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2-Methoxyestradiol promotes radiosensitivity of esophageal squamous cell carcinoma by suppressing hypoxia-inducible factor-1α expression

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Abstract. – OBJECTIVE: Acquired radioresistance remains the primary obstacle to improving the survival of esophageal cancer (EC). Related hypoxia factors play crucial roles in radioresistance such as hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), and angiogenic factor with G-patch and FHA domains 1 (AGGF1). The aim of this study was to investigate the effect of 2-methoxyestradiol (2ME2) on the radiosensitivity of EC.

PATIENTS AND METHODS: The expression of HIF-1α, VEGF, and AGGF1 in 70 EC patients was detected using immunohistochemistry. The relation between the expression levels of the above genes with clinicopathologic characteristics was explored as well. Human ECA-109 cells were subjected to hypoxia and/or radiation in the presence or absence of 2ME2. Subsequently, cell growth, colony formation, and apoptosis were evaluated. Moreover, the mRNA and protein expression levels were determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blotting, respectively.

RESULTS: HIF-1α, VEGF, and AGGF1 were significantly in EC tissues and were associated with cancer aggressiveness. 2ME2 treatment significantly increased the radiosensitivity of ESCC cells in a dose-dependent manner, which was related to the inhibited expression of HIF-1α. Subsequent immunohistochemical staining results showed that the VEGF expression was positively correlated with the HIF-1α expression in EC.

CONCLUSIONS: High levels of HIF-1α, VEGF, and AGGF1 in EC are indicators of poor prognosis in patients treated with radiotherapy, which can offer new ideas and methods for the treatment of ESCC. In addition, 2ME2 inhibits the expression of HIF-1α and confers radiosensitivity in ECA-109 cells. Our findings suggest that 2ME2 has the potential to be applied as an adjuvant treatment with radiotherapy for EC.

Key Words:
2ME2, HIF-1α, VEGF, AGGF1, Radiosensitivity, Esophageal cancer (EC).

Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer mortality worldwide[1]. The most prevalent histologic type of EC in China is esophageal squamous cell carcinoma (ESCC)[2]. Due to the lack of reliable early detection methods and the absence of early symptoms, most EC patients have already been in advanced-stage when first diagnosed. Currently, complex multidisciplinary treatment, especially concurrent chemoradiotherapy, has become a common practice. However, the rate of local recurrence and distant metastasis of ECSS remains high[3,4]. Acquired radioresistance during radiotherapy (RT) has been considered as one of the most important reasons for treatment failure[5]. Previous studies have indicated that many factors affect radioresistance, including tumor size, hypoxia, and intrinsic radiosensitivity.

Tumor hypoxia is an important factor leading to radioresistance and poor clinical outcomes. Oxygen during RT generates free oxygen radicals that induce DNA damage and kill tumor cells[6,7]. Hypoxic environments can activate a specific set of tumor promoting transcription factors such as hypoxia-inducible factor 1 (HIF-1)[8]. HIF-1α is an oxygen-sensitive factor that is capable of regulating genes involved in tumor cell survival, metabolism, proliferation, and invasion[9]. The vascular endothelial growth factor (VEGF), one of its downstream genes, is transcriptionally activated by HIF-1α in

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the hypoxic environment[10-12]. Meanwhile, tumor progression is often correlated with the up-regulation of angiogenesis due to increased VEGF[11].

The angiogenic factor with G-patch and FHA domains 1 (AGGF1) encodes an angiogenic factor with 714 amino acid residues and is also a factor that has been implicated in angiogenesis[13]. Increased AGGF1 expression is associated with congenital vascular disorder Klippel-Trenaunay syndrome (KTS)[13]. AGGF1 can promote angiogenesis in chicken embryo angiogenesis assays and mouse hindlimb ischemia models[13,14]. Furthermore, the up-regulation of AGGF1 reportedly contributes to tumor angiogenesis in HCC[15].

Recently, it is well known that tumor growth requires angiogenesis. Some agents can sensitize cancer cells to radiation by inhibiting HIF-1α[16]. 2-methoxyestradiol (2ME2) is a naturally occurring derivative of estradiol. Also, it has been shown to be an orally active, well-tolerated small molecule that possesses anti-tumor and anti-angiogenic activities[17]. In addition, 2-ME2 has been used to block HIF-1α nuclear accumulation through oxygen- and proteasome-independent pathway involving the disruption of microtubules[18]. However, the effects of 2ME2 on ESCC cells and the underlying mechanism have not been fully elucidated.

In this study, we aimed to investigate whether 2ME2 could radiosensitize ESCC cells under hypoxia by inhibiting HIF-1α expression. The effects of 2ME2 and/or radiation on ESCC cells exposed to normoxia or hypoxia were explored *in vitro*. Furthermore, we determined whether AGGF1 could be used as a potential therapeutic target for ESCC.

