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Epigenetic downregulation of RUNX3 by DNA methylation induces docetaxel chemoresistance in human lung adenocarcinoma cells by activation of the AKT pathway

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ABSTRACT

The RUNX3 gene has been shown to function as a tumor suppressor gene implicated in various cancers, but its association with tumor chemoresistance has not been fully understood. Here, we investigated the effect of epigenetic downregulation of RUNX3 in docetaxel resistance of human lung adenocarcinoma and its possible molecular mechanisms. RUNX3 was found to be downregulated by hypermethylation in docetaxel-resistant lung adenocarcinoma cells. Its overexpression could resensitize cells to docetaxel both in vitro and in vivo by growth inhibition, enhancement of apoptosis and G1 phase arrest. Conversely, knockdown of RUNX3 could lead to the decreased sensitivity of parental human lung adenocarcinoma cells to docetaxel by enhancing proliferative capacity. Furthermore, we showed that overexpression of RUNX3 could inactivate the AKT/GSK3β/β-catenin signaling pathway in the docetaxel-resistant cells. Importantly, co-transfection of RUNX3 and constitutively active Akt1 could reverse the effects of RUNX3 overexpression, while treatment with the MK-2206 (AKT inhibitor) mimicked the effects of RUNX3 overexpression in docetaxel-resistant human lung adenocarcinoma cells. Immunohistochemical analysis revealed that decreased RUNX3 expression was correlated with high expression of Akt1 and decreased sensitivity of patients to docetaxel-based chemotherapy. Taken together, our results suggest that epigenetic downregulation of RUNX3 can induce docetaxel resistance in human lung adenocarcinoma cells by activating AKT signaling and increasing expression of RUNX3 may represent a promising strategy for reversing docetaxel resistance in the future.

1. Introduction

Lung cancer is the leading cause of all cancer-related deaths worldwide (Siegel et al., 2011). The risk of disease recurrence is high, even for patients treated by surgery in the early stages, and continuous efforts are being made to identify molecular markers that predict prognosis and response to additional therapy (Andrews et al., 2011). Docetaxel is one of several first-line chemotherapy agents used to treat advanced non-small cell lung cancer (NSCLC), with genotoxic effects attributed to induction of apoptosis via microtubule bundling and stabilization and inhibition of Bcl-2 (Jung et al., 2007). Unfortunately, there are several problems associated with docetaxel-based chemotherapy, with many patients non-responsive to docetaxel or acquiring resistance during treatment.

The molecular mechanisms underlying initial or acquired docetaxel resistance result from both genetic and epigenetic dysregulation of key genes, involving drug transporters, changes in drug metabolism and pathway alterations affecting cell cycle and inhibition of apoptosis (Hopper-Borge et al., 2004). DNA methylation has been shown to play a crucial role during the development of acquired chemoresistance, leading to the transcriptional inactivation of such key genes (Wilting and Dannenberg, 2012). Previous studies have demonstrated the relationship between chemotherapy resistance and DNA methylation (Brandes et al., 2009; Strathdee et al., 1999). To date, there have been few studies focused on the association of DNA methylation with docetaxel resistance in tumor cells. Previously, Kastl et al. (2010) demonstrated that DNA methylation is associated with resistance to docetaxel in breast cancer cells. However, to the best of our knowledge, there have been no such reports in human lung adenocarcinoma (LAD).

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To gain further insight into the molecular mechanisms underlying docetaxel resistance and explore novel potential therapeutic targets for reversing this resistance in LAD, we established two LAD cell lines with docetaxel resistance (SPC-A1/DTX and H1299/DTX) by continuous exposure to increasing concentrations of docetaxel. Global array analysis of DNA methylation and gene expression (Wang et al., 2012) identified epigenetic downregulation of human runt-related transcription factor 3 (RUNX3), suggesting an important role for this gene in docetaxel resistance of SPC-A1/DTX cells. The RUNX3 gene, which encodes a protein belonging to the runt domain family of transcription factors, is a candidate tumor suppressor gene located at 1p36 (Subramaniam et al., 2009). Loss of RUNX3 expression by DNA methylation is associated with the genesis and progression of various types of human cancers (Shiraha et al., 2011; Chuang and Ito, 2010). Moreover, RUNX3 has been shown to sensitize cancer cells to chemotherapeutic drugs including etoposide, 5-fluorouracil and cisplatin (Xu et al., 2012; Guo et al., 2005).

In the present study, we use combinations of cell lines, nude mouse xenograft models and human tumor tissues to demonstrate that epigenetic downregulation of RUNX3 contributes to docetaxel resistance in LAD. Also, the potential molecular mechanisms need to be further evaluated.

