Screening for Functional Tumor Suppressor Activity in Pancreatic Cancer*

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Abstract

Pancreatic cancer has a well-known reputation as one of the leading causes of cancer deaths worldwide. Thus, acquisition of efficient approaches and markers for accurate detection at the earlier stages of the disease should be prioritized. We have been focusing on tumor suppressor genes (TSGs) activity in pancreatic cancer to find effective methods for its genetic diagnosis and/or treatment.

In this study, we utilized the technique of micro-cell-mediated chromosome transfer (MMCT) to introduce a normal copy of human chromosome 18 individually into some pancreatic cancer cells. Subsequently, the tumorigenic ability of the resulting hybrids was assessed in vitro and in vivo. In vitro growth of the hybrid clones was significantly delayed as compared to the parental cells. This was paralleled by the hybrid cells promotion of invasive carcinomas in nude mice at a significantly lower rate and with a longer latency than the parental tumor cells.

This study provides evidence that MMCT is an efficient tool for screening of tumor suppressor activity in pancreatic cancer. The functional data emerging from this study bring into sharp relief the implication of chromosome 18 as a putative location for new TSG(s), yet to be identified in this region.

Key words

Pancreatic cancer - tumor suppressor genes - chromosome 18

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Cancerul pancreatic are o binecunoscută reputaţie ca fiind una din cauzele majore de deces in întreaga lume. In acest sens, dezvoltarea unor procedee si markeri de detecţie precoce ai acestui tip de neoplazie trebuie sa fie o prioritate a lumii medicale.

In acest studiu, am utilizat tehnica de transfer cromozomal mediat microcelular pentru a introduce o copie normală a cromozomului 18 in câteva linii celulare de cancer pancreatic. Proprietăţile tumorigene ale hibrizilor rezultaţi au fost analizate atât in vitro cât si in vivo. Rata de multiplicare in vitro a clonelor hibride a fost semnificativ întârziată comparativ cu cea a celulelor parentale. In acelaşi timp, celulele hibride au fost capabile sa genereze tumori în şoareci imunodeficienţi, având o rată de dezvoltare mai redusă şi o perioadă de latenţă semnificativ mai lungă in comparaţie cu cea a celulelor tumorale parentale.

Acest studiu arată că această tehnică de transfer cromozomal este o metodă eficientă de screening al activităţii supresoare tumorale codată de cromozomul 18 în cancerul pancreatic. Datele prezentate indică faptul ca o importantă activitate de supresie tumorală este localizată pe cromozomul 18, care poate fi sediul unor noi gene supresoare tumorale neidentificate până în prezent.

Introduction

Studies of somatic cell hybrids formed between tumor and normal cells have demonstrated the recessive nature of tumor- associated traits including tumorigenicity and/or in vitro transformed phenotypes. When tumor cells are fused to normal cells, tumor phenotypes are virtually suppressed. The re-expression of tumor phenotypes correlates with the loss of specific normal chromosomes. Thus, putative tumor suppressor genes (TSGs) have been localized on these normal chromosomes. A direct approach to identify the chromosome carrying TSGs is the introduction of individual normal chromosomes into tumor cells via micro-cell fusion (1, 2).

The chromosomal regions in which the most frequent losses are found implicate locations of essential TSGs and DNA repair genes that may be involved in the pathogenesis of several tumor types. Tumor suppressor genes are attractive candidates as markers for early genetic diagnosis because, by definition, their loss should be followed by switching to a tumorigenic phenotype (1, 3).

