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Silencing of FOXA2 decreases E-cadherin expression and is associated with lymph node metastasis in oral cancer

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Abstract

Objectives: FOXA2 gene methylation links to the progression of cancers, but has not been documented in oral cancer. Herein, we explore the role of FOXA2 in the migration of oral cancer cells.

Material and Methods: Methylation-specific PCR was applied for gene methylation. Wound healing and transwell experiments were tested for cell migration. FOXA2 expression in oral cancer tissues was addressed by immunohistochemistry, followed by statistical analysis of its association with clinical manifestations and patient survival.

Results: FOXA2 bound to the promoter of CDH1 and enhanced the expression of its gene product E-cadherin, and decreased the cancer cell migration activity. High FOXA2 expression in oral cancer tissues was associated with high E-cadherin expression, decreased lymph node metastasis, and increased patient survival.

Conclusion: FOXA2-E-cadherin link is involved in regulation of oral cancer cell metastasis and provides a new insight for the tumor suppressor activity of FOXA2 in oral cancer.

KEYWORDS

cell migration, E-cadherin, FOXA2, gene methylation, oral cancer

Bow and Wang contributed equally to this article.

1 | INTRODUCTION

Oral cancer is a major cause of morbidity and mortality worldwide (Chinn & Myers, 2015) with lymph node metastasis as the most important prognostic factor (Amit et al., 2013; Godeny, 2014; Liao et al., 2010). It is the seventh most common cancer and fifth most lethal cancer in Taiwan, with an increase in both incidence and death rates in recent years (Chen et al., 2008; Liao et al., 2017). While the clinical outcome of oral cancer has been improved, overall survival rate remains poor for the progressive and metastatic cases (Godeny, 2014; Krishna Rao, Mejia, Roberts-Thomson, & Logan, 2013).

Cancer development is a multi-stage process involving genetic and epigenetic changes that lead to the activation of oncogenes and inactivation of tumor suppressor genes (Montenegro et al., 2015; Morris & Chan, 2015). DNA hypermethylation is an epigenetic regulation mechanism which causes silencing in different genes including tumor suppressor genes, which has been detected in a high percentage of human cancers (Akhavan-Niaki & Samadani, 2013; Ehrlich, 2009; Esteller, 2008; Shaw et al., 2013). Epigenetic regulation mechanism is also involved in the process of epithelial–mesenchymal transition (EMT)-related genes including CDH1 and FOXA2 (Liu et al., 2005; Sakamoto et al., 2012; Schmalhofer, Brabletz, & Brabletz, 2009). Epithelial marker E-cadherin, the gene product for CDH1, is a well-characterized cell adhesion molecule and is involved in the process of epithelial–mesenchymal transition (Nieto, Huang, Jackson, & Thiery, 2016; Polyak & Weinberg, 2009; Singh & Settleman, 2010; Talmadge & Fidler, 2010). FOXA2 belongs to the forkhead box protein 2A/winged-helix family of transcriptional factors (Myatt & Lam, 2007). It is involved in embryonic development and broadly expressed in multiple adult tissues derived from endoderm layer (Bahar Halpern, Vana, & Walker, 2014; Kaestner, Knochel, & Martinez, 2000). Previous study reported that FOXA2 expression is downregulated by DNA methylation to promote breast cancer progression (Zhang et al., 2015). The dysregulation or downregulation of FOXA2 has been directly linked to the progression of certain cancers (Chinn & Myers, 2015; Song, Washington, & Crawford, 2010), but the role of FOXA2 in oral cancer is not clear. In this study, we found that high FOXA2 expression in oral cancer tissues was associated with high E-cadherin expression, decreased lymph node metastasis, and increased patient survival. In vitro study showed that FOXA2 prevented the migration of oral cancer cells. Furthermore, a positive association between the expression of FOXA2 and E-cadherin was observed, and downregulation of FOXA2, through suppression of CDH1 transcription, promoted the migration of oral cancer cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Oral cancer cells (Ca9-22 and SAS) were purchased from American Type Culture Collection (ATCC). Ca9-22 and SAS were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 1% L-glutamine. Both cell lines were incubated at 37°C with 5% CO_2.

2.2 | Clinical samples

We retrospectively reviewed the clinical records of 43 consecutive OSCC patients who underwent surgery at the Department of Oral and Maxillofacial Surgery, Kaohsiung Medical University Hospital, Taiwan, during the period from October 2000 to July 2014. Pathologic diagnosis of OSCC was independently confirmed by two dental pathologists. Clinical cancer stage was determined according to the TNM (tumor–node–metastasis) staging system of the American Joint Committee on Cancer (AJCC; Godeny, 2014). All patients were followed until March 2015. Overall survival (OS) was defined as the interval between the time of diagnosis and death. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-20130300), and informed consent was obtained from all patients.

2.3 | Extraction of genomic DNA

Oral cancer cells and cancer tissues were concentrated by centrifugation and were solubilized in cell lysis solution. The cellular lysate was digested with proteinase K (20 μg/ml) at 55°C for overnight. The cellular DNA was finally precipitated by isopropanol and redissolved in sterile water until further study. DNA extraction method was followed according to the manufacturer's protocol for Genomic DNA Purification Kit (Invitrogen).

