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KSRP suppresses cell invasion and metastasis through miR-23a-mediated EGR3 mRNA degradation in non-small cell lung cancer

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A R T I C L E I N F O

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A B S T R A C T

KH-type splicing regulatory protein (KSRP) is a single-strand RNA binding protein which regulates mRNA stability either by binding to AU-rich elements (AREs) of mRNA 3′UTR or by facilitating miRNA biogenesis to target mRNA. Unlike its well-characterized function at the molecular level in maintaining RNA homeostasis, the role of KSRP in cancer progression remains largely unknown. Here we investigate the role of KSRP in non-small cell lung cancer (NSCLC). We first examined KSRP expression by immunohistochemistry in a cohort containing 196 NSCLC patients and observed a strong positive correlation between KSRP expression and survival of NSCLC patients. Multivariate analysis further identified KSRP as an independent prognostic factor. Manipulating KSRP expression significantly affected *in vitro* cell mobility and *in vivo* metastatic ability of NSCLC cells. Microarray analysis identified an ARE-containing gene, *EGR3*, as a downstream effector of KSRP in NSCLC. Interestingly, we found that KSRP decreased *EGR3* mRNA stability in an ARE-independent manner. By screening KSRP-regulated miRNAs in NSCLC cells, we further found that miR-23a directly binds to *EGR3* 3′UTR, reducing EGR3 expression and thereby inhibiting NSCLC cell mobility. Our findings implicate a targetable KSRP/miR-23a/EGR3 signaling axis in advanced tumor phenotypes.

1. Introduction

K-homology (KH) splicing regulatory protein (KSRP, also known as KHSRP) is a multi-functional RNA binding protein that is involved in different steps of mRNA metabolism. The RNA binding ability of KSRP is central to its various functions and is mediated by the four KH domains that occupy the protein central region [1]. In addition to its originally identified function in neuronal RNA splicing [2], KSRP was also found to recognize AU-rich elements (AREs) within the 3′-untranslated regions (3′UTRs) of a subset of mRNAs and to control their turnover in the cytoplasm [3]. After targeting these mRNAs, KSRP recruits the exosome and other de-adenylation factors, resulting in polyA shortening followed by 3′ to 5′ exonucleolytic digestion of the mRNAs [1,4]. Several distinct ARE-containing mRNA targets of KSRP have been identified and found to participate in multiple cellular functions, including organogenesis [5], muscle differentiation [6], lipid metabolism [7], inflammation [8,9], and parathyroid hormone (PTH) expression [10]. However, although some of the KSRP targeted mRNAs, such as β-catenin and IL-8, are involved in tumor development and progression [11,12], the functional significance of KSRP in cancer development and progression has not been investigated.

In addition to directly targeting the ARE sites of mRNA to facilitate

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mRNA decay, KSRP has also been found to control mRNA turnover by promoting biogenesis of a subset of microRNAs (miRNAs) [13]. KSRP-mediated miRNA processing is also involved in diverse cellular functions such as cytokine expression, lipid metabolism, cell-fate decisions, tissue regeneration, and DNA damage response [14–17]. Furthermore, some KSRP-regulated miRNAs also play critical roles in cancer development. For example, the tightly regulated cellular concentration of the prototypical tumor suppressor let-7, was shown to be positively regulated by KSRP and negatively regulated by Lin28 and hnRNP A1 [18]. On the other hand, Ruggiero et al. have demonstrated that KSRP is required for the cell-specific maturation of the oncogenic miRNA, miR-155, in mouse macrophages in response to a variety of pro-inflammatory stimuli [19]. Taken together, KSRP may promote biogenesis of different sets of miRNAs in different cell contexts or under different stimuli. KSRP was reported to serve as a component of both Drosha and Dicer complexes and to regulate the biogenesis of a subset of miRNAs [13]. Previous studies have found Dicer and Drosha to be dysregulated in cancer cells. Decreased expression of Dicer, Drosha, or both enzymes is associated with decreased patient survival in ovarian, breast, and lung carcinoma [20]. Abnormalities in the copy number of the Dicer gene have also been described in human melanoma, breast, and ovarian cancers [21]. Even though KSRP is as necessary as other miRNA processing components, the functional roles of KSRP and its regulated miRNAs in cancer pathogenesis has not been fully characterized.

Although the accumulated evidence suggests that KSRP may act in multiple steps of cancer development through its known mRNA and miRNA targets, a comprehensive study to functionally evaluate the roles of KSRP in cancer is still lacking. In the current study, we demonstrate that KSRP expression positively correlates with better survival of patients with lung adenocarcinoma and that KSRP suppresses cell mobility and tumor metastasis by promoting *EGR3* mRNA degradation, indirectly *via* facilitation of miR-23a maturation, instead of directly *via* targeting the ARE sites of *EGR3* 3′UTR.

