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Induction of miR-224 by Reactive Oxygen Species Regulates RASSF6 and Thus Modulates Malignant Behaviors and Chemosensitivity in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Esophageal cancer is one of the most common malignant tumors of the digestive tract, and miR-224 can promote the hypoxia tolerance of esophageal cancer cells. The expression of miR-224 and HIF-1α in esophageal cancer cells under hypoxic induction and their relationship with ROS was studied using RT-qPCR and Western Blot assays; cell viability and apoptosis under hypoxia, as well as the effects of miR-224 on cell proliferation and drug resistance, were investigated using CCK8, Annexin V-FITC/PI, H2DCFDA staining and Western Blot assays. Under hypoxic induction, miR-224 and HIF-1α expressions were upregulated, with the upregulation of miR-224 being related to ROS accumulation, while HIF-1α upregulation was not affected by ROS. Furthermore, the upregulation of miR-224 facilitated the survival of esophageal cancer cells under hypoxic conditions and reduced their chemosensitivity to CDDP. This effect was also validated in vitro, as miR-224 overexpression promoted the malignant behaviors in ESCC cells. Under hypoxic induction, ROS accumulation can lead to the upregulation of miR-224. MiR-224 facilitates the survival of esophageal cancer cells under hypoxic conditions and induces chemotherapeutic drug resistance.

1 | Introduction

Esophageal cancer is a common malignancy of the digestive tract that originates from the epithelial cells or submucosal tissues of the esophagus. Esophageal cancer is one of the most aggressive tumors, ranking seventh in incidence and sixth in mortality worldwide [1]. The disease is most prevalent in East Asia, with over 70% of the deaths occurring in China [2]. In China, the majority of diagnosed esophageal cancer cases are esophageal squamous cell carcinoma (ESCC), accounting for about 90%. The prognosis for ESCC is extremely poor, with a 5-year survival rate of only 10%–25% [3]. Chemotherapy is crucial for ESCC patients, as it plays an irreplaceable role in postoperative adjuvant treatment and first-line therapy [4].

Core tip:

Esophageal cancer is a common malignancy of the digestive system that arises from the epithelial cells or the submucosal tissues of the esophagus. It stands as one of the most aggressive forms of cancer, ranking seventh in terms of incidence and sixth in mortality across the globe. Hypoxia-induced oxidative stress is one of the primary factors leading to the progression of chemoresistance in esophageal squamous cell carcinoma (ESCC). Therefore, we aim to investigate the effects and mechanisms by which hypoxia exposure, or even oxidative stress, directly cause the malignancies of ESCC. MicroRNA-224 can characteristically target the RASSF6 gene. In this study, we examined the expression of the RASSF6 gene caused by overexpression of miR-224 under hypoxic and oxidative stress conditions, as well as changes in tumor chemoresistance.

Currently, cisplatin (CDDP) is the drug of choice for treating ESCC, and CDDP-based chemotherapy regimens remain the most common strategy for treating unresectable or recurrent tumors [4]. However, chemotherapy resistance limits the effectiveness of CDDP, thereby worsening the prognosis of ESCC patients [5]. Thus, studying the chemoresistance of ESCC to CDDP is beneficial for further improving the prognosis of ESCC patients.

Hypoxia is one of the important hallmarks of malignant tumors and is associated with a more aggressive tumor phenotype, higher likelihood of metastatic progression, and resistance to radiotherapy and chemotherapy, among other malignant behaviors [6, 7]. Rapid and uncontrollable tumor growth requires a large amount of nutrients, thus triggering new blood vessel formation. However, the resulting tumor neovasculature is highly disorganized and inefficient. Therefore, a state of chronic hypoxia may exist within tumor tissues for an extended period. Studies have found that many chemotherapy drugs (such as CDDP, etoposide, doxorubicin, etc.) require oxygen to exert their maximum therapeutic activity. Hence, under hypoxic conditions, many tumor cells show reduced sensitivity to chemotherapy drugs. This resistance to chemotherapy drugs is mainly achieved through mechanisms such as upregulation of glucose and oxygen regulatory proteins, DNA over-replication, cell cycle arrest, alterations in cellular metabolism, and increased drug efflux pumps [8, 9]. In the clinical treatment of ESCC, hypoxia is considered a key factor in patients' treatment resistance and poor prognosis [10].

miRNAs can regulate gene expression post-transcriptionally and control the translation or induce degradation of their target genes. Therefore, miRNAs play a significant role in basic cellular physiological processes such as cell growth, differentiation, apoptosis, energy metabolism, and immune response [11]. Studies have found that miR-224 is dysregulated in different human malignancies and may affect many cellular processes related to cancer, including gene transcription, proliferation, differentiation, and cell death [12–14]. Research by He et al. showed that miR-224 is overexpressed in ESCC tissues and promotes the proliferation and inhibits apoptosis of ESCC cells [15]. Research by Chang et al. suggested that miR-224 is associated with CDDP resistance in ESCC, and downregulating miR-224 can enhance the chemosensitivity of ESCC cells to CDDP [16]. In our previous studies, we found that miR-224 expression is upregulated in ESCC under hypoxic conditions. However, the effects of miR-224 on the proliferation of ESCC cells and their chemoresistance to CDDP under hypoxia-induced conditions, as well as the underlying mechanisms, are currently unclear.

