased from the American Type Culture Collection (Manassas, VA, USA). Chromosome 12 was introduced in these cells by the microcell-mediated chromosome transfer (MMCT) method (see below). Cells were cultured according to the protocols of the suppliers. The parental cell lines were previously well mutationally characterized.[15] The normal human fibroblast cell line MRC-5 (American Type Culture Collection) and the mouse A9 cell line (provided by Japanese Cancer Research Resources Bank) were maintained according to the suppliers' protocols. All cells were routinely monitored for Mycoplasma as well as for mouse hepatitis, Sendai, and pneumonia viruses and were consistently negative.

MMCT

MRC-5 fibroblast cells were transfected with pSV2neo plasmid DNA and then selected in DMEM medium containing 400 μg/ml G418 (GibcoBRL, Grand Island, NY, USA). Cell hybrids of G418-resistant human fibroblast cells and mouse A9 cells were fused, selected, and pooled as described elsewhere.[16] MMCT experiments were performed as described[16,17] using as donors A9H(12) hybrids containing an MRC5 human chromosome 12 tagged with a neomycin-resistance gene, thus allowing clonal selection and expansion in medium containing 400 μg/ml of G418. The resulting final hybrids, five stable clones for each recipient, were named as follows: PCI-35H(12)-1 and -2, and MIAPaCa2H(12)-1 through -3, respectively.

Microsatellite Analysis

Genomic DNA from the A9H(12), parental cell lines, their hybrids, and the corresponding nude mice tumors was analyzed with highly polymorphic microsatellite markers, as described previously.[9] A panel of microsatellite markers was selected that spaced at approximately 10-cM intervals along the long arm of chromosome 12 as follows: *D12S1701* (12q12), *D12S88* (12q21), *D12S1719* (12q21), *D12S360* (12q22), *D12S78* (12q23), and *D12S366* (12q24). *D12S336* on 12p was also used as the control for the short arm marker. Nucleotide sequences of the markers and conditions for PCR have been described previously.[18] The PCR products were separated by running in 6% polyacrylamide/8 M urea/32% formamide gel, followed by fixation in 5% acetic acid/5% methanol for 30 min, drying on a 3 mm filter paper (Whatman Inc., Clifton, NJ, USA) and autoradiography. For each marker, two independent PCR amplifications labelling forward and reverse primers, respectively, were carried out to confirm the results.

Fluorescence *In Situ* Hybridization (FISH)

FISH analysis was carried out as previously described.[10] Briefly, the parental cells and their hybrids were prepared in a metaphase spread by hypotonic treatment and fixation in Carnoy's solution. Dual-color FISH was performed by using two different regional probes for 12q21: b605B21 and b759H8.[19] As the corresponding centromere-specific probe, we used α12H8 corresponding to *D12Z3* (purchased from ATCC, Rockville, MD, USA). BAC DNAs were labelled with biotin–16-dUTP (green signals), and the centromeric probe was labelled with digoxigenin–11-dUTP (red signals). Fluores-

cence detection of the signals was performed with antidigoxigenin–tetramethylrhodamine isothiocyanate (TRITC) and avidin–fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Mannheim, Germany) followed by counterstaining using 4′,6-diamino-2 phenylindole (DAPI) in an antifade solution. At least 100 nuclei for every spread were analyzed, and an average TRITC/FITC ratio profile was estimated.

Proliferation Assays

Anchorage-dependent proliferation was monitored by an MTT assay for 5 days in the absence of G418, and a daily proliferation index (PI) was calculated for each parental and corresponding hybrid cell line by the methods described by van Golen *et al*.[20] In all assays, 1000 cells in 100 μl suspension of each cell type were plated and incubated in wells of five flat-bottomed 96-well plates. The conversion of MTT to formazan dye was spectrometrically measured for absorbance at 590 nm using a multiwell plate ImmunoReader System (Molecular Dynamics, Inc., Sunnyvale, CA, USA). All experiments were performed in duplicates of eight and repeated at least twice. For each cell line, the PI was estimated as previously described.[21] Data from two independent experiments were pooled, averaged, and then statistically analyzed.

For anchorage-independent proliferation, 10 000 cells of each parental and hybrid line were plated in 1 ml medium containing 0.3% Bacto-agar (Becton Dickinson, Sparks, MD, USA) with 10% fetal bovine serum (FBS) as an upper layer into 30-mm dishes. Another 1 ml medium with 0.7% Bacto-agar and 10% FBS was used for the bottom layer. Dishes were maintained in a humidified 5% CO_2 atmosphere at 37°C and fed biweekly with 0.3 ml medium. After 21 days, 0.3 ml of 1 mg/ml INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride) solution (Dojindo Laboratories, Kumamoto, Japan) was added in each dish and further incubated for another 3 h. The viable, red-stained colonies were photographed using a Zeiss microscope (Carl Zeiss, Göttingen, Germany). Both colony number and size were measured and averaged on three randomly chosen photographs from each plate by using public domain NIH1.62 software. Every anchorage-independent growth was assessed in triplicate by two independent experiments.

Tumorigenicity in SCID Mice

Female SCID mice, 5 weeks old, purchased from Clea Japan Inc. (Tokyo, Japan) were maintained under pathogen-free conditions and used in accordance with NIH and Tohoku University Medical School institutional guidelines. Logarithmically growing cells trypsinized from subconfluent monolayers were suspended in medium containing 25% Matrigel Growth Factor Reduced (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 1×10^7 cells/ml. For each inoculation, 3×10^6 cells in 0.3 ml suspension were injected s.c. into the hind flanks of nude mice. For every pair of cells, inoculations were performed in three mice. The tumor volume was estimated by the formula: $V = 0.4Dd^2$ (V = tumor volume, D = longitudinal diameter, and d = latitudinal diameter) at the time of biweekly measurements. Data from two independent experiments were pooled for statistical analysis.

