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The Krüppel-like factor 6 represses proliferation and invasion of cutaneous malignant melanoma

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All authors have equal implication in this study.

Cutaneous carcinoma is one of the most common neoplasm tumors in the West. Its incidence rate is one of the fastest growing tumors in China. The Krüppel-like factor 6 (KLF6) is a latent tumor suppressor. Decreased KLF6 is related to the occurrence and progression of many cancers in human. Our previous studies have demonstrated that KLF6 was down-regulation in cutaneous malignant melanoma (CMM), and was significant correlated with ulcer, lymph node metastasis and clinical stage, suggesting that KLF6 loss is expected to become a biological indicator of poor prognosis in CMM patients. In this research, we would further study the features of KLF6 in the malignant progression of CMM. The expression of KLF6 was up-regulated by lentivirus infection containing KLF6, and short hairpin RNA (shRNA) was used for knockdown of KLF6 in CMM cells. Western blot, RT-qpcr, CCK8 assay, transwell migration assays, wound healing assay and flow cytometry were used to test the role of KLF6 in the CMM. We found that reduced expression of KLF6 significantly enhanced proliferation, migration and invasion. Moreover, KLF6 induced CMM cell apoptosis and G1 cycle arrest. The decreased KLF6 expression is expected to be a biological indicator of poor prognosis in CMM patients.

Key words: Krüppel-like factor 6, cutaneous malignant melanoma, proliferation, migration, invasion.

Introduction

Cutaneous carcinoma has become the most common malignant tumor in the West, and the overall 5 year survival rate is 95% [1]. However, cutaneous malignant melanoma (CMM) is associated with much greater morbidity and mortality, accounting for 65% of all skin cancer deaths [1]. Its incidence rate is one of the fastest growing tumors in China. At present, TNM stage is still the main index for clinicians to judge the prognosis of CMM patients. However, due to the heterogeneity of tumors, patients with similar differentiation or the same TNM stage sometimes have different prognosis. Therefore, there are still some limitations in using the above indicators to judge the prognosis of patients.

The development of tumor molecular biology provides a new means for the study of the prognosis of tumor patients. If we can explore the changes of genes related to CMM invasion and metastasis

at the molecular level, look for sensitive and specific prognostic indicators, combine multiple factors and multiple indicators, and comprehensively analyze the prediction of CMM patients, we can make up for these limitations to a certain extent, comprehensively and accurately reflect the actual situation of patients, which is of great significance for clinicians to identify high-risk CMM patients with metastasis, deterioration or recurrence and implement individualized treatment.

The complete form of Krüppel-like factor 6 (KLF6) is a tumor suppressor. Loss of heterozygotes, somatic mutations, transcriptional silencing, and alternative splicing dysregulation are important causes of KLF6 inactivation [2-8], which regulates cancer development and progression [9]. KLF6 expression is significantly associated with the prognosis of prostate cancer [10], pancreatic cancer, pulmonary carcinoma [11] and squamous cell carcinoma of head and neck [12]. Although KLF6 is known to play a key role in CMM, its specific role in CMM remains unknown. Whether inactivation of KLF6 is involved in the occurrence and development of CMM has few reported at home and abroad.

Firstly, lentiviruses overexpressing KLF6 was used to increase its expression in the CMM cell line A375 and M14; KLF6 silencing in A375 and M14 cells was achieved by transfection with KLF6 shRNA. We examined the cell survival, growth and migration of KLF6-overexpresing and KLF6-silenced A375 and M14 cells.

Material and methods

Cell culture

A375 (Procell, Wuhan, China) and M14 (BeNa Culture Collection, Jiangsu, China) were placed in the DMEM medium serum (Procell, Wuhan, China) which was added 10% FBS (Thermo Fisher Scientific, USA). Then it was incubated in an incubator containing 5% CO_2 at a temperature of 37°C, changing fresh medium daily. The logarithmic growth cells was taken for experimentation.

Lentivirus containing KLF6 was used to infecte A375 and M14 cell line. A375 and M14 were transfected with lipofectamine containing specific shRNA.

Real-time quantitative-PCR (RT-qPCR)

Ultrapure RNA Kit was used to extract mRNA from cells (ComWin Biotech Co., Ltd, Beijing). Total RNA was extracted with trizol and standard phenol-chloroform extraction methods from CMM cells. Reverse transcription of the same amount of RNA was carried out, and quantitative real time PCR was carried out using the SYBR ® Green qPCR kit (Accurate Biotechnology Co., Hunan). The following primer (Beijing Dingguochangsheng BIOTECHNOLOGY Co., Ltd, Beijing) were used; KLF6 sense primer (5'GTGACAAGGGAAATGGCGATG3') and KLF6 antisense primer (5'CTCACACCCTTCCCATGAGC3').