The RASSF family includes 10 genes in the human genome, six of which (RASSF1-6) belong to the C-RASSF subgroup [17, 18]. The expression of C-RASSF is often suppressed in human cancers, and cancers with low C-RASSF expression are more malignant and have a poorer prognosis, thus considered tumor suppressor molecules. Studies have shown that RASSF6 is related to the regulation of the NF-κB signaling pathway. Research by Zhou et al. indicated that the miR-524/RASSF6 regulatory axis is associated with chemoresistance to doxorubicin in ovarian cancer [19]. Research by Tang et al. showed that the interaction between miR-524 and RASSF6 promotes chemotherapy resistance in osteosarcoma [20]. However, the impact of RASSF6 on chemoresistance in ESCC and its related mechanisms remain to be studied. In summary, this article focuses on the interaction between miR-224 and RASSF6 under hypoxia induction, exploring the effect of miR-224/RASSF6 on the proliferation of ESCC cells and their chemoresistance to CDDP, seeking new targets and approaches to improve the treatment of ESCC.

2 | Materials and Methods

2.1 | Antibody

The following antibodies were used in this study: Actin antibody (Abcam, UK, ab8226, 1:1000); HIF-1α antibody (Abcam, UK, ab179483,1:1000); PARP antibody (Abcam, UK, ab139417, 1:1000); cleaved PARP antibody (Abcam, UK, ab32064, 1:1000); caspase 3 antibody (Abcam, UK, ab145046, 1:1000); cleaved caspase 3 antibody (Abcam, UK, ab2302, 1:1000); GAPDH antibody (Abcam, UK, ab8245, 1:1000); RASSF6 antibody (Abcam, UK, ab138067, 1:1000); Ki67 antibody (Abcam, UK, ab16667, 1:1000).

2.2 | Cell Culture

Deferoxamine (Sigma, USA, D9533); Cobalt Chloride (Sigma, USA, 232696); ROSUP (Beyotime, China, S0033S); NAC (Sigma, USA, 1009005); CDDP (Sigma, USA, 232120). EC9706 and TE-1 (DMEM-F12 medium + 10%FBS); Cultured under normoxia 5% CO_2, 20% O_2, 37°C and hypoxia 5% CO_2, 1% O_2, 94% N_2, 37°C.

2.3 | Cell Transfection

The full-length sequences of HIF-1α and RASSF6 were obtained using the human genome as a template and constructed on pcDNA3.1(+) by adding *Sa*lI and *Bam*HI restriction enzyme sites. After synthesizing the promoter sequence of the target gene miR-224, it was constructed on the pGL3 fluorescence vector using *Kpn*I/*Hin*dIII restriction enzyme to obtain the miR-224 promoter luciferase reporter gene. At the same time, according to the binding sites of miR-224 promoter and HIF-1α predicted by software, Stratagene point mutation kit was used to mutate these sites to obtain the mutant vector Mut of miR-224 promoter HIF-1α binding site and extract plasmid DNA. One day before transfection, 3.0-5.0×105 cells were inoculated into 6-well plates, and 10% fetal bovine serum containing DMEM fresh medium without antibiotics was added. When the cell density reached 70%–80% of the well plate area, the cells were divided into three groups: normal culture group, miR-224 mimic group, and miR-224 inhibitor group. On the day of transfection, the preconstructed plasmid DNA was added into the opti-MEM medium without serum and double antibody; after the plasmid DNA was prepared, the vortex was separated instantly. MiR-224 mimics (miR-224 mimic group) or miR-224 inhibitor (miR-224 inhibitor group) was added into the opti-MEM medium without serum and double antibody, and

Lipofectamine2000 (normal culture group) was added into the opti-MEM medium without serum and double antibody, respectively. The above three solutions were mixed and allowed to stand at room temperature for 20 min, so that the plasmid DNA, miRNA-224, and Lipofectamine2000 formed a complex. The complex was slowly added into the well plate incubated the day before, and after 4 h, the final concentration of serum was 10%. After 48 h of culture under the required conditions, the cell samples were collected for relevant detection.

2.4 | Western Blot

Cells were lysed with RIPA lysing buffer, washed with PBS after lysing, and centrifuged. Protein abundance was detected using a BCA protein detection kit (Thermo Scientific, USA, 23225). The samples were loaded onto SDS-PAGE for electrophoresis and transferred to the PVDF membrane. Then, antibodies were added and incubated. Protein bands were detected using ECL-Pus/Kit (Thermo Scientific, USA, 34579).

2.5 | RT-qPCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA, 15596026CN), and cDNA was reverse-transcribed using an RT-qPCR kit (Invitrogen, USA, 12594100). Then, RNA expression was quantified by qPCR. The primer sequences used in this study are shown in Table T1. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6 | Cell Viability Assay

The cells were seeded in 96-well plates (1500 cells/well). Then, cell viability was detected using the CCK-8 kit (brand, place, article number) according to the instructions.

2.7 | Cell Cycle Assay

The cells were collected and fixed with 70% ethanol at 4°C overnight. The cells were washed, stained in the dark with 50 μg/mL PI and 50 μg/mL RNase A for 1 h, and then analyzed by flow cytometry to determine the percentage of cells in each specific cell cycle stage. Flow cytometry analysis was performed using FACS Calibur flow cytometry apparatus. Each sample was evaluated, and cell cycle distribution was determined using Cell Quest software (Becton Dickinson, Heidelberg, Germany). Results were presented in terms of the number of cells as indicated by fluorescence intensity and the amount of DNA. All experiments were performed three times. Annexin V-FITC/PI double staining.

The assay was performed using the Annexin V-FITC/PI kit. In this assay, Annexin V-FITC binds phosphatidylserine, which is transported to the outer layer of the cell membrane during the early stages of apoptosis. Apoptotic cells were therefore specifically stained with Annexin V-FITC, while necrotic cells were double stained with Annexin V-FITC and PI. The cells were suspended in binding buffer to a final cell concentration of 1×105 cells/mL and incubated in the dark with Annexin V-FITC and PI for 25 min. The DNA content of the stained cells was analyzed using Cell Quest software and FACS Calibur flow cytometry.

