U2AF2 Promote the Proliferation and Migration of Esophageal Adenocarcinoma Cells by Sustaining the Mrna Stability of SNORA21

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Research Article

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Abstract

Background

Esophageal adenocarcinoma has gains predominance with squamous carcinoma moving to a second place in the western world. The 5-year survival rate of patients with esophageal cancer (EC) is still between 15–25%, although the treatment strategies for EC have been improved. RNA-binding proteins (RBPs) are key players in post-transcriptional events. Particularly, the U2 snRNP auxiliary (U2AF2), as a canonical RBP, has been reported to play an important role in the development, progression, and metastasis of several human cancers. However, the biological role of U2AF2 in the context of malignancies, including EC, has not yet been reported. This study is aimed to investigate the role and mechanism of U2AF2 in esophageal adenocarcinoma cell line.

Methods

The expression level of U2AF2 and SNORA21 was analysed by TCGA database. The expression level of U2AF2 and SNORA21 in esophageal adenocarcinoma cells was determined by Western Blot and RT-qPCR assay. The cell viability and proliferation was detected by CCK8 assay and BrdU incorporation assay. The apoptosis was examined by caspase3 immunocytofluorescense. The protein levels of Bax, Bcl-2 and E-cadherin were explored by Western Blot assay. The interaction between U2AF2 and SNORA21 was predicted by Starbase 3.0 and confirmed by RIP assay. The RNA regulated by U2AF2 was through RNA-seq analysis on the basis of downregulation of U2AF2.

Results

The expression of U2AF2 and SNORA21 was found to be upregulated and served as an unfavourable factor which negatively related with overall survival of esophageal adenocarcinoma patients. Silencing the expression of U2AF2 or SNORA21 could suppress the proliferation and migration of OE19 and OE33 cells and stimulate its apoptosis. Moreover, we identified that the U2AF2 could bind and interact with SNORA21 to sustain its stability. Furthermore, we show that overexpression of SNORA21 could rescue the proliferation inhibition phenotype caused by si-U2AF2.

Conclusion

Our study provides several lines of evidence supporting the intriguing concept that U2AF2 could promote the proliferation of OE19 and OE33 cells by directly targeting SNORA21, which might serve as a novel candidate biomarker and a new target for the treatment of esophageal adenocarcinoma.

Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer-related death worldwide and thus becoming a main global health concern[1, 2]. EC mainly includes two subtypes: squamous cell carcinoma (ESCC) and adenocarcinoma (EAC)[3]. Previously, ESCC was considered to be the pathological type with the highest incidence in the world outside of the United States, accounting for about 90% of the total incidence[4]. However, recent years, there has been a sharped and steady rise in the morbidity of esophageal adenocarcinoma in the western world[5]. Especially in United states, Australia, United Kingdom and Western Europe, the adenocarcinoma gains dominance with squamous carcinoma moving to second place[6]. The 5-year survival rate of patients with EC is still between 15–25%, although the treatment strategies for EC have been improved [7]. As such, it is urgent to discovery new therapeutic targets for EC treatment.

RNA-binding proteins (RBPs) play an important role in post-transcriptional events, determining the fate and function of each transcript within the cell and maintaining the stability of the intracellular environment. They interact dynamically with other proteins mRNAs and long non-coding RNAs to create a ribonucleoprotein complexes, which is a functional unit to regulate RNA alternative splicing, polyadenylation, stability, localization, translation, and degradation[8, 9], etc. Previous evidence has supported that RBPs are involved in several biological processes which contribute to tumour development, including sustained proliferation capacity[10], resistance of cell death[11], and enhanced invasiveness[11, 12]. Correspondingly, several studies reported that RBPs are dramatically overexpressed in tumorous tissues and increased expression of RBPs is negatively correlated with prognosis of patients. [13–15]. Thus, elucidating the intricate interaction networks of RBPs with their cancer-related RNA targets may help to understand their important role in tumorigenesis, development and treatment, and may reveal new targets for cancer therapy.

The U2 small nuclear RNA auxiliary factor (U2AF2, also called U2AF65), as a canonical RBP in conjunction with U2AF35 (also called U2AF1), which is found to play a key role in tumour development. In recent years, studies have shown that U2AF2 promotes the tumorigenesis, recurrence, metastasis, and poor survival in non-small cell lung cancer via the AKT/mTOR signalling pathway[16], as well as the colorectal cancer [17], melanoma [18]. However, the regulatory mechanism of U2AF2 and target RNAs in human cancers is remain unclear, especially in esophageal cancer. snoRNA is a kind of small non-coding RNAs which length is about 60-300nt. It has to do with the chemical modification of rRNA and act as a guide to post-transcriptional modification of rRNA and snRNA. snoRNA has a very conservative structure and falls into two main categories: C/D box snoRNA and H/ACA box snoRNA. Recent evidence suggested that snoRNA was identified to participate in many other physiological and pathological processes[19], especially involved in tumour invasion, metastasis, angiogenesis and proliferation.

Aiming to explore the function of U2AF2 in esophageal cancer, we firstly found that U2AF2 expression is upregulated in esophageal adenocarcinoma tissues compared with normal tissues, and the higher expression of U2AF2 was dramatically correlated with poor overall survival compared to lower U2AF2 expression. Knockdown the U2AF2 could inhibit the proliferation and migration and promote apoptosis of OE19 and OE33 esophageal adenocarcinoma cell line. Also, in order to explore the molecular mechanism

of U2AF2, we used RNA-Seq to detect changes in gene expression resulting from U2AF2 downregulation and identified the differentially expressed genes (DEGs) regulated by U2AF2. Then we searched the Starbase database and found that U2AF2 could interact with SNORA21, and SNORA21 was down regulated as shown in the result of RNA-sEq. The RNA Binding Protein Immunoprecipitation result showed that U2AF2 can bind with SNORA21 and sustain the mRNA stability of SNORA21. And knockdown of SNORA21 could inversely inhibit the proliferation, invasion and the enhance apoptosis rate. In addition, overexpression of SNORA21 could rescue the phenotype caused by si-U2AF2. Altogether these results demonstrated that U2AF2 could promote the proliferation of esophageal adenocarcinoma cells via binding with SNORA21.

