Neoplasma 2024; 71(2): 180–192

doi:10.4149/neo_2024_240116N24

Calreticulin regulates the expression of MMP14 and ADAR1 through EIF2AK2 signaling to promote the proliferation and progression of malignant melanoma cells

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Received January 16, 2024 / Accepted March 18, 2024

It has been demonstrated that calreticulin (CALR) is expressed abnormally in various tumors and is involved in the occurrence and development of tumors. In this study, CALR and EIF2AK2 expression was measured in the clinical specimens of 39 patients with melanoma. Then, we constructed knockdown and overexpression cell models of CALR and EIF2AK2 and used wound healing and Transwell assays to observe cell migration and invasion. Apoptosis, EDU, and ROS assays were used to measure cell apoptosis and proliferation, as well as ROS levels. The effect of CALR on endoplasmic reticulum stress was detected using endoplasmic reticulum fluorescent probes. Western blotting was used to detect protein levels of CALR, EIF2AK2, ADAR1, and MMP14. The results indicated that CALR and EIF2AK2 expression levels were significantly higher in human melanoma tissues than in adjacent non-tumor tissue. In addition, we found a correlation between CALR and the expression of EIF2AK2 and MMP14, and the experimental results indicated that overexpression of CALR significantly upregulated the expression of EIF2AK2, MMP14, and ADAR1, while knockdown of CALR inhibited their expression. Notably, the knockdown of EIF2AK2 in the CALR overexpression group blocked the upregulation of MMP14 and ADAR1 expression by CALR, and the knockdown of both CALR and EIF2AK2 significantly inhibited MMP14 and ADAR1 expression. In conclusion, CALR and EIF2AK2 play a promoting role in melanoma progression, and knockdown of CALR and EIF2AK2 may be an effective anti-tumor target, and its mechanism may be through MMP14, ADAR1 signaling.

Key words: CALR; EIF2AK2; malignant melanoma; progression

Melanoma is a tumor originating from malignant melanocytes, characterized by high-grade malignancy and rapid progression [1, 2]. Malignant melanoma (MM) represents approximately 4.0% of all skin cancers, and exhibits an extremely poor prognosis, with a dramatic increase in recurrence over a 5-year follow-up period and a less than 10% 5-year survival rate [3, 4]. Furthermore, it demonstrates a propensity for metastasis to the small intestine while also exhibiting affinity for accumulation in the stomach and large intestine [5, 6]. Therefore, early diagnosis and intervention are imperative for MM management [7, 8]. Despite significant advancements in high-throughput analysis in various tumors, the genetic diagnosis of melanoma still lags behind [9]. Recent studies have demonstrated a high number of somatic genome mutations associated with melanoma [10–12]. Therefore, it is crucial to explore new specific molecular mechanisms and potential molecular biomarkers for early diagnosis and clinical treatment of melanoma [13].

The endoplasmic reticulum (ER) homeostasis may be disrupted by physiological and pathological effects, leading to the accumulation of misfolded and unfolded proteins in the ER lumen, a situation known as ER stress [14]. In recent years, the role of adaptive ER stress in the occurrence, progression, and drug resistance of melanoma has been well confirmed, and this compartment has been identified as a potential therapeutic target [15]. Calreticulin (CALR), an ER-chaperone protein, plays a vital role in cellular homeostasis in healthy cells [16, 17]. Accumulating evidence suggests that CALR is involved in tumorigenesis and tumor inhibition across different types of cancer [16, 18, 19]. For example, loss-of-function CALR mutations impair anticancer immunosurveillance and promote oncogenesis

while gain-of-function of CALR mutations rapidly worsen the disease course of acute lymphoid leukemia [16, 20]. Therefore, CALR may be a protein of interest in the outcome of melanoma and plays an important role in ER stress.

Eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2) is a cellular stress kinase activated by ER stress [21]. EIF2AK2 plays a role in regulating cellular processes such as cell proliferation and differentiation, signal transduction, and apoptosis [22]. Studies have shown that EIF2AK2 shRNA downregulates EIF2AK2 expression in B16-F10 melanoma cells and reduces the metastatic potential of these tumor cells [22]. EIF2AK2 and GCN2 are key mediators of eIF2a phosphorylation, promoting the translocation of CALR on the surface of melanoma cells under drug stimulation [15]. However, the relationship between CALR and EIF2AK2 during ER stress is not clear.

Matrix metalloproteinases (MMPs) are a family of enzymes that degrade proteins in the extracellular matrix of tumor cells [23]. MMPs are widely involved in various stages of cancer progression, including degradation of tumor cell basement membranes and stroma. ER stress induces an increase in MMP9 gene expression and precursor secretion. When ER stress is inhibited, it can reduce the expression, secretion, and activity of MMP9 gene [24]. In cells transfected with Ad-CALR/MAGE-A3, the expression of MMP2 and MMP9 was downregulated, which may indicate that these MMPs are downstream products of cellular signaling induced by CALR and MAGE-A3 [23]. MMP14, a membrane-bound, plays an essential role in tissue homeostasis and development [25]. MMP14, as the main collagenase in skin homeostasis, is associated with poor prognosis in different types of cancer in terms of expression and activity. In addition to maintaining tissue homeostasis, MMP14 is upregulated in most tumors, including breast cancer, ovarian cancer, and melanoma, and promotes tumor progression by regulating tumor cell proliferation, invasion, and migration [26]. However, there are still few reports on research on MMP14 and ER stress.

Matrix metalloproteinase was detected as an aberrantly expressed gene along with ADAR1 in melanoma cells [27]. Adenosine deaminase acting on RNA 1 (ADAR1) is a group of RNA editing enzymes that catalyze the deamination of double-stranded RNA adenosine to produce hypoxanthine [28]. Knockdown of ADAR1 increases MMP2 and MMP9 mRNA stability and inhibits aortic wall mechanism degradation [29]. ADARs have multiple functions, such as editing protein-coding regions to cause changes in protein function, controlling mRNA levels and translation efficiency, controlling tissue development and hematopoiesis, etc. [30]. The catalytic activity of ADAR1 is essential for maintaining ER homeostasis [31]. It has been shown that ADAR1 deficiency triggers ER stress, leading to the loss of intestinal stem cells and disturbing the stability of the intestinal internal environment [32].

In this study, we first detected the expression of CALR in tumor tissue and adjacent tissues of 39 melanoma patients. We established a cell model of CALR overexpression and knockdown to investigate the effects of CALR function acquisition or loss on cell proliferation, migration, invasion, apoptosis, ROS levels, and ER stress in melanoma. In addition, the mechanistic relationship between CALR and the expression of EIF2AK2 and ADAR1 in malignant melanoma was explored to identify potential new targets for the treatment of the disease.

Patients and methods

Bioinformatical analyses. The correlation between CALR and EIF2AK2 or CALR and MMP14 was assessed using the online bioinformatics tool GEPIA (http://gepia.cancer-pku.cn/).