2.8 | Soft Agar Colony Formation

A 0.6% agar base was added to a 6-well plate, followed by 8×103 cells in each well and a top layer of complete growth medium containing 0.3% agar. The plates were incubated for 2 weeks until colony formation. Finally, the colony was quantitatively analyzed using ImageJ.

2.9 | Plate Colony Formation

The cells were seeded in six-well plates (500 cells/well). The cells were cultured for 13 days and washed with PBS. The cells were fixed with 1 mL of 4% paraformaldehyde for 30–60 min. Then, the cells were stained with Giemsa dye for 10–20 min. The cells were washed with ddH_2O several times and dried at 24°C. The cell colonies were scanned under a microscope.

3 | Result

3.1 | Hypoxia-Induced Expression of miR-224 and Hif-1A

We selected esophageal cancer EC9706 and TE-1 cells, treated them with 1% O_2, deferoxamine (DFO), cobalt chloride ($CoCl_2$) for 12 and 24 h, and detected the expression of HIF-1α by Western Blot (Figure 1A). In EC9707 cells, the three methods including 1% O_2, DFO, and $CoCl_2$ could effectively increase the protein level of HIF-1α; in TE-1 cells, except DFO, 1% O_2, and $CoCl_2$ could effectively increase the protein level of HIF-1α, indicating that we could successfully obtain the hypoxia model. Subsequently, we detected the expression of miR-224 by RT-qPCR. At the same time, H2DCFDA staining was used to observe the ROS content in cells after different treatments. The results showed that the three methods of 1% O_2, DFO, and $CoCl_2$ significantly increased the ROS content in cells (Figure 1B). The results showed that the three modeling methods could significantly increase the level of miR-224 after 12 and 24 h of modeling ($p < 0.05$). To confirm whether the increased expression of miR-224 was caused by ROS accumulation, we treated the cells with ROSUP, which causes ROS accumulation, and NAC, which scavenges ROS, and then detected the levels of HIF-1α and miR-224. The results showed that (Figure 2A-B), the presence of ROS was necessary for the upregulation of miR-224; the presence of ROS was related to ROS accumulation, while the upregulation of HIF-1α was not affected by ROS. H2DCFDA staining also showed the same result (Figure 2C).

3.2 | Effects of Hypoxia-Induced miR-224 on Cell Proliferation and Cddp Resistance

To explore whether miR-224 induced by 1% O_2, DFO, and $CoCl_2$ has an impact on cell viability, we treated cells with

