Original Article

Functional Analysis of Chromosome 18 in Pancreatic Cancer: Strong Evidence for New Tumour Suppressor Genes

Liviu P. Lefter,[1,2] Makoto Sunamura,[1] Toru Furukawa,[2] Toshimasa Yastsuoka,[1] Hisashi Abe,[1] Hiroko Inoue,[1] Tadayoshi Abe,[1] Shinichi Egawa,[1] Koh Miura,[1] Rina Morita,[1] Akira Horii[2] and Seiki Matsuno,[1] Departments of [1]Gastrointestinal Surgery and [2]Molecular Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan.

BACKGROUND: In a previous work, we demonstrated that loss of heterozygosity of 18q is a frequent event significantly associated with poor prognosis in pancreatic cancer. We hypothesized that restoration of heterozygosity of chromosome 18 in pancreatic cancer cells would reduce their tumorigenicity. This study was intended to provide functional evidence for the existence of new tumour suppressor gene(s) located on chromosome 18.

METHOD: Restoration of heterozygosity was achieved by introducing a normal copy of chromosome 18 into pancreatic ductal carcinoma using a microcell-mediated chromosome transfer technique. The tumorigenicity and metastatic ability of both the parental cells and resulting hybrids were assessed *in vitro* and *in vivo*.

RESULTS: *In vitro* growth of hybrid clones was significantly delayed compared to parental cells. This was paralleled by a significantly lower rate of promoting invasive carcinoma in nude mice and a longer latency with hybrid cells compared with parental tumour cells. Hybrid clones showed significant suppression in the number of surface lung metastases when compared with parental cells.

CONCLUSION: These data represent strong functional evidence that chromosome 18q encodes strong tumour and metastasis suppressor activity that is able to switch human pancreatic cancer cells to a dormant phenotype.
[*Asian J Surg* 2004;27(2):85–92]

Introduction

Tumour 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. The molecular pathology of pancreatic carcinogenesis is characterized by a broad spectrum of distinct gene mutations and chromosomal alterations, some of which seem to be non-random. Recent advances in molecular biology have increased our understanding of the pathophysiology of, and the frequent genetic alterations in, this disease.[1]

Many studies employing different molecular techniques have consistently outlined the loss of chromosome 18q as an early event in pancreatic carcinogenesis. Chromosome 18 harbours a cluster of either tumour or metastasis suppressor genes such as *SMAD2*, *SMAD4*, *DCC*, *maspin* and *PAI-2*. Loss of heterozygosity (LOH) of 18q is a common event in more than 90% of pancreatic carcinomas, while only 50% are characterized by bi-allelic inactivation of the *SMAD4* genes.[2,3] This high frequency of losses affecting 18q caused special interest in this region. We reported the loss of 18q in 92% of pancreatic juice samples collected from patients with clinically early pancre-

Address correspondence and reprint requests to Dr. Makoto Sunamura, Department of Gastrointestinal Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan.
E-mail: msun-thk@umin.ac.jp • Date of acceptance: 29th June, 2003

atic neoplasia and emphasized this approach as a useful tool in early detection of this deadly disease.[4] In a recent study, we found that LOH of 18q is significantly associated with a poor prognosis in pancreatic cancer.[5] Loss of *SMAD4* expression occurs biologically late in neoplastic progression; therefore, even when clinically early infiltrating pancreatic cancers are detected, they could, in fact, be considered genetically late.[6,7] On the other hand, adenovirus-mediated transfection with *SMAD4* inhibits mouse tumorigenesis by halting angiogenesis but fails to inhibit *in vitro* growth of pancreatic ductal adenocarcinoma cells with completely inactivated *SMAD4*.[8,9]

To date, there is no strong evidence that implicates *SMAD2* or *MADH2*, another candidate tumour suppressor gene residing on chromosome 18q, in colon and other cancers; they are inactivated only in a small fraction, accounting for 5% of colon and other cancers.[10] Loss of *DCC* expression was initially found to be involved in colon cancer,[11] where it could play a role in tumour progression. However, only one recent study reported inactivation of *DCC* by homozygous deletions in a subset of pancreatic and biliary cancers, as an isolated event or in addition to *SMAD4* alterations.[12]

Maspin is initially expressed in normal human mammary and prostate epithelial cells, but is down-regulated during cancer progression. *Maspin* inhibits cell motility, invasion and metastasis in breast and prostate cancers.[13] *Maspin* expression is up-regulated in pancreatic cancer, in contrast to its absence in normal pancreatic tissue.[14,15] However, mutations in these genes alone cannot explain the whole process of pancreatic carcinogenesis; there may be some other genes that play important roles. We have focused on tumour suppressor genes in pancreatic cancer to find effective methods for genetic diagnosis and/or treatment. Given that a few different known and probably unknown tumour and metastasis suppressor genes are clustered on 18q, we presumed that correction of these defects could restore quasi-normal growth status to pancreatic cells.