Patients and Methods

Clinical Samples

70 tumor tissues and 30 non-tumor tissues were collected from patients who received diagnosis in the Affiliated Tumor Hospital of Xinjiang Medical University from 2011 to 2015. All patients had symptoms such as nausea, feeding disturbance, sour regurgitation, but were finally diagnosed as esophagitis. Histological diagnoses were performed by expert pathologists. Detailed clinicopathologic data were collected and recorded. No patient received surgery before the study. However, the patients received RT using 6-MV X-rays at 1.8-2.0 Gy per fraction (5 times per week), with a total dose of 46-66 Gy in 4-6 weeks. Informed consent was obtained from each subject before the study. This investigation was approved by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University.

Chemicals and Reagents

Roswell Park Memorial Institute-1640 (RPMI-1640) medium modified, phosphate-buffered saline (PBS), fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were purchased from Hyclone Laboratories (HyClone, Logan, UT, USA). Cobalt (II) chloride ($CoCl_2$) was from Sigma-Aldrich (St. Louis, MO, USA), and 2ME2 from MedChem Express (Princeton, NJ, USA). They were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 mmol/L, and stored at -20°C for use. Rabbit antibodies against HIF-1α, VEGF, and AGGF1 were from Abcam Biotechnology Inc (Abcam, Cambridge, UK), while rabbit antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular technologies, Inc (Dojindo Laboratories, Kumamoto, Japan).

Cell Culture

Human ESCC line ECA-109 was provided by the Central Experimental Laboratory of Xinjiang Medical University (Urumqi, China). All cells were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Hypoxic conditions were mimicked using a hypoxia-mimetic agent, $CoCl_2$[19]. Prior to IR, cells were pre-treated and/or 2ME2.

Cell Growth by CCK-8 Assay

The effect of 2ME2 on cell viability with IR was monitored by the CCK-8 assay. Cells were first seeded into 96-well plates at a density of 4×10^5 cells/well and pre-treated with various concentrations of 2ME2 for 24 h, followed by incubation for 24 h after IR. Approximately, 10 μL of CCK-8 reagent was added to each well. The absorbance at 450 nm was detected by a microplate reader.

Clonogenic Survival Assay

Different densities (200-2,000) of cells were first seeded into six-well plates. IR and/or 2ME2 were placed under normoxic or hypoxic conditions for 24 h and irradiated by various doses (0, 2, 4, 6, 8, and 10 Gy). After 10 days of culture, the medium was aspirated, and the plates were washed with PBS. Formed colonies were fixed with methanol for 30 min and stained with Giemsa. Finally, the number of colonies containing more than 50 cells was calculated.

Apoptosis Analysis

Cells were treated with 0.5 or 1 mM 2ME2 for 24 h under normoxic or hypoxic conditions before IR. After 24 h, cells floating in the supernatant and adherent cells harvested by trypsin were gathered to produce single cell suspension. After centrifugation, the cells were washed with PBS, and suspended in 500 μL of buffer solution, followed by staining with 5 μL annexin V and 5 μL propidium iodide (BD Bioscience, Franklin Lakes, NJ, USA). Apoptosis analysis was performed on a Beckman flow cytometer. Hoechst 33342 working solution was used to identify apoptosis-like death rate by the formula: apoptosis-like cell death rate = number of apoptosis-like dead cells/total number of cells.

Protein Extraction and Western Blot Analysis

Cells were digested with trypsin, transferred into microtubes, and centrifuged at 2000 rpm at room temperature for 5 min. The cell pellet was washed with ice-cold PBS (–) twice and homogenized in 40 μL of protein extraction buffer with chilling. Then, the solution was centrifuged at 12,000 rpm and 4°C for 20 min. The concentration of extracted protein was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). After that, the mixture was boiled at 90°C for 5 min. Equal amounts (40 μg) of protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). For the molecular weight standard, 3 μL of Prestained Protein Marker was loaded in the left lane and 1 μL in the right lane. Subsequently, the membranes were blocked with 5% of non-fat dried milk in PBS containing 1% Tween-20 for 1 h at room temperature. Then, they were incubated with primary antibodies (1:500), or rabbit monoclonal anti-GAPDH antibody (1:1000) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. Finally, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL) Western blotting detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemical Analysis