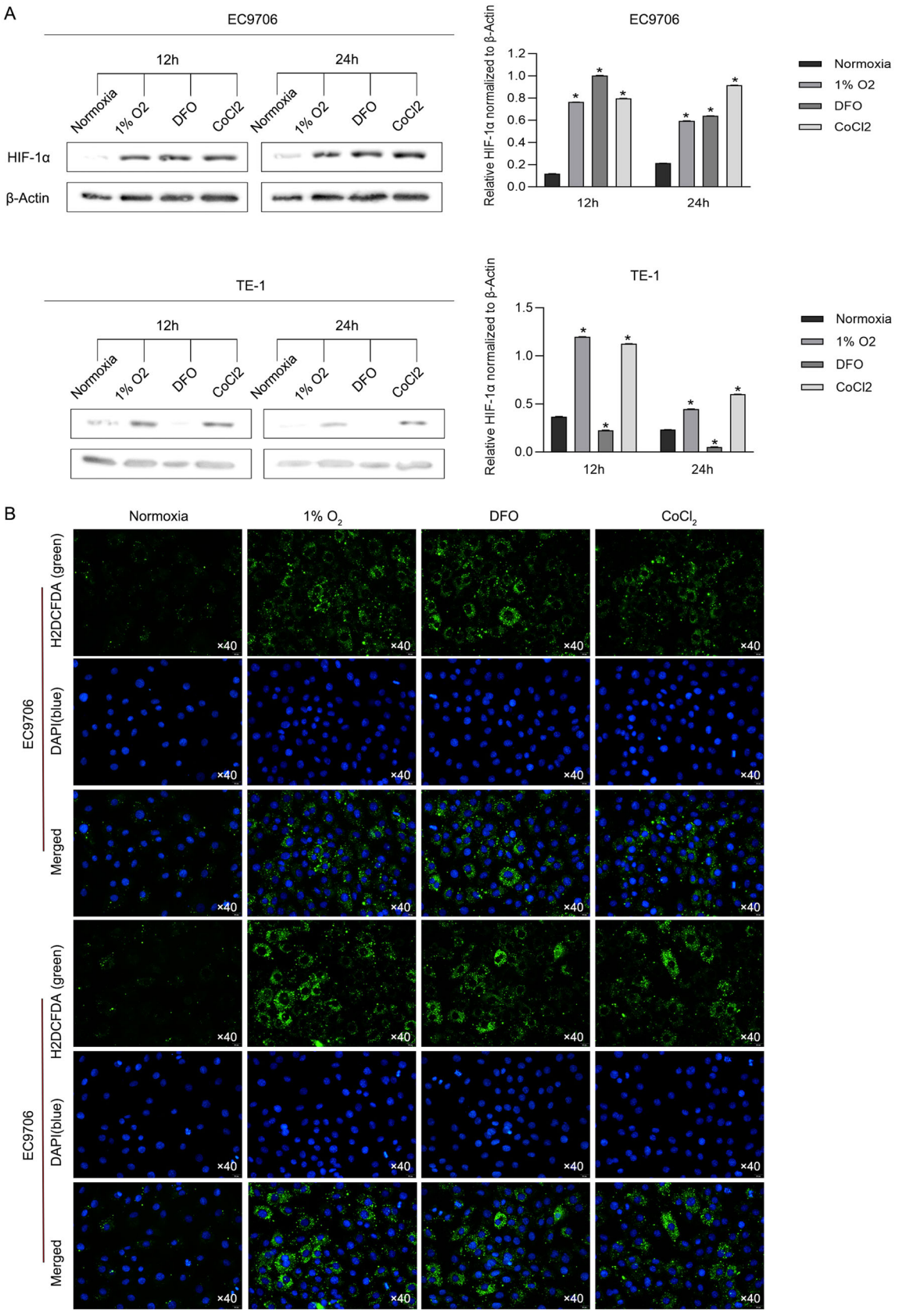

FIGURE 1 | Hypoxia treatment induces the upregulation of miR-224. (A) after being exposed to 1% O_2 (hypoxic condition), DFO, or $CoCl_2$ for 12 or 24 h, protein level of HIF-1α was measured by western blot in EC9706 and TE-1 cells. *$p < 0.05$, versus Normoxia control group. (B) After being treated as mentioned above, the expression level of ROS was measured by H2DCFDA staining in EC9706 and TE-1 cells.

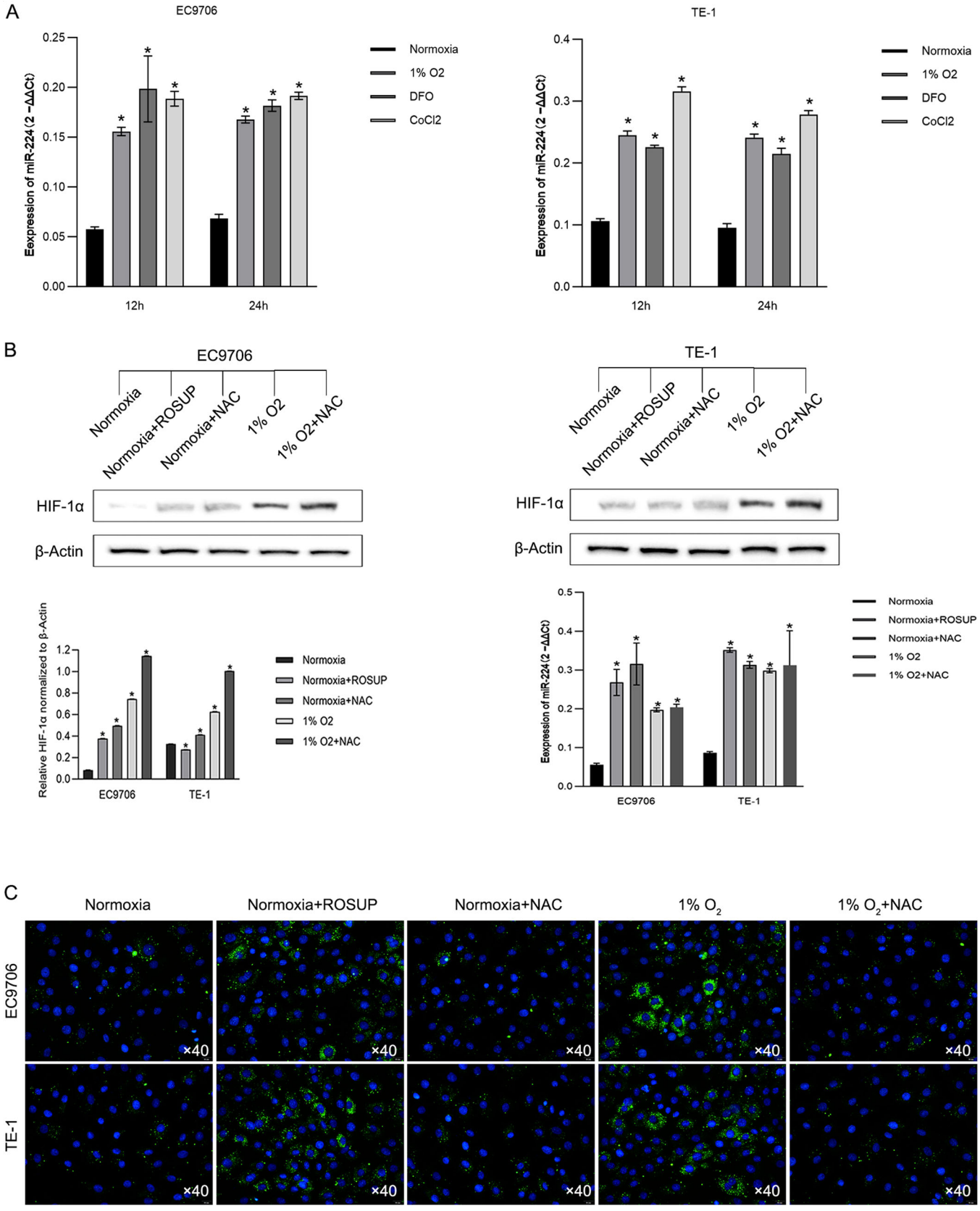

FIGURE 2 | Hypoxia treatment induces the upregulation of miR-224. (A) After being treated as mentioned above, expression level of miR-224 was measured by RT-qPCR in EC9706 and TE-1 cells. *$p < 0.05$, versus Normoxia control group. (B) To evaluate the effect of ROS on HIF-1α and miR-224, after ROS was induced by ROSUP or scavenged by addition of NAC, HIF-1α was measured by performing western blot and miR-224 was measured by RT-qPCR. *$p < 0.05$, versus Normoxia group. (C) After being treated as mentioned above, expression level of ROS was measured by H2DCFDA staining in EC9706 and TE-1 cells.

these three methods, respectively, and transfected miR-224 inhibitor, and then observed the changes in cell viability after 1–3 days. The results are shown in Figure 3A–C. Treatment with 1% O_2, DFO, and $CoCl_2$ can significantly reduce cell viability, while transfection with miR-224 inhibitor can significantly restore the decrease in cell viability caused by the three treatments.

