

Meaning of tumor protein 53-induced nuclear protein 1 in the molecular mechanism of gemcitabine sensitivity

NAOKI NAKAYA^{1*}, YASUHIITO ISHIGAKI^{2*}, HIDEO NAKAJIMA^{1,3}, MANABU MURAKAMI², TAKEO SHIMASAKI¹, TAKANOBU TAKATA², MAMORU OZAKI², NELSON J. DUSSETTI⁴, JUAN L. IOVANNA⁴ and YOSHIHARU MOTOO¹

¹Department of Medical Oncology, ²Medical Research Institute, Kanazawa Medical University, Uchinada, Ishikawa; ³Department of Oncology, Ageo Central General Hospital, Ageo, Saitama, Japan; ⁴Stress Cellulaire, INSERM U624, Marseille, France

Received May 22, 2012; Accepted July 10, 2012

DOI: 10.3892/mco.2012.8

Abstract. Stress proteins of the pancreas, such as tumor protein 53-induced nuclear protein 1 (TP53INP1), are important factors in the invasion and metastasis of pancreatic cancer. TP53INP1 is a pro-apoptotic factor and is transcriptionally regulated in p53-dependent and -independent manners. A previous study proved that gemcitabine induces TP53INP1 expression in pancreatic cancer cells and the pancreatic cancer cell line (PANC-1). The present study aimed to clarify the association between TP53INP1 and gemcitabine sensitivity. The expression of TP53INP1 and its related factors, such as cell growth and cell cycle status in TP53INP1-knockout mouse embryonic fibroblasts [TP53INP1^{-/-}-mouse embryonic fibroblasts (MEFs)] to those in wild-type counterparts (TP53INP1^{+/+}-MEFs) were compared. Flow cytometric analysis demonstrated no difference of the checkpoint function in TP53INP1^{-/-}-MEFs and TP53INP1^{+/+}-MEFs when exposed to 10 ng/ml of gemcitabine. No significant difference was found in the level of p53 expression in the cell types, although the base level and gemcitabine-induced expression of p21 were significantly decreased in TP53INP1^{-/-}-MEFs, compared to those in wild-type counterparts. Results showed that gemcitabine induced the p21 expression in TP53INP1^{+/+}-MEFs, although not in TP53INP1^{-/-}-MEFs. However, their respective cell-cycle checkpoints were not different. Therefore, TP53INP1 was found to be associated with drug sensitivity through control of the cell cycle.

Introduction

Pancreatic cancer is a highly lethal disease. Approximately 227,000 individuals succumb to the disease annually throughout the World. The prognosis of pancreatic cancer is poor, with a 5-year survival rate of 5% or less (1). The surgery adaptation is 20% or less in newly diagnosed cases of pancreatic cancer (2). Pancreatic cancer cells have aggressive biological characteristics, while the antitumor effects of radiation and chemotherapy are insufficient to treat the disease (3).

Gemcitabine [2',2'-difluorodeoxycytidine (dFdC)], a nucleic acid analogue, is one of a few chemotherapeutic drugs used in the treatment of pancreatic cancer. Regarding the action mechanism, gemcitabine is metabolized to dFdC diphosphate (dFdCDP) and dFdC triphosphate (dFdCTP) in the cells (4). Since dFdCTP are incorporated into the DNA strand by DNA polymerase, DNA replication is obstructed. After that, apoptosis is induced (5). Consequently, the dCTP density in the cells is decreased. In addition, gemcitabine inhibits ribonucleotide reductase (6). Consequently, DNA synthesis is decreased.

Deletion, amplification and methylation of DNA are important for pancreatic cancer tumor development and progression (1,2). Pancreatitis-associated protein (PAP), a pancreatic stress protein, is induced in pancreatic acinar cells in acute pancreatitis, demonstrating anti-apoptotic as well as anti-inflammatory actions (7). Ectopic PAP expression was detected in pancreatic ductal adenocarcinoma (8). Additionally, PAP levels in serum (9) and pancreatic juice (10) were increased in pancreatic cancer. Pancreatic stress proteins, other than PAP, include p8 and tumor protein 53-induced nuclear protein 1 (TP53INP1). The actions of p8 resemble those of PAP. Notably, p8 is crucial in the gemcitabine resistance (11).

