Hepatocyte Nuclear Factor 6 Suppresses the Migration and Invasive Growth of Lung Cancer Cells through p53 and the Inhibition of Epithelial-Mesenchymal Transition*

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**Background:** The role of HNF6 in lung cancer growth and progression remains to be characterized.

**Results:** HNF6 up-regulates p53 and inhibits EMT, cell migration, and invasive growth in lung cancer cells.

**Conclusion:** HNF6 suppresses EMT and invasive growth of lung adenocarcinoma cells through p53.

**Significance:** HNF6 is potentially a molecular marker and target for diagnostic and therapeutic purpose for lung adenocarcinoma cancers.

Epithelial-mesenchymal transition plays an important role in many patho-physiological processes, including cancer invasion and metastatic progression. Hepatocyte nuclear factor 6 (HNF6) has been known to be an important factor for both physiological and pathological functions in liver and pancreas. However, its role in EMT and lung cancer progression remains unidentified. We observed that HNF6 level can be down-regulated by TGF-β1 in human lung cancer cells. Knockdown of HNF6 induced EMT and increased cell migration. In contrast, ectopically expression of HNF6 inhibited cell migration and attenuated TGF-β1-induced EMT. The data suggest that HNF6 plays a role in maintaining epithelial phenotype, which suppresses EMT. HNF6 also inhibits both colony formation and proliferation of lung cancer cells. It pronouncedly reduced the formation of tumor xenografts in nude mice. In addition, HNF6 can activate the promoter activity of p53 by directly binding to a specific region of its promoter and therefore increase the protein level of tumor suppressor p53. p53 knockdown induced EMT and increased cell migration, whereas the opposite effect was generated by p53 over-expression. p53 knockdown also inhibited the effect of HNF6 on EMT and cell migration, indicating that p53 is required for the functions of HNF6 herein. Moreover, there is a high positive correlation among the expression levels of HNF6, p53, and E-cadherin in human lung cancer cells and tissues. The data suggest that HNF6 inhibits EMT, cell migration, and invasive growth through a mechanism involving the transcriptional activation of p53.

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‡ The abbreviations used are: EMT, epithelial-to-mesenchymal transition; HNF6, hepatocyte nuclear factor 6; ZEB1/2, zinc finger E-box-binding homebox 1/2; HNF4α, hepatocyte nuclear factor 4α; FoxA1/2, forkhead box protein A2; qPCR, quantitative PCR.
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signaling. Both HNF6 and FoxA2 have been implicated in colorectal liver metastases (12). The expression level of HNF6 was found to be much lower in both pancreas cancer tissues and cell lines compared with normal pancreas tissues and cell lines, implying a possible tumor suppressor role of HNF6 in pancreas (17).

p53 is a very important tumor suppressor gene, which is mainly involved in cell cycle arrest, apoptosis, senescence, DNA repair, and the inhibition of angiogenesis (18–20). Recent studies indicate that p53 is involved in the inhibition of EMT through regulation of micro-RNAs, such as miR-34, miR-200, and miR-130b by targeting ZEB1/ZEB2. Mutation or loss of p53 expression may induce EMT and metastasis in cancer cells (21–23). However, the regulation of p53 by HNF6s and its biological significance in the control of EMT and relevant events have not been identified. In exploring the role of HNF6 in tumor growth and progression, we observed that HNF6 is implicated in maintaining the epithelial phenotype of lung cancer cells and is a positive regulator for p53 expression. Further studies showed that HNF6 plays suppressive role in the control of EMT, migration and invasive growth of lung cancer cells. These roles of HNF6 have been closely related with its regulatory effect on p53 expression.

EXPERIMENTAL PROCEDURES

Materials

293T, A549, NCI-H446, NCI-H358, and NCI-H1650 cells were originally purchased from the American Type Culture Collection (Manassas, VA). DMEM and RPMI 1640 were bought from HyClone (Thermo Fisher Scientific). FBS and transfection reagents were purchased from Invitrogen. TGF-β1 was from Chemicon (Millipore, Billerica, MA). Rabbit anti-HNF6 antibody (ab83564) were purchased from Abcam (Cambridge, MA), Rabbit anti-p53 antibody was from Chemicon (Millipore, Billerica, MA). Rabbit anti-E-cadherin, N-cadherin, γ-catenin, vimentin, β-actin, and FLAG were all from BD Biosciences. Lentivirus plasmids pCDH-CMV-MCS-EF1-Puro, Δ8.9, VSV-G, and pLKO.1-TRC were purchased from Addgene (Cambridge, MA).

