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Original Article

Human nonsense-mediated RNA decay regulates EMT by targeting the TGF-ß signaling pathway in lung adenocarcinoma

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ABSTRACT

Nonsense-mediated mRNA decay (NMD) is a highly conserved pathway that selectively degrades aberrant RNA transcripts. In this study, we proved that NMD regulates the epithelial–mesenchymal transition (EMT) of lung adenocarcinoma (ADC). Moreover, we found that NMD core factor UP-frameshift 1 tends to be expressed at lower levels in human ADC tissues than in normal lung tissues, thereby raising the possibility that NMD may be downregulated to permit ADC oncogenesis. Our experiments in human ADC cell lines showed that downregulating NMD can promote EMT. Moreover, EMT can be inhibited by upregulating NMD. We tested the role of TGF-ß signaling and found that NMD influences EMT by targeting the TGF-ß signaling pathway. Our findings reveal that NMD is a potential tumor regulatory mechanism and may be a potential therapeutic target for ADC.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Traditional treatments are ineffective in lung cancer, and the 5-year survival rate for lung cancer patients is lower than other cancers. Lung adenocarcinoma (ADC) is the most common histological type of primary lung cancer. Moreover, the related mechanisms in ADC have hindered the success of lung cancer therapy [1]. Epithelial–mesenchymal transition (EMT) is a key mechanism in tumorigenesis and neoplasm metastasis [2,3]. Transforming growth factor (TGF)-ß1 regulates various cellular responses and it is the key EMT inducer [4,5].

NMD is an mRNA surveillance process. It selectively eliminates aberrant transcripts that contain premature termination codons (PTCs) [6,7]. The mutated mRNAs that contain PTCs result from various events, such as gene mutations, alternative splicing, and programmed DNA rearrangements in immune cells [6,8]. The cellular transcripts recognized by the NMD apparatus may have at least one of the following features: contain PTCs, upstream open-reading frames, long 3′ UTRs, and alternatively spliced isoforms [9–11]. The mutations the generate mRNA harbor PTCs that are NMD pathway targets [12]. However, during tumorigenesis, NMD regulation often becomes aberrant [13]. The core of the NMD apparatus is composed of some polypeptides called UP-frameshift 1 (UPF1), UPF2, UPF3, Y14, SMG1, SMG5, SMG6, and SMG7 [14,15]. UPF1 is a key player among RNA degradation pathways [16]. Moreover, quantitative expression of UPF1 can determine NMD regulation [15,17]. However, NMD is a constitutive quality control mechanism and degrades multiple non-mutated transcripts [18]. This unexpected finding resulted from a large proportion of altered transcripts when NMD was disabled or downregulated by siRNA knockdown of UPF1 [19].

NMD could be degraded by various cellular stresses, including amino acid deprivation, cellular hypoxia, and cell exposure to reactive oxygen [13,18,20]. Moreover, NMD can be regulated when

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cells are exposed to hostile microenvironments for a long time, and several NMD-targeted transcripts will be generated and increased to promote cellular adaptation in response to these environmental stresses [21,22]. Microenvironment adaptation is crucial in tumorigenesis, and NMD targets numerous mutated tumor suppressor genes. Therefore, NMD regulation may have important implications in cancer [20,23].

In this study, we found that NMD is downregulated in ADC cells and tissues. Furthermore, reducing NMD may be due to tumor microenvironment stresses, such as starvation and hypoxia. Finally, we found that NMD suppresses tumorigenesis and EMT in ADC by targeting the TGF-ß signaling pathway.

Materials and methods

Clinical specimens

A total of 160 lung ADC specimens and their corresponding adjacent tissues were collected from Tianjin Medical University Cancer Institute and Hospital from January 1 to December 31, 2011. All resources were characterized and contained patients' clinical and pathological data, including age, primary tumor size, histological tumor type, TNM stage, and development of lymphatic and distant metastases. None of the patients had received chemotherapy or radiotherapy before their operation. The diagnosis of lung cancer was histopathologically confirmed. The protocols of this study were approved by the hospital's Protection of Human Subjects Committee. Overall survival (OS) was defined as the last follow-up visit or the interval between resection and death. Recurrence-free survival (RFS) was defined as the interval between treatment and the first diagnosis of recurrence or metastasis. In this study, all cases were followed up. Follow-ups ranged from 54 to 66 months and ended in June 1, 2016.