2.4 | Bisulfite modification

The methylation status of the promoter regions was determined by sodium bisulfite reaction using the MethyCode bisulfite conversion kit (Invitrogen). The purified cellular DNA was added to a cytosine conversion reagent provided by commercial supplier (Invitrogen). Bisulfite treatment of DNA converted unmethylated cytosine to uracil, while methylated cytosine remained unchanged.

2.5 | Methylation-specific polymerase chain reaction

Primers and the reaction conditions for CDH1 were described in previous study (Kang, Lee, Lee, & Hwang, 2004). Methylated forward primer for FOXA2 was 5′CGCGTTAGGTGGGATTTTG, and reverse primer was 5′CGCGAAAACCGTACCTATC. Unmethylated forward primer was 5′TATGTGTTAGGTGGGATTTTGTG, and reverse primer was 5′TATCTCCACAAAAACCATACCTATC. The annealing temperature for CDH1 was started at 65°C, decreased 1°C for each cycle until 55°C, and amplified at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The

polymerase chain reaction for FOXA2 is 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, for 40 cycles. Two microliter of bisulfite-modified DNA was added to 25 μl of polymerase chain reaction (PCR) reaction mixture which contained 1×PCR buffer, 0.2 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 0.7 units of HotStart Taq polymerase (Promega). The amplified PCR products were visualized by the 1.8% agarose gel electrophoresis. Unmethylated control is sperm DNA, and methylated control is the DNA from H1299 non-small-cell lung cancer cell line.

2.6 | In vitro migration assay

For wound healing assay, 8×10^4 cells of SAS or Ca9-22 were seeded on 6-well plate for 12 hr, treated with 5 μM 5-aza (Sigma) for 48 hr, and then, wound closure was analyzed every 6 hr after a straight line was scraped. For transwell assay, 5×10^4 cells were treated with 5-aza and seeded onto top chamber with 8 μm pore size (Millicell Hanging Cell) for each insert for overnight incubation. After incubation for 48 hr, the non-migrated cells were gently wiped from the upper chamber with a cotton swab. Migrated cells were stained with 0.5% crystal violet or 1 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma) and counted under microscope. The experiment was repeated three times independently.

2.7 | Transfection and luciferase activity assay

Two micrograms of pGL4.14-CDH1 reporter plasmid were reacted with 200 μl prime buffer and 4 μl jet PRIME reagent (Polyplus transfection) for 10 min before added to the cells. Transfection of SAS or Ca9-22 cells (2×10^5) was carried out following the instructions from the supplier. After 18 hr of incubation, cells were harvested and lysed, the supernatant was reacted with luciferin (Steady-Glo Luciferase Assay System; Promega) according to manufacturer's protocol, and luciferase activity was measured with a luminometer (Plate Chameleon V Multilabel Detection Platform, HIDEX). Experiments were done triplicate independently.

2.8 | siRNA transfection and luciferase activity assay

1×10^5 cells of SAS or Ca9-22, after pretreatment with 5 μM 5-aza for 48 hr, were transfected with a mixture of FOXA2 siRNA 5 μM (GeneDireX Inc) and 4 μl GeneJet transfection reagent (SignaGen Laboratories). After transfection of FOXA2 siRNA (GE, Healthcare Dharmacon) for 48 hr, cells were collected for Western blot and luciferase assay.

2.9 | Chromatin immunoprecipitation assay

SAS cells were transfected with His-tagged Foxa2 expression plasmid before harvest for chromatin immunoprecipitation. Chromatin immunoprecipitation assay (ChIP) assay was performed using magnetic ChIP kit according to the manufacturer's instructions (Thermo Fisher Scientific). Rabbit anti-His FOXA2 antibody or rabbit IgG was used to immunoprecipitate DNA-containing complexes. Precipitated DNA was analyzed by polymerase chain reaction with primers for CDH1 promoter.

2.10 | Lentiviral expression of FOXA2 protein

The lentivirus particles containing pReceiver-lv151 vector cloned with FOXA2 gene were obtained from Genecopoeia (Rockville). The vector also expressed neomycin resistance gene. SAS cells (5×10^4) were seeded on a 6-well plate (Corning Life Sciences) and incubated at 37°C overnight. Cells were infected by virus particles with MOI of 5. After infection for 48 hr, antibiotic G418 (A.G. Scientific) at concentration of 500 μg/ml was added for selection. Selected cells were maintained in the growth medium containing G418 at 500 μg/ml. FOXA2 overexpression in the cells was determined by immunoblotting. Cells infected with lentivirus particles containing empty vector were used as control for the experiments.

2.11 | Western blot

The cells were homogenized and lysed in RIPA buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing complete protease inhibitor mixture (Roche). Total protein of 50 μg was loaded onto 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and was transferred to polyvinylidene fluoride membranes (PVDF). Protein expression was detected using primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The intensity of each band was visualized by enhanced chemiluminescence ECL Western blotting detecting reagents (Pierce). Protein expression was normalized by using β-actin as internal control.

