Benzo(a)pyrene enhances the EMT-associated migration of lung adenocarcinoma A549 cells by upregulating Twist1

XI CHEN[1], HONGBING PENG[2], JIAN XIAO[2], ANQI GUAN[2], BIN XIE[2], BIXIU HE[2] and QIONG CHEN[2]

Departments of [1]Respiratory Medicine and [2]Geriatrics, Respiratory Medicine, Xiangya Hospital of Central South University, Changsha, Hunan 410008, P.R. China

Received February 28, 2017; Accepted July 20, 2017

DOI: 10.3892/or.2017.5874

Abstract. Benzo(a)pyrene (BaP), an important toxic component of cigarette smoke, can cause lung cancer and lead to the progression of lung cancer. In the present study, we investigated the effect of BaP on the migration of lung adenocarcinoma A549 cells. BaP (1 μM) promoted the migration of A549 cells in a time-dependent manner and upregulated the expression of the Twist family BHLH transcription factor 1 (Twist1). BaP also induced upregulation of the mesenchymal markers N-cadherin and vimentin and downregulation of the epithelial marker E-cadherin. When the expression of Twist1 was knocked down in A549 cells that were treated with BaP for 4 weeks ($A549^{BaP-4w}$), the expression of Twist1 decreased, which inhibited the migration capacity of $A549^{BaP-4w}$ cells, the expression of N-cadherin and vimentin was downregulated and the expression of E-cadherin was upregulated. In addition, morphological observations of $A549^{BaP-4w}$ cells revealed that the epithelial characteristics of A549 cells became mesenchymal characteristics. When the expression of Twist1 was knocked down, the $A549^{BaP-4w}$ cells were transformed back to cells with epithelial characteristics. In conclusion, the results from the present study indicate that BaP enhances the epithelial-mesenchymal transition-associated migration of lung adenocarcinoma A549 cells by upregulating Twist1.

Introduction

Cigarette smoking is strongly associated with lung cancer incidence and mortality (1,2). As an important toxic component of cigarette smoke (3), benzo(a)pyrene (BaP) alone is sufficient to induce lung cancer (4, 5). However, BaP promotes lung cancer through different mechanisms (6-10), and one of these mechanisms is the induction of epithelial-mesenchymal transition (EMT) (10).

EMT is a process by which epithelial cells gain migratory and invasive characteristics to become motile mesenchymal stem cells. In physiological conditions, EMT is integral in embryo implantation, organ development and wound healing (11,12). However, under pathological conditions, EMT contributes to fibrosis and cancer progression (11,12). It is an important process in the development and progression of lung cancer induced by tobacco smoke (13), and it is an important cause of drug resistance and metastasis in lung cancer (14). In addition, the EMT secretory phenotype can also predict survival in patients with lung cancer (15).

Twist family BHLH transcription factor 1 (Twist1) is a basic helix-loop-helix domain-containing transcription factor that is encoded by the Twist1 gene (16), and is essential for embryonic differentiation during physiological conditions (16). Upregulated Twist1 expression is associated with the progression of lung cancer (17) and other types of cancer, such as gastric (18), breast (19), colorectal (20), endometrial (21) and prostate (22) cancer. In addition, Twist1 can induce EMT in cancer cells (23,24). Particularly, inhibiting the expression of Twist1 in lung cancer cells suppresses cell proliferation (17,25) and metastasis (25), activates oncogene-induced senescence (26) and suppresses the EMT process (25).

In the present study, we aimed to study the effects of BaP on migration ability, the EMT process and Twist1 expression in adenocarcinoma A549 cells. We found that BaP can enhance the migration of lung adenocarcinoma A549 cells through the promotion of EMT by upregulation of Twist1.

Materials and methods

Cell culture and treatments. Human A549 cells (Xiangya Cells Center, Central South University, Changsha, Hunan, China) were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel) and 1% penicillin/streptomycin and incubated in a humidified incubator at 37˚C with 5% CO_2.

BaP (purity ≥96%; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium at a final concentration of 1 μM. Before

Correspondence to: Professor Bixiu He or Professor Qiong Chen, Department of Geriatrics, Respiratory Medicine, Xiangya Hospital of Central South University, Changsha, Hunan 410008, P.R. China
E-mail: hebixiu000@tom.com
E-mail: qiongch@163.com

Key words: benzo(a)pyrene, epithelial-mesenchymal transition, migration, lung cancer, Twist1

proceeding with further experiments, A549 cells were treated with 1 μM BaP for 24, 48 and 72 h; 1, 2 and 4 weeks.

Wound healing assay. Wound healing assays were conducted to evaluate the lateral migration capacity of cells. After cells were seeded into 24-well plates and had grown into a monolayer, a 'scratch' was scraped in a straight line using a p200 pipet tip. The cells were washed 3 times with growth medium to smooth the edges of the scratch and remove debris, and the medium was replaced with fresh culture medium. The cells were then allowed to recover for 12 h. Before and after the 12 h incubation, the initial scratch and the recovery area, respectively, were photographed under a microscope. The wound closure rate was equal to the recovered distance divided by the original width of the scratch.

Transwell migration assay. Transwell plates (6.5 mm in diameter at the lower surface and 8-μm pore filters; Corning Costar, Cambridge, MA, USA) without Matrigel were used to detect the longitudinal migration ability of cells. Briefly, $5x10^4$ cells in 200 μl of 0.1% FBS culture medium were seeded in the upper chamber and 800 μl of medium with 10% FBS was placed in the lower chamber. Following incubation for 24 h, a cotton swab was used to remove the cells adhering to the membrane of the upper chamber. The migrated cells on the lower side of the filter membrane surface were stained with 0.1% crystal violet, and the cells were counted under a light microscope.

