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Suppression of cleavage factor Im 25 promotes the proliferation of lung cancer cells through alternative polyadenylation

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) is a life-threatening disease that has a poor prognosis and low survival rate. Cleavage factor Im 25 (CFIm25) is a RNA-binding protein that if down-regulated causes 3′UTR shortening and thus promotes the transcript stability of target genes. It is not clear whether CFIm25 and alternative polyadenylation (APA) play a role during cancer development. The purpose of this study is to explore the role of CFIm25 in lung cancer cell proliferation.
Methods: CFIm25 was knocked down in A549 cells. Western blots were carried out to determine the protein expression of CFIm25, insulin growth factor 1 receptor (IGF1R), CyclinD1 (CCND1) and TP53. Real-time qRT PCR was performed to determine the total transcript levels of CFIm25 targets and the normalized fold changes in their distal PAS (dPAS) usage. Immunofluorescence was carried out to check the expression of CFIm25, IGF1R and CCND1. Cell proliferation over time was determined using the WST-1 reagent.
Results: The transcript levels of CCND1 and GSK3β were significantly increased and the dPAS usage of several oncogenes (IGF1R, CCND1 and GSK3β) were decreased after CFIm25 knockdown. The protein level of IGF1R was increased, and we detected increased percentage of CCND1 positive cells and cell proliferation over time in CFIm25 knockdown cells. In addition, the mRNA and APA analysis of IGF1R using patient RNA-seq data from the Cancer Genome Atlas indicated that IGF1R is shortened in both lung adenocarcinoma and lung squamous cell carcinoma compared to normal controls.
Conclusions: Our findings suggest that CFIm25 plays an important role in lung cancer cell proliferation through regulating the APA of oncogenes, including IGF1R, and promoting their protein expression.

1. Introduction

The occurrence and development of lung cancer is a complicated process related to many signal pathways. Among the many classifications of lung cancers, non-small cell lung cancer (NSCLC) is the most common type that accounts for about 85% of all lung cancers [1]. Lung squamous cell carcinoma (LUSC, 30% of NSCLC) and lung adenocarcinoma (LUAD, 50% of NSCLC) are the two major subtypes of NSCLC. NSCLC has a poor prognosis, low 5-year survival rates (14% for stage IIIA and 5% for stage IIIB) and no effective preventive measures [2]. Lung cancer cells are highly proliferative by escaping cell cycle check points as well as programed cell death, a process that is normally mediated by mutations or changed expression of oncogenes, tumor-suppressor genes or microRNAs [3]. However, the underlying molecular mechanisms that account for altered oncogene expression and promote the abnormal proliferation of lung cancer cells are extremely complex and remain unknown.

Polyadenylation is an important RNA process for messenger RNA (mRNA) maturation. More than half of mammalian mRNA have more than one polyadenylation sites (PAS), thus usage of different PAS can result in transcripts with different sizes of 3′-untranslated regions (3′UTRs) or different transcripts, a process called alternative polyadenylation (APA) [4,5]. Transcripts with shorter 3′UTR are normally more stable due to the loss of binding sites for miRNAs and other regulatory proteins, and normally have higher protein

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output. APA was recently identified as a widespread mechanism controlling gene stability and expression, and the importance of APA has been emphasized in many cancers including glioblastoma tumor, hepatocellular carcinoma, prostate cancer and breast cancer [6–8]. APA can be regulated by multiple proteins involved in the polyadenylation machinery. Among these, cleavage factor Im 25 (CFIm25) was identified as the top regulator that suppresses the usage of proximal PAS, thus its depletion promotes 3′UTR shortening of target genes and normally enhances target gene stability and expression. CFIm25 down-regulation was also identified as a mechanism that promotes glioblastoma tumor growth [6]. However, it is not known whether CFIm25 also plays a role in lung cancer cell proliferation and lung cancer progress.

To understand the role of CFIm25 and APA in lung cancer cell proliferation, we used siRNA to knockdown CFIm25 expression in A549 cells, a well characterized lung adenocarcinoma cell line, and analyzed the expression of several proteins involved in cell proliferation and apoptosis. We observed increased A549 proliferation upon CFIm25 knockdown, possibly due to enhanced expression of IGF1R and CCND1. In addition, we confirmed that both IGF1R and CCND1 have 3′UTR shortening after CFIm25 depletion, and this may be sufficient to increase their protein expression. The 3′UTR shortening of IGF1R was also observed in lung cancer samples compared to normal tissues, suggesting APA is an important novel mechanism for increased IGF1R signaling in lung cancers. In summary, we have determined that CFIm25 down-regulation has a significant impact on lung cancer cell proliferation through APA.