However, TP53INP1 expression decreases in cancer cells, as well as having a tumor suppressor gene character (12-14). TP53INP1 protein expression is induced in a gemcitabine-treated pancreatic cancer cell line (PANC-1) (15), therefore, an association between the gemcitabine sensitivity of pancreatic cancer and TP53INP1 was suspected. The p53 gene is mutated in most human cancer cells, including pancreatic cancer. TP53INP1 is closely correlated with p53, with a pro-

Correspondence to: Dr Naoki Nakaya, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan
E-mail: n-naoki@kanazawa-med.ac.jp

*Contributed equally

Key words: gemcitabine, tumor protein 53-induced nuclear protein 1, p21, p53, cell cycle

apoptotic potential (12). TP53INP1 controls transcriptional activities of p53 by homeodomain-interacting protein kinase 2 (HIPK2) (16). Overexpression of TP53INP1 stops the cell cycle, while inducing cell apoptosis (17).

The present study was conducted to clarify the molecular mechanisms of gemcitabine sensitivity regarding TP53INP1. Moreover, the significance of TP53INP1 in cell cycles, check points and gemcitabine sensitivity was delineated.

Materials and methods

TP53INP1^{-/-} and TP53INP1^{+/+} mouse embryonic fibroblasts (MEFs). TP53INP1-deficient mice were generated as described previously (18). At 14.5 days *post coitum*, TP53INP1^{-/-}-MEF and TP53INP1^{+/+}-MEF were prepared from embryos derived from the homozygous breeding of TP53INP1-deficient mice and of their wild-type littermates, according to earlier reports in the literature (12,14,19). These cells were transformed and immortalized by transduction with the pBabe-E1A/*ras*V12 retroviral vector, encoding the constitutively active *ras*. The TP53INP1 genotypes of the respective MEFs were determined by polymerase chain reaction (PCR). Genotype analyses were conducted on genomic DNA from MEF, with the following PCR primers F, R1 and R2: F, 5'-AATGTATGCAATCTTAGCTGATGC-3'; R1: 5'-TCTTGAGGTAACATAGTGAAATGC-3'; and R2: 5'-CCAAACACTGTCACTGTATTGATA-3'. PCR analysis was performed as described in an earlier study (18).

Cell culture and treatment. TP53INP1^{-/-}-MEF and TP53INP1^{+/+}-MEF were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen Corp., Groningen, The Netherlands) at 37°C under 5% in CO₂ atmosphere. The *ras*-transformed MEFs at early passages were used for subsequent experiments.

TP53INP1^{-/-}-MEF and TP53INP1^{+/+} MEF were treated with 0, 5, 10 and 20 ng/ml gemcitabine (Eli Lilly and Co., Indianapolis, IN, USA) for 24 h.

Western blot analysis. Whole cell lysates were prepared using RIPA buffer with a protease inhibitor cocktail (Roche Diagnostics KK, Basel, Switzerland). A 25 µg aliquot of each cellular protein sample was diluted in loading buffer (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). The proteins were separated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P polyvinylidene fluoride 7 (PVDF) membranes (Millipore Corp., Billerica, MA, USA). Subsequent to blocking with 5% skimmed milk solution, the membrane was treated with monoclonal antibodies against p21, p53, TP53INP1 (Abcam, Cambridge, UK), Rb (Cell Signaling Technology, Inc., Beverly, MA, USA), and β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA). A Cell Cycle/Checkpoint Antibody Sampler kit (Cell Signaling Technology, Inc.) was also used. The respective bound antibodies were detected with an IgG rabbit monoclonal antibody (SouthernBiotech, Birmingham, AL, USA), then visualized with Immune Star LD (Wako Pure Chemical Industries, Ltd., Osaka, Japan), using Fuji Xerox LAS4000 image analyzer (Fujifilm Corp., Tokyo, Japan). The intensity of each protein signal was measured using Multi Gauge® (Fujifilm Corp.).

Reverse transcription-PCR (RT-PCR). Total RNA was extracted from TP53INP1^{-/-}-MEF and TP53INP1^{+/+}-MEF, using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). The RT reaction was conducted with a SuperScript™ III First-Strand Synthesis System (Invitrogen Corp.) using random hexamers to generate complementary DNA (cDNA) according to the manufacturer's instructions. RT-PCR was conducted using TaqMan probe; TaqMan® Fast Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA). Sequences of the primers were CDKN1A (p21) mixed primers; Mm00432448_m1 (Applied Biosystems), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mixed primers; Mm99999915_g1 (Applied Biosystems). RT-PCR was conducted, using a Real-Time PCR system (Applied Biosystems 7900HT Fast; Applied Biosystems) as well as the ΔΔCT method. The result was analyzed with the SDS software ver. 2.3 (Applied Biosystems).

