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Study on Mechanism for P21/53 Promoting Invasion of Human Glioma Stem Cells by Inhibiting TIMP-2 Expression

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Abstract: To study the role of P21/53 in directly regulating and controlling TIMP-2 and the important mechanism for promoting invasion of GSCs by P21/53. Transwell *in vitro* invasion experiment was used to test invasion ability of GSCs and GCs as well as effect of P21/53 on GSCs invasion. Techniques of qRT-PCR and Western blot were used to test and transfect expression variation of TIMP-2, MMP2 and MMP9 in GSCs of P21/53 inhibitor. The luciferase reporter gene experiment was used to test the role of P21/53 in directly regulating and controlling TIMP-2. The number of invasive GSCs significantly decreased following transfection of P21/53 inhibitor ($p<0.05$). The expression levels of TIMP-2 mRNA in the U87 cell line and GSCs from the two primary glioma specimens rose compared with the control group, respectively while the expression levels of MMP2 mRNA and MMP9 mRNA fell compared with the control group ($p<0.05$) following transfection of P21 inhibitor. Expression of protein level of TIMP-2, MMP2 and MMP9 were consistent with mRNA level and had significant difference compared with those of the control group following transfection of P21 inhibitor and P53 inhibitor. The luciferase activity of the reporter vector containing wiled-type TIMP-2-3'UTR increased significantly following transfection of P21/53 while the luciferase activity of the reporter decreased significantly following transfection of P21/53 simulator ($p<0.05$). High expression of P21/53 promotes invasiveness of GSCs and its mechanism is achieved by down-regulating expressions of TIMP-2 and up-regulating expressions of MMP2 and MMP9 as a minimum.

Key words: P21, P53, TIMP-2, human glioma stem cells, invasion

INTRODUCTION

Spongioblastoma is the most common primary malignant brain tumour. One of the important characteristics of malignant glioma is invasive growth of tumour cells which gives rise to difficulties in resecting tumor tissues and a high post-operative recurrence rate (Aboody *et al*., 2013). It is significant to understand the mechanism of spongioblastoma invasion and repress invasion. In the theory of Cancer Stem Cells (CSCs), its is belived that tumour cells have heterogeneity and consist of a group of cells with functional heterogeneity, among which a very small number of tumour cells have self-renewal capability infinite multidirectional differentiative potential and neoplasia capability which play a decisive role in initiating neoplasia, maintaining tumor proliferation, invasion, transfer, recurrence, etc. (Lee *et al*., 2013). In the existing studies, it was reported that CSCs have been preliminarily separated from leukemia, breast carcinoma and glioma (Jin *et al*., 2013). CSC may be not only the source of tumorigenesis but also an important cause for tumor invasion and tumor metastasis (Cao *et al*., 2013). Some studies have demonstrated that Glioma Stem Cells (GSC) have high invasiveness and treatment resistance (Dietrich *et al*., 2010).

Invasion and metastasis of tumor is a complex multi-step process including origin separation, reaching Extracellular Matrix (ECM), ECM reconstruction, metastasis, etc. (Jin *et al*., 2013). A number of studies have shown that the most important step is tumour cells penetrating ECM and basement membranes in tumor invasion and metastasis. Matrix Metalloproteinase (MMPs) mediated ECM degradation plays a critical role and MMPs damaging basement membrane completeness is an significant cause of tumour cell invasion (Velpula *et al*., 2012). The degradation process of ECM primarily depends on MMP2 and MMP9 with capacity to degrade the critical composition of the basement membrane, IV type collagen (Filatova *et al*., 2013). Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) is a natural inhibitor for MMPs and it is significant to study the role of MMPs in human brain glioma.

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P21 gene is a member of cell Cycle Inhibitory Protein (CIP) family which is associated with tumor growth, infiltration and metastasis and has the function of prognosis prediction (Bexell *et al*., 2013). *P53* is a cancer suppressor gene involved in negative regulation of cell growth and inhibition of tumour cell growth and closely assoicated with apoptosis; P53 mutation can prevent apoptosis and promote proliferation (Xu *et al*., 2012).