Reverse transcription PCR (RT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using a PrimeScript First Strand cDNA Synthesis kit (Takara Biotech, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. The following are the primers used for PCR amplification: Twist1 forward, 5'-CTCAGCTACGCCTTCTCGGT-3' and reverse, 5'-AGTCCATAGTGATGCCTTTC-3'; N-cadherin forward, 5'-ATCCAGACCGACCCAAACAG-3 and reverse, 5'-GGAA TTCCATTGTCAGAAGC-3; vimentin forward, 5'-CCAGG CAAAGCAGGAGTC-3 and reverse, 5'-CGAAGGTGACGA GCCATT-3; E-cadherin forward, 5'-CTGAGAACGAGGCT AACGTCG-3 and reverse, 5'-AGTGTAGGATGTGATTTC CTG-3; β-actin forward, 5'-AGAGCTACGAGCTGCCTG AC-3 and reverse, 5'-AGCACTGTGTTGGCGTACAG-3.

Western blotting. Whole cell protein was prepared using ice-cold RIPA buffer (Millipore, Temecula, CA, USA) mixed with a protease inhibitor cocktail. The protein concentrations were determined after cell lysates were centrifuged at 12,000 x g at 4˚C for 20 min to clear the cell debris. Equal amounts of protein (50 μg) from each sample were loaded/lane and separated on 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore) via electroblotting. Then, 5% non-fat milk was used to block the membranes for 2 h. Primary antibodies, diluted according to the manufacturer's recommendations, were incubated with the membranes overnight at 4˚C. The antibodies used included antibodies against Twist1 (Santa Cruz, Heidelberg, Germany), N-cadherin (BD Biosciences, San Jose, CA, USA), vimentin (Santa Cruz) and E-cadherin (BD Biosciences), and β-actin (Cell Signaling Technology, Beverly, MA, USA) served as the loading control. The membranes were washed 3 times (10 min/time) in Tris-buffered saline with Tween-20 (TBST) buffer, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The protein bands were detected using an enhanced chemiluminescence (ECL) reagent kit (Thermo Scientific Pierce, Rockford, IL, USA).

Construction of plasmid-expressing short hairpin RNA targeting Twist1. A short hairpin RNA sequence (GCTGAGC AAGATTCAGACCCTTTCAAGAGAAGGGTCTGAATCT TGCTCAGC) targeting Twist1 (Twist1-shRNA) was inserted into a pGCsilencer™ U6/Neo/GFP/RNAi plasmid (GeneChem, Shanghai, China). A negative control shRNA (TTCTCCGAAC GTGTCACGT) vector was also constructed using a pGCsilencer™ U6/Neo/GFP/RNAi plasmid to verify the sequence specificity of Twist1-shRNA. The cells were transfected with plasmids using Lipofectamine™ 2000 reagent (Invitrogen). The transfection efficiency was observed under a fluorescence microscope after transfection for 24 h. The knockdown efficiency of Twist1-shRNA was detected with western blotting after transfection for 48 h.

Statistical analysis. Statistical analyses were conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) with a Windows operating system. Repeated measurement data were presented as the mean ± standard deviation (SD). When two sets of data were compared, differences were calculated using Student's t-test (two-tailed). Statistical significance was defined as $p<0.05$.

Results

BaP promotes the migration of A549 cells. To study the effect of BaP on the migration of A549 cells, we first treated A549 cells with 1 μM BaP for different lengths of time (24, 48 and 72 h; 1, 2 and 4 weeks). Thereafter, we used a wound healing assay to study the lateral migration ability of BaP-treated A549 cells. As shown in Fig. 1, after the cells in the wound healing assay were allowed to recover for 12 h, BaP enhanced the lateral migration ability of A549 cells when the treatment time was at least 48 h. Furthermore, we used Transwell migration assays to research the longitudinal migration ability of BaP-treated A549 cells. As shown in Fig. 2, after the Transwell migration assay was performed for 24 h, BaP enhanced the longitudinal migration ability of A549 cells when the intervention time was at least 48 h, similar to the enhancement effect observed on lateral migration. In addition, we found that the effect of BaP on the migration of A549 cells was gradually enhanced with prolonged treatment time (Figs. 1 and 2).

Effect of BaP treatment on the expression of Twist1, N-cadherin, vimentin and E-cadherin in A549 cells. As shown in Figs. 3 and 4, after A549 cells were exposed to 1 μM BaP for different durations of time (24, 48 and 72 h; 1, 2 or 4 weeks), both the mRNA (Fig. 3) and protein expression (Fig. 4) of Twist1 were gradually increased with prolonged intervention times. Accordingly, the expression of N-cadherin and vimentin were also gradually increased with prolonged BaP intervention (Figs. 3 and 4). However,

