Original Articles

Targeting c-Myc: JQ1 as a promising option for c-Myc-amplified esophageal squamous cell carcinoma

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ABSTRACT

c-Myc amplification-induced cell cycle dysregulation is a common cause for esophageal squamous cell carcinoma (ESCC), but no approved targeted drug is available so far. The bromodomain inhibitor JQ1, which targets c-Myc, exerts anti-tumor activity in multiple cancers. However, the role of JQ1 in ESCC remains unknown. In this study, we reported that JQ1 had potent anti-proliferative effects on ESCC cells in both time- and dose-dependent manners by inducing cell cycle arrest at G1 phase, cell apoptosis, and the mesenchymal-epithelial transition. Follow-up studies revealed that both c-Myc/cyclin/Rb and PI3K/ AKT signaling pathways were inactivated by JQ1, as indicated by the downregulation of c-Myc, cyclin A/E, and phosphorylated Rb, AKT and S6. Tumor suppression induced by JQ1 in c-Myc amplified or highly expressed xenografts was higher than that in xenografts with low expression, suggesting its potential role in prediction. In conclusion, targeting c-Myc by JQ1 could cause significant tumor suppression in ESCC both *in vitro* and *in vivo*. Also, c-Myc amplification or high expression might serve as a potential biomarker and provide a promising therapeutic option for ESCC.

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1. Introduction

Esophageal squamous cell carcinoma (ESCC) is the dominant subtype of esophageal cancer in China, accounting for more than 90% of cases [1]. Although patients with advanced disease partially benefit from chemotherapy, the prognosis is still poor with a 5-year survival rate ranging from 10% to 25% [2]. The challenging reality of limited chemotherapeutic drugs, susceptibility to drug resistance and lack of targeted therapies underscore the urgent need to discover novel therapeutic targets for ESCC.

Recently, next-generation genomic profiling has shown that cell cycle dysregulation is frequent in ESCC [3–7], which has provided insights into potential therapeutic strategies. Our previous research demonstrated that a cyclin-dependent kinase 4/6 inhibitor exerted potent anti-tumor activity in ESCC by inhibiting phosphorylated retinoblastoma (Rb) and inducing cell cycle arrest at G1 phase [8]. In addition, another cell cycle regulator, c-Myc, is frequently amplified (about 12%) or highly expressed (about 37%) in ESCC [3–6,9], suggesting its potential role in ESCC pathogenesis. In addition, our unpublished data showed that c-Myc was amplified in 3 out of 23 ESCC patient-derived xenografts (PDXs) (the genetic features of xenografts were shown in Fig. S1A, the raw data of genetic features were shown in Table S1), which enabled the following study.

c-Myc plays important roles in multiple cellular processes, including cell proliferation, cell cycle and apoptosis, cell progression and metabolism, and is an oncogene involved in multiple

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cancers [10]. Although the importance of c-Myc in carcinogenesis is well documented, therapies targeting c-Myc are not available currently. c-Myc was considered 'undruggable' due to its lack of innate enzymatic function, small molecule interactions and inaccessibility to any antibody-based therapies. Some compounds targeting MAX, the best known heterodimeric partner for c-Myc, including 10058-F4, 10074-G5 and KJ-Pyr-9, showed limited efficacy in vivo [11–13]. However, JQ1, which targets the bromodomain and extra-terminal (BET) domain family, has emerged as an alternative method to suppress oncogenic transcription factors mainly through inhibiting the function of c-Myc [14]. Notably, as a BET protein inhibitor, JQ1 could decrease c-Myc expression and exert promising anti-tumor activities in hematological malignancies, pancreatic cancer and other solid tumors [15–26]. Following this rationale, BET protein inhibitors are currently being investigated in phase I and II clinical trials (such as NCT02419417, NCT02543879 and NCT03068351) in advanced malignancies.

This study was designed to elucidate the potential anti-tumor effects of JQ1 in ESCC both in vitro and in vivo. Furthermore, we also explored possible mechanisms underlying the anti-tumor activity of JQ1 and evaluated eligible patients who could benefit from JQ1.