Based on the research progress in this field both at home and abroad, researchers assume that: P21/53 promotes GSC invasion by negatively regulating the expression of TIMP-2. In the research subject, specific molecular action mechanism is discussed through a study on effect of P21/53 on invasion ability of GSCs and regulating effect of P21/53 on TIMP-2.

MATERIALS AND METHODS

The DMEM medium was purchased from Shanghai Jianglai Biotechnology Co., Ltd., the fetal calf serum was purchased from Shanghai Boye Biotechnology Co., Ltd., the mouse anti-human CD133 monoclonal antibody was purchased from US sigma company the restriction endonuclease Hind III, restriction endonuclease Bam I and restriction endonuclease Spe I were purchased from US SantaCruz Company; T4 DNA ligase and Lipofectamine 2000 transfection reagent were purchased from US Labnet Company; the microplate reader was purchased from US Medica Company; the Vertical Electrophoresis System was purchased from Germany Heraeus Company; the high speed centrifuge was purchased from Beijing Jingli Centrifuge Co., Ltd., the laser scanning confocal microscope was purchased from US Hach Company.

Cell culture: Culture of U87 cell line, human glioma cell line U87 was placed in an incubator containing 5% CO_2 at 7°C for culture with fetal calf serum DMEM medium. The interval for solution replacement is 2-3 days and passage time is 3-5 days; mixed digestive juice consisting of 0.25% trypsin+0.02% EDTA was used for 2-4 min digestion before passage.

Culture of primary glioma cells: The 0.01M PBS was used to clean the primary tissue block for 3 times so as to carefully remove surface fiber and necrotic tissues. The tissue block was immersed into a small amount of DMEM medium and the tumor tissue block was cut into fragments of 1 mm^3 with a pair of ophthalmic scissors. The mixed collagenase was added for 20-30 min digestion, a pipettor was used to obtain single-cell suspension while digesting, the suspension was placed in the DEM medium (containing 10% fetal calf serum) for culture. The solution was replaced and the erythrocytes were removed after 24 h and CD133+cells were sorted when the cells grew to 80~90%.

Cell transfection MMP2: Concentrated solutions of P21/53 mimics and inhibitors were prepared, 250 μL DEPC water was added to dissolve P21/53 mimics, inhibitors and negative control to maintain the concentration of 20 μM, i.e., 20 pmol μL^{-1}, the RNA-OptiMEM mixed solution I was prepared: 5 μL of the above RNA concentrated solutions was added to the 250 μL OptiMEM medium and mixed well gently, the Lipofectamine 2000-OptiMEM concentrated solution II was prepared: 5 μL Lipofectamine 2000 was added to 250 μL OptiMEM medium and mixed well gently, the mixed solution I and mixed solution II were mixed well and placed for 20 min at room temperature, the above mixed solutions were added to cells in each well as per number and 1.5 mL stem cell medium without antibiotic and serum was added, it was replaced with a complete stem cell medium 5 h later.

Transwell *in vitro* invasion experiment: The medium for the cells was discarded and washed for 3 times with PBS, the anchorage-dependent cells was centrifuged after being digested by trypsin + EDTA digestive juice and suspended again with the medium to adjust the concentration of cells to 5×10^5 mL^{-1}. The 10 μL Matrigel mixture was pipetted and applied onto the transwell membrane, the inserts and 24 well microplate were placed in the incubator for at 37°C for 30 min, 500 μL complete stem-cell medium was added to the lower compartments of the insert and 100 μL cell suspensions were added to the upper compartments of the inserts, respectively they were placed in a incubator at 37°C for incubation. The U87 cells and stem cells were taken out after 24 h and primary glioma cells and stem cells were taken out after 48 h, the media in the upper and lower compartments of the inserts were pipetted, the cells were washed with PBS for 3 times and 500 μL 4% paraformaldehyde was added to the lower compartments of the inserts and fixed for 20 min, washed for 3 times with PBS for 5 min each time, 500 μL crystal violet was added to the lower compartments of the inserts stained for 20 min, washed with tap water to terminate staining after the staining was finished, the number of cells penetrating the inserts was observed under the microscope and calculated.