Specimen of human pathological tissue. Tumor tissues and paracancer tissues of 39 melanoma patient clinical specimens were collected from the General Hospital of Ningxia Medical University from March 4, 2022 to April 6, 2023. Among the 39 tissue samples, 23 were male and 16 were female. There were 18 cases older than 65 years old and 21 cases younger than or equal to 65 years old.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was guaranteed by the Ethics Committee of Ningxia Medical University (No. KYLL-2021-639).

Immunohistochemical and immunofluorescent staining. Melanoma tissue and adjacent cancer tissue were taken as clinical specimens, and the specimens were fixed in formalin and then embedded in paraffin. The specimens that were cut into 4 μm thicknesses were deparaffinized using xylene and the antigen was restored under high pressure. For immunohistochemistry, the specimens were blocked with 3% hydrogen peroxide at 37 °C for 20 min, and goat serum was used for immunofluorescence and then incubated at 4 °C overnight with anti-CALR (1:200, Cat. 27298-1-AP, Proteintech, China) and anti-EIF2AK2 (1:100, Cat. 18244-1-AP, Proteintech, China) as primary antibodies, at 4 °C overnight. The following day, for immunohistochemical assay, samples were incubated with a second antibody (PV-6000; ZSGB-BIO, China) for 20 min at 37 °C. After staining with 3,3-diaminobenzidine (DAB) and hematoxylin, the slides were sealed with neutral resin and observed under a light microscope. For immunofluorescence, the slides were incubated with a secondary antibody (Alexa Fluor® 488 Goat Anti-Rabbit IgG) at a dilution of 1:200 for 1 h at 37 °C. The sections were then stained with 4,6-diamidino-2-phenylindole (DAPI) for fluorescence observation.

The staining intensity score criteria were as follows: 0, no staining; 1, light yellow staining; 2, yellow staining; and 3, brown staining. The following scores were assigned for different percentages of tumor-positive tissues: 0, negative; 1, 1–25% positive tissues; 2, 25–50%; and 3, >50%. The staining intensity, percentage of positive samples, and tumor grades were scored between 0 and 9 (with 0 indicating a lack

of brown immunoreactivity and 9 reflecting intense dark brown staining) and divided into the following categories: 0–1, negative; ≥2, +; 3–4, ++; and ≥ 5, +++, corresponding to low, moderate and high expression, respectively.

Cell culture and transfection. Human malignant melanoma cell lines A375 were obtained from the Cell Libraries of the Chinese Academy of Sciences (Cat. SCSP-533, Shanghai, China). The authenticity of A375 cells was confirmed by the short tandem repeat (STR) analysis. A375 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cat. 11960044, Invitrogen) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco), GlutaMAX (Cat. 35050061, Invitrogen), and 1 mM sodium pyruvate (Cat. 11360-070, Invitrogen) under a 5% CO_2 air atmosphere at 37 °C.

Plasmid construct and transfection. The full-length of human CALR CDS was inserted into the pcDNA3.1 vector. Recombinant plasmid pcDNA3.1-CALR was verified by BGI sequencing (Beijing). A375 cells were transfected for 48 h with pcDNA3.1 or pcDNA3.1-CALR plasmids using Lipo8000 reagent (Cat. C0533, Beyotime Biotech, Shanghai) according to the manufacturer's protocol, respectively.

Small RNA interference and transfection. Human CALR siRNA (sense 5'-CCAGUAUCUAUGCCUAUGAUATT-3', antisense 5'-UAUCAUAGGCAUAGAUACUGGTT-3'), EIF2AK2 siRNA (sense 5'-GCCGCUAAACUUGCAUAUCUUTT-3', antisense 5'-AAGAUAUGCAAGUUUAGCGGCTT-3') and negative control (sense 5'-CCAGCACUUGUGCCUGUACCAGAAA-3', antisense 5'-UUUCUGGUACAGGCACAAGUGCUGG-3') were synthesized by the Sangon Biotech (Shanghai) Co., Ltd. Cell were transfected with 100 nM negative control or indicated siRNAs for 36–48 h using Lipo8000 reagent according to the manufacturer's protocol.

Western blotting. After corresponding treatment, A375 cells were lysed with RIPA cell lysis buffer (Cat. 9806, Cell Signaling Technology) containing protease and phosphatase inhibitor (Cat. P0013C, Beyotime Biotech), and protein concentration was quantified by a BCA protein assay kit (Cat. P0010S, Beyotime Biotech). The same amounts of 30 μg protein were separated by 8–10% SDS-PAGE gel and transferred to PVDF membranes (Cat. IPVH00010, Millipore). The membranes were incubated with indicated primary antibodies after being blocked in 5% non-fat milk solution for 1 h at room temperature. The primary antibodies were anti-rabbit CALR antibody (1:2000, Cat. 27298-1-AP, Proteintech), anti-rabbit EIF2AK2 antibody (1:5000, Cat. 18244-1-AP, Proteintech), anti-rabbit phospho-EIF2AK2 antibody (1:1000, Cat. ab32036, Abcam), anti-rabbit MMP14 (1:1000, Cat. 14552-1-AP, Proteintech), anti-mouse ADAR1 (1:200, Cat. sc-271854, SANTA CRUZ, USA), anti-rabbit GRP78 (1:2000, Cat. 11578-1-AP, Proteintech), and anti-mouse β-actin antibody (1:2000, Cat. 66009-1-Ig, Proteintech) overnight at 4 °C. Then, the blots were incubated with HRP-conjugated goat anti-rabbit or mouse IgG secondary antibody (1:6000, Cat. SA00001-2, Proteintech) for 1 h and then were exposed to the SuperSignal West Pico Substrate (Cat. D3308-1, Beyotime Biotech). The gray value of the protein bands was evaluated by ImageJ software.

Transwell invasion assay. A Transwell chamber (Corning) per-coated with Matrigel (Cat. BME001, R&D systems) was placed in a 24-well plate. The bottom of each chamber was supplemented with 500 μl complete DMEM containing 20% FBS per well. Transfected A375 cells and untreated cells were suspended with 100 μl serum-free DMEM and then planted onto the top of the transwell chamber at a density of 5×10^5 cells/well. After incubation at 37 °C for 24 h, the cells on the top of the chamber were removed, and the remaining cells were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet solution (Cat. G1062, Solarbio, China) for 30 min. The invaded cells were counted and photographed under a light microscope.

Cell migration assay. A375 cells were cultured in an incubator after being seeded and transfected. When the cells reached 90–100% confluence, the cell layer was wounded by scraping with a sterile pipette tip. Then cells were exchanged in a serum-free medium and cultured for 24 h in an incubator. After washing in PBS, the cells were photographed at 0 h and 24 h by a light microscope, and randomly selected fields were used for counting wound size.

