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Autophagy-dependent Bidirectional Regulation of EMT by NFκB in Non-Small Cell Lung Cancer Cells

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Abstract

Background: Non-small cell lung cancer (NSCLC) is the most common type of lung cancer worldwide. Autophagy and epithelial-to-mesenchymal transition (EMT) are key processes in maintaining cellular homeostasis; their dysregulation can accelerate carcinogenesis. The nuclear factor-kappa B (NFκB) transcription factor regulates genes involved in proliferation, survival, and development. Although crosstalk between autophagy and EMT, and NFκB's involvement in each process, have been reported, evidence for its regulation of both mechanisms together in NSCLC is limited.

Methods: We investigated the role of NFκB (p65 subunit) in EMT regulation under autophagy-induced and non-induced conditions in NSCLC cell lines. Previously generated NFκB-overexpressed and -suppressed NSCLC samples from our earlier work were used. Cells were subjected to autophagy-induced or non-induced conditions, and quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess expression levels of EMT-related genes: positive regulators (Snail, Slug, N-cadherin) and the epithelial marker E-cadherin.

Results: Under autophagy-induced conditions, NFκB overexpression significantly increased EMT-promoting genes and suppressed E-cadherin. In non-induced conditions, NFκB overexpression suppressed EMT-promoting genes and increased E-cadherin. NFκB suppression produced opposite effects, confirming its regulatory role. These results suggest a bidirectional influence of NFκB on EMT, dependent on autophagic status.

Conclusions: NFκB, previously shown by us to positively regulate autophagy, acts as an autophagy-associated EMT regulator in NSCLC. It promotes EMT under autophagy-induced conditions, potentially contributing to progression and metastasis in advanced disease, while under non-induced conditions it may help maintain epithelial characteristics. Targeting the NFκB/autophagy/EMT axis could support the development of personalized NSCLC therapies.

Keywords: NSCLC, NFκB, autophagy, EMT

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Küçük Hücre Dışı Akciğer Kanseri Hücrelerinde NFκB Tarafından EMT'nin Otofaji-bağımlı Çift Yönlü Düzenlenmesi

Öz

Giriş: Küçük hücreli dışı akciğer kanseri (KHDAK), dünya genelinde en yaygın akciğer kanseri tipidir. Otofaji ve epitel-mezenkimal geçiş (EMT), hücresel homeostazın korunmasında kritik süreçlerdir; bu süreçlerin bozulması, karsinogenezi hızlandırabilir. Nükleer faktör kappa B (NFκB) transkripsiyon faktörü, proliferasyon, hayatta kalma ve gelişimle ilişkili genleri düzenler. Otofaji ile EMT arasındaki etkileşim ve NFκB'nin her iki süreçteki rolü bildirilmiş olsa da NFκB'nin bu iki mekanizmayı birlikte düzenleyip düzenlemediğine dair KHDAK özelinde sınırlı kanıt vardır.

Yöntemler: NFκB (p65 alt birimi)'nin, otofaji indüklenmiş ve indüklenmemiş koşullar altında EMT düzenlenmesindeki rolü KHDAK hücre serilerinde araştırıldı. Daha önceki çalışmamızda oluşturulan NFκB aşırı eksprese edilmiş ve baskılanmış KHDAK örnekleri kullanıldı. Hücreler otofaji indüklenmiş veya indüklenmemiş koşullara tabi tutuldu ve EMT ile ilişkili genlerin (pozitif düzenleyiciler: Snail, Slug, N-kaderin ve epitel belirteci E-kaderin) ekspresyon seviyeleri kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile analiz edildi.

Bulgular: Otofaji indüklenmiş koşullarda NFκB aşırı ekspresyonu, EMT'yi teşvik eden genlerde belirgin artışa ve E-kaderin düzeyinde azalmaya yol açtı. Otofaji indüklenmemiş koşullarda ise NFκB aşırı ekspresyonu EMT'yi teşvik eden genleri baskıladı ve E-kaderin düzeyini artırdı. NFκB baskılanması ise ters yönde etkiler gösterdi. Bu sonuçlar, NFκB'nin EMT üzerinde otofaji durumuna bağlı çift yönlü bir etkisi olduğunu göstermektedir.