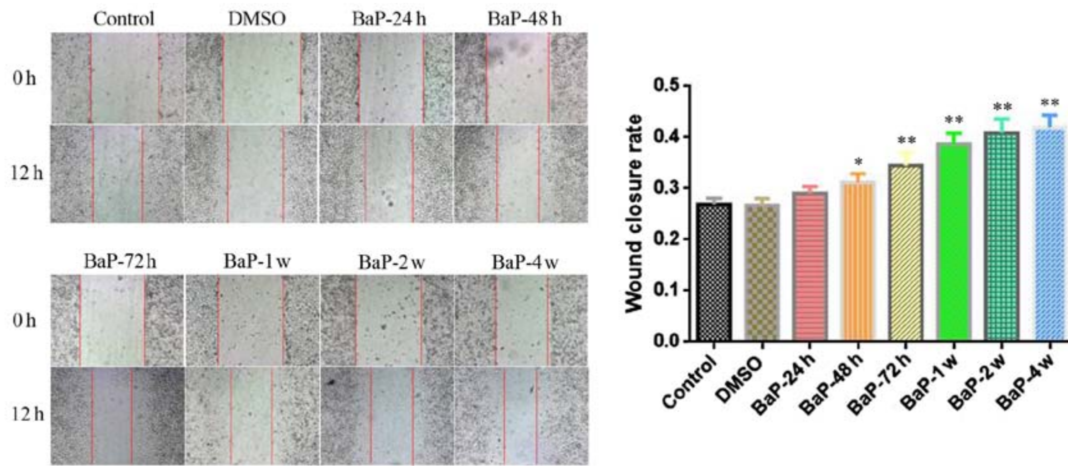

Figure 1. BaP (1 μM) stimulates A549 cell migration, shown by a wound healing assay. After intervention with BaP for different lengths of time (24, 48 and 72 h; 1, 2 and 4 weeks), wound healing assays were performed with a 12-h recovery time. Graphics present the wound closure rate of cells among different treatment groups, and the results are presented as the mean ± SD of 3 independent experiments; $^{*}p<0.05$, $^{**}p<0.01$. BaP, benzo(a)pyrene.

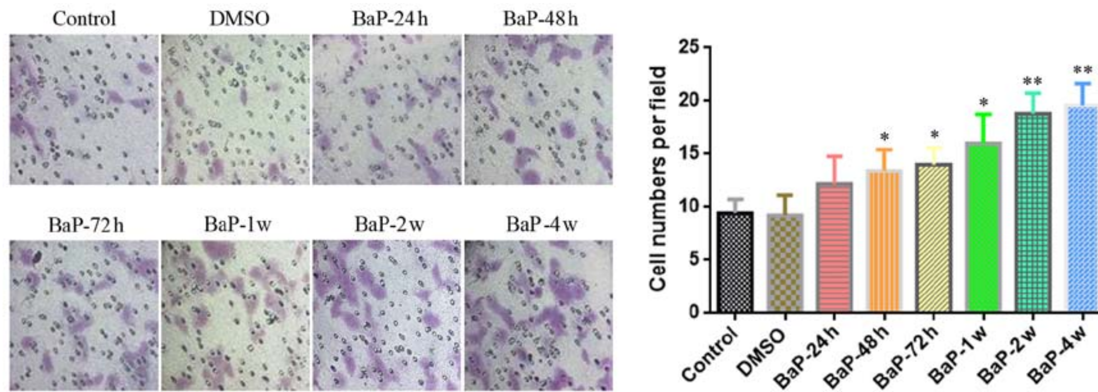

Figure 2. BaP (1 μM) stimulates A549 cell migration, as revealed by a Transwell migration assay. After intervention with BaP for different lengths of time (24, 48 and 72 h; 1, 2 and 4 weeks), Transwell migration assays were performed for 24 h (the microscopy fields are shown at a magnification of x200). Graphics display the number of cells/field between different treatment groups, and the results are presented as the mean ± SD of 3 independent experiments; $^{*}p<0.05$, $^{**}p<0.01$. BaP, benzo(a)pyrene.

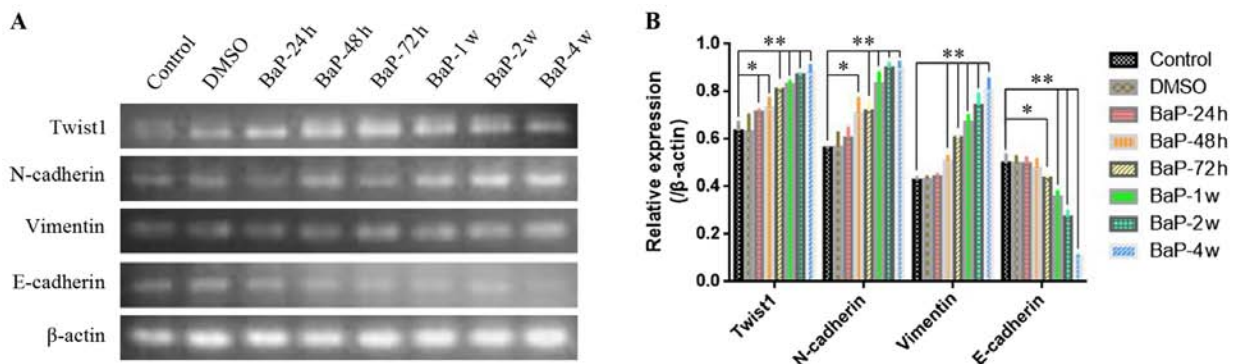

Figure 3. Effect of BaP on the mRNA expression of Twist1, N-cadherin, vimentin and E-cadherin in A549 cells. (A) After A549 cells were exposed to 1 μM BaP for 24, 48 and 72 h; 1, 2 or 4 weeks, the mRNA levels were determined using RT-PCR. (B) The statistical results are presented as the mean ± SD of 3 independent experiments; $^{*}p<0.05$, $^{**}p<0.01$. BaP, benzo(a)pyrene; Twist1, Twist family BHLH transcription factor 1.

conversely, the expression of E-cadherin gradually decreased as the BaP intervention time was extended (Figs. 3 and 4).