This study was undertaken to provide functional evidence for the existence of new tumour suppressor genes located on chromosome 18 that play a role in pancreatic tumorigenesis.

Materials and methods

Cell lines

Two-pancreatic cancer cell lines, PK-1 (established in our department from liver metastasis of pancreatic cancer)[16] and Panc-1 (American Type Culture Collection, Rockville, MD, USA), were cultured according to the protocols of the suppliers. The cell lines are well characterized.[17]

Briefly, PK-1 is homozygous for deletion of *SMAD4* (*SMAD4*$^{-/-}$), whereas Panc-1 expresses normal *SMAD4* (*SMAD4*^{wt}) (Table). For each cell line, three stable hybrids containing a normal copy of chromosome 18 (detected by fluorescence *in situ* hybridization, FISH, using mouse DNA as probes) were established: PK-1H(18)-1 through -3 and Panc-1H(18)-1 through -3, respectively. These hybrids were generated by the microcell-mediated chromosome transfer (MMCT) technique and expanded in medium containing 400 μg/mL of G418, as previously described.[18–20] The A9H(18) mouse fibroblast cell line, which carries a single copy of human chromosome 18 and an integrated neomycin resistance 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.

Table. Oncogenic properties of stable hybrid cells

	PK-1	PK-1H(18)	Panc-1	Panc-1H(18)
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
Tumour size (mm^3 at day 45)†	1945 ± 245	366 ± 98	756 ± 168	142 ± 39
Latency period (d)†§	8 ± 2	14 ± 3	9 ± 3	18 ± 3
Lung metastatic lesion (number/lung)†‖	51 ± 11.8	5.2 ± 2.6	29 ± 9	6 ± 3.4
Proliferation index (PCNA-positive cells/x 40 field)†	57 ± 11.6	16 ± 5.1	42 ± 8.3	9 ± 3.4
SMAD4 status of parental cells	*SMAD4*$^{-/-}$		*SMAD4*$^{+/+}$	

*Colony number and size were measured and averaged from three randomly chosen photographs from each plate; †averaged results ± standard deviation from three independent experiments; ‡apoptotic cells were detected by annexin V/EGFP staining; §latency period is defined as time course when tumours became palpable (reached 4–5 mm in diameter); ‖surface lung metastatic tumours counted and microscopically confirmed on day 30. PCNA = proliferating cell nuclear antigen.

Microsatellite analysis

Genomic DNA from cells and nude mouse tumours was extracted using the Nucleon BACC3/ST kit (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England) and analysed using highly polymorphic microsatellite markers located on 18q, as described previously.[20,21]

Briefly, seven microsatellite markers, D18S1104, D18S463, D18S72, D18S35, D18S1144, D18S483 and D18S58 (http://gdbwww.gdb.org), are spaced at approximately 10 cM intervals (mean, 9.96 cM) along the long arm of chromosome 18. Microsatellite primers were designed to amplify approximately 100 bp to 150 bp products. The forward primers of each pair were end-labelled with ^{32}P-g-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). Polymerase chain reaction (PCR) amplification consisted of initial denaturation at 95°C for 2 minutes, 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 60–62°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. The PCR products were separated in 8 M urea-polyacrylamide gel and autoradiographed. For each marker, PCR amplification was carried out at least twice.

In vitro proliferation

Anchorage-dependent proliferation was monitored using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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.[22] Conversion of MTT to formazan dye was measured using absorbance at 590 nm in a multi-well plate immunoreader system. Data from three independent experiments were pooled, averaged and analysed.