Considering the close impact of hypoxia on the chemotherapy sensitivity of tumor cells, we hope to detect the impact of

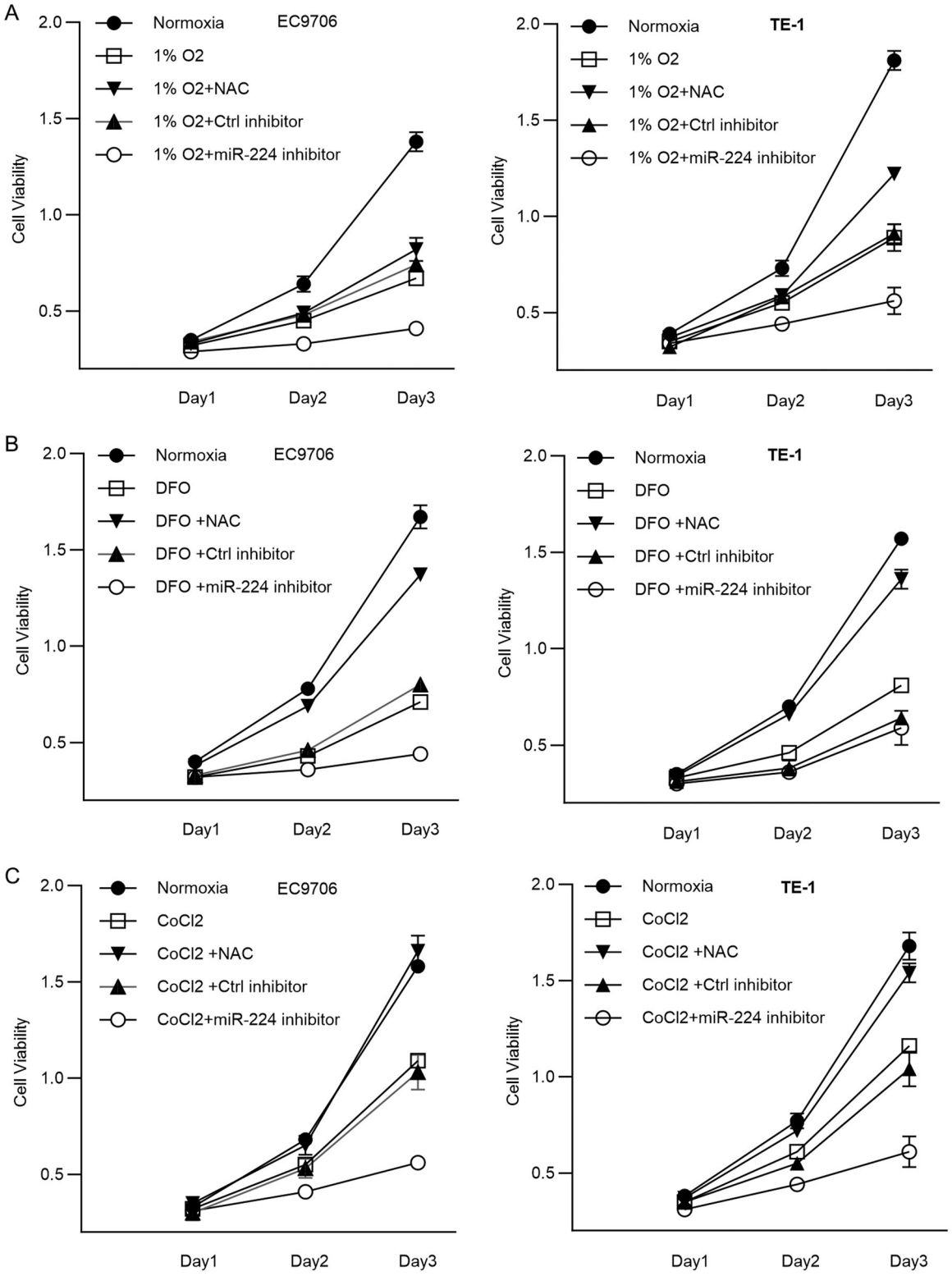

FIGURE 3 | The effects of miR-224 on cell viability affected by different conditions. After being treated by hypoxia (A), DFO (B), or $CoCl_2$ (C) for 12 h, NAC was added for 12-h extra treatment, or miR-224 inhibitor was introduced for 12-h extra culture. Then, cell viability was measured on Day 1, Day 2, and Day 3, by performing CCK-8 assay.