Cell apoptosis assay. Cell apoptosis assay was performed using Apoptosis and Necrosis Detection Kit with YO-PRO-1 and PI dye (Cat. C1075, Beyotime Biotech) in accordance with the manufacturer's protocol. Cells were seeded in a 96-well plate and transfected with indicated plasmids. After transfection for 36–48 h, the cells were washed with cold PBS buffer and then labeled with 100 μl working buffer containing 1 μl YO-PRO-1 and 1 μl PI, followed by incubation in the dark environment at 37 °C for 10 min. The treated cells were photographed, counted, and measured as the proportion of apoptotic cells.

EdU assay. The EdU assay was utilized to investigate the proliferation of transfected A375 cells. Briefly, cells were planted in a 6-well plate and cultured at 37 °C. After treatment, cells/well were incubated with 10 μM EdU (Cat. C10071L, Beyotime Biotech) for 2 h according to the manufacturer's protocol. Subsequently, the cells were fixed with 4% PFA and permeabilized with 0.3% Triton X-100 for 10 min. Cells per well were supplemented with 500 μl reaction solution, followed by incubation in the dark environment at room temperature for 30 min. After washing, the nuclei were stained with 5 μg/ml DAPI (Cat. ZLI-9557, ZSGB-BIO, China). Cells were imaged under a fluorescence microscope and counted by ImageJ software.

ER-Tracker Red. A375 cells were stained utilizing an ER-Tracker Red kit (Beyotime, China) according to the manufacturer's protocol. After washing the cells twice with PBS, add the prepared ER Tracker Red staining solution pre-incubated at 37 °C and co-incubated with the cells at 37 °C for 30 min. Subsequently, the stained cells were observed using a fluorescence microscope.

Cellular immunofluorescence. The transfected cells were seeded onto the coverslips treated with 0.1 mg/ml poly-L-lysine (Cat. A3890401, Gibco) and fixed with 4% PFA for 10 min at room temperature, and then permeabilized with 0.1% Triton X-100 for 10 min. Next, cells were incubated with 1% BSA (Cat. ST2249, Beyotime Biotech), 0.1% Tween 20 in PBS for 30 min to prevent unspecific binding of the antibodies, and then incubated with CALR antibody (1:200), EIF2AK2 antibody (1:200), phospho-EIF2AK2 (1:200), ADAR1 (1:200), or MMP14 antibody (1:200) overnight at 4 °C. Afterward, cell samples were incubated with FITC or Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:200, Cat. ZF-0311/0512, ZSGB-BIO, China), and then nuclei were stained with 5 μg/ml DAPI. Cell samples were observed under a Zeiss microscope, and then the fluorescence intensity was analyzed by ImageJ software.

ROS production analysis. Cells were cultured and transfected with plasmids. They were subsequently incubated with a 10 μM DHE probe (Cat. S0033S, Beyotime Biotech) for 20 min at 37 °C. Unspecific binding fluorescent dyes were removed with a serum-free medium. The cells were then observed under a fluorescence microscope, and intracellular ROS production was evaluated and counted by ImageJ software.

Statistical analysis. GraphPad 9.0 software (San Diego, CA, USA) was used for statistical analysis. All representative data from at least three independent experiments were presented as mean ± SEM. The correlation between CALR and EIF2AK2 or CALR and MMP14 was analyzed using Pearson's correlation analysis. Differences between the independent two groups were analyzed using Student's t-test. Significant multiple-group differences were evaluated using One-way analysis of variance (ANOVA). A p-value <0.05 was considered to indicate statistical significance.

Results

The expression of CALR and EIF2AK2 in malignant melanoma tissue and their effects on melanoma cell migration and invasion. We found through immunohistochemistry and immunofluorescence that the expression of CALR and EIF2AKA in melanoma tissue was significantly higher than that in adjacent normal tissues (Figures 1C–1H; Tables 1 and 2). These findings suggest the clinical relevance of CALR and EIF2AK2 in the diagnosis and treatment of melanoma.

To investigate the function of CALR in melanoma cells, we generated a cell model that both overexpressed and silenced the CALR gene. The western blot analysis revealed that CALR siRNA 1 and CALR siRNA 2 markedly decreased CALR protein expression when compared to siRNA NC, with the most significant decrease being observed with CALR siRNA#2. Consequently, CALR siRNA#2 was employed as a knockdown tool. However, in contrast to the OE-NC group, transfection of CALR overexpression plasmids markedly increased CALR protein expression levels (Figures 1I–1L).

We conducted a comprehensive investigation into the impact of CALR on the migration and invasion abilities of human melanoma cells (A375). The results from transwell and wound healing assays revealed that there was a significant reduction in the migration and invasion capacity of A375 cells following the knockdown of CALR in comparison with siRNA-NC. Conversely, overexpression of CALR increased the migration and invasion ability of A375 cells (Figures 2A–2D). These findings imply that CALR facilitates the progression of melanoma.

Effects of CALR on apoptosis, proliferation, and ROS of malignant melanoma cells. We examined the impact of CALR on melanoma cell proliferation and apoptosis. Knockdown of CALR encouraged apoptosis in A375 cells, whereas overexpression of CALR hindered apoptosis (Figures 3A, 3B). The EDU experiment noted that overexpression of CALR promoted the proliferation of melanoma cells, and conversely, knockdown of CALR inhibited the proliferation of melanoma cells (Figures 3C, 3D). In the experiment on ROS, it was discovered that overexpression of CALR increased the production of ROS in cells, and knockdown of CALR decreased the fluorescence intensity of ROS (Figures 3E, 3F). These findings suggest a close relationship between the regulation of melanoma cell proliferation and apoptosis by CALR and the regulation of ROS production.

CALR regulates ER stress. Using an ER fluorescent probe, it was observed that overexpression of CALR upregulated the fluorescence intensity of the ER probe. Conversely, knockdown of CALR downregulated the fluorescence intensity of the ER probe (Figures 3G, 3H). We measured the protein level of the ER stress marker GRP78 in cells by western blot and showed that overexpression of CALR significantly increased the expression level of GRP78, whereas knockdown of CALR significantly decreased the expression level of GRP78 (Figures 3I, 3J). This indicated that CALR regulates ER stress.

Effects of CALR on the expression of EIF2AK2 and MMP14. GEPIA data demonstrated a positive correlation between CALR expression and EIF2AK2 and MMP14 expression in melanoma tissues (Figures 4A, 4B). The results of cellular immunofluorescence experiments showed that CALR was mainly expressed in the cytoplasm, and overexpression could be accompanied by expression in the nucleus

Table 1. Immunohistochemical detection of CALR expression in melanoma and adjacent tissues.

Group	n	Positive	Negative	χ^2	p-value
Melanoma	39	27 (69.23%)	12 (30.77%)	5.214	0.022
Adjacent	39	17 (43.59%)	22 (56.41%)		

Table 2. Immunohistochemical detection of EIF2AKA expression in melanoma and adjacent tissues.