Western blot analysis

The cells were dissolved for 30 minutes on ice, then centrifuged to remove the supernatant. The BCA protein quantitation was used for the preparation of the protein sample, which was then transferred to the PVDF membrane by SDS-polyacrylamide gel electrophoresis (PAGE). After sealing with 5% skimmed milk, primary antibody against KLF6 (1:1000; rabbit; proteintech) and GAPDH (1:5000; rabbit; Abclonal) were added at 4°C overnight, then adding secondary antibody with peroxidase-labelled (1:1000; HRP labeled Goat anti rabbit; ZSGB, Beijing, China). Determination of KLF6 protein by chemiluminescence assay system (Tanon, Shanghai, China).

CCK8 assay

To evaluate the proliferation of cells, the cell counting kit-8 (CCK-8) was used (Servicebio, Wuhan, China). At one, two, three, and four days after transfection, each well was adding 10 μl CCK-8 reagent and measurement of absorbance at 450 nm, the mean values of the 5 wells were calculated, and the test was repeated 3 times.

Transwell migration assay

The CMM cells after treatment were diluted to 10×10^5/ml. The upper transwell chamber was adding 200 μl cell suspension, and a 500 μl culture medium containing 10% FBS was added in the lower chamber. The upper chamber was steeped in the liquid of the lower chamber and incubated for 24 hours in 37°C incubator. 24 hours later, the upper chamber was put into an container containing 600 μl of PBS three times. Under the electron microscope, the upper chamber was observed and the image was taken after crystal violet staining. Each experiment make three replicates.

Wound healing assay

Cells were placed in a twelve-well plate with a cell density of 1.5×10^5/well. The cells were attached to the wall in a single layer, and the six well plates were cut vertically with a 10 μl tube tip. PBS was used to clean the suspension cells, and the culture was carried out in 5% carbon dioxide incubator at 37°C. Photographs were taken at 0, 12, 24, and 48 h, and repeated 3 times.

Flow cytometry

Cell Cycle Analysis kit (cat. no. MA0334; Meilune) was used to detect cell cycle. After two PBS washing, the cells were subjected to trypsin treatment and centrifuged. After washing with D-Hanks, Cells were fixed at 4 ℃ with 70% alcohol for 1 hour. The cells were stained with the addition of RNase A for 30 minutes at room temperature. The FACScan flow cytometer (ACEA NovoCyte, China) was used to assess the cell cycle and the CellQuest software was used to analyze.

Cell apoptosis was analyzed with Annexin V-PE/7-AAD (Meilunebio). After PBS washing, the cells were treated with trypsin and centrifuged. The cells were diluted in 1 × binding buffer to 1 × 10^6 cells. The binding buffer was incubated with 5 μl of PE-conjugated Annexin V (BD) and 5 μl 7-AAD (BD) at room temperature for 15 minutes. The flow cytometer (ACEA NovoCyte, China) was used to analyze the sample and the NovoExpress (ACEA NovoCyte) was used to analyze. Based on the double labeling of Annexin V-PE and 7-AAD, it is possible to distinguish early apoptosis (PE positive, 7-AAD negative), late apoptosis, and death cells (PE positive, 7-AAD positive).

Statistical analysis

The statistical analysis was carried out with SPSS software (SPPS, Inc.). Data are presented as the mean ± standard deviation. The difference of $p < 0.05$ was regarded as statistically significant.

Results

KLF6 expression in CMM cell lines

We established A357 and M14 cell lines which overexpressing KLF6. The KLF6 protein and RNA of lentivirus-infected cells are significantly overexpressed compared to control cell lines (Fig. 1A). Inhibition of KLF6 in A375 and M14 cells by shRNA. Compared to control cell lines, KLF6 were significantly inhibited in shRNA transfected cells (Fig. 1B).

KLF6 modulates Proliferation, migration, and invasion of cells

The purpose of this study was to study the influence of KLF6 on the growth of CMM cells. Firstly, the cell viability of A375 cells and M14 cells were detected by CCK8 method. The cell viability of cells overexpressing KLF6 was significantly reduced compared with control cells (Fig. 2A). In contrast, KLF6 inhibited cells were significantly more viable than control cells (Fig. 2B).

Next, We examined whether over-expression or suppression of KLF6 might affect the migration and invasion of A375 and M14 cells. Compared with NC cells, over-expression of KLF6 significantly inhibited A375 and M14 cells (Fig. 3A); conversely, knocking out KLF6 could enhance the migration of A375 and M14 cells (Fig. 3B). We also analyzed the effect of KLF6 overexpression or silencing on the invasive capacity of CMM cells. Compared to the NC control cells, the KLF6 overexpression cells were less aggressive (Fig. 3C). The KLF6 inhibitor cells exhibited higher aggressiveness than NC control cells (Fig. 3D). It was concluded that over-expression of KLF6 could inhibit the proliferation, migration and invasion of A375 and M14 cells, whereas KLF6 silencing could promote the proliferation, movement and invasion of CMM cells.

KLF6 induces CMM cell apoptosis and G1 cycle arrest

It was found that over-expression of KLF6 increased the apoptosis of A375 and M14 cells (Fig. 4A, B). In contrast, the KLF6 suppression cells showed decreased apoptosis compared with NC control cells (Fig. 4C, D). Flow cytometry to detected the cell cycle. The results indicated that the over-expression of KLF6 in G1 cell cycle stage was higher than that of the control group (Fig. 4E, F). In summary, KLF6 can induced apoptosis and G1-stage cell cycle block in CMM cells.