Serial sections (4 μm) subjected to immunohistological staining were first fixed with fresh 3% H_2O_2 containing 0.1% of sodium azide. Then, the sections were treated with antigen retrieval solution for 15 min. After 15 min of incubation in blocking reagent, the sections were incubated with primary antibodies (1:500) overnight at 4°C. On the next day, they were incubated with corresponding secondary antibody and extravidin-conjugated horseradish peroxidase. The expression level was quantified based on the intensity of staining (scored as 0-no, 1-weak, 2-moderate, and 3-strong staining) and the percentage of positive tumor cells (scored as 0, ≤5%, 1, 5-25%, 2, 26-50%; 3, ≥51%). The final score was calculated as the product of two parameters, and at least 3 points were considered positive.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues and cells was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, the extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript™ RT Reagent Kit (Perfect Real Time, TaKaRa, Otsu,Shiga, Japan). Quantitative real-time PCR was performed with a 20 μL system containing SYBR Green (SYBR® Premix ExTaq™ II; TaKaRa, Otsu, Shiga, Japan) on an Applied 7600 Fast quantitative PCR system. Primers used in this study were as follows: HIF-1α, F, 5'-TGCAACATGGAAGGTATTGC-3'/R, 5'-TTCACAAATCAGCACCAAGC-3'; VEGF, F, 5'-CCTTGCTGCTCTACCTCCAC-3'/R, 5'-AGCTGCGCTGATAGACATCC-3'; AGGF1, F, 5'-TGGAGAAGATGGGTTGGAAG-3'/R, 5'-ATGAGGATGGTTTGCCTGTC-3'; GAPDH, F, 5'-TCCCATCACCATCTTCCAGG-3'/R, 5'-GATGACCCTTTTGGCTCCC-3'.

Radiotherapy for Cultured Cells

Cells subjected to radiotherapy were exposed to doses of 0-10 Gy, with X-ray from a linear accelerator (Varian 600C/D, Salt Lake City, UT, USA) at an average dose rate of 100 cGy/min. The operation was performed at 100 kV and 5 mA, and the distance to the IR source was 100 cm.

Diagnostic Criteria for Tumor Response

Tumor response was assessed using X-ray images after completion of IR based on Response Evaluation Criteria in Solid Tumors (RECIST) 18. Complete response (CR) was defined as the disappearance of the target lesion. Partial response (PR) was defined as a reduction of at least 50% in the product of the longest diameter and longest vertical diameter of target lesions. Progressive disease (PD) was defined as an increase of at least 25% in the product of longest diameter and longest vertical diameter of target lesions, or new

lesions appeared. Stable disease (SD) was defined as a reduction of less than 25% in the product of the longest diameter and longest vertical diameter of target lesions, or no new lesions appeared.

Statistical Analysis

GraphPad Prism program version 5.0 (GraphPad Software, La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) statistical version 17.0 (SPSS Inc. Chicago, IL, USA) were performed for all statistical analysis. Experimental data were presented as mean ± Standard Deviation (SD). All experiments were performed at least three times. Continuous variables were compared by analysis of variance (ANOVA) and Student's *t*-test, while categorical variables were compared by the Chi-square test. Correlation analysis was performed using the Spearman analysis. $p<0.05$ was considered statistically significant.

Results

Expression of HIF-1α, VEGF, and AGGF1 in EC Tissues

To investigate the roles of HIF-1α, VEGF, and AGGF1 in the progression of EC, we first detected their expressions in EC tissues and non-tumor tissues by immunohistochemical (IHC) staining. Results indicated that HIF-1α protein was mainly located in the cytoplasm and nucleus, while VEGF and AGGF1 were confined to the cytoplasm (Figure 1). HIF-1α, VEGF, and AGGF1 proteins were positively expressed in 60%, 37.14%, 54.29% of EC tissues, and 10.0%, 16.67%, 23.33% in non-tumor tissues, respectively. There was a statistically significant difference in the positive rate of the three proteins between EC and non-tumor tissues group (χ^2=21.212, 4.116, 8.129, p=0.000, 0.042, 0.004).

Association of HIF-1α, VEGF, and AGGF1 Expression with Clinicopathologic Parameters

The expression rates of HIF-1α, VEGF, and AGGF1 in EC with respect to several standard clinicopathologic features were listed in Table I. No significant difference was observed between their expressions and clinicopathologic features such as patient gender, age, nationality, etc. However, their expression levels were found significantly down-regulated in patients with well differentiation and earlier clinical stage.