Group	n	Positive	Negative	χ^2	p-value
Melanoma	39	25 (64.10%)	14 (35.90%)	4.165	0.041
Adjacent	39	16 (41.03%)	23 (58.97%)		

(Figures 4C, 4D), suggesting that nuclear translocation occurred in the localization of positive expression after CALR overexpression. Knocking down CALR significantly reduced the expression of CALR in the cytoplasm and ER, blocking the nuclear translocation of CALR. MMP14 is expressed predominantly in the cell membrane and cytoplasm. The overexpression of CALR resulted in a significant increase in MMP14 expression, whereas the knockdown of CALR resulted in a decrease in MMP14 expression (Figures 4C, 4E). EIF2AK2 and its phosphorylated form p-EIF2AK2 are primarily expressed in the cytoplasm and nucleus. Phosphorylated EIF2AK2 is one of the pro-apoptotic factors in the ER stress process [33]. CALR overexpression enhanced their expression levels while knockdown of CALR decreased their expression (Figures 4C, 4F, 4G). Collectively, these findings have sparked our speculation that CALR may have a regulatory effect on the expression of EIF2AK2 and MMP14.

The role of EIF2AK2 in the development of melanoma cells. To investigate the role of EIF2AK2 in melanoma cell development, a knockdown EIF2AK2 cell model was constructed. The western blot analysis demonstrated that the knockdown of EIF2AK2 could downregulate the expres-

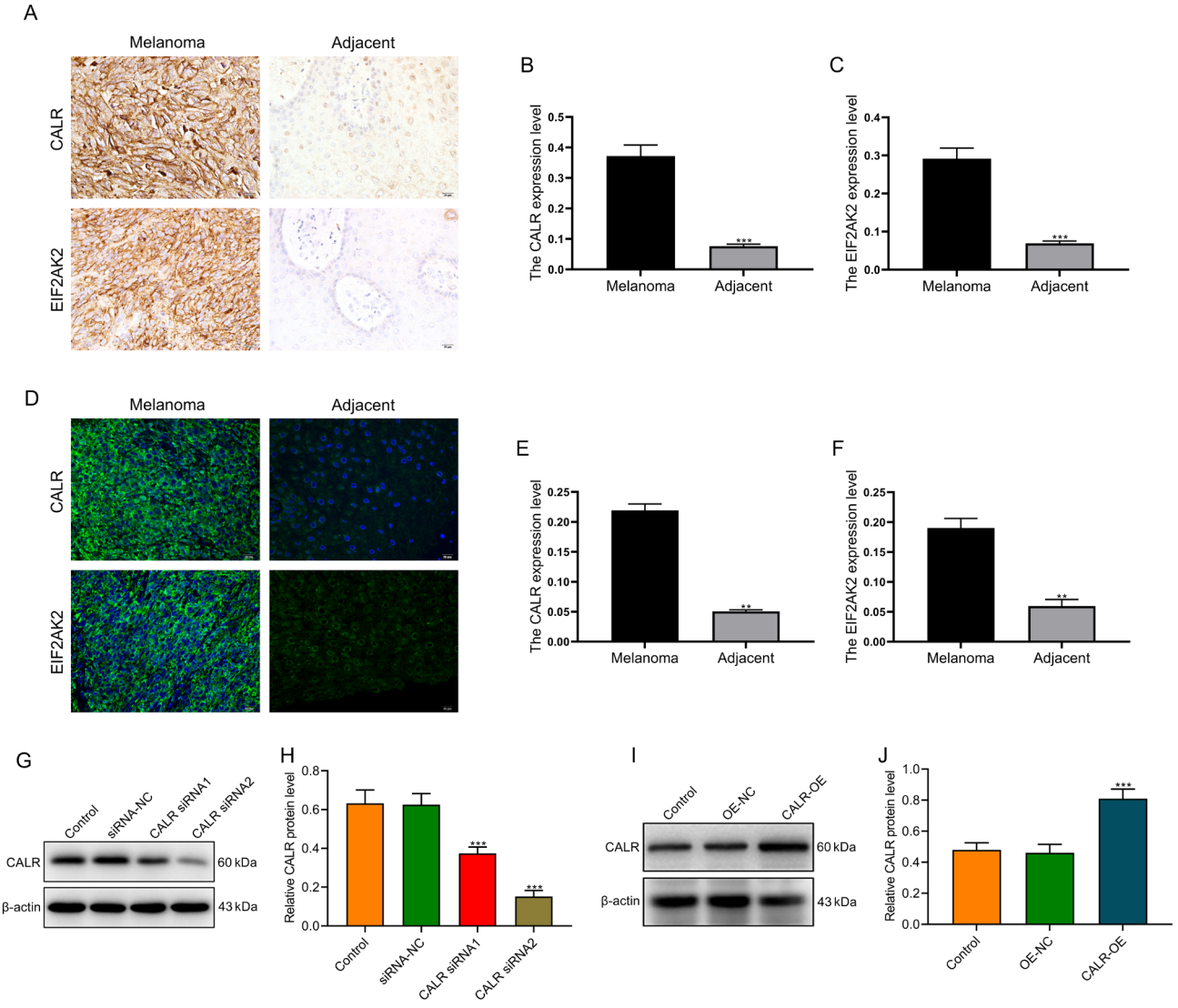

Figure 1. CALR and EIF2AK2 overexpression in malignant melanoma tissue. A-C) Immunohistochemical detection of CALR and EIF2AK2 expression in malignant melanoma tissue and normal skin tissue. Data are presented as the mean ± SD (n=3). **p<0.01, ***p<0.001. Scale bar, 20 μm, 400×. D-F) Immunofluorescence detection of CALR and EIF2AK2 expression in malignant melanoma tissue and normal skin tissue. Data are presented as the mean ± SD (n=3). **p<0.01, ***p<0.001. Scale bar, 20 μm, 400×. G) The CALR siRNAs were designed and transfected for 36 h into A375 cells, and then accessed using western blot assay. H) The gray blots were analyzed with ImageJ software (n=3). ***p<0.001 vs. siRNA-NC. I) A375 cells were transfected with control pcDNA3.1 or CALR overexpression plasmid for 48 h, and then cell lysates were performed using western blot assay. J) The gray blots were analyzed with ImageJ software (n = 3). ***p<0.001 vs. OE-NC