Materials And Methods

U2AF2 silencing assay

siRNA clones for the *U2AF2* gene were downloaded from the Sigma website or designed using an online software SnapGene.

The U2AF2 silencing sequence was as follows:

5’- CCGCGCGGCCGUGAAGUGGGG-3

The SNORA21 silencing sequence was as follows:
5’-UUUCUUGACGGCUCAAUAGGU-3’

Cell culture and tissue

The OE19 and OE33 esophageal adenocarcinoma cell lines were acquired from American Type Culture Collection (ATCC). Both cell lines were maintained at 37°C in a cell incubator with 5% CO_2, and cultured in PRMI 1640 replenished with 10% fetal bovine serum (FBS), 1% streptomycin, and 1% penicillin. Four paired tumour and adjacent para-carcinoma tissues were collected from Renmin hospital of Wuhan university. And the study was permitted by the Institutional Ethics Committee of Renmin hospital of Wuhan University.

Transfection assay

Prepare plasmid mixture and PEI mixture, then mix the two mixtures, let stand for 30 minutes, then add to the cells, change the medium after 12 hours.

RNA Extraction

The OE19 and OE33 cells were scraped off from the plate in the first place. Using TRIzol reagent (Ambion) to extract total RNA. Using phenol chloroform to obtain high-purity RNA. Then the samples were dealed with RQ1 DNase (Promega, Madison, WI, USA) to eliminate contaminated DNA. By using Smartspec Plus (BioRad, USA), the absorbance of the sample at 260nm and 280nm (A260/A280) was

detected to evaluate the quality and concentration of the total RNA. Then, examining the integrity of the RNA on a simple native 1.5% agarose gel. RNA purification was also performed according to the manufacturer's instruction (NucleoSpin).

Library Preparation and High-Throughput Sequencing

By using the VAHTS Stranded mRNA-seq Library Prep Kit (Vazyme), 1μg RNA per sample was needed for RNA-seq library establishment according to the manufacturer's instruction. Then using the Illumina HiSeq X Ten system to sequence the samples. The library was prepared from 200–500 bp size selected cDNA fragments and was sequenced to generate 150 nt paired-end sequences.

Western blot analysis

Using the RIPA lysis buffer (Pierce Biotechnology) to extract total proteins on the ice for 30 min, then 100°C for 10 min with loading buffer. The proteins of different molecular weights were separated on a 10% SDS-PAGE gel, and then transferred to a PVDF membrane. The latter was then blocked in 10% skimmed milk for 1h and incubated with the primary antibodies of U2AF2 (1:1000, A1936, AbClonal) and GAPDH (15000, AC002, ABclonal) at 4°C overnight. Then washing three times with TBST buffer for 10 minutes each time and subsequently incubating with HRP-conjugated secondary antibodies (ABclonal) at room temperature for 1h. Afterwards, washing the membrane three times with TBST for 10 min each time. Finally, protein expression was analysed using chemiluminescence and normalized to GAPDH.

Differentially Expressed Genes (DEG) analysis

Using the EB-Seq (Differential expression analysis for sequence count data) algorithm to assess the expression values of different genes between the two groups. Using R Bioconductor package edgeR [20] to filter out the DEGs. Genes with FDR of < 0.05 and fold change of > 2 or < 0.5 were considered DEGs.

Transwell assay

The transwell plate diam of 6.5mm (Costar, pore size 8.0μm, Corning) was used for Transwell assay. Starving the esophageal adenocarcinoma cells 30 min with Earle"s Balanced Salt Solution (EBSS) at first. We put 20,000 cells in each transwell plate with non FBS in the upper and 10% FBS in the below. After 48h, wiping the cells in the upper of the transwell plate and washing the cells under the transwell plate, then fix the cells 10 min with 4% PFA. After washing 4 times with colding PBS, 5 min per times, we dye the cells with crystal violet for 10 min. Finally washing the cells 3 times with ddH_2O, 5min per times. Take a microscope and count the cells passing through the transwell plate.

CCK8 assay

The Cell Counting Kit-8 (Beyotime) was used to do CCK8 assay to explore the proliferation rate of OE19 and OE33. Adding 2000 cells per well in a 96-well plate, three replicate holes for each sample, which cultured in a cell incubator for 24h, 48h, 72h and 96h. Then add 10% CCK-8 solution to each well at each time node, and sequentially incubate for 30min-1h in the cell culture. Finally, using a multifunctional

microplate reader to measure the absorbance of each well at 450nm and calculate the rate of inhibition of cell proliferation in the laboratory relative to the control group.

Immunocytofluorescense

Washing the cell with colding PBS and fixing the cells 10min with 4% PFA. Then washing 4 times with colding PBS, 5min per time, subsequently incubate 40min with blocking fluid. Then incubate the primary antibody (1:100) overnight at room temperature, recycle the primary antibody. Washing the cells three times with colding PBS, 5min per times. Afterwards, incubate second antibody (1:1000) 2h at room temperature. Washing the cells with colding PBS once again. In the end, DAPI staining for 10min.

BrdU staining assay

Prepare a 10mM stock solution of BrdU by dissolving 3mg of BrdU in 1mL aseptic ddH_2O. Dilute the 10mM BrdU stock solution in cell culture medium to make a 10uM BrdU labelling solution. Filter the 10 uM BrdU labelling through a 0.2uM filter under sterile conditions. Remove the existing culture medium from the cells and replace with 10uM labelling solution. Incubate the cells in the BrdU labelling solution for 2 hours at 37℃ in a CO_2 incubator. Remove the BrdU labelling solution from the cells and wash twice in PBS for about 5 seconds per wash. Wash three more times with PBS for two minutes each. Incubate cells in 1M HCL for 30 minutes at room temperature. Then wash three times in PBS. And incubate 40min with blocking fluid. Then continue with immunostaining according to standard immunocytochemistry protocols.

RNA stability measurement

The cells were cultured in the medium which was contained 2μg/mL actinomycin D to impede the de novo RNA synthesis. And the expression of SNORA21 was detected by RT-qPCR at different times. The half-life of SNORA21 is defined as the time it takes for RNA expression levels to degrade to 50% of their initial level in the presence of actinomycin D treatment.