Colony formation assay

Ten thousand cells were plated in 1 mL medium containing 0.3% Bacto-agar (Becton Dickinson, Sparks, MD, USA) with 10% fetal bovine serum as an upper layer into 30 mm dishes. Another 1 mL medium with 0.7% Bacto-agar was used for the bottom layer. After 2 weeks, viable colonies were stained red by incubation for 3 hours with 0.3 mL of 1 mg/mL 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride (INT; Dojindo Laboratories, Kumamoto, Japan). Red colonies were photographed using a Zeiss microscope (Carl Zeiss, Jena, Germany) with × 5 objective. Both the number and size of colonies were measured and averaged on three randomly chosen photographs from each plate using National Institutes of Health (NIH) 1.62 software. Independent anchorage growth was assessed in triplicate from two independent experiments.

Determination of apoptosis

Apoptotic cells were detected using annexin V/enhanced green fluorescent protein (EGFP) staining with an ApoAlert Annexin V-EGFP kit (Clontech, Palo Alto, CA, USA). Stained cells were quantified using a Becton Dickinson FACScan, and data were analysed using CellQuest software (version 3.1, Becton Dickinson).

Animal experiments

Eight-week-old male athymic nude (BALB/c-nu/nu) mice (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 10% Matrigel Growth Factor Reduced (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 10^7 cells/mL. Then, 3×10^6 cells in 300 μL suspension were injected subcutaneously into the hind flanks of nude mice.

Tumour volume was estimated after biweekly measurements using the formula $V = D \times d^2 \times 0.4$, where V = tumour volume, D = largest dimension and d = smallest dimension. Mice were sacrificed at week 8, when tumours from parental cells reached approximately 2,000 mm^3. Tumours were resected, weighed and bisected; half of the tissue was snap-frozen in liquid nitrogen for molecular investigation and the other half was fixed in neutral buffered formalin for further investigation.

To estimate the metastatic ability of hybrids, we used a lung colonization model.[23] Briefly, parental and hybrid cells were prepared as single-cell suspensions in sterile phosphate-buffered saline at a concentration of 5×10^6/mL, then 250 μL (1.25×10^6 cells, viability 95% as determined by trypan blue exclusion) was injected intravenously via the tail vein of the mice. Animals were sacrificed on day 30, when most control mice became moribund. Surface lung metastatic tumours were counted and microscopically confirmed. Each hybrid clone was assessed using two mice, and data from three independent experiments were pooled for statistical analysis. No spontaneous mortality or dropouts due to incomplete tumour growth were recorded.

Immunohistochemical analysis

Sections (5 μm) were prepared from formalin-fixed, paraffin-embedded specimens. Immunohistochemical reactions were

performed as described previously,[1] using mouse anti-proliferating cell nuclear antigen antibody (anti-PCNA, clone PC10, Dako Corporation, Copenhagen, Denmark), and developed using an Immunomouse kit (Zymed Laboratories Inc, South San Francisco, CA, USA). Proliferating cells were quantitated by counting PCNA-positive cells as well as total cells in 10 arbitrarily selected fields at × 40 magnification in a double-blinded manner. The percentage of PCNA-positive cells per 10 × 40 fields was determined from the number of PCNA-positive cells × 100/total number of cells. Negative control slides were prepared by omitting the primary antibody.

Statistical analysis

A two-tailed Student's *t* test calculated with GraphPad Prism 3.0 software (GraphPad Software Inc, San Diego, CA, USA) was used to determine the statistical significance of measured differences. The level of significance was established at *p* less than 0.05.

Results

Molecular characterization of parental and hybrid cells

The efficiency of chromosome transfer into hybrids was explored using microsatellite analysis; typical examples are shown in Figure 1. A complete copy, or at least a great majority, of human chromosome 18 was transferred and maintained in each hybrid cell; the band originating from A9H(18) was seen in the DNA of each hybrid cell line. To assess whether the transferred chromosome was maintained indefinitely, we repeated this analysis at the end of the experiment using sample DNA from both the hybrids (after 10–15 passages) and subcutaneous tumours generated in nude mice (Figure 1). The results confirmed that the additional copy of chromosome 18 was efficiently transferred and maintained indefinitely.

Phenotypic assessments

In vitro growth of hybrid cells was significantly suppressed compared with parental cells, regardless of initial *SMAD4* status (Figure 2). No morphological changes were apparent throughout the hybrid clones when compared with either parental or control cells (Figures 2B and C). This suggests that a product of a chromosome 18 gene other than *SMAD4* plays a role in this behaviour of hybrids cells.