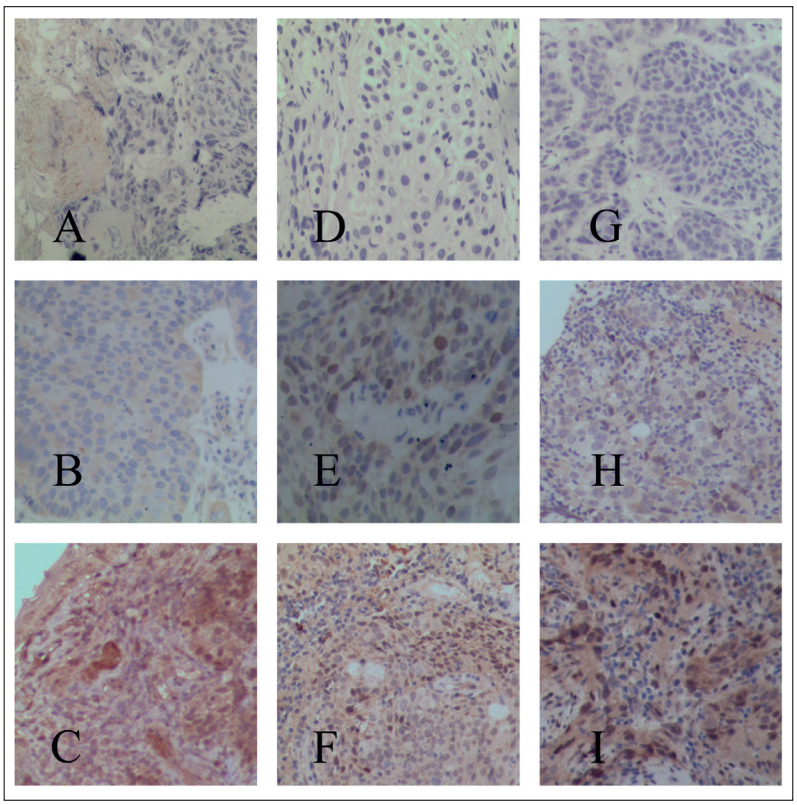

Figure 1. Immunohistochemical analysis of HIF-1α, VEGF, and AGGF1 in EC. HIF-1α, VEGF, and AGGF1 staining, **A-C**, Negative. **D-F**, Weak positive. **G-I**, Strong positive. Original magnification×100.

Table I. Expression of HIF-1α, VEGF and AGGF1, and the relation with the clinicopathologic features in EC.

Variables	No.	HIF-1α		*p*-value	VEGF		*p*-value	AGGF1		*p*-value
		positive	negative		positive	negative		positive	negative	
Gender				0.338			0.502			0.563
Male	39	25	14		14	25		21	18	
Female	31	17	14		12	19		17	14	
Nationality				0.371			0.389			0.325
Han	33	21	12		15	18		21	12	
Uygur	31	19	12		9	22		14	17	
Ha	6	2	4		2	4		3	3	
Age (years)				0.365			0.326			0.366
≤65	28	18	10		9	19		14	14	
>65	42	24	18		17	25		24	18	
Tumor location†				0.171			0.505			0.720
Upper	18	8	10		5	14		9	10	
Middle	38	25	13		16	22		21	17	
Lower	14	10	4		5	8		8	5	
Tumor length (cm)				0.921			0.411			0.668
≤5.0 cm	54	33	21		18	36		29	25	
5.0-7.0 cm	11	6	5		5	6		7	4	
≥7.0 cm	5	3	2		3	2		2	3	
Differentiation				0.045			0.009			0.029
Well	6	4	2		4	2		4	2	
Moderate	43	21	22		10	33		18	25	
Poor	21	17	4		12	9		16	5	
T category				0.011			0.017			0.014
T2	19	6	13		10	9		5	14	
T3	37	27	10		8	29		23	14	
T4	14	9	5		8	6		10	4	
N category				0.004			0.028			0.016
N0	15	4	11		2	13		4	11	
N1	55	38	17		24	31		34	21	
M category				0.009			0.019			0.013
M0	54	28	26		16	38		25	29	
M1	16	14	2		10	6		13	3	
Clinical stage				0.014			0.009			0.005
II	24	9	15		8	16		7	17	
III	30	23	7		7	23		22	8	
IV	16	10	6		11	5		9	7	
Radiotherapy response				0.014			0.039			0.182
CR+PR	43	21	22		12	31		21	22	
NR+PD	27	21	6		14	13		17	10	
Survival status				0.022			0.022			0.018
Alive	31	14	17		7	24		12	19	
Dead	39	28	11		19	20		26	13	

†Lower/Middle/Upper, lower/middle/upper thoracic EC;

Table II. The relationship of HIF-1α, VEGF and AGGF1 in EC tissues.

Variables	VEGF		r	*p*-value	AGGF1		r	*p*-value
	positive	negative			positive	negative		
HIF-1α			0.266	0.026			0.070	0.563
positive	20	22			24	18		
negative	6	22			14	14		

Further analysis demonstrated that low expressions of HIF-1α, VEGF, and AGGF1 were associated with improved IR response in EC patients, with 51.2%, 72.1%, and 51.2% of patients achieving CR+PR after IR for 3 months, respectively. Alternatively, a higher expression of HIF-1α and VEGF indicated a significant greater resistance of EC patients to radiotherapy.

Relationship Between HIF-1Ð, VEGF, and AGGF1 Expression in EC Tissues

As shown in Table II, we found that VEGF was positively correlated with HIF-1α. However, there was no statistically significant relation between AGGF1 and HIF-1α expression in EC tissues.

2ME2 Inhibited Proliferation of ESCC Cells

The effect of various concentrations of 2ME2 on ESCC cells was determined by the CCK-8 assay. Meanwhile, it was explored whether 2ME2 was cytotoxic for cell proliferation. As indicated in Figure 2A, 2ME2 produced cytotoxicity in a dose-dependent manner. The 24-h survival rates of 0.5 and 1 mM 2ME2-treated groups were 89.52% and 71.61%, respectively. The results showed that 2ME2 exhibited a low toxicity effect on the growth of ECA-109. Thus, low concentrations of 2ME2 (0.5 and 1 mM) were chosen for the following assays.