Sonuçlar: Önceki çalışmamızda otofajiyi pozitif düzenlediğini gösterdiğimiz NFκB, KHDAK'ta otofaji ile ilişkili bir EMT düzenleyicisi olarak rol oynamaktadır. Otofaji-indüklü koşullarda EMT'yi teşvik ederek ileri evre hastalıkta tümör progresyonu ve metastaza katkıda bulunabilirken, otofaji indüklenmemiş koşullarda epitel özelliklerin korunmasına yardımcı olabilir. NFκB/otofaji/EMT ekseninin hedeflenmesi, KHDAK'ta kişiselleştirilmiş tedavi yaklaşımlarının geliştirilmesine katkı sağlayabilir.

Anahtar kelimeler: KHDAK, NFκB, otofaji, EMT.

INTRODUCTION

According to the most recent estimates published by the International Agency for Research on Cancer (IARC) in 2022, lung cancer remains the most commonly diagnosed cancer and the leading cause of cancer-related deaths worldwide. With approximately 2.5 million new cases reported in 2022, lung cancer accounts for nearly one in every eight cancer diagnoses[1]. There are two main types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 85% of lung cancer cases and tends to grow and spread more slowly than SCLC[2]. Although targeted therapies, immunotherapies, and biomarkers have been developed, there is still a need to clarify molecular mechanisms underlying disease progression.

Autophagy, initially defined as a degradation and recycling pathway for survival under stress conditions, is now known to regulate processes such as metabolism, differentiation, aging, immune response, and cell death[3,4]. It plays context-dependent roles in cancer, functioning as either a tumor suppressor or promoter depending on tumor stage, cellular context, and microenvironment[5–7]. Another key mechanism in cancer biology is epithelial–mesenchymal transition (EMT), during which epithelial cells lose polarity and adhesion, acquire motility and invasiveness, and adopt mesenchymal characteristics[8]. EMT occurs in embryogenesis (Type I), tissue regeneration (Type II), and cancer progression/metastasis (Type III)[9]. Hallmarks include downregulation of E-cadherin, a central adhesion protein, and upregulation of N-cadherin, which is prevalent

in non-epithelial tissues[10–12]. EMT initiation is driven by transcription factors such as Snail and Slug, which repress E-cadherin expression[11]. In NSCLC tissues, EMT-related proteins show expression patterns consistent with EMT induction[13,14]. Targeting EMT may also offer therapeutic benefit in NSCLC with coexisting COPD[14].

Cellular mechanisms often share pathways that may act synergistically or antagonistically. This study addresses whether NFκB regulates the potential interplay between autophagy and EMT in NSCLC and, if so, how. The NFκB transcription factor family regulates genes involved in development, proliferation, survival, inflammation, immunity, and aging[15,16]. In mammals, the family includes RelA (p65), c-Rel, RelB, p50, and p52, encoded by RELA, REL, RELB, NFKB1, and NFKB2, respectively[17]. While NFκB's role in cancer is often linked to chronic inflammation, it also promotes tumorigenesis by enhancing proliferation, inhibiting apoptosis, regulating angiogenesis, promoting metastasis, and altering tumor metabolism[18].

Studies examining NFκB's simultaneous regulation of autophagy and EMT in cancer are limited. Some report that autophagy inhibition promotes EMT via the NFκB (RELA/p65) pathway, whereas others show autophagy induces EMT through NFκB activation in hepatocellular carcinoma cells[19,20]. NFκB has also been implicated in promoting metastasis in breast cancer by regulating EMT-associated genes[21]. Our previous findings demonstrate that NFκB positively regulates autophagy in NSCLC cell lines[22]. Based on this, we sought to contribute to clarifying the unexplored relationship between NFκB, NSCLC, EMT, and autophagy by measuring EMT-related gene expression in samples from the same study.

