Effect of $P15^{INK4b}$ expression on the cell cycle and G_1 phase related regulatory genes in human melanoma cells*

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Abstract Using the transfection technique, $P15^{INK4b}$ was introduced into $P15^{INK4b}$ gene deleted human melanoma A375 cells, and a cell model MLIK6 overexpressing $P15^{INK4b}$ was constructed. Comparing with the control cells MLC2, MLIK6 cells in G_1 phase increased by 11%, but those in S phase decreased by 15% by FCM. By the method of thymidine (TdR) and N_2O arresting, the proportions of synchronized M phase cells of MLIK6 and MLC2 were measured and found to be 89.1% and 76.8%, respectively, and the cells in G_1 phase were 74.3% for MLIK6 and 76.4% for MLC2. The result of ^{3}H-TdR incorporation indicated that the transition of G_1/S of MLIK6 cell was delayed 2 h as compared with that of MLC2 cells, and incorporation rate also decreased. The observation on expressions of some G_1/S-related relatory regulating genes showed that in MLIK6 cells the protein level of $P27^{KIP1}$ increased with the decreasing expressions of cyclinD1, cyclinE and c-myc, especially cyclinD1 in late G_1 phase. The expression of cyclinE obviously decreased at G_1/S transition, and c-myc was inhibited throughout all the process of $G_1 \rightarrow$S phase. All the results suggest that $P15^{INK4b}$ can delay G_1/S transition of MLIK6 cells by inhibiting the cell cycle engine molecule, and by increasing the expression of Cdk inhibitor $P27^{KIP1}$ in different stages of G_1 phase.

Keywords: $P15^{INK4b}$, CKI, cell cycle, G_1 phase-related gene, cell synchronization.

$P15^{INK4b}$ is a member of INK4 family which consists of $P16^{INK4a}$, $P15^{INK4b}$, $P18^{INK4c}$ and $P19^{INK4d}$[1] and belongs to CKI (cyclin-dependent kinase inhibitor)[2]. The target of all INK4 proteins is cyclinD-CDK4/6 compound[3]. All of these proteins control cell's proliferation and differentiation, and even apaptosis[4]. They affect also G_1/S transition in cell cycle. The deletion, point mutation and high level methylation of $P15^{INK4b}$ gene have been found in several cancer cell lines[3]. It is known that $P15^{INK4b}$ can bind to CDK4/6 and down-regulate the phosphorylation of pRb[5], but the regulatory function of $P15^{INK4b}$, especially in the different stages from G_1 to S phase has not been elucidated. We construct in this paper a cell model that overexpresses $P15^{INK4b}$ using a human melanoma cell line A375, and study the effect of $P15^{INK4b}$ on the related regulator in G_1/S transition.

1 Materials and methods

1.1 Cell culture

Human melanoma cell line A375 (provided by Beijing Institute for Cancer Research) and

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transfected cells were maintained at 37℃ in DMEM, supplemented with 10% calf serum in 5% CO_2 incubator.

1.2 Transfection

PBS.SK$^+$ P15 plasmid containing $P15^{INK4b}$ gene was a gift from Prof. Beach (Cold Spring Harbor Laboratory, USA) and an eukaryortic-expressing plasmid PXJ-41-P15 was constructed in our laboratory. PXJ-41-P15 and PXJ-41-neo[6] were introduced into A375 cells respectively by Lipofectamine (Gibco BRL). After being incubated for another 24 h the cells were selected by G418 (Sigma). The clones of G418-resistant cells were isolated after 2 weeks.

1.3 PCR

According to Ref. [7] two primers located within the exon 1 region of $P15^{INK4b}$ gene were synthesized. The sequences are 5′-CCAGAAGCAATCCAGGCGCG-3′ and 5′-AATGCACACCTCGCCAACG-3′. The cells in logarithmic phase were lysed at 55℃ for 30 min, then at 100℃ for 5 min. The lysate was centrifuged at 12 000 g for 5 min, and 2 μL of the product was taken as the template. The PCR program condition was 30 cycles of 95℃ 2 min, 95℃ 30 s, 59℃ 1 min, 70℃ 1 min, and a final extension of 72℃ 10 min. The PCR product was analyzed in a 1.5% argarose gel.