Methods

Cell Culture and Transfection

A549 and 293T cells were cultured in DMEM containing 10% FBS supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml). H446, H358, and H1650 cells were grown in RPMI 1640 medium containing 10% FBS supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Transfection was carried out using Lipofectamine 2000 as transfection reagent according to the manufacturer's instructions.

Western Blotting and Immunofluorescence Staining

Western blotting and immunofluorescence staining was performed as described previously (24).

Plasmids and shRNAs

For knockdown of HNF6 and p53, two independent shRNA sequences targeting human HNF6 and p53 were used respectively, with one scramble shRNA sequence as a control. shRNAs were inserted into pLKO.1-TRC plasmid following the Addgene instructions. All shRNAs were from Generay Biotech Co. (Shanghai, China). shRNA sequences targeting human HNF6 are as follows: 5′-GGCTCCCATGAATAACCTCTAT-3′ and 5′-TGGAGAATATTCAGGGCAGAT-3′, and the latter sequence was used for most experiments. shRNAs for human p53 were as follows: 5′-GTCCAGATGAACTCCTCCAGAA-3′, and 5′-CACCATCCACTACACTAT-3′, and the latter sequence was used for most experiments. The scramble shRNA sequence is 5′-CCTAAGGTATTGCTGCCCTCG-3′. For overexpression of HNF6 and p53, N-terminal FLAG-tagged full-length human wildtype HNF6 and p53 gene sequences were inserted into the pCDH-CMV-MCS-EF1-Puro plasmid, respectively.

Lentivirus Transduction

Lentiviruses for knockdown and overexpression were produced as described (25).

Real Time qPCR

mRNA were extracted from cells with TRIzol reagent (Invitrogen), and 1 µg for each were used for reverse transcription by the ReverTra Ace (Toyobo, Japan) following the product instructions. Real time qPCR was carried out in the ABI-7500 systems by following the instructions from ABI. In brief, cycles were as follows: 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 1 min, 40 cycles, and adding the annealing curve. For real time qPCR, primer sequences were as follows: p53, 5′-GGCTGAGCACTTGCCCAACAAC-3′ (forward) and 5′-GGATCTGAAAGGTGAATATTCTC-3′ (reverse); GAPDH-F, 5′-GCGCGTGCTTTACATCAC-3′ (forward) and 5′-TCTGGCCACAAAGTGCGTAG-3′ (reverse).

Cell Migration Assays

Scratch Assay—Cells were scratched by a pipette tip when cell confluence reached ~90% and further incubated with fresh medium; media were changed every day. Photographs were taken just after scratching (0 h) and at time points of 24 h and 48 h, at ×40 magnifications.

Transwell Assay—Cell migration assays were performed using Transwell migration chambers (8-µm pore size, Costar) according to the manufacturer's instructions. For all cells, 2 × 10⁴ cells were plated into the insert of the well, and representative photos were taken at ×100 magnifications.

Colony Formation in Soft Agar

Base agar was made by mixing of volume 1:1 of melted 1.2% agar (DNA grade) and 2 × DMEM, 1 ml for each 35-mm dish. Top Agar final concentration in the DMEM-agar mixture was 0.6%, and 5 × 10³ cells were seeded in top agar in each dish. After the top agar melted, 1 ml of DMEM with 10% of FBS was added to each dish. Cells were scored after 2 weeks.

Animal Experiments

All animal experiments were performed under the ethical guidelines of the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy
of Sciences). For each mouse, 1 × 10^7 cells in 200 μl of DMEM were injected subcutaneously. And the mice were narcotized and photographed after 6 weeks. To obtain the blood of the mice injected subcutaneously with lung cancer cells, mice were narcotized, and blood was gathered by cutting the neck. Then tumors were finally weighed and statistically analyzed. Then, RNA for whole blood was extracted and reverse-transcribed into cDNA. Human cells in circulating blood were examined by real time qPCR by detecting the ratio of human GAPDH expression. The specific primers for human tubulin expression to mouse GAPDH expression. The specific primers for human tubulin expression were as follows: 5'-CAGATGCCAGTGACAGACAAGC-3' (forward) and 5'-CAATGCCCATCAGGCTGAT-3' (reverse); and for mouse GAPDH, the specific primers were as follows: 5'-AGGTGCGTTGGAACGATT GGTTG-3' (forward) and 5'-GGCCTCACCCCATTTGGAGT GT-3' (reverse).