Downregulation of Twist1 inhibits the migration ability of A549^{BaP-4w} cells. Compared with A549 cells without BaP intervention, Twist1 was highly expressed in A549 cells treated with BaP for 4 weeks (A549^{BaP-4w}) (Figs. 3 and 4), and the migration capacity of A549^{BaP-4w} cells was significantly enhanced (Figs. 1 and 2). We hypothesized that downregulation of Twist1 expression may decrease the migration of A549^{BaP-4w}

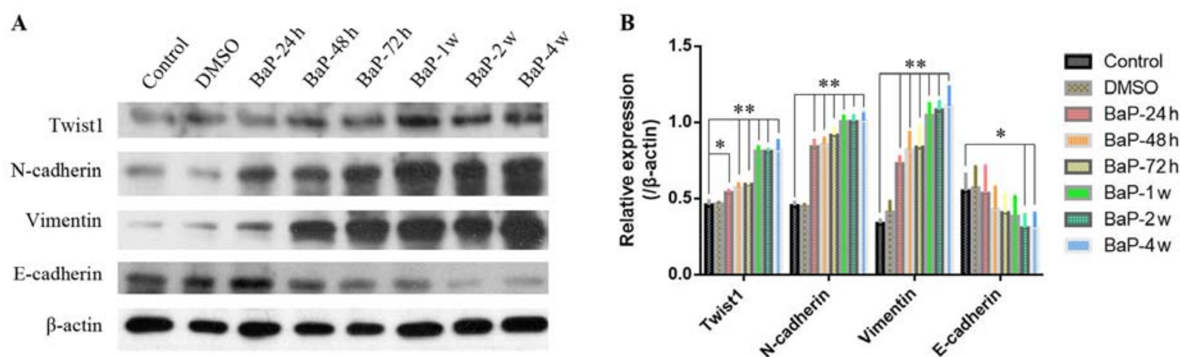

Figure 4. Effect of BaP on the protein expression of Twist1, N-cadherin, vimentin and E-cadherin in A549 cells. (A) After A549 cells were exposed to 1 μM BaP for 24, 48 and 72 h; 1, 2 or 4 weeks, the protein levels were determined with western blotting. (B) The statistical results are presented as the mean ± SD of 3 independent experiments; *p<0.05, **p<0.01. BaP, benzo(a)pyrene.

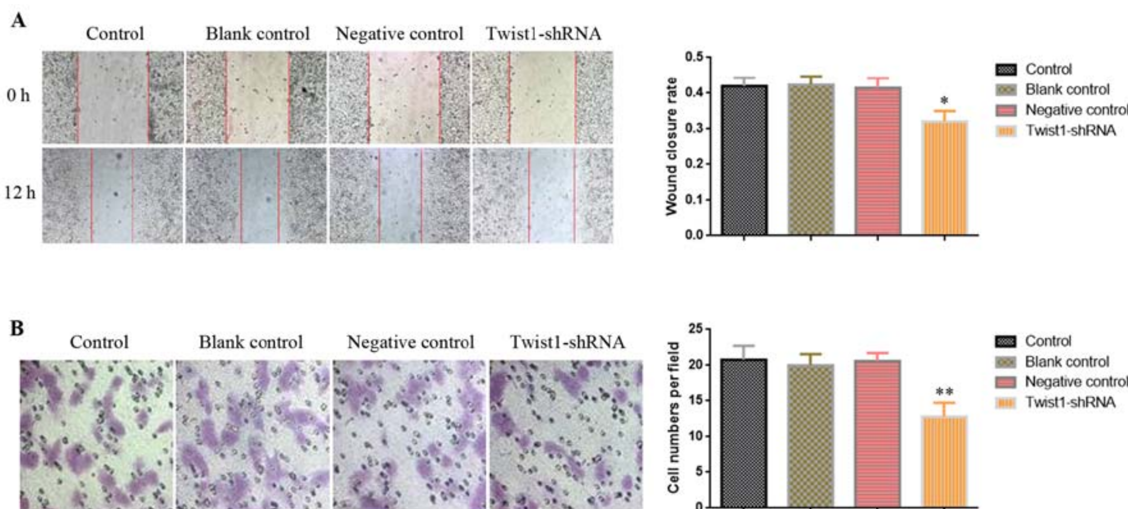

Figure 5. Downregulation of Twist1 in A549^{BaP-4w} cells inhibits the migration ability. After downregulation of Twist1 using Twist1-shRNA, (A) a wound healing assay was performed with 12 h of recovery and (B) a Transwell migration assay was performed at 24 h (the microscopic fields are shown at a magnification of x200). The graphics display the (A) wound closure rate and (B) the number of cells/field between the different groups. The statistical results are presented as the mean ± SD of 3 independent experiments; *p<0.05, **p<0.01. A549^{BaP-4w}, A549 cell model incubated with 1 μM BaP for 4 weeks, resulting in the upregulation of the expression of Twist1; control, A549^{BaP-4w} cell group without plasmid transfection; blank control, A549^{BaP-4w} cell group transfected with blank plasmid; negative control, A549^{BaP-4w} cell group transfected with negative plasmid; Twist1-shRNA, A549^{BaP-4w} cell group transfected with recombinant plasmid containing short hairpin RNA to downregulate Twist1 gene expression. BaP, benzo(a)pyrene; Twist1, Twist family BHLH transcription factor 1.

cells. Thus, we applied wound healing and Transwell migration assays to assess A549^{BaP-4w} cells that were transfected with recombinant plasmid containing short hairpin RNA (Twist1-shRNA). The results of both the wound healing (Fig. 5A) and Transwell migration assay (Fig. 5B) revealed that downregulation of Twist1 inhibited the migration ability of A549^{BaP-4w} cells.