1.4 Flow cytometry (FCM)

The cells in logarithmic phase were harvested, pelleted and fixed in 70% ethanol. After incubation with RNase (50 μg/mL), the cells were stained with PI (650 μg/mL) for 1 h and their DNA content was analyzed by FCM.

1.5 Cell synchronization and cell cycle analysis by ^{3}H-TdR incorporation

N_2O-TdR double arresting method[8] was used for synchronization. The synchronized cells in M phase were released for 4, 7, 9, 11, 13, 15, 17, 19 and 21 h, repectively, and incorporated with ^{3}H-TdR, then determined by liquid scintillation counting[9].

1.6 Western blot

Protein extracted from the cells was separated by polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose filters[10], the level of protein was determined using an ECL Western blotting analysis system with antibodies of CKI P15, cyclinD1, CDK4, cyclinE, P27 and c-myc purchased from Santa Cruz Corporation.

2 Results

2.1 Construction and identification of the human melanoma cell model overexpressing $P15^{INK4b}$

The reconstructed plasmid PXJ-41-P15 and vector PXJ-41-neo were introduced into A375 cells respectively and several G418-resistant clones (named MLIK) and the clones containing vector PXJ41-neo only (named MLC) were obtained. After identified by Western blot and PCR with their genomic DNA (Fig. 1(a)), MLIK6 and MLC2 were selected as the samples for further experiments.

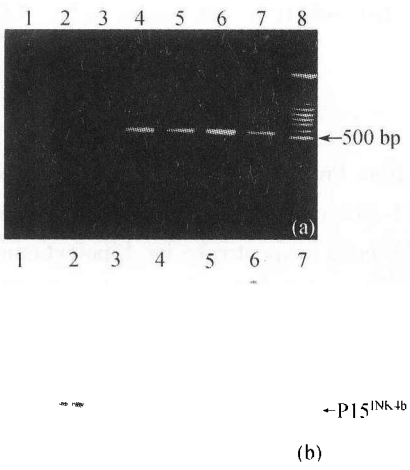

Fig. 1 Identification of the cell model overexpressing $P15^{INK4b}$ in human melanoma cells. (a) PCR analysis 1 ~ 8, H_2O, MLC_2, MLC_1, $MLIK_9$, $MLIK_8$, $MLIK_6$, $MLIK_2$, 100bp DNA ladder respectively; (b) western blot analysis 1 ~ 7, A375, MLIK6, MLIK8, MLC1, MLC2, MLC3, MLC4, respectively.

The result of Western blot (Fig. 1(b)) showed that $P15^{INK4b}$ was expressed stably in MLIK6 cells, but it was not expressed in MLC2 cells, which suggested that the human melanoma cell model expressing $P15^{INK4b}$ was constructed successfully.

2.2 Effect of $P15^{INK4b}$ on progression of cell cycle and synchronization

Comparing with the control MLC2 cells, the proportion of MLIK6 cells in G_1 phase increased by 11%, but the proportion of the cells in S phase decreased by 14%, as measured by flow cytometry (Fig. 2(a)). Using the method of thymidine (TdR) and N_2O arresting, the proportions of synchronized MLIK6 and MLC2 in M phase were 89.1% and 76.8% respectively, and that of the synchronized cells in G_1 phase was 74.3% for MLIK6 and 76.4% for MLC2 (Fig. 2(b)). The ^{3}H-TdR incorporation indicated that the incorporation level of MLIK6 was decreased, the transition of G_1/S of MLIK6 was delayed 2 h and S phase of MLIK6 was shortened as compared with that of MLC2 cell (Fig. 2(c)), suggesting that the G_1/S progression in MLIK6 was inhibited.