To study proliferation and morphology under anchorage-independent conditions, we transferred both parental and hybrid cells from a mono- to a bi-layered Bacto-agar suspension. The size and number of colonies observed among hybrids were significantly smaller than among parental cells (mean, 49 ± 3.6 vs 228 ± 19.36 colonies/wheel and 106 ± 19.61 vs 392 ± 37.22 μm for PK-1H(18) and PK-1, respectively). In addition, parental cells formed tight, densely packed, multicellular spheroids (MCS) easily detected by the naked eye, while hybrids formed smaller, loose spheroids (Figures 2D and E). Because the ability of cells to grow in soft agar suspension very closely correlates with their tumorigenic potential *in vivo*,[24] these features strongly suggest that chromosome 18 transfer is associated with major changes in hybrid cell behaviour.

The percentage of annexin V-positive parental cells (2.1 ± 0.5%) was very low (Table); the percentage increased slightly among hybrid clones (3.9 ± 0.7%). Thus, apoptosis is unlikely to participate in the chromosome 18-mediated growth suppression observed in hybrid cells.

Despite inherent interclonal variation, the hybrids showed a significant reduction in tumour volume and a longer latency compared to parental cells (Figures 3A–D). We found that 16% of tumour cells stained positive for PCNA in hybrid tumours compared with 57% in parental tumours (Figures 3E and F). Thus, proliferation was decreased in hybrid tumours compared with parental tumours (Table), indicating that retardation of tumour growth is caused by diminished proliferation of hybrid cells.

The number of surface metastases in mice lungs injected with hybrids was significantly decreased compared with that in mice injected with parental cells (Table). Furthermore,

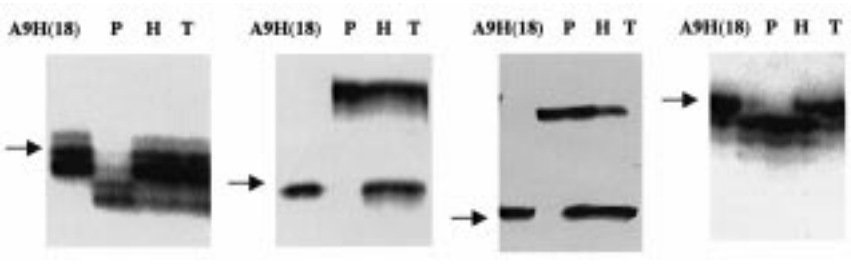

Figure 1. Microsatellite analysis. Representative scanned autoradiographs showing restoration of heterozygosity for the different 18q-microsatellite markers after chromosome 18 transfer into PK-1 cells. Arrows indicate new bands of the same size as those obtained from A9H(18)H used as donor cells. The generic names and cytogenetic position (Genome Database, http://gdbwww.gdb.org) along 18q are indicated below every panel. P = parental cells; H = hybrids; T = nude mouse tumours.