Molecular pathology of pancreatic carcinogenesis could be characterized by a broad spectrum of distinct gene mutations and chromosomal alterations, of which some seem to be non-random (4-8). So far, many studies, employing different molecular techniques, have consistently outlined the loss of chromosome 18q as an early event in pancreatic carcinogenesis. However, functional evidence showing the tumor suppressor activity of chromosome 18 was desirable (8, 9). Chromosome 18 harbors a cluster of either tumor or metastasis suppressor genes such *SMAD2, SMAD4, DCC*, maspin and *PAI-2*. The band 18q21 includes two known tumor suppressor genes, *DPC4* (*SMAD4*), Maspin (10, 11) and *DCC*. *DPC4* (deleted in pancreatic carcinoma 4), a member of the *MAD* gene family, is involved in signal transduction of serine threonine kinase receptors. Its inactivation occurs in almost half of pancreatic carcinomas but is uncommon in other tumor types (6, 12). However, studies in colorectal cancer cells and *DPC4* mutated mice have suggested that *DPC4* has a role in the progression of colorectal tumors. The *DCC* gene (deleted in colorectal cancer) encodes a netrin receptor. Originally, deletions and mutations of *DCC* have been found in colorectal carcinomas (5, 12). Moreover, inactivation of *DCC* has been found in breast, prostate, pancreatic and gastric cancer, in glioma and osteosarcoma, and in some haematological malignancies (6, 7, 13, 14). The 18q21 region harbors also another member of the *MAD* gene family, *MADR2* (*SMAD2*), which has been suggested to be a tumor suppressor gene (15). No currently known TSG has been assigned to 18p (1). In a recent study, we found that LOH (loss of heterozygosity) of 18q is significantly associated with a poor prognosis in pancreatic cancer (16). This high frequency of losses affecting 18q in pancreatic cancer prompted us to take a special interest in this region.

However, mutations of these known genes alone cannot explain the whole process of pancreatic carcinogenesis; there may be some other genes that play important roles. In this regard, we have been focusing on TSGs in pancreatic cancer to find effective methods for genetic diagnosis and/or treatment. As long as a few different TSGs are clustered on the 18q we presume that correction of these defects could restore to a quasi-normal growth status of pancreatic cells.

Material and method

Cell lines

Two pancreatic cancer cell lines: PK-1 (established in our department) and PANC-1 (purchased from American Type Culture Collection, Rockville, MD) were used as recipients in MMCT experiments and cultured according to the protocols of the suppliers. These cell lines are well mutational characterized (17). Briefly, PK-1 is homozygous deleted for *SMAD4* (*SMAD4*$^{-/-}$) whereas PANC-1 expresses normal *SMAD4* (*SMAD4*^{wt}). For each cell line, three stable hybrids containing a normal copy of chromosome 18 were established: PK-1H(18)-1 through -3 and PANC-1H(18)-1 through -3, respectively. These hybrids were generated by the micro-cell-mediated chromosome transfer (MMCT) technique and expanded in medium containing 400 µg/ml of G418, as previously described (2, 18-23). The A9H(18) mouse fibroblast cell line carrying a single copy of human chromosome 18 tagged with an integrated neomycin resistant gene, was maintained in the same selective medium. All cells were routinely monitored for Mycoplasma as well as for mouse hepatitis, Sendai and pneumonia viruses, and were consistently negative.

Microsatellite analysis. Genomic DNA from cells and nude mice tumors was extracted using Nucleon BACC3/ST Kit (Amersham, Buckinghamshire, England) and analyzed with highly polymorphic microsatellite markers located on 18q, as described previously (20, 24). Each PCR amplification and analyzing were carried out at least twice.

In vitro proliferation. Anchorage-dependent proliferation was monitored by an MTT assay for five days and a daily proliferation index (PI) was calculated for each parental and corresponding hybrid cell line (25). Conversion of MTT to formazan dye was measured for absorbance at 590 nm using a multi-well plate ImmunoReader System. Data from 3 independent experiments were pooled, averaged and then statistically analyzed.

Colony Formation Assay. Ten thousands cells 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 Bacto-agar 0.7% was used for the bottom layer. After two weeks, the viable colonies were stained by incubation for 3h with 0.3 ml of 1 mg/ml INT (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride, Dojindo Laboratories, Japan). The viable red stained colonies were photographed using a Zeiss microscope with x5 objective. Both colony number and size were measured and averaged on three randomly chosen photographs from each plate by using NIH 1.62 software. Every independent anchorage growth was assessed in triplicate by two independent experiments.

Determination of Apoptosis. Apoptotic cells were detected by annexin V/EGFP staining using an ApoAlert Annexin V- EGFP kit obtained from Clontech (Palo Alto, CA). Stained cells were quantified using a Becton Dickinson FACScan and data were analyzed using the CellQuest software (version 3.1, Becton Dickinson).