In Vivo Microscopy

The dorsal transparent chamber and *in vivo* microscopy system are described elsewhere.[22] A total of 1×10^6 cells of either parental cells or their corresponding hybrids were implanted into the mice. Tumor vessel formation was observed for 3 weeks after tumor cell implantation. Images were captured by a CCD camera (TEC-470 Optronics Co., Chelmsford, MA, USA) attached to a microscope (Nikon, Tokyo, Japan), and recorded on a Super VHS video recorder (Victor, Kanagawa, Japan). Finally, the images were analyzed and prepared off-line using Avid VideoShop 3.0.2 (Avid Technology Inc., Tewksbury, MA, USA) and Adobe Photoshop 5.0.2 (Adobe Systems Inc., San Jose, CA, USA) software.

Immunohistochemistry

Resected specimens were fixed with 4% paraformaldehyde solution overnight. After embedding in OCT compound, the specimens were frozen at −80°C. We used 4 μm sections from frozen specimens for immunohistochemical staining. Anti-mouse CD31 antibody (BD Biosciences-Pharmingen, San Diego, CA, USA) diluted 100-fold in PBS was used as the primary antibody and incubated for 1 h at room temperature. Then the peroxidase-conjugated anti-rat IgG antibody (BD Biosciencies-Pharmingen) was used for the secondary antibody reaction and incubated for 30 min at room temperature, followed by a reaction with AEC reagents (Vector Laboratories, Burlingame, CA, USA) for 10 min at room temperature. AEC reagents were used as chromogens, and hematoxylin was used for counterstaining.

Microarray Analysis

Total RNAs were extracted from the cultured hybrids and their corresponding parental cells using an RNeasy Midi Kit (QIAGEN, Valencia, CA, USA), and the messenger RNAs (mRNAs) were refined from the total RNAs with an Oligotex-dt30 mRNA purification kit (TAKARA, Kyoto, Japan) according to the suppliers' protocols. Cy3- and Cy5-labelled

complementary DNA (cDNA) probes for hybridization were prepared from refined mRNAs with a CyScribe First-Strand cDNA Labelling kit and a CyScribe GFX Purification kit (Amersham Biosciences, Piscataway, NJ, USA) according to the supplier's protocols. We used cDNA microarray slides fabricated by spotting 23 040 unique cDNAs purchased from Amersham Biosciences on Type 7 slides using the Gen III Array Spotter (Amersham Biosciences). The cDNAs were selected from UniGene database,[23] including expressed sequence tags (ESTs), and prepared by PCR-amplification with unique primers. The Lucidea Microarray ScoreCard system (Amersham Biosciences) containing 32 control samples including 11 human housekeeping genes and several artificial cDNAs was used for checking dynamic range and variations of signal intensities according to the supplier's instructions. Each cDNA was spotted in duplicate. Each 10 pmol of labelled probes were hybridized by using Automated Slide Processor according to the supplier's instructions (Amersham Biosciences). The hybridized slides were scanned with a GenePix 4000A scanner (Axon Instruments, Union City, CA, USA). The scanned image was converted to intensity values using GenePix Pro software (Axon Instruments). Duplicated hybridization experiments were carried out for all samples to confirm the results. Standardization of signals was carried out by using the Lucida Microarray ScoreCard software according to the supplier's instructions. The standardized data were analyzed using the GeneSpring software (Silicone Genetics, Red Wood City, CA, USA). Interspot normalization was carried out by dividing sample signal values by control channel values. Interslide normalization was carried out by dividing each signal value by median values of 11 housekeeping genes. We selected genes whose expression showed more than 1.5-fold difference, either higher or lower, with a statistical significance of less than 0.05 in probabilities by ANOVA and *t*-test. Significant differences between parental cells and hybrids were accepted to be less than 0.05 by ANOVA and *t*-test as provided in the software. Annotated information about genes was obtained by using the GeneSpider program provided in the software. Grouping of genes according to ontology was carried out by using the GeneOntology program provided in the software.

Reverse Transcription-PCR

Total RNAs extracted from cell pellets were used for reverse transcription reactions with SuperScript II RNase H-reverse transcriptase (Invitrogen, Inc., San Diego, CA, USA) according to the method described previously.[24] For semiquantitative reverse transcription-PCR, concentrations of template cDNAs were adjusted to give the same quantity by *β2-microglobulin* mRNA measured by ethidium bromide staining in agarose gel electrophoresis. Sequences of primer and optimized conditions for reactions are available upon request. For quantitative RT-PCR, we designed specific primers and fluorescence-labelled probes for the *RAB21* with the primer express

