Biological Effects of CRISPR/Cas9-mediated Knockout of RAB27A in SCLC

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ABSTRACT

Small cell lung cancer (SCLC) is characterized by rapid growth and early metastasis. Identifying new molecular targets are important in the pathogenesis of SCLC in order to develop new treatment strategies. RAB27A is the critical protein for intracellular exosome trafficking and is a driver of tumour progression. However, demonstrating the potential impact of suppressing RAB27A in SCLC as therapeutic approach is an important deficiency. RAB27A gene knockout SCLC cell lines were generated using a CRISPR/cas9 system. qRT-PCR, Western blotting and Sanger sequencing were performed to confirm RAB27A knockout in SCLC cells. TEM and EXOCET assays were used to detect the alteration of exosomes. Proliferation and colony formation were detected by MTT and microscopy. Subsequently, we intrapulmonally injected N417 and H524 SCLC cells (control and RAB27A knockout for each cell) into SCID mice. The effects of RAB27A knockout on mouse tumor model were analysed using 18F-FDG PET/CT scans. Knocking out RAB27A significantly decreased the expression of CD9, CD63, Tsg101, exosome secretion and exosomal protein in SCLC($p<0.0001$). We found that RAB27A knockout dramatically reduced proliferation and colony formation in SCLC cells ($p< 0.001$, $p<0.0001$). Furthermore, RAB27A knockout decreased proliferation and especially metastasis in mouse model ($p<0,0001$). These studies clearly demonstrated that RAB27A plays an important role in the pathogenesis of SCLC, and targeting the RAB27A gene in SCLC cell lines significantly reduces the activity of the exosomal pathway. RAB27A, therefore, can be a promising cancer therapeutic strategy.

Keywords: RAB27A, exosome, SCLC, CRISPR/Cas9, Carcinogenesis

Küçük Hücreli Akciğer Kanseri'nde RAB27A'nın CRISPR/Cas9 Sistemi Aracılığıyla Susturulmasının Biyolojik Etkileri

ÖZET

Küçük hücreli akciğer kanseri (KHAK) hızlı büyüme ve erken metastaz ile karakterizedir. Yeni tedavi stratejileri geliştirmek için KHAK patogenezinde yeni moleküler hedeflerin belirlenmesi önemlidir. RAB27A, hücre içi eksozom trafiği için kritik bir proteindir ve tümör progresyonunda rol oynar. Bununla birlikte, KHAK'de RAB27A'nın baskılanmasının terapötik yaklaşım olarak potansiyel etkisinin gösterilmesi önemli bir eksikliktir. RAB27A geni nakavt KHAK hücre hatları CRISPR/cas9 sistemi kullanılarak üretildi. qRT-PCR, Western blotlama ve Sanger sekanslama KHAK hücrelerinde RAB27A nakavtını doğrulamak için gerçekleştirildi. Eksozomların nicel ve nitel değişimini tespit etmek için TEM ve EXOCET testleri kullanıldı. Proliferasyon ve koloni oluşumu MTT ve mikroskopi ile tespit edildi. Daha sonra, N417 ve H524 KHAK hücrelerini (her hücre için kontrol ve RAB27A nakavtı) SCID farelerine intrapulmonal olarak enjekte edildi. RAB27A nakavtının fare tümör modeli üzerindeki etkileri 18F-FDG PET/BT taramaları kullanılarak analiz edildi. RAB27A'nın nakavt edilmesi KHAK'de CD9, CD63, Tsg101, eksozom salgısı ve eksozomal protein ekspresyonunu önemli ölçüde azaltmıştır ($p<0.0001$). RAB27A nakavtının KHAK hücrelerinde proliferasyonu ve koloni oluşumunu önemli ölçüde azalttığını bulduk ($p< 0.001$, $p<0.0001$). Ayrıca, RAB27A nakavtının fare modelinde proliferasyonu ve özellikle metastazı azalttığı görülmüştür ($p<0,0001$). Bu çalışma, RAB27A'nın KHAK patogenezinde önemli bir rol oynadığını ve KHAK hücre hatlarında RAB27A geninin hedeflenmesinin eksozomal yolağın aktivitesini önemli ölçüde azalttığını açıkça göstermiştir. Bu nedenle RAB27A, umut verici bir kanser terapötik stratejisi olabilir.