Discussion

Cutaneous malignant melanoma (CMM) is a highly aggressive cutaneous tumor originating from melanocytes [13, 14], which is the most life-threatening tumor in the skin. CMM is also seen as a major health problem, with both incidence and death rates increasing. KLF6 is a tumor suppressor gene. Although the precise mechanism of action of KLF6 is not fully understood, a number of highly correlated and overlapping pathways have been described: p21 performed reverse transcriptional activation in a p53-independent manner [15], reduced the cyclin D1/cdk4 complexes by interacting with cyclin D1 [16], inhibited the activity of c-Jun protooncoprotein, reduced the expression of VEGF [17], and induced apoptosis [18]. Loss of heterozygotes, somatic mutations, transcriptional silencing, and alternative splicing dysregulation are important causes of KLF6 inactivation [2-8]. Recent studies have found that the splicing variant KLF6-SV1 of KLF6 can resist the growth inhibition of KLF6 and promote the growth and proliferation of tumor cells. Our past research has demonstrated high expression of KLF6-SV1 in non-small cell lung cancer (NSCLC), especially lung adenocarcinoma, and is significantly associated with tumor progression [19]. KLF6 as an indicator of tumor prognosis has also been gradually revealed in recent years of research. The expression of KLF6 in prostate [10], lung [11], head and neck

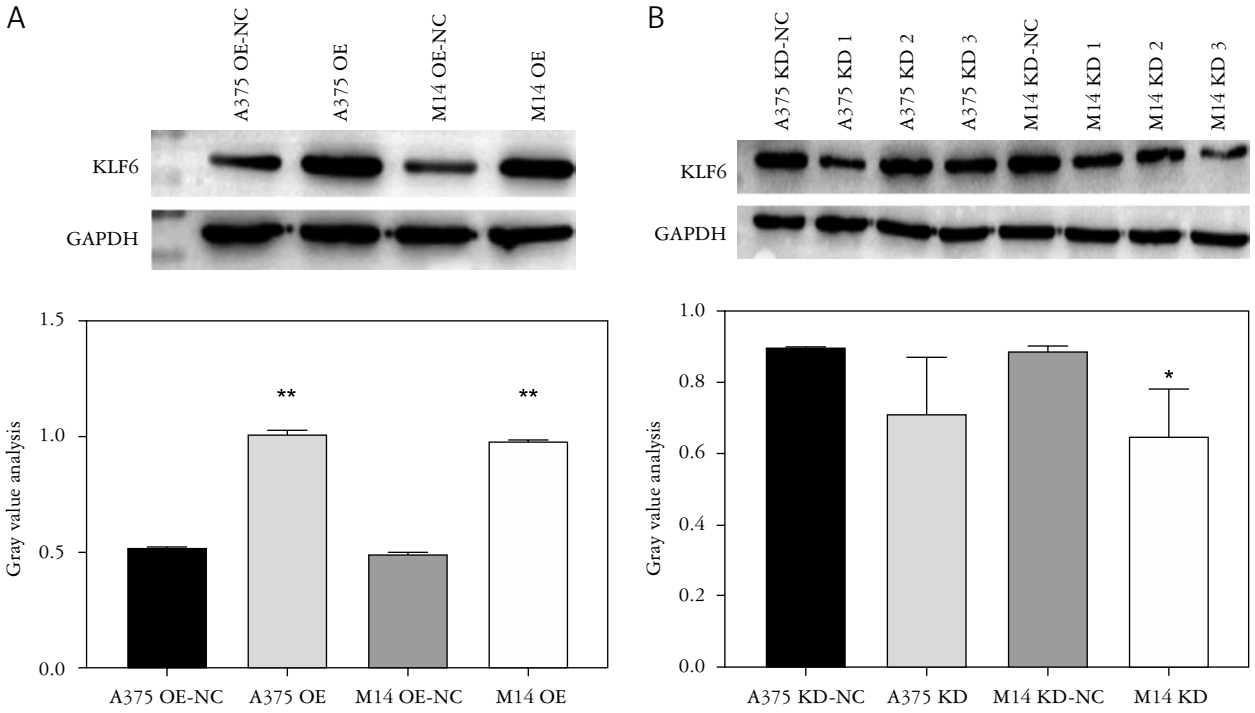


Fig. 1. Expression of KLF6 in cutaneous malignant melanoma cell line. **A**) Western blot and RT-qpcr detected that the expression of KLF6 was upregulated by lentivirus transfection into A375 cells and M14 cells. A375 OE-NC and M14 OE-NC were used as negative controls. **B**) The expression of KLF6 was down regulated by transfecting A375 cells and M14 cells with shRNA-KLF6. A375 KD-NC and M14 KD-NC were used as negative controls. OE, cells were transfected with LV-KLF6 as the overexpression group (OE); OE-NC, cells were transfected with LV-NC as the negative control (NC); KD, cells were transfected with shRNA-KLF6 as the knockdown group (KD); KD-NC, cells were transfected with a scramble sequence as the negative control. Data are expressed as the mean ± SD from three independent experiments. **p < 0.01 vs. the control group