2.12 | Tissue microarray and immunohistochemical analysis

To determine the expression of FOXA2 and E-cadherin in OSCC, FFPE (formalin-fixed, paraffin-embedded) tumor tissues were placed in a tissue microarray. Slides from hematoxylin–eosin-stained sections were reviewed by a pathologist to select the representative areas of tumor and normal regions for core punches within the primary paraffin block by the technicians. Tissue microarray was constructed following standard tissue array-producing protocols as described previously (Song et al., 2010). Four-micrometer-thick sections were cut from the recipient tissue microarray block by using a microtome with an adhesive-coated tape sectioning system (Alphelys). Immunohistochemical analyses were performed using Bond-Max autostainer (Leica Microsystems). Sections on microscopic slides were deparaffinized at 72°C and rehydrated in Bond Wash solution. Heat-induced antigen retrieval was

carried out with Bond Epitope Retrieval Solution 2 for 20 min at 100°C and peroxide block placement on the slides for 5 min at room temperature. Slides were then incubated with FOXA2 (1:200, ab08422; Abcam) or E-cadherin (1:100, ab1416; Abcam) antibodies for 30 min at room temperature. Antibody detection was carried out with the Bond Polymer placement, and color development was performed with DAB (3,3′-diaminobenzidine tetrahydrochloride). Slides were counterstained with hematoxylin, followed by mounting of the slides. Images of IHC-stained sections were captured by a Nikon Eclipse Ti microscope and processed by Nikon NIS-Elements version 4.30 software. The intensity of immunostaining for FOXA2 was determined as score 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). For further statistical analysis, scores 0 and 1 were categorized as low expression, and scores 2 and 3 were categorized as high expression (cut point at median). The staining was determined separately for each specimen by two independent experts simultaneously under the same condition. E-cadherin immunostaining was determined by staining intensity (scores 0 to 3) multiplied by percentage of positively stained tumor cells (score 0, 0%–4%; score 1, 5%–24%; score 2, 25%–49%; score 3, 50%–74%; and score 4, 75%–100%; Sarbia et al., 1998). The expression of E-cadherin was further categorized as low (<4) and high (≧4) expression using the median as cutoff point.

2.13 | Statistical analysis

Comparisons of high-expression and low-expression groups of FOXA2 with tumor grade, tumor size, lymph node metastasis,