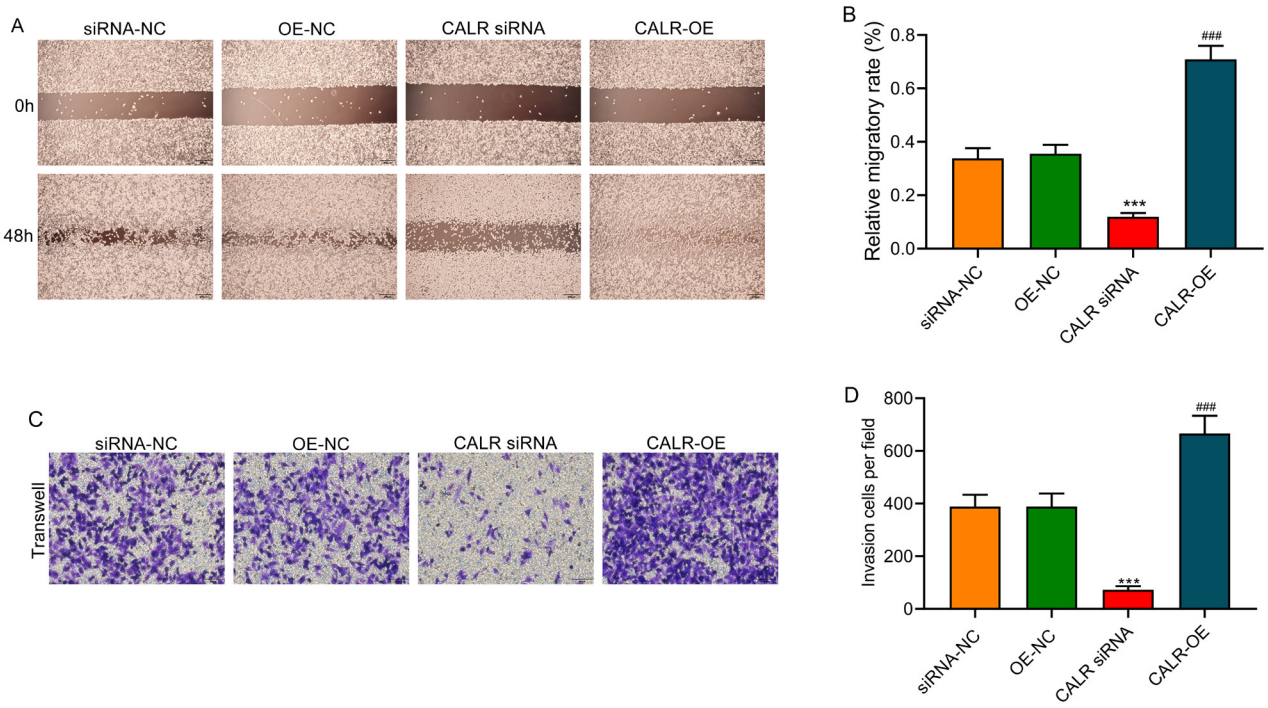

Figure 2. The effects of CALR knockdown and overexpression on migration and invasion of A375 cells. A) A wound healing assay was used to evaluate the migration ability at 48 h after CALR knockdown or overexpression in A375 cells. Scale bar, 200 μm. B) Different fields of cells were randomly selected, and then wound size was calculated (n=3). ***p<0.001 vs. siRNA-NC, ###p<0.001 vs. OE-NC. C) Transwell assay was used to detect the invasion ability after CALR knockdown or overexpression in A375 cells. Scale bar, 50 μm. D) Cells of different fields were randomly selected and counted (n=3). ***p<0.001 vs. siRNA-NC, ###p<0.001 vs. OE-NC

sion of phosphorylated EIF2AK2, EIF2AK2, MMP14, and ADAR1, with EIF2AK2 siRNA2 having the most significant effect, so further experiments would continue to use it (Figures 5A–5F). Transwell assays showed that the knockdown of EIF2AK2 significantly inhibited the invasion and migration of melanoma cells (Figures 5G–5J). These findings indicate that EIF2AK2 plays a crucial role in promoting melanoma cell progression.

We further downregulated EIF2AK2 expression on the basis of overexpressing and knocking down CALR in melanoma cells. Our findings demonstrated that the knockdown of EIF2AK2 blocked the upregulation of phosphorylated EIF2AK2, EIF2AK2, MMP14, and ADAR1 expression caused by overexpression of CALR. Interestingly, CALR expression remained heightened. However, the simultaneous knockdown of both CALR and EIF2AK2 expression decreased the expression of MMP14 and ADAR1 significantly (Figures 5K–5P). These results suggest that EIF2AK2 may function as a downstream effector of CALR.

Discussion

For advanced melanoma, many new medications have been developed, such as immunotherapy, gene therapy, and bio-chemotherapy [13, 34]. However, the five-year survival rate for melanoma patients is only 10% [35, 36]. Furthermore, the absence of clear indications and symptoms in the early stages of melanoma leads to the diagnosis of around 80% of patients only at advanced stages of the illness [36, 37]. These issues highlight the pressing requirement for the creation of innovative and effective therapeutic approaches. Hence, comprehending the molecular mechanisms of melanoma phenotypes is imperative to attain a beneficial diagnosis and treatment for patients. In this study, we have identified a new signaling pathway, known as the CALR/EIF2AK2 pathway, which has a pivotal role in the pathogenesis of melanoma. Specifically, we observed that CALR and EIF2AK2 were overexpressed in human melanoma tissues and that melanoma cell proliferation, apoptosis, invasion, migration, ROS production, and ER stress could be regulated by the knockdown and the overexpression of CALR. More importantly, CALR could regulate MMP14 and ADAR1 expression by mediating EIF2AK2 phosphorylation.

In recent years, there have been rapid advancements in immunotherapy and molecular-targeted therapies for melanoma [38]. For patients with mutated melanoma, the combination of BRAF and MEK inhibitors in clinical settings offers a promising option to consider [35, 39]. The potential viability of utilizing inhibitors targeting the PI3K/Akt/mTOR pathway for the management of melanoma is presently under

