

# The Effect of Wild Type P53 Gene Transfer on Growth Properties and Tumorigenicity of PANC-1 Tumor Cell Line

Marzieh Hagi-Sharifia Taghavi<sup>1</sup>, Jamshid Davoodi<sup>2</sup> and Manouchehr Mirshahi<sup>\*1</sup>

<sup>1</sup>Dept. of Biochemistry, Faculty of Basic Sciences, Tarbiat Modares University, Tehran, Iran; <sup>2</sup>Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran P.O. Box 13145-1384

Received 9 May 2005; revised 13 August 2006; accepted 30 August 2006

## ABSTRACT

**Background:** The p53 protein function is essential for the maintenance of the nontumorigenic cell phenotype. Pancreatic tumor cells show a very high frequency of p53 mutation. To determine if restoration of wild type p53 function can be used to eliminate the tumorigenic phenotype in these cells, pancreatic tumor cell lines, PANC-1 and HTB80, differing in p53 status were stably transfected with exogenous wild type p53 gene. **Methods:** The transfection was performed using Polybrene/DMSO-Assisted Gene Transfer method. The wild type p53 gene integration into genomic DNA was detected by Southern blot and PCR. Furthermore, the expression of wild type p53 protein was detected in selected clones by immunohistochemistry and Western blot. **Results:** While HTB80 cell line failed to produce a stable p53 expressing clone, the PANC-1 cells produced stable lines. Following characterization of clones, the growth rate and tumorigenicity of PANC-1 wild type p53 clones were compared to the control cells. Our data showed that the expression of wild type p53 decreased the growth rate of PANC-1 cells. It was also observed that the expression of wild type p53 in PANC-1 cells suppressed its potential for tumor formation in nude mice, completely, while the parental line leads to the formation of a relatively large tumor. **Conclusion:** Our results suggest that gene therapy based on restoration of wild type p53 protein function in pancreatic tumor cells with high amount of mutant p53 is a feasible option in pancreatic cancer treatment. *Iran. Biomed. J. 11 (1): 1-6, 2007*

**Keywords:** p53, pancreatic cancer, tumor formation

## INTRODUCTION

The p53 tumor suppressor gene is the most common known mutated sequence in human cancers, 50% of all human cancers lacks a wild type (wt) p53 gene allele and thus generates a functionally defective p53 protein [1, 2] and it is now thought that p53 protein function is essential for the maintenance of the nontumorigenic cell phenotype [3]. The p53 protein is a sequence specific transcription factor that activates a variety of cellular genes in response to DNA damage, hypoxia, stress, and many pathological states [4-6]. Human pancreatic cancer is the fifth leading cause of cancer death in the North America [7, 8]; it presents its symptoms late in the course of the disease and is usually fatal within a year. Pancreatic cancer is refractory to radiation and chemotherapy. Therefore, alternative therapeutic strategies are

urgently required for pancreatic cancer. Pancreatic carcinomas display P53 mutations at relatively high frequency [9, 10]. Sequence analysis of exon 5-8 of the P53 gene in 13 human pancreatic cell lines revealed missense mutations in seven cell lines that were in the evolutionarily conserved regions of P53 gene [11]. These results confirm the p53 inactivation as an important event in human pancreatic tumorigenesis. Therefore, it is important to define whether the expression of p53 is compatible with the transformed phenotype of pancreatic cells. Studies with p53 gene transfer have been conducted in many cancer types including cervical, ovarian, prostatic, bladder, brain and head and neck tumors [12-15]. Restoration or overexpression of p53 is known to achieve various antitumor activities, which includes induction of apoptosis, inhibition of cell proliferation, antiangiogenesis, and repair of damaged genes. These antitumor mechanisms of p53

\*Corresponding Author; Tel. (+98-21)8801-1001 ext 4408; Fax: (+98-21) 8800-9730; E-mail: mirshahi@modares.ac.ir

are known to be dependent in part on the tumor cell type and on the level of p53 expressed [16]. The effect of over expression of p53 protein is not known in the pancreatic tumor cells. In the present study, we examine the effects of introducing wt p53 gene on the growth properties and tumorigenicity of pancreatic tumor cells, differing in p53 status. Two pancreatic tumor cell lines, PANC-1 and HTB80 were selected. The p53 gene of PANC-1 pancreatic tumor cell line has a mutation of Arg to His at codon 273. While PANC-1 cells express high amount of mutant p53, HTB80 tumor cells express very low levels of wt p53 protein. We present data, which show wt p53 gene functions as a suppressor of cellular growth in the PANC-1 pancreatic tumor cell line, and that it completely suppresses its ability for tumor formation in nude mice.

## MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Company (USA) unless otherwise stated. Anti-p53 antibody Ab-5 (PAb1620) recognizes a conformational epitope of wt p53 and therefore will not recognize mutant or denatured p53. Ab-2 (PAb1801) recognizes amino acids 46-55 of both mutant and denatured wt p53. They were both from (Oncogene, USA). Polybrene: (1, 5-dimethyle 1, 5-undecamethylene polymethobromide) was from Aldrich, USA. Pancreatic tumor cell lines, PANC-1 and HTB80 were obtained from American Type Culture (USA) Collection. The plasmid pcNXRS containing human wt p53 cDNA under the control of cytomegalovirus (CMV) promoter/enhancer (Invitrogen, USA) was used for the transfection experiments [17].

**Cell culture.** The pancreatic tumor cell lines were grown in DMEM (10% fetal bovine serum, FBS), supplemented with 1mM L-glutamine and 100 U/ml penicillin. All cell cultures were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO<sub>2</sub>. Growth medium was replaced every 4 days.

**Transfection experiments.** Transfection was performed according to reference [18]. Cells (1 × 10<sup>6</sup> per 100 mm diameter dish) of pancreatic tumor cell line PANC-1 were transfected with 1-10 µg of pcNXRS plasmid DNA in the presence of 5 µg/ml polybrene in culture medium supplemented with 10% FBS and 30% DMSO (Fisher Scientific) for

permeabilization. Following incubation for 24 h, cells were washed and fed with fresh selection medium contain 0.8-1 µg/ml of G418 (Geneticin; Invitrogen, USA). After 2-3 weeks G418 resistant individual colonies were picked and expanded for further analysis.

### *Expression of p53 protein in selected clones.*

Analysis of exogenous p53 expression was performed by Western blotting and immunohistochemistry techniques (Oncogene, USA) as described below.

### *Immunohistochemistry with cultured cells.*

Cultured cells were fixed on cover slips with a freshly prepared solution containing 4% paraformaldehyde, 0.1% glutaraldehyde in PBS for 15 minutes on ice, and rinsed 3 times with PBS. Cells were then permeabilized with 0.1% Triton X-100 and blocked with a mixture of 10% goat serum and 2% BSA in PBS for 1 hour. This was followed by overnight incubation at room temperature with anti-nitrotyrosine in 10% goat serum containing 0.1 % Triton X-100 in PBS. Following three PBS washes, samples were incubated with biotin-conjugated secondary antibody of choice in 10% goat serum for 30 minutes and rinsed. Finally, DAB-peroxidase substrate was added and samples were washed with PBS and distilled water and mounted in glycerol.

**Western blots.** Whole-cell extracts were prepared by rinsing the cells twice with ice-cold PBS and then adding cell lysis buffer (50 mM Tris HCl, 0.1% SDS, 10% glycerol, 0.72 M 2-mercaptoethanol, 0.1M phenylmethanesulfonyl fluoride and 6 M urea). The cells were scraped off the dish using a rubber spatula and scraping was done over ice. Total proteins (20-50 µg) were separated on SDS-PAGE gel and transferred into PVDF membrane (Millipore). Membranes were blocked in 0.5% blocking solution (0.5% milk in Tris buffered saline, TBS) at 25°C for 1 h. Then, they were incubated with primary antibody in 0.1% blocking solution at 4°C overnight. Membranes were washed four times in TBST (0.1% Tween 20 in TBS) and then incubated with secondary antibody at 25°C for 1 h. Finally, membranes were washed three times in TBST, and primary antibody-antigen complex was visualized by enhanced chemiluminescence using horseradish peroxidase coupled secondary anti-mouse antibodies (Boehringer Mannheim, USA).

