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ORIGINAL ARTICLE

AGAP2-AS1 affects TNM staging and prognosis of lung cancer patients by acting on SLC7A11 mRNA stability and ferroptosis*

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Abstract

Objective The initiation and progression of lung carcinomas are critically regulated by long non-coding RNAs (lncRNAs). However, the role of lncRNAs in the pathways causing lung cancer remains unknown.

Methods Cell morphology was regularly observed using an inverted phase-contrast microscope. Cell viability was assessed using CCK-8 according to the manufacturer's instructions. Total RNA was retrotranscribed from each specimen using the RNAiso Plus Kit. The RT-PCR data were calculated using the Ct approach for comparison. Flow cytometric analyses were prepared by Click-iT™ Plus TUNEL Assay for In Situ apoptosis detection, with Alexa Fluor™ 594 dye, as instructed. RNA immunoprecipitation assays were used to determine RNA concentration.

Results Activated natural killer cells repeat and PH domain-containing protein 2 antisense RNA 1 (AGAP2-AS1) levels in cancerous tissues were significantly correlated with cancerous tumor node metastasis (TNM) stage, with cancerous AGAP2-AS1 levels being higher in cancerous tissues than healthy tissues. Patients withelevated AGAP2-AS1 levels had considerably worse outcomes than those with reduced AGAP2-AS1 levels,regardless of the progression-free or overall survival. Functionally, AGAP2-AS1 downregulation represseslung cancer cell growth. AGAP2-AS1 elimination induces erastin-mediated ferroptosis in lung cancer cells.However, the ferritin inhibitor FERSINT-1 negated this result, whereas ERASTIN induced lung cancer cellmortality. After AGAP2-AS1 silencing, erastin-treated lung cancer cells showed a remarkable decrease inGSH levels. These results indicated that AGAP2-AS1 enhanced the stabilization of SLC7A11 mRNA via Recombinant Insulin Like Growth Factor Binding Protein 2(IGF BP2). Patients with elevated AGAP2-AS1 had considerably worse outcomes. Down-regulating AGAP2-AS1 was able to repress lung cancer cell growth and induce greater Erastin-mediated ferroptosis. Lungcancer cells treated with Erastin exhibited a remarkable decrease inglutathione (GSH) levels. The mechanical findingsindicated that AGAP2-AS1 enhanced the stabilization of SLC7A11 mRNA via the IGF2BP2.

Conclusion We identified a novel effect of AGAP2-AS1 on TNM staging and the prognosis of patientswith lungcancer by modulating SLC7A11 mRNA stability and ferroptosis.

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Key words: AGAP2-AS1; ferroptosis; lung cancer; mRNA stability

Lung carcinoma is a malignant neoplasm characterized by mutations in lung cells and their proliferation. Patients with advanced metastatic lung cancer have a low survival rate [1, 2]. Lung cancer is responsible for over 30% of all malignant mortalities [3]. Factors that can induce these conditions include environmental factors (e.g. air pollution) and genetics. Early stage patients with lung cancer have a higher five-year survivability after prolonged resection and lymph node dissection [4]; however, the five-year survival rate for patients with distant metastasis is extremely low, and the prognosis is poor. In metastatic lung cancer patients with advanced chemotherapy and radiotherapy, 15%–20% of treatments succeed, but their survival time is short [5].

Iron toxicity is a relatively new form of controlling cell death (RCD). Ergot inhibits the onset of RCD, particularly in cancer cells with mutations in the RAS signaling pathway [6]. Increasing tumorigenesis by suppressing

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Table 1 PCR primer Sequence

Gene	F Sequence (5'-3')	R Sequence (5'-3')
AGAP2-AS1	TACCTTGACCTTGCTGCTCTC	TACCTTGACCTTGCTGCTCTC
U6	CTCGCTTCGGCAGCACAT	TTTGCGTGTCATCCTTGCG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
SLC7A11	TCCTGCTTTGGCTCCATGAACG	AGAGGAGTGTGCTTGCGGACAT

ferroptosis, which plays a critical role in the progression of malignancies, enhances the initiation of neoplasms [7]. Ferroptosis is not fully understood as the mechanism underlying lung cancer growth [8, 9].

