FOXD1 Promotes EMT and Cell Stemness of Head and Neck Squamous Cell Carcinoma by Transcriptional Activation of SNAI2

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Research Article

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Abstract

Background

Epithelial-mesenchymal transition (EMT) and cell stemness are implicated in the initiation and progression of head and neck squamous cell carcinoma (HNSCC). Revealing the intrinsic regulatory mechanism may provide effective therapeutic targets for HNSCC.

Results

In this study, we found Forkhead box D1 (FOXD1) was upregulated in HNSCC when compared with normal samples. Patients with higher FOXD1 expression had poorer overall survival and disease-free survival. Immunohistochemistry results showed that FOXD1 expression was related to the clinical stage and relapse status of HNSCC patients. When knockdown the expression of FOXD1 in CAL27 and SCC25 cells, the migration, invasion, colony formation, sphere formation, and proliferation abilities decreased. Moreover, the EMT and stemness-related markers changed remarkably, which indicated the EMT process and cell stemness were inhibited. Conversely, overexpression of FOXD1 promoted EMT and cell stemness. Further study demonstrated that FOXD1 could bind to the promoter region and activate the transcription of SNAI2. The elevated SNAI2, in turn, affected the EMT and cell stemness. The in vivo study showed FOXD1 overexpressed CAL27 cells possessed stronger tumorigenic ability.

Conclusions

Our findings revealed a novel mechanism in regulating EMT and cell stemness, and proposed FOXD1 as a potential marker for diagnosis and treatment of HNSCC.

Background

Head and neck squamous cell carcinoma (HNSCC) ranks the 7th most common cancer worldwide, and over 430,000 deaths related to HNSCC are reported annually [1,2]. Despite dramatic advances in the diagnosis and therapy strategies have been made, the prognosis of HNSCC remains poor owing to the high recurrence and metastasis rate [3]. Therefore, finding key genes and regulatory pathways controlling the progression of HNSCC is especially imperative.

Forkhead box D1 (FOXD1), a member of Forkhead family, is first identified in forebrain neuroepithelium and has been demonstrated as a vital gene participating in the development of kidney and retina [4]. Previous studies showed that FOXD1 also participated in the development of various cancer, including liver cancer [5], cervical cancer [6], pancreatic cancer [7], breast cancer [8], and glioma [9]. For instance, Sun et al. found LncRNA NORAD promoted cell stemness and angiogenesis of liver cancer via regulating miR-211-5p/FOXD1/VEGF-A axis [5]; Cheng et al. found FOXD1 could determine the renewing ability and tumorigenicity of glioma through transcriptional regulation of ALDH1A3 [9]. Recently, FOXD1 was found
to be significantly highly-expressed in OSCC tissues and related to overall survival, disease-free survival, and metastasis status [10]. Nevertheless, the function of FOXD1 in HNSCC remains unclear.

Epithelial-mesenchymal transition (EMT) is a process during which epithelial tumor cells lose their polarity and cell-cell adhesions, then transform into mesenchymal cell phenotype. Cancer cells that have undergone EMT display lower E-cadherin and higher N-cadherin and Vimentin expression, and possess stronger migration and invasion abilities [11]. Recent studies have demonstrated EMT process is associated with cell stemness in various cancers. For example, Pastushenko et al. revealed the initiation, progression, invasiveness, metastasis, and stemness of squamous cell carcinoma were promoted in a hybrid EMT state, which was inducted by the function loss of FAT1 [12]. Our previous study also demonstrated the interaction between CCL21/CCR7 could regulate EMT and cell stemness [13]. Tumor cells with enhanced stemness possess stronger self-renewal ability and tumorigenicity [14]. However, whether FOXD1 participates in regulating EMT and stemness of HNSCC remains unknown at present.