**Characterization of P53 gene integration in transfectants using Southern Blot.** Genomic DNA (10 µg) was digested with the restriction endonucleases HindIII and electrophoresed on 1% agarose gels. Southern transfer was performed using 0.4 M NaOH and Pall Biodyne B nylon membrane. Membranes were prehybridized with HYBSOL (1.5 × SSPE, 7% SDS, 10% Poly Ethylene glycol) supplemented with 200 µg/ml Herring sperm DNA at 65°C for 2-4 h. Hybridization was performed in HYBSOL supplemented with 32P-p53 labeled probe for 16-20 h at 65°C. Membranes were washed with 2 × SSC/0.5% SDS, 0.2 × SSC/0.5% SDS, and 0.1 × SSC/0.1% SDS, respectively.

**PCR analysis.** Two neomycin phosphotransferase oligonucleotide primers, neo forward (5'-CAAGATGGATTGCACGCAGG-3') and neo reverse (5'-CCCGCTCAGAAGAACTCGTC-3') were synthesized. Conditions for amplification of a 790-bp fragment of the neo gene were as follow: 1 µg of genomic DNA was incubated in a 100 µL of PCR reaction buffer (20 mM Tris-HCL, Ph 8.9, 50 mM KCl), 200 µM each dNTP, 1.5 mM MgCl<sub>2</sub> and 0.5 µM each primer, at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and in the last cycle, 72°C for 10 min, through 35 cycles. Ten µL of amplification product was then loaded on 1% agarose gel.

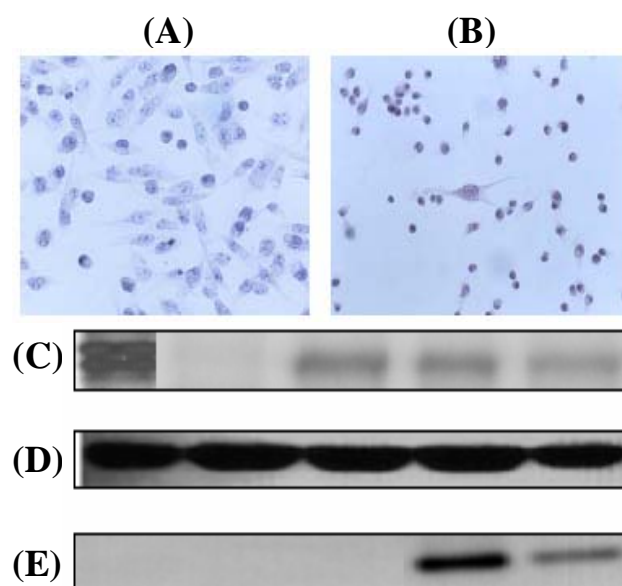
**Growth rate analysis.** Equal numbers of cells from individual clones ( $1 \times 10^5$ ) were seeded onto 60 mm dishes into regular medium as described earlier. Cells were counted every 2 days by culture counter.

**Tumorigenicity assays.** Young adult female, 4-7 weeks old nude mice were used. Cells from tissue culture with viability greater than 98% were used and equal number of cells ( $3-6 \times 10^6$ ) from each clone was injected into flanks of thymic nude mice. Tumor formation and tumor size were measured and recorded weekly until 12 weeks postinjection.

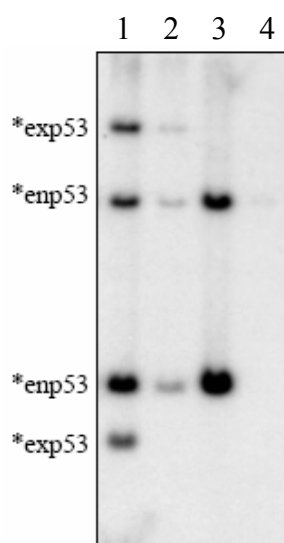
## RESULTS

**Establishment of stable wt p53 expressing cell line.** Human pancreatic cell lines, PANC-1 and HTB80 were chosen for this study. The former cell line contains mutant P53 gene that has mutation in exon 7 (codon 273, Arg to His mutation) [10] and expresses high amount of mutant P53 protein detectable by both immunohistochemistry and Western blot. The latter express very low amount of

p53 protein. In the first experiment, the above cell lines were transfected with pcNXRS construct, which carried the cloned human p53 cDNA, coupled to the neo gene under CMV promoter control. G418-resistant cells were recovered 3-4 weeks post-transfection and single-cell clones were then isolated and expanded. Unfortunately, having isolated very few HTB80 clones, they all died in the expansion step. The PANC-1 clones on the other hand produced viable colonies. Thus, these clones were screened for the expression of p53 protein by immunohistochemistry and Western blot using monoclonal antibody PAB1620 directed against wt forms of p53 (Fig. 1 A-C). The densitometric evaluation of exogenous p53 expression in Figure 1C, lanes 2-5, showed the 0, 170, 140, 103, values (sum above background) for each band, respectively. The β-actin was used as loading control (Fig. 1D). Integration of P53 gene fragment into clones was also examined by PCR amplification of neo gene, selection gene, (Fig. 1E). As an additional confirmatory tool, Southern blot analysis of exogenous genomic P53 was performed (Fig. 2). The positive clones confirmed by three methods were selected for the following experiments.