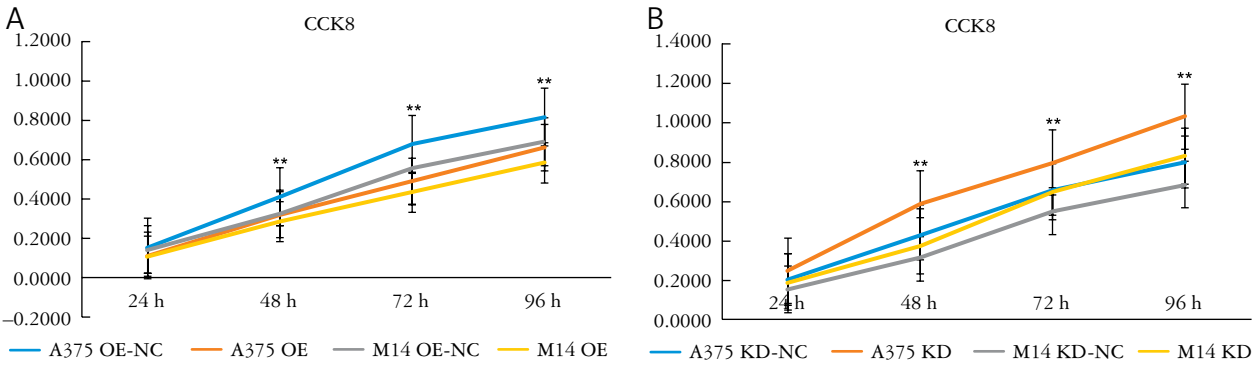


Fig. 2. Low expression of KLF6 can increase the survival rate of CMM cells. Cells were transfected for 24 h. A and B. CCK8 assay was used to assess cell viability in KLF6 overexpression (**A**) and KLF6 low expression (**B**) cells. Data are expressed as the mean ±SD from three independent experiments; *p < 0.05 vs. the control group

squamous cell cancer [12], and pancreatic cancer [13] is related to poor prognosis. Our study also demonstrated that high levels of KLF6-SV1 is closely related to poor prognosis in NSCLC [20]. The biological complexity of CMM makes it very difficult to study the prognosis. It is one-sided and limited to judge the prognosis only by a certain biological phenomenon or a single molecular index. Although many proteins have been identified as prognostic markers of CMM, they lack high sensitivity and specificity. Therefore, new CMM combined targets with high sensitivity and specificity need to be discovered. At present, there are few studies on whether the inactivation of KLF6 is related to the prognosis of CMM at home and abroad. In our previous studies, it was found that the decrease of KLF6 in CMM was associated with ulcer, lymph