2. Materials and methods

2.1. Patients and specimens

Lung specimens were obtained from a total of 196 consecutive patients who underwent surgical resection at Kaohsiung Veterans General Hospital. None of the patients had received preoperative neoadjuvant chemotherapy or radiation therapy. Clinical information and pathology data were collected *via* retrospective review of the medical records. All cases were staged according to the cancer staging manual of the American Joint Committee on Cancer (AJCC) and the histological cancer type was classified according to World Health Organization (WHO) 2004 classification. Paraffin-embedded, formalin-fixed surgical specimens from all 196 patients were collected for immunohistochemical (IHC) staining for KSRP. Fresh, flash-frozen paired specimens of lung cancer and adjacent normal tissue were also obtained from 24 patients for Western blot analysis of KSRP expression. The study was conducted on the basis of approved protocols by the Institutional review board (IRB) at National Taiwan University Hospital (Taipei, Taiwan) and Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). Informed consent was obtained for experimentation with human subjects.

2.2. Immunohistochemistry

Anti-KSRP (A302-021A, Bethyl Laboratories) at a dilution of 1:1000 was used for IHC staining. The IHC staining assessment was independently conducted by 2 pathologists (Chia-Yi Su and Michael Hsiao) blinded to patient outcome. Only expression of the tumor cells in the cores was evaluated. Both the immunoreactivity intensity and percentage were recorded. The intensity of staining was scored using a four-tier scale and defined as follows: 0, no staining; 1 +, weak staining; 2 +, moderate staining; 3 +, strong staining. The extent of staining was scored by the percentage of positive cells (0–100%). The final IHC scores were obtained by multiplying staining intensity by the percentage of positive cells. All cases were divided into two groups according to the final IHC scores. High expression level was defined as a score ≥ 100 and a score < 100 was defined as low expression.

2.3. Cell culture

A series of lung adenocarcinoma cell lines, CL1-0, CL1-1, CL1-3, CL1-5, and CL1-5/F4, in ascending order of invasiveness, was established by Chu et al. [22,23]. PC14 and PC9 were developed by Lee and colleagues at National Cancer Center Hospital, Tokyo, Japan [24]. Other lung cancer cell lines (H441, A549 and H1650) were obtained from the American Type Culture Collection (ATCC). All cells were cultured in RPMI-1640 supplemented with 10% FBS. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.4. Two-chamber migration and invasion assay

Invasion and migration assays were performed using Transwell inserts for a 24-well plate that contained 8-mm pores (Millipore). 1×10^4 cells (for migration assays) or 5×10^4 (for invasion assays) were plated into the top chamber onto a Matrigel-coated (for invasion assays) or non-coated (for migration assays) filter and allowed to migrate or invade into the lower chamber for 24 h. Invaded cells were fixed and stained with 0.2% crystal violet. Stained cells were quantified by counting.

2.5. Immunofluorescence staining

Cells were grown on coverslips and fixed in 4% paraformaldehyde, permeabilized, and stained with primary antibodies followed by secondary Alexa 633-conjugated rabbit antibodies. F-actin was detected with FITC-phalloidin (Invitrogen). Slides were examined and photographed using a Zeiss Axiophot fluorescence microscope. Nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI).

2.6. Animal studies

NOD.CB17-Prkdc^{scid}/NcrCrl (NOD/scid) mice were maintained in a germ-free environment and had unlimited access to food and water. For the orthotopic model, CL1-0 cells (1×10^6) with stable luciferase expression were immersed in 10 μL of 50% Matrigel/PBS and injected into the left lung lobe. For the experimental metastasis model, CL1-5 cells (1×10^6) with stable luciferase expression were immersed in 100 μL of PBS and injected into the tail vein of the mice. 6 mice per group were used in both models. After 6 weeks, the mice were sacrificed and luciferase activity in the excised lungs and livers was determined using an *in vivo* bioluminescence system (IVIS-Spectrum, PerkinElmer, Waltham, MA, USA). Mouse lungs and livers were also fixed, sectioned, and stained with hematoxylin and eosin (H & E). All animal work was performed according to protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

2.7. RNA isolation, reverse-transcription polymerase chain reaction (RT-PCR), and microarray

Total RNA was isolated using TRizol (Invitrogen) according to the manufacturer's instructions. Reverse-transcription of RNA isolated from cells was performed in a final reaction volume of 20 μL containing 5 μg of total RNA in Moloney murine leukemia virus (MMLV) reverse-transcriptase buffer (Promega), and 200 U of MMLV reverse transcriptase (Promega). The reaction mixture was incubated at 37 °C for 2 h, and the

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