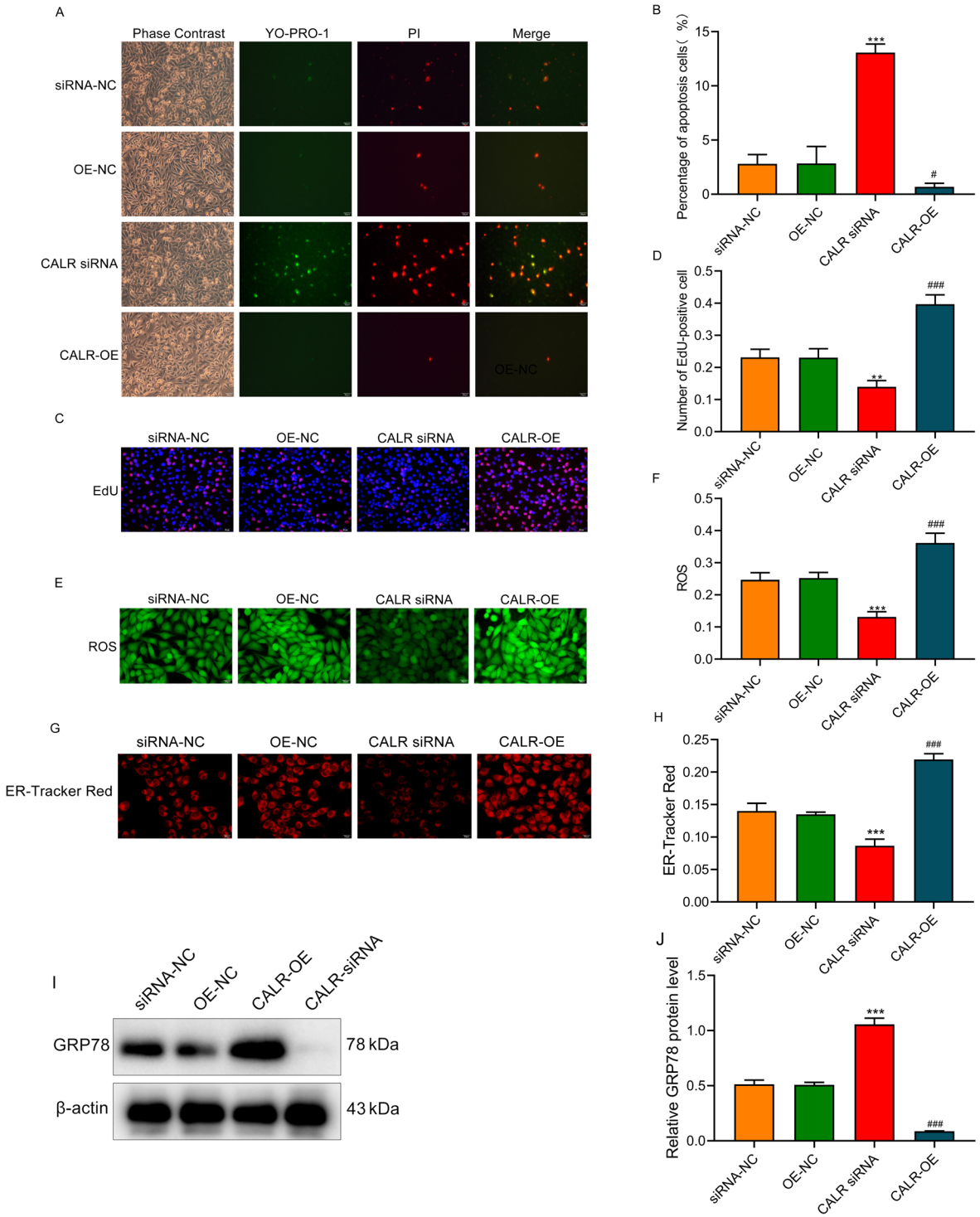

Figure 3. The effect of CALR on apoptosis, proliferation, and ROS of malignant melanoma cells. A) A375 cells were transfected with CALR siRNA and overexpression plasmid, respectively, and then stained with both YO-PRO-1 and PI dye. Scale bar, 50 μm. B) The proportion of green-labeled apoptotic cells was measured according to randomly selected cell fields (n=5). ***p<0.001 vs. siRNA-NC, #p<0.05 vs. OE-NC. C) EdU assay was used to evaluate the proliferation ability after CALR knockdown or overexpression in A375 cells. Scale bar, 200 μm. D) Different fields of cells were randomly selected, and then positive cell numbers were calculated (n=3). ***p<0.001 vs. siRNA-NC, ##p<0.01 vs. OE-NC. E, F) The intracellular ROS production was determined after CALR knockdown or overexpression in A375 cells. Scale bar, 50 μm. ***p<0.001 vs. siRNA-NC, ###p<0.001 vs. OE-NC (n=3). G, H) The ER stress was determined after CALR knockdown or overexpression in A375 cells. Scale bar, 50 μm. ***p<0.001 vs siRNA-NC, ###p<0.001 vs. OE-NC (n=3). I) A375 cells were transfected with CALR plasmid, CALR siRNA, respectively, and then examined using western blot. J) The gray blots were analyzed with ImageJ software (n=3), ***p<0.001 vs. OE-NC, ###p<0.001 vs. siRNA-NC

