RESEARCH ARTICLE

2-Methoxyestradiol Promotes Radiosensitivity of Esophageal Squamous Cell Carcinoma by Suppressing Hypoxia-Inducible Factor-1α Expression

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Abstract

Background: Acquired radioresistance remains a primary obstacle to improving survival of patients with esophageal cancer(EC). Factors related to hypoxia play crucial roles in such 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). However, evidence clarifying the role of AGGF1 in EC is limited. We here aimed to investigate the effects of 2-methoxyestradiol(2ME2) on the radiosensitivity of EC. **Methods:** In this study, we investigated the expression of HIF-1α,VEGF and AGGF1 in 70 ECs from patients using immunohistochemistry, to determine how expression levels might correlate with clinicopathologic characteristics. Then, human esophageal squamous cell carcinoma (ESCC) ECA-109 cells were subjected to hypoxia and/or irradiation (IR) in the presence or absence of 2ME2. Cell growth, colony formation, and apoptosis were subsequently evaluated. Moreover, mRNA and protein expression levels of HIF-1α, VEGF and AGGF1 in ESCC cells were determined using qRT-PCR and Western blotting. **Results:** We found that HIF-1α, VEGF and AGGF1 proteins were overexpressed in EC tissues and that this overexpression was associated with cancer aggressiveness. 2ME2 treatment increased the radiosensitivity of ESCC cells in a dose-dependent manner, and this increase was correlated with inhibited expression of HIF-1α and VEGF. The immunohistochemical staining results of the clinical tissue samples clearly showed that expression of VEGF and HIF-1α positively correlated in EC. However, no obvious correlation was found between AGGF1 and HIF-1α. **Conclusions:** High levels of HIF-1α, VEGF and AGGF1 expression in EC are indicators of poor prognosis in patients treated with radiotherapy. AGGF1 may contribute to EC tumor angiogenesis and represent a potential therapeutic target. In addition, 2ME2 may inhibit the expression of HIF-1α and VEGF and thus confer radiosensitivity on ESCC cells. As such, 2ME2 has potential applications as an adjuvant treatment with radiotherapy for EC cases.

Keywords: 2ME2- HIF-1α- VEGF- AGGF1- radiosensitivity- esophageal cancer

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Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer mortality worldwide (Jemal et al., 2011). The most prevalent EC histologic type in China is esophageal squamous cell carcinoma (ESCC) (Fu et al., 2013).

Due to lack of reliable early detection methods and absence of early symptoms, most EC patients are diagnosed with relatively advanced-stage disease. Although complex multidisciplinary treatment, especially the concurrent chemoradiotherapy, has recently become a common practice, the rate of local recurrence and distant metastasis remains high (Sjoquist et al., 2011; Van et al., 2012). Acquired radioresistance during radiotherapy (RT) has been considered as one of the most important reasons for treatment failure (Linkous and Yazlovitskaya, 2012). Many factors affect radioresistance, including tumor size, hypoxia, and intrinsic radiosensitivity.

Tumor hypoxia is an important factor leading to radioresistance and poor clinical outcomes. The oxygen occurred during RT generates free oxygen radicals that induce DNA damage and kill tumor cells (Yang et al., 2013; Zhang et al., 2014). Hypoxic environments can activate a specific set of tumor promoting transcription factors, such as hypoxia-inducible factor 1 (HIF-1) (Cummins and Taylor, 2005). HIF-1α is an oxygen-sensitive factor that regulates genes involved in tumor cell survival, metabolism, proliferation, and invasion (Keith et al., 2011). Vascular endothelial growth factor (VEGF), one of its downstream genes, is transcriptionally activated by HIF-1α in hypoxic environments(Belozerov and Van, 2005; Meijer et al., 2012; Yang et al., 2013). Tumor

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progression is often correlated with an up-regulation of angiogenesis because of an increase in VEGF(Yang et al., 2013).