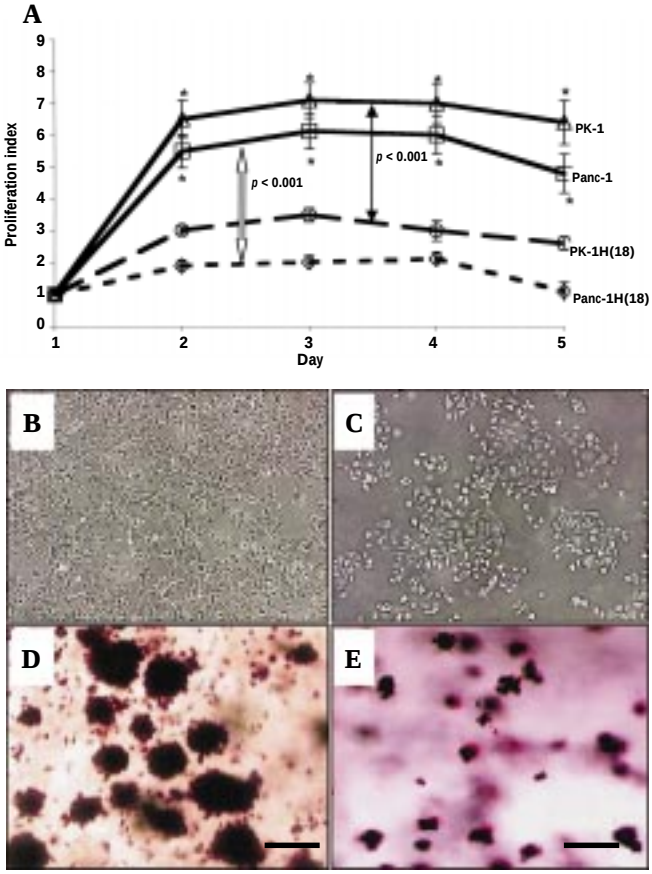

Figure 2. *In vitro* proliferation. A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays indicated that hybrids (dashed lines) derived from *SMAD4*^{wt} cells demonstrated the ability to suppress *in vitro* growth to a greater extent than hybrids derived from *SMAD4*$^{-/-}$ recipients (*$p < 0.05$). Note the same trend for parental cells. The calculated standard deviations (bars) were too low to be resolved in some curves. Up-to-down arrows reflect mean differences recorded between hybrid and parental cell tumours. B, C) PK-1 parental cells and their hybrids at 96 hours under a phase-contrast microscope at original magnification × 20. D, E) Anchorage-independent growth at day 21 shows that hybrids have an increased ability to suppress growth in soft agar (D, PK-1; E, PK-1H(18)). Bar = 500 μm.

lungs showing no metastases at surface examination had few detectable micro-metastases; some of them appeared to be dormant. These results demonstrate that the introduced chromosome 18 provides an important factor that reduces metastatic activity.

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.[25] At the time of the initial detection of clinical cancer, several genetic alterations have already accumulated in tumour cells. In pancreatic cancer, *KRAS*, *TP53*, *p16* and *SMAD4* are thought to play key roles in tumorigenesis.[26] Several lines of accumulated evidence have clearly shown that loss of 18q is one of the more consistent findings among chromosomal abnormalities identified in a variety of cancers, and that this event is associated with a poor clinical outcome.[5] However, functional evidence implicating chromosome 18 in pancreatic tumorigenesis is desirable.

In this study, we used MMCT to introduce a normal copy of human chromosome 18 individually into two pancreatic cancer cell lines. MMCT is a useful tool that has provided functional evidence of the location of tumour suppressor genes in a variety of cancers including melanoma[27] and prostate cancer.[28] We first performed MMCT and then checked the efficiency of chromosome transfer by microsatellite analyses. Although corresponding normal tissues were not available for each cell line, we demonstrated the introduced chromosome 18 by observing the additional band in hybrid cells. Because these cell lines do not have high microsatellite instability,[29] additional bands for each marker are likely to be derived from the introduced 18q copy. Although some microdeletions cannot be totally excluded, it is reasonable to assume that the great majority of the 18q arm was maintained in our hybrid cells. However, in this system, based on a semi-quantitative PCR method, it is impossible to estimate the percentage of cells retaining the transferred chromosome copy.[28]

In vitro growth of the hybrid clones was significantly delayed compared to that of parental cells, apparently regardless of initial *SMAD4* status. The latter fact is not surprising since over-expression of *SMAD4* itself does not affect the *in vitro* proliferative rate of pancreatic cancer cells.[8] 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.[24] The size and number of colonies were significantly smaller among hybrids than among parental cells. In addition, parental cells formed tight, densely packed MCS easily detected by the naked eye, while hybrids formed smaller, loose spheroids. This is in keeping with changes in the adhesion properties of hybrids, but should be examined in the light of further specific investigation. The ability of cells to grow in soft agar suspension very closely correlates with their tumorigenic potential *in vivo*, so the results of this experiment showed that chromosome 18 transfer is associated with major changes in tumour cell behaviour.

Cells undergoing apoptosis were detected using an annexin V assay, which measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event

typically associated with apoptosis. In comparison with traditional tests, this assay is sensitive and offers the possibility of detecting early phases of apoptosis before the loss of cell-membrane integrity, and permits measurement of the kinetics of apoptotic death in relation to the cell cycle.[30] The percentages of annexin V-positive cells were very low in parental cells and slightly increased among the hybrid clones (Table). Thus, apoptosis is unlikely to be involved in chromosome 18-mediated growth suppression observed throughout the hybrid clones.

Tumorigenesis in nude mice is one of the most stringent tests and yields highly valuable information about cancer-cell behaviour. In order to shorten tumour latency and enhance tumour growth, we mixed cells in a suspension containing Matrigel extract. Despite inherent interclonal variation, hybrids showed a significant reduction in tumour volume and a longer latency compared to parental cells. Remarkably, there was a significant difference in tumour volumes related to *SMAD4* status. Specifically, *SMAD4*$^{+/+}$ cells generated significantly bigger tumours with a shorter latency than *SMAD4*$^{-/-}$ cells. This has recently been explained by the fact that *SMAD4* inhibits angiogenesis via putative down-regulation of vascular endothelial growth factor.[8] We found that 16% of tumour cells stained positive for PCNA in hybrid tumours compared with 57% in parental tumours. Thus, proliferation was decreased in hybrid tumours compared with parental tumours (Table), which indicated that retardation of tumour growth is caused mainly by slower proliferation.