Decreased expression of Twist1 in A549^{BaP-4w} cells results in downregulation of N-cadherin and vimentin and upregulation of E-cadherin. As shown in Fig. 6, the expression of Twist1 in A549^{BaP-4w} cells was obviously downregulated after transfection with Twist1-shRNA. Accordingly, the expression of N-cadherin and vimentin was also markedly decreased after A549^{BaP-4w} cells were transfected with Twist1-shRNA (Fig. 6A and B). However, decreased expression of Twist1 in A549^{BaP-4w} cells resulted in the upregulation of E-cadherin expression (Fig. 6A and B).

BaP induces EMT in A549 cells by upregulating Twist1. Under a microscope, epithelial cells are characterized by a flat and polygonal shape, and mesenchymal cells are characterized by a relatively small cell body that is long and thin. As shown in Fig. 7A, after 4 weeks of 1 μM BaP intervention, most of the of A549^{BaP-4w} cells transformed from cells with epithelial characteristics to cells with mesenchymal characteristics. However, after transfection of A549^{BaP-4w} cells with Twist1-shRNA (Fig. 7B) to downregulate the expression of Twist1 (Fig. 7C), the A549^{BaP-4w} cells were transformed from cells with mesenchymal characteristics back into cells with epithelial characteristics (Fig. 7D). Consequently, we concluded that BaP induced EMT in A549 cells by upregulating Twist1.

Discussion

In the present study, we first found that BaP promotes the migration of lung adenocarcinoma A549 cells in a time-dependent manner. Increased expression of Twist1 in A549 cells resulted in upregulation of N-cadherin and vimentin

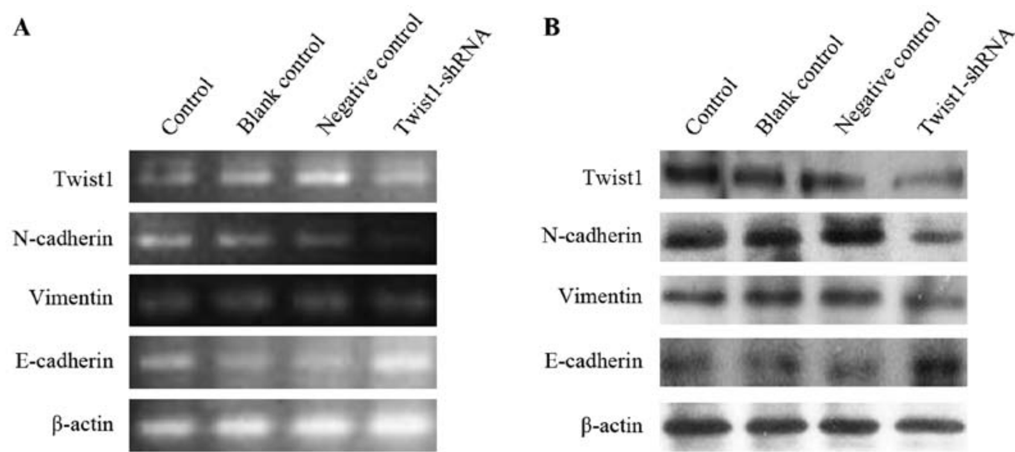

Figure 6. Decreased expression of Twist1 in A549^{BaP-4w} cells results in the downregulation of N-cadherin and vimentin and upregulation of E-cadherin. (A) The mRNA levels and (B) protein expression of Twist1, N-cadherin, vimentin and E-cadherin were determined with RT-PCR and western blotting, respectively. The results are grouped by A549^{BaP-4w} cells with different transfection statuses (control, A549^{BaP-4w} cell group without plasmid transfection; blank control, A549^{BaP-4w} cell group transfected with blank plasmid; negative control, A549^{BaP-4w} cell group transfected with negative plasmid; Twist1-shRNA, A549^{BaP-4w} cell group transfected with recombinant plasmid containing short hairpin RNA to downregulate Twist1 gene expression). BaP, benzo(a)pyrene; Twist1, Twist family BHLH transcription factor 1.