Angiogenic factor with G-patch and FHA domains 1 (AGGF1), encodes an angiogenic factor with 714 amino acid residues and is a factor that has been implicated in angiogenesis (Tian et al., 2004). Increased AGGF1 expression is associated with the congenital vascular disorder Klippel–Trenaunay syndrome (KTS) (Tian et al., 2004). AGGF1 can promote angiogenesis in chicken embryo angiogenesis assays and in mouse hindlimb ischaemia models (Tian et al., 2004; Lu et al., 2012). The up-regulation of AGGF1 reportedly contributes to tumor angiogenesis in HCC (Wei et al., 2015).

Tumor growth require angiogenesis. Some agents can sensitize cancer cells to radiation by inhibiting HIF-1α (Muh et al., 2014). 2-methoxyestradiol (2ME2) is a naturally occurring derivative of estradiol and has been shown to be an orally active, well-tolerated small molecule that possess antitumor and antiangiogenic activity (Pribluda et al., 2000). In addition, 2-ME2 has been used to block HIF-1α nuclear accumulation through an oxygen- and proteasome-independent pathway that involves the disruption of microtubules (Mabjeesh et al., 2003). However, the effects of 2ME2 on ESCC cells and the underlying mechanisms have not yet been reported. In this study, we aimed to investigate whether 2ME2 could radiosensitize ESCC cells under hypoxia by inhibiting HIF-1α expression. We examined the effects of 2ME2 and/or radiation on ESCC cells exposed to normoxia or hypoxia in vitro. Furthermore, we determined whether AGGF1could be a potential therapeutic target for ESCC.

Materials and Methods

Clinical samples

Tumor tissues of 70 patients and non-tumor tissues from 30 patients who had the symptoms such as nausea, feeding disturbance, sour regurgitation but finally diagnosed as esophagitis were obtained from the Affiliated Tumor Hospital of Xinjiang Medical University from 2011 to 2015. Histological diagnoses were performed by expert pathologists. Detailed clinicopathologic data were recorded. All of the patients didn't have surgery. They 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. All the samples were obtained following patient consent and approval by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University.

Chemicals and reagents

RPMI medium modified (RPMI-1640), Phosphate buffered saline (PBS), fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were purchased from Hyclone Laboratories (HyClone, Logan, UT, USA). Cobalt (II) chloride (CoCl2) was from Sigma-Aldrich (St. Louis, MO, USA), while 2ME2 from MedChem Express (Princeton, NJ, USA), they all dissolved in DMSO at a concentration of 10 mmol/L, and stored at -20 °C. The rabbit antibodies against HIF-1α, VEGF and AGGF1 were from Abcam Biotechnology Inc (Abcam, Cambridge, UK) , while the rabbit antibody against 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 cultures

Human ESCC line ECA-109 were provided by the Central Experimental Laboratory of Xinjiang Medical University (Urumqi, China) and cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100U/mL), and streptomycin (100mg/mL). Hypoxic conditions were mimicked using a hypoxia-mimetic agent, CoCl2 (Mori et al., 2009). Prior to IR, cells were pretreated and/or 2ME2.

Measurement of cell growth by CCK-8 assay

The effect of 2ME2 on cell viability with IR was monitored by CCK-8 assay, which was carried out in 96-well plates. Cells (4×10^5 cells/well) were pretreated with various concentrations of 2ME2 for 24 h and incubated for 24 h after IR. Approximately, 10 μL of CCK-8 reagent was added to each well.

Clonogenic survival assay

Different densities (200-2,000) of cells were seeded in six-well plates, IR and/or 2ME2 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. Colonies were fixed in methanol for 30 min following Giemsa staining. We counted the colonies containing more than 50 cells.

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 a single cell suspension. The cells were centrifuged, washed with PBS, suspended in 500 μL of buffer solution, and stained with 5 μL annexin V and 5 μL propidium iodide (BD Bioscience, Oxford, UK) for analysis on a Beckman flow cytometer. We used Hoechst 33342 working solution to identify apoptosis-like death rate by the formula (apoptosis-like cell death rate = number of apoptosis-like dead cells/total number of cells) (Brady, 2004).