2. Material and methods

2.1. Cell culture

Lung carcinoma from human cells (A549) were purchased from ATCC (Manassas, VA). A549 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplement with 10% fetal bovine serum (FBS) and antibiotics. Cells were cultured at 37 °C in a humidified 5% carbon dioxide atmosphere.

2.2. Transfection

For siRNA transfection, cells were cultured in antibiotic-free media, transfected with 50 ng/ml miRNA mimic using Lipofectamine® RNAiMAX (Life technology, Grand Island, NY) on day 0 and day 2, and collected for Western Blot or real-time PCR analysis on day 4.

2.3. Western blot

A549 cells were collected and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; and 1% Nonidet P-40) containing a protease inhibitor cocktail (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Equal amounts of protein were separated on SDS-PAGE and transferred to nitrocellular membranes. The membranes were then blocked with 5% (w/v) nonfat milk, washed with Tris-buffered saline–Tween-20 (TBST), and incubated with primary rabbit anti-CFIm25 (Proteintech, Chicago, IL), rabbit anti-IGF1R or mouse anti-β-Actin antibodies (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C, and then incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (Cell Signaling, Danvers, MA) for 1 h at room temperature. Membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA).

2.4. RNA purification and real-time quantitative PCR

A549 cells were lyzed using TRIzol (Life Technologies) and total RNA was isolated using RNeasy Mini Kit (Quagen, Valencia, CA). For real-time PCR analysis, RNA was treated with DNase and reverse-transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad). Real-time PCR was performed under Lightcycler 96 (Roche, Indianapolis, IN) using primers listed in Table 1 and data were quantified using the comparative Ct method and presented as mean ratio to β-actin RNA.

The percentage of dPAS usage was examined using a PCR-based method as described. Two pairs of primers were designed with one targeting the open reading frame to represent the total transcript level, and the other targeting sequences just before the dPAS to detect long transcripts that used the dPAS. Percentage of dPAS usage was calculated as $\Delta CT = CT_{distal} - CT_{total}$. Data were presented as fold changes normalized to control by calculating $\Delta\Delta CT = \Delta CT_{average\ target} - \Delta CT_{average\ of\ control}$.

2.5. Immunofluorescence

A549 cells were fixed in 1% formaldehyde in PBS overnight. After washing with PBS, cells were blocked with Avidin/Biotin Blocking System (VectorLabs) and incubated in 5% normal goat serum for 1 h. A549 cells were then incubated with primary antibodies against CFIm25 (1:200, proteintech), CCND1 and IGF1R (1:200, Cell signaling) overnight at 4 °C, and with biotinylated anti-Rabbit antibodies (1:1000, VectorLabs) for 1 h at room temperature (RT). After washing, Vector® Red Substrate (VectorLab) was used for CFIm25/CCND1/IGF1R immunofluorescence dual-staining for 5 s at RT. Cells were finally mounted with DAPI (Life Technologies).

2.6. APA analysis

The RNA-seq BAM file of 503 LUSC and 51 normal samples, 515 LUAD and 59 normal samples were downloaded from the national cancer institute's Genomic Dac Commons (GDC) (http://gdc.cancer.gov) The gene expressions were recalculated as reads per kilobase per million mapped reads (RPKM) across all samples. The expression data were then normalized using quartile normalization. To characterize the dynamic APA events of IGF1R in cancer and normal tissues, we used a well-established algorithm named Dynamic analysis of Alternative PolyAdenylation from RNA-seq (Dapars, https://github.com/ZhengXia/dapars) to identify the alternative proximal polyA sites and calculate the Percentage of Distal poly(A) Site Usage Index (PDUI) for IGF1R [9]. T-tests were then used to compare the PDUI of IGF1R in cancers and normal samples.

3. Results

3.1. CFIm25 knockdown promotes the IGF pathway

To understand the role of CFIm25 and APA in lung cancer development, we first silenced CFIm25 expression in A549 cells using CFIm25-specific siRNA. Western blot and immunofluorescence show that CFIm25 protein expression is successfully depleted in A549 cells transfected with CFIm25 siRNA (Fig. 1A and B). The insulin-like growth factor 1 receptor (IGF1R) has recently been identified as a potential therapeutic target in NSCLC [10]. IGF1R has increased expression in many NSCLC patients and is involved in promoting the tumor transformation, growth and survival of cancer cells. We found that pre-IGF1R expression is increased in A549 cells with CFIm25 KD. Similarly, immunofluorescence also showed an increased IGF1R expression in CFIm25 KD A549 cells (Fig. 1C). Taken together, our results indicate that KD of CFIm25 enhances IGF1R

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