Metastasis-regulatory genes can be broadly categorized as either metastasis-promoting or metastasis-suppressing. Analogous to the role of oncogenes in tumorigenesis, metastasis promoters drive conversion from non-metastatic to metastatic cells.[31] As expected, tumour suppressors inhibit both phenotypes because tumorigenicity is a prerequisite for metas-

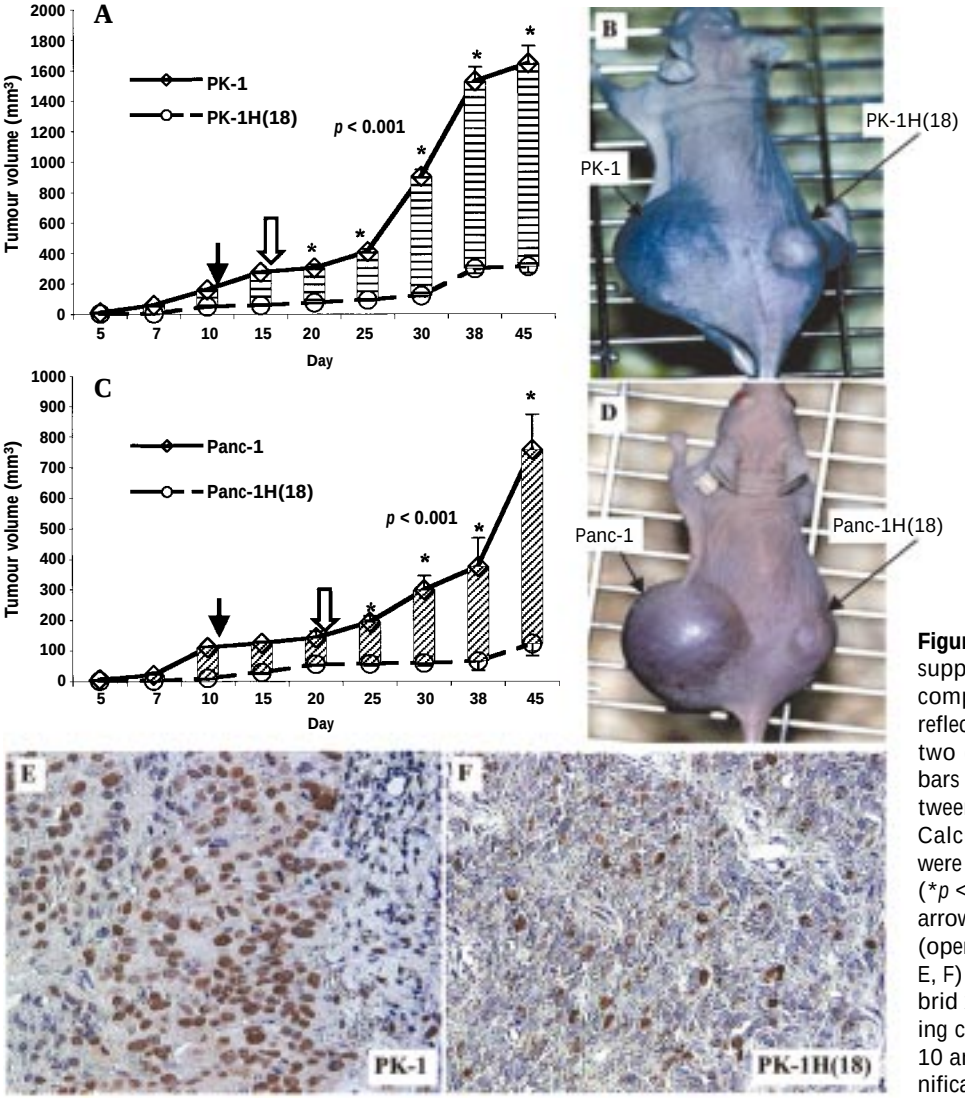

Figure 3. *In vivo* growth. A–D) Significant suppression of tumorigenesis in hybrids compared to parental cells. Each point reflects the average of three tumours from two different experiments. Up-to-down bars reflect mean differences recorded between hybrid and parental cell tumours. Calculated standard deviations (bars) were too low to be resolved in some curves (*$p < 0.001$). In A and C, the up-to-down arrows reflect the latency period for hybrid (open arrows) and parental cell tumours. E, F) Decreasing proliferation index in hybrid xenografts. Percentage of proliferating cell nuclear antigen-positive cells per 10 arbitrarily selected fields at × 40 magnification (E, PK-1; F, PK-1H(18)).