Testing expression of TIMP-2, MMP2 and MMP9 mRNA by qRT-PCR: The primer sequence was designed on the basis of the references and GAPDH served as internal reference substance (Mo *et al*., 2013). They are compounded by Shanghai Ricky Biotechnology Co., Ltd. The primer sequences are as follows; TIMP-2: Upstream

Primer 5'-GAATCAGGTGGGCGGTGAA-3', Downstream Primer 5'-CACTTTGCCACCCAGCTG-3'; MMP2: Upstream Primer 5'-TACAGCCTCCCACTTCGA-3', Downstream Primer 5'-ACTCCCATCTTTCGGAGAA A-3'; MMP9: Upstream Primer 5'-GGAGCCGAGTTGTTCT GA-3', Downstream Primer 5'-CAAAGTACACTTTGGACTGCC-3'; GAPDH: Upstream Primer 5'-TGTTCCATGCT TCTTCTCAC-3', Downstream Primer 5'-TACTACGCTT ACCCGGCTC-3'. The reaction system is as follows: Premix Ex Taq II (2×) 12.5 μL, PCR Forward Primer (10 μM) 0.5 μL, PCR Reverse Primer (10 μM) 0.5 μL, cDNA 2.5 μL, ddH_2O 9.0 μL, 25.0 μL in total. The qPCR conditions are as follows: reaction 95°C 2 min, 95°C 15 sec, 60°C 15 sec, 72°C 20 sec, 40 cycles in total.

Testing expression of TIMP-2, MMP2 and MMP9 proteins by western blot: The medium was pipetted carefully, washed for 3 times with PBS for a 6 well microplate, 200 μL M-PER reagent was added to each well and shaken for 5 min to allow mixing of cells and liquid, the cell lysis buffer was pipetted to a new EP tube, 10000 g, centrifuged for 10 min at 4°C, the supernatant was pipetted into a new clean EP tube and cryopreserved for back up at -70°C.

The glass plate was aligned and vertically clamped on the rack for casting gel, the water on the upper layer was poured after the separation gel was completely solidified and a clear common boundary formed, the liquid was absorbed with a piece of filter paper, the spacer gel was added to the glass plate immediately after preparation, a comb was inserted and the spacer gel was placed at room temperature, the comb was pulled out after solidification, the glass plate was washed with water, then it was put into the electrophoresis tank, the sample was taken with a microsyringe and the needle of the microsyringe was inserted into the hole to add sample slowly. Based on the conditions of 80 V and 30 min for spacer gel and 120 V and 120 min for separation gel, the power was switched on and electrophoresis continued until the bromophenol blue was about to escape from the spacer gel, the electrophoresis can be ended and the power can be switched off.

Luciferase gene experiment: The 150 μL PLB was added to each well containing CD133+GSCs transfected, it was shaken for 1 h at room temperature, the cell lysis buffer was collected with a clean EP tube, 10000 g cell lysis buffer was centrifuged for 1 min, 50 μL supernatant was added to another new clean EP tube, 100 μL LARα was added to the centrifuge tube containing supernatant, a dual-luciferase tester was used to test the visible light intensity for 10 sec, the centrifuge was taken out and 100 μL Stop&GLO reagent was added and pipetted up and down, the dual-luciferase tester was used to test the intensity of visible light for 10 sec. The ratio of sea pansy fluorescence/firefly fluorescence was calculated and the regulatory effect of P21/53 on TIMP-2 was analysed (Sun *et al*., 2012).

Statistical analysis: SPSS 13.0 Mathematical Statistics Analysis Software was used to process the experimental data and the data was expressed with $\bar{X}\pm S$, the t-test was used to compare the significance of difference between the two groups and the one way ANOVA test was used to compare the significance of difference among multiple groups, $p<0.05$ indicates a statistical difference and $p<0.01$ indicates a very significant difference.

RESULTS AND DISCUSSION

Vector construction: After double digestion, the correct positons of the two strips should be 6400 and 240 bp approximately. It can be seen from Fig. 1 that both strips are in the correct positions indicating a successful vector construction.