RNA immunoprecipitation (RIP) assay

The EZ-magna RIP Kit (Millipore) and primary antibodies against AGO2 and IgG (Millipore) were used to conduct the RIP assay according to the manufactures' instructions. Cells in different experimental groups were lysed using RIP lysis buffer, which was subsequently conjugated with IgG or AGO2. After digesting by proteinase K, we extracted the RNA for analysis by RT-qPCR assay.

Reverse transcription qPCR assay

In order to further verify the relative expression of U2AF2 and SNORA21, quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was conducted. Using M-MLV Reverse Transcriptase (Vazyme) reversed transcribe RNA into cDNA. Using the StepOne Real Time PCR System and SYBR Green PCR kit (Yeasen) to perform real-time PCR. The following PCR cycling conditions were 95°C for 10 min, subsequently 40 cycles of 95°C for 15 s, and annealing and extension at 60°C for 1 min. Repeat 3

replicate wells for each sample. The RNA expression levels were normalized to GAPDH. The primers of GAPDH were forward 5'-CATCATCCCTGCCTCTACTGG-3', reverse 5'-GTGGGTGTCGCTCTTGAAGTC-3'. The primers of SNORA21 were forward 5'-AGCAGTCAGTAGTTGGTCCTTTG-3', reverse 5'-CCATCAGTCCCGTCTTGAAAC-3'. The primers of U2AF2 were forward 5'-TACGGGCTTGTCAAGTCCATCG-3', reverse 5'-CTGGCAGTCAAACACAGAGGTG-3'.

Statistical analysis

All data was presented as mean ± SEM. Statistic difference between groups was analysed using Student's t-text. The Kaplan-Meier method was used to draw the survival curve, and the log-rank test was used for analysis. All reported *P* values were two-sided and $P < 0.05$ was considered statistically significant.

Results

1. U2AF2 mRNA levels correlate with poor prognosis in EAC

To investigate whether U2AF2 plays a role in EC, we firstly downloaded RNA-seq data for all existing esophageal related samples in the Cancer Genome Atlas (TCGA) database, including EAC tissue samples and normal tissue samples and we then calculated the difference in U2AF2 expression level between esophageal cancer tissues and normal esophageal tissues. Surprisingly, we found that U2AF2 was overexpressed in 89 EAC samples when compared to 10 normal tissue samples (Fig. 1.A). Meanwhile, among the EAC samples, the lower expression of U2AF2 had a better OS (Fig. 1.C). In addition, we further classified the esophageal cancer samples to different stages, which include IA, IIA, III, IIIA, IIIB, IIIC and IV, IVA. The esophageal cancer was staged according to the standards of the eighth edition of the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) classification. We selected at least 5 samples in each phase of EAC to analyse the relationship between U2AF2 expression and OS (Fig. 1.B). We further examined the U2AF2 expression in four esophageal adenocarcinoma tissues compared to para-carcinoma tissue, we found that U2AF2 was overexpressed in esophageal adenocarcinoma tissues (Figure. 1D). Statistical analysis of D (Fig. 1.E)

2. Silencing of U2AF2 suppress the proliferation, migration of OE19 and OE33 cells and promotes apoptosis

In order to identify the role of U2AF2 in esophageal adenocarcinoma cells, we knocked it down in OE19 and OE33 cells. The expression of U2AF2 was significantly downregulated compared with the control group. (Figure.2A). The cell viability was dramatically suppressed in the shU2AF2 group compared with the control by CCK8 assay in OE19 and OE33 cells in a time dependent manner (Figure.2B, C). The enhanced proliferate rate was significantly reduced in the shU2AF2 group when it comes to control by BrdU incorporation assay in OE19 and OE33 cells (Fig. 2.D, E, F, G). In addition, the apoptosis rate was higher due to downregulation of U2AF2 in OE19 and OE33 cells presented as the higher caspase 3 foci in the shRNA group (Fig. 2.H, I). Moreover, we observed a significance reduction of OE19 and OE33 cells

migration rate in the shU2AF2 group compared with the control (Fig. 2.J, K). In order to further conform the results above, western blot assay was used. The protein level of Bax and E-cadherin was significantly increased by shU2AF2 and the Bcl-2 was decreased in both OE19 and OE33 cells (Fig. 2.L, M). In a word, all the results presented here demonstrated that the absence of U2AF2 has a critical impact on the proliferation and migration, apoptosis of OE19 and OE33 cells.

3. U2AF2 could bind and sustain the mRNA stability of SNORA21

RBPs are known to bind and interact with RNAs to regulate the letter expression to play an important role in tumour progression. To identify candidate genes directly regulated by U2AF2, we analysed RNA-seq results which was on the basis of U2AF2 knockdown demonstrated that SNORA21 was downregulated (Fig. 3. B). Through searching the starbase 3.0 software, we found that there were two binding sites between U2AF2 and SNORA21, which is 5'-CCACAGGCC-3',5'-AUUGAAGCAAACAACACAUGUUGCC-3' (Fig. 3. A). In addition, quantification of the expression levels of U2AF2 and SNORA21 indicated that when the U2AF2 expression was upregulated, the expression of SNORA21 was enhanced. Synchronously, the downregulation of U2AF2 also impede the SNORA21 expression in both OE19 and OE33 cells (Fig. 3.C, D, E, F). To further verify the interaction between U2AF2 and SNORA21, then, the RIP assay results showed that U2AF2 knockdown reduced the enrichment of SNORA21 in OE19 and OE33 cells, while upregulation of U2AF2 expression further enhanced the enrichment of SNORA21(Fig. 3.G, H, I, J). Interestingly, we found that the half-life of SNORA21 was significantly shortened following U2AF2 down expression through RNA stability measurement assay in both OE19 and OE33 cells (Fig. 3.K, L). Overall, the results suggested that U2AF2 can bind and interact with SNORA21 to sustain its stability.