2ME2 Sensitized ESCC Cells to IR

The clonogenic survival assay was performed to investigate the effect of 2ME2 on the proliferative ability of ESCC cells. 2ME2 displayed significant radiosensitivity to colony formation ability under hypoxia. The clonogenic surviving fraction of 2ME2 and IR was shown in Figure 2B. In IR group alone, the survival of ESCC cells decreased significantly with the increase in. IR. However, it decreased markedly in IR+2ME2 group, showing a statistically significant difference (p<0.01). According to the click multitarget model, the main parameters of ESCC cell dose-survival curves were obtained. The radiosensitization effect of 2ME2 in ESCC cells was summarized in Table III.

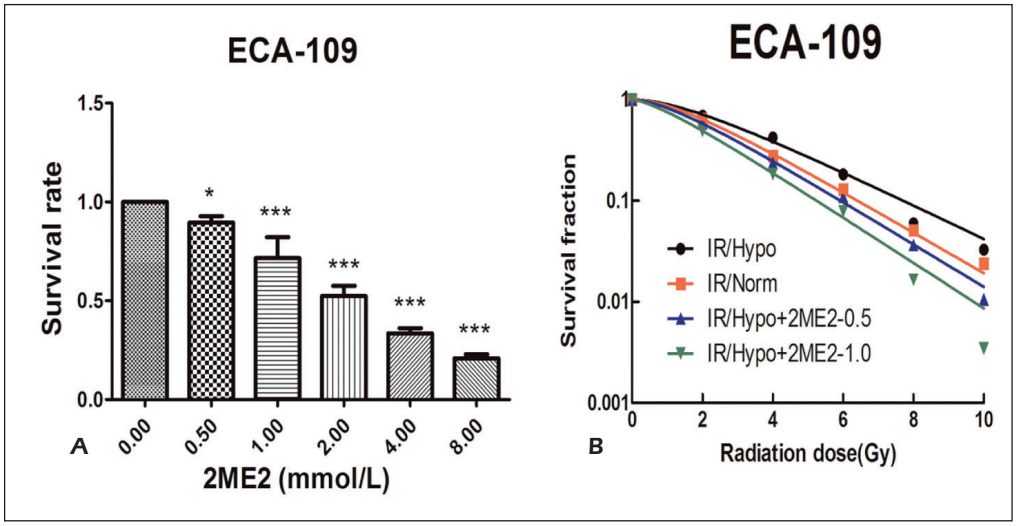

Figure 2. **A**, 2ME2 inhibited the proliferation of human ESCC cells. Cell viability was determined by CCK-8 assay. 2ME2 (0.5-8 mM) was added before IR. After 24 h, the effects of 2ME2 were tested (*p<0.05). **B**, Clonogenic survival of human ESCC cells following IR. ESCC cells were seeded into six-well plates and subjected to different doses of IR. Cells were treated with 2ME2 and/or IR. Clonogenic survival curves were plotted as the log of the surviving fraction versus the IR dose.

Table III. The radiosensitization of 2ME2 in ESCC cells.

	Do (Gy)	Dq (Gy)	SF2	SER
Hypoxia IR	2.59	2.65	0.69	
Normoxia IR	2.15	2.22	0.64	1.20
Hypoxia IR+2ME2 0.5 mM	2.05	1.71	0.58	1.26
Hypoxia IR+2ME2 1 mM	1.93	1.06	0.49	1.34

Sensitization enhancement ratio (SER) = Do (without sensitizer) / Do (with sensitizer)
Do mean lethal dose, Dq quasi-threshold dose, SF2 survival fraction values at 2Gy

With the decrease of the mean lethal dose in combination group, we found that 2ME2 produced radiosensitive effects on ESCC cells under hypoxia.

Effect of 2ME2 on Radiosensitivity to Apoptosis of ESCC Cells Induced by IR

24 h after treatment with 2ME2 and/or IR, the apoptosis of ESCC cells was assessed, and the proportion of apoptotic cells was determined by flow cytometry. The results showed that 2ME2+IR group exhibited a significantly greater cell apoptosis than that of the single IR group. Meanwhile, significant radiosensitivity was observed under hypoxic conditions (Figure 3A-3E).

Subsequently, we investigated apoptosis-like cell and total cell death rate of ECA109 cells treated with IR (0, 2, 5, 8 Gy) + 2ME2 (0, 0.5, 1 mM). Treatment with various concentrations of 2ME2 resulted in a dose-dependent inhibition of cell proliferation (Figure 3F-3G).

2ME2 Inhibited the Up-Regulation of HIF-1α Induced by Hypoxia and IR in ESCC Cells

The expressions of HIF-1α, VEGF, and AGGF1 mRNAs in ESCC cells were determined by qRT-PCR (Figure 4A-4C). Results indicated that when the three mRNAs were generated based on the trend of radiosensitivity, their expressions increased with the cell resistance to IR hypoxic condition. During the treatment with 0.5 and 1 mM 2ME2, both the expressions of HIF-1α and VEGF decreased, approximately 30% of that with IR alone (Figure 4A and 4B, $p<0.001$). Spearman correlation analysis verified a negative correlation between radiosensitivity capacity and the mRNA expressions of HIF-1α, VEGF, and AGGF1 (r = -0.974, -0.394, -0.604, p=0.001, 0.001, 0.000).