In this study, we find FOXD1 is upregulated in HNSCC and correlated with poor clinical outcomes. Then, we demonstrate FOXD1 can promote EMT and cell stemness of HNSCC. Further study showed that FOXD1 promotes the transcriptional activity of SNAI2, which is a key regulatory gene related to EMT and cell stemness. This study reveals the role and mechanism of FOXD1 in regulating tumor progression and proposes FOXD1 as a novel therapeutic target for HNSCC.

**Materials And Methods**

**Specimen collection**

Total 60 HNSCC and 8 normal specimens were collected from the Hospital of Stomatology, Wuhan University. Our research was permitted by the Ethics Committee of Wuhan University. Written informed consent was obtained from each participant.

**Cell lines and culture**

The CAL27, SCC25, and HN4 cell lines were cultivated with culture medium containing 10 % fetal bovine serum (FBS, Natocor, Córdoba, Argentina). Above cell lines were obtained from China Center for Type Culture Collection (Shanghai, China). Human Immortalized Oral Epithelial Cell (HIOEC) was generously donated by Professor Chengzhang Li and cultivated in KGM-goldTM keratinocyte cell basal medium (Lonza, Walkersville, MD) with its supporting growth factors. All cells were cultured at 37 °C in moist circumstances with 5% CO₂.

**Cell transfection**

Recombinant lentivirus (Genechem, Shanghai, China) was used for FOXD1 overexpression. Short hairpin RNAs (shRNAs; Genechem, Shanghai, China) were used to knockdown FOXD1 expression (Additional file 1: Table S1). Small interference RNAs (siRNAs; Hanbio, Shanghai, China) were applied to silence SNAI2
expression (Additional file 1: Table S2). Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA) was used to transfect siRNAs and shRNAs. To screen out stable FOXD1 overexpression cell lines, the transfected cells were cultured with 2 μg/mL puromycin for 7 days.

**Real-Time PCR (RT-PCR)**

We conducted the experiments as already described [13]. Primers that we employed in this research were shown in Table S3 (Additional file 1: Table S3).

**Western blot**

The experiment was carried out following our previous protocol [13]. Anti-FOXD1 antibody was purchased from Genetex (1:1000; CA, USA). Anti-E-cadherin, anti-N-cadherin, anti-Vimentin antibodies were purchased from Cell Signaling Technology (1:1000; MA, USA); anti-CD44, anti-ALDH1A1, anti-BMI1, anti-SNAI2 antibodies were purchased from Proteintech (1:1000; Wuhan, China).

**Wound healing and Matrigel invasion assays**

The experiments were processed in the light of previously designated [13]. The images of migration were acquired at 0 and 24 h after scratch. The results of invasion were acquired 24 h after the inoculation. The migration areas and the number of invasion cells were measured by Image-ProPlus 6.0 (Media Cybernetics, Inc., USA).

**Sphere forming and colony formation assays**

400 cells were sown into each well of 12-well plates and then cultured for 10 days to observed colony formation ability. Then the colonies were fixed and stained with crystal violet. Colonies were photographed and counted under a light microscope.

The 6-well culture plates were pretreated with polyHEMA like previously described to construct low adhesion dishes [13]. Cells were seeded into the pretreated plate (1000 cells/well) and cultivated with a standard cancer stem cells medium. After 12 days' cultivation, the quality of spheres was observed under a microscope.

**CCK-8 assay**

The experiment was conducted as previously described [13]. In a few words, cells were sowed into 96-well plates and incubated with the mixture of 100 μl medium and 10 μl Cell Counting Kit-8 (CCK-8, Biosharp, Hefei, China) for 24, 48, and 72 h. After that, the proliferation ability of the cells was determined by the optical density (OD) at 450 nm.

**EdU incorporation assay**
EdU detection was performed using BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Shanghai, China). The HNSCC cells (1 × 10^4) were evenly sown into each well of 24-well culture plates and then cultivated with Edu reagents. 2 h later, the cells were fixed with 4% paraformaldehyde (Servicebio, Wuhan, China). And then, cell staining was progressed following the manufacturer’s explanatory memorandum. Finally, images were attained with the fluorescent microscope (Biozero BZ-8000, Keyence, Osaka, Japan).