4. SNORA21 expression was upregulated in esophageal cancer tissues and the absence of SNORA21 inversely reduced the proliferation, and migration of OE19 and OE33 cells and promotes apoptosis

TCGA database analysis suggested that SNORA21 was upregulated in esophageal cancer tissues (Figure.4A). To investigate the role of SNORA21 in OE19 and OE33 cells, we transfected si-SNORA21 to both OE19 and OE33 cells. The transfection efficiency was verified by RT-qPCR (Figure.4B). Interestingly, we found that the cell viability and proliferate was attenuated by downregulation of SNORA21 by CCK8 and BrdU incorporation assay in both OE19 and OE33 cells (Fig. 4. C, D, G, H, I, J). Also, the apoptosis rate was enhanced, accompanied by increased caspase 3 foci in both two cells (Fig. 4. E, F). Moreover, as expected, the migration rate was decreased because of the blockage of SNORA21 expression (Fig. 4. K, L). To further verify the above results, the western blot indicated that the protein expression level of Bax and E-cadherin was increased whereas the Bcl-2 was decreased when downregulation of SNORA21 (Fig. 4. M, N). These data suggested that si-SNORA21 can inhibit the proliferation and migration of esophageal adenocarcinoma cells and promotes apoptosis.

5. SNORA21 overexpression rescues the anti-proliferative, anti-migrative and pro-apoptotic phenotype of si-U2AF2

To explore the effect of the interaction between U2AF2 and SNORA21, we transiently transfected SNORA-OE plasmid to si-U2AF2 OE19 and OE33 cells. When the U2AF2 was knocked down, the proliferation and migration rate was reduced accompanied with increased apoptosis rate by CCK8, BrdU incorporation, transwell and caspase3 immunocytofluorescense assays. Simultaneously, when the SNORA21 was overexpression on the basis of downregulation of U2AF2, the proliferate, invasive and apoptosis rate was rescued by increasing the fraction of proliferating, migrated cells and reducing the fraction of apoptotic cells (Fig. 5. A-L). All the results indicated that SNORA21 overexpression can overcome OE19 and OE33 cell growth suppression induced by si-U2AF2.

Discussion

U2AF2 plays an important role in tumour progression. It is demonstrated upregulated in several kinds of cancers, and is corelated with prognosis. The stability and ubiquitination of U2AF2 is associated with the increased migration of melanoma[18]. And U2AF2 is upregulated in primary non-small cell lung tissues and largely associated its progression[16], and is also upregulated in colorectal tumours[21]. Consistently, supporting this theory, our study from four independent EAC tissues shown here presents that U2AF2 was overexpression in EAC, and was negatively corelated with OS of EAC patients.

It has been shown that the snoRNAs is frequently upregulated in tumours, and acted as a biomarker which was negatively corelated with survival. For example, SNORD33, SNORD66, and SNORD76, SNORD42 were overexpressed in NSCLC patients, which expression levels were oppositely associated with the survival of patients[22, 23]. SNORD42 and SNORD21 were also demonstrated to be promising predictive biomarkers for prognosis of CRC patients[24], so as SNORA23 in HCC[25]. Moreover, previous study proved that small nuclear RNA host gene expression was upregulated in ESCC tissues and cell lines, and inhibit which can impair the proliferation, invasion of cancer cells [26]. [27]. [28–30]. SNORA21 was demonstrated upregulated in gallbladder cancer[31], gastric cancer[32] and colorectal cancer[33], which is a predictor of poor survival of the latter two. Recently, more and more evidence has confirmed that overexpression of SNORA21 could inhibit the proliferation, migration and invasion and enhance apoptosis of gallbladder cancer cells. In addition, overexpression of SNORA21 could inhibit the growth of gallbladder tumours in vivo. However, the downregulation of SNORA21 could decrease the proliferation and invasion of colorectal cells. Unfortunately, the function of SNORA21 in esophageal cancer has not been investigated before. In our study, we found that U2AF2 has some binding sites with SNORA21, which indicated that SNORA21 might been regulated by U2AF2 to influence the progression of tumour. Moreover, our finding that SNORA21 was overexpression in EC, and loss of which could weaken the proliferation, migration, and enhance the apoptosis of esophageal adenocarcinoma cells is in line with previous studies. Further, we reported that U2AF2, a canonical RBP, could bind and sustain the stability of SNORA21 in OE19 and OE33 cells. And the phenotype caused by si-U2AF2 could reversed by overexpression SNORA21.

In summary, this is the first report found that U2AF2 could bind to SNORA21 to promote the proliferation, migration of esophageal adenocarcinoma cells and inhibit the apoptosis rate of cells, which raise the

possibility that targeted inhibition of U2AF2/SNORA21 axis might provide insight into esophageal adenocarcinoma treatment. However, as a clinical marker for clinical diagnosis and prognosis, the role of U2AF2 has not been confirmed. we will further explore the expression of the U2AF2 and SNORA21 in a large amount of esophageal cancer tissues to identify its efficiency in tumour diagnosis. Moreover, *U2af2* knock-out mice may be utilized to explore its function in esophageal cancer. The aim of these future studies would be to utilize U2AF2 for the prompt detection of the occurrence of tumours and early initiation of appropriate treatment to improve the survival of esophageal adenocarcinoma patients.

Conclusions

Our study provides several lines of evidence supporting the intriguing concept that U2AF2 could promote the proliferation of OE19 and OE33 cells by directly targeting SNORA21, which might serve as a novel candidate biomarker and a new target for the treatment of esophageal adenocarcinoma.

Declarations

Ethics approval and consent to participate: All methods were carried out in accordance with relevant guidelines and regulations of Renmin hospital of Wuhan University. All experimental protocols were approved by Renmin hospital of Wuhan University. Informed consent was obtained from all subjects.

Consent for publication: Not applicable.

Availability of data and materials: The datasets generated and analysed during the current study are available in the TCGA and Starbase repository.

Competing interests: None

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Authors' contributions: Chen Yongshun have made substantial contributions to the conception, Gao Lijuan contributes to the design of the work and drafted the work and substantively revised it. Li Bin contributes to the creation of new software used in the work. All authors read and approved the final manuscript.