Cell culture conditions and treatments

Lung ADC cell lines A549 and H1299 included in the study were purchased from the Type Culture Collection of the Chinese Academy of Sciences, in Shanghai, China. Both cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA). A549 and H1299 were treated with 100 ng/mL cycloheximide (CHX), 5 ng/mL TGF-β1 for different durations, and activin doses for 24 h. CHX was obtained from Sigma-Aldrich (St. Louis, MO, USA). TGF-β1 and activin were purchased from PeproTech (Rocky Hill, NJ, USA). Throughout the experiments, except those in hypoxic conditions, cells were incubated with the appropriate culture media in a humidified incubator at 37 °C with 5% CO_2. For experiments in hypoxic conditions, cells were maintained inside a modular incubator chamber (Billups-Rothenberg, CA, USA), wherein humidity was maintained by placing a 60 mm petri dish containing 5 mL water. Prior to each use, the chamber was flushed with 5% CO_2, 1% O_2, and N_2 before being sealed and incubated at 37 °C. Cells were removed from the chamber for not more than 1 h to conduct experiments. For exposure to starvation conditions, cells were cultured in standard culture medium without FBS (serum-free) for different durations.

Western blot

Each cellular protein sample was electrophoresed with SDS-polyacrylamide gel electrophoresis (4% stacking and 10% separating gels), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and incubated with primary antibodies overnight at 4 °C. On the second day, the PVDF membranes incubated with secondary antibodies were subsequently subjected to immunoblotting analysis using the ECL immunoblotting kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol. The following primary antibodies were used: E-cadherin (Cell Signaling Technology, CST), N-cadherin (CST), ZEB-1 (CST), snail (CST), vimentin (CST), Smad2/3 (Abcam), P-Smad2/3 (Abcam), UPF1 (Santa Cruz Biotechnology), and Y14 (Santa Cruz Biotechnology).

Immunohistochemistry (IHC)

For IHC, paraffin-embedded tissues were cut into 4 μm sections. The sections were deparaffinized, rehydrated, and stained with primary antibodies overnight at 4 °C. The slides were treated with a broad-spectrum secondary antibody and then treated with DAB. Finally, the slides were counterstained with hematoxylin and visualized under a light microscope. Two pathologists independently quantified the expression of the antibody without knowledge of other clinic pathological parameters. The multiplier of the positive percentage and staining intensity of the stained area as a result of the total immunostaining score ranged from 0 to 6. The percent positivity was scored as "0" (0%–25%), "1" (26%–50%), and "2" (>50%). The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), and "3" (strongly stained). A total score of >0 and $\leq$ 3 indicates low expression of protein, and a total score $\geq$4 for high expression protein and 0 for negative expression.

Immunofluorescence

For immunofluorescence staining, cells were cultured in 24-well plates and treated with drugs. Then, the cells were washed with PBS twice, fixed in 4% paraformaldehyde, permeabilized using 0.2% Triton X-100, and incubated overnight at 4 °C with primary and secondary antibodies used according to the manufacture's protocol. The cells were rinsed and incubated with DAPI. A confocal laser-scanning microscope was used for immunofluorescence analysis.

Transwell and wound healing assay

The upper chambers of Transwell were coated with matrigel (BD, USA) for the invasion assay. Briefly, lung cancer cells (1×10^5) were incubated with the indicated concentrations of CHX or TGF-β1 for 24 h and seeded in the upper well with RPMI 1640 medium containing no FBS and a medium containing 10% FBS in the lower chamber. After incubating for 24 h at 37 °C, cells in the upper chamber were removed with a cotton swab, and the rest of the membrane had invaded the cells. The invaded cells were fixed with 4% paraformaldehyde and stained with Giemsa. Cell migration was assessed using a wound healing assay. The lung cancer cells (1×10^6) were treated with the indicated concentrations of CHX or TGF-β1, and wounds were made using a 100 μl plastic pipette tip. After 48 h of wound formation, the wound size was measured and photographed.