Animal experiments. Eight-week-old male athymic nude (BALB/c-nu/nu) mice (purchased from Clea Japan Inc., Tokyo, Japan) were maintained under pathogen-free conditions and used in accordance with NIH guidelines. Logarithmically growing cells trypsinized from subconfluent monolayers were suspended in medium containing 10% Matrigel Growth Factor Reduced (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 10^7 cells/ml. Then, 3 x 10^6 cells in 300 ml suspension were injected s.c. into the hind flanks of nude mice. The tumor volume was estimated by the formula: $V=D \times d^2 \times 0.4$ (V= tumor volume, D= largest dimension and d= smallest dimension) after biweekly measurements (26, 27). Data from 3 independent experiments were pooled for statistical analysis. In week 8, when the tumors from parental cells reached approximately 2000 mm^3, the mice were sacrificed. The tumors were resected, weighted and bisected; half of tissue was either snap-frozen in liquid nitrogen for molecular or fixed in neutral buffered formalin for the further investigations.

Immunohistochemical analysis

Five-μm-thick sections were prepared from formalin-fixed paraffin-embedded specimens. Immunohistochemical reactions were performed (28) using a mouse anti-proliferating cell nuclear antigen (anti-PCNA, clone PC10, Dako Corporation) and developed with a Zymed Immunomouse kit (San Francisco, CA). The quantitation of proliferating cells was made by counting the PCNA-positive cells as well as the total cells at 10 arbitrarily selected fields at x40 magnification in a double-blinded manner. The percentage of PCNA-positive cells per 10 x40 fields was determined as number of PCNA-positive cells x 100/total number of cells. Negative control slides were prepared by omitting the primary antibody.

Statistical analysis

A two-tailed t Student computed by GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA) was used to determine the statistical significance of measured differences. The level of significance was established at $p < 0.05$.

Results and Discussion

Pancreatic cancer, like other cancers, is a genetic disease, arising from an accumulation of mutations that promote clonal selection of cells with increasingly aggressive behaviour (4). At the time of the initial detection of clinical cancer, several genetic alterations have already accumulated in the tumor cells. In pancreatic cancer, *KRAS*, *TP53*, *p16*, and *SMAD4* have been considered as molecules that play key roles in tumorigenesis (5). Several lines of cumulated evidence had clearly shown that the loss of 18q is one of the more consistent findings among the chromosomal abnormalities identified in a variety of cancers and this event is associated with a poor clinical outcome (29). Furthermore, loss of *SMAD4* expression occurs biologically late in the neoplastic progression therefore even when those clinically “early” infiltrating pancreas cancers are detected, this fact could be considered as genetically late (30, 31). On the other hand, adenovirus-mediated transfection of *SMAD4* inhibits mice tumorigenesis through halting of angiogenesis but fails to do it for in vitro growth of pancreatic ductal adenocarcinoma cells with completely inactivated *SMAD4* (26, 27).

In this study, we utilised the technique of micro-cell-mediated chromosome transfer to introduce a normal copy of human chromosome 18 individually into two pancreatic cancer cells. MMCT has been proved to be a useful tool providing functional evidence for location of TSGs in a variety of cancers including melanoma (32) and prostate cancer (9). A successful transfer of a particular single chromosome to other cells via micro-cell fusion is substantiated by using chromosome donor cells which have two major characteristics. Firstly, a large number of small-sized micronuclei should be introduced in the donor cells by colcemid treatment. Secondly, the chromosome to be transferred should be marked with a selectable gene (2). In this study, we first obtained somatic cell hybrids between human fibroblast cells and A9 mouse cells. These resulted clones were used to deliver a normal copy of chromosome 18 into some pancreatic cancer cell lines. Using 7 highly polymorphic microsatellite markers spaced along to 18q, we detected restoration of heterozygosity for 18q in all hybrid clones (Fig.1, lanes H). The transferred chromosome is apparently indefinitely maintained, as we detected similar signals at the end of experiment (after 15-20 passages) and also in tumors generated in nude mice (Fig.1, lanes T). However, in this system, based on a semi-quantitative PCR method, it is impossible to appreciate the percentage of cells retaining transferred chromosome copy (20).