Luciferase Assay

The 2.8 kb (−2810 bp to 0 bp), 300 bp (−2810 bp to −2446 bp), and 2.5 kb (−2446 bp to 0 bp) of p53 promoters was cloned into pGL3-basic plasmid. The primers used for PCR were as follows: −2810 bp to 0 bp, 5'-GACTGTTACCCCTGTCGTCCTC-3' (reverse); primer-1 (forward) and 5'-GCTACCTGCAAGACTGCC-3' (reverse); and for mouse GAPDH, the primers used were as follows: 5'-GTACCTGAGTTCAACCCGGTAC-3' (forward) and 5'-GTCATCTGGTCACAAGCCGCTT-3' (reverse).

ChIP

ChIP assays were performed according to the manufacturer’s instructions (Millipore). HNF6 antibody was used to precipitate DNAs cross-linked with HNF6 in the nuclear extracts of A549 cells, and normal rabbit IgG (Santa Cruz Biotechnology) performed in samples in parallel as controls. Enriched DNAs were then used as templates to assess the binding intensity of HNF6 to putative binding sites in p53 promoters. Binding intensity was detected by real time qPCR, and relative binding intensity was calculated by the ratio of ChIP-enriched DNA over control IgG input DNA and normalized. Primers used in this assay are as follows: primer-1, 5'-GTCTGATCTTTGCTGTTT-3' (forward) and 5'-TGGCCAGGGCTATCATCAAT-3' (reverse); primer-2, 5'-CTTGGCAAAACATGCTCCGTTT-3' (forward) and 5'-GGAGCAAGTGGAATATGCAACCTAAGGCAAGAATCTCT-3' (forward) and 5'-GAGGAGCAGGCAAGACACCATGACGTAAGGCGCAG-3' (reverse). Luciferase assays were performed according to the manufacturer’s instructions by Promega (Madison, WI).

Tissue Microarray Immunohistochemistry

Human lung adenocarcinoma tissue microarrays were from National Engineering Center For Biochip at Shanghai, China, and the product ID of the tissue microarray is Hlug-Ade060PG-01. The concentrations of antibodies were as follows: HNF6, 1/150; E-cadherin, 1/500; and p53, 1/75. The immunohistochemistry was done as described (26).

Statistical Analysis

Quantitative data are reported as means ± S.D. Statistical significance was determined by the Student’s t test. A p value of < 0.05 was considered statistically significant. *, p < 0.05; **, p < 0.01.
Overexpression of HNF6 Represses Anchorage-independent Growth, Cell Proliferation, and Tumor Xenograft Growth and Invasion—Anchorage-independent growth is an important indicator of cancer malignancy, and EMT increases the anoikis resistance ability of cells thus increases the anchorage-independent growth of cancer cells. Our investigations showed that HNF6 can significantly decrease the colony formation of cells in soft agar (Fig. 3A). In contrast, HNF6 knockdown increased an anchorage-independent growth of cells (Fig. 3B). Tumor formation ability is an important indicator of the malignancy of cancer cells, so we next detected the effect of HNF6 on tumor formation by subcutaneously A549 cell injection of mice. Surprisingly, overexpression of HNF6 strongly inhibited tumors growth (Fig. 3C, left). The invasion ability of lung cancer cells from primary tumor to circulating blood was also examined by detecting the human tubulin mRNA levels compared with mouse GAPDH mRNA levels in the whole blood of mice by real time qPCR. The results showed that HNF6 can significantly decrease the cancer cells invasion to blood vessels (Fig. 3C, right). We also detected the effect of HNF6 overexpression on cell proliferation rate of A549 cells. As shown in Fig. 3D, overexpression of HNF6 significantly inhibited cell proliferation in A549 cells,
suggesting that tumor suppressive effect of HNF6 may also be linked with its effect on cell proliferation besides on EMT.

Correlation between the Level and Effect of HNF6 and p53—p53 has been in recent years reportedly implicated in the inhibition of EMT. Interestingly, p53 levels are not only positively correlated with that of HNF6 in A549, H1650, and H446 lung cancer cells but also positively correlated with the levels of E-cadherin and reversely correlated with N-cadherin (Fig. 4A). The data suggest that p53 functions in maintaining epithelial phenotype. This observation is consistent with the reported work that shows a loss of wild type p53 in A549 cells activated EMT (28). Knockdown of p53 in A549 cells induced a morphological change with the characteristic of EMT (Fig. 4B, left). Decrease in E-cadherin and increase in N-cadherin level was also detected (Fig. 4B, right). Correspondingly, a marked increase in cell migration was observed as detected by Tran-
swell and scratch assays (Fig. 4C). In contrast, overexpression of wide type p53 in A549 cells increased E-cadherin and γ-catenin levels and decreased N-cadherin level (Fig. 4D, left). In line with the above effect, p53 overexpression suppressed cell migration (Fig. 4D, right). Similar effects of p53 overexpression in H446 cells were also observed (Fig. 4E).