PCR and quantitative real-time PCR (qRT-PCR)

The RNA used in this study was extracted from cells and lung cancer tissues using the RNAiso Plus (Takara Biotechnology) according to the manufacturer's instructions. Reverse transcription was performed using high-capacity cDNA reverse transcription kits (Takara Biotechnology) according to the manufacturer's instructions. RT-PCR was done using 2 × PCR Solution Premix Taq (R004A, Takara Biotechnology). qRT-PCR was performed using a standard protocol from the SYBR Green PCR kit (Toyobo, Osaka, Japan) on an iQ5 quantitative PCR system (Bio-Rad, USA). ß-actin was used as an internal control, and $2^{-\Delta\Delta CT}$ values were normalized to β-actin levels. Each sample was analyzed in triplicate. The primers were the following: UPF1:5′-*CTGCAACGGACGTGGAAATAC*-3′ and 5′-*ACAGCCGCAGTTGTAGCAC*-3′; MIXL1:5′-*GGCGTCAGAGTGGGAAATCC*-3′ and 5′-*GGCAGGCAGTTCACATCTACC*-3′; SOX17: 5′-*GGCGCAGCAGAATCCAGA*-3′ and 5′- *CCACGACTTGCCCAGCAT*-3′; and ß-actin: 5′-*AGCGAGCATCCCCCAAAGTT*-3′ and 5′-*GGGCACGAAGGCTCATCATT*-3′.

Plasmid constructions and transfection assay

The UPF1 plasmid (Genbank: NM_001297549.1) was cloned into a pcDNA3.1 vector (Life Technology). UPF1-siRNAs were designed and synthesized by Ruibo Biotech (Guangzhou, China). The si-RNA sequences are as follows: UPF1-siRNA 1: 5′-*GCGAGAAGGACUUCAUCAUTT*-3′ and 5′-*AUGAUGAAGUCCUUCUCGCTT*-3′; UPF1-siRNA 2: 5′-*GCAGCCACAUUGUAAAUCATT*-3′ and 5′-*UGAUUUACAAUGUGGCUGCTT*-3′; UPF1-siRNA 3: 5′-*CCUACCAGUACCAGAACAUTT*-3′ and 5′-*AUGUUCUGGUACUGGUAGGTT*-3′; and UPF1-scramble: 5′-*UUCUCCGAACGUGUCACGUTT*-3′, and 5′-*ACGUGACACGUUCGGAGAATT*-3′. UPF1 expression was confirmed by qRT-PCR and Western blot. Transfection was done using a Fugene HD transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol.

Xenograft mouse model

Ten nude mice (Wei Tong Li Hua Experimental company, Beijing, China) were randomly divided into two groups and received 3×10^6 A549 cells stably transfected with pcDNA3.1-UPF1 or an empty vector via subcutaneous injection in the groin. All animal care and procedures were approved by the Institutional Animal Use and Care Committee of Tianjin Medical University. Tumor volumes were measured 7 days after tumor cell injection.

Tail vein injections into nude mice

A549 cells stably transfected with pcDNA3.1-UPF1 and the empty vector were suspended at 5×10^6 cells/mL. Suspended cells (100 μl) were injected into the tail veins of mice (5 weeks old), which were sacrificed 5 months after injection. The visceral organs were removed and subjected to hematoxylin-eosin staining (H&E) for further analysis.

H&E staining

Tissues were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin wax, cut 4 μm thick, deparaffinized with xylene, and processed with a graded ethanol series. Sections were stained with H&E and observed using an Olympus BX51 microscope.

Statistical analysis

Data are presented as the means ± standard deviation from at least three independent experiments. Student's *t*-test, $\chi 2$ test, and Fisher's exact tests were used to compare the two groups using SPSS 22.0 (IBM, Chicago, IL, USA). The Kaplan–Meier test was used to estimate the OS and RFS. A value of $p < 0.05$ was considered significant.