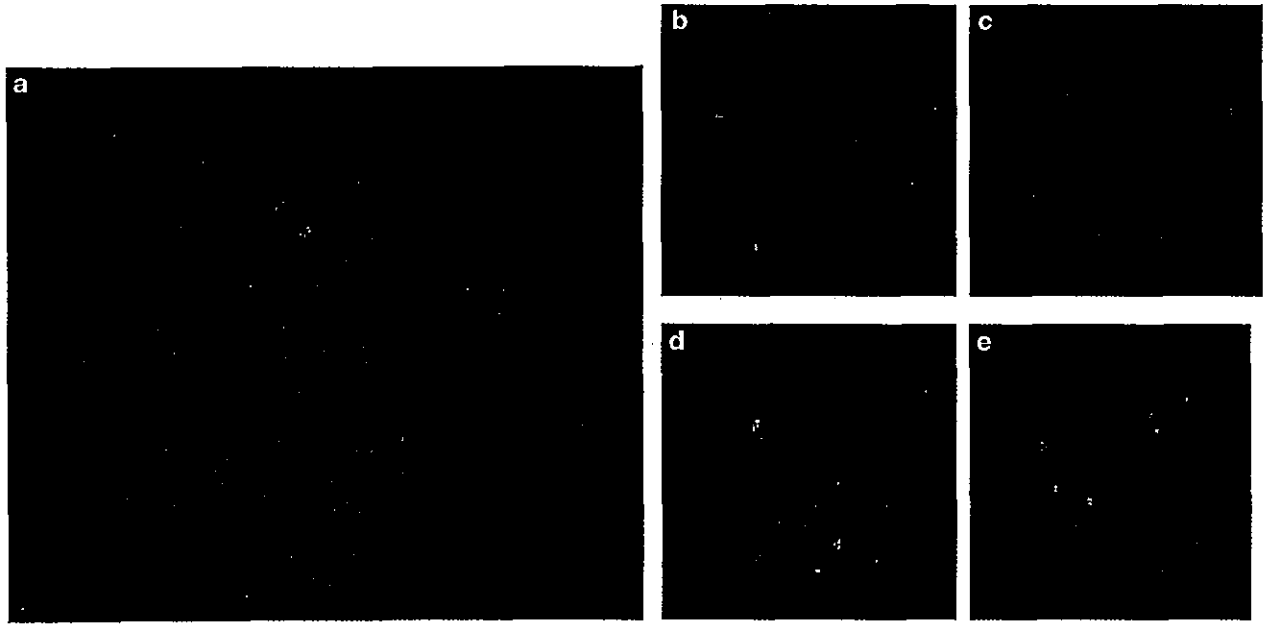

Figure 1 Representative images of FISH analysis for pancreatic cancer cells and their chromosome 12-hybrid clones. Red signals, fluorescence detection with TRITC for the centromere of chromosome 12 (pα12H8); green signals, fluorescence detection with FITC for D12S88 localizing at 12q21 (b759H8). (a) Chromosome spread of the metaphase of normal cells. (b–e) Interphase nuclei of MIAPaCa2 (b), MIAPaCa2H(12)-3 (c), PCI-35 (d), and PCI-35H(12)-1 (e).

Table 1 Alteration in copy number detected by FISH

12cen/12q21 ratio[a]	*2/2*	*3/1*	*3/2*	*3/3*	*4/1*	*4/2*	*4/3*	*4/4*	*5/4*	*5/5*
PCI-35	—	—	—	6	—	—	20	***74***	—	—
PCI 35 H(12)-1	—	—	—	10	—	—	30	12	18	***30***
PCI 35 H(12)-2	—	—	—	6	—	—	10	8	30	***46***
MIAPaCa2	6	14	***60***	8	2	8	2	—	—	—
MIAPaCa2 H(12)-1	6	2	14	4	0	22	***38***	—	14	—
MIAPaCa2 H(12)-2	4	16	***31***	2	6	12	29	—	—	—
MIAPaCa2 H(12)-3	8	2	16	8	0	24	***40***	—	2	—

[a]12cen/12q21 ratio was determined by observation of at least 100 nuclei in duplicate.
In each cell line, the most frequently observed 12cen/12q21 ratio is shown in bold italic face.

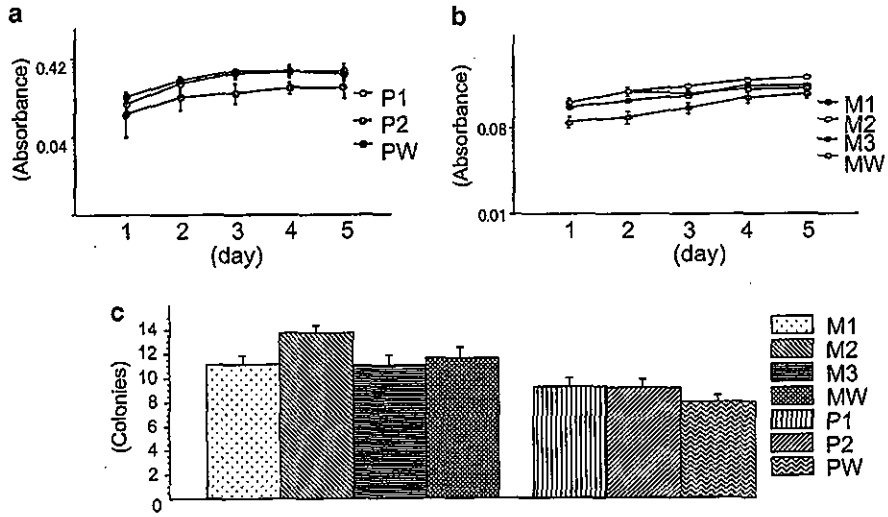

Figure 2 *In vitro* anchorage-dependent proliferation assay. The data were plotted in logarithmic scale. (**a**) PCI-35 (PW) and PCI-35 hybrid clones (P1 and P2). (**b**) MIAPaCa2 (MW) and MIAPaCa2 hybrid clones (M1, M2 and M3). (**c**) *In vitro* anchorage-independent proliferation assay. Each solid bar indicates the number of the colonies in the medium with Bacto-agar after 1 month of culture of cells of PCI-35 (PW), PCI-35 hybrid clones (P1 and P2), MIAPaCa2 (MW), and MIAPaCa2 hybrid clones (M1, M2 and M3).

software (Applied Biosystems, Foster City, CA, USA). Each cDNA was subjected to 40 cycles of the PCR using the ABI PRISM 7700 (Applied Biosystems) according to the supplier's instructions.

Statistical Analysis

All experiments were performed in duplicate or triplicate. A two-tailed Student's *t*-test was performed by using the StatView software 5.0 (SAS Institute Inc., Cary, NC, USA) to determine the statistical significance of differences. The level of significance was established at $P<0.05$.

