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Activation of Akt involves resistance to NF-κB inhibition and abrogation of both triggers synergistic apoptosis in lung adenocarcinoma cells

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ABSTRACT

Objectives: Although nuclear factor (NF)-κB and phosphoinositide 3-kinase (PI3K)-Akt-mTOR comprise key pathways, their interrelationship in lung cancer cell survival is poorly understood and needs further analyses.
Materials and methods: We examined the activation of the NF-κB and Akt-mTORC1-p70 S6 kinase (S6K) pathways and the effect of inhibitors for NF-κB, mTORC1, and Akt using fresh lung adenocarcinoma cells.
Results: The cases used for this study showed constitutive NF-κB activity; however, all cases but one showed resistance to NF-κB inhibition. Further examination revealed that the resistant cases were also active in the Akt-mTORC1-S6K pathway. These cases were insensitive to mTORC1 inhibition but sensitive to Akt inhibition. Akt inhibition recovered sensitivity to NF-κB inhibition and dual inhibition showed a synergistic effect on apoptosis induction.
Conclusion: These results indicate that the activation of Akt involves resistance to NF-κB inhibition and both pathways synergistically support the survival of lung adenocarcinoma cells. The results also indicate that inhibition of the mTORC1-S6K pathway does not inhibit the survival of these cells.

1. Introduction

Lung cancer is a bronchogenic carcinoma and is histologically subdivided into small cell lung cancer (SCLC) and non-small cell lung cancers (NSCLCs). Adenocarcinoma is the most prevalent NSCLC. Chemotherapy is the major treatment approach for lung cancer, although options for localized disease are surgery and radiotherapy. Regardless of most treatment attempts, the prognosis of lung cancer remains very poor [1].

Molecular-targeted therapy with small-molecule drugs is a promising treatment strategy for cancer. This strategy targets a molecule that is responsible for the survival of cancer cells [2]. In lung cancer, tyrosine kinase inhibitor (TKI) for epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) have been shown to be effective for patients with lung cancer bearing mutated EGFR and chimerical ALK, respectively. However, the development of cellular resistance to these drugs hampers better long-term prognosis. Importantly, these genetic abnormalities are found in very few patients with lung cancer [3–6]. Therefore, a new strategy developed from a biological basis shared by lung cancers is required.

The transcription factor nuclear factor (NF)-κB plays central roles in cancer biology, i.e. proliferation, anti-apoptosis, vascular regeneration, inflammation, metastasis, and infiltration [7]. Activation of NF-κB is triggered by degradation of the regulatory factor inhibitor of κB (IκB). Constitutive activation of NF-κB represents a hallmark of various types of cancers, including lung cancer [8]. This pathway may also contribute to lung cancer cell survival and proliferation [9,10]. The phosphoinositide 3-kinase (PI3K)-Akt-mTORC1 pathway influences the survival and proliferation of cells [11,12]. Recent studies in cancer cell biology indicated that cancer cells depend upon a small number of deregulated pathways, although they bear numerous genetic abnormalities [13]. Therefore, it is important to clarify the contributions of the NF-κB and PI3K-Akt-mTORC1 pathways in the proliferation of lung cancer cells to develop a new treatment strategy.

Dehydroxymethylepoxyquinomicin (DHMEQ) is a unique NF-κB inhibitor that is a 5-dehydroxymethyl derivative of the novel compound epoxyquinomicin C. We have shown that DHMEQ

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Table 1
Clinical information of patients with lung adenocarcinoma in this study.

Case no.	Age	M/F	Previous treatment	Sampling from diagnosis (Mo)	Post treatment	Treatment duration (Mo)	Outcome	EGFR mutations in exons 18–21	TNM stage
3	64	F	Cisplatin + vinorelbine radiation, docetaxel	15	Gefitinib, erlotinib, TS-1 pemetrexed, amrubicin gefitinib	39	Dead of disease	Ex19: L747-T751 (del)	cT4N3M1 (M:HEP,PLE)
5	59	M	–	0	TS-1	2	Dead of disease	No mutation	cT4N0M1 (M:PLE)
6	52	M	Operation carboplatin + paclitaxel	60	Cisplatin + gemcitabine erlotinib, TS-1, amrubicin	70	Dead of disease	No mutation	cT4N2M1 (M:PLE)
9	77	F	–	0	Gefitinib, radiation	10	Dead of disease	Ex21: L858R	cT4N3M1 (M:PLE,OSS,HEP)

Abbreviations: M, male; F, female; Mo, month; Ex, exon; L, leucine; T, tyrosine; R, arginine; del, deletion; M, metastasis; HEP, hepatic; PLE, pleural; OSS, osseous.

specifically binds to NF-κB and inhibits NF-κB activation at the level of nuclear translocation and DNA binding [14,15].