The same experiments were performed 3 times. The mean number of cells in each experiment was calculated with standard deviations (SDs).

Flow cytometry (FCM). The cells at the exponential growth phase were labeled with 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich Corp.) [1 mM dissolved in phosphate-buffered saline (PBS)] in a final concentration of 10 µM in the medium. BrdU labeling and subsequent handling of the cells were conducted under subdued light. Subsequent to 15 min of incubation at 37°C, the BrdU-containing medium was removed, and the cells were rinsed twice with PBS. The labeled cells were trypsinized and counted, using a hemocytometer and centrifuged at 5,000 x g for 5 min at 4°C. The pelleted cells were suspended and fixed in ice-cold 70% ethanol (~2x10⁶ cells/ml) and stored at -20°C until analysis.

The fixed cells were resuspended in 1 ml PBS containing 0.5% Triton X-100, treated with 2N-HCl for 30 min at room temperature and neutralized with 0.1 M sodium tetraborate (pH 8.5). Subsequent to washing twice with washing solution [PBS containing 0.5% Tween-20 and 1% bovine serum albumin (BSA)], the cells were stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-BrdU antibody or FITC-labeled mouse IgG1κ isotype as a negative control (both antibodies included in FITC Mouse Anti-Human BrdU set; Becton-Dickinson, Franklin Lakes, NJ, USA) overnight at 4°C. The stained cells were washed twice with 1 ml ice-cold PBS and incubated with 0.25% RNase (Sigma-Aldrich Corp.) for 30 min at room temperature. The cells were then stained with 5 µg/ml propidium iodide (PI; Sigma-Aldrich Corp.) and examined using FCM (FACSCalibur; Becton-Dickinson) for green and red fluorescence, which determined the relative number of BrdU-labeled cells and cells examined, respectively.

Colony formation assay. A total of 200 exponentially growing TP53INP1^{-/-}-MEFs or INP1^{+/+}-MEFs were seeded on a 6-cm dish and grown in DMEM Glutamax medium, containing 10% FBS at 37°C in a 5% CO₂ atmosphere. After a week, the colonies per dish were counted; each cell line was counted immediately.

When cells were confluent, gemcitabine was added to the medium 3 days subsequent to cell seeding. TP53INP1^{-/-} and TP53INP1^{+/+} cells were treated with 0, 5, 10 and 20 ng/ml

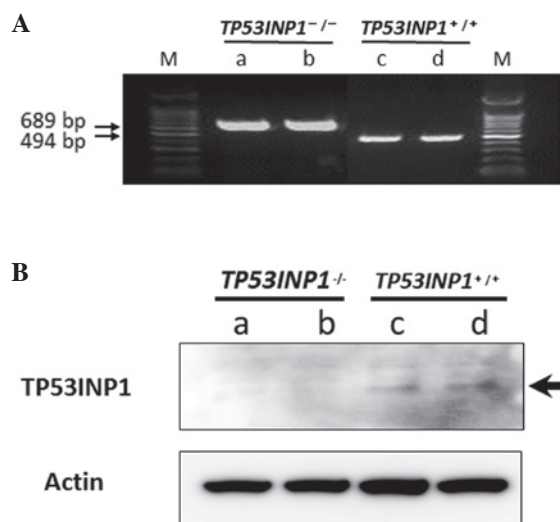


Figure 1. Confirmation of TP53INP1 expression. (A) Genotyping is shown. A band of 689 bp in TP53INP1^{-/-} cells and 494 bp in TP53INP1^{+/+} cells were detected. (B) Western blot analysis of TP53INP1 protein. Western blot analysis demonstrates the expression of TP53INP1 protein of 27 kDa in TP53INP1^{+/+} cells, although no expression was found in TP53INP1^{-/-} cells.