2. Materials and methods

2.1. Reagents and antibodies

JQ1 was purchased from MedChem Express (Princeton, NJ, USA). The following antibodies were purchased from Cell Signaling Technology (Boston, Massachusetts, USA): AKT (#4691), p-AKT (#4060), S6 (#2217), p-S6 (#4858), p21 (#2947), Rb (#9313), p-Rb (#9307), Cleaved Caspase 9 (#20750), Cleaved caspase 8 (#8592), Cleaved PARP (#5625), Bcl-2 (#15071), E-cadherin (#3195), Vimentin (#5741) and β-catenin (#8480S). The c-Myc (ab32072) and p27 (ab32034) antibodies were purchased from Abcam (Cambridge, UK). The Cyclin A (#SC-751) and Cyclin E (#SC-198) antibodies were purchased from Santa Cruz Biotechnology (Beijing, China). The Ki-67 (ZM-0165) and β-actin (#014M4759) antibodies were purchased from ZSJB-BIO (Beijing, China) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. Cell lines and cell culture

The human ESCC cell lines EC 109, TE-1, KYSE 450, KYSE 510 and KYSE 140 were obtained from certificated cell bank of Peking Union Medical College (Beijing, China; http://sbm.pumc.edu.cn/) and maintained in our department. All cells were identified by short tandem repeat analysis and confirmed no mycoplasma. Cells were cultured in RPMI-1640 (Gibco-BRL, Gaithersburg, MD, USA) medium containing 10% fetal bovine serum (GIBCO) and 1% penicillin and streptomycin (GIBCO) and incubated at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cell viability assay

Cells (about 3000 cells per well) were plated onto 96-well plates and cultured overnight in complete medium. Cells were treated with serial concentrations of JQ1 for 24–72 h and then evaluated for cell viability using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) following the manufacturer's instructions. The absorbance was measured at 490 nm using a spectrophotometer. All experiments were repeated at least three times.

2.4. Cell cycle and cell apoptosis evaluation

For cell cycle analysis, cells after 500 nM JQ1 or vehicle treatment for 48 h were harvested and fixed in 75% cold ethanol overnight at 4 °C, followed by staining with 50 μg/mL propidium iodide (PI, BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 30 min in the dark. A FACS Calibur system (BD Biosciences) was employed to detect the cell cycle distribution and data were analyzed using ModFit 3.0 software (BD Biosciences).

For cell apoptosis determination, cells after 500 nM JQ1 or vehicle treatment for 48 h were stained with phycoerythrin (PE)–annexin V and 7-amino-actinomycin (7-AAD) (BD Biosciences) at room temperature for 15 min in the dark, followed by flow cytometry within 1 h (BD Biosciences). Data analysis was performed using WinMDI 2.9 software (BD Biosciences).

2.5. Cell migration and invasion assays

Cell migration assay was performed in 24-well CIM plates (BD Biosciences, CA, USA) as previously described [27]. Briefly, 10,000 to 30,000 cells per well were seeded in serum-free medium with 500 nM JQ1 or vehicle treatment in the upper compartment of the CIM plates. Serum-complemented medium was added to the lower compartment of the chamber. After 12 h incubation, cells that passed through the septum were fixed and stained with crystal violet. The number of cells permeating septum was counted in five random microscopic fields. The average number of cells in five fields was calculated, which represented the ability of cells to migrate.

Cell invasion assay was performed in 24-well CIM plates (BD Biosciences, CA, USA) coated with matrigel. Other steps were the same as cell migration assay. The average number of cells permeating the matrigel in five fields was calculated to determine cell invasion activity.

2.6. Western blotting analysis

Cells and tissues were harvested and total protein was extracted from cell pellets or xenografts using RIPA Lysis Buffer (Beyotime Biotechnology, Jiangsu, China) on ice, supplemented with complete protease inhibitor and phosphatase inhibitor cocktail (Roche, Switzerland). Protein concentration was measured using the BCA Protein Assay Kit (Applygen Technologies Inc., Beijing, China), and 40 μg protein from each sample was separated on 10% or 12% SDS-PAGE gels. After transfer, the nitrocellulose membrane was incubated with the corresponding primary antibodies (the dilutions of primary antibodies were shown in Table S2) at 4 °C overnight, followed by secondary antibodies at room temperature for 1 h. Proteins were visualized with a chemiluminescent detection system (GE Healthcare), using ECL plus Western Blotting Detection Reagents (GE Healthcare). The protein expressions were quantified and normalized by Image J software.

2.7. RNA extraction and quantitative real-time PCR

Total RNA was extracted from cell pellets using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RNA samples with an OD260/OD280 ratio between 1.9 and 2.0 were used for the following cDNA synthesis using High Capacity RNA to cDNA kits (Applied Biosystems). Quantitative PCR was performed using SYBR Green master mix (Applied Biosystems) with the housekeeping gene GAPDH as the internal control. The relative expression of c-Myc was calculated using the comparative Ct method. The primers of c-Myc and GAPDH were as follows:
c-Myc forward, 5′-TCCTGTACCTCGTCCGATTC-3′,