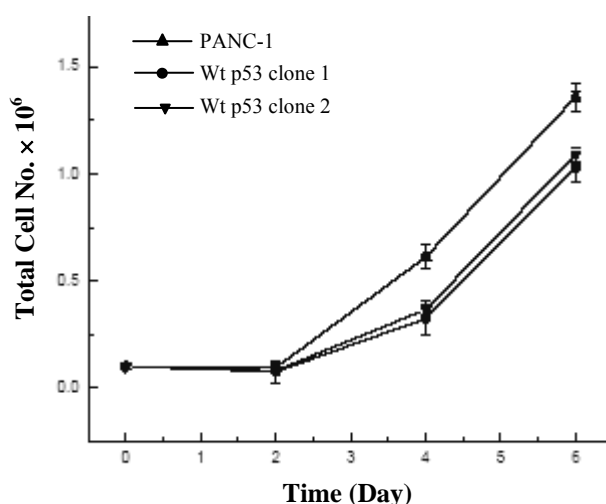
**Fig 1.** Analysis of wild type p53 expression in pancreatic tumor cell line, PANC-1. **A** and **B** are the immunohistochemistry analysis of the untransfected and wild type p53 transfected PANC-1 cells, respectively. **C** shows the Western blot of the PANC-1 and wild type p53 transfectants using wild type p53 antibody (1620 antibody): Lane 1, PANC-1 positive control (1801 antibody); lane 2, PANC-1 negative control (Ab 1620); lanes 3-5, wild type p53 expressing transfectants. **D**, β-actin control. **E**, PCR amplification of the selection gene, neo gene: Lane 1, negative control without DNA; Lanes 2 and 3, PANC-1 cell line; Lanes 4 and 5, wild type p53 transfected clones.



**Fig 2.** Southern blot analysis of exogenous p53 gene integration in pancreatic tumor cell line, PANC-1. Lane 1 and 2, positive transfectants; lane 3, PANC-1; lane 4, vector. Genomic DNA for each sample was digested with HindIII. \*ex, exogenous; \*en, endogenous.

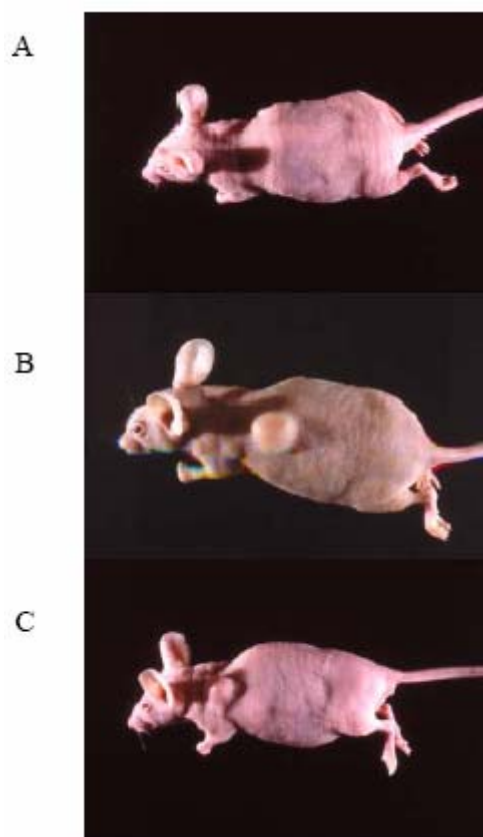
#### Growth properties of wt p53 transfectants.

Several studies have shown that introduction of wt P53 gene to tumor cell lines induces growth arrest. This effect, however, depends on both protein dosage and cell type. In order to test whether the expression of P53 may induce a suppression of cellular growth in pancreatic tumor cell line, PANC-1, the growth rates of each clone was measured. As presented by the slopes of growth curves (Fig. 3), the growth rates of PANC-1 transfectants were consistently slower than parent cells.