Results

In this study, we utilized the technique of MMCT to introduce a normal copy of human chromosome 12 individually into two pancreatic cancer cell lines, MIAPaCa2 and PCI-35. In a previous investigation, MIAPaCa2 showed a loss of chromosome 12q arm, whereas PCI-35 did not.[18] We established three independent hybrid clones for MIAPaCa2, MIAPaCa2H(12)-1, -2 and -3, and two independent clones for PCI-35, PCI-35H(12)-1 and -2. To elucidate portions of retained alleles, we performed a microsatellite analysis using a panel of highly polymorphic markers on chromosome 12. However, we could not distinguish between existing alleles and the introduced alleles for most of markers we analyzed because of the few heterozygosities of the microsatellite markers (data not shown). Therefore, we performed a dual-color FISH analysis in duplicate to monitor both the number of introduced chromosomes and the percentage of cells maintaining the introduced chromosome. Markers used were as below: chromosome 12 centromere, 12q21, and 12q23.1. These markers were selected because their high frequencies of losses in primary pancreatic cancer have been reported.[18] Previous works reported that MIAPaCa2 was hypotriploid with losses of 12q21 and 12q23.1, whereas PCI-35 was mainly hypotetraploid without those losses.[18,25] Consistent with the previous results, our FISH analyses, as shown in Figure 1, indicated losses of 12q21

(b759H8) and 12q23.1 (b339F2) in the majority of cells of MIAPaCa2 (Table 1). On the other hand, we found additional signals in the hybrid cells indicating one more copy of portions of the centromere, 12q21 and 12q23.1, as shown in Figure 1; the most frequently observed signal pattern for cen/12q21 or cen/12q23.1 was 3/2 in MIAPaCa2, whereas that in the hybrid was 4/3. Notably, we detected loss of the introduced chromosome in the MIAPaCa2H(12)-2, showing the 3/2 pattern in the majority of cells ($P<0.05$), although the cells maintained resistance to G418. Confirming our previous results,[18] PCI-35 shows an apparently nondefective status of 12q: predominant ratios were 4/4 and 5/5 in parental and PCI-35H(12) cells, respectively. These results are summarized in Table 1. As clearly indicated in Table 1, the hybrid cells we employed were mixed populations of various status of the transferred chromosome because the transferred chromosomes are not stable and sometimes partial loss occurs during the course of serial passages. Each population may express different phenotypes, but we could only observe the mixed phenotypes because of technical difficulties in isolating a pure population.

Once the presence of the introduced chromosome 12 in the hybrids was verified, we estimated their *in vitro* proliferation in either an anchorage-dependent or -independent manner. The results as outlined in Figure 2 show that the *in vitro* growth of the hybrid clones was not significantly different from that of parental cells.

Next, we examined the *in vivo* tumorigenesis phenotypes of the hybrid cells by inoculating them into SCID mice and comparing them with parental cells. In order to shorten tumor latency and enhance tumor growth, we mixed the cells in a suspension containing Matrigel extract. As shown in Figure 3, hybrids MIAPaCa2H(12)-1 and -3 showed significant reductions in tumor volume and a longer latency when compared with their parental cells. MIAPaCa2H(12)-2 and the hybrid clones derived from PCI-35 did not show any significant reduction in tumor volume. Microscopically, tumors generated of the hybrids MIAPaCa2H(12)-1 and -3 tended to form fibrotic changes and showed significant reductions in vessel number as proven by the quantitative vessel counting assay comparing numbers with those in tumors grown from the parental cells (Figure 4). In addition, to compare angiogenesis between MIAPaCa2 and the hybrids, we monitored tumor vessel formation for 3 weeks after tumor cell implantation in a dorsal transparent chamber and an *in vivo* microscopy system. An intense blood vessel formation implying active vascularization