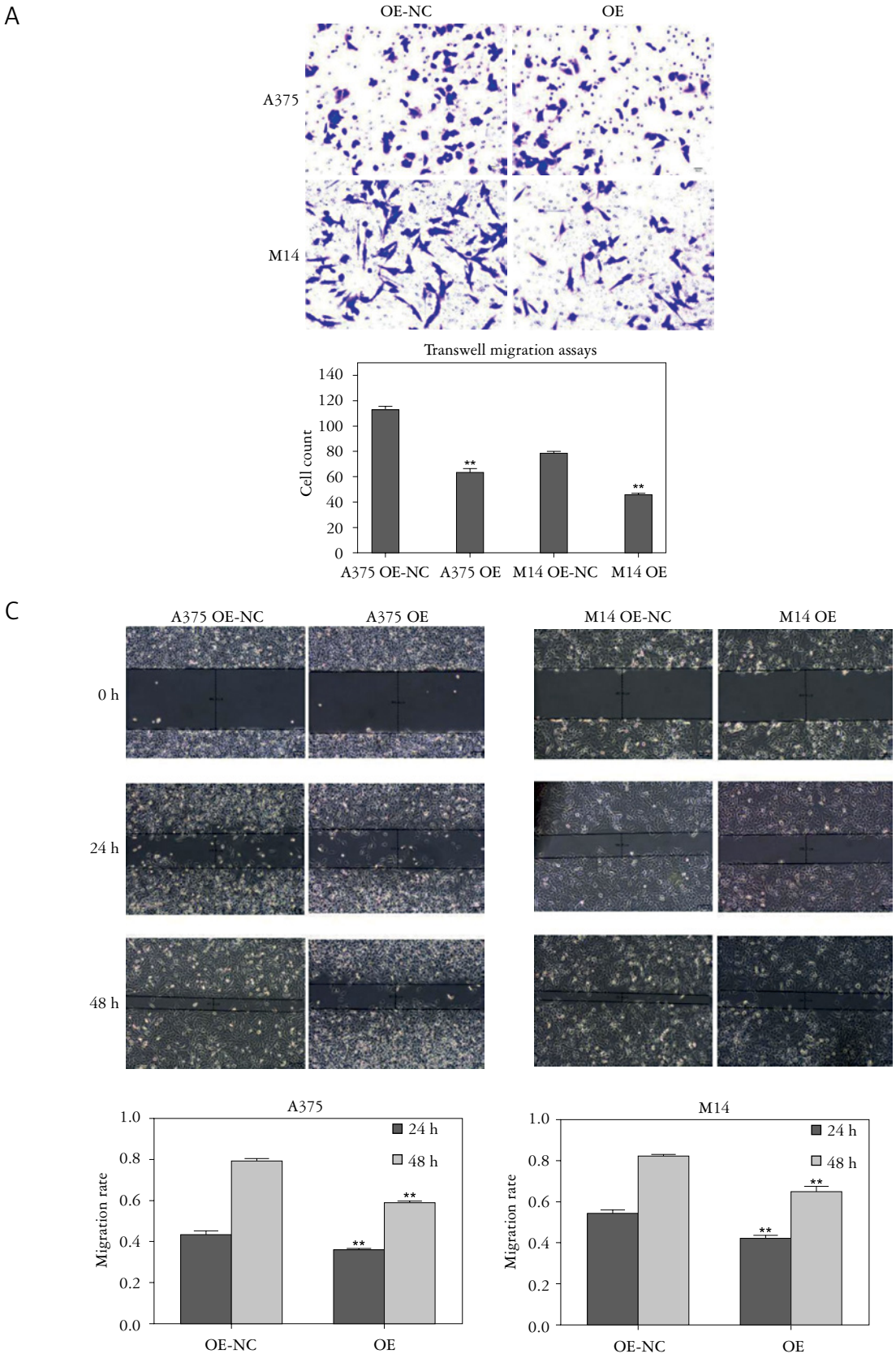
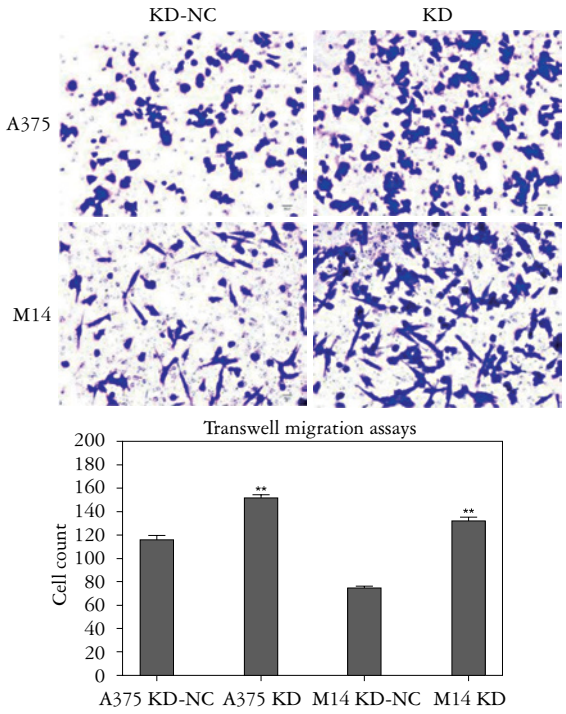


Fig. 3. Low expression of KLF6 promotes the invasion and migration of CMM cells in vitro. Cells were transfected for 24 h. **A, B**) Transwell migration assay was used to detect the migration ability of overexpressing (**A**) or inhibiting (**B**) KLF6 in A375 and M14 cells *in vitro*. **C, D**) The ability of overexpression (**C**) or inhibition (**D**) of KLF6 on the migration of A375 cells and M14 cells *in vitro* was evaluated by wound healing assay. Data are expressed as the mean ± SD from three independent experiments; *$p < 0.05$ vs. the control group