Long non-coding RNAs (lncRNAs) have been consistently regarded as "noise" in transcription [10]. Studies have shown lncRNAs could regulate genomic expression and affect organism growth and development [11]. It has been found that lncRNAs are involved in several crucial processes and regulate gene expression at various levels [12]. An increasing amount of data has shown that lncRNAs are the most diverse functional ncRNAs [13]. LncRNA malfunction has a significant influence on cytoplasmic integrity. Non-expression of *AGAP2-AS1* contributes to GXP4 mRNA deterioration via m5C-dependent regulation as well as the modification of SLC7A11 mRNA by m5C and its binding to *AGAP2-AS1*.

Materials and methods

TCGA database retrieval

From the TCGA (http://tcga-adtaa. nci. nih. gov/tcga/) database using the Epicalc function package of R 2.15.3 software to download and preprocess the *AGAP2-AS1* data from the lung cancer data set for analysis.

Cell culture

Human lung cancer cells (A549 cells) were cultured in DMEM medium containing 10% fetal bovine serum (FBS). An incubator with 5% CO_2 and 95% humidity at 37°C was used for all of lung-cancer cells. Regular observations of cell morphology were performed using an inverted phase-contrast microscope.

CCK-8 assay

The cells were trypsinized, rinsed and incubated at 5000 cells/well in 96-well plates. Erastin was applied at various concentrations to the cells. After incubation for approximately 24 h, supernatants were collected. Cell viability was assessed using the CCK-8 (Enzo Life Sciences) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader. Proliferation assays were performed independently and repeatedly in triplicate. Culture conditions were as follows (1) The 6-well plate was laid according to the steps of instructions, and cells were transfected overnight according to the steps of instructions. (2) After 12 h of transfection, the cells were counted and the transfected cells were inoculated on a 96-well plate with a density of 2×10^3/well, with three wells in each group. (3) The 96-well plates of A549 cells were cultured in a 5% CO_2 incubator at 37°C. Then cell proliferation was measured at 0, 24, 48, 72 and 96 h using the CCK-8 kits (Tokyo Dojindo, Japan). The CCK-8 working solution was added as gently as possible to prevent air bubbles from interfering with the subsequent absorbance measurements. The ratio of CCK-8 to serum-free medium was approximately 1:10. (4) A 96-well plate with the CCK-8 working solution was incubated in a cell incubator for 2 h, and the absorbance at 450 nm was measured using an enzyme-labeling instrument, which was used for the CCK-8 analysis to indirectly reflect the number of living cells (American Bole).

Real-time (RT) quantitative PCR test

After 24 h plasmid transfection, total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen) and purified using RNase R reagent (Epicenter). Total RNA was reverse-transcribed into cDNA using the Prime Script RT Reagent Kit (Takara, Japan). Total RNA was reverse transcribed from each specimen using an RNAiso Plus Kit (Takara, Japan). Following the completion of the RT-PCR reactions, the data were calculated using the Threshold cycle approach for comparison, and each specimen's expression of β-actin served to standardize the results. The primer sequences used are listed in Table1. The relative expression level of total RNA was analyzed by $2^{-\Delta Ct}$ method.

Flow cytometric analysis

Following a 48-h transfection, flow cytometric measurements were prepared by Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 594 dye (Invitrogen) as instructed. Individual stages were classified, and the proportions of dying, presorting, and apoptotic cells were evaluated based on standards. The cells were cultured for 48 h, rinsed with PBS, 500μl binding buffer was added, and then mixed with 10μL

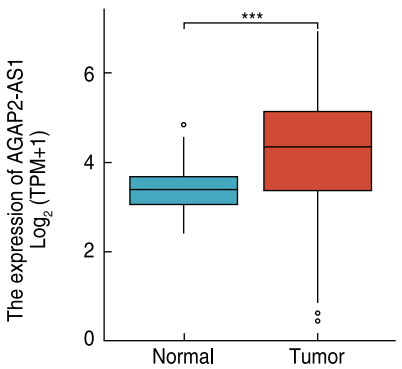

Fig. 1 The levels of *AGAP2-AS1* in normal and lung cancer cases in TCGA. *** $P < 0.001$ for comparison between groups