**Fig. 3.** Effect of p53 expression on the growth rate of PANC-1 tumor cells. Equal number of cells ( $1 \times 10^5$ ) was seeded onto 60 mm culture dishes. Cells were trypsinized and counted every two days. Results are the mean of three independent experiments.

**Tumorigenic potential of wt p53 expressing cells in nude mice.** To further assess the growth suppression potential of the wt p53 gene in pancreatic tumor cells, a tumorigenic assay in nude mice was performed. The results showed that wt p53 expressing PANC-1 transfectants were not tumorigenic in nude mice (Fig. 4).



**Fig. 4.** Tumorigenicity of p53 positive and negative clones in nude mice. PANC-1 cells transfected with wild type p53 (A); vector without p53 (B) and untransfected PANC-1 (C) were injected into nude mice.

## DISCUSSION

Ductal pancreatic adenocarcinoma represents 80-85% of all pancreatic malignancies and is one of the most lethal cancers in the Western world. Despite recent advances in diagnostics, staging and therapy, the long-term survival of patients remains dismal. In fact, pancreatic carcinoma is refractory to chemotherapy and radiotherapy [19- 21]. Several protooncogenes and tumor suppressor genes have been implicated in pancreatic tumors, including tumor suppressor p53 [22]. P53 mutations are the most commonly observed gene alterations in human cancers. As a central regulator of cell growth, p53 is essential for the maintenance of the normal balance

between cell proliferation and cell death. Given the importance of p53 in regulating cell growth and its frequent loss in human cancers, scientists began to explore the idea of reintroducing wt p53 into cancer cells to induce growth arrest, apoptosis or even to increase their sensitivity to chemotherapeutic agents. Inactivation of p53, a constant feature of pancreatic carcinoma, [23, 24], could account for resistance to therapy. In fact, p53 function is required for growth arrest or apoptosis after administration of DNA-damaging agents [21]. In this study, we evaluated the efficacy of pancreatic cancer gene therapy by examining the effect of expression of wt tumor suppressor p53 on the treatment of pancreatic cancer cells, PANC-1, with high amount of mutant p53 and HTB80 with very low amount of wt p53 protein. HTB80 cells did not produce viable colonies and died in the expansion stage. Since these cells contain wt p53, we speculate that p53 is functional in these cells and reasons other than p53 alteration caused their tumorigenic phenotype. Furthermore, it seems that the elevated level of p53 protein by gene transfer can cause these tumor cells to undergo cell death due to the fact that they are unable to bear extra amount of p53. Wt p53 transfectants of PANC-1 cells, which express high amount of mutant p53 (codon 273) protein, exhibited a reduced growth rate of pancreatic carcinoma cells in comparison to the parental cells. Furthermore, introduction of p53 into these cells resulted in complete suppression of tumor formation in the nude mice. The results reported herein also suggest that gene therapy based on restoration of wt p53 protein function in pancreatic tumor cells with high amount of mutant p53 is a feasible option in pancreatic cancer therapy. Obviously, combining this therapy with conventional chemotherapeutic agents may yield a more beneficial response than conventional treatments alone. With improving gene delivery systems and novel drugs that restore p53 function, it is likely that combining wt p53 based gene therapy with conventional therapies will be the most effective cancer therapy regimen in the future.