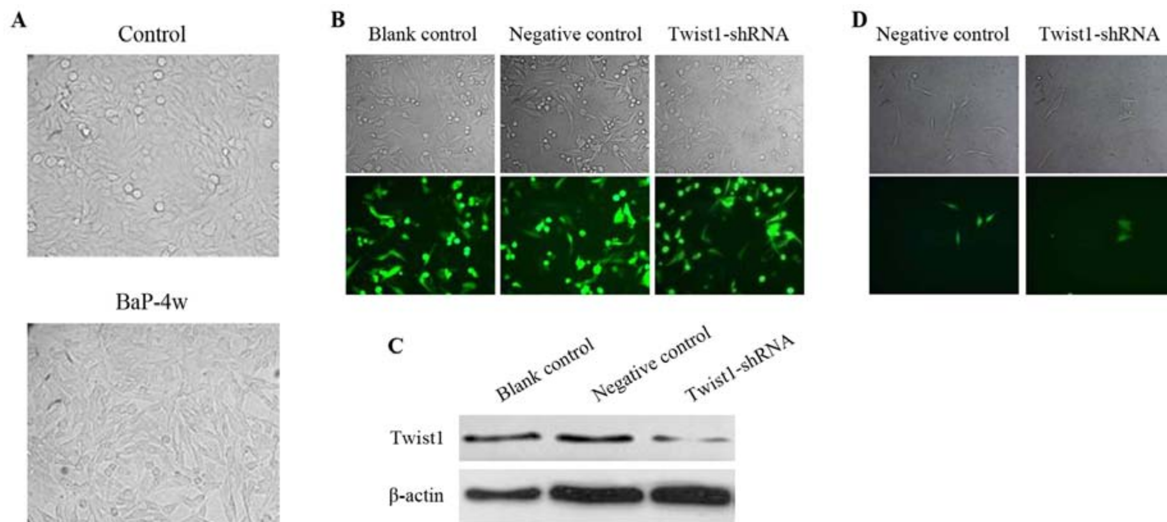

Figure 7. (A) Morphological observation reveals that most of the A549^{BaP-4w} cells were transformed from cells with epithelial characteristics to cells with mesenchymal characteristics after 4 weeks of 1 μM BaP intervention. (B) After transfection with recombinant plasmid, (C) containing short hairpin RNA to downregulate Twist1 gene expression, (D) A549^{BaP-4w} cells were transformed into cells with epithelial characteristics from cells with mesenchymal characteristics. Blank control, A549^{BaP-4w} cell group transfected with blank plasmid; negative control, A549^{BaP-4w} cell group transfected with negative plasmid; Twist1-shRNA, A549^{BaP-4w} cell group transfected with recombinant plasmid containing short hairpin RNA to downregulate Twist1 gene expression. BaP, benzo(a)pyrene; Twist1, Twist family BHLH transcription factor 1.

and downregulation of E-cadherin. Then, after the expression of Twist1 was knocked down in A549 cells that were treated with BaP for 4 weeks (A549^{BaP-4w}), the resulting decrease in the expression of Twist1 inhibited the migration capacity of A549^{BaP-4w} cells, downregulated the expression of N-cadherin and vimentin and upregulated the expression of E-cadherin. Finally, along with the morphological results observed under the microscope, we concluded that BaP enhanced the migration of lung adenocarcinoma A549 cells through the promotion of EMT by upregulation of Twist1.

Prior to the present study, Yoshino *et al* (10) reported that BaP can induce the EMT of lung cancer cells. In addition, D'Angelo *et al* (23) and Yoon *et al* (24) found that Twist1 can induce the EMT of cancer cells. We therefore hypothesized that Twist1 may be the target of BaP-treated lung adenocarcinoma A549 cells, and we thus demonstrated this hypothesis in the present study. However, as the mechanism of cigarette smoking-induced lung cancer is very complex, we surmised that Twist1 cannot be the only target of BaP in lung cancer cells. More studies on the mechanism of cigarette smoking/BaP-induced lung cancer need to be conducted in the future.

Yoshino *et al* (10) exposed A549 cells to 1 μM BaP for a long period of time (24 weeks) and used a gene chip to conduct a microarray analysis. They reported that the mRNA expression of Twist was upregulated and the mRNA expression of E-cadherin was downregulated. However, they did not observe morphological changes under these experimental

conditions (10). In the present study, by treating A549 cells with 1 μM BaP for different lengths of time (24, 48 and 72 h; 1, 2 and 4 weeks), we found that the expression of Twist1, N-cadherin and vimentin were increased at both the mRNA and protein levels, while the expression of E-cadherin was decreased. Furthermore, we discovered through observation under a microscope that most of the A549 cells were transformed from cells with epithelial characteristics to cells with mesenchymal characteristics when treated with BaP for 4 weeks. Through a comparative analysis, we hypothesized that a relatively short period of BaP intervention can promote EMT in A549 cells, whereas a long duration of intervention may reverse this effect. However, the related mechanisms of this phenomenon remain to be clarified.

Wang *et al* (27) incubated A549 cells with 8 μM BaP for 24 h and reported that BaP promoted A549 cell migration. They found that the mRNA and protein expression of Twist were upregulated in A549 cells with treatment with 8 μM BaP for 24, 48 and 72 h (27). In addition, when the expression of Twist was knocked down in A549 cells, the migration capacity was blocked by intervention with 8 μM BaP for 24 h (27). In the present study, after treating A549 cells of 1 μM BaP for at least 48 h, we obtained results similar to those of Wang *et al*, specifically, migration promotion and Twist1 upregulation. Similarly, we also revealed that downregulation of Twist1 can inhibit the migration capacity of $A549^{BaP-4w}$ cells, indicating that Twist1 indeed plays an important role in the progression of smoke-induced lung cancer.

Generally, N-cadherin and vimentin are considered to be mesenchymal markers (28-30) while E-cadherin is regarded as an epithelial marker (28,29,31). In the present study, notably, we found that the expression trend of Twist1 was consistent with that of N-cadherin and vimentin but contrary to that of E-cadherin. Furthermore, we also noted a similar situation in many previous cancer studies (32-35). Therefore, we speculate that Twist1 is a new potential mesenchymal biomarker in the process of cancer progression. However, future studies are warranted to confirm this speculation.

Previous studies have shown that Twist1 is an oncogene (36-38). It promotes proliferation (17,39-41) and inhibits apoptosis (42) of cancer cells. Furthermore, emerging evidence suggests that Twist1 significantly enhances EMT-associated cell migration and invasion to promote cancer metastasis (16). It also plays a role in chemotherapeutic resistance (16,43). Therefore, Twist1 is considered to be a potential therapeutic target for cancer (16,44,45). Since lung cancer is reported to be the leading cause of cancer-related deaths (46), and cigarette smoking is the main factor leading to lung cancer (47), the development of therapeutic strategies targeting Twist1 is particularly important in the treatment of lung cancer.

In conclusion, the results from the present study indicate that BaP enhances EMT-associated migration of lung adenocarcinoma A549 cells by upregulating Twist1.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81572284) and the Important Research and Development Plan of Hunan Provincial Science and Technology Department (grant no. 2015SK20662).