**Regulation of p53 by HNF6 and Its Involvement in EMT and Cell Migration**—The fact that p53 is abundant in A549 cells, and its levels are positively correlated with that of HNF6 in A549, H446, and H1650 lung cancer cells suggests that HNF6 may act as an upstream regulator of p53 expression in the control of EMT and cell migration and growth. To test this possibility, we examined the effect of HNF6 on p53 level in A549 cells and found that HNF6 knockdown apparently reduced p53 mRNA and protein levels (Fig. 5A). While overexpression of HNF6 increased p53 mRNA and protein levels (Fig. 5B). The increase of p53 protein levels by overexpression of HNF6 can also be observed in H446 cells and H358 cells (Fig. 5C). We also tested the effect of HNF6 on EMT and p53 expression in xenograft tumors. The results showed that HNF6 overexpression increased the protein levels of E-cadherin and γ-catenin. In contrast, a weak decrease in the level of mesenchymal marker protein fibronectin in HNF6 overexpressed tumors was observed. In addition, increased p53 level can be detected in HNF6 overexpressed tumors (Fig. 5D). To examine whether p53 is implicated in the function of HNF6, we knocked down p53 in HNF6-overexpressing cells. As shown, p53 knockdown fundamentally abolished MET-like change and the inhibition of cell migration mediated by HNF6 (Fig. 5, E and F).

**HNF6 Regulates p53 through Directly Transcriptional Activation of Its Promoter**—Because HNF6 is a transcription factor, it is likely that HNF6 regulates the p53 level through transcriptional...
tional activation p53 gene. By an assay using a Dual-Reporter pGL3 luciferase assay system, we found that HNF6 significantly increased p53 promoter activity (Fig. 6A). We also determined whether HNF6 can directly bind to the p53 gene promoter by ChIP assay. There are two putative HNF6 binding sites in the upstream of the p53 cDNA sequence region, which are AAGTCAATCA and ATTGAT (13, 30). HNF6 can directly bind to p53 promoter through the AAGTCAATCA sequence (Fig. 6B).

In addition, the region of /H11002/2.8 kb /H11011/2.5 kb of the p53 promoter, which contains the AAGTCAATCA sequence, was identified to be essential for the activation of p53 promoter activity by HNF6 (Fig. 6C).

Correlation of the E-cadherin and p53 Levels with HNF6 Levels in Human Lung Cancer Tissues—To confirm the relationship between the expression levels of HNF6 and E-cadherin or p53 protein levels, we also examined and analyzed their expression levels in human lung adenocarcinoma tissue microarrays by immunohistochemistry. As shown in Fig. 7A, the HNF6 and E-cadherin levels are highly correlated in human lung adenocarcinoma tissues. Surprisingly, the high expression level of HNF6 always correlated with high levels of E-cadherin in 21 cases examined. Similarly, Fig. 7B showed a high correlation between the HNF6 and p53 levels. These data further suggest that HNF6 is a regulator for p53 expression and a suppressor of EMT. Analysis of one microarray data set from NCBI GEO profiles revealed that during colorectal cancer metastasis, HNF6 expression was decreased in lymph node metastasis, as compared with primary tumor (Fig. 7C). In addition, during the multistep pancreatic carcinogenesis, HNF6 expression was also obviously decreased in intraductal papillary mucinous adenoma, intraductal papillary mucinous carcinoma, and invasive cancer of intraductal papillary mucinous neoplasm, as compared with normal pancreatic tissues (Fig. 7D). These data suggest that HNF6 can function as an important suppressor for tumor progression.

DISCUSSION

HNF6 was initially been identified as an important factor in liver and pancreas development and organogenesis, and it is also essential in supporting the normal functions of these organs. In recent years, a few reports showed that decreased expression levels of HNF6 are associated with the tumor metastasis and increased cell proliferation, which suggest a possibility that HNF6 may be implicated in the control of tumor growth.
and metastasis. However, no experimental evidence and further investigation have been reported. The functions of HNF6 in EMT, migration, cancer cell growth, tumor formation, and invasion remain unclear. In this study, we determined the role and potential mechanism of HNF6 in EMT, migratory ability, and invasive growth of A549 lung adenocarcinoma cells. The disappearance of epithelial phenotype and acquisition of mesenchymal phenotype constitute a basic molecular and morphological manifestation of EMT. This process increases cell mobility and constitutes a critical step in cell migration, which is associated with various biological processes, including cancer invasion and metastasis. Thus, the maintenance of epithelial phenotype and suppression of EMT have been increasingly recognized to be important for preventing cancer progression.