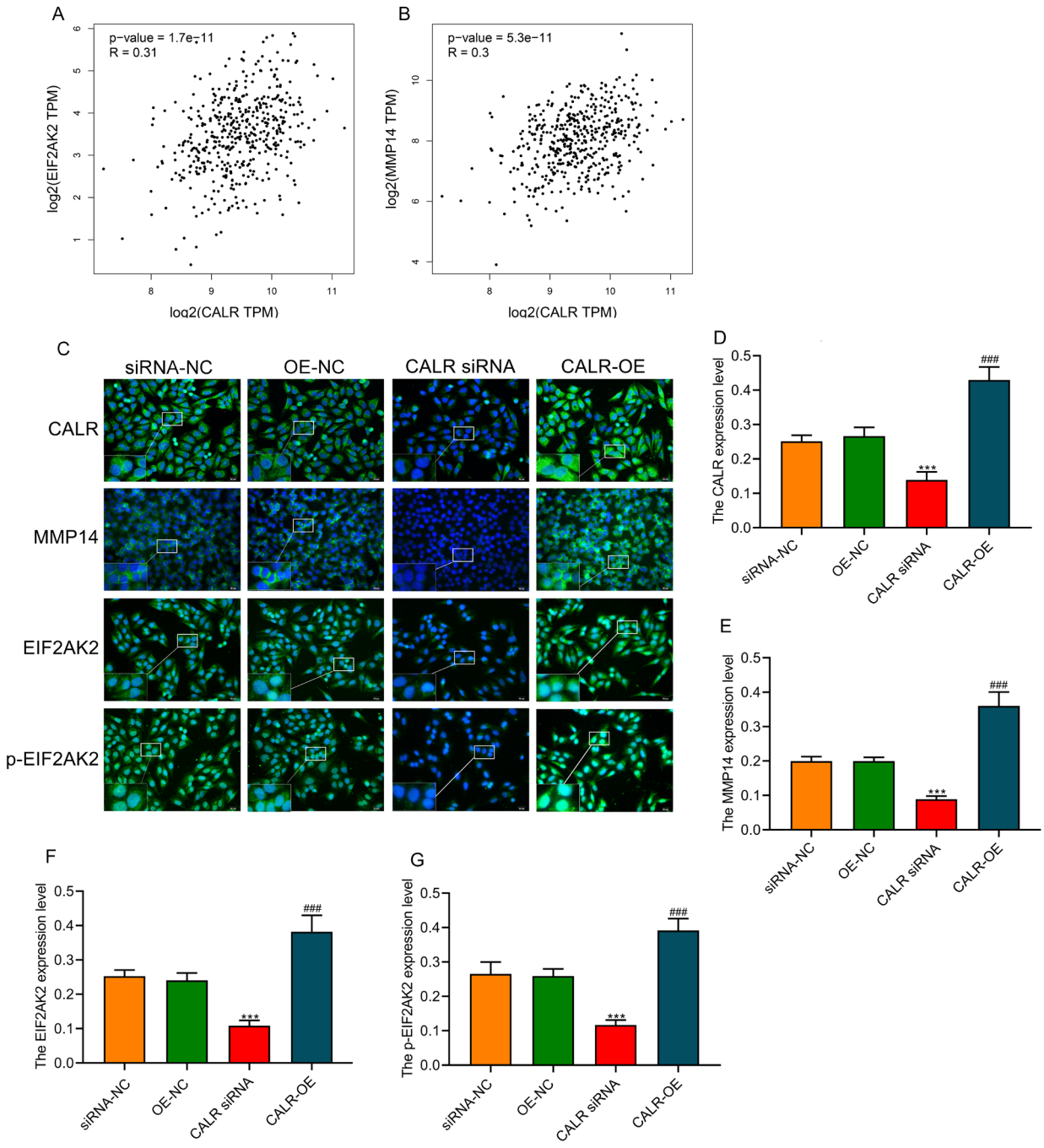

Figure 4. The effect of CALR on the expression of EIF2AK2 and MMP14. A) GEPIA database showed that the expression of CALR was positively associated with the expression of EIF2AK2 in malignant melanoma tissues. p<0.001, R=0.31. B) GEPIA database showed that the expression of CALR was positively associated with the expression of MMP14 in malignant melanoma tissues. p<0.001, R=0.3. C) A375 cells were immunostained with CALR, MMP14, EIF2AK2, and phospho-EIF2AK2 antibodies after CALR knockdown or overexpression. Scale bar, 50 μm. D-G) The gray blots were analyzed with ImageJ software (n=3). ***p<0.001 vs. siRNA-NC, ###p<0.001 vs. OE-NC

investigation [35, 40, 41]. Although significant efforts and promising developments have been made to treat melanoma, there has been a continued increase in both the incidence and mortality rates of this disease [37]. Furthermore, the precise molecular mechanisms behind advanced melanoma are as of yet largely uncharacterized. Therefore, the identification of new, specific molecular mechanisms and potential biomarkers is crucial for the effective diagnosis and treatment of this disease [10]. Molecular chaperones play a critical role in controlling cellular processes and maintaining

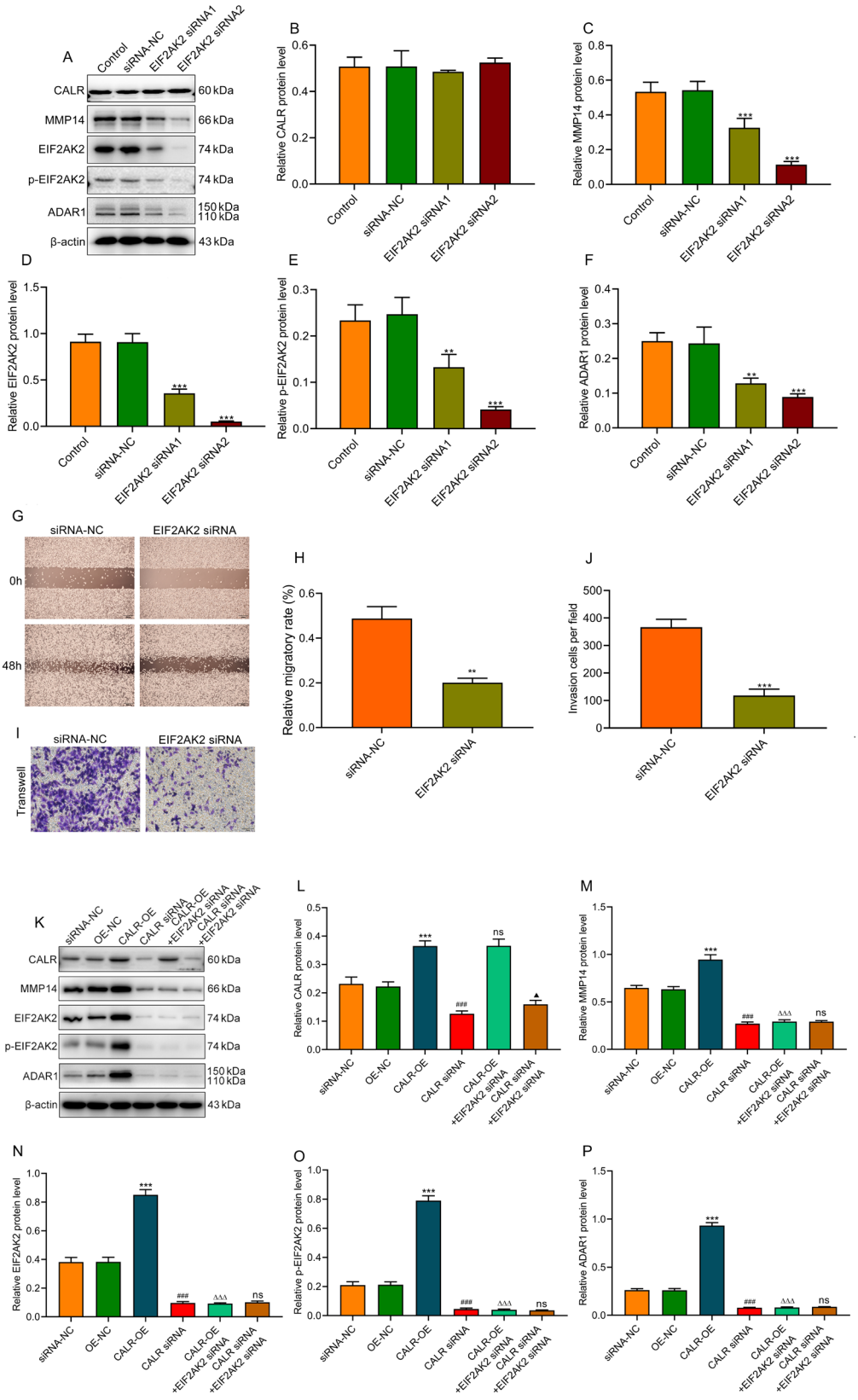

Figure 5. The role of EIF2AK2 in the development of melanoma cells. A) EIF2AK2 siRNAs were transfected for 36 h into A375 cells, and then accessed using western blot assay. B-F) The gray blots were analyzed with ImageJ software (n=3). **p<0.01, ***p<0.001 vs. NC siRNA. ***p<0.001, **p<0.01 vs. siRNA-NC. G) A wound healing assay was used to evaluate the migration ability at 48 h after CALR knockdown or overexpression in A375 cells. Scale bar, 200 μm. H) Different fields of cells were randomly selected, and then wound size was calculated (n=3). **p<0.01 vs. siRNA-NC. I) Transwell assay was used to detect the invasion ability after CALR knockdown or overexpression in A375 cells. Scale bar, 50 μm. J) Cells of different fields were randomly selected and counted (n=3). ***p<0.001 vs. siRNA-NC. K) A375 cells were transfected or co-transfected with CALR plasmid, CALR or EIF2AK2 siRNA, respectively, and then examined using western blot. L-P) The gray blots were analyzed with ImageJ software (n=3), ***p<0.001 vs. OE-NC, ###p<0.001 vs. siRNA-NC, ΔΔΔp<0.001 vs. CALR-OE, ▲p<0.05 vs. CALR siRNA