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Figures

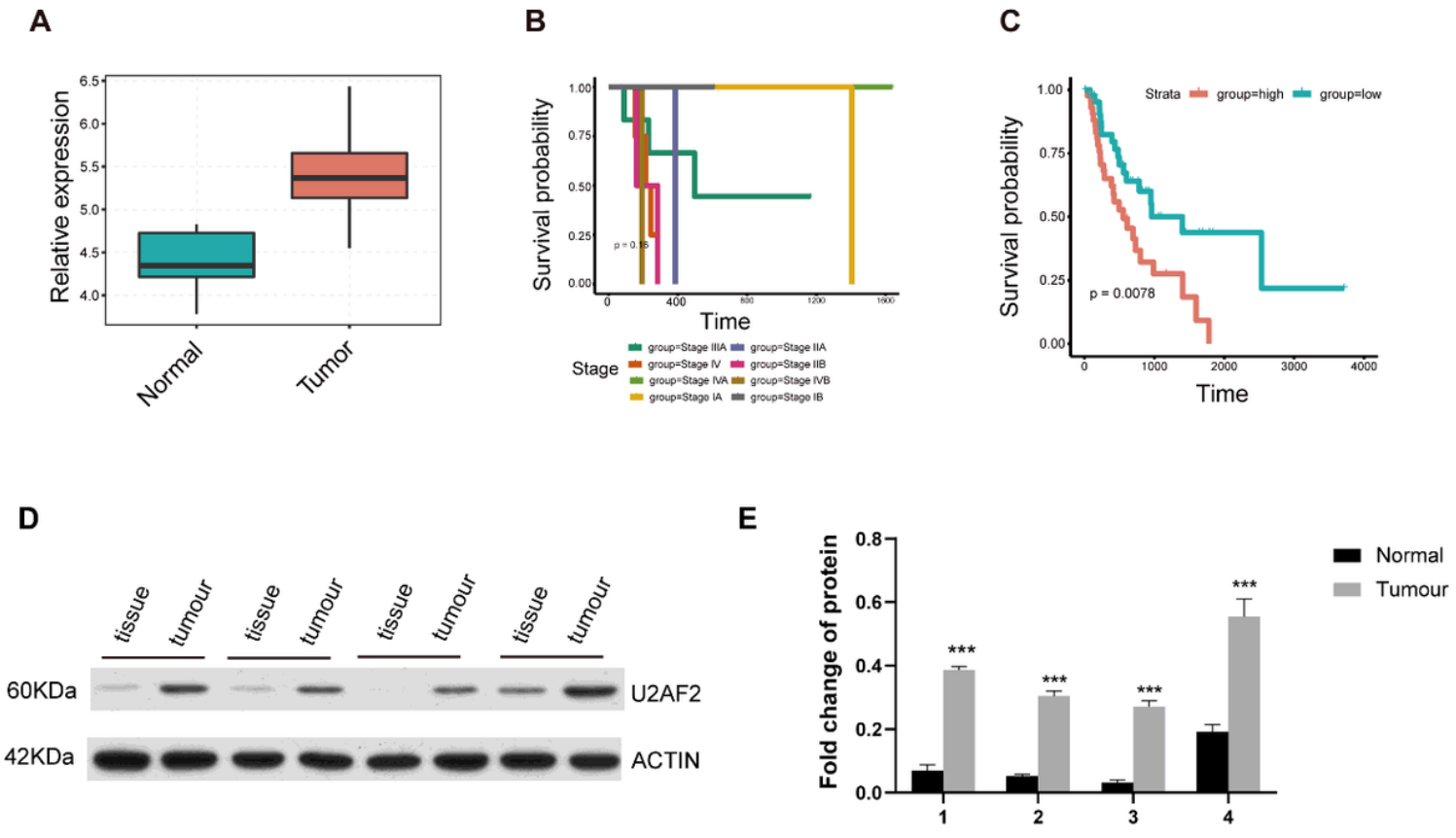

Figure 1

U2AF2 mRNA levels correlate with poor prognosis in EAC. (A) U2AF2 expression in normal and EAC samples. The data was downloaded from the TCGA database. (B, C) The survival curves of EAC patients, and the differences in U2AF2 expression correlated with the survival of EAC patients. (D) The U2AF2 expression level of EAC tissues and para-carcinoma tissues. The grouping of gels was cropped from different parts of the same gel. (E) Statistical analysis of the protein level in D. Error bars represent the mean±SEM. Statistical analysis was generated from unpaired t-test, asterisks denote statistical significance as *p□0.05, **p□0.01, ***p□0.001.