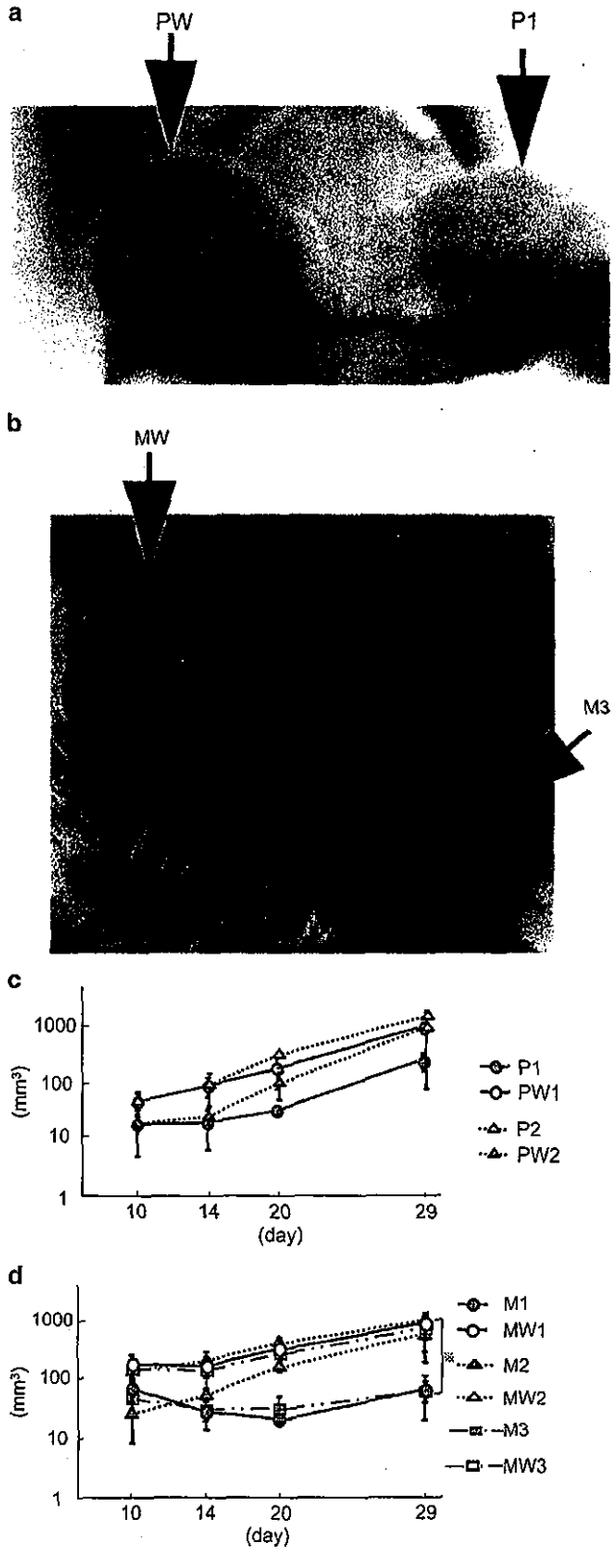

Figure 3 *In vivo* tumorigenic assay employing a total of 3×10^6 tumor cells inoculated into subcutaneum of SCID mice. (**a**, **b**) Representative pictures taken at 1 month after the inoculation: (**a**) PCI-35 (PW1) and PCI-35H(12)-1 (P1), (**b**) MIAPaCa2 (MW3) and MIAPaCa2H(12)-3 (M3). (**c**, **d**) Growth curves of the inoculated tumors. The volume of tumors were plotted in logarithmic scale. (**c**) PCI-35 clones (PW1 and PW2) and PCI-35 hybrid clones (P1 and P2); (**d**) MIAPaCa2 clones (MW1, MW2 and MW3) and MIAPaCa2 hybrid clones (M1, M2 and M3). Asterisks denote statistically significant differences ($P<0.05$).

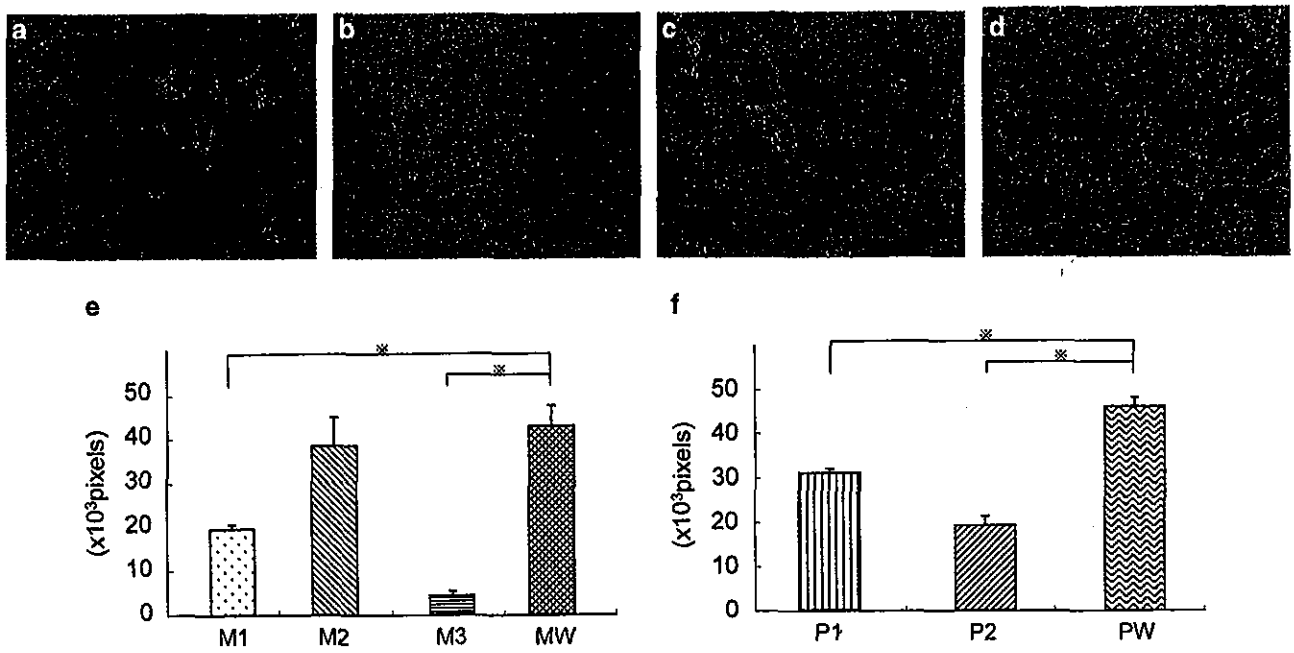

Figure 4 (a–d) Immunohistochemistry employing with anti-CD31 antibody for specimens of inoculated tumors of MIAPaCa2 (a), MIAPaCa2H(12)-3 (b), PCI-35 (c) and PCI-35H(12)-1 (d) (× 400). (e, f) Quantification of CD31-positive areas by counting pixels in digitally imported images. (e) MIAPaCa2 (MW) and MIAPaCa2 hybrid clones (M1, M2 and M3). (f) PCI-35 (PW) and PCI-35 hybrid clones (P1 and P2). Asterisks denote statistically significant differences ($P<0.05$).