Comparison of invasion ability between GSCs and GCS: To study the difference of invasion ability between GSCs and GCs researchers have adopted a transwell *in vitro* invasion experiment to observe the invasion ability of GSCs and GCs and the number of invading GSCs is significantly higher than that of GCs ($p<0.05$) as shown in Fig. 2 and 3.

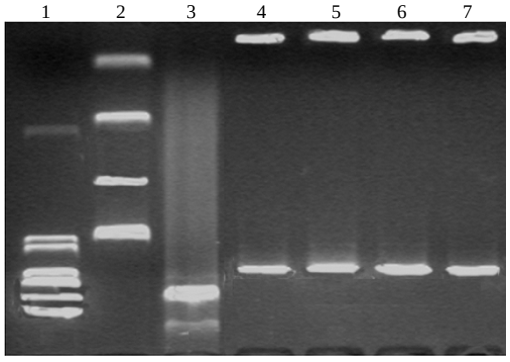

Fig. 1: pMIR-P21/53-TIMP2-WT pMIR-P21/53-TIMP2-MUT plasmid digestion assay. 1: DNA Marker λ-Hind III digest; 2: DNA Marker DL2000; 3: pMIR-Reporter plasmid; 4-5: pMIR-P21/53-TIMP2-WT plasmid following double digestion; 6-7: pMIR-P21/53-TIMP2-MUT plasmid following double digestion

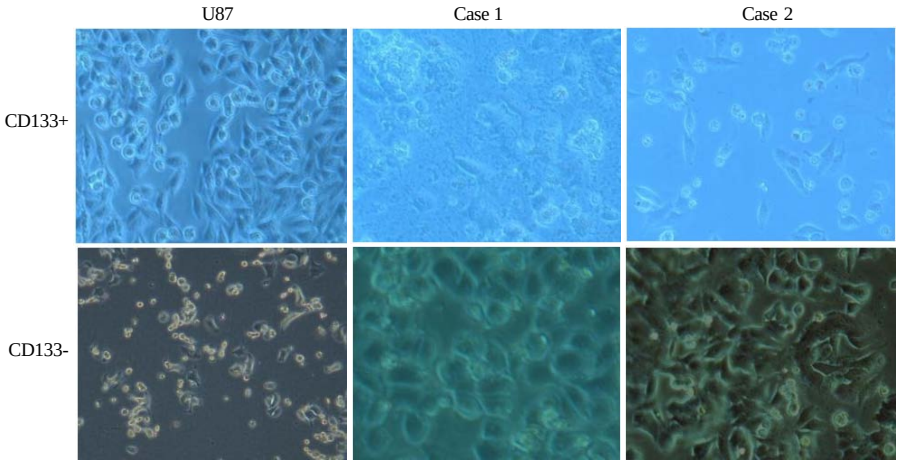

Fig. 2: The number of cells in transwell invasion experiment for invasion ability of GSCs and GCs

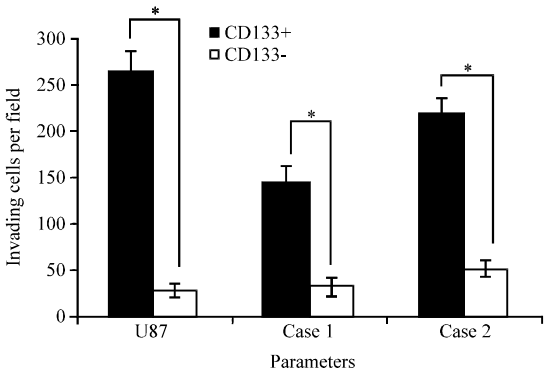

Fig. 3: Result of quantitative analysis on invasion ability of GSCs and GCs. The comparison between the two groups indicates that *p<0.05

Effect of P21/53 on GSCs invasion ability: To study effect of P21/53 on GSCs invasion, researchers have adopted a transwell *in vitro* invasion experiment to observe variation of the invasion ability of GSCs after P21/53 inhibitor being transfected, the number of invading GSCs significantly decreases ($p<0.05$) as shown in Fig. 4 and 5.