METHODS

Design of Experimental Groups, Cell Culture, RNA Isolation and cDNA Synthesis

In this study, cDNA samples of H1299 cell lines obtained from our previous study were used[23]. The cell culture study was designed and conducted as follows: The cells were divided into two main groups: 1) Autophagy-induced group (treated with Torin1), 2) Control group (untreated with Torin1). Each main group was further genetically modified into the following subgroups: 1) p65 overexpressed groups (via pcDNA3.1-p65 plasmid vector gifted from Prof. Dr. Osman Nidai OZES), 2) p65 knockdowned groups (via p65 spesific siRNA transfection (Cat. No: 6261, CST)). A negative control siRNA (Cat. No: 6568, CST) and empty pcDNA3.1 vector (gifted from Prof. Dr. Prof. Dr. Osman Nidai OZES), were used for mock controls. Cells were seeded at a density of 0.5×10^6 cells per well, and when they reached approximately 80% confluency, transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions.

Total RNA from each experimental group was isolated using the QIAGEN miRNeasy Kit following the manufacturer's protocol (Cat. No: 217084, Qiagen). RNA concentration and purity were assessed with a NanoDrop spectrophotometer. For cDNA synthesis, 2 µg of total RNA from each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Cat. No: 00342824, Applied Biosystems). The synthesized cDNA samples were stored at −80 °C until further use.

Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

Gene expression analysis was performed on a Bio-Rad CFX96 system using RT-PCR Syber green master mix (M3003L, New England Biolabs). cDNA samples from the eight

experimental groups were used to determine the expression levels of Snail, Slug, E-cadherin, and N-cadherin genes by qRT-PCR. β-Actin (ACTB) was employed as the housekeeping gene for normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method based on the obtained Ct values. Each sample was analyzed in triplicate. The qRT-PCR cycling protocol began with an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s and 60 °C for 1 min. The base sequences of the primers used are presented in Table 1.

Table I: Primers used for qRT-PCR.

Gene Name	Primer sequence (5'→3')
E-cadherin (Forward) (Human)	GCCTCCTGAAAAGAGAGTGGAAG
E-cadherin (Reverse) (Human)	TGGCAGTGTCTCTCCAAATCCG
N-cadherin (Forward) (Human)	CCTCCAGAGTTTACTGCCATGAC
N-cadherin (Reverse) (Human)	GTAGGATCTCCGCCACTGATTC
Snail (Forward) (Human)	TGCCCTCAAGATGCACATCCGA
Snail (Reverse) (Human)	GGGACAGGAGAAGGGCTTCTC
Slug (Forward) (Human)	ATCTGCGGCAAGGCGTTTTCCA
Slug (Reverse) (Human)	GAGCCCTCAGATTTGACCTGTC
B-actin (Forward) (Human)	AGAGCTACGAGCTGCCTGAC
B-actin (Reverse) (Human)	AGCACTGTGTTGGCGTACAG

Statistical Analysis

All qRT-PCR data were analyzed using SPSS version 25.0. Differences between independent groups were evaluated using Student's t-test and one-way analysis of variance (ANOVA). Experiments were performed in triplicate, and results are presented as the mean ± standard deviation (SD).

RESULTS

NFκB Overexpression Promotes E-cadherin Under Non-autophagy-induced Conditions, While Suppressing E-cadherin Under Autophagy-induced Conditions

qRT-PCR analysis revealed that under non-autophagy-inducing conditions (Torin(−)), silencing of p65 (NFκB) via siRNA led to a significant reduction in E-cadherin mRNA expression ($p<0.03$). In contrast, E-cadherin levels increased by approximately 2.8-fold in the p65-overexpressing group, and this increase was highly significant ($p< 0.01$) (Figure 1). These findings suggest that, under basal conditions, NFκB may exert a positive regulatory effect on E-cadherin expression. However, under autophagy-inducing conditions (Torin (+)), this regulatory pattern was reversed. Specifically, NFκB silencing resulted in a marked increase in E-cadherin expression (approximately 2.3-fold, $p<0.01$), whereas NFκB overexpression caused a pronounced suppression of E-cadherin levels, reducing them to below basal levels ($p<0.03$) (Figure 1). Taken together, the data presented in Figure 1 indicate that the regulatory effect of NFκB on E-cadherin is bidirectional and dependent on autophagy activation. Under autophagy-inducing conditions, p65 overexpression significantly suppresses E-cadherin expression, suggesting a potential mechanism by which NFκB may promote the loss of epithelial characteristics and facilitate the EMT process.