In this report, we examine the activation of the NF-κB and Akt-mTORC1-p70 S6 kinase (S6K) pathways and the effect of inhibition on NF-κB, mTORC1, and Akt using fresh lung adenocarcinoma cells. To this end, we discuss the roles of these pathways in lung cancer biology and their significance in treatment.

2. Materials and methods

2.1. Samples

Fresh lung cancer cells were obtained from the pleural effusion fluid of patients. The diagnosis of lung cancer was confirmed clinically and pathologically. Tissue sections were prepared from formalin-fixed biopsy samples at the time of the initial diagnosis as lung cancer. The ethics committee of Kitasato University approved the collection of the samples and the procedure. Samples were obtained following informed consent from each patient. Fresh cells were separated by centrifugation and stocked at −80 °C in Cell Banker 1 cell cryopreservation medium (BIOLABO, Tokyo, Japan). The purity of the cancer cells in the pleural fluid was more than 90% and the viability measured by trypan blue dye exclusion test following cryopreservation was more than 80%. For the experiments, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.2. Reagents

The NF-κB inhibitor DHMEQ was dissolved with dimethylsulfoxide (DMSO). The mTORC1 inhibitor rapamycin purchased from Cell Signaling Technology, Inc. (Danvers, MA) and Akt inhibitor KP372-1 purchased from STEMGENT (San Diego, CA) were dissolved with DMSO. Bisbenzimide H33342 trihydrochloride (Hoechst 33342) was purchased from Calbiochem (Bad Soden, Germany).

2.3. Electrophoretic mobility shift analysis

Electrophoretic mobility shift analysis (EMSA) was carried out as described [16]. For detection, a double-stranded oligonucleotide containing the κB site of the promoter for NF-κB and that containing the Oct-1 consensus sequence were purchased from Promega (Madison, WI).

2.4. Cell viability assay

The effects on cell viability were assayed by color reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay) as described [17]. In several experiments trypan blue dye exclusion test was included to corroborate MTT findings.

2.5. Immunohistochemistry

Immunostaining was performed on paraffin-embedded specimens. After deparaffinization, samples were subjected to autoclave antigen retrieval in 10 mM citrate buffer (pH 6.0) for 2 min. Sections were then treated with 3% H_2O_2 for 15 min, blocked using 5% normal goat serum for 1 h, and incubated with indicated antibodies listed in supplementary data 1 at 4 °C overnight. Bound antibodies were detected using the Histofine Simple Stain MAX PO (MULTI) and DAB substrate kits (both from Nichirei Biosciences, Tokyo, Japan).

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2013.10.018.

2.6. Immunoblot analyses

Aliquots (30 μg) of cell lysates were resolved on a SDS-PAGE gel and transferred onto a PVDF membrane by iBlot blotting system (Invitrogen, Carlsbad, CA). The membrane was probed with the indicated primary antibodies and horseradish alkaline phosphatase-conjugated secondary antibody. Immunoreactive proteins were visualized by Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega). The antibodies used were described in supplementary data 1.

2.7. Statistical analysis

Differences between mean values were assessed by two-tailed *t*-test. A P-value < 0.05 was considered to be statistically significant. Cobination index (CI) was calculated as described using CalcuSyn 2.0 (Biosoft, Cambridge, UK) [18].

3. Results

3.1. Constitutive NF-κB activation in fresh lung adenocarcinoma cells and the effect of NF-κB inhibition by DHMEQ

Previous reports indicated that the inhibition of NF-κB induced by TNF-α, radiation, or chemotherapy sensitizes lung cancer cells to apoptosis [19–21]. A recent report showed that IκB kinase (IKK)β inhibition inhibits the proliferation of tumor cells and prolongs survival in a lung cancer mouse model [22]. However, previous studies used cell lines or mouse models and as far as we know, no experiment has used fresh lung cancer cells. Therefore, using fresh lung cancer cells, we examined constitutive NF-κB activation and the effect of NF-κB inhibition by a specific NF-κB inhibitor, DHMEQ. We