2.3 Effect of $P15^{INK4b}$ on G_1 phase related regulatory factors in human melanoma cells

After being arrested in M phase, the synchronized MLIK6 and MLC2 cells were released for 4, 6, 8 and 10 h respectively, then the protein was extracted and analyzed for determination of the levels of cyclinD1, CDK4, cyclinE, P27 and c-myc. The results (Fig. 3) showed that the expression of cyclinD1 was inhibited. It could not be detected at 8 h time point, but after 10h releasing, its expression became detectable at a low level. Compared with MLC2 cells, a decreasing tendency of CDK4 level in MLIK6 through G_1 progression was observed. CyclinE expression in MLIK6 was also decreased, paralleled to G_1 progression and reached to the lowest level at 10 h time point. The c-myc was inhibited through out the $G_1 \rightarrow S$ process. The level of P27 increased significantly in MLIK6 after 6, 8 and 10h released from synchronization and the overexpression of $P15^{INK4b}$ could increase P27 level and inhibit the expression of cyclinD1 simultaneously (especially in middle of G_1), but CyclinE was inhibited markedly at G_1/S transition. The inhibition of c-myc expression went through whole G_1 phase.

3 Discussion

$P15^{INK4b}$ gene lies in homo sapiens chromosome 9p21 and neighbors to $P16^{INK4a}$ gene, showing the important role of 9p21 during the regulation of cell cycle. Hannan and Beach reported that the $P15^{INK4b}$ level increased 30-fold in the G_1 arresting of HaCaT cells induced by TGF-β[11]. Later on the researches were mostly focused on the mechanism of $P15^{INK4b}$ gene mutation, including deletion, point mutation and methylation in carcinoma cell lines. But little is known about the downstream regulation