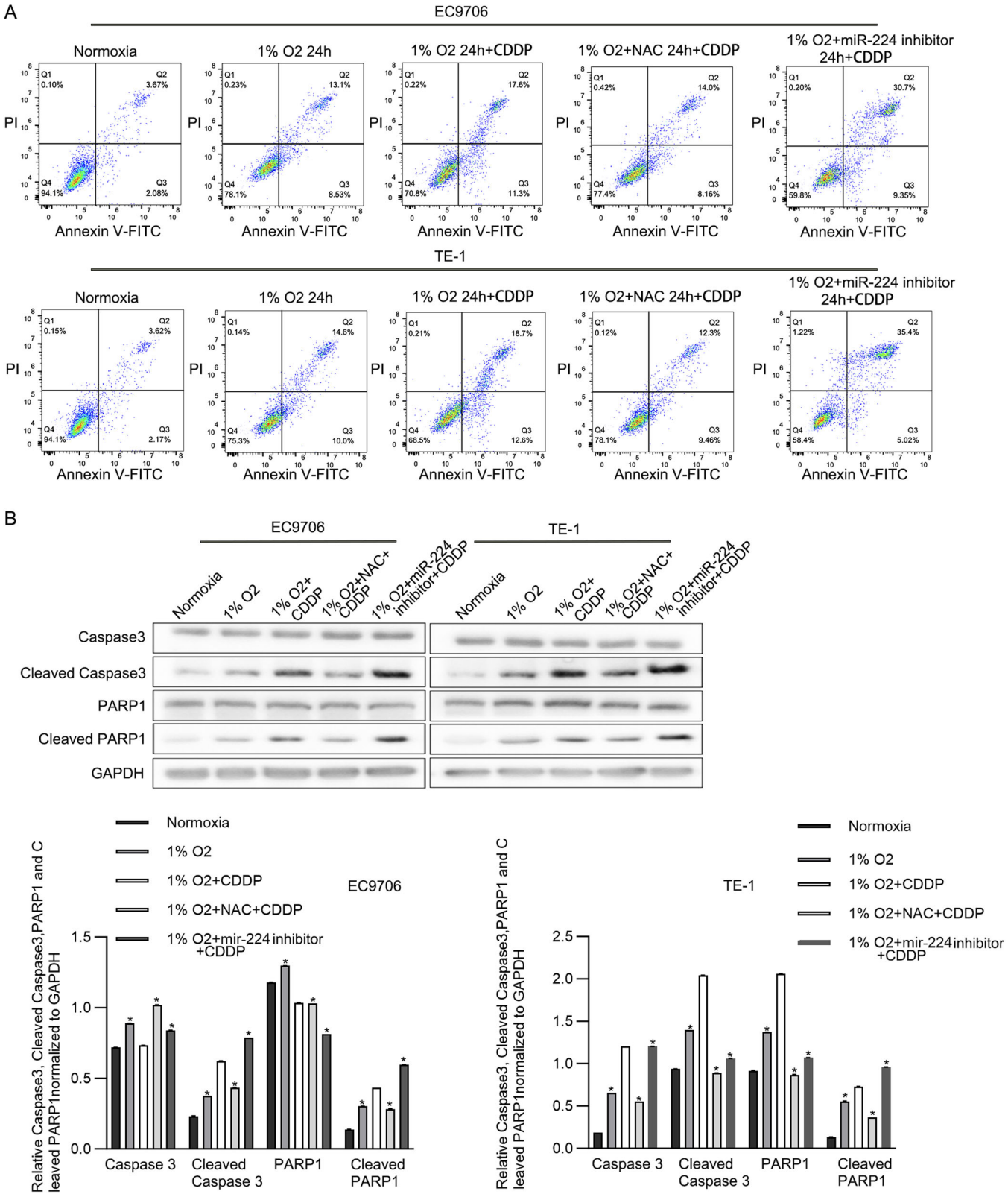

FIGURE 4 | The effects of miR-224 on apoptosis induced by CDDP affected by hypoxia treatment. After being cultured by hypoxia condition for 24 h, with or without CDDP, cell apoptosis was measured by performing Annexin V-FITC/PI double staining followed by flow cytometry assay (A). Apoptotic hallmarkers, including cleaved caspase 3 and cleaved PARP 1 were measured by performing western blot. $*p < 0.05$, versus normoxia group (B).

hypoxia-induced increased expression of miR-224 on chemotherapy sensitivity. Therefore, we selected CDDP, whose sensitivity is enhanced by hypoxia, for subsequent experiments. As shown in Figure 4A, after 24 h of hypoxia and CDDP treatment at the same time, the apoptosis rates were 21.6% ± 1.3% for EC9706 and 30.4 ± 2.2 for TE-1, while after interfering with the expression of miR-224, the apoptosis rates were increased to 39.4% ± 3.1% for EC9706 and 40.6% ± 1.7% for TE-1. To further confirm the effect of miR-224 on apoptosis induced by CDDP, we detected cleaved caspase-3 and cleaved PARP. The results showed that (Figure 4B) after interfering with miR-224, CDDP treatment could significantly increase cleaved caspase-3 and cleaved PARP. These results indicate that hypoxia-induced miR-224 can significantly inhibit CDDP-induced apoptosis, indicating its protective effect on tumor cells under hypoxic conditions.

3.3 | Hypoxia-Induced Effects of miR-224 on RASSF6 Gene Expression

Considering that miR-224 can specifically target RASSF6 mRNA and interfere with its expression level, we detected the protein level of RASSF6 after 12 and 24 h of treatment with 1% O_2, DFO, and $CoCl_2$ and found that the three treatment methods could significantly increase the protein level of RASSF6 (Figure 5A, $p < 0.05$). To further confirm the effect of miR-224 on the expression of RASSF6, we overexpressed miR-224 mimics and then detected the protein level of RASSF6, and the results showed that the protein level of RASSF6 was significantly decreased after overexpression of miR-224 (Figure 5B, $^*p < 0.05$).

3.4 | The Impact of Overexpressed miR-224 on the Viability and Proliferation of ESCC Cells

By constructing ESCC cell lines overexpressing miR-224/RASSF6, we investigated the impact of miR-224/RASSF6 on the viability and proliferation of ESCC cells. Flow cytometry analysis revealed cell cycle arrest at G1/G0 phase upon RASSF6 overexpression, which was rescued by miR-224 overexpression (Figure 6A). Soft agar colony formation assays demonstrated that ESCC colony formation was inhibited by RASSF6 overexpression, which was restored upon miR-224 overexpression (Figure 6B). These results were consistent with the results of plate colony formation assays (Figure 6C).

4 | Discussion

In this study, we examined the effects of miR-224 overexpression on ESCC cells by observing the biological consequences of downregulating the RASSF6 gene under hypoxic conditions. We found that this phenomenon leads to enhanced cell vitality, accelerated cell proliferation, and reduced sensitivity to cisplatin (CDDP) chemotherapy in ESCC cells. Additionally, this process also contributes to tumor formation and growth. The RASSF6 gene plays a significant role in the development and progression of many malignant tumors [21, 22], yet its role in ESCC is not well understood. Our findings suggest that RASSF6 is an effective tumor suppressor in ESCC. The expression of RASSF6 can effectively inhibit ESCC cell proliferation and induce G1/G0 phase arrest.