Protein extraction and Western blot analysis

Briefly, cells were digested by trypsin, transferred into the microtube, and centrifuged at 2000 rpm at room temperature for 5 min. The cell pellet was washed in ice-cold PBS (−) twice and homogenized in 40 μL of the protein extraction buffer with chilling. Finally, the solution was centrifuged at 12,000 rpm at 4 °C for 20 min. The concentration was determined by BCA protein assay. The mixture was boiled at 90 °C for 5 min. Equal amounts (40 μg) of samples were loaded into each lane, separated by SDS-PAGE, and then transferred onto PVDF membranes. For the molecular weight standard, 3 μL of Prestained Protein Marker was loaded in the left lane and 1μL in the right lane. The membranes were blocked with 5% non-fat dried milk in PBS containing 1% Tween 20 for 1 h at

room temperature and incubated with primary antibodies (1:500), or with rabbit monoclonal anti-GAPDH antibody (1:1,000) overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1h and visualized by super ECL Western Blotting Detection System (Thermo Scientific, Rockford, USA).

Immunohistochemical analysis

Serial sections (4 μm) subjected to immunohistological staining were fixed with freshly prepared 3% H_2O_2 with 0.1% sodium azide and then treated with antigen retrieval solution for 15 min. After placing in blocking reagent for 15 min, the sections were incubated in primary antibodies (1:500) overnight at 4 °C, followed by incubation with the secondary antibody and extravidin-conjugated horseradish peroxidase. Their expression was quantified based on the intensity of staining (scored as: 0-no, 1-weak, 2-moderate, 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 was considered as positive.

RNA extraction and quantitative Real-Time PCR

Total RNA was extracted by Trizol, and cDNA was synthesized using the PrimeScript TM RT Reagent Kit (Perfect Real Time, TaKaRa, Japan). Quantitative real-time PCR was performed in a 20ul total volume containing SYBR Green (SYBR® Premix ExTaq TM II, TaKaRa, Japan) on an Applied 7600 Fast quantitative PCR system. Primer pairs 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 (Vairan 600C/D, Salt Lake, USA) at an average dose rate of 100 cGy/min, operating at 100 kV and 5 mA, and the distance to 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 disappearance of target lesion . Partial response (PR) was defined as a reduction of at least 50% in the product of 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 longest diameter and longest vertical diameter of target lesions, no new lesions appeared.

Statistical data analyses

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism program version 5.0 (GraphPad Software, CA, USA) and SPSS statistical version 17.0 (SPSS Inc. IL, USA). Data were presented as mean ±SD. Continuous variables were compared by analysis of variance (ANOVA) and Student's t test, categorical variable were compared by Chi-square test. Correlation analysis was performed using Spearman analysis. Difference was considered significant at values of $P < 0.05$.

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 detected the expression of three proteins in EC and non-tumor tissues by IHC staining. In our immunostaining results, the location of HIF-1α protein was mainly in the cytoplasm and nucleus, while VEGF and AGGF1 was confined to the cytoplasm (Figure 1). HIF-1α,VEGF and AGGF1 protein were expressed positively in 60% , 37.14% , 54.29% of EC

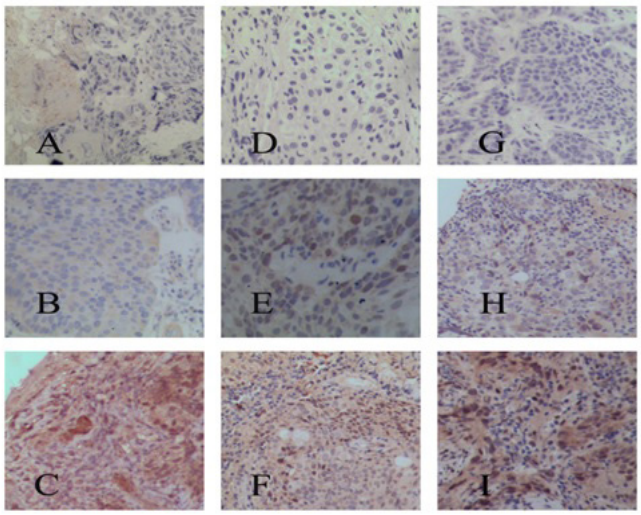

Figure 1. Immunohistochemical Analysis of HIF-1α, VEGF and AGGF1 in Esophageal Cancer. HIF-1α, VEGF and AGGF1 staining, A-C, Negative; D-F, Weak positive; G-I, Strong positive; Original magnification×100.