Western blot was performed to investigate the effect of 2ME2 on HIF-1α-induced VEGF activity. To evaluate the expression of HIF-1α and VEGF induced by hypoxia, ECA-109 cells were first treated with hypoxia. As shown in Figure 4D and 4E, HIF-1α and VEGF levels reached highest level at 48 h after hypoxia, while AGGF1 level was at 24 h. Subsequently, we analyzed their expressions under hypoxia and 2ME2. Results demonstrated that 2ME2 inhibited hypoxia-induced up-regulation of HIF-1α and VEGF expressions in ECA-109 cells.

Discussion

EC is one of the most common gastrointestinal carcinomas worldwide. Yoshimura et al[20] have found that EC leads to hypoxic environments due to the increased oxygen consumption of extensively growing tumor cells and decreased oxygen delivery from disorganized tumor blood vessels. Ultimately, this imbalance may result in radiation resistance in cancer cells. However, radiotherapy is now a major treatment component for locally advanced EC.

HIF-1α is a key transcription factor in tumor development. A better understanding of the molecular mechanism of HIF-1α may be beneficial for exploring novel and promising therapeutic strategies for EC. In our study, results showed that HIF-1α was over-expressed in EC tissues. Meanwhile, the high expression level of HIF-1α was correlated with cancer aggressiveness.

Radioresistance is a challenging obstacle in the treatment of EC, and researches addressing this problem are of great significance. Strategies to enhance tumors' radiosensitivity without further toxicity are currently needed. Li et al[21] have demonstrated that the development of radioresistance is complicated, involving multiple molecular mechanisms. Recently, numerous new hypoxic radiosensitizers have been developed, and some have already been clinically evaluated. Pre-clinical studies have shown that the suppression of HIF-1α can sensitize tumors to chemotherapy and radiotherapy. A landmark report has suggested that 2ME2 inhibits angiogenesis, which shows an