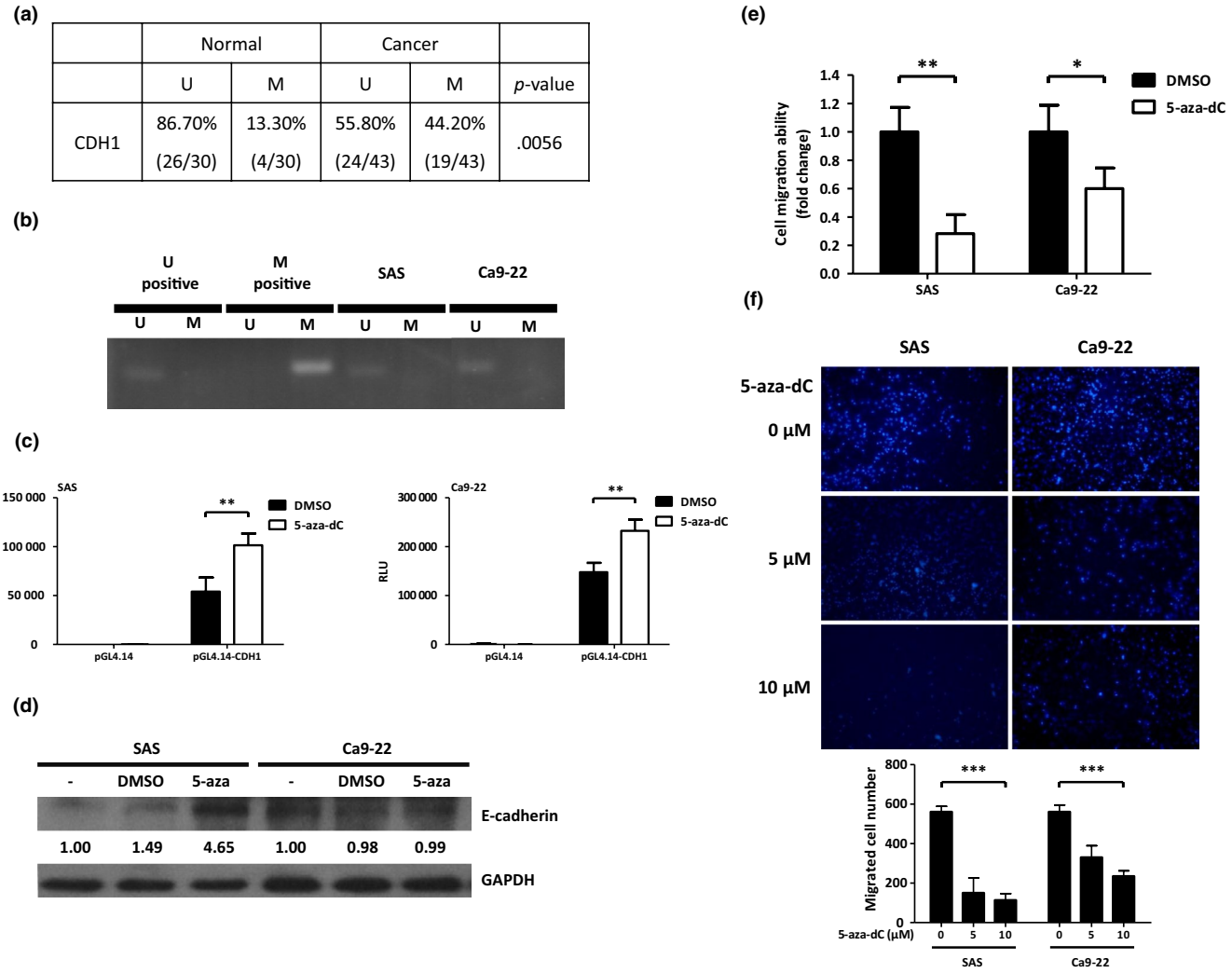

FIGURE 1 CDH1 gene promoter was methylated in oral cancer cells. (a) Methylation-specific polymerase chain reaction result of CDH1 gene in oral cancer specimens. U, unmethylated; M, methylated. (b) Methylation-specific polymerase chain reaction result of CDH1 gene in oral cancer cell lines SAS and Ca9-22. U, unmethylated; M, methylated. (c) Luciferase reporter assay result showing CDH1 gene promoter activity in SAS and Ca9-22 cells treated with 5-aza-2′-deoxycytidine. RLU, relative luciferase unit; DMSO, dimethyl sulfoxide; 5-aza-dc, 5-aza-2′-deoxycytidine. (d) Western blot result of E-cadherin in SAS and Ca9-22 cells treated with 5-aza. GAPDH, glyceraldehyde phosphate dehydrogenase. (e) Cell migration activity was assayed by wound healing, and migration area was calculated by TScratch software. DMSO, dimethyl sulfoxide; 5-aza-dc, 5-aza-2′-deoxycytidine. (f) Cell migration activity was analyzed by transwell assay, and the number of migrated cells was stained with DAPI and counted under microscope. DAPI, 4′,6-diamidino-2-phenylindole [Colour figure can be viewed at wileyonlinelibrary.com]