P21/53 regulating expression of TIMP-2 pathway: The expression level of TIMP-2 mRNA in the U87 cell line and GSCs orginated from two primary glioma specimens was approximately 2.1, 2.7 and 2.6 times of the control group, respectively and the expression level of MMP2 mRNA approximately fell by 35, 45 and 60% compared with the control group, the expression level of MMP9 mRNA also fell by 60, 70 and 45% approximately compared with the control group after P21 inhibitor was transfected, the expression level of TIMP-2mRNA was approximately 1.7, 2.4 and 2.1 times of the control group, the expression level of MMP2 mRNA fell by 50, 55 and 48% and expression level of MMP9 mRNA fell by 70, 80 and 30% compared with the control group after the P53 inhibitor was transfected. Expression of protein level of TIMP-2, MMP2 and MMP9 were consistent with mRNA level and had significant difference compared with those of the control group following transfection of P21 inhibitor and P53 inhibitor as shown in Fig. 6 and 7.

Research on mechanism for directly regulating TIMP-2 by P21/53: To study whether P21/53 directly regulating the expression of TIMP-2, researchers established luciferase reporter gene vectors and the luciferase reporter gene experiment was used for test. The luciferase activity of the reporter vector containing wild TIMP-2-3'UTR significantly decreased compared with that of the reference vector and mutant TIMP-2-3'UTR reporter vector ($p<0.05$). The luciferase activity of the reporter vector containing wiled-type TIMP-2-3'UTR increased significantly following transfection of P21/53 while the luciferase activity of the reporter decreased significantly following transfection of P21/53 simulator ($p<0.05$). And the inhibitor and simulator of P21/53 had no significant effect on the luciferase activity of reporter vector containing mutant TIMP-2-3'UTR ($p<0.05$) as shown in Fig. 8.

Currently, only a few studies have reported existence of CSCs. Similar to normal stem cells, CSCs were first separated by using the surface marker of stem cells, CD133. CD133 was called AC133 orignially and considered the most appropriate candidate marker due to the fact that it is the important symbol of the stem nature of primitive cells in nerve cells, epithelial cells and hemopoietic tissues (Gao *et al*., 2013). At present, CSCs

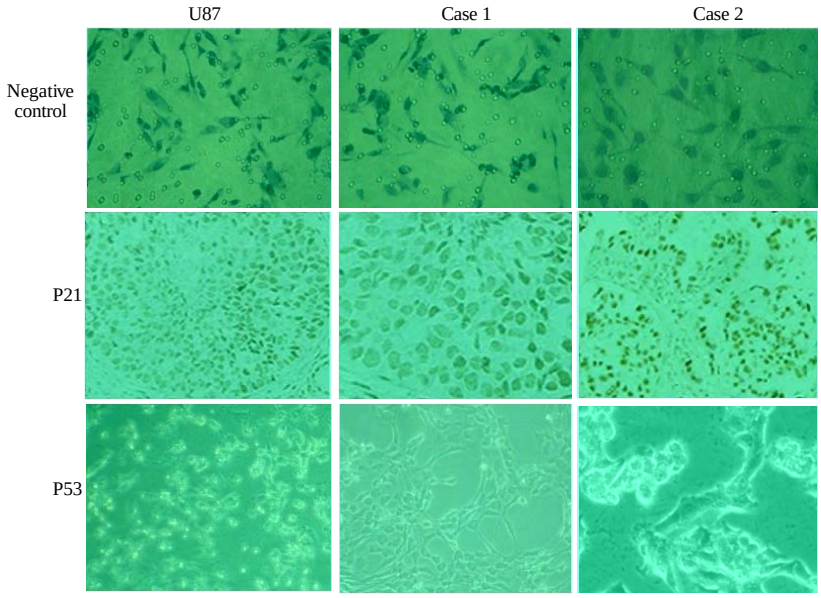

Fig. 4: The number of cells in transwell invasion experiment for invasion ability of GSCs (x200) after P21/53 being transfected