Table 1. 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			0.72
Upper	17	8	11		5	14		9	10	
Middle	38	25	13		16	22		21	17	
Lower	13	9	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
I	24	9	15		8	16		7	17	
II	30	23	7		7	23		22	8	
III	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

tissues and 10.0 %, 16.67%, 23.33% of non-tumor tissues. There was a significant difference in the positive rate of the three proteins between EC and non-tumor tissues group ($\chi 2 = 21.212, 4.116, 8.129$, $P = 0.000, 0.042, 0.004$).

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

Table 2. 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			0.07	0.563
positive	20	22			24	18		
negative	6	22			14	14		

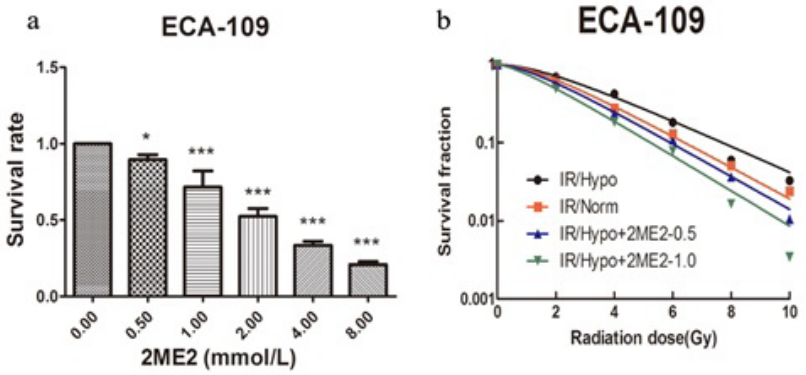

Figure 2 a. 2ME2 Inhibits the Proliferation of Human ESCC Cells. Cell viability was determined by CCK-8 assay. 2ME2(0.5-8mM)was added before IR. After 24 h, the effects of 2ME2 were tested. (*p < 0.05).b. Clonogenic survival of human ESCC cells following IR. ESCC cells were seeded in six-well plates 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.

Association of HIF-1α,VEGF and AGGF1 protein 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 1. No significant difference was observed between their expression levels and clinicopathologic features such as patient gender, age, nationality etc. However, their expression levels were found to be significantly lower in patients with well differentiation, earlier clinical stage.