B


KD-NC
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Transwell migration assays
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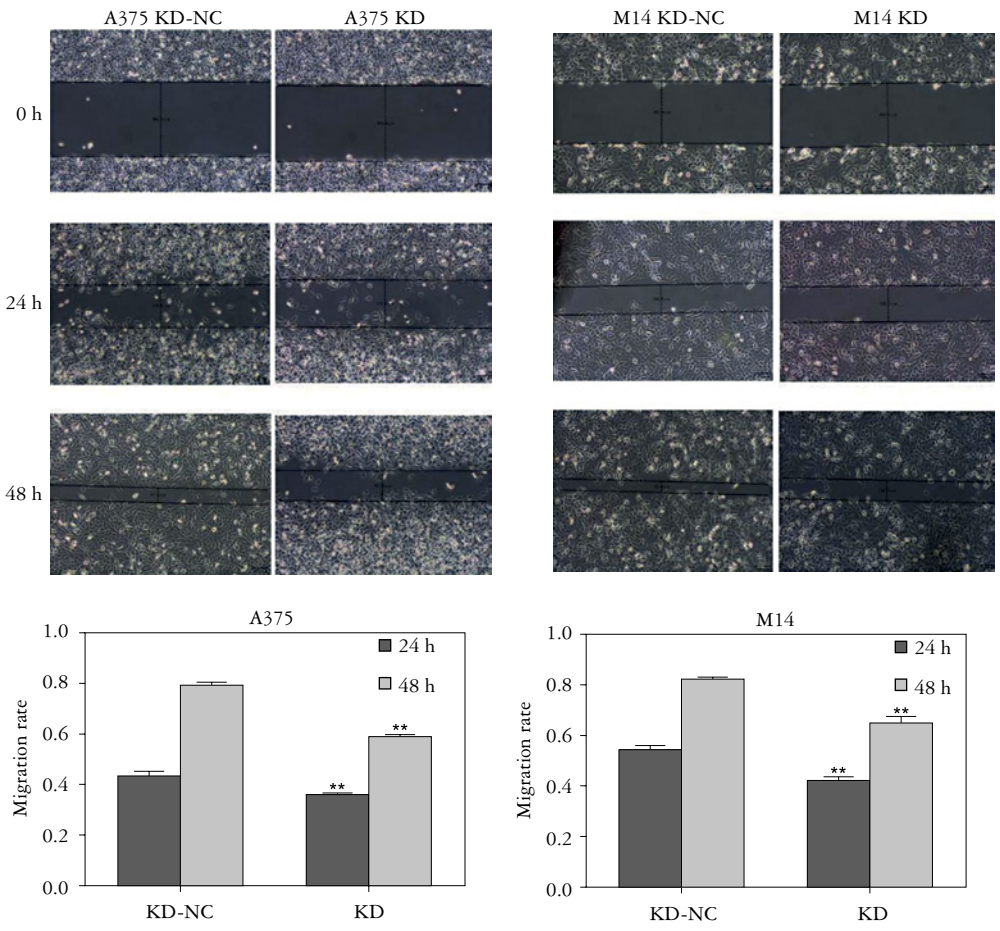