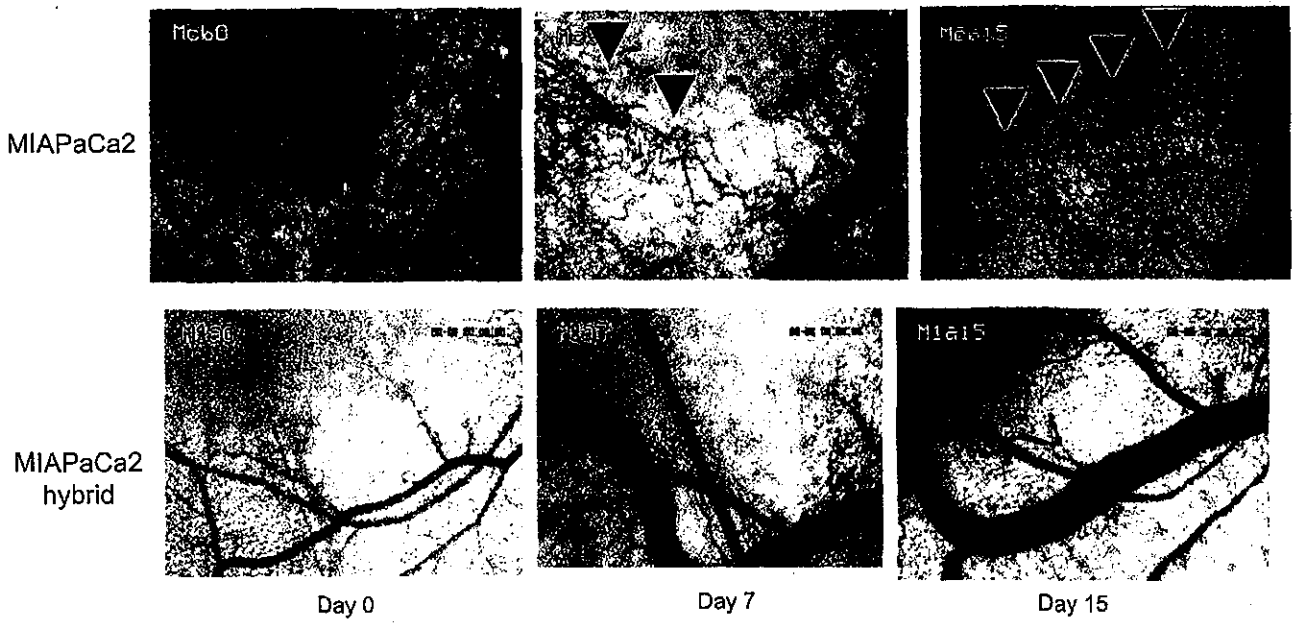

Figure 5 *In vivo* microscopy images of the dorsal skin chamber to monitor angiogenesis. The formation of microvessels indicated active vascularization (arrowheads). Upper panels, MIAPaCa2. Lower panels, MIAPaCa2 hybrid.

was observed in tumors derived from MIAPaCa2 parental cells. On the other hand, tumors of hybrid clones showed very sparse vessel formation (see Figure 5). These results indicated that some of the hybrid cells had a significant reduction in *in vivo* tumorigenic activity, which could be accounted for by the suppression of angiogenesis.

Next, we performed a cDNA microarray analysis to determine the differences in gene expression profiles between the parental MIAPaCa2 and its hybrid clones, because significant phenotypic differences were observed in this set. We employed a cDNA microarray platform consisting of 23 040 genes commercially available from Amersham

Table 2 Differentially expressed genes between MIAPaCa2 clones-1 and -3 and their parental cell

Accession no.	*P-value*	*Normalized ratio*	*Locus*	*Description*	*GO ontology*
NM_033111.2	0.046	1.7	13q12	LOC88523: CG016	Unknown
NM_005347.2	0.044	2.1	9q33	*HSPA5*: heat shock 70 kDa protein 5	ATP binding
NM_006067.3	0.042	0.6	16q24	*NOC4*: neighbor of COX4	Mitochondrion
BX647106.1	0.040	2.5	6q27	*MRNA*: cDNA DKFZp686N23124	Unknown
NM_006088.3	0.036	1.9	6p25	*TUBB2*: tubulin,beta,2	Structural constituent of cytoskeleton
NM_006082.1	0.026	1.6	12q13	*K-ALPHA-1*: tubulin, alpha, ubiquitous	Microtuble
NM_001614.2	0.022	1.8	17q25	*ACTG1*: actin, gamma 1	Structural constituent of cytoskeleton
AK057366.1	0.021	1.6	7q11	*Homo sapiens* cDNA FLJ32804 fis	Unknown
NM_002392.1	0.014	1.8	12q14	*MDM2*: Mdm2, transformed 3T3 cell double minute 2,	Cell growth and maintainance
NM_006203.2	0.011	1.5	5q12	*PDE4D*: phosphodiesterase 4D, cAMP-specific	Signal transduction
NM_002898.1	0.010	1.6	12q13	*RBMS2*: RNA-binding motif, single-stranded interacting protein 2	RNA binding
NM_002715.1	0.010	1.9	5q23	*PPP2CA*: protein phosphatase 2, catalytic subunit, alpha isoform	RNA splicing
NM_014865.2	0.009	1.6	12p13	*CNAP1*: chromosome condensation-related SMC-associated protein1	Cell cycle
NM_003977.1	0.008	0.4	11q13	*AIP*: aryl hydrocarbon receptor interacting protein	Signal transduction
NM_001763.1	0.007	1.6	1q22	*CD1A*: CD1A antigen, a polypeptide	Immune response
NM_005914.2	0.006	1.6	8q12	*MCM4*: minichromosome maintenance deficient 4	ATP binding
NM_006824.1	0.005	2.0	1q35	*EBNA1BP2*: EBNA1-binding protein2	Membrane fraction
NM_021947.1	0.005	1.8	17p13	*SRR*: Serin racemase	Amino-acid metabolism
NM_003057.2	0.005	2.4	6q26	*SLC22A1*: solute carrier family 22, member 1; synonyms	Membrane fraction
NM_003380.1	0.004	0.7	10p13	*VIM*: vimentin	Structural constituent of cytoskeleton
NM_005159.2	0.003	2.4	15q11	*ACTC*: actin,alpha,cardiac muscle	Actin filament
BC063863.1	0.001	1.7	19p13	*KIAA0892* protein	Unknown
NM_001712.2	0.001	1.6	19q13	*CEACAM1*: carcinoembryonic antigen-related cell adhesion molecule 1	Immune response
NM_001743.3	0.001	1.7	2p21	*CALM2*: calmodulin 2 (phosphorylase kinase, delta)	Calcium ion binding

Biosciences (Piscataway, NJ, USA) and performed a comparative hybridization analysis between the parental cells and their hybrids with suppressed tumorigenicity phenotype. The results give us information about differentially expressed genes, theoretically caused by the introduction of chromosome 12. We selected genes with differential values of more than 1.5-fold and showed statistically significant differences. Among the results, we found that 24 genes met the criteria (see Table 2). Predicted functions were annotated based on the Gene Ontology database.