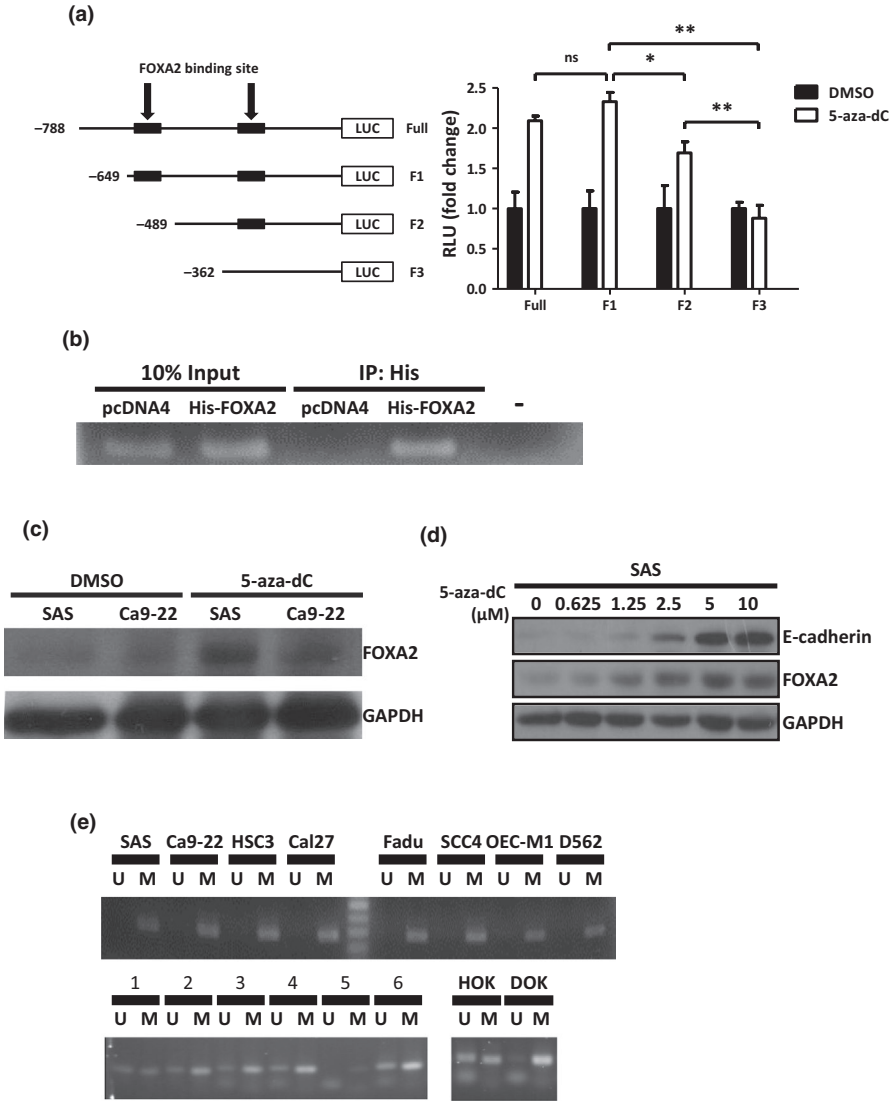

FIGURE 2 CDH1 promoter activity and gene expression in oral cancer cell lines and oral cancer specimens. (a) Constructs of CDH1 gene promoter linked with luciferase reporter plasmid. The black box in the reporter constructs contains two FOXA2 binding sites. CDH1 gene promoter activity of luciferase reporter assay in SAS and Ca9-22 cell lines treated with 5-aza. RLU; relative luciferase unit, DMSO; dimethyl sulfoxide, 5-aza-dc; 5-aza-2′-deoxycytidine. (b) Chromatin immunoprecipitation of CDH1 promoter. His-tagged FOXA2 vector was transfected into SAS cells and precipitated with anti-His antibody. Immunoprecipitate was detected by PCR with CDH1 primers set. (c and d) Western blot result of FOXA2 and E-cadherin in SAS and Ca9-22 cell lines treated with 5-aza-dc. FOXA2 and E-cadherin were expressed concomitantly in a dose-dependent manner of 5-aza-dc treatment. 5-aza-dc, 5-aza-2′-deoxycytidine; GAPDH, glyceraldehyde phosphate dehydrogenase. (e) Methylation-specific polymerase chain reaction result of FOXA2 gene in different oral cancer tissues (1–6) and oral cancer cell lines (SAS, CA9-22, HSC3, Cal27, FaDu, and OCEM), HOK, human normal oral cells; DOK, human oral precancer cell line; U, unmethylated; M, methylated

pathologic stage, sex, alcohol drinking, betel quid chewing, and cigarette smoking status were analyzed by the chi-square test. The overall survival (OS) analysis was estimated using the Kaplan–Meier product-limit method and log-rank tests. JMP version 9.0.1 for Windows (SAS Institute) was used for all the statistical analysis. *p*-Values <.05 were considered statistically significant. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ were used in the whole manuscript.

3 | RESULTS

3.1 | CDH1 gene promoter was hypermethylated in oral cancer tissues

We examined the methylation status of CDH1 gene in oral cancer tissues by methylation-specific polymerase chain reaction (MSP). The methylation frequency of CDH1 gene promoter was 44.2% (19/43) in cancer tissues, whereas only 13.3% (4/30) of the adjacent normal tissues were methylated ($p = .0056$; Figure 1a). The methylation rate of CDH1 was elevated 3.4-fold in cancer tissues versus the adjacent normal tissues.

3.2 | E-cadherin expression was regulated by DNA methylation

To determine whether the epigenetic change was responsible for the regulation of CDH1 expression, we analyzed the DNA methylation in different oral cancer cell lines, and CDH1 promoter was unmethylated in both SAS and Ca9-22 oral cancer cells (Figure 1b). A luciferase reporter plasmid linked with CDH1 promoter sequence was constructed and transfected into SAS and Ca9-22 cells. The result showed that transcriptional activity of CDH1 promoter in SAS and Ca9-22 cells was upregulated by the addition of methylation inhibitor, 5-aza-2′-deoxycytidine (Figure 1c). Consistently, Western blot result showed the expression of E-cadherin, CDH1 gene product, in oral cancer cells was induced when treated with 5-aza-2′-deoxycytidine. SAS cells expressed higher level of E-cadherin than Ca9-22 cells did (Figure 1d).