References

1. Freedman ND, Leitzmann MF, Hollenbeck AR, Schatzkin A and Abnet CC: Cigarette smoking and subsequent risk of lung cancer in men and women: Analysis of a prospective cohort study. Lancet Oncol 9: 649-656, 2008.
2. Islami F, Torre LA and Jemal A: Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res 4: 327-338, 2015.
3. Xu D, Penning TM, Blair IA and Harvey RG: Synthesis of phenol and quinone metabolites of benzo[*a*]pyrene, a carcinogenic component of tobacco smoke implicated in lung cancer. J Org Chem 74: 597-604, 2009.
4. Kalabus JL, Cheng Q, Jamil RG, Schuetz EG and Blanco JG: Induction of carbonyl reductase 1 (*CBR1*) expression in human lung tissues and lung cancer cells by the cigarette smoke constituent benzo[a]pyrene. Toxicol Lett 211: 266-273, 2012.
5. Kasala ER, Bodduluru LN, Barua CC, Sriram CS and Gogoi R: Benzo(a)pyrene induced lung cancer: Role of dietary phytochemicals in chemoprevention. Pharmacol Rep 67: 996-1009, 2015.
6. Kometani T, Yoshino I, Miura N, Okazaki H, Ohba T, Takenaka T, Shoji F, Yano T and Maehara Y: Benzo[a]pyrene promotes proliferation of human lung cancer cells by accelerating the epidermal growth factor receptor signaling pathway. Cancer Lett 278: 27-33, 2009.
7. Shinmura K, Iwaizumi M, Igarashi H, Nagura K, Yamada H, Suzuki M, Fukasawa K and Sugimura H: Induction of centrosome amplification and chromosome instability in *p53*-deficient lung cancer cells exposed to benzo[a]pyrene diol epoxide (B[a]PDE). J Pathol 216: 365-374, 2008.
8. Tang SC, Sheu GT, Wong RH, Huang CY, Weng MW, Lee LW, Hsu CP and Ko JL: Expression of glutathione S-transferase M2 in stage I/II non-small cell lung cancer and alleviation of DNA damage exposure to benzo[a]pyrene. Toxicol Lett 192: 316-323, 2010.
9. Wang BY, Wu SY, Tang SC, Lai CH, Ou CC, Wu MF, Hsiao YM and Ko JL: Benzo[a]pyrene-induced cell cycle progression occurs via ERK-induced Chk1 pathway activation in human lung cancer cells. Mutat Res 773: 1-8, 2015.
10. Yoshino I, Kometani T, Shoji F, Osoegawa A, Ohba T, Kouso H, Takenaka T, Yohena T and Maehara Y: Induction of epithelial-mesenchymal transition-related genes by benzo[a]pyrene in lung cancer cells. Cancer 110: 369-374, 2007.
11. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428, 2009.
12. Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15: 178-196, 2014.
13. Dasari V, Gallup M, Lemjabbar H, Maltseva I and McNamara N: Epithelial-mesenchymal transition in lung cancer: Is tobacco the 'smoking gun'? Am J Respir Cell Mol Biol 35: 3-9, 2006.
14. Nurwidya F, Takahashi F, Murakami A and Takahashi K: Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. Cancer Res Treat 44: 151-156, 2012.
15. Reka AK, Chen G, Jones RC, Amunugama R, Kim S, Karnovsky A, Standiford TJ, Beer DG, Omenn GS and Keshamouni VG: Epithelial-mesenchymal transition-associated secretory phenotype predicts survival in lung cancer patients. Carcinogenesis 35: 1292-1300, 2014.
16. Qin Q, Xu Y, He T, Qin C and Xu J: Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. Cell Res 22: 90-106, 2012.
17. Han Z, Liu L, Liu Y and Li S: Sirtuin SIRT6 suppresses cell proliferation through inhibition of Twist1 expression in non-small cell lung cancer. Int J Clin Exp Pathol 7: 4774-4781, 2014.
18. Sakamoto A, Akiyama Y, Shimada S, Zhu WG, Yuasa Y and Tanaka S: DNA methylation in the exon 1 region and complex regulation of Twist1 expression in gastric cancer cells. PLoS One 10: e0145630, 2015.
19. Xu Y, Qin L, Sun T, Wu H, He T, Yang Z, Mo Q, Liao L and Xu J: Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. Oncogene 36: 1157-1166, 2017.
20. Galván JA, Helbling M, Koelzer VH, Tschan MP, Berger MD, Hädrich M, Schnüriger B, Karamitopoulou E, Dawson H, Inderbitzin D, *et al*: TWIST1 and TWIST2 promoter methylation and protein expression in tumor stroma influence the epithelial-mesenchymal transition-like tumor budding phenotype in colorectal cancer. Oncotarget 6: 874-885, 2015.