protein homeostasis [16, 36]. The roles of a number of chaperone molecules, including those involved in cell proliferation, apoptosis, and tumorigenesis, have been identified as increasingly implicated in human cancer studies [16, 36]. Recent evidence has shown that CALR functions as a conserved chaperone and plays a crucial role in tumorigenesis and tumor suppression across various tumor subtypes [18]. For instance, the impairment of cellular protein folding due to loss-of-function of CALR mutations results in oncogenesis. Thus, CALR can be considered as a gene that inhibits the development of cancer [16]. Additionally, the controversial roles of CALR in tumor progression and the possible role of CALR mutated in other human cancers are also addressed [20]. For instance, CALR was upregulated and acted as an oncogene in gallbladder cancer [18]. The specific role of CALR in most human cancers, including melanoma, remains unclear. Our study outlined the expression of CALR in melanoma and found that the expression of CALR was significantly higher in melanoma than in adjacent tissues, indicating the clinical value of CALR in melanoma. In the face of different views of the role CALR plays in different malignancies, our experimental results highlight the critical role of CALR in regulating melanoma cell proliferation, invasion, migration, and ROS production. We speculate that the enhanced invasiveness of melanoma cells by CALR may be related to the nuclear translocation of its expression, which needs further verification in the future. This experiment highlights that CALR may be a new target for the treatment of melanoma, which provides a new basis for the development of targeted drugs. Additionally, immunogenic cell death is a stress-dependent cell death induced by ER stress combined with the production of reactive oxygen species, which is increasingly attracting attention in the treatment of tumors [42]. It was shown that the absence of CALR translocation on the membrane of mouse melanoma cells hinders the stimulation of effective ICD responses *in vivo* [43]. The above findings indicated the role of CALR from the perspective of tumor immunity but did not elaborate on the regulatory mechanism of CALR. The results of the present study preliminarily suggest that the knockdown of CALR significantly reduced EIF2AK2 phosphorylation, which may be related to the regulation of tumor immunity. Because in cancer studies, DFNA5 of the Gasdermin family can regulate immune infiltration through EIF2AK2 [44]. All these results reflected that EIF2AK2 played a key role in regulating tumor progression.

EIF2AK2, also known as PKR, is an IFN-stimulated gene, and it has been widely reported that EIF2AK2 plays an inhibitory role in cancer progression, including breast and lung cancer [45, 46]. However, some studies have shown that activation of EIF2AK2 is involved in the progression of breast and pancreatic cancer, promoting cell migration and invasion [47, 48]. Faced with the contradiction of EIF2AK2 in cancer, we found that the expression level of EIF2AK2 in human melanoma tissues was higher than that in adjacent non-tumor tissue, indicating the clinical significance of EIF2AK2 in melanoma research. Studies have reported that the expression of EIF2AK2 in pancreatic cancer tissues was confirmed to be significantly higher than that in the adjacent pancreatic tissues in clinical samples from pancreatic cancer patients, and the use of pancreatic cancer cells validated that the knockdown of EIF2AK2 inhibited cell proliferation, migration, and invasion, demonstrating that EIF2AK2 can be used as a diagnostic and prognostic biomarker for pancreatic cancer patients. Studies have reported that the clinical samples from pancreatic cancer patients confirmed that the expression of EIF2AK2 was significantly higher in pancreatic cancer tissue than that in adjacent pancreatic tissue. The pancreatic cancer cells were used to validate that knocking down EIF2AK2 inhibited cell proliferation, migration, and invasion, proving that EIF2AK2 could be used as a diagnostic and prognostic biomarker for pancreatic cancer patients [49]. EIF2AK2 is implicated in the proliferation of colorectal cancer cells and has the ability to regulate resistance to oxaliplatin [50]. In this study, knocking down EIF2AK2 significantly reduced invasion and migration ability in melanoma cells, indicating that knocking down EIF2AK2 can independently hinder the progression of melanoma. Importantly, knocking down CALR and EIF2AK2 respectively can significantly reduce MMP14 expression, suggesting that they may exert anti-tumor effects by improving the melanoma cell matrix.

We conducted a search of the GEPIA database and identified a close association between EIF2AK2 and the expression of MMP14 and ADAR1. Prior to cancer cell metastasis, the primary tumor releases certain factors, including extracellular matrix (ECM) structural proteins that actively stimulate the metastatic site, while MMP14 plays a regulatory role in the ECM remodeling process [51]. In studies on melanoma, MMP14 is a regulatory factor for invasion [52], and the invasiveness of melanoma cells can be decreased by inhibiting MMP14 [53]. The MMP14 knockdown model in mice illustrated normal development and reproduction but reduced growth and metastasis of melanoma, and decreased vascular permeability [54], this indicates that MMP14 is a pivotal factor in melanoma progression. It has been proposed that matrix metalloproteinase-2 can potentially impact mitochondrial function by modifying ER-mitochondrial Ca^{2+} signaling through calreticulin hydrolysis [55]. Based on the results of this study, it was found that CALR and EIF2AK2 could regulate the expression of MMP14 in A375 cells, describing the regulatory relationship between CALR and EIF2AK2 and MMP14 in melanoma. We speculate that MMP14 is one of the downstream effectors of CALR and EIF2AK2 regulation, providing a reference for possible pathways of ER stress-related signaling to regulate the extracellular matrix.

ADAR1, as an RNA editor, promotes tumor occurrence and development [28, 56]. In non-tumor studies, ADAR1 promotes Zika virus viral protein translation by impairing the

activation of protein kinase PKR [57]. During the response to type I interferon, ADAR1 blocks translation shutdown by inhibiting PKR hyperactivation [58]. The study revealed that knockdown of EIF2AK2 could downregulate the expression of ADAR1 in melanoma cells, suggesting that EIF2AK2 may be involved in melanoma progression by affecting ADAR1 expression. In the presence of a mechanism of multiple molecular signals cross-talking each other in tumor cells [59], our experimental findings also prompt a reconsideration of ADAR1 as a target. In many cancer studies, ADAR1 can be used as a standalone target for anti-cancer therapy [60]. However, in this experiment, it was found that CALR or EIF2AK2 could affect the expression of ADAR1, which is likely related to ER stress. In the future, we will further explore their possible mechanisms. It is worth noting that knocking down the expression of CALR or EIF2AK2 significantly downregulated the expression of ADAR1 in melanoma cells. Interestingly, knocking down EIF2AK2 expression prevented CALR from regulating the expression of MMP14 and ADAR1. Therefore, we assume that CALR exerts its regulatory effect through the downstream factor EIF2AK2.

In summary, this study suggests that CALR and EIF2AK2 contribute to the pro-carcinogenic processes involved in melanoma progression. Additionally, the knockdown of CALR and EIF2AK2 may be an effective anti-tumor approach, the mechanism of which may be mediated by MMP14 and ADAR1 signaling.

Acknowledgments: This study was supported by the Ningxia Natural Science Foundation (No. 2022AAC03588).

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