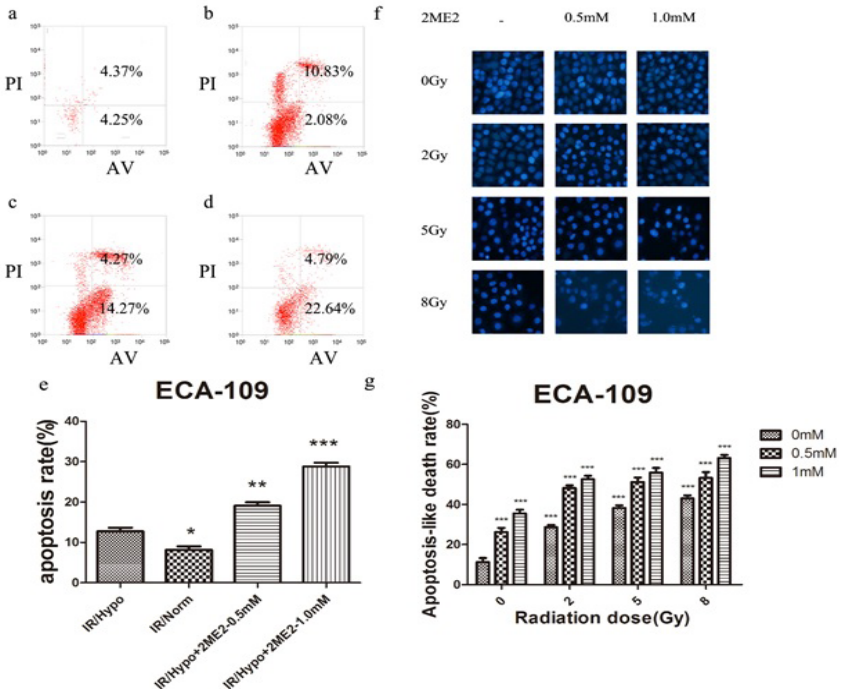

Figure 3. 2ME2 Promotes IR-Induced Apoptosis in ESCC Cells. Cells were pretreated with 2ME2 for 24 h before IR. At IR under 8 Gy, ESCC cells were incubated for another 48 h and then digested by trypsin. (a-e) Flow cytometric analysis showing that 2ME2 induced apoptosis of hypoxic ESCC cells. a. normoxia 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. represents the morphologic changes of the ESCC cells treated with 0.5 and 1mM 2ME2, 1 day after IR on diverse doses. g. apoptosis-like death rate of ESCC cells treated with 0.5 and 1mM 2ME2, and IR on diverse doses. (*p < 0.05).

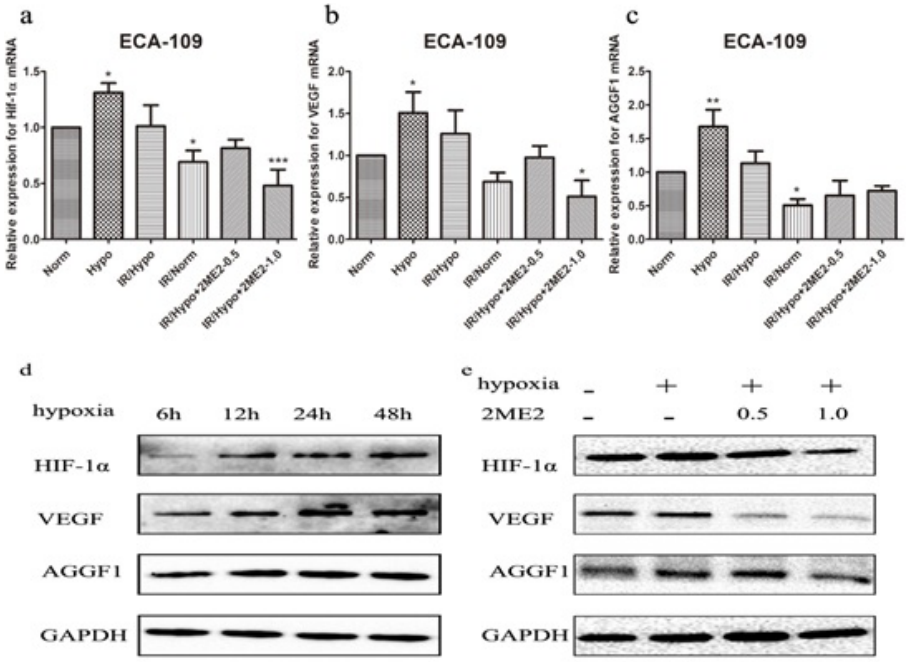

Figure 4 a-c. The HIF-1α, VEGF and AGGF1 mRNAs in ESCC Cells were Detected Using qRT-PCR. Cells were treated with 2ME2 and/or IR. (* $p < 0.05$). d-e. Expression of HIF-1α, VEGF and AGGF1 in ESCC cells, 2ME2 inhibits the expression of HIF-1α and VEGF induced by hypoxia and IR. d. exposed to hypoxia for 6, 12, 24, 48 h. e. treated with 2ME2 (0.5 or 1 mM) for 24 h in hypoxic conditions.