tasis.[32] As chromosome 18 harbours a cluster of candidate tumour and metastasis suppressor genes, i.e. *SMAD2*, *SMAD4*, *DCC*, *maspin* and *PAI-2*, and because poor prognosis is significantly associated with 18q-LOH,[5] it is of great interest to examine whether genes on chromosome 18 play roles in metastatic processes. To estimate the metastatic ability of hybrids, we used a lung colonization model. We recorded a significant suppression in the number of surface metastases in mice injected with hybrids compared to mice injected with parental cells (mean, 5 vs 51 for PK-1(18) and PK-1, respectively) (Table). This demonstrated that 18q encodes an important metastasis suppressor factor and confirmed our previous study that clearly correlated LOH of 18q with poor prognosis in patients with pancreatic cancer.[5]

The MMCT technique means that hybrid cells usually do not contain mouse chromosomes,[19] but there is nevertheless a small possibility that the observed growth suppression could be attributed to the effects of remaining mouse chromosomes. However, we obtained three independent hybrid clones for each parental cell line, and the possibility that these individual clones would harbour the same particular undetected mouse chromosome or chromosome fragments is unlikely. Hence, it is likely that the tumour suppression phenotype is caused by the introduction of normal human chromosome 18 into pancreatic cancer cells.

Nevertheless, whole chromosome transfer raises at least two issues: the direct effects of known or unknown genes located on chromosome 18, and indirect effects through possible interactions among the transferred chromosome and other genes. Although in this setting, these effects could not be clearly delineated, it is conceivable that introduction of an extra copy of chromosome 18 confers a less aggressive tumour phenotype on pancreatic cancer cells. In other words, the metastatic inhibition encoded by 18q could explain the dormant status of the hybrids and, in turn, the presence of micro-metastases unable to form macro-metastases. Although this study clearly implicates the important role of gene(s) on chromosome 18, the precise sub-chromosomal localization of the metastasis suppressor gene(s) remains an open question. In the absence of spontaneous revertant hybrids, the precise localization and identification of a putative metastasis suppressor gene will require further effort. In an attempt to define new presumable interactions between genes on chromosome 18 and others, a microarray analysis is currently under way in our department.

Briefly, we achieved significant suppression of both *in vitro* and *in vivo* growth of pancreatic cancer cells by transfer of chromosome 18. The suppression was observed regardless of *SMAD4* status, and induction and restoration of *SMAD4* could not prevent *in vitro* growth, regardless of *SMAD4* mutational background.[8] These functional data bring into sharp relief the implication of chromosome 18 in pancreatic carcinogenesis, but new research will be able to locate tumour suppressor gene(s) in this region.

Acknowledgements

We are grateful to Emiko Shibuya, Hiroko Fujimura and Keiko Inabe at Tohoku University for their expert technical assistance. Liviu P. Lefter is a recipient of the Hong Kong International Cancer Congress Young Investigator Award. Part of this article reflects data presented at the 18th World Congress of Digestive Surgery in Hong Kong, 9–12 December 2002.