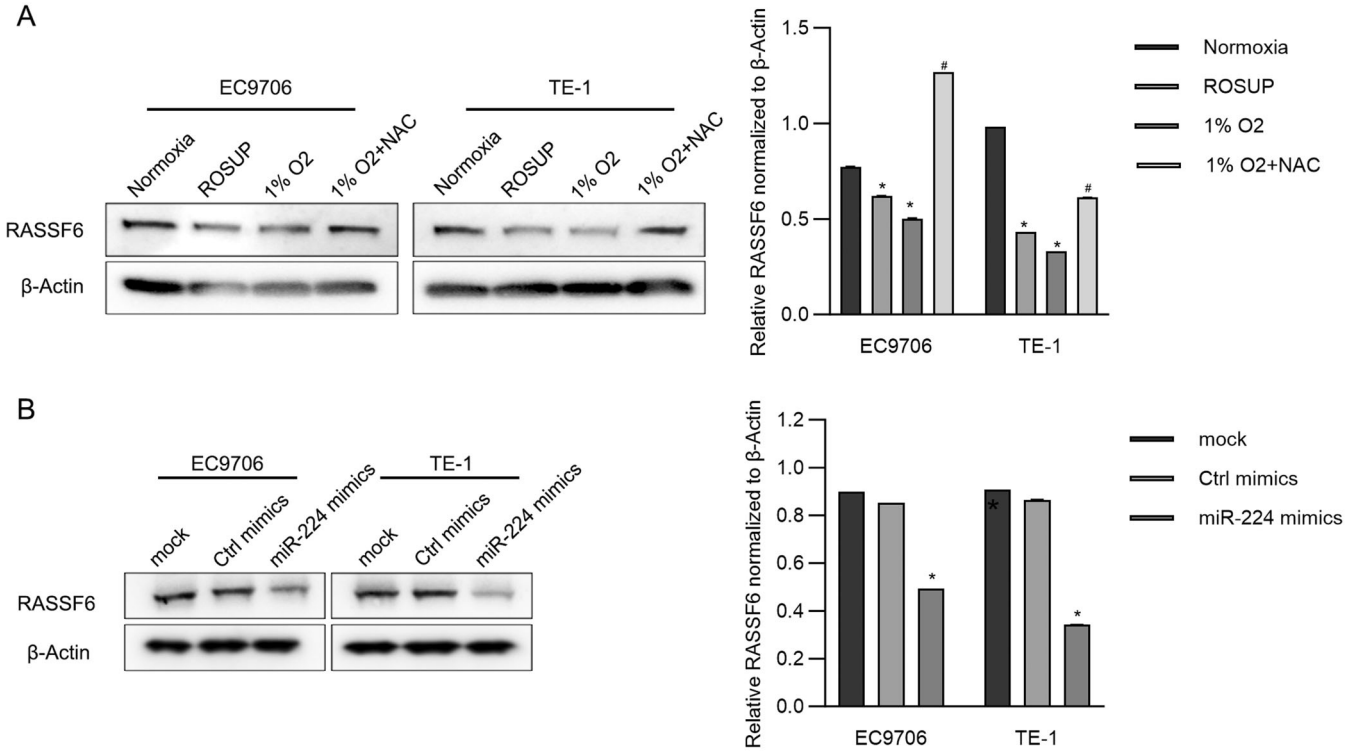

FIGURE 5 | Hypoxia treatment decreased RASSF6 potential via inducing mir-224. (A) After being treated by ROSUP or hypoxia condition, RASSF6 protein level was measured by performing Western Blot. $^*p < 0.05$, versus Normoxia group; $\#p < 0.05$, versus 1% O_2 group. (B) After introducing miR-224 mimics, RASSF6 protein level was measured. $^*p < 0.05$, versus Ctrl mimics group.