Loss of HNF6 in A549 cells causes the loss of epithelial phenotype and the gain of mesenchymal phenotype and also the increase of cell migration. Overexpression of HNF6 can shift the epithelial-mesenchymal balance toward the side of epithelial phenotype and decrease the intensity of EMT induced by TGF-β. These data indicate that HNF6 is essential for the maintenance of epithelial phenotype and changes in its expression level will disrupt the balance between the epithelial-mesenchymal phenotypes. Similar to HNF6, some other molecules have been shown to be critical for maintaining the epithelial phenotype of cells, such as IRS1, FoxA2, and HNF4α, suggesting that a signaling network is orchestrated to regulate the balance between the epithelial-mesenchymal properties. Down- and up-regulation of molecule(s) required for the maintenance of the epithelial phenotype causes a loss or gain of the epithelial phenotype. Likewise, down- and up-regulation of molecule(s) important for the mesenchymal phenotype such as Snail, Slug, Twist, ZEB1, and ZEB2 (31) will cause a converse shift between the balance of epithelial-mesenchymal phenotype. In most epithelial cells, the expression levels or activity of these two groups

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**FIGURE 5. Regulation of p53 by HNF6 and its involvement in EMT and cell migration.**

A, the effect of HNF6 knockdown on p53 mRNA level and protein level in A549 cells were detected by real time qPCR and Western blotting. B, the effect of HNF6 overexpression on p53 mRNA and protein levels in A549 cells were detected by real time qPCR and Western blotting. C, overexpression of HNF6 in H446 cells and H358 cells increased p53 protein levels. D, the effect of HNF6 overexpression on E-cadherin, γ-catenin, fibronectin, and p53 in A549 xenograft tumors were detected by Western blotting. E and F, depletion of the effect of p53 on epithelial and mesenchymal marker proteins level and cell migration in HNF6 overexpressed A549 cells were detected by Western blotting and Transwell assays.

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of molecules are stably balanced, with the epithelial gatekeeper molecules activated and the mesenchymal inducers repressed at a relative low level, keeping the tissue and organ in a state of homeostasis. In this report, we show for the first time that, HNF6 is a gatekeeper molecule for epithelial phenotype, adding a new member to this group.

Overexpression of HNF6 increased the epithelial phenotype and inhibited the EMT and the invasive growth of lung tumor cells. Because anchorage-independent growth is an important indicator of cancer malignancy, and it could often be increased after the EMT of cells, HNF6-mediated suppression of anchorage independent growth in soft agar and the tumor formation and invasion in vivo is therefore more likely due to its inhibitory effect on EMT and cell proliferation.

p53 is an important tumor suppressor gene. It plays important roles in apoptosis, DNA repair, and cell proliferation inhibition, and it has been emerged in recent years a critical inhibitor of EMT. A large number of molecules have been reported to be regulated by p53 (32), and many molecules are shown to control the stability and activity of p53 (33). While much less molecules have been reported to regulate p53 expression through transcriptional regulation of its mRNA level. In this report, we found that HNF6 can positively regulate p53 expression by directly activate its promoter activity, suggesting the roles of HNF6 on EMT, cell migration, cell proliferation, and tumor growth may at least partially through its up-regulation of p53. Besides the roles of p53 mentioned above, stemness inhibition is also an important function of p53 reported in recent years (34, 35). The inhibitory effect of p53 on cell stemness may also be related to its inhibitory effect on EMT because EMT was considered to increase stemness in some circumstances (22, 36). However, as an upstream molecule of p53, whether HNF6 is involved in the regulation of cell stemness remains to be investigated.

E-cadherin is one of the most important indicators of epithelial phenotype. In clinical diagnosis, E-cadherin could be used as a prognostic factor in some types of cancers (16, 29). High E-cadherin expression level correlated with less metastatic ability of tumors. HNF6 expression level was highly correlated with E-cadherin not only in lung cancer cell lines but also in human
lung cancer tissues, and HNF6 can up-regulate E-cadherin in several lung cancer cell lines. High expression of HNF6 correlated with more epithelial phenotype and less metastatic ability and decreased proliferation. These observations suggest a potential diagnostic value of HNF6 in early clinical cancer diagnosis. In addition, factors that are able to restore or up-regulate the expression of HNF6 may be considered as potential therapeutic candidate molecules in the treatment of some cancers.

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