## REFERENCES

- Hainaut, P., and Hollstein, M. (2000) p53 and human cancer: the first ten thousand mutations. *Adv. Cancer Res.* 77: 81-137.
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science* 253: 49-53.
- Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.P., Pinkel, D., Gray, J., Bradley, A., Medina, D. and Varmus, H.E. (1996) The role of p53 loss in genomic instability and tumor progression in a murine mammary cancer model. *Prog. Clin. Biol. Res.* 395: 1-11.
- Harris, S.L. and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops. *Oncogene* 24: 2899-2908.
- Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* 88: 323-331.
- Prives, C. and Hall, P.A. (1999) The p53 pathway. *J. Pathol.* 187: 112-126.
- Carriaga, M.T. and Henson, D.E. (1995) Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 75: 171-190.
- Trapasso, F., Sarti, M., Cesari, R., Yendamuri, S., Dumon, K.R., Aqeilan, R.I., Pentimalli, F., Infante, L., Alder, H., Abe, N., Watanabe, T., Viglietto, G., Croce, C.M. and Fusco, A. (2004) Therapy of human pancreatic carcinoma based on suppression of HMGA1 protein synthesis in preclinical models. *Cancer Gene Ther.* 11: 633-641.
- Bardeesy, N. and DePinho, R.A. (2002) Pancreatic cancer biology and genetics. *Nat. Rev. Cancer* 2: 897-909.
- Ruggeri, B., Zhang, S.Y., Caamano, J., DiRado, M., Flynn, S.D. and Klein-Szanto, A.J. (1992) Human pancreatic carcinomas and cell lines reveal frequent and multiple alterations in the p53 and Rb-1 tumor-suppressor genes. *Oncogene* 7: 1503-1511.
- Barton, C.M., Staddon, S.L., Hughes, C.M., Hall, P.A., O'Sullivan, C., Kloppel, G., Theis, B., Russell, R.C., Neoptolemos, J., Williamson, R.C. Lane, D.P. and Lemoine, N.R. (1991) Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. *Br. J. Cancer* 64: 1076-1082.
- Ganjavi, H., Gee, M., Narendran, A., Parkinson, N., Krishnamoorthy, M., Freedman, M.H. and Malkin, D. (2006) Adenovirus-mediated p53 gene therapy in osteosarcoma cell lines: sensitization to cisplatin and doxorubicin. *Cancer Gene Ther.* 13: 415-419.
- Yoshida, K., Monden, M., Nakamura, Y. and Arakawa, H. (2004) Adenovirus mediated p53AIP1 gene transfer as a new strategy for treatment of p53-resistant tumors. *Cancer Sci.* 95: 91-97.
- Lawler, S.E., Peruzzi, P.P. and Chiocca, E.A. (2006) Genetic strategies for brain tumor therapy. *Cancer Gene Ther.* 13: 225-233.
- Irie, A., Matsumoto, K., Anderegg, B., Kuruma, H., Kashani-Sabet, M., Scanlon, K.J., Uchida, T. and Baba, S. (2006) Growth inhibition efficacy of an adenovirus expressing dual therapeutic genes, wild-type p53, and anti-erbB2 ribosome, against human bladder cancer cells. *Cancer Gene Ther.* 13: 298-305.
- Sugrues, M.M., Shin, D.Y., Lee, S.W. and Aaronson, S.A. (1997) Wild-type p53 triggers a rapid

- senescence program in human tumor cells lacking functional p53. *Proc. Natl. Acad. Sci. USA* 94: 9648-9653.
17. Takahashi, T., Carbone, D., Nau, M.M., Hida, T., Linnoila, I., Ueda, R. and Minna, J.D. (1992) Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res.* 52: 2340-2343.
18. Aubin, R.A., Weinfeld, M., Taghavi, M., Mirzayans, R. and Paterson, M.C. (1997) Highly effective delivery of foreign DNA to adherent cells via polybrene/DMSO-assisted gene transfer. *Methods Mol. Biol.* 62: 319-342.
19. Warshaw, A.L. and Fernandez-Del Castillo, C. (1992) Pancreatic carcinoma. *N. Engl. J. Med.* 326: 455-465.
20. Yeo, C.J., Cameron, J.L., Lillemoe, K.D., Sitzmann, J.V., Hruban, R.H., Goodman, S.N., Dooley, W.C., Coleman, J. and Pitt, H.A. (1995) Pancreaticoduodenectomy for cancer of the head of the pancreas. 201 patients. *Ann. Surg.* 221: 721-731.
21. Landis, S.H., Murray, T., Bolden, S. and Wingo, P.A. (1999) Cancer statistics, 1999. *CA Cancer J. Clin.* 49: 8-31.
22. Jaffee, E.M., Hruban, R.H., Canto, M. and Kern, S.E. (2002) Focus on pancreas cancer. *Cancer Cell* 2: 25-28.
23. Pellegata, N.S., Sessa, F., Renault, B., Bonato, M., Leone, B.E., Solcia, E. and Ranzani, G.N. (1994) K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. *Cancer Res.* 54: 1556-1560.
24. Redston, M.S., Caldas, C., Seymour, A.B., Hruban, R.H., Da Costa, L., Yeo, C.J. and Kern, S.E. (1994) p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. *Cancer Res.* 54: 3025-3033.