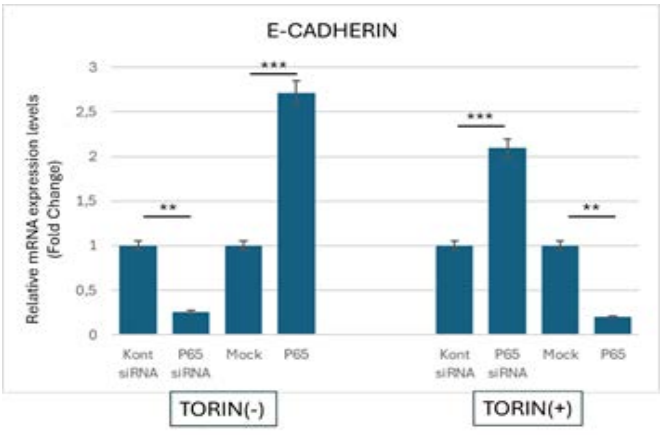

Figure 1. Effect of p65 (NFκB) expression level on E-cadherin mRNA levels in autophagy-induced (Torin(+)) and non-induced (Torin(-)) H1299 cells. Data are presented as mean ± SD, statistical significance levels are indicated as **p < 0.03 and ***p < 0.01.

NFκB Overexpression Suppresses Positive Regulators of EMT in Non-Autophagy-Induced Conditions, While Supporting It in Autophagy-Induced Conditions

When the effect of p65 (NFκB) expression on genes positively associated with EMT was examined, it was observed that under non-autophagy-inducing conditions, silencing p65 resulted in a significant increase in the expression levels of Snail, Slug, and N-cadherin, whereas p65 overexpression significantly reduced the expression of these genes. This finding suggests that, under basal conditions, NFκB may exert a negative influence on EMT. Interestingly, upon induction of autophagy with Torin1, the effect of NFκB on these genes was markedly enhanced. The mRNA levels of Snail and Slug were significantly increased in the p65-overexpressing groups ($p < 0.01$) (Figure 2a, b). Similarly, N-cadherin expression, which reduces cell–cell adhesion and supports the mesenchymal phenotype, was also significantly elevated in the NFκB-overexpressing groups ($p < 0.01$) (Figure 2c).

These findings demonstrate that NFκB activation in the context of autophagy induction upregulates positive regulators of the EMT process. Increased p65 (NFκB) expression under autophagy-inducing conditions led to a significant rise in both transcriptional drivers of EMT (Snail and Slug) and the phenotypic marker of mesenchymal transition (N-cadherin).

Overall, these results identify NFκB as a key regulator capable of activating EMT under autophagic stress, suggesting that NFκB may cooperate with autophagy to promote the transition of tumor cells toward a mesenchymal phenotype.