References

1. Zhu Z, Kleeff J, Kayed H, et al. Nerve growth factor and enhancement of proliferation, invasion, and tumorigenicity of pancreatic cancer cells. *Mol Carcinog* 2002;35:138–47.
2. Hahn SA, Hoque AT, Moskaluk CA, et al. Homozygous deletion map at 18q21.1 in pancreatic cancer. *Cancer Res* 1996;56:490–4.
3. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;271: 350–3.
4. Fukushige S, Furukawa T, Satoh K, et al. Loss of chromosome 18q is an early event in pancreatic ductal tumorigenesis. *Cancer Res* 1988;58:4222–6.
5. Yatsuoka T, Sunamura M, Furukawa T, et al. Association of poor prognosis with loss of 12q, 17p, and 18q, and concordant loss of 6q/17p and 12q/18q in human pancreatic ductal adenocarcinoma. *Am J Gastroenterol* 1995;8:2080–5.
6. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000;60:2002–6.
7. Inoue H, Furukawa T, Sunamura M, et al. Exclusion of SMAD4 mutation as an early genetic change in human pancreatic ductal tumorigenesis. *Genes Chromosomes Cancer* 2001;31:295–9.
8. Schwarte-Waldhoff I, Volpert OV, Bouck NP, et al. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:9624–9.
9. Duda DG, Sunamura M, Lefter LP, et al. Restoration of SMAD4 by gene therapy reverses the invasive phenotype in pancreatic adenocarcinoma cells. *Oncogene* 2003;22:6857–64.
10. Takagi Y, Koumura H, Futamura M, et al. Somatic alterations of the SMAD-2 gene in human colorectal cancers. *Br J Cancer* 1998;78: 1152–5.
11. Fearon ER, Cho KR, Nigro JM, et al. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* 1990;

247:49–56.
12. Hilgers W, Song JJ, Haye M, et al. Homozygous deletions inactivate DCC, but not MADH4/DPC4/SMAD4, in a subset of pancreatic and biliary cancers. *Genes Chromosomes Cancer* 2000;27:353–7.
13. Sheng S, Carey J, Seftor EA, et al. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc Natl Acad Sci USA* 1996;93:11669–74.
14. Maass N, Hojo T, Ueding M, et al. Expression of the tumor suppressor gene Maspin in human pancreatic cancers. *Clin Cancer Res* 2001; 7:812–7.
15. Lefter LP, Sunamura M, Furukawa T, et al. Inserting chromosome 18 into pancreatic cancer cells switches them to a dormant metastatic phenotype. *Clin Cancer Res* 2003;9:5044–52.
16. Kobari M, Matsuno S, Sato T, et al. Establishment of a human pancreatic cancer cell line and detection of pancreatic cancer associated antigen. *Tohoku J Exp Med* 1984;143:33–46.
17. Sun C, Yamato T, Furukawa T, et al. Characterization of the mutations of the K-ras, p53, p16, and SMAD4 genes in 15 human pancreatic cancer cell lines. *Oncol Rep* 2001;8:89–92.
18. Fournier RE, Ruddle FH. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. *Proc Natl Acad Sci USA* 1977;74:319–23.
19. Koi M, Morita H, Yamada H, et al. Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. *Mol Carcinog* 1989;2:12–21.
20. Lefter LP, Furukawa T, Sunamura M, et al. Suppression of the tumorigenic phenotype by chromosome 18 transfer into pancreatic cancer cell lines. *Genes Chromosomes Cancer* 2002;34:234–42.
21. Kimura M, Abe T, Sunamura M, et al. Detailed deletion mapping on chromosome arm 12q in human pancreatic adenocarcinoma: identification of a 1-cM region of common allelic loss. *Genes Chromosomes Cancer* 1996;17:88–93.
22. van Golen KL, Wu ZF, Qiao XT, et al. RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res* 2000;60:5832–8.
23. Luu HH, Zagaja GP, Dubauskas Z, et al. Identification of a novel metastasis-suppressor region on human chromosome 12. *Cancer Res* 1998;58:3561–5.
24. Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* 1988;240:177–84.
25. Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science* 1997;278:1043–50.
26. Sunamura M, Yatsuoka T, Motoi F, et al. Gene therapy for pancreatic cancer based on genetic characterization of the disease. *J Hepatobiliary Pancreat Surg* 2002;9:32–8.
27. Trent JM, Stanbridge EJ, McBride HL, et al. Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science* 1990;247:568–71.
28. Padalecki SS, Johnson-Pais TL, Killary AM, Leach RJ. Chromosome 18 suppresses the tumorigenicity of prostate cancer cells. *Genes Chromosomes Cancer* 2001;30:221–9.
29. Kondo E, Furukawa T, Yoshinaga K, et al. Not hMSH2 but hMLH1 is frequently silenced by hypermethylation in endometrial cancer but rarely silenced in pancreatic cancer with microsatellite instability. *Int J Oncol* 2000;17:535–41.
30. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995;184:39–51.
31. Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. *Cancer Res* 2000;60: 2764–9.
32. Welch DR, Rinker-Schaeffer CW. What defines a useful marker of metastasis in human cancer? *J Natl Cancer Inst* 1999;91:1351–3.