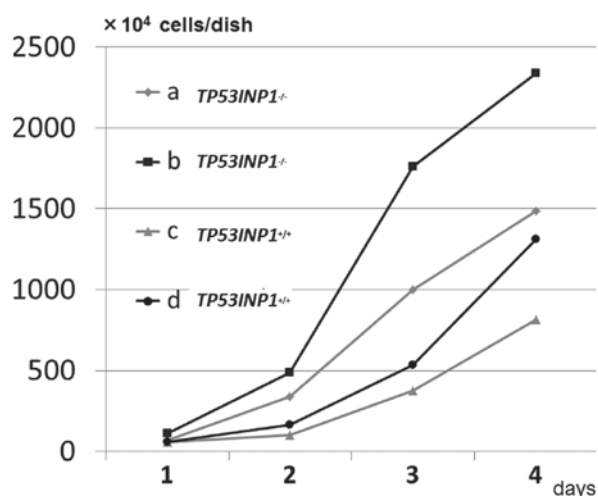


Figure 2. Cell growth curve. TP53INP1^{-/-} cells tend to grow faster than TP53INP1^{+/+} cells.

gemcitabine for 24 h. Cell growth was determined using the colony formation assay. The same experiments were performed 3 times. The mean number of colonies in each experiment was calculated.

Statistical analysis. Data were analyzed using the Student's t-test. Values were given as the mean \pm SEM and $P < 0.05$ was considered to indicate a statistically significant difference. The results were analyzed using Excel (Microsoft Corp.).

Results

Characteristics of TP53INP1-deficient and the wild-type MEFs. The TP53INP1, a nuclear localization protein gene, was amplified using PCR. Null mutation mice were gener-

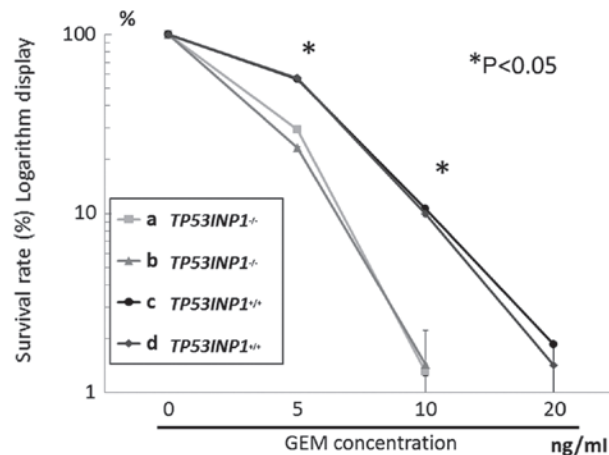


Figure 3. Cell viability after gemcitabine treatment. TP53INP1^{-/-} cells were significantly more sensitive to gemcitabine than TP53INP1^{+/+} cells ($P < 0.05$).

ated by genotyping (18). As confirmed by PCR (Fig. 1A) and western blot analysis, TP53INP1 was deficient in TP53INP1^{-/-} MEFs, but expressed in TP53INP1^{+/+} MEFs, respectively (Fig. 1B).

TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs were cultured and the colonies were confirmed. Subsequently, several subclones were established from the TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs. Two subclones from cells of each type (subclones a and b from the TP53INP1^{-/-} MEFs; subclones c and d from the TP53INP1^{+/+} MEFs) were selected.

Proliferation of TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs. Cell proliferation was measured by colony formation assay. The proliferation of TP53INP1^{-/-} MEFs tended to be faster compared to TP53INP1^{+/+} MEFs (a, b and c, d, respectively) (Fig. 2).

Sensitivities of TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs to gemcitabine. Cells were treated with gemcitabine for 24 h, then cell growth was assayed. Gemcitabine inhibited a significantly greater TP53INP1^{-/-} cell growth compared to TP53INP1^{+/+} cells, in a concentration-dependent manner (Fig. 3) ($P < 0.05$). According to the curve indicating the survival rate, IC₅₀ of TP53INP1^{-/-} was estimated as 2-3 ng/ml, while that of TP53INP1^{+/+} was 5-10 ng/ml.