In vitro proliferation was assessed by MTT method over five days. The results showed that in *vitro* growth of the hybrid clones was significantly delayed as compared to the parental cells, apparently regardless of initial *SMAD4* status (Fig. 2 A, B, C). However, the growth rates were significantly different between *SMAD4* ^{wt} and *SMAD4* $^{-/-}$ parental cells.

To study their proliferation and morphology under anchorage-independent conditions, we transferred both parental and their hybrid cells from monolayer to a double layer Bacto-agar suspension. The in vitro culture of cells in suspension is believed to more closely mimic in vivo conditions than the culture of cells in a two-dimensional monolayer (33). After two weeks, for an accurate assessment, the viable colony was stained by incubation with INT solution. In addition to a significantly lower size and number of colonies (Fig. 2, D and E, Table I) observed among the hybrids compared with parental cells (mean colony number and size 49 colonies/wheel $\pm$ 3.6 versus 228 $\pm$ 19.36 and 106mm$\pm$19.61 versus 392mm$\pm$37.22 for PK-1H(18) and PK-1, respectively), other notable features could be seen.

Namely, parental cells formed tight densely packed multicellular spheroids easily detected by the naked eye, while hybrids formed smaller and loose spheroids. The latter observation could be supported by changes in adhesion abilities of the hybrids but this aspect should be examined in the light of further specific investigation. Because the ability of cells to grow in the soft agar suspension very closely correlates with their tumorigenic potential in vivo,

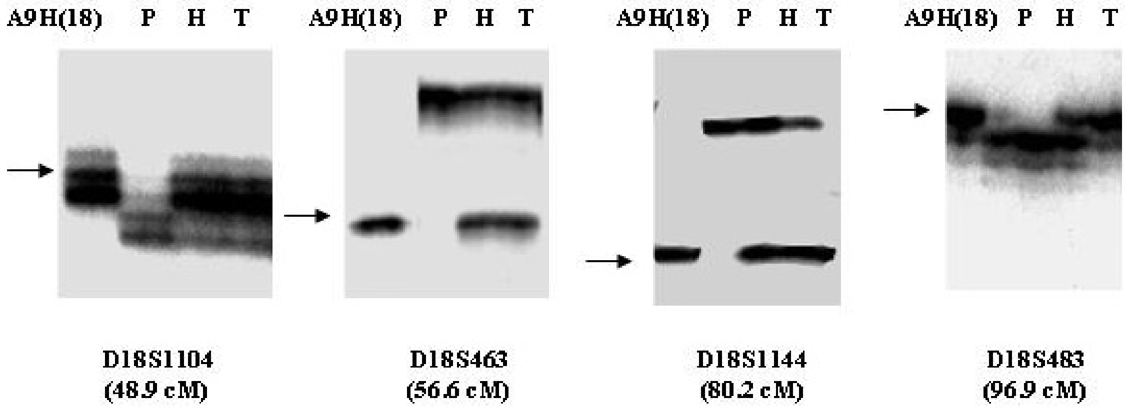

Fig.1 ***Microsatellite analysis***. Representative scanned autoradiographs showing restoration of heterozygosity for the different 18q-microsatellite markers after chromosome 18 transfer in PK-1 cells. The arrows indicate the newly appeared bands with the same size as obtained from A9H(18)H used as donor cell in MMCT experiment. The generic names and cytogenetic position (Genome Database, http://gdbwww.gdb.org)) along the 18q are indicated below to every panel. Lanes: P-parental cells, H-hybrids, T-nude mice tumors.