Next, we compared the expression profiles between MIAPaCa2H(12)-2 and MIAPaCa2H(12)-3. Although both were MMCT hybrid clones, the former lost its growth-suppressive activity, while the latter retained it after inoculation into SCID mice. This comparison may give significant information of genes accounting for the tumor-suppressive phenotype without a noise of MMCT technique itself. We found that 18 genes showed more than a 1.5-fold difference in expression level, which is a statistically significant difference (Table 3). These genes could account for the differences in tumorigenic and angiogenic phenotypes between the clones.

Not only genes on chromosome 12 that were expressed differentially beyond our criteria but also those that were expressed below the criteria could produce the tumor-suppressive phenotype, because the addition of one allele to the existing three alleles on chromosome 12 may not result in a significant difference in expression levels in some genes, especially in those supposed to be functionally altered by structural alteration. Therefore, we searched for all expressed genes beyond background levels in hybrids of MIAPaCa2H(12)-1 and -3, both of which showed the suppressed tumorigenic phenotype, and found 25 genes on chromosome 12 according to the annotated information as listed in Table 4.

We validated the results of alterations of expressions detected in the microarray experiment by the semiquantitative RT-PCR method (see Figure 6). Although the results of semiquantitative RT-PCR were not completely consistent with the corresponding data of microarray experiment in the magnitude of change in expression level, the direction of change, either upregulation or downregulation, in each case was retained. Among these, the *RAB21* gene, one of the candidate genes selected by microarray analysis and located on chromosome

Table 3 Differentially expressed genes between suppressed and unsuppressed tumorigenic phenotypes

Accession no.	*Locus*	P-*value*	*Fold change*	*Description*	*GO ontology*
NM_015004.2	3p21	0.0273	32.5	*KIAA0116*: Human mRNA for *KIAA0116* gene, partial cds	Exonuclease activity
AF086240.1	18q21	0.01	5.03	*Homo sapiens* full-length insert, cDNA clone ZD28F11	Unknown
BX116634.1	1p21–p22	0.007	3.91	*Homo sapiens* transcribed sequense	Unknown
NM_001776.2	10q24	0.0277	3.90	*ENTPD1*: ectonucleoside triphosphate diphosphohydrolase 1	Cell–cell signalling
BC039676.1	11q24	0.0269	3.65	*Homo sapiens*, clone IMAGE: 5173389, mRNA	Unknown
AI827562.1	15q22	0.0061	3.01	*Homo sapiens* transcribed sequence	Unknown
NM_005736.2	10q24	0.0377	2.92	*ACTR1A*: ARP1 actin-related protein 1 homolog A, centractin alpha (yeast)	Structual constituent of cytoskeleton
NM_004745.3	8p23	0.0168	2.60	*DLGAP2*: discs, large (*Drosophila*) homolog-associated protein 2	Protein binding
NM_005276.2	12q12	0.0425	2.10	*GPD1*: glycerol-3-phosphatede hydrogenase 1	Carbohydrate metabolism
NM_005244.3	20q13	0.0391	2.08	*EYA2*: eyes absent homolog 2 (*Drosophila*);	Development
L08438.1	5q35	0.0026	1.96	Human autonomously replicating sequence (ARS)	Unknown
NM_194261.1	16p13	0.0035	1.91	*UBE1*: ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	Ubiquitin cycle
NM_005159.2	15q11	0.0452	1.89	*ACTC*: Action,alpha,cardiac muscle	Structual constituent of cytoskeleton
NM_005594.1	12q23	0.0097	1.84	*NACA*: nascent-polypeptide-associated complex alpha polypeptide	Unknown
NM_006067.3	16q24	0.0223	1.70	*NOC4*: neighbor of *COX4*	Mitochondrion
NM_004595.2	Xp22	0.0148	1.52	*SAS*: spermine synthase	Transferase activity
NM_005720.2	7q22	0.0153	0.63	*ARPC1B*: actin-related protein 2/3 complex, subunit 1B, 41 kDa	Structual constituent of cytoskeleton
XM_371546	2q12	0.0454	0.51	Human sequence similar to elongation factor-1 alpha (ef-1) mRNA, 3′ end	Unknown

arm 12q, showed a higher expression in MIAPaCa2 hybrids than in their parental cells. The results of microarray analysis were reconfirmed by the quantitative real-time RT-PCR method (see Figure 7).

Discussion

Several lines of evidence, as we described in the Introduction, have suggested that chromosome 12q may carry a TSG(s) that plays a role in the development and/or progression of pancreatic cancer. We aim to gather functional evidence for the existence of TSG(s) and refine candidate(s) yet to be identified on the 12q arm. We transferred a normal copy of chromosome 12 into pancreatic cancer cell lines by the microcell-mediated chromosome transfer (MMCT) technique[16,17] and analyzed its phenotype. MMCT has been proven to be a useful tool providing functional evidence for identification of TSG in a variety of cancers such as pancreatic cancer,[21] colon cancer,[26,27] prostate cancer,[28] Wilms' tumor,[29] and melanoma.[30] This technique also led the way to the isolation of the *NBS* gene.[31]

The derived hybrids of chromosome 12 showed clear differences from parental cells not in *in vitro* but *in vivo* tumorigenic study. The *in vitro* studies of anchorage-dependent and -independent cell proliferations showed no remarkable differences. However, the inoculation of the hybrid cells MIAPaCa2H(12) into SCID mice strikingly showed a significant suppression of tumorigenesis when compared with parental cells of MIAPaCa2. The hybrid cells of the PCI-35 lineage did not show such a phenotype. These results were of particular interest because MIAPaCa2 was partially defective for chromosome 12q, but PCI-35 was not.[18] These results suggested that newly introduced genes on chromosome 12 overcame defective functions of existing genes in MIAPaCa2 but not in PCI-35. One clone of hybrid of MIAPaCa2, MIAPaCa2H(12)-2, did not show a suppressive phenotype. We suspected that this clone lacked some important portions of the introduced allele. However, we could not detect differences in genotypes regarding chromosome 12 among hybrids derived from MIAPaCa2 in our panel of microsatellite analysis, mainly because of similarities in the number of repeats, which consisted of the microsatellites between the existing alleles and the introduced allele.