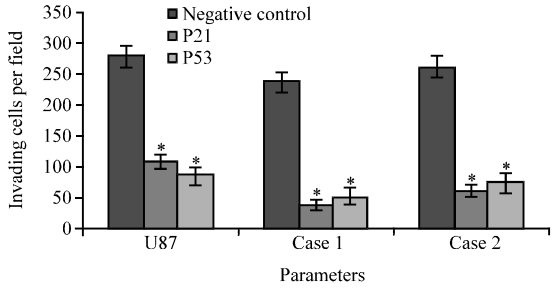

Fig. 5: Result of quantitative analysis on invasion ability of GSCs after P21/53 being transfected. The comparison with the negative control indicates that *p<0.05

have been found in different blood systems and solid tumors such as leukemia, brain tumor, colon cancer, liver cancer, prostatic cancer, etc. (Guvenc *et al*., 2013). CD133 seems to be the most-likely extensive stem nature candidate marker. Glycosylation can occur on both epitopes of CD133 molecules and these monoclonal antibodies can identify glycosylated CD133 molecules. Thus, it is highly possible for false negative or false positive antibody binding to occur. A false negative result may occur if the monoclonal antibody can not identify the non-glycosylated CD133 molecules (Gao *et al*., 2012). In addition, different glycosylations of the epitope may also cause inconsistent results (Ji *et al*., 2013).

Malignant tumors are distinguished benign tumors from by their invasiveness and migration capability. Migration may occur due to that fact that they can invade and survive in the adjacent tissues (Yang *et al*., 2013).

Therefore, invasion is the first step for tumor progression and changing the invasion ability of tumor cells provides a treatment orientation for limiting tumor progression and subsequent death due to vicious circle. Many target spots have been found to promote invasion of tumor cells (Ying *et al*., 2013). In recent years, some studies have shown that P21 and P53 have play a critical role in invasion of tumor cells they have become a research object and considered to be a crucial role in invasion of tumor cells (Morgan *et al*., 2012). CSC is a new concept proposed in oncobiology studies in recent years. In CSC theories, it is believed that only a extremely small number of tumor cells have self-renewal capability, infinite multidirectional differentiative potential and neoplasia capability and play a decisive role in initiating neoplasia, maintaining tumor proliferation, invasion, transfer, recurrence, etc. (Firat *et al*., 2012). Thus, CSC may be not only the source of tumorigenesis but also an important cause for tumor invasion and tumor metastasis (Hu *et al*., 2012).

The *P53* gene has been the gene most highly correlated with the human tumor gene up to now and people's understanding of P53 have changed from oncoprotein antigen, oncogene to cancer suppressor gene since the gene was first reported (He *et al*., 2012). At first, people thought the *P53* gene is a oncogene but the subsequent studies showed that P53, more importantly,