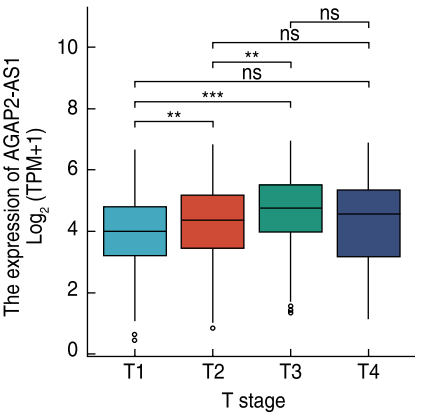

Fig. 2 According to the TCGA database, levels of *AGAP2-AS1* correlations were analyzed relative to lung cancer specimens and the clinical features of the patients. * $P < 0.05$ and ** $P < 0.01$ for comparison between groups

AnnexinV-FITC and 5μL PI solution. The solution was incubated for 10 min out of direct sunlight. The rate of apoptosis was determined using flow cytometry.

Staining of reactive oxygen species assay

To remove serum, the cells were rinsed with PBS at least twice, then resuspended in Hanks' Balanced Salt Solution (HBSS) (containing Mg^{2+} and Ca^{2+}) at a density of 1×10^6 /mL. Afterwards, the cell suspension was mixed and maintained at 37°C in a darkroom for 10 min, before being labeled by adding 5–6 liters of HBSS. After rinsing once with ice-cold complete media, all cells were assessed simultaneously using a FACSCalibur.

RNA immunoprecipitation (RIP) assay

The RIP test was performed using an RIP kit (Bersin Bio) as per the manufacturer's instructions. When cells reached 85% confluency, they were lysed with RIP buffer. Multiwell spectrophotometers (Bio-Rad, USA) were used to calculate RNA concentration, and specific foci (Agilent, USA) were used to determine RNA integrity. Finally, the immunoprecipitated RNA was harvested and analyzed by qRT-PCR.

Statistical analysis

All data collected from several tests are expressed as mean ± SD. In addition to one-way and two-way ANOVA, Student's *t*-tests were used to determine the levels of significance. All statistical analyses were performed using Graphpad Prism 9.0 Software.

Results

Upregulated *AGAP2-AS1* could predict patient prognosis

First, the levels of *AGAP2-AS1* in normal and lung carcinoma specimens were obtained from the TCGA database. There was a dramatic difference in *AGAP2-AS1* levels in normal tissues compared to lung cancer tissues,

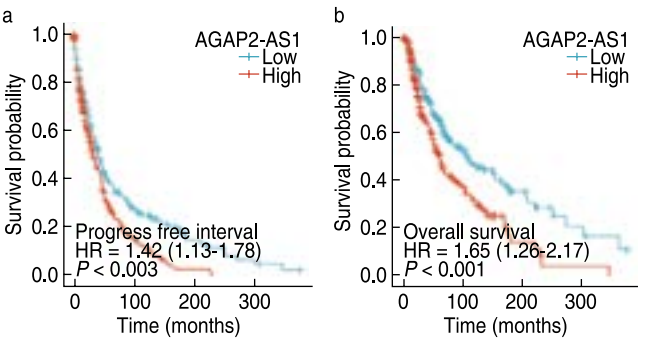

Fig. 3 PFS and OS of lung-cancer patients with various *AGAP2-AS1* levels

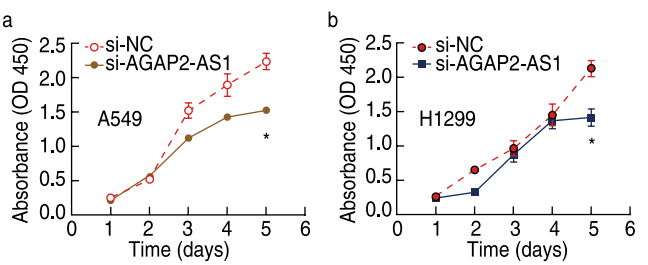

Fig. 4 *AGAP2-AS1* can promote proliferation of lung-cancer cells. * $P < 0.05$ for comparison between groups

where *AGAP2-AS1* levels were lower in cancerous samples (Fig. 1).

According to our findings, *AGAP2-AS1* levels in cancerous tissues were remarkably related to the TNM stage (Fig. 2).