We found a remarkable suppression of angiogenesis in and surrounding the inoculated tumors of hybrids in examinations employing quantitative vessel counting with immunohistochemical labelling and an *in vivo* microscopy system. The suppression of angiogenesis could account for the suppressive phenotype of *in vivo* tumorigenesis. These facts indicated a potential interposition of

Table 4 Differentially expressed genes on chromosome 12 among MIAPaCa2 hybrids

Accession no.	*Locus*	*Description*	*GO ontology*
NM_004982.2	12p11	*KCNJ8*: potassium inwardly rectifying channel, subfamily J, member 8	Voltage-gated ion channel activity
NM_003213.1	12p13	*TEAD4*: TEA domain family member 4	RNA polymerase II transcription factor activity
NM_005768.4	12p13	*C3F*: putative protein similar to nessy (*Drosophila*)	Unknown
NM_006170.1	12p13	*NOL1*: nucleolar protein 1, 120 kDa	Positive regulation of cell proliferation
NM_002831.3	12p13	*PTPN6*: protein tyrosine phosphatase, nonreceptor type 6; synonyms	Protein tyrosine phosphate activity
L16783.1	12p13	*FOXM1*: forkhead box M1	RNA polymerase II transcription factor activity
NM_005276.2	12q12	*GPD1*: glycerol-3-phosphate dehydrogenase 1	Carbohydrate metabolism
NM_02898.1	12q13	*RBMS2*: RNA-binding motif, single-stranded interacting protein 2	RNA-binding activity
NM_021019.2	12q13	*MYL6*: myosin, light polypeptide 6, alkali, smooth muscle and nonmuscle; synonyms	Structural constituent of muscle
NM_002475.2	12q13	*MLC1SA*: myosin light chain 1 slow a	Structural constituent of muscle
NM_006576.2	12q13	*AVIL*: advillin	Actin binding
NM_006082.1	12q13	*K-ALPHA-1*: tubulin, alpha, ubiquitous	Structural molecule activity
NM_000289.3	12q13	*PFKM*: phosphofructokinase, muscle	Transferase activity
NM_000239.1	12q14	*LYZ*: lysozyme (renal amyloidosis)	Hydrolase activity
NM_002392.1	12q14	*MDM2*: Mdm2, transformed 3T3 cell double minute 2, p53-binding protein (mouse); synonym	Oncogenesis
NM_014999.1	12q15	*RAB21*: RAB21, member RAS oncogene family	GTP-binding activity
NM_005123.1	12q23	*NR1H4*: nuclear receptor subfamily 1, group H, member 4	Transcription factor
NM_006700.1	12q23	FLN29 gene product	Unknown
NM_000970.2	12q24	*RPL6*: ribosomal protein L6	Ribosome
NM_031954.2	12q24	*KCTD10*: potassium channel tetramerization domain containing 10(MSTP028)	Voltage-gated ion channel activity
NM_001516.3	12q24	*GTF2H3*: general transcription factor IIH, polypeptide3	Damaged DNA binding
NM_000617.1	12q24	*SLC11A2*: solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	Iron ion transporter
NM_005594.2	12q24	*NACA*: nascent-polypeptide-associated complex alpha polypeptide	Protein biosynthesis
NM_019086.2	12q24	Hypothetical protein FLJ20674	Unknown
NM_020993.2	12q24	*BCL7A*: B-cell CLL/Lymphoma 7A, mRNA	Actin binding

putative TSGs at chromosome 12 playing suppressive roles, not in proliferation of the tumor itself in an early phase, but in angiogenesis in a later phase of tumorigenesis. Angiogenesis is a key factor for tumorigenesis, and its suppression plays a major role in a tumor-suppressive activity.[32–34] The suppressive activity of introduction of chromosome 12 in the later phase of pancreatic tumorigenesis could explain our previous finding of significant association of loss of chromosome 12q with poor prognoses in patients with pancreatic cancer.[12]

We further analyzed a total of 23 040 unique human genes in this study to search for genes closely associated with tumorigenesis by altering expression and successfully grasped gene expression profiles of hybrids in comparison to parental cells. Although the possibility of missing important genes cannot be excluded because of the limited number we examined, about two-thirds of the total human genes that were analyzed, this method is one of the best ways to explore the genes that play important roles in pancreatic carcinogenesis. Using this method, detection of genes that lose their function by structural alterations cannot be detected either. However, this technique enabled us to obtain valuable information from various aspects such as examinations of the TGF-beta/SMAD4 pathway[35] or introduction of a DNA methylation inhibitor or a selective COX-2 inhibitor in pancreatic cancer cells.[36,37] Discovery of various overexpressed genes in pancreatic cancer cells was also reported by this method; sea urchin fascin homolog, heat shock protein,[38] *ABL2*, *Notch4*, *SOD1*,[39] c-*myc* and *Rad51*.[40] We report herein the first results of microarray analysis of the comparison between parental cells and their hybrids after introduction of chromosome 12 using the MMCT technique.

For analyzing the data of the microarray, we first selected genes whose expressions were significantly different statistically and more than 1.5-fold differentially expressed genes between the parental cell and hybrid clones were picked up. These data gave information about alteration of gene expression by introduction of the additional copy of chromosome 12. According to our FISH results, the majority of hybrid cells harbored one additional copy of the chromosome 12 to tri- and tetraploid cells, MIAPaCa2 and PCI-35, respectively. Although we could not precisely estimate alterations of expressions of genes between parental cells with three or four alleles and cells with one additional allele in them, it is probable that the differences may be small. Therefore, we used only statistical methods for data analysis without cutting off the data in large-fold