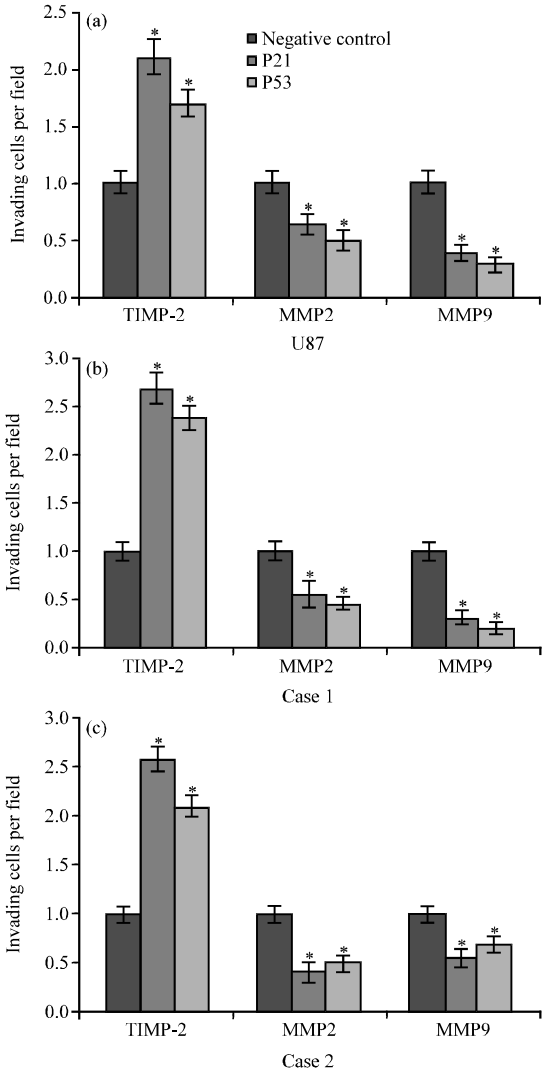

Fig. 6: Expression variation of TIMP-2, MMP2 and MMP9 mRNA level after P21/53 inhibitor transfection. The comparison with the negative control indicates that *p<0.05

serve as a cancer suppressor gene (Heddleston *et al*., 2011). It has been demonstrated that the P53 protein is the product from gene mutation and a tumor promoting factor which can eliminate the functions of normal P53 (Lee *et al*., 2011). The P53-mediated cell signaling transduction pathway plays an important role in regulating the normal cellular vital activities and its connection with other cell signaling transduction pathways is very complex. The number of types of genes regulated and controlled by P53 has exceeded 160 and the concept of *P53* gene network emerged accordingly (Ahmed *et al*., 2011). In the viewpoint, it is considered that biological functions of individual genes should not be observed separately and should be regarded as a whole. For events of activating molecules upstream the *P53* gene network, new progress has been made on regulation of expression level of P53 protein and study of *P53* gene functions (Frosina, 2011).

In a cell cycle, the regulatory function of P53 is mainly reflected in monitoring calibration points in phase G1 and G2/M. There are 2 P53 binding domains upstream the P21 coding region, mutual regulation between P53 and P21 holds significance to blocking the injured cells in phase G1 (Marsh *et al*., 2012). In a new research result, it is believed that for cells following DNA damage, the

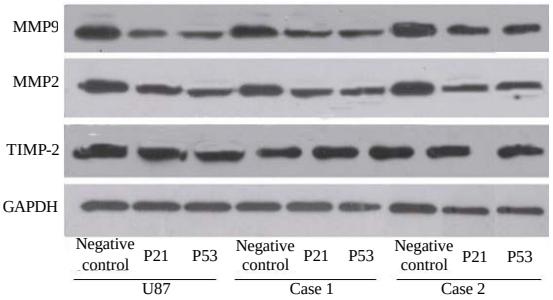

Fig. 7: Variation of expression levels of TIMP-2, MMP2 and MMP9 protein level after P21/53 inhibitor transfection

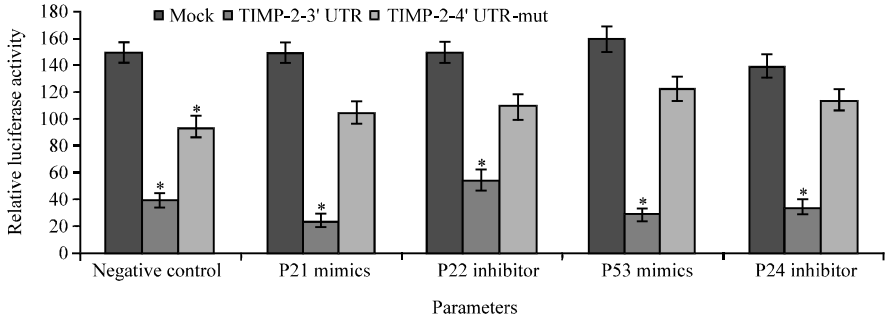

Fig. 8: Testing the role of P21/53 in directly regulating and controlling TIMP-2 by luciferase reporter gene experiment. The comparison with the Mock group indicates that *p<0.05

regulatory effect of P53 and P21 is necessary for blocking cells in phase G2 (Hua *et al*., 2011). In a study on the *P53* gene in chronic granulocytic leukemia, it was also discovered that chronic granulocytic leukemia may have P53 inhibiting factors or binding protein resulting in loss of p53 function.

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