2. Materials and methods

2.1. Cell culture

Two human lung adenocarcinoma cell lines (SPC-A1 and NCI-H1299) were purchased from the Tumor Cell Bank of Chinese Academy of Medical Science (Shanghai, China) and cultured in RPMI 1640 medium containing 10% fetal bovine serum and ampicillin and streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The docetaxel-resistant SPC-A1 cell line (SPC-A1/DTX) and H1299 cell line (H1299/DTX) were established and preserved in 5.0 μg/L docetaxel.

2.2. Microarray-based DNA methylation analysis

Bisulfite-converted genomic DNA was analyzed using Illumina's Infinium Human Methylation27 Beadchip Kit (WG-311-1202) (Nakabayashi et al., 2011). Data were analyzed using Bioconductor (http://www.bioconductor.org). A prediction analysis of microarrays and a two-sided *t*-test were used to identify differentially methylated CpG loci.

2.3. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA). RT was performed using PrimeScript™ RT reagent Kit (Takara, Otsu, Japan) following the manufacturer's instructions. PCR amplification of RUNX3 and GAPDH was performed for 20 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, annealing/extension at 60 °C for 30 s in ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA), using the SYBR Premix Ex Taq kit (Takara). The specific primer sequences for each gene are as follows: RUNX3: Forward 5′-*TCTGTAAGGCCCAAAGTGGGTA*-3′, Reverse 5′-*ACCTCAGCATGACAATATGTCACAA*-3′; GAPDH: Forward 5′-*TGAAGGTCGGAGTCAACGGATT*-3′, Reverse 5′-*CCTGGAAGATGGTGATGGGATT*-3′. Data analysis was performed using the $2^{\Delta\Delta}$CT method for relative quantification and all samples were normalized to GAPDH.

2.4. Methylation-specific PCR (MSP) and real-time quantitative MSP (qMSP)

The MSP and qMSP assays were performed as previously described (Zheng et al., 2011; Chou et al., 2010). Primers targeting the RUNX3 promoter region were as follows: 5′-*ATAATAGCGGTCGTTAGGGCGTCG*-3′ and 5′-*GCTTCTACTTTCCCGCTTCTCGCG*-3′ for methylation status; 5′-*ATAATAGTGGTTGTTAGGGTGTTG*-3′ and 5′-*ACTTCTACTTTCCCACTTCTCACA*-3′ for unmethylation status.

2.5. Stable transfection of cells

RUNX3-expressing plasmid (GFP-RUNX3), negative control (GFP-NC), RUNX3 gene interfering plasmid (pGPU-shRUNX3), negative control (pGPU-NC) and a constitutively active Akt1-expressing plasmid (Akt1-Myr) were purchased from GenePharma (Shanghai, China). The sequence of the RUNX3 shRNA was 5′-*TCAGTAGTGGGTACCAATCTT*-3′. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. G418 (600 μg/mL, Sigma) was used to select stably transfected cells.

2.6. Cell viability assay

Standard tetrazolium bromide (MTT) assays were performed as previously described (Wang et al., 2012). All assays were repeated at least three times.

2.7. Colony formation assay

Cells were seeded into six-well plates (1000 cells/well) in a final volume of 2 mL RPMI-1640 medium supplemented with 10% FBS and incubated at 37 °C in 5% CO_2 for 14 days. Visible colonies were manually counted. The cloning efficiency was calculated as follows: (the number of clones/the number of seed cells) × 100%.

2.8. Flow cytometric analysis

Cells were harvested directly or 48 h after transfection and washed with ice-cold PBS for cell cycle detection. Apoptosis and cell cycle assays were performed as previously described (Wang et al., 2012).

2.9. Xenograft transplantation

Animal studies were performed as previously described (Liu et al., 2009). Immunostaining analysis for RUNX3 protein and proliferating cell nuclear antigen (PCNA) expression were performed according to the manufacturer's instructions. The animal study protocol was approved by the Animal Experimentation Ethics Committee of the Jinling Hospital.

2.10. Real-time quantitative PCR array

A customized PCR array from CT Bioscience (Changzhou, China) (Zheng et al., 2012) was used to compare the expression profile of a selected group of genes in RUNX3-transfected versus NC-transfected SPC-A1/DTX cells. Target mRNAs included 88 potential molecules involved in cancer signaling pathways (listed in Supplement Table S1). GAPDH, b2-MG, b-actin, RPL27, HPRT1 and OAZ1 were used as housekeeping genes for normalization.