A375 KD-NC
A375 KD
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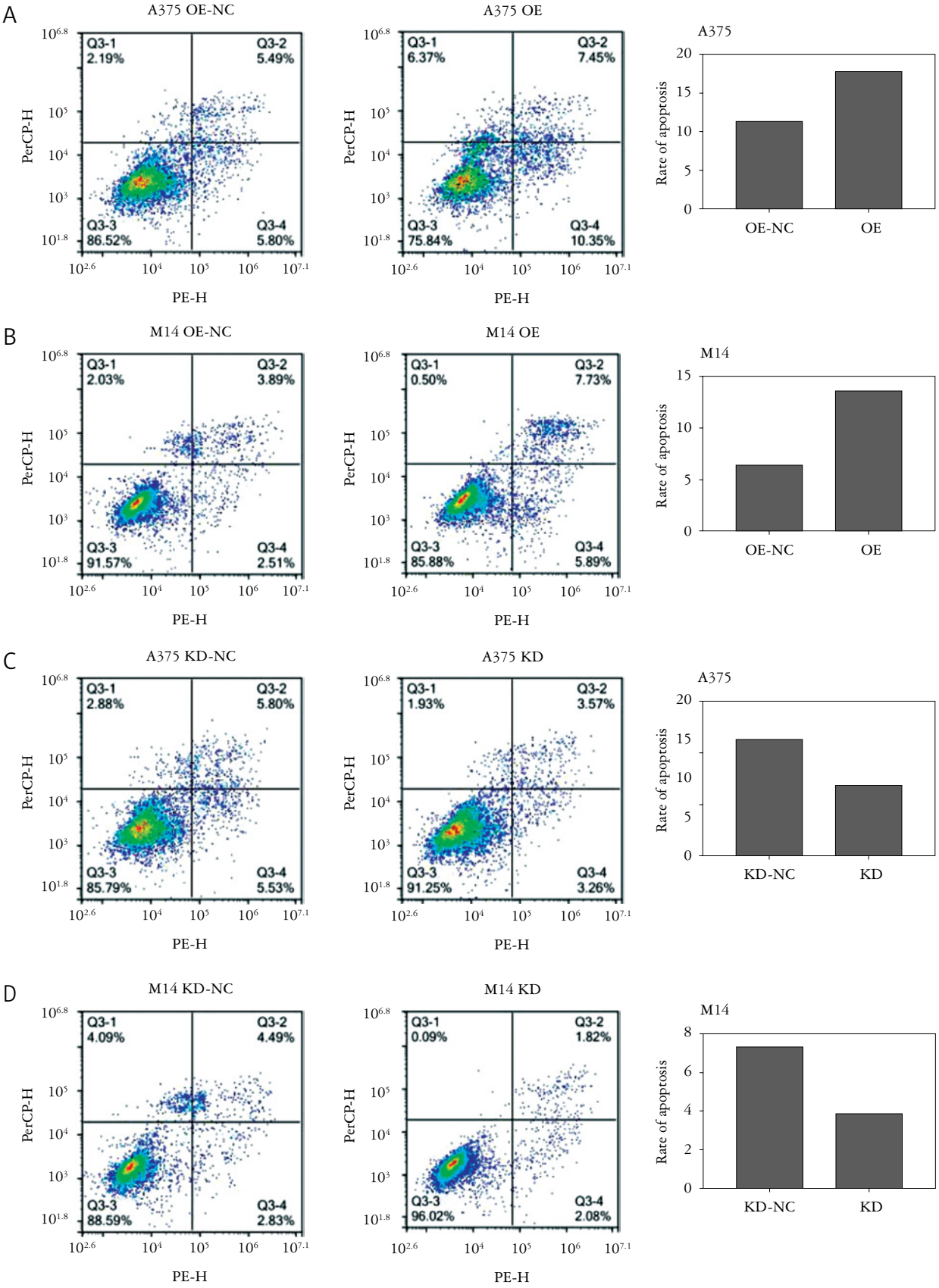
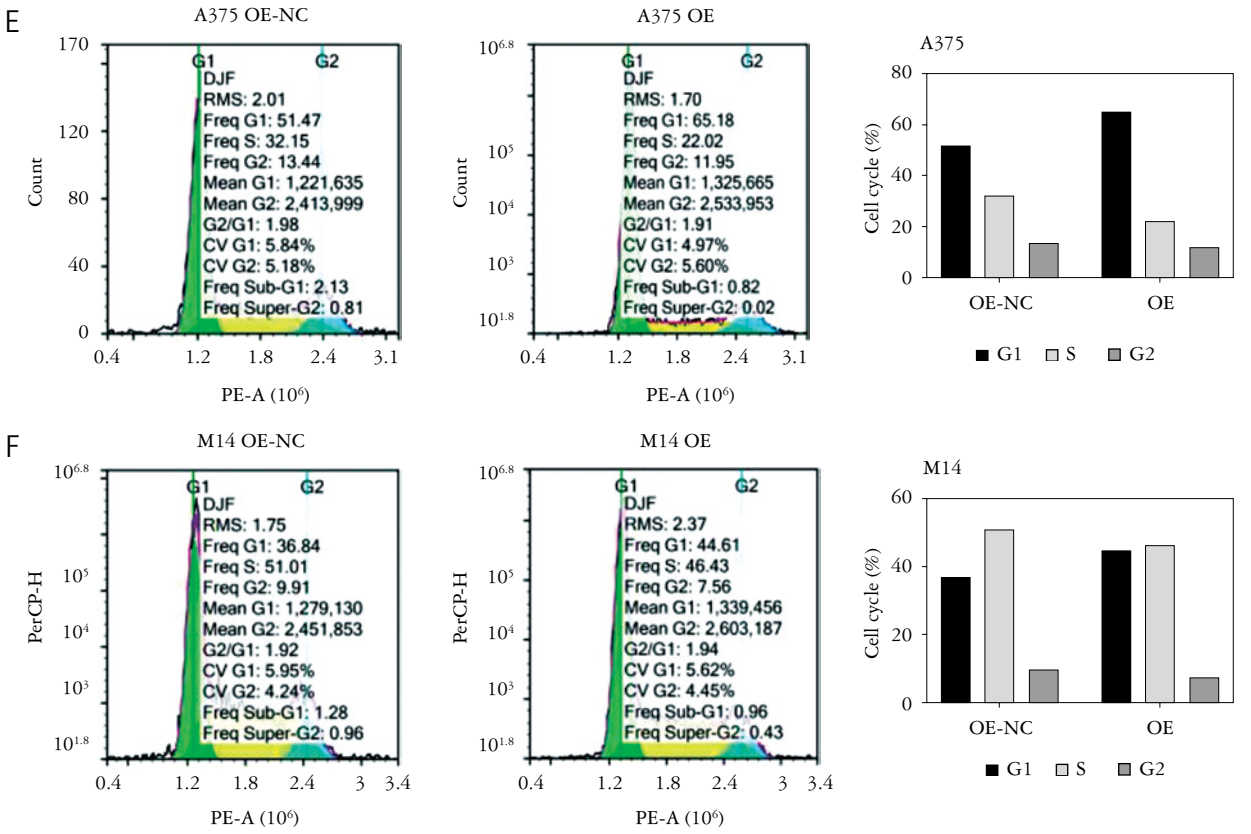