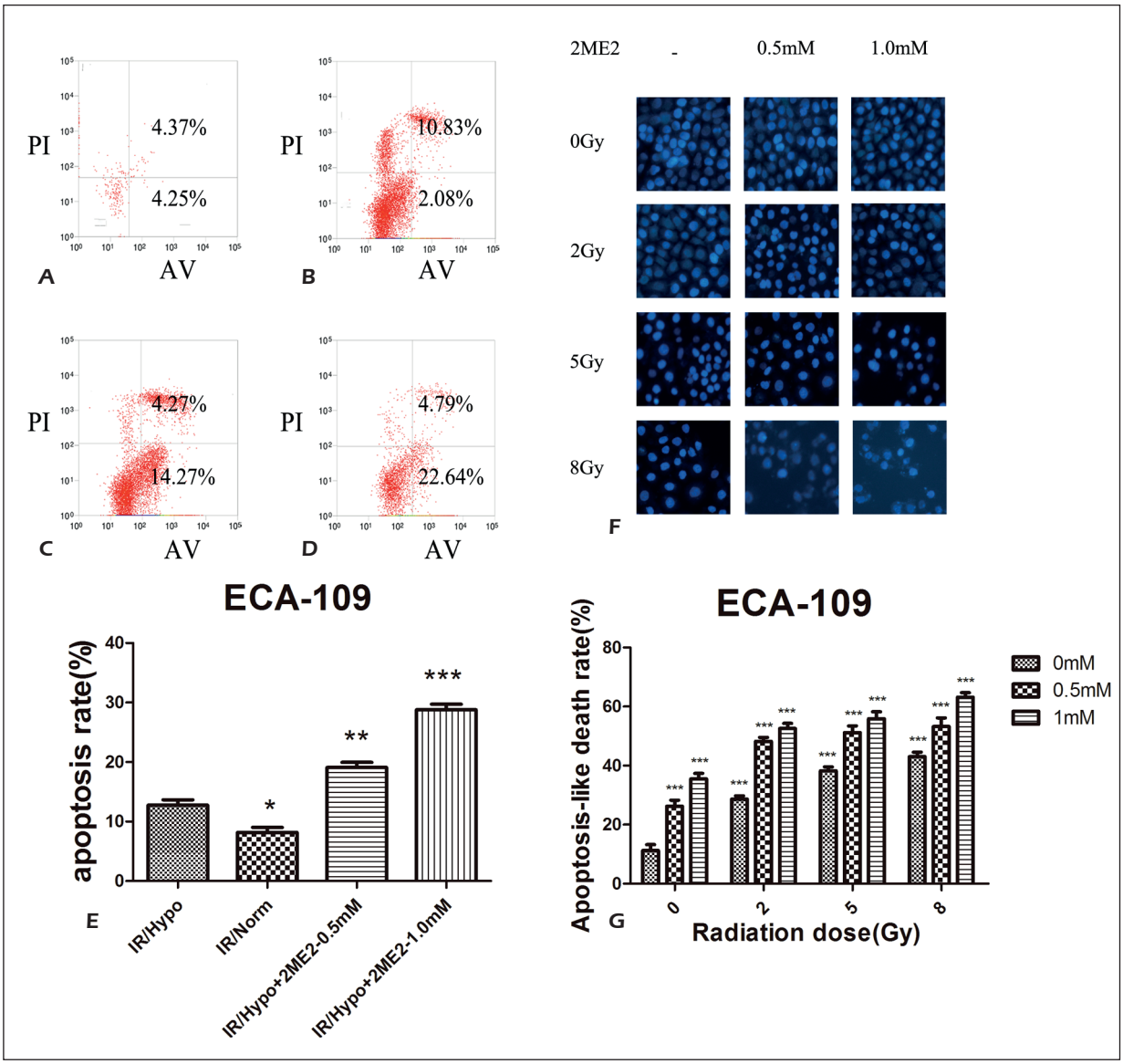

Figure 3. 2ME2 promoted IR-induced apoptosis in ESCC cells. Cells were pretreated with 2ME2 for 24 h before IR. At IR under 8 Gy, ESCC cells were incubated for another 48 h and digested with trypsin. **A**-**E**, Flow cytometric analysis showed 2ME2 induced apoptosis of hypoxic ESCC cells. **A**, normoxia IR, **B**, hypoxia IR, **C**, hypoxia IR+2ME2-0.5 mM, **D**, hypoxia IR+2ME2-1 mM, **E**, apoptosis rate in four groups, **F**-**G**, ESCC cells stained with Hoechst 33342 dye. **F**, Represented the morphologic changes of ESCC cells treated with 0.5 and 1 mM 2ME2, 1 day after IR on diverse doses (magnification: 100×). **G**, Apoptosis-like death rate of ESCC cells treated with 0.5 and 1 mM 2ME2, and IR on diverse doses. (*p<0.05).

important function in tumor growth, metastasis, and invasion, by dysregulating HIF[22]. Moreover, increased levels of HIF-1α are required for angiogenesis[23]. So far, 2ME2 has been proved to be low toxic and effective in several cancers, including GBM and HCC[16, 24].

Our results indicated that 2ME2 pretreatment decreased cell viability, reduced cell apoptosis and the cloning survival rate under hypoxic and IR conditions. Meanwhile, this study revealed for the first time that 2ME2 could significantly promote radiosensitivity of ESCC cells under hypoxic conditions by inhibiting HIF-1α. 2ME2 inhibited the HIF-1α protein accumulation in ESCC cells, thereby suppressing the expressions of related genes involved in tumor cell proliferation. Therefore, we speculated that VEGF, an important regulator of angiogenesis[25], might be associated with this effect. Our further results indicated that HIF-1α signaling contributed, at least in