21. Bing L, Hong C, Li-Xin S and Wei G: MicroRNA-543 suppresses endometrial cancer oncogenicity via targeting FAK and TWIST1 expression. Arch Gynecol Obstet 290: 533-541, 2014.
22. Cho KH, Jeong KJ, Shin SC, Kang J, Park CG and Lee HY: STAT3 mediates TGF-β1-induced TWIST1 expression and prostate cancer invasion. Cancer Lett 336: 167-173, 2013.
23. D'Angelo RC, Liu XW, Najy AJ, Jung YS, Won J, Chai KX, Fridman R and Kim HR: TIMP-1 via TWIST1 induces EMT phenotypes in human breast epithelial cells. Mol Cancer Res 12: 1324-1333, 2014.
24. Yoon NA, Jo HG, Lee UH, Park JH, Yoon JE, Ryu J, Kang SS, Min YJ, Ju SA, Seo EH, *et al*: Tristetraprolin suppresses the EMT through the down-regulation of Twist1 and Snail1 in cancer cells. Oncotarget 7: 8931-8943, 2016.
25. Li L and Wu D: miR-32 inhibits proliferation, epithelial-mesenchymal transition, and metastasis by targeting TWIST1 in non-small-cell lung cancer cells. Onco Targets Ther 9: 1489-1498, 2016.
26. Burns TF, Dobromilskaya I, Murphy SC, Gajula RP, Thiyagarajan S, Chatley SN, Aziz K, Cho YJ, Tran PT and Rudin CM: Inhibition of *TWIST1* leads to activation of oncogene-induced senescence in oncogene-driven non-small cell lung cancer. Mol Cancer Res 11: 329-338, 2013.
27. Wang Y, Zhai W, Wang H, Xia X and Zhang C: Benzo(a)pyrene promotes A549 cell migration and invasion through up-regulating Twist. Arch Toxicol 89: 451-458, 2015.
28. Hänze J, Henrici M, Hegele A, Hofmann R and Olbert PJ: Epithelial mesenchymal transition status is associated with anti-cancer responses towards receptor tyrosine-kinase inhibition by dovitinib in human bladder cancer cells. BMC Cancer 13: 589, 2013.
29. Li B, Zheng YW, Sano Y and Taniguchi H: Evidence for mesenchymal-epithelial transition associated with mouse hepatic stem cell differentiation. PLoS One 6: e17092, 2011.
30. Shirakihara T, Saitoh M and Miyazono K: Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. Mol Biol Cell 18: 3533-3544, 2007.
31. Zeisberg M and Neilson EG: Biomarkers for epithelial-mesenchymal transitions. J Clin Invest 119: 1429-1437, 2009.
32. Chen SF, Nieh S, Jao SW, Wu MZ, Liu CL, Chang YC and Lin YS: The paracrine effect of cancer-associated fibroblast-induced interleukin-33 regulates the invasiveness of head and neck squamous cell carcinoma. J Pathol 231: 180-189, 2013.
33. Feng J, Fu Z, Guo J, Lu W, Wen K, Chen W, Wang H, Wei J and Zhang S: Overexpression of peroxiredoxin 2 inhibits TGF-β1-induced epithelial-mesenchymal transition and cell migration in colorectal cancer. Mol Med Rep 10: 867-873, 2014.
34. Guo G, Yao W, Zhang Q and Bo Y: Oleanolic acid suppresses migration and invasion of malignant glioma cells by inactivating MAPK/ERK signaling pathway. PLoS One 8: e72079, 2013.
35. Lee YJ, Han ME, Baek SJ, Kim SY and Oh SO: MED30 regulates the proliferation and motility of gastric cancer Cells. PLoS One 10: e0130826, 2015.
36. Gort EH, van Haaften G, Verlaan I, Groot AJ, Plasterk RH, Shvarts A, Suijkerbuijk KP, van Laar T, van der Wall E, Raman V, *et al*: The *TWIST1* oncogene is a direct target of hypoxia-inducible factor-2alpha. Oncogene 27: 1501-1510, 2008.
37. Laursen KB, Mielke E, Iannaccone P and Füchtbauer EM: Mechanism of transcriptional activation by the proto-oncogene Twist1. J Biol Chem 282: 34623-34633, 2007.
38. Nairismägi ML, Füchtbauer A, Labouriau R, Bramsen JB and Füchtbauer EM: The proto-oncogene TWIST1 is regulated by microRNAs. PLoS One 8: e66070, 2013.
39. Chen H, Hu L, Luo Z, Zhang J, Zhang C, Qiu B, Dong L, Tan Y, Ding J, Tang S, *et al*: A20 suppresses hepatocellular carcinoma proliferation and metastasis through inhibition of Twist1 expression. Mol Cancer 14: 186, 2015.
40. Qian J, Luo Y, Gu X, Zhan W and Wang X: Twist1 promotes gastric cancer cell proliferation through up-regulation of FoxM1. PLoS One 8: e77625, 2013.
41. Qiang L, Zhao B, Ming M, Wang N, He TC, Hwang S, Thorburn A and He YY: Regulation of cell proliferation and migration by p62 through stabilization of Twist1. Proc Natl Acad Sci USA 111: 9241-9246, 2014.
42. Orlandella FM, Di Maro G, Ugolini C, Basolo F and Salvatore G: *TWIST1*/miR-584/*TUSC2* pathway induces resistance to apoptosis in thyroid cancer cells. Oncotarget 7: 70575-70588, 2016.
43. Ren H, Du P, Ge Z, Jin Y, Ding D, Liu X and Zou Q: TWIST1 and BMI1 in cancer metastasis and chemoresistance. J Cancer 7: 1074-1080, 2016.
44. da Silva SD, Alaoui-Jamali MA, Soares FA, Carraro DM, Brentani HP, Hier M, Rogatto SR and Kowalski LP: TWIST1 is a molecular marker for a poor prognosis in oral cancer and represents a potential therapeutic target. Cancer 120: 352-362, 2014.
45. Tran PT, Shroff EH, Burns TF, Thiyagarajan S, Das ST, Zabuawala T, Chen J, Cho YJ, Luong R, Tamayo P, *et al*: *Twist1* suppresses senescence programs and thereby accelerates and maintains mutant *Kras*-induced lung tumorigenesis. PLoS Genet 8: e1002650, 2012.
46. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
47. Dela Cruz CS, Tanoue LT and Matthay RA: Lung cancer: Epidemiology, etiology, and prevention. Clin Chest Med 32: 605-644, 2011.