Fig. 4. KLF6 induces CMM cell apoptosis and G1 cycle arrest. **A-D**) The effect of overexpressing (**A, B**) or inhibiting (**C, D**) KLF6 in A375 and M14 cells apoptosis with Annexin V-PE/7-AAD stained flow cytometry method. **E, F**) Cell cycle distribution was determined via flow cytometry and quantified. Data are expressed as the mean ±SD from three independent experiments; *$p < 0.05$ vs. control group



node metastasis and clinical stage. CMM patients with low KLF6 had a lower survival rate than that with high KLF6 [21]. However, it is not known how KLF6 is regulated in cutaneous melanoma cells. The purpose of this study was to investigate the molecular mechanism of KLF6 in cutaneous malignant melanoma.

Firstly, we infected A375 and M14 cell lines with lentivirus containing KLF6 and KLF6 protein levels increased significantly. Then, the effects of the expression of KLF6 on proliferation, migration, invasion and survival in CMM cells were investigated. We also designed shRNA to inhibit KLF6 expression and the effects of KLF6 silencing on A375 and M14 cells were investigated.

Our data indicate that over-expression of KLF6 in A375 and M14 cells is effective in inhibiting the proliferation, migration and invasion of cells, which has few reported in CMM. Conversely, KLF6 silencing showed the opposite result in A375 and M14 cells.

Many studies have shown that KLF6 was involved in cancer cell cycles and apoptosis. Our studies showed that overexpression of KLF6 could induce apoptosis in A375 and M14 cells. In addition, we further demonstrated that overexpression of KLF6 could also induce G1 cell cycle block.

Finally, we provide evidence for the detailed mechanisms of KLF6 in regulating growth, migration, invasion and survival of CMM. Our research indicates that KLF6 is important in CMM and provides a new evidence for the study of the prognosis of CMM patients at the cell level. Detecting the expression of KLF6 may be a prognostic index of CMM and intervention strategies for the inactivation of KLF6 might be one approach to the treatment of spontaneous malignant melanoma.

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The authors declare no conflict of interest.

References

1. Cummins DL, Cummins JM, Pantle H, et al. Cutaneous malignant melanoma. Mayo Clin Proc 2006; 81: 500-507.
2. Reeves HL, Narla G, Ogunbiyi O, et al. Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. Gastroenterology 2004; 126: 1090-1103.
3. Camacho-Vanegas O, Narla G, Teixeira MS, et al. Functional inactivation of the KLF6 tumor suppressor gene by loss of heterozygosity and increased alternative splicing in glioblastoma. Int J Cancer 2007; 121: 1390-1395.
4. Kremer-Tal S, Narla G, Chen Y, et al. Downregulation of KLF6 is an early event in hepatocarcinogenesis, and stimulates proliferation while reducing differentiation. J Hepatol 2007; 46: 645-654.

5. Cho YG, Kim CJ, Park CH, et al. Genetic alterations of the KLF6 gene in gastric cancer. Oncogene 2005; 24: 4588-4590.
6. Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002; 2: 485-495.
7. Camacho-Vanegas O, Narla G, Teixeira MS, et al. Functional inactivation of the KLF6 tumor suppressor gene by loss of heterozygosity and increased alternative splicing in glioblastoma. Int J Cancer 2007; 121: 1390-1395.
8. DiFeo A, Martignetti JA, Narla G. The role of KLF6 and its splice variants in cancer therapy. Drug Resist Update 2009; 12: 1-7.
9. Narla G, Heath KE, Reeves HL, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. Science 2001; 294: 2563-2566.
10. Kettunen E, Anttila S, Seppänen JK, et al. Differentially expressed genes in nonsmall cell lung cancer: expression profiling of cancer-related genes in squamous cell lung cancer. Cancer Genet Cytogenet 2004; 149: 98-106.
11. Teixeira MS, Camacho-Vanegas O, Fernandez Y, et al. KLF6 allelic loss is associated with tumor recurrence and markedly decreased survival in head and neck squamous cell carcinoma. Int J Cancer 2007; 121: 1976-1983.
12. Hartel M, Narla G, Wente MN, et al. Increased alternative splicing of the KLF6 tumour suppressor gene correlates with prognosis and tumour grade in patients with pancreatic cancer. Eur J Cancer 2008; 44: 1895-1903.
13. Cummins DL, Cummins JM, Pantle H, et al. Cutaneous malignant melanoma. Mayo Clin Proc 2006; 81: 500-507.
14. Chen C, Hyytinen ER, Sun X, et al. Deletion, mutation, and loss of expression of KLF6 in human prostate cancer. Am J Pathol 2003; 162: 1349-1354.
15. Benzeno S, Narla G, Allina J, et al. Cyclin-dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. Cancer Res 2004; 64: 3885-3891.
16. Slavin DA, Koritschoner NP, Prieto CC, et al. A new role for the Kruppel-like transcription factor KLF6 as an inhibitor of c-Jun proto-oncoprotein function. Oncogene 2004; 23: 8196-8205.
17. Ito G, Uchiyama M, Kondo M, et al. Krüppel-like factor 6 is frequently down-regulated and induces apoptosis in non-small cell lung cancer cells. Cancer Res 2004; 64: 3838-3843.
18. Glinsky GV, Glinskii AB, Stephenson AJ, et al. Gene expression profiling predicts clinical outcome of prostate cancer. J Clin Invest 2004; 113: 913-923.
19. Zhang N, Yan QQ, Lu L, et al. The KLF6 splice variant KLF6-SV1 promotes proliferation and invasion of non-small cell lung cancer by up-regulating PI3K-AKT signaling pathway. J Cancer 2019; 10: 5324-5331.
20. Zhang N, Li Z, Xiao W, et al. KLF6-SV1 is a new prognostic biomarker in postoperative patients with non-small cell lung cancer. Cancer Manag Res 2018; 10: 3937-3944.
21. Zhang N, Qiu LY, Yang F, et al. Clinical and prognostic significance of Krüppel-like transcription factor 6 expression in 67 patients with cutaneous malignant melanoma in China. Pol J Pathol 2021; 72: 245-251.

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