Table 3. 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	1.2
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

Further analysis demonstrated that low HIF-1α, VEGF and AGGF1 expression was associated with an improved IR response in EC patients, with 51.2%, 72.1% and 51.2% of patients achieving CR+PR after IR for 3 months in each group. Alternatively, the higher the expression of HIF-1α and VEGF the more resistant EC patients were to radiotherapy .

Relationship between HIF-1α,VEGF and AGGF1 protein expression in EC tissues

In Table 2, we found VEGF was positively related to HIF-1α, but there was no significant relationship between AGGF1 and HIF-1α in EC tissues.

2ME2 inhibits proliferation of ESCC cells

We investigated the effect of various concentrations of 2ME2 on ESCC cells by CCK-8 assay to detect whether it is cytotoxic to cell proliferation. Figure 2a shows 2ME2 produced a cytotoxic effect in a dose dependent manner. The survival rates in the 0.5 and 1 mM 2ME2-treated group for 24 h were 89.52% and 71.61%, which showed a low toxicity effects on the growth of ECA-109. Thus, we selected these two low 2ME2 concentrations (0.5 and 1 mM) for the following assays.

2ME2 sensitizes ESCC cells to IR

We performed clonogenic survival assays to investigate the effect of 2ME2 on the proliferative ability of ESCC cells. 2ME2 displayed significant radiosensitivity on the clonogenic effect under hypoxia. Figure 2b shows the clonogenic surviving fraction of 2ME2 and IR. In the IR alone group, ESCC cells survival decreased with increasing IR, but significantly decreased in the IR+2ME2 group. This difference was statistically significant ($p<0.01$). According to the click multi-target model, we obtained the main parameters of ESCC cells dose-survival curves. The radiosensitization effects of 2ME2 in ESCC cells were summarized in Table 3. As the mean lethal dose of the combination group decreased, we indicate 2ME2 produced radiosensitive effects on ESCC cells under hypoxia.

Figure 2 a. 2ME2 inhibits the proliferation of human ESCC cells. Cell viability was determined by CCK-8 assay. 2ME2(0.5-8mM)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 in 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.

Effect of 2ME2 on radiosensitivity to apoptosis induced by IR on ESCC cells

We assessed the apoptosis of ESCC cells 24 h after treatment with 2ME2 and/or IR. The proportion of apoptotic cells was determined by flow cytometry. The graph shows 2ME2+IR group exhibited more cell apoptosis than single IR group. Significant radiosensitivity

was observed under hypoxic conditions(Figure 3a-e).

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

Figure 3 2ME2 promotes 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 then digested by trypsin. (a-e) Flow cytometric analysis showing that 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. represents the morphologic changes of the ESCC cells treated with 0.5 and 1mM 2ME2, 1 day after IR on diverse doses. g. apoptosis-like death rate of ESCC cells treated with 0.5 and 1mM 2ME2, and IR on diverse doses. (*$p < 0.05$).

2ME2 inhibits the upregulation of HIF-1α induced by hypoxia and IR in ESCC cells

The expressions of HIF-1α,VEGF and AGGF1 mRNAs were tested in ESCC cells by qRT-PCR (Figure 4a-c). As a result, when these 3 mRNAs were generated based on the trend of radiosensitivity, their expressions were rising as cells in resistance to IR hypoxic condition. While they were treated with 0.5 and 1mM 2ME2, both the expression of HIF-1α and VEGF mRNAs decreased, approximately 30% fold of that only with IR (Figure 4a-b, $P < 0.001$). The Spearman correlation analysis verified a negative correlation with radiosensitivity capacity and expression of HIF-1α,VEGF and AGGF1 mRNAs ($r = -0.974, -0.394, -0.604$, $P = 0.001, 0.001, 0.000$).

Western blot was performed to confirm 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 treated with hypoxia. Figure 4d-e shows that HIF-1α and VEGF level was highest at 48h after hypoxia, while AGGF1 level was at 24h. We analyzed their expressions under hypoxia and 2ME2. 2ME2 inhibited hypoxia-induced upregulation of HIF-1α and VEGF expressions in ECA-109 cells.