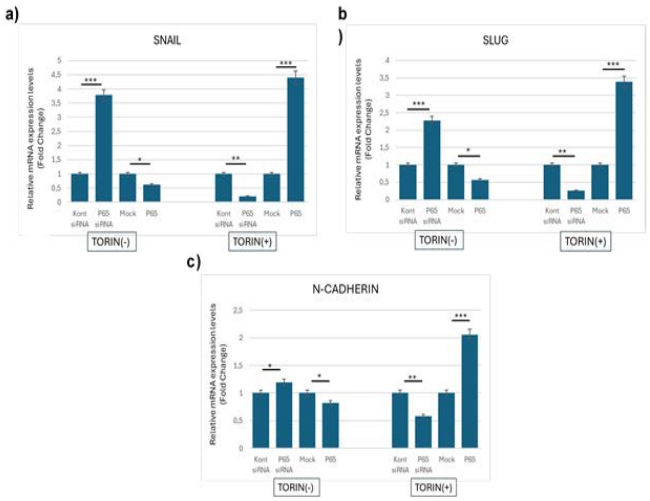

Figure 2. Effect of p65 (NFκB) expression level on EMT-associated Snail (a), Slug (b), and N-cadherin (c) mRNA levels in autophagy-induced (Torin (+)) and non-induced (Torin (-)) H1299 cells. Data are presented as mean ± SD, and statistical significance levels are indicated as *$p < 0.05$, **$p < 0.03$, and ***$p < 0.01$.

DISCUSSION

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer worldwide. Elucidating the cellular mechanisms involved in NSCLC carcinogenesis is crucial for developing effective preventive and therapeutic strategies. Autophagy and EMT are fundamental processes that maintain cellular homeostasis under physiological conditions, and their dysregulation can accelerate carcinogenesis[24]. The transcription factor NFκB regulates the expression of genes involved in proliferation, survival, development, and aging[25]. Although there is evidence that autophagy and EMT can influence each other and that NFκB is modulated by these pathways, studies directly assessing whether NFκB regulates both mechanisms in conjunction within NSCLC are scarce[26]. To provide a limited contribution to this deficiency, we investigated the role of NFκB (p65 subunit) in the regulation of EMT under different autophagy conditions in an NSCLC cell line.

Our findings show that under autophagy-induced conditions, NFκB overexpression increased the expression of EMT-promoting genes such as Snail, Slug, and N-cadherin, while significantly suppressing E-cadherin, a key molecule in cell-cell adhesion. In contrast, when autophagy was not induced, NFκB overexpression suppressed EMT-promoting genes and elevated E-cadherin expression. These results suggest that NFκB exerts a bidirectional effect on EMT, dependent on the cellular context and microenvironmental signals. When compared with the limited available literature, our results expand current knowledge. One study reported that autophagy inhibition enhanced EMT via the RELA/p65 pathway, whereas another demonstrated that autophagy activated NFκB signaling to induce EMT in hepatocellular carcinoma cells[19,20]. Although both studies highlight autophagy as a regulator of EMT via the NFκB pathway, their conclusions differ: one associates autophagy suppression with EMT induction, while the other links autophagy activation to EMT promotion. Our findings provide an additional perspective by showing that NFκB can regulate EMT in both directions, depending on whether autophagy is induced or non-induced. These discrepancies may arise from differences in cancer type, experimental models, or disease stage, and underline the complexity of the NFκB/autophagy/EMT regulatory network.

Based on our previous findings, NFκB positively regulates autophagy in NSCLC. Combined with the results of the present study, we conclude that NFκB exerts a context-dependent effect on EMT: it suppresses EMT under non-autophagy-induced conditions but promotes EMT when autophagy is induced. In this context, NFκB emerges as an autophagy-related EMT regulator with potentially divergent roles in different stages of tumor progression. It is known in the literature that autophagy can exert tumor-suppressive effects in the early stages of cancer and tumor-promoting effects in advanced stages[22]. Similarly, in our study, NFκB-mediated EMT promotion in cases where autophagy is active suggests a mechanism that may increase disease progression and metastatic potential in advanced-stage NSCLC.

In conclusion, this study demonstrates that NFκB, which acts as a positive regulator of autophagy in NSCLC, regulates EMT bidirectionally in an autophagy-dependent manner. It suggests that targeting the NFκB/autophagy/EMT axis may have potential for cancer treatment. In advanced lung cancer, the combined assessment of autophagy, EMT, and NFκB activity may guide the development of personalized treatment strategies.

Ethics Committee Approval: Not applicable. No animals/humans were used for studies that are base of this research.

Conflict of Interest: The authors declare no conflict of interest, financial or otherwise.

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