Anahtar kelimeler: RAB27A, eksozom, KHAK, CRISPR/Cas9, Karsinogenezis

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INTRODUCTION

Small cell lung cancer (SCLC) is one of the histopathological subtypes of lung cancer. SCLC is of neuroendocrine origin and accounts for 15% of lung cancers. High proliferative index, early metastasis and poor prognosis. The average survival time for patients with SCLC is ~ 10 months, with a 2-year survival rate of only 6%. Many oncogenic genes and signalling pathways are involved in the development of SCLC and intra-tumour heterogeneity is high (Rudin, et.al., 2021). The Rab27 subfamily is composed of two isoforms as RAB27A and RAB27B. Essential proteins, which are critical for regulating the tumour microenvironment and carcinogenesis are involved in the biogenesis of exosomes. (Ostrowski et.al., 2010; Guo et.al., 2019; Van Solinge et.al., 2020; Tang et.al, 2016) However, there is a lack of information in the literature on the impact of targeting RAB27A in the development of a treatment strategy for SCLC. Accordingly, to investigate the biological changes that may occur in SCLC when the RAB27A gene is silenced, in vitro and in vivo studies are being conducted.

MATERIALS AND METHODS

Cell Culture, Generating of SCLC cell lines knocked out of RAB27A and Validation

The cell lines H889, H1963 and H524 were obtained from the American Type Culture Collection (ATCC). H889, H524, H1963 and N417 small cell lung cancer cell lines were cultured in RPMI-1640 media with 10% fetal bovine serum (FBS) and 1% Penisilin/Streptomycin (Gibco, USA). All cells were grown in 5% CO_2 humidified air at 37ºC. SCLC cell lines were co-transfected with RAB27A-targeting gRNA+Cas9+GFP and HDR+RFP vectors. Sanger sequencing, qRT-PCR, and Western blotting were used to validate the application of CRISPR/Cas9 to the *RAB27A* gene.

EXOCET Assay

The amount of SCLC cell-exosomes were quantified using the EXOCET assay (System Biosciences, USA) according to the manufacturer's protocol. For the Exocet assay, 80 µl of lysis buffer, SCLC-20 µl of derived exosome samples were added to a total volume of 100 µl. Incubated at 37°C for 5 minutes to liberate exosome proteins. Centrifuged at 1500 x g for 5 minutes to remove debris. The EXOCET Reaction Buffer fresh just before using by combining Buffer A with Buffer B depending upon the number of reactions prepared. We used 50 µL of Buffer A plus 0.5 µL Buffer B per reaction. We calculated plate using a spectrophotometric plate reader immediately at 405 nm.

Proliferation assay

The effect of knocking out RAB27A on proliferation of SCLC cells was investigated. To determination effect RAB27A knock out on proliferation of SCLC cells, H889, H889 RAB27A KO, N417, N417 RAB27A KO, H524 RAB27A KO, H1963, H1963 RAB27A KO cells were plated onto 6-

well plates (1×10^5 per well). After 48h, the population doubling time assay was performed as previously described (Tokgun O., et.al., 2020).

In Vivo SCLC model and 18F-FDG PET/CT Analysis

All procedures performed in animal studies were in accordance with the ethical standards of the Boğaziçi University Local Ethics Committee for Institutional Animal Experiments (BÜHADYEK), İstanbul, Turkey. The in vivo experimental phase of our study was realised by intrapulmonary injection of the N417 control and N417 RAB27A KO group cell lines into SCID mice. PET-CT analyses at the end of the 6th week to analyse possible changes in tumour growth and metastatic activity of the N417 cell lines in vivo after RAB27A manipulation.

RESULTS

There was a statistically significant reduction in RAB27A gene expression in SCLC cell sequences after the CRISPR/Cas9 procedure.