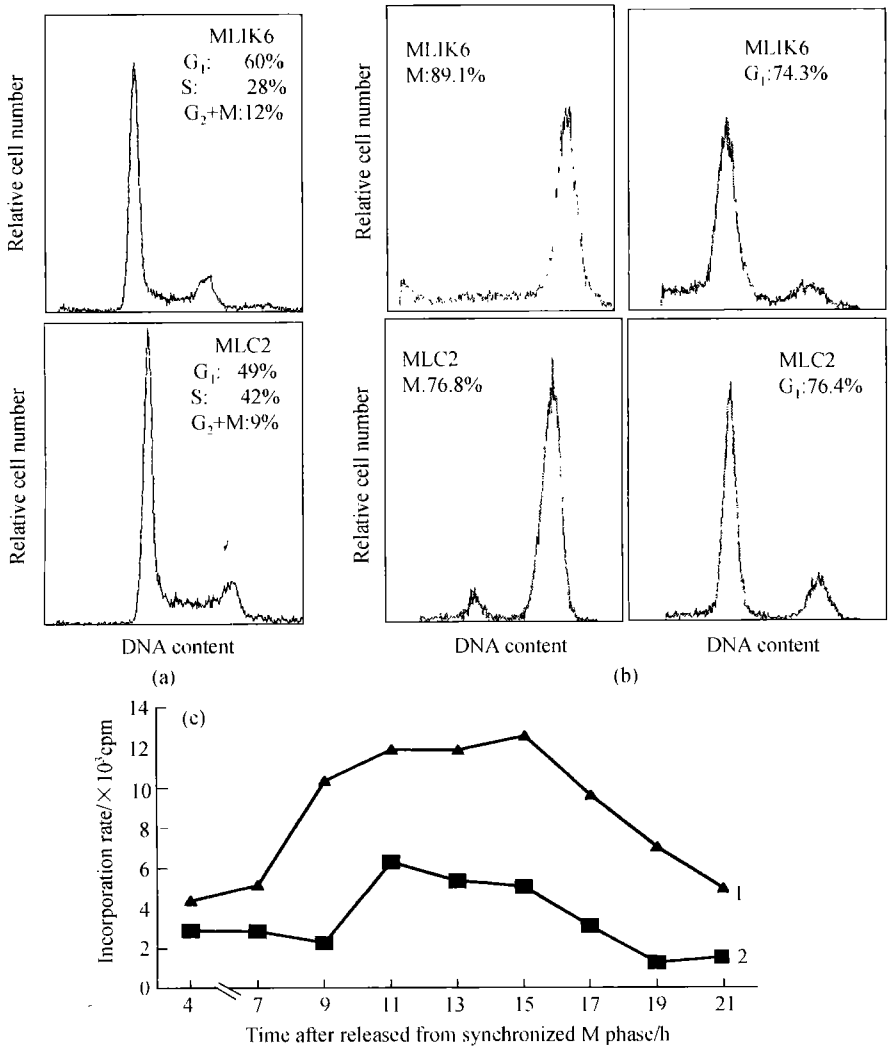

Fig. 2 Effect of overexpressing P15^{INK4b} on the progression of cell cycle. (a) Phase distribution of MLIK6 and MLC2 by FCM; (b) percentage of synchronized M and G_1 phase cells by N_2O-TdR arresting; (c) $G_1 \rightarrow S$ progression by ^{3}H-TdR incorporation. Curve 1, MLC2; curve 2, 2 MLIK6.

of P15^{INK4b}, especially about the function of P15^{INK4b} during the different progressions in G_1 phase.

We found in this study that in the constructed cell model MLIK6, the proliferation of the cells was inhibited when P15^{INK4b} expressed. FCM analysis provided the evidence that the overexpression of P15^{INK4b} could inhibit $G_1 \rightarrow S$ progression in human melanoma cells. Due to CDK4 and CDK6 are the target proteins of P15^{INK4b}[11], binding of P15^{INK4b} with CDK4/6 can prevent pRb phos-

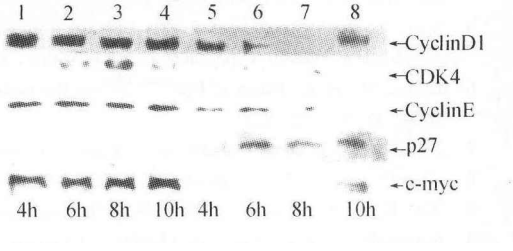

Fig. 3 Western blot analysis 1 ~ 4, MLC2 cells; 5 ~ 8, MLIK6 cells.

phorylation from cyclinD-CDK4/6, which affects G_1/S transition[11, 12]. But the effect of $P15^{INK4b}$ on the G_1-related regulatory factor in different stages of G_1 phase remains unclear. The result from synchronization experiment displayed that $P15^{INK4b}$ could reduce the expression level of cyclinD1 (the activator of CDK4/6) in G_1 phase, especially after being released from synchronization for 8 h (about late G_1). This result suggests that $P15^{INK4b}$ not only strongly inhibits CDK4/6 activity, but also reduces the expression level of CyclinD1 to delay the transition of G_1/S.

CyclinE is another G_1 phase cyclin. It binds to CDK2 to promote G_1 progression[13]. The activity of cyclinE-CDK2 reaches to a peak at G_1/S transition[14]. Our result showed the level of cyclinE was decreased when $P15^{INK4b}$ was overexpressed, especially at G_1/S transition. Different from cyclinD1, cyclinE was strongly inhibited at late G_1, which leads to the delay of G_1/S transition. P27 was first found in the cells blocked by TGF-β treatment. It can bind cyclinE-CDK2 to form a complex and inhibit the activity of cyclinE-CDK2[15], delaying the $G_1 \rightarrow$S transition. We found that when cyclinE decreased in the $P15^{INK4b}$ overexpressed MLIK6 cells, the level of P27 increased obviously, suggesting that high level of $P15^{INK4b}$ can not only inhibit the activity of cyclinE-CDK2, but also delay or block G_1 phase by affecting the expression of cyclinE and P27.

Recently some reports showed that there might be a pathway that directly affects cell cycle motoring molecule and regulates cell cycle[16]. The transition of G_1/S was found blocked and the expression of c-myc was down-regulated by TGF-β in MvlLu cell[17], whereas these could be inverted by overexpressing c-myc[17]. As was observed, the level of c-myc decreased obviously in G_1 phase when $P15^{INK4b}$ was overexpressed, suggesting that CKI can inhibit progression of cell cycle by affecting the expression of some oncogenes, and that c-myc should be involved in the disordered cell's proliferation and in carcinogenesis through regulation of cell cycle.

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