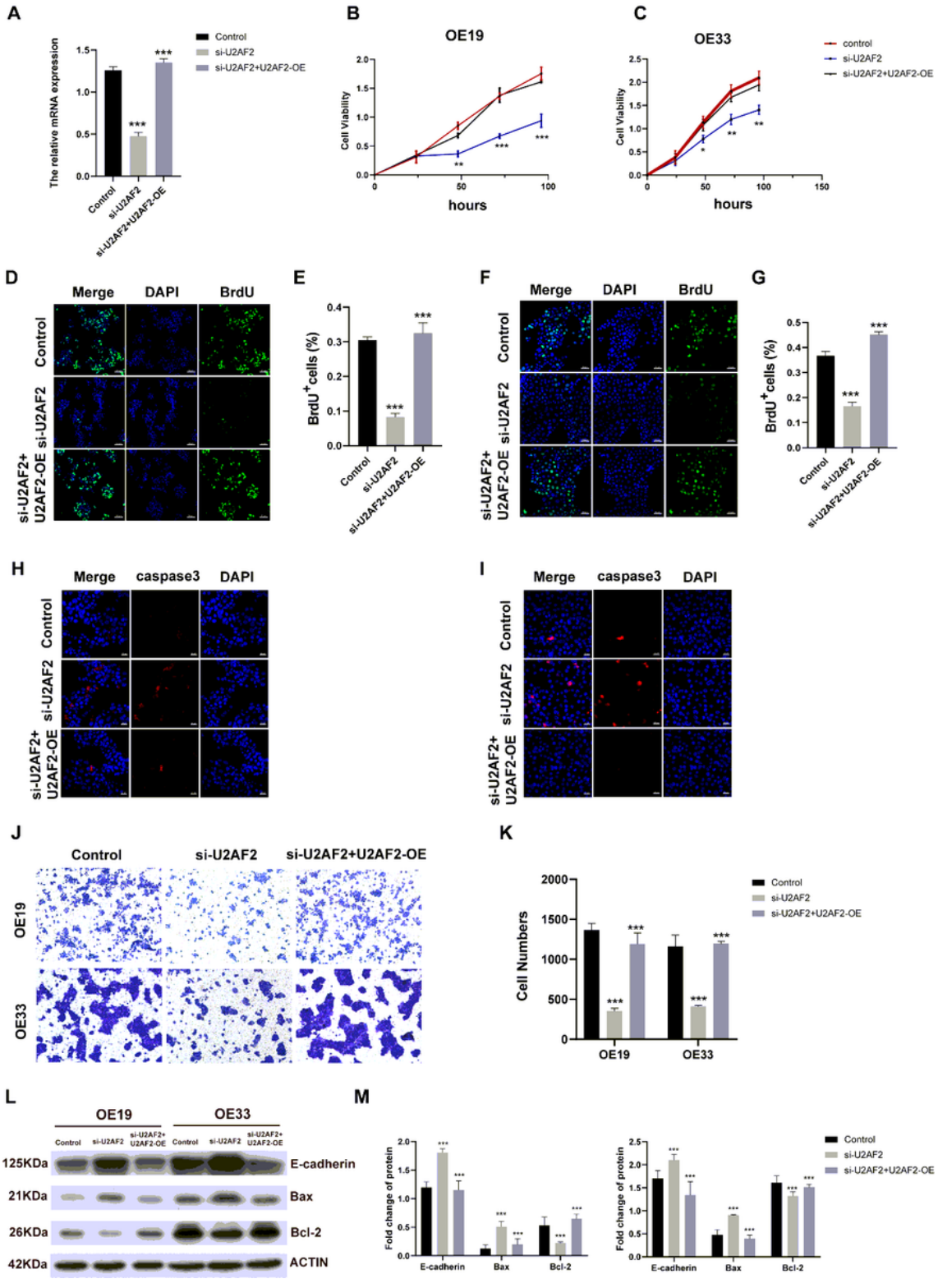

Figure 2

Silencing of U2AF2 suppress the proliferation, migration of OE19 and OE33 cells and promotes apoptosis. (A) The transfection efficient was verified by qRT-PCR assay. (B, C) The cell viability after silencing U2AF2 was detected by CCK8 assay. (D, F) The proliferation rate was explored by BrdU incorporation assay. (E, G) Statistical analysis of the BrdU positive cells' percentage. (H, I) The apoptosis rate was invested by caspase 3 immunofluorescence assay. (J) The migratory ability was detected by transwell assay. (K) Statiscal analysis of the cell numbers which migrate through the membrane. (L) The protein level of Bax, Bcl-2, E-cadherin between control group and si-U2AF2 group was detected by western blot assay. The E-cadherin, Bax, Actin was cropped from different parts of the same gel, and Bcl-2 was cropped from different gels. (M) Analysis Statistical analysis of the protein level in L. Error bars represent the mean±SEM. Statistical analysis was generated from unpaired t-test, asterisks denote statistical significance as *p□0.05, **p□0.01, ***p□0.001.