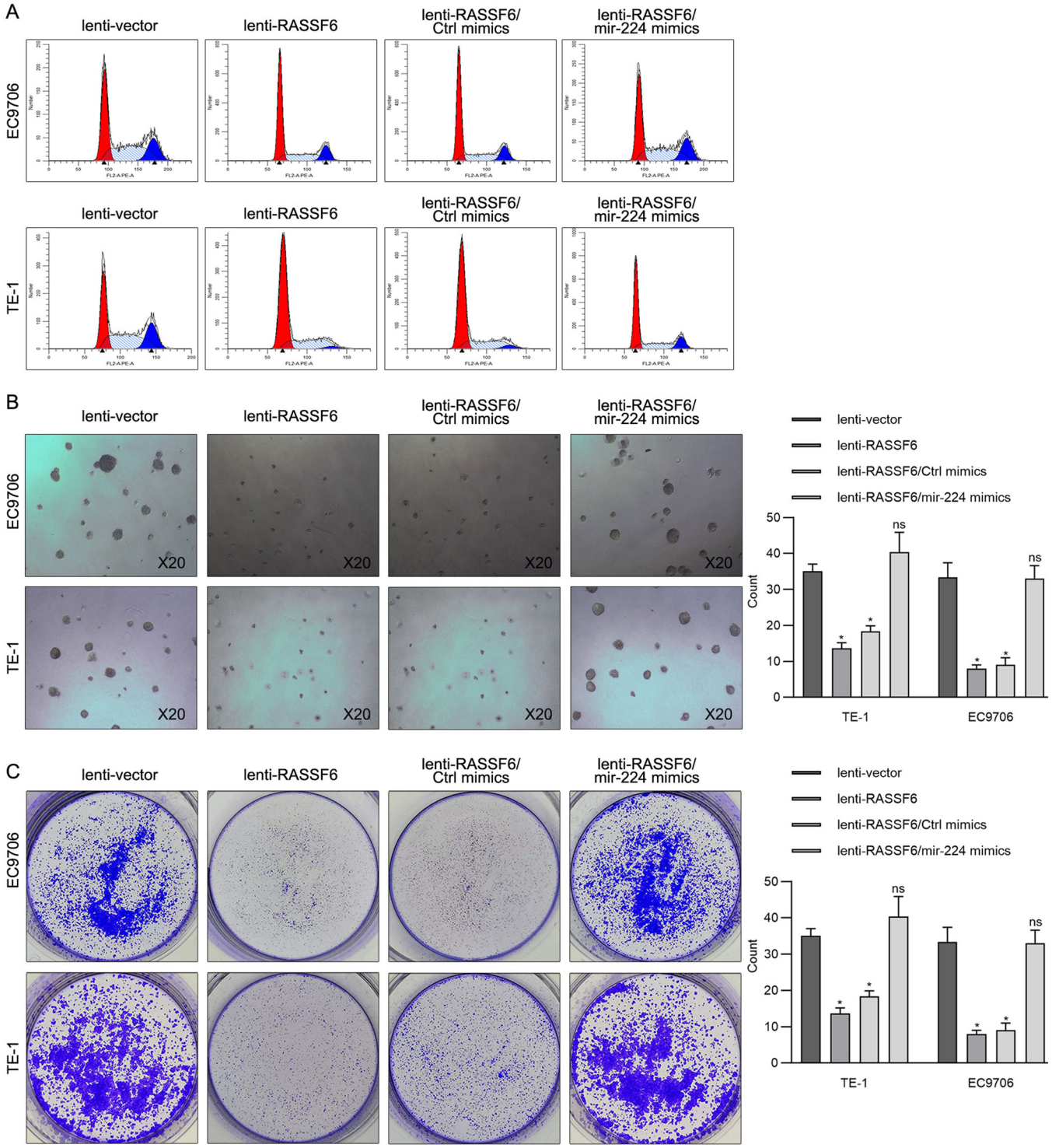

FIGURE 6 | The effects of miR-224 on malignancies exert potentially via regulating RASSF6. After overexpressing RASSF6 with or without cotransfection of miR-224 mimics, cell cycle phase distribution was measured by performing PI staining followed by flow cytometry analysis (A), tumor formation capacity was measured by performing soft agar tumor formation assay (B, $*p < 0.05$, vs. lenti-vector group), and colony formation capacity was performed (C, $*p < 0.05$, vs. lenti-vector group).

Furthermore, we explored the relationship between miR-224 and RASSF6. The results indicate that under hypoxic conditions, the expression of miR-224 is upregulated, and it can inhibit the expression of RASSF6. Considering that hypoxia, DFO treatment, and $CoCl_2$ treatment all lead to increased ROS, we hypothesized that accumulated ROS induce the upregulation of miR-224. The results showed that after clearing ROS, the expression level of miR-224 significantly decreased, suggesting a close relationship between miR-224 expression and ROS accumulation caused by hypoxia. This finding provides a new direction for the treatment of ESCC. Notably, it is observed that overexpression of RASSF6 inhibited cell cycle progression, colony formation, and tumor formation. After miR-224 introduction, the inhibitory effects of RASS6 were reversed.

These results indicate that mir-224 might exert critical roles mainly via targeting to RASSF6.

In most cases of ESCC, hypoxia is a norm, and it is one of the indicators of poor prognosis. We believe that this poor tumor prognosis caused by hypoxia may be realized through the miR-224/RASSF6 regulatory axis. Although we introduced three methods to induce increased ROS, due to the significant cytotoxicity of DFO and CoCl2 treatments on cells, prolonged treatment led to massive cell death. Therefore, these two cell models were not included in the subsequent functional recovery experiments, which is one of the limitations of this study.

In our research, we also demonstrated that the upregulation of miR-224 caused by hypoxia leads to reduced sensitivity of ESCC to CDDP chemotherapy, and this inhibitory effect can be counterbalanced by introducing an miR-224 inhibitor. It is evident that miR-224/RASSF6 plays a significant role in chemotherapy resistance induced by hypoxia. However, the effects of hypoxia are continuous, affecting many malignant behaviors of ESCC, such as metabolism, malignant transformation, invasion, and metastasis. The exact role played by miR-224/RASSF6 in these processes requires further investigation and validation. Next, we will explore the impact of miR-224/RASSF6 on the invasion and metastasis of ESCC under hypoxia induction. The treatments with DFO and CoCl2 caused massive cell death in EC9706 and TE-1 cells, which could not be alleviated by clearing ROS or inhibiting miR-224. This may be due to the treatments' strong cytotoxicity, which is irreversible. This also suggests that the effects of miR-224 on cells might be masked by factors other than ROS. Although we focused on the effects of mir-224 or RASSF6 in ESCC under hypoxia condition, it is still unclear the exact roles of miR-224 or RASSF6 in ESCC under normoxia condition, which is worth to be investigated in further study.

5 | Conclusion

Under hypoxia induction, the accumulation of ROS can lead to the upregulation of miR-224. MiR-224 facilitates the survival of esophageal cancer cells under hypoxic conditions and promotes chemotherapy resistance. This effect is mainly achieved through the downregulation of the RASSF6 gene. Through this mechanism, overexpressed miR-224 can increase the vitality of esophageal cancer cells and promote cell proliferation, thereby accelerating tumor formation and growth.

Author Contributions

Yang Wang and Yi Liu conceived and designed the study. Yina Wang, Adili Salai, and Yueying Yang conducted the cell culture–related experiments. Yi Liu analyzed the data and wrote the manuscript. Yang Wang confirms the authenticity of all the raw data. All authors read and approved the final manuscript.

Acknowledgments

Mr. Tao Hong checked the manuscript.

Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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