Figure 4 a-c. The HIF-1α, VEGF and AGGF1 mRNAs in ESCC cells were detected using qRT-PCR. Cells were treated with 2ME2 and/or IR. (* $p < 0.05$). d-e. Expression of HIF-1α, VEGF and AGGF1 in ESCC cells, 2ME2 inhibits the expression 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.

Discussion

As one of the most common gastrointestinal carcinomas, EC leads to hypoxic environments due to increased oxygen consumption of extensively growing tumor cells and decreased oxygen delivery from disorganized tumor blood vessels(Yoshimura et al., 2013), this imbalance causes radiation resistance in cancer cells. Yet, 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 new, promising therapeutic strategies for the treatment of EC. In our study, the results showed HIF-1α was overexpressed in EC tissues and the high expression levels were correlated with cancer aggressiveness.

Radioresistance is a challenging obstacle in the treatment of EC, and research addressing this problem is essential. Strategies that enhance tumors' radiosensitivity without causing further toxicity are needed. Previous studies have demonstrated that the process of developing radioresistance is complicated and involves multiple molecular mechanisms (Li et al., 2013). Numerous new hypoxic radiosensitizers have recently been developed, and some have been clinically evaluated. Preclinical studies have shown that the suppression of HIF-1α can sensitize tumors to chemotherapy and radiotherapy. A landmark report suggested that 2ME2 inhibits angiogenesis, which has an important function in tumor growth, metastasis, and invasion, by dysregulating HIF (Mabjeesh et al., 2003), as increased levels of HIF-1α are required for angiogenesis (Shi et al., 2012). So far, 2ME2 has demonstrated low toxicity with promising, but mixed efficacy in several cancer types, including GBM and HCC (Carrie et al., 2014; Ma et al., 2014).

Our results indicate that 2ME2 pretreatment decreased cell viability, reduced apoptosis and cloning survival rate under hypoxic and IR conditions. This study revealed for the first time that 2ME2 could significantly promote radiosensitivity of ESCC cells under hypoxic conditions by inhibiting HIF-1α. It is possible that 2ME2 inhibited HIF-1α protein accumulation in ESCC cells, then suppressed the expression of related genes, which are involved in tumor cell proliferation. We hypothesized that VEGF, an important regulator of angiogenesis (Forsythe et al., 1996), may be associated with this effect. Thus, our results indicate that HIF-1α signalling contributes, at least in part, to 2ME2-induced cell growth inhibition, i.e. 2ME2 functioned as a highly effective agent for sensitizing ESCC cells by significantly decreasing the expression of HIF-1α. However, further studies are needed to confirm these phenomena.

Previous studies have shown that purified human AGGF1 promoted angiogenesis as potently as VEGF and that the knockdown of AGGF1 expression inhibited endothelial vessel formation (Tian et al., 2004). Our results showed AGGF1 was significantly higher in EC tissues compared with non-tumor tissues and its overexpression was significantly associated with poor clinicopathologic characteristics. Similarly, malignant pleural mesothelioma exhibits AGGF1 overexpression (Roe et al., 2010). Further Western blotting analysis showed hypoxia induced an increase in AGGF1 protein level in human ESCC cells, which may possibly result in the development of ESCC.

The statistical analysis identified a significant association between the up-regulation of AGGF1 and the development of ESCC. Collectively, these findings suggest AGGF1, similar to VEGF, may 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, statistical analysis showed no association between HIF-1α and AGGF1, suggesting that additional studies are required to further 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 for treating ESCC.

In conclusion, our data showed 2ME2 could significantly promote radiosensitivity of ESCC cells under hypoxic conditions by inhibiting HIF-1α expression, suggesting 2ME2 could potentially be used against hypoxia. We report, for the first time, that there is a significant association between AGGF1 overexpression and various clinicopathological characteristics, as well as prognosis of ESCC patients. We also report the hypoxia-induced up-regulation of AGGF1 expression. While further confirmation of these results is required, our data suggest that AGGF1 may contribute to tumor angiogenesis and represent a potential therapeutic target for ESCC.

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