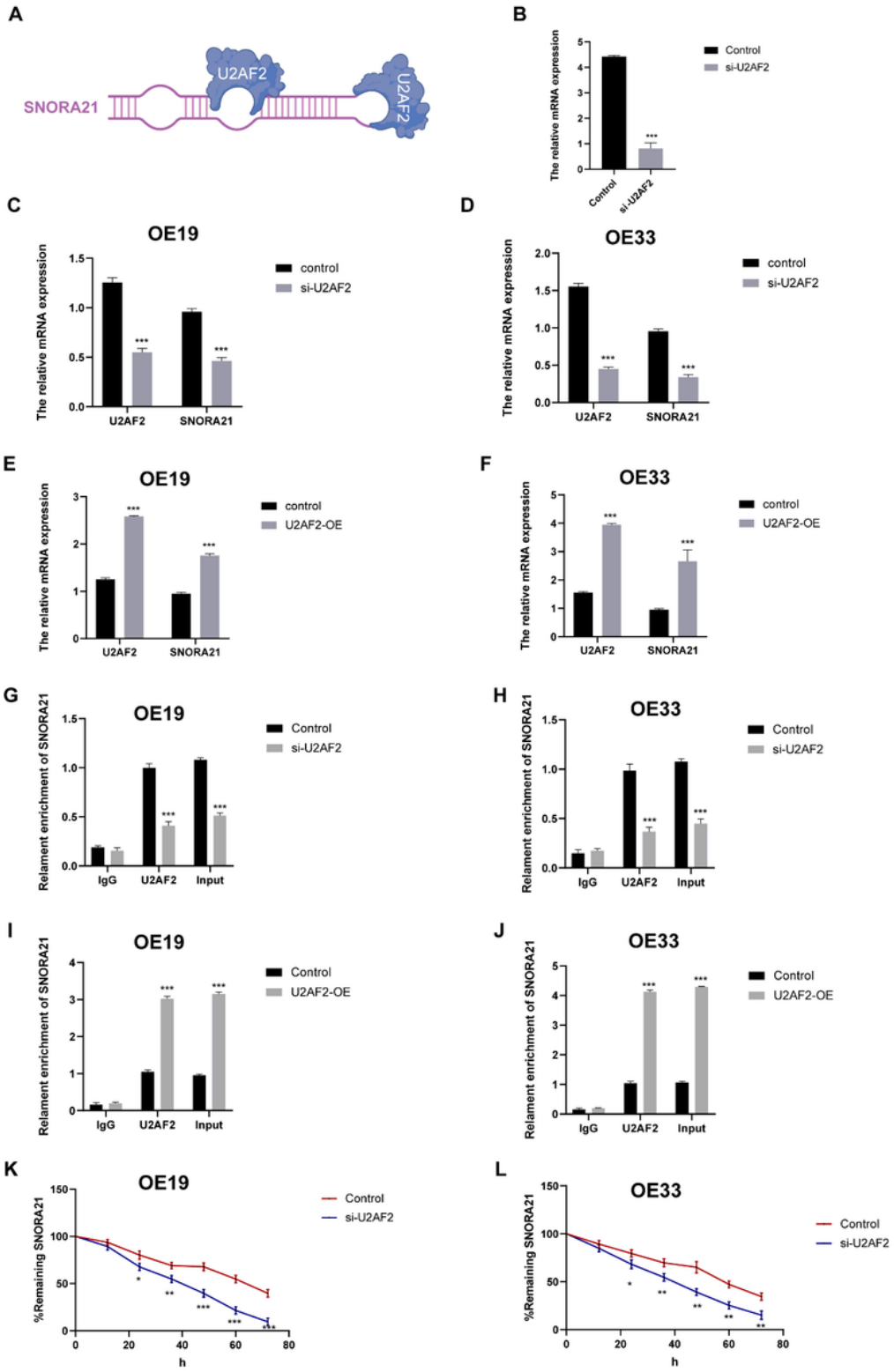

Figure 3

U2AF2 could bind to and sustain the mRNA stability of SNORA21. (A) there were two binding sites between U2AF2 and SNORA21. (B) The relative expression of SNORA21 in RNA-seq by knockdown of U2AF2. (C, D) The relative expression of SNORA21 after si-U2AF2 in OE19 and OE33 cells, which was conducted by RT-qPCR assay. (E, F) The relative expression of SNORA21 after U2AF2-OE in OE19 and OE33 cells, which was conducted by RT-qPCR assay. (G, H, I, J) The binding and interaction between

U2AF2 and SNORA21 was confirmed by RIP assay. (K, L) The half-life time of SNORA21 by shU2AF2 was detected by RNA stability measurement assay. Error bars represent the mean±SEM. Statistical analysis was generated from unpaired t-test, asterisks denote statistical significance as *p□0.05, **p□0.01, ***p□0.001.