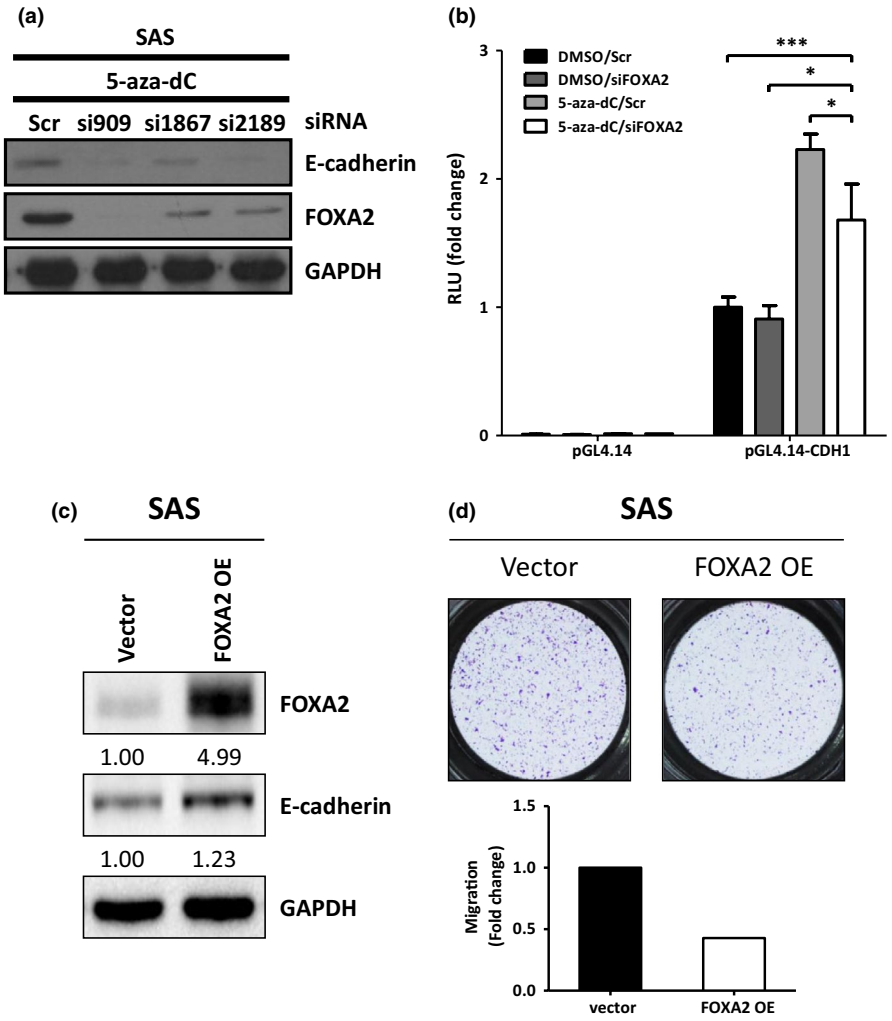

FIGURE 3 FOXA2 regulated the expression of E-cadherin. (a) Silencing of FOXA2 downregulated the expression of E-cadherin. SAS cells were pretreated with 5-aza-dc and followed by transfection of siRNAs, including FOXA2 siRNAs si909, si1867, and si2189, and scrambled siRNA (Scr). (b) Luciferase reporter activity of CDH1 promoter in SAS cells pretreated with 5-aza and followed by transfection of FOXA2 siRNA. RLU, relative luciferase unit; DMSO, dimethyl sulfoxide; 5-aza-dc, 5-aza-2′-deoxycytidine. (c) Western blot result of FOXA2 and E-cadherin expression in SAS cells after lentiviral overexpression of FOXA2. (d) Cell migration activity was assayed by transwell assay, and the migrated cells was stained by crystal violet and calculated. DMSO, dimethyl sulfoxide; 5-aza-dc, 5-aza-2′-deoxycytidine [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Cell migration was related to DNA methylation

Wound healing experiment was carried out to determine the effect of methylation inhibitor 5-aza-2′-deoxycytidine on oral cancer cell migration. The migration activity was significantly decreased in SAS and Ca9-22 cells after 5-aza-2′-deoxycytidine treatment (Figure 1e), suggesting that methylation enhanced oral cancer cell migration, while hypomethylation reduced it. Similar result was also observed in the transwell cell migration assay. The number of the migrated cells was significantly reduced in SAS and Ca9-22 cells when treated with 5-aza-2′-deoxycytidine (Figure 1f).

3.4 | FOXA2 was involved in the regulation of CDH1 transcription

The upstream region of CDH1 promoter was rich in CG dinucleotide sequences and possessed binding sites for FOXA2 transcriptional factor. To demonstrate FOXA2 was responsible for the activation of CDH1 promoter, different deletion plasmids of CDH1 promoter region were constructed for luciferase reporter assay. The constructs included the full length of CDH1 promoter sequence and constructs with one or two binding regions of FOXA2 missing (Figure 2a). Transcriptional activity of CDH1 promoter was reduced when one FOXA2 binding region was deleted (F2 construct), and the activity was completely eliminated when both FOXA2 binding regions were removed (F3 construct; Figure 2a). Chromatin immunoprecipitation assay was used to detect the interaction between FOXA2 and the promoter of CDH1, and the result demonstrated that FOXA2 enhanced CDH1 promoter activity through direct binding (Figure 2b). In addition, Western blot showed that protein expression of FOXA2 in SAS and CA9-22 cells was upregulated upon 5-aza-2′-deoxycytidine treatment (Figure 2c). Of note, protein expression of both E-cadherin and FOXA2 in SAS cells was concomitantly increased in a dose-dependent manner when treated with 5-aza-2′-deoxycytidine (Figure 2d). The methylation status of FOXA2 in different oral cancer cell lines was further analyzed, and the result revealed that FOXA2 promoter was methylated in all the tested cell lines (Figure 2e). While a regulatory pathway of CDH1 by classical transcriptional factors Snail and Twist was reported (Batlle et al., 2000; Thiery & Sleeman, 2006), the expression of Snail and Twist proteins in SAS cells was not changed by 5-aza-2′-deoxycytidine treatment in our study (Figure S1). Moreover, 5-aza-2′-deoxycytidine treatment had no effect on cell viability in SAS cells (Figure S2).