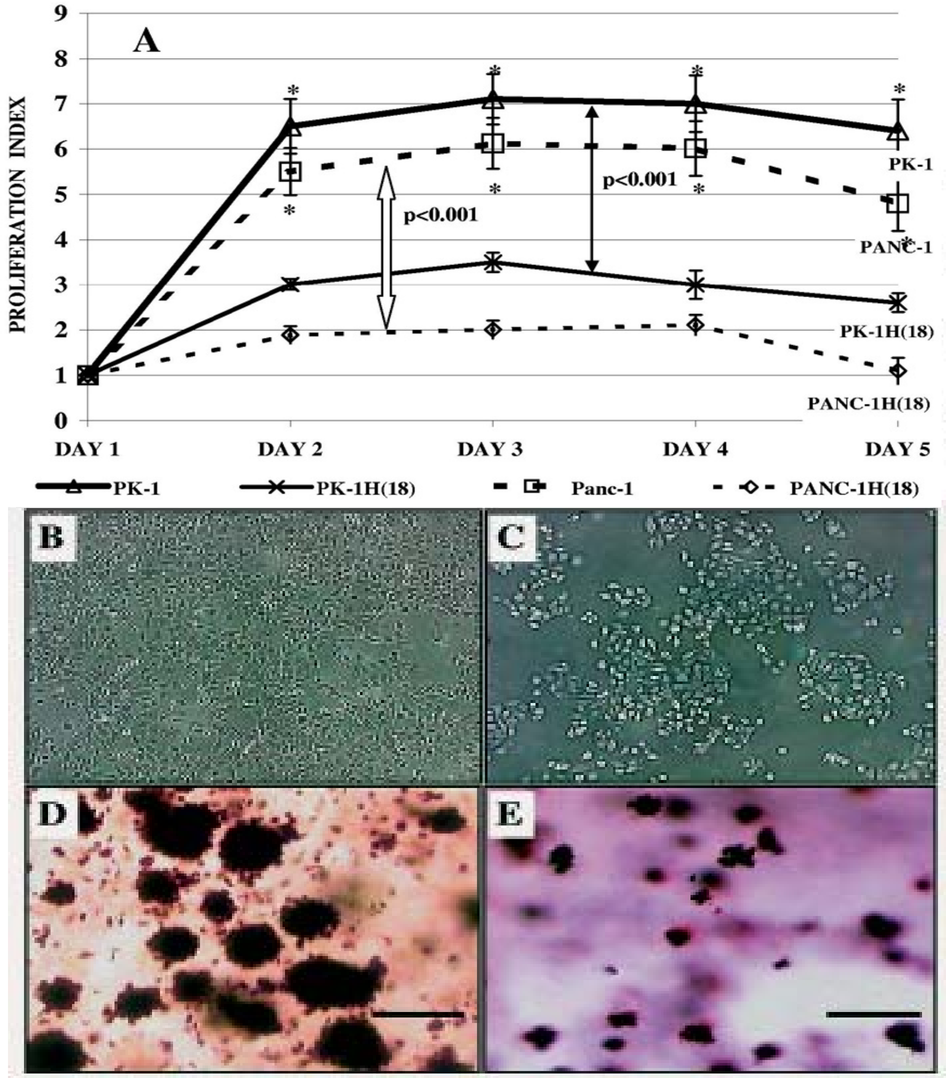

Fig.2 ***In vitro proliferation***. **(A) MTT assays** were conducted as described in "Materials and Methods". The hybrids derived from *SMAD4*^{wt} cells demonstrated the ability to suppress *in vitro* growth to a greater extent than the hybrids derived from *SMAD4*$^{-/-}$ recipients ($^*p<0.05$). Note the same trend for the parental cells. Values from three independent experiments were pooled, averaged and plotted. The calculated SD (bars) is too low to be resolved in some curves. Up-to-down arrows reflect mean differences recorded between hybrid and parental cells tumors. **(B, C) Microscopic appearances of PK-1 parental cells and their hybrids.** One hundred thousand indicated cells were plated in separate 10-cm dishes. Pictures were taken at 96 hr with a phase contrast microscope at original magnification x20. (**D, E**) **Anchorage-independent growth.** Cells were transferred in a double layer suspension containing Bacto-agar. At the day 21 colonies were stained with INT, photographed and counted as described in "Materials and Methods". The hybrids demonstrated an increased ability to suppress growth in soft agar (D, E, PK-1 and PK-1H(18), respectively). Bars reflect 500mm.