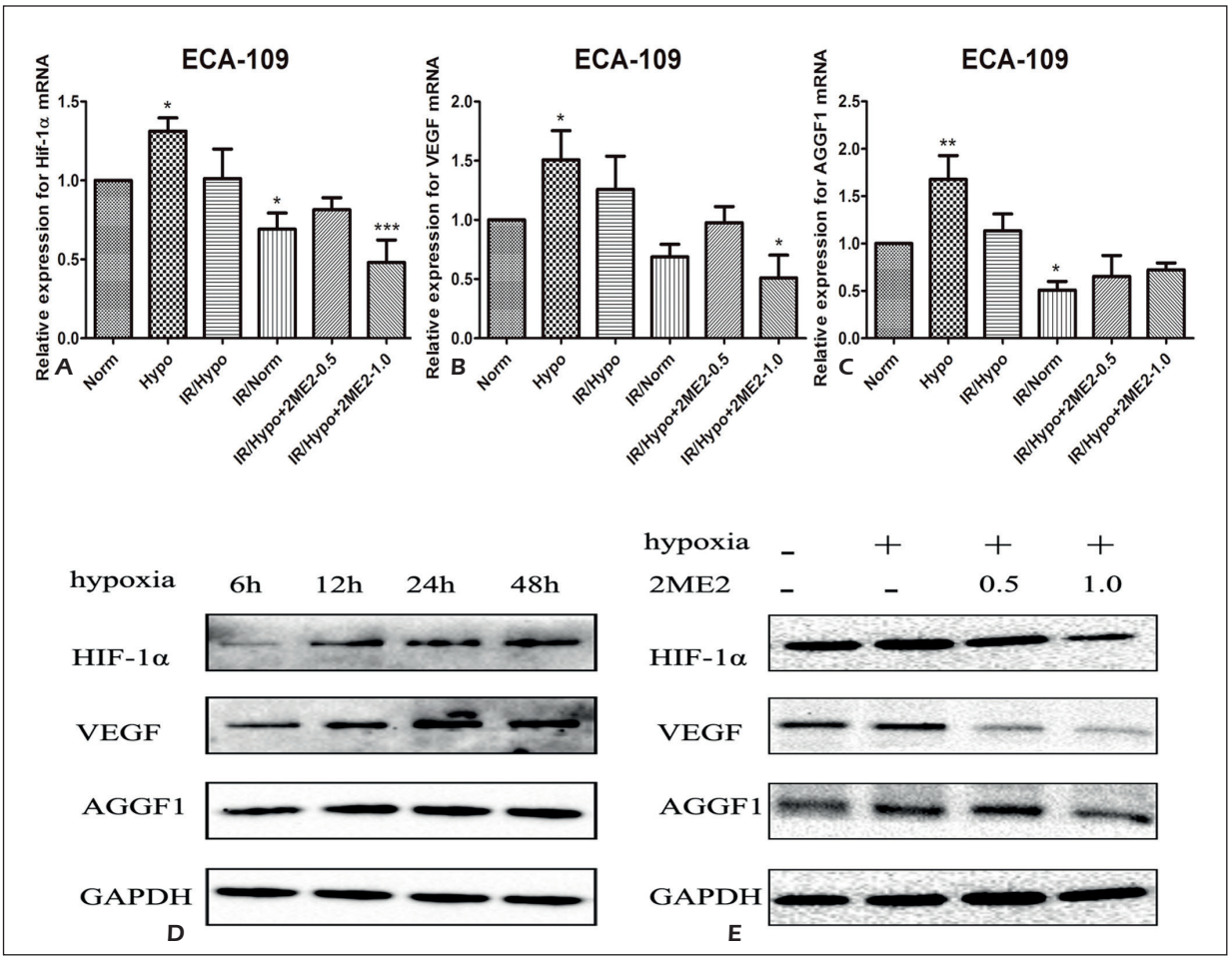

Figure 4. A-C, MRNA expressions of HIF-1α, VEGF and AGGF1 in ESCC cells were detected using qRT-PCR. Cells were treated with 2ME2 and/or IR. (*p<0.05). **D-E**, Expressions of HIF-1α, VEGF and AGGF1 in ESCC cells. 2ME2 inhibited the expressions of HIF-1α and VEGF induced by hypoxia and IR. **D**, Exposed to hypoxia for 6, 12, 24, 48 h. **E**, Treated with 2ME2 (0.5 or 1 mM) for 24 h in hypoxic conditions.

part, to 2ME2-induced cell growth inhibition. For example, 2ME2 functioned as a highly effective agent for sensitizing ESCC cells by significantly reducing the expression of HIF-1α; however, further studies were needed to confirm these findings.

Previous studies have shown that purified human AGGF1 promoted angiogenesis as effectively as VEGF. Meanwhile, the knockdown of AGGF1 expression inhibited endothelial vessel formation[13]. Our results showed that AGGF1 was significantly up-regulated in EC tissues when compared to non-tumor tissues. AGGF1 overexpression was found to be significantly associated with poor clinicopathologic characteristics. Similarly, malignant pleural mesothelioma exhibited AGGF1 overexpression[26]. Further Western blotting analysis showed that hypoxia induced an increase in the protein level of AGGF1 in human ESCC cells, which might possibly result in the development of ESCC. Statistical analysis identified a significant association between the up-regulation of AGGF1 and the development of ESCC. Collectively, these findings suggested that AGGF1, similar to VEGF, might play an important role in tumor development and progression. However, more studies are needed to elucidate the exact roles of AGGF1 in EC angiogenesis.

Nevertheless, the statistical analysis showed no association between HIF-1α and AGGF1, suggesting that further studies were required to verify these findings. Similarly, future clinical studies investigating therapeutic angiogenesis using AGGF1 with a plasmid-based gene delivery system are needed to unequivocally establish the efficacy of AGGF1 in the treatment of ESCC.

Conclusions

2ME2 could significantly promote the radiosensitivity of ESCC cells under hypoxic conditions by inhibiting HIF-1α expression. Results suggested that 2ME2 could potentially be used against hypoxia. The expressions of HIF-1α, VEGF, and AGGF1 mRNA/protein increased significantly after $CoCl_2$-induced hypoxia. The radiation could reduce the mRNA/protein expressions of HIF-1α, VEGF, and AGGF1 induced by $CoCl_2$-induced through hypoxia. Cell cycle arrest in the G0/G1 phase in hypoxia might be associated with decreased radiosensitivity in ESCC cells. Moreover, AGGF1, HIF-1α, and VEGF were highly expressed in ESCC and were closely related to the clinicopathological features of ESCC. Overexpression of AGGF1, HIF-1α, and VEGF might have an influence on the curative effect of radiotherapy and the survival prognosis of ESCC patients. Our findings could offer new ideas and methods for the treatment of ESCC. In addition, AGGF1 might contribute to tumour angiogenesis and could be used as a potential therapeutic target for ESCC.

Conflict of Interests

The authors declared that they have no conflict of interests.

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