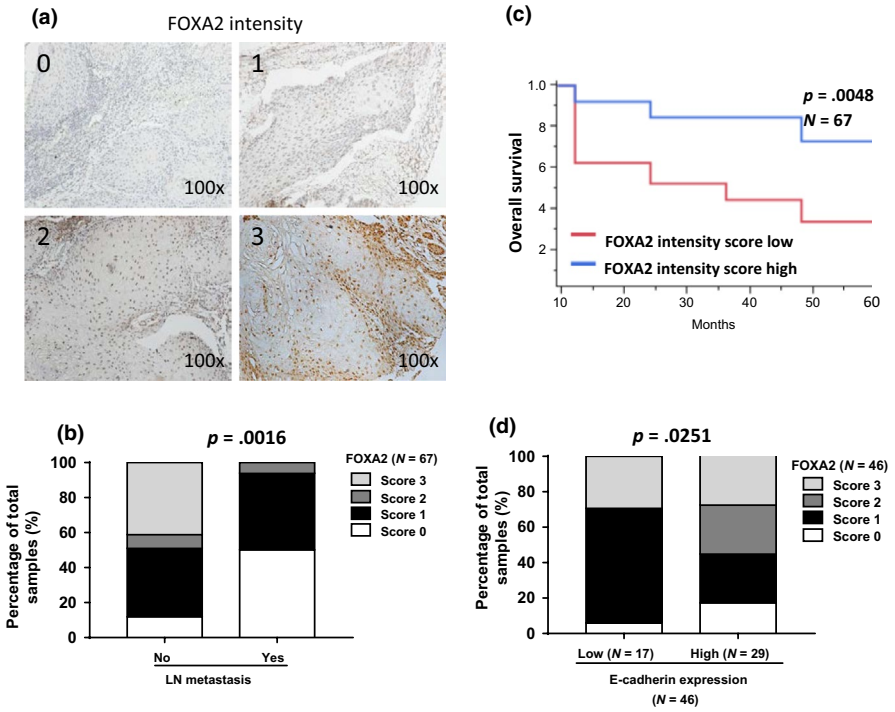

FIGURE 4 Low FOXA2 expression in oral cancer tissues was associated with increased lymph node metastasis and poor overall survival. (a) Scoring of FOXA2 intensity after immunohistochemical staining of oral squamous cell carcinoma (OSCC) tissues. Staining was scored according to the staining intensity in four semi-quantitative categories (score 0, absent staining; score 1, weak staining; score 2, moderate staining; and score 3, strong staining). The representative photographs were shown with ×100 magnification. (b) FOXA2 expression was significantly higher in OSCC tissues without lymph node metastasis than those with lymph node metastasis. (c) Kaplan–Meier analysis of FOXA2 protein in 67 OSCC patients, categorized as having low (scores 0 and 1) and high expression (scores 2 and 3) of FOXA2 based on immunohistochemistry score. The differences in survival rates were analyzed by log-rank test. Overall survival was analyzed according to the FOXA2 expression levels in OSCC samples. (d) Association between E-cadherin staining and FOXA2 staining [Colour figure can be viewed at wileyonlinelibrary.com]

To further investigate the relationship between CDH1 and FOXA2, we treated SAS cells with 5-aza-2′-deoxycytidine followed by transfection of FOXA2 siRNA. Knockdown of FOXA2 decreased the expression of E-cadherin in SAS cells pretreated with 5-aza-2′-deoxycytidine (Figure 3a). Luciferase reporter assay result showed that FOXA2 siRNA was able to downregulate the activity of CDH1 promoter (Figure 3b). On the other hand, overexpression of FOXA2 in SAS cells by lentiviral infection significantly inhibited cell migration ability in transwell assay (Figure 3c,d).

3.5 | Expression of E-cadherin and FOXA2 in oral cancer tissues

The expression of E-cadherin and FOXA2 proteins in oral squamous cell carcinoma (OSCC) tissues was determined by immunohistochemical staining. In this study, scores 0, 1, 2, and 3 indicated the intensity of FOXA2 expression from low to high in cancer tissues (Figure 4a). We compared the intensity of FOXA2 staining in cases with or without lymph node metastasis. In the population of no lymph node metastasis, 25 (49.1%) patients had high intensity of FOXA2 (scores 2 and 3). However, in the metastasis population, 15 (93.8%) patients had low intensity of FOXA2 (scores 0 and 1; p = .0016; Figure 4b). We also examined the relationship of FOXA2 expression with clinicopathological features and survival in oral cancer patients. The expression of FOXA2 was negatively associated with lymph node metastasis (p = .002; Table 1). In addition, low FOXA2 expression (scores 0 and 1) in OSCC was associated with poor overall survival (p = .0048; Figure 4c). Further study showed the expression of E-cadherin was associated with the expression of FOXA2 in OSCC tissues (p = .0251; Figure 4d).

4 | DISCUSSION

Aberrant DNA methylation occurs in many types of cancer and contributes to malignant transformation by silencing tumor suppressor genes (Chakravarthi, Nepal, & Varambally, 2016; Esteller, 2008; Feinberg & Tycko, 2004). Previous studies have reported DNA methylation changes in cancer-related genes (Esteller, 2008; Feinberg & Tycko, 2004). In particular, changes in hypermethylation of promoter regions have been strongly implicated with the onset and progression of cancers. Therefore, DNA methylation has been increasingly recognized as a promising epigenetic and diagnostic biomarker.