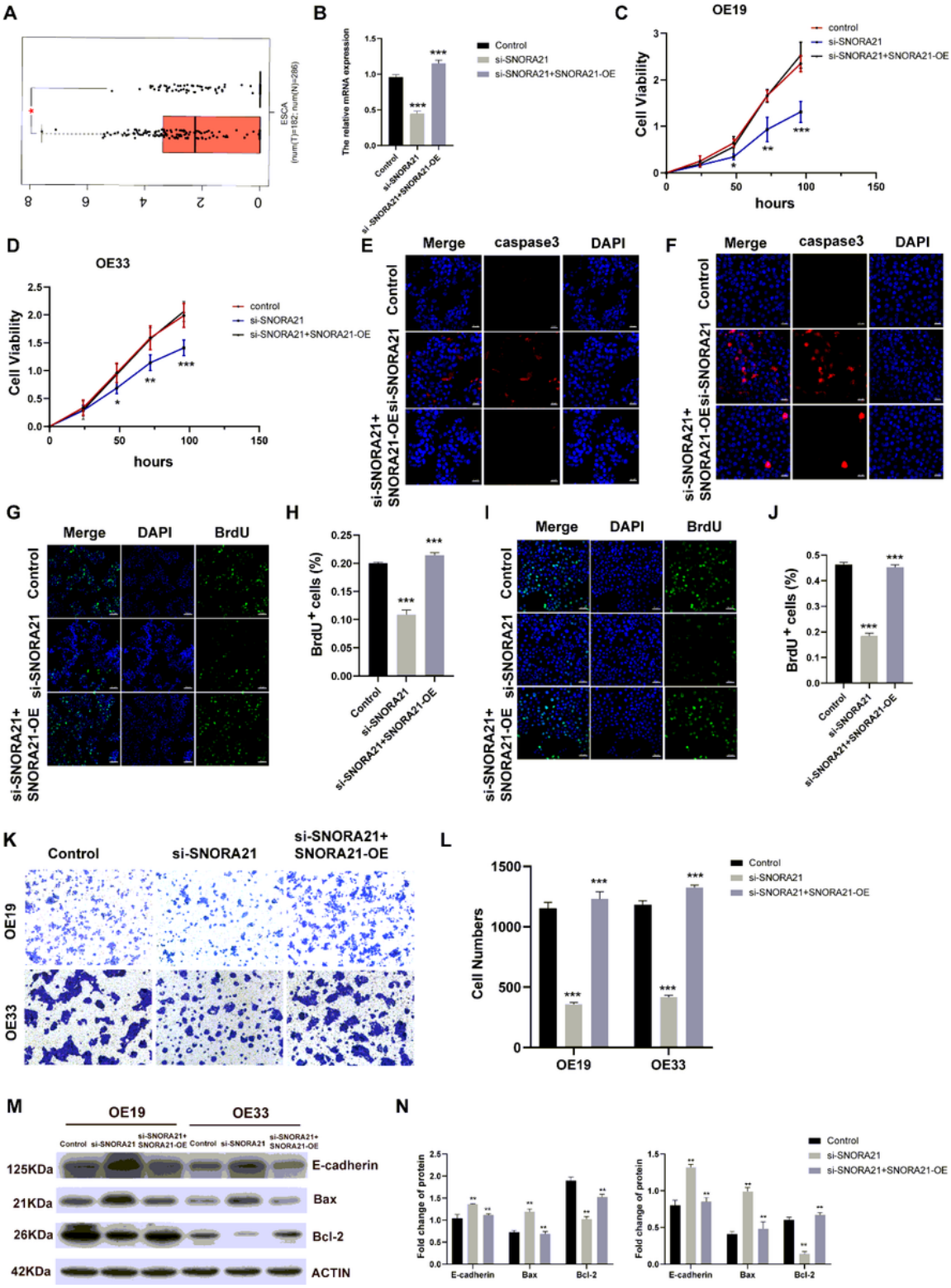

Figure 4

SNORA21 expression was upregulated in esophageal cancer tissues and the absence of SNORA21 inversely reduced the proliferation, and migration of OE19 and OE33 cells and promotes apoptosis. (A) TCGA data analysis of SNORA21 expression in EC tissues. (B) Transfection efficient of si-SNORA21 and SNORA21-OE plasmid was detected by RT-qPCR. (C, D) The cell viability in the different groups was detected by CCK8 assay. (G, I) The proliferation rate was explored by BrdU incorporation assay. (H, J) Statistical analysis of the BrdU positive cells' percentage. (E, F) The apoptosis rate was investigated by caspase 3 immunofluorescence assay. (K) The migratory ability was detected by transwell assay. (L) Statistical analysis of the cell numbers which migrate through the membrane. (M) The protein level of Bax, Bcl-2, E-cadherin between different groups was detected by western blot assay. The E-cadherin, Bax, Actin was cropped from different parts of the same gel, and Bcl-2 was cropped from different gels. (N) Statistical analysis of protein level in M. Error bars represent the mean±SEM. Statistical analysis was generated from unpaired t-test, asterisks denote statistical significance as *p□0.05, **p□0.01, ***p□0.001.

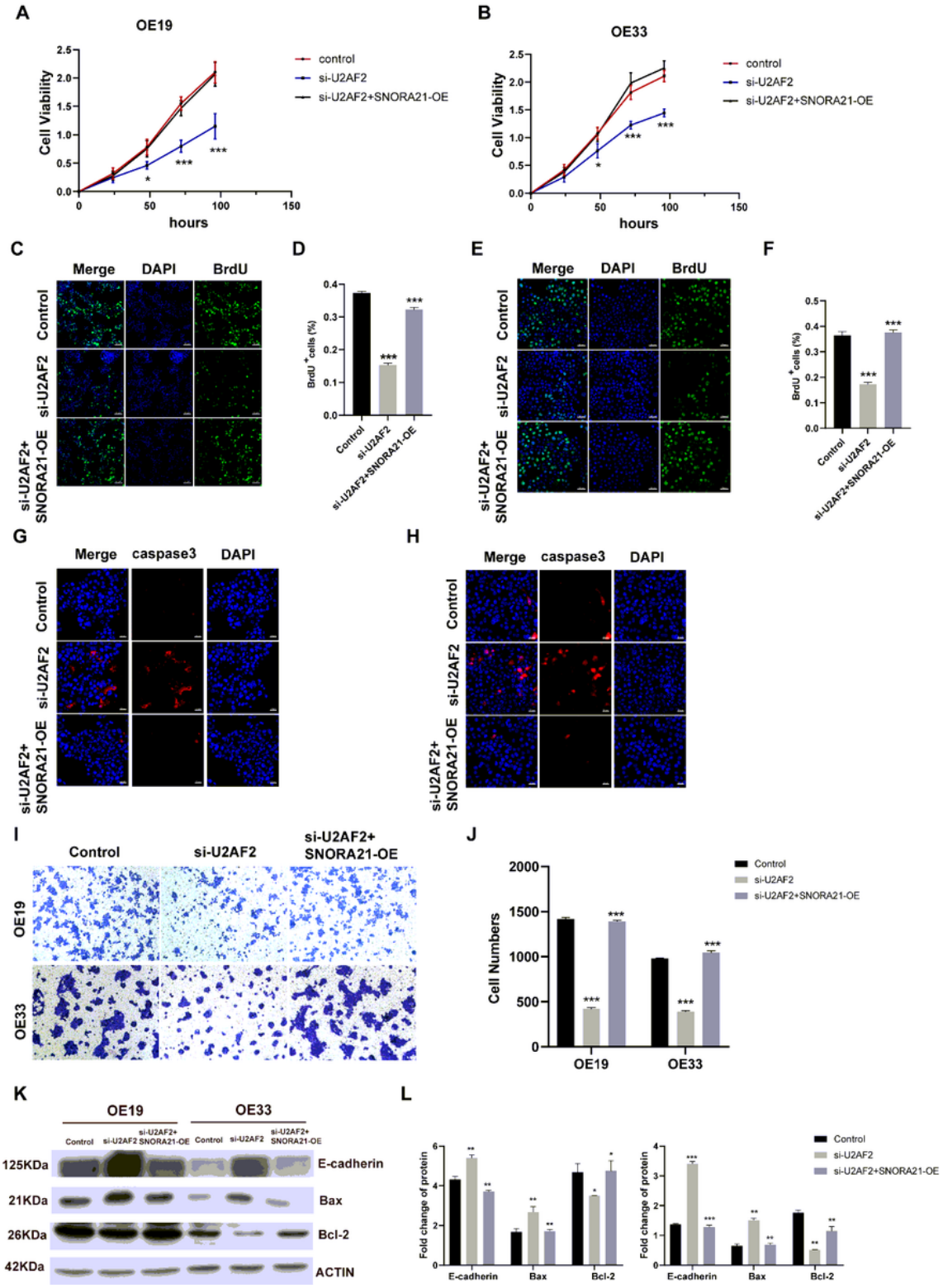

Figure 5

SNORA21 overexpression rescues the anti-proliferative, anti-migrative and pro-apoptotic effects of si-U2AF2. There were three groups control, si-U2AF2+SNORA21-OE, si-U2AF2. (A, B) The cell viability in the different groups was detected by CCK8 assay in OE19 and OE33 cells. (C, E) The proliferation rate of all groups was explored by BrdU incorporation assay. (D, F) Statistical analysis of the BrdU positive cells' percentage. (G, H) The apoptosis rate of all groups was investigated by caspase 3 immunofluorescence

assay. (I) The migration ability of all groups was detected by transwell assay. (J) Statistical analysis of the cell numbers which migrate through the membrane. (K) The protein level of Bax, Bcl-2, E-cadherin between different groups was detected by western blot assay. The E-cadherin, Bax, Actin was cropped from different parts of the same gel, and Bcl-2 was cropped from different gels. (L) Statistical analysis of protein level in K. Error bars represent the mean±SEM. Statistical analysis was generated from unpaired t-test, asterisks denote statistical significance as *p□0.05, **p□0.01, ***p□0.001.