A clear association was observed between higher *AGAP2-AS1* levels and shorter life expectancy. Patients with higher *AGAP2-AS1* levels had not only a significantly shorter progression-free survival (PFS) but

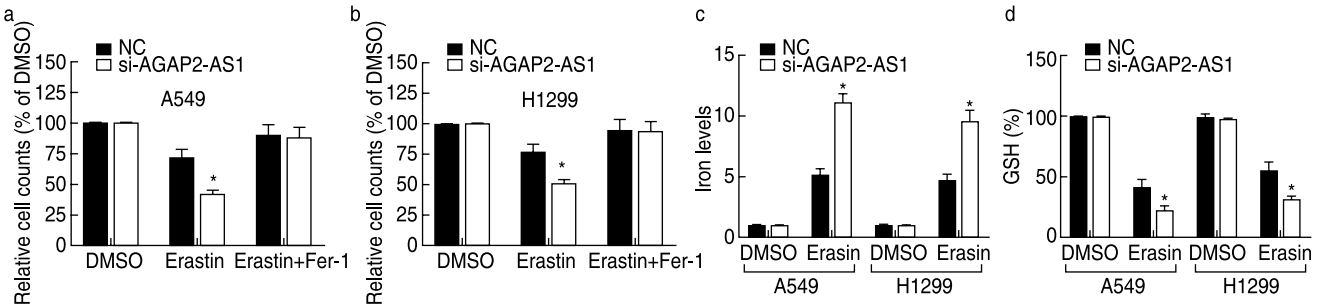

Fig. 5 *AGAP2-AS1* prevents ferroptosis of lung cancer cells. * $P < 0.05$ for comparison between groups

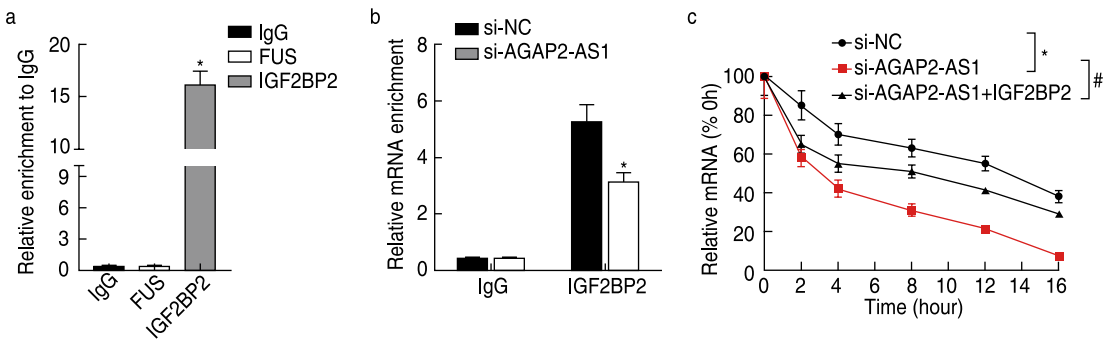

Fig. 6 *AGAP2-AS1* was able to regulate the mRNA stabilization of SLC7A11 via IGF2BP2. * $P < 0.05$ for comparison between IgG and IGF2BP2 groups; # $P < 0.05$ for comparison between si-AGAP2-AS1 and si-AGAP2-AS1+IGF2BP2 groups

also a significantly shorter overall survival (OS; Fig. 3).

AGAP2-AS1 promotes the proliferation of lung-cancer cells

After 48 and 72 h, CCK-8 data showed that the uptake rate of *AGAP2-AS1* was considerably lower than that of the other two groups, with increasing differences over time. These findings suggest that the downregulation of *AGAP2-AS1* inhibits the growth of lung cancer cells (Fig. 4).

AGAP2-AS1 could prevent ferroptosis of lung cancer cells

We tested the effects of the ferroptosis inducer erastin on the activity of tumor cells. After transfection and erastin treatment, the knockdown of *AGAP2-AS1* amended Erastin-mediated ferroptosis (Fig. 5a and 5b). Concomitantly, we found that the ferroptosis inhibitor FERSINT-1 restored this effect, whereas erastin caused lung cancer cell mortality (Fig. 5a and 5b). This indicates that *AGAP2-AS1* downregulation enhances ferroptosis. These results suggest that *AGAP2-AS1* silencing inhibited the proliferation of lung cancer cells and enhanced ferroptosis. Subsequently, the influence of *AGAP2-AS1* accumulation on ferroptosis and GSH levels was assessed. Because Fe^{2+} is an essential contributor to ferroptosis, we initially evaluated the effects of *AGAP2-AS1* on intracellular Fe^{2+} concentrations. The findings showed that intracellular iron and Fe^{2+} levels were upregulated upon *AGAP2-AS1* silencing with erastin (Fig. 5c). Furthermore, *AGAP2-AS1* knockdown led to a remarkable decrease in GSH content in rastin-treated lung cancer cells (Fig. 5d). Blocking *AGAP2-AS1* facilitates ferroptosis in lung cancer cells, implying that *AGAP2-AS1* contributes to anti-ferroptosis effects.