Effects of gemcitabine on the cell cycle status of TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs. TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs were treated with gemcitabine at 10 ng/ml for 24 and 48 h. Subsequent to treatment, each cell cycle status was determined using FCM. Differences in each peak of G1, S and G2/M of TP53INP1^{-/-} MEF and TP53INP1^{+/+} MEF were impossible to confirm based on the results of FCM. Gemcitabine treatment generated almost equal apoptosis in both cell lines.

p21 and p53 expression in TP53INP1^{-/-} MEFs and TP53INP1^{+/+} MEFs. A previous report (16) demonstrated that TP53INP1 overexpression induced p21 expression, while findings of the present study confirmed the decrease of the

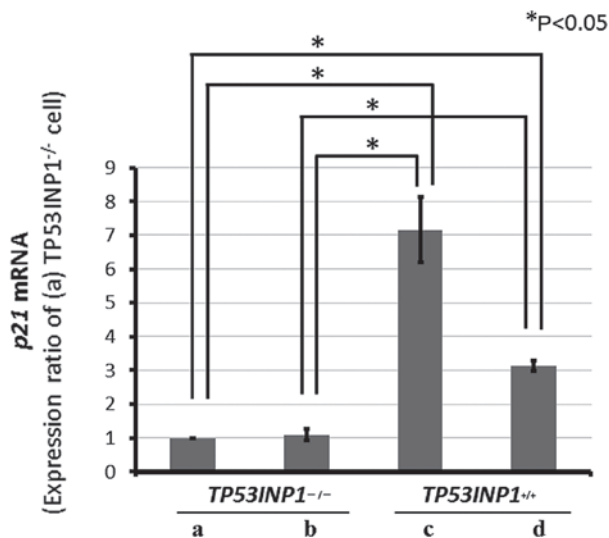


Figure 4. Real-time RT-PCR for p21mRNA. Real-time RT-PCR shows a significant decrease in p21 mRNA expression in (a and b) TP53INP1^{-/-} cells, compared to (c and d) TP53INP1^{+/+} cells (*P<0.05).

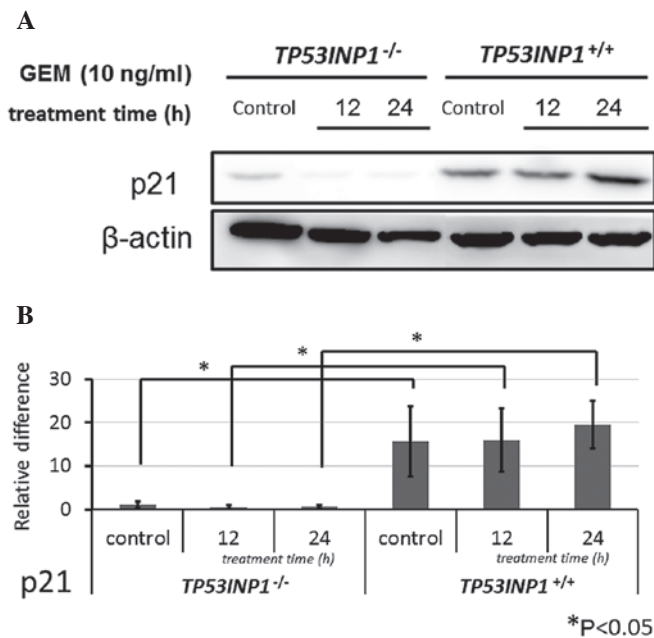


Figure 5. (A) Western blot analysis of p21 expression. TP53INP1^{-/-} cells do not express p21, although TP53INP1^{+/+} cells show p21 expression treatment with gemcitabine. (B) The p21 protein revelation amount is analyzed quantitatively. The expression of p21 decreases significantly in both TP53INP1^{-/-} and TP53INP1^{+/+} cells (*P<0.05).

mRNA and protein expressions induced by the TP53INP1 knockout. Moreover, another study described that oxidative stress decreased the p21 expression in TP53INP1 knockout cells; thus, the change in p21 expression was examined using gemcitabine treatment.

Subsequent to gemcitabine treatment in both cell lines, in RT-PCR, the p21 mRNA expression was significantly decreased in the TP53INP1^{-/-}-MEFs compared to TP53INP1^{+/+}-MEFs (P<0.05) (Fig. 4).

Subsequent to the same treatment in both cell lines, western blot analysis revealed that the protein expression of

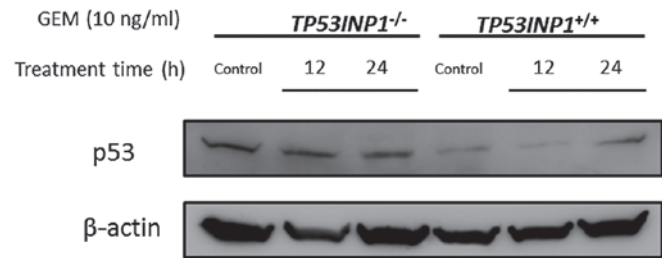


Figure 6. Western blot analysis of p53 expression. Western blot analysis shows no change in p53 expression in either TP53INP1^{-/-} or TP53INP1^{+/+} cells, subsequent to gemcitabine treatment.

p21 was significantly lower in TP53INP1^{-/-}-MEFs compared to TP53INP1^{+/+}-MEFs (P<0.05) (Fig. 5).