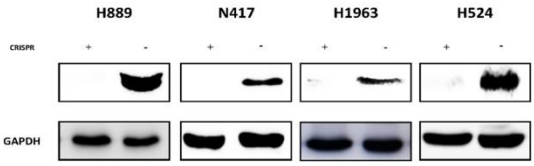

Figure 1. Expression of RAB27A protein in SCLC cell lines after CRISPR/Cas9 application.

In small-cell lung cancer cell lines, the Rab27A gene was found to be dramatically decreased in expression at both protein and RNA levels (Figure1). Mutation in the 6th exon DNA region of the RAB27A gene was detected by Sanger sequencing after knockout by CRISPR/Cas9 application.

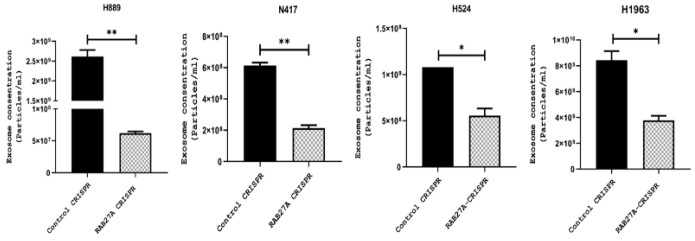

Figure 2. Change in the amount of exosomes.

There was also a statistically significant decrease in the total amount of exosomal protein secreted by the cells following suppression of the exosomal pathway by targeting RAB27A in CHAC cell sequences ($p<0.01$; $p<0.001$). We determined that the size of exosomes secreted by the RAB27A KO SCLC cell lines was reduced in comparison to the control groups (Figure 2).

Knockout of RAB27A in SCLC cell lines results in reduction of cell proliferation.

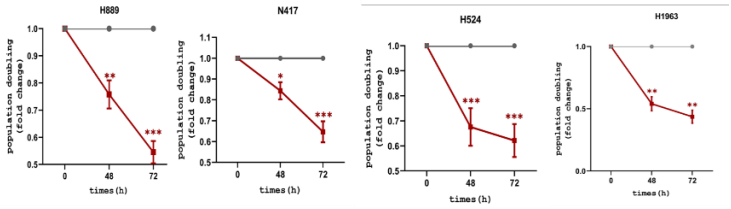

Figure 3. Proliferative activity of SCLC cell lines.

To investigate the function of RAB27A on SCLC cell proliferation, Cell Counting Kit-8 (CCK-8) was used to measure the growth of the established cell lines. As shown in Figure 3, the optical density (OD) value in RAB27A knockout N417, H889, H524 and H1963 cells was significantly lower than that of the control cells 48 hours after cell seeding, indicating that RAB27A knockout inhibits the proliferative activity of SCLC cell lines.

In vivo SCLC Mouse model studies

At the end of 6 weeks, 18F-FDG PET-CT analyses were performed on 3 mice from each group and the macro- and micro-metastatic status of the mice was analysed. In SCID mice injected with control vector-bearing N417 cells, higher than normal 18F-FDG uptake was observed in distant lymph nodes and bones. Furthermore, It was found that the 18F-FDG uptake of metastatic tumours was lower in mice that were injected with RAB27A knockout cells. Therefore, RAB27A gene silencing reduced metastatic activity in the SCLC in vivo model.

DISCUSSION AND CONCLUSION

Small cell lung cancer (SCLC) is a subtype of lung cancer with aggressive neuroendocrine characteristics. The success rate of translating potential treatment strategies into clinical practice is low due to its genetic structure. The Rab27 protein has been implicated in several human cancers. RAB27A play essensial roles in exosome production and release. It is known that Rab27A dysfunction causes human Griscelli type 2 syndrome, proliferation, migration and metastasis in melanoma and colorectal cancer (Li et.al., 2023). Based on our findings, the RAB27A gene has the potential to become a new targetable molecule for the treatment of SCLC. Suppression of the exosome pathway by inhibition of RAB27A may be an effective treatment strategy for SCLC.

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