AGAP2-AS1 can enhance SLC7A11 mRNA stabilization via IGF2BP2

Therefore, we investigated the modulatory association between SLC7A11 and *AGAP2-AS1*. Initially, RIP assays were performed to examine the interplay between SLC7A11 and IGF2BP2. SLC7A11 and IGF2BP2 could interact, however *AGAP2-AS1* knockdown reduced this interaction. Moreover, subsequent mRNA degradation revealed that *AGAP2-AS1* knockdown reduced SLC7A11 mRNA stabilization, while over-expression reversed the functionality of *AGAP2-AS1*. These findings revealed that *AGAP2-AS1* enhanced the stabilization of SLC7A11 mRNA via IGF2BP2.

Discussion

While immunosuppressive therapy significantly improved survival rates in some populations with lung

cancer, it does not help about 40–60% of patients [14]. The same group of chemotherapeutic drugs has shown remarkable benefits in the treatment of lung cancer subtypes [10]. LncRNAs are integral to the multiplication, differentiation, and apoptotic processes of various malignancies. LncRNAs have been shown to be involved in the neoplastic cell cycle, cell signaling pathways, and genetic modifications, leading to the emergence of lncRNAs as potential candidates for investigation [15].

An lncRNA is a protein-coding gene that is unable to encode a protein, although it directly performs biofunctions in the form of RNA [16]. With a length of over 200 of nucleotides, it is incomplete in its open read frame [17, 18]. LncRNAs are then transcribed by RNA Polymerase II and subsequently amended by 5' end-capping, splicing, and 3' end-polyadenylation [19]. Primarily, they are believed to serve non-biological roles; however, the findings of numerous assays revealed that lncRNAs mediate genetic regulation in the form of RNA along numerous layers, including transcription, post-transcriptional profiling, and epigenetics [20, 21]. LncRNAs mainly perform their effects in four ways: first, by induction, lncRNAs are able to associate with particular miRNAs and hence regulate targeted genes obliquely; second, lncRNAs can serve as signaling agents working on pathways to modulate associated genes up and downstream of their targeted genes [22, 23]. Furthermore, lncRNAs can regulate specific target genes by guiding associated RNA-binding proteins via cis- or trans-regulation of protein complexity sites [24, 25]. Finally, in scaffolding, lncRNAs may serve as a platform on which a diverse combination of relevant regulatory compounds can act to strengthen messaging and the interplay between molecular complexities [26].

We investigated the functions of *AGAP2-AS1* by examining the clinical characteristics of patients with lung carcinoma and lung carcinoma cell lines. We found that *AGAP2-AS1* was more highly expressed in lung carcinoma than in normal and healthy neighboring tissues, while *AGAP2-AS1* was more strongly expressed in the lung metastasis group than in the non-distant metastasis group. This is in agreement with the results of other investigators who analyzed lncRNA expression levels in other neoplastic specimens, indicating that *AGAP2-AS1* might be involved in the initiation and promotion of lung carcinoma [27]. Additionally, the presence of *AGAP2-AS1* in lung carcinoma samples may influence the OS of patients with lung carcinoma. The greater the expression of *AGAP2-AS1* in lung carcinoma, the worse the prognosis. In vitro experiments demonstrated that elimination of *AGAP2-AS1* expression suppressed cell growth and caused ferrotoxicity in cells. The use of ROCK suppressors has gained attention in contemporary medicine with increasingly efficient targeted approaches [17].

In conclusion, this study identified a novel behavior of *AGAP2-AS1* and the underlying mechanisms of ferroptosis and lung cancer initiation, which may aid in the development of new lung cancer therapies. We identified a novel effect of *AGAP2-AS1* on TNM staging and prognosis of patients with lung cancer by acting on SLC7A11 mRNA stability and ferroptosis.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

All authors contributed to data acquisition and interpretation and reviewed and approved the final version of this manuscript.

Data availability statement

The data supporting the findings of this study are available from the first author upon request.

Ethical approval

Not applicable.

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