It is already widely acknowledged that p53 is located upstream of p21. A previous study described that a decreased p21 expression resulted from a decreased p53 expression (21). In TP53INP1^{-/-} cells, the p21 expression decreased in RNA and protein. Whether or not these results were derived from the status of p53 expression was investigated. The expression of p53 was verified by western blotting in both TP53INP1^{-/-}-MEF and TP53INP1^{+/+}-MEF. This experiment demonstrated that p53 expression was not decreased in TP53INP1^{-/-}-MEFs (Fig. 6).

Discussion

This study is the first addressing the role of TP53INP1 in the molecular mechanisms of gemcitabine sensitivity.

TP53INP1 is considered to suppress cell proliferation, while reducing gemcitabine sensitivity, since TP53INP1^{-/-}-MEF proliferation is faster and gemcitabine sensitivity of INP1^{-/-} is higher compared to the wild-type MEFs. Flow cytometry analysis indicated no existing difference between INP1^{+/+} and INP1^{-/-} in the cell-cycle-checkpoint. It is known that DNA replication processes with disrupted DNA repair leads to unsuccessful cell proliferation. Moreover, TP53INP1 is known to be a pro-apoptosis gene. An earlier study demonstrated that TP53INP1 expression is induced by gemcitabine treatment in a pancreatic cancer cell line, PANC-1 (15).

The gemcitabine action was expected to be stronger in the INP1^{+/+} compared to INP1^{-/-} cells, the results, however, revealed the opposite. This phenomenon differed from those delineated in previous studies on gemcitabine sensitivity in pancreatic cancer, since the cells used were immortalized MEF instead of pancreatic cancer cells. TP53INP1 has a tumor suppressor gene character, while TP53INP1 expression is decreased in cancer cells (20), while being decreased or eradicated in the course of pancreatic carcinogenesis (14). In this study, the p21 expression was delineated to be suppressed and no change was observed subsequent to gemcitabine treatment in INP1^{-/-} cells, thereby not contradicting the p21 suppression in INP1^{-/-} cells treated with antioxidant stresses (21). These phenomena suggest that decreased or disrupted functions of the checkpoints engender a rapid cell growth without the arrest of DNA replication at the G2/M phase. Apoptosis might be promoted due to the wrong DNA synthesis, whereas these mechanisms might partially explain gemcitabine sensitivity.

Regarding p53, results demonstrate p53-independent regulation of TP53INP1 expression in addition to the p53-dependent, confirming that p21 expression is independent of p53.

The INP1^{-/-} cells grew more rapidly compared to the INP1^{+/+} cells. Rb phosphorylation was considered to be involved in this phenomenon. A preliminary analysis of the expression of Rb protein and its phosphorylation was conducted, however, no phosphorylation of Rb was found in the INP1^{+/+} and INP1^{-/-} cells (data not shown). When Rb is phosphorylated, p21 induces G2 arrest and inhibits DNA replication (22). In the present study, no difference was found between INP1^{+/+} and INP1^{-/-} cells in the cell-cycle-checkpoint. The activity of TP53INP1, however, should be analyzed at each cell-cycle-checkpoint, based on the information presented above.

Notably, SMG-1, a phosphatidylinositol 3-kinase (PI3K), has recently been found to be involved in the gemcitabine-induced expression mechanism of microRNA-155/BIC in pancreatic PANC-1 cancer cells (23). As described above, TP53INP1 is suppressed by microRNA-155/BIC in pancreatic carcinogenesis (14), while microRNA-155 is considered to be associated with gemcitabine sensitivity. Consequently, the correlation among TP53INP1, microRNA-155 and gemcitabine sensitivity requires to be further investigated.

Additional studies are required regarding the role of TP53INP1 in the molecular mechanisms of gemcitabine sensitivity, including that of micro-RNA.

Acknowledgements

This study was supported in part by the Grant for Collaborative Research from the Kanazawa Medical University (C2008-3) and by the Project Research Grant from the High-Tech Research Center of Kanazawa Medical University (H2010-11).

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