While CDH1 gene was methylated in 44% oral cancer tissues, it was unmethylated in the oral cancer cell lines we tested. Futhermore,

TABLE 1 The association of FOXA2 expression with the clinical demography of OSCC patients

		FOXA2		
		Low	High	
Variables	Categories	*N* (%)	*N* (%)	*p*-Value
Grade	I	36 (59.0)	25 (41.0)	.39
	II–IV	5 (83.3)	1 (16.7)	
Tumor size	T1 + T2	28 (58.3)	20 (41.7)	.58
	T3 + T4	13 (68.4)	6 (31.6)	
Lymph node metastasis	No	26 (51.0)	25 (49.0)	.002
	Yes	15 (93.8)	1 (6.2)	
Pathologic stage	I + II	21 (55.3)	17 (44.7)	.32
	III + IV	20 (69.0)	9 (31.0)	
Sex	Female	3 (75.0)	1 (25.0)	1.00
	Male	38 (60.3)	25 (39.7)	
Alcohol drinking	No	9 (52.9)	8 (47.18)	.57
	Yes	32 (64.0)	18 (36.0)	
Betel quid chewing	No	10 (76.9)	3 (23.1)	.23
	Yes	31 (57.4)	23 (42.6)	
Cigarette smoking	No	9 (69.2)	4 (30.8)	.75
	Yes	32 (59.3)	22 (40.7)	

the cell migration ability was decreased in SAS and Ca9-22 cells after treatment with 5-aza-2′-deoxycytidine. It was reported that CDH1 was downregulated by classical transcriptional factors Snail and Twist (Krishna Rao et al., 2013), whose expression was not changed by the treatment of demethylation agent, suggesting that the regulation of E-cadherin expression by Snail and Twist was through DNA methylation independent mechanism.

The promoter of CDH1 contains FOXA2 binding sites, and previous studies have demonstrated that FOXA2 is hypermethylated in various cancer cell lines (Basseres et al., 2012; Halmos et al., 2004; Miyamoto et al., 2005; Song et al., 2010; Yu et al., 2011). The treatment of a demethylation agent 5-aza-2′-deoxycytidine was able to upregulate FOXA2 expression in oral cancer cell lines, suggesting that DNA methylation is a possible mechanism for regulating FOXA2 expression in oral cancer cells. Of note, while FOXA2 was fully methylated in all the tested oral cancer cell lines, it was less methylated in HOK normal human oral epithelial cells.

FOXA2 belongs to the family of transcriptional factors critical for the development of endoderm-derived tissues and plays essential role in the embryonic development (Kaestner et al., 2000; Myatt & Lam, 2007; Simpson & Pruitt, 1989). It has also been shown to function as a tumor suppressor, since loss of FOXA2 promotes epithelial–mesenchymal transition in pancreatic cancer and higher expression of FOXA2 is associated with good prognosis (Song et al., 2010). FOXA2 is frequently downregulated in lung cancer cells as a result of DNA methylation (Mao, Keller, Garfield, Shen, & Wang, 2013), and the induced expression of FOXA2 in lung cancer cells decreased cell growth and increased apoptosis, suggesting that FOXA2 has potential tumor suppressive activity (Mao et al., 2013). The role of FOXA2 in regulating metastasis in various cancers, such as lung cancer (Batlle et al., 2000), liver cancer (Chinn & Myers, 2015; Miyamoto et al., 2005), and breast cancer (Zhang et al., 2015) has been studied, but little information in oral cancer is documented. In this study, overexpression of FOXA2 increased the expression of E-cadherin and resulted in the reduced cell migration activity. Also, a direct interaction between FOXA2 and the promoter of CDH1 enhanced CDH1 promoter activity in oral cancer cells. All these results provide a novel finding for the involvement of FOXA2-E-cadherin link in the control of oral cancer cell metastasis.

Finally, the clinical relevance of FOXA2 was addressed by immunohistochemistry analysis, followed by statistical analysis of its association with clinical manifestations and survival in oral cancer patients. The results showed that high FOXA2 expression in cancer tissues was negatively associated with lymph node metastasis and was a good prognosis factor. In conclusion, this study provides a new insight for the tumor suppressor activity of FOXA2 in oral cancer. Further extensive studies are required to consolidate the potential of FOXA2 as a prognosis marker as well as a therapeutic target for oral cancer.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y-D Bow, Y-Y Wang, Y-K Chen, C-W Su, C-W Hsu, S-S Yuan, and R-N Li conceived and designed the experiments. Y-D Bow, Y-Y Wang, Y-K Chen, C-W Su, C-W Hsu performed the experiments. Y-D Bow, Y-Y Wang, R-N Li, and S-S Yuan involved in data analysis and discussion. S-S Yuan and R-N Li contributed to reagents/materials/analysis tools. Y-D Bow, Y-Y Wang, L-Y Xiao, R-N Li, and S-S Yuan prepared the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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