Table I Oncogenic properties of the stable hybrid cells

Cell lines / Properties	PK-1	PK-1H(18)	PANC—1	PANC-1H(18)
*A. Anchorage -independent growth in soft agar, Proliferation and Apoptosis Index. The averaged results ±SD from three independent experiments are represented **				
Colony Formation (number/3-cm dish)	228±19.36	49±3.61	196±18.21	34±2.89
Colony Size (μm)	392±39.22	106.4±19.61	293±32.93	32±29.66
Apoptosis Index (%)	2.1±0.5	3.9±0.7	1.9±0.3	4.1±0.5
*B. In Vivo Tumorigenitciy and Proliferating Index of Hybrids in Athymic Nude Mice ***				
Size of Tumors (mm^3 at day 45)	1645±245	326±98	756±168	142±39
Latency Period (days)	8±2	14±3	9±3	18±3
Proliferating Index (PCNA positive cells/x40 field)	57±11.6	16±5.1	42±8.3	9±3.4

* The colony number and size were measured and averaged on three randomly chosen photographs from each plate by using NIH 1.62 software. Apoptotic cells were detected by annexin V/EGFP staining. Data were quantified using a FACScan and analyzed using the CellQuest software. ** Representative averaged results ±SD from the triplicate experiments are shown. Latency period is defined as the time course when tumors became palpable (reaching 4-5 mm diameter).

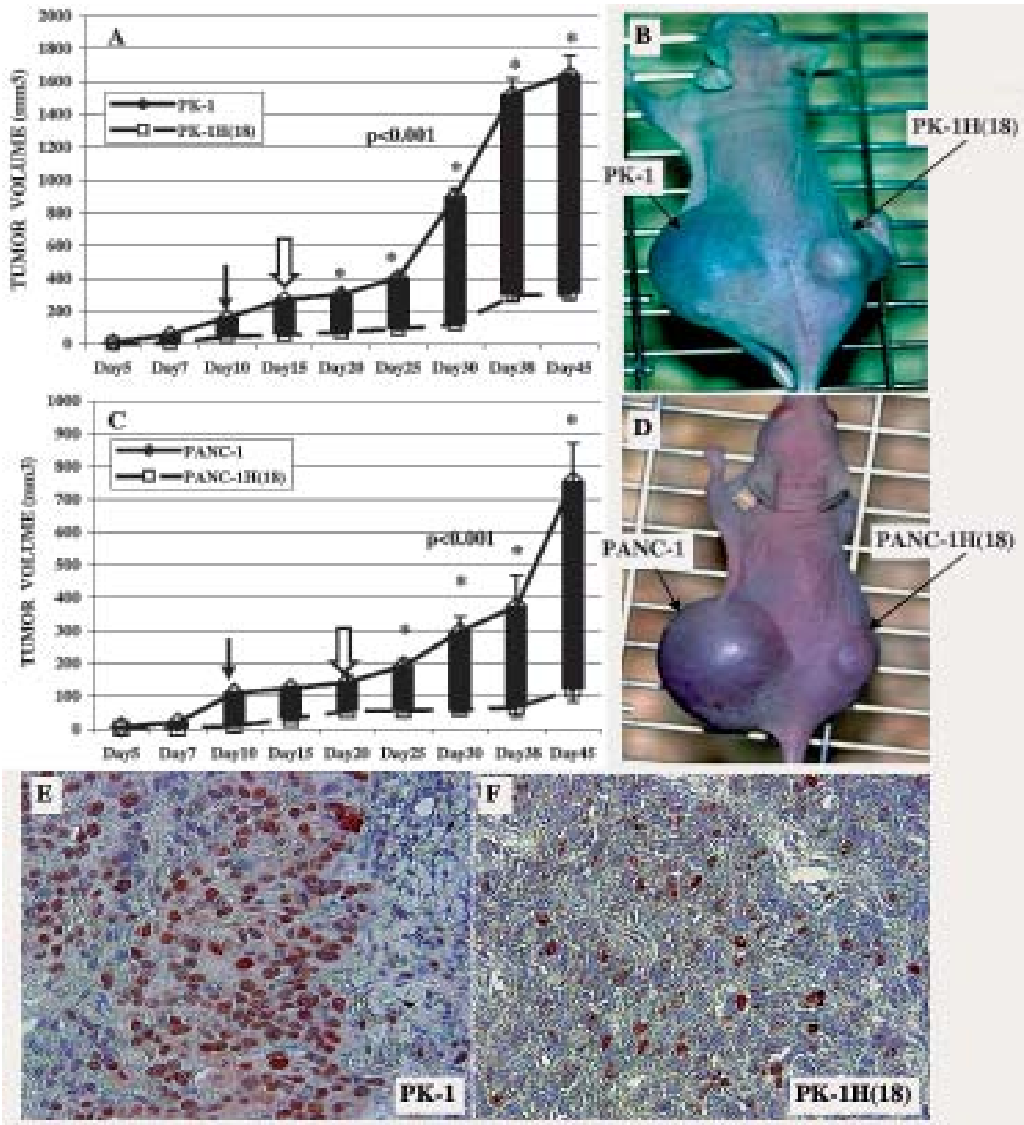

Fig.3 ***In vivo growth***. (A-D) 3 x 10^6 of indicated cells in 300 ml suspension were injected s.c. into the hind flanks of 8 week-old nude mice. Each point reflects the average of three mice tumors from two different experiments. Up-to-down bars reflect mean differences recorded between hybrid and parental cells tumors. Note that calculated SD (bars) is too low to be resolved in some curves; *, P<0.001. In A and C the up-to-down arrows reflect latency period for hybrid (open arrows) and parental cell tumors. (E, F) **Decreasing of proliferation index in hybrid xenografts**. Five-μm-thick sections were prepared from the indicated formalin-fixed paraffin-embedded specimens and were immunohisto-chemically stained with anti-PCNA antibody for analysis of PCNA positive proliferating cells. The positive cells were estimated as an average of percentage PCNA-positive cells per 10 arbitrarily selected fields at x40 magnification. Representative data are shown at x40 original magnification (PK-1, PK-1H(18) in A and B, respectively).

these features outline that chromosome 18 transfer is associated with major changes in the tumor cells' behaviour. Cells undergoing apoptosis were detected by annexin V-EGFP. The annexin V assay measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event typically associated with apoptosis (34). The percentages of cells that were annexin V positive (Table I) were very low in parental cells (2.1 ± 0.5%) and slightly increased among the hybrid clones (3.9 ± 0.7%). Thus, apoptosis was not involved in the chromosome 18-mediated growth suppression observed throughout the hybrid cells.

Tumorigenesis in nude mice is one of the most stringent tests and gives us highly valuable information about cancer cells' behaviour. In order to shorten tumor latency and enhance tumor growth we mixed the cell in a suspension containing Matrigel extract. Despite of inherent interclonal variation, hybrids showed a significant reduction in tumor volume and a longer latency when compared to parental cells (Fig.3 A-D, Table I).

The expression of PCNA, a nuclear marker of cell proliferation was also analyzed in tumor sections (Fig. 3 E, F). We found that 16% stained positive for PCNA in the hybrid tumors compared with 57% in the parental tumors. Thus, proliferation was decreased in hybrid tumors compared with the parental group (Table I) indicating that retardation of tumor growth is caused by a significant lower proliferation rate.

To summarize, this study provides evidences that MMCT is an efficient tool for screening of TSG activity in pancreatic cancer. Moreover, the use of libraries of micro-cell hybrids containing single human chromosomes will facilitate human gene mapping and other genetic studies including gene dosage and gene deletion effects. We are currently investigating the tumor suppressor activity of chromosome 12 in pancreatic cancer. The functional data emerging from this work bring into a sharp relief the implication of chromosome 18 as a putative location for new TSG(s), yet to be identified in this region.

Conclusion

The complexity of the process associated with malignant tumor development and metastasis is a major hurdle in attempting to cure this fatal disease. We achieved significant suppression of both in vitro and in vivo growth of pancreatic cancer cells by transferring a normal copy of chromosome 18. Moreover, this suppression was observed regardless of the *SMAD4* status. However, induction and restoration of SMAD4 could not prevent in vitro growth, regardless of SMAD4 mutational background (26, 27). Overall, these results suggested that introduction of chromosome 18 induces significant suppression of the tumorigenic phenotype of pancreatic cancer cells. Although our and others' studies clearly implicate the important role of gene(s) on chromosome 18, the precise sub-chromosomal localization of the tumor or metastasis suppressor gene(s) is still an open question. The precise localisation and identification of a putative TSG will require further and additional research.

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