**Luciferase reporter assay**

The pGL4-basic plasmids containing SNAI2 promoter, pGL4-basic luciferase plasmids, phRL-TK plasmids were bought from Miaolingbio (Wuhan, China). The pGL4-basic plasmids containing SNAI2 promoter and phRL-TK plasmids were used to co-transfect into cells with Lipofectamine™ 3000. The cells transfected with phRL-TK plasmid and pGL4-basic luciferase plasmid were used as negative control. 48 h later, luciferase activity was determined by the Luciferase Assay System Kit (Promega, USA) according to the specification.

**Immunohistochemistry (IHC)**

Paraffin-embedded HNSCC tissues, normal mucosae, and xenograft tumors sections were IHC stained using anti-FOXD1 (1:100), anti-E-cadherin (1:400), anti-CD44 (1:200), anti-SNAI2 (1:400) antibodies. The IHC process was carried out as our previous study [13]. The IHC score was determined by Image-ProPlus 6.0.

**Mouse xenografts**

All the animal experiments were approved by the Ethics Committee of the Hospital of Stomatolgy at Wuhan University. The BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). CAL27 cells transfected with FOXD1 overexpression lentivirus or empty vectors were used for this assay. 45 days later, the tumors were removed and analyzed.

**Statistical analysis**

Student t-tests and one-way analysis of variance were used in this research. All statistical analyses were performed using GraphPad Prism 6 software (San Diego, CA, USA). Experimental results were presented as the mean ± standard error of the mean. The statistical results were showed as: ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Results**

**FOXD1 is upregulated in HNSCC and correlated with poor clinical outcomes**

First, FOXD1 expression of HNSCC was revealed. TCGA data from Gene Expression Profiling Interactive Analysis (GEPIA) web tool [15] showed that the expression of FOXD1 in HNSCC samples was
significantly higher than normal samples (Fig. 1A). Also, the overall survival and disease-free survival analysis acquired from GEPIA demonstrated that patients with higher FOXD1 expression possessed a worse prognosis (Fig. 1B, C). Next, FOXD1 expression in 60 HNSCC tissues and 8 normal mucosae were detected using IHC. And we found the expression of FOXD1 was related to the clinical stage and relapse status of HNSCC patients (Fig. 1D, E). Then, results from western blot and PCR revealed HNSCC cell lines CAL27, SCC25, and HN4 possessed higher FOXD1 expression than normal oral epithelial cell line HIOEC (Fig. 1F, G). In general, the above-mentioned results indicated that FOXD1 was upregulated in HNSCC and forecast poor clinical outcomes.

**FOXD1 knockdown inhibits EMT of HNSCC**

To reveal the role and intrinsic regulatory mechanism of FOXD1 on HNSCC progression, we knockdown FOXD1 expression in CAL27 and SCC25 cells using three different shRNAs. 48 h after transfection, western blot and PCR were used to detected FOXD1 expression. And we found shFOXD1#3 was the most effective sequence for interfering with FOXD1 expression in both CAL27 and SCC25 cells (Fig. 2A, B). Then, wound healing and Matrigel invasion assays were employed. And we found the migration and invasion abilities were decreased after FOXD1 knockdown (Fig. 2C, D). EMT is a process closely correlated with the occurrence and development of HNSCC [16]. Here, we found knockdown of FOXD1 significantly promoted E-cadherin expression and reduced N-cadherin and Vimentin expression, which implying an inhibited EMT status of tumor cells (Fig. 2E, F). Collectively, these results demonstrated silencing FOXD1 inhibited the migration, invasion, and EMT of HNSCC.

**FOXD1 knockdown decreases cell stemness of HNSCC**

In our previous study, we demonstrated the EMT process of tumor cells was closely related to their cell stemness [13]. Here, we found CSCs-related markers CD44, ALDH1A1, and BMI1 were decreased in protein and mRNA levels after FOXD1 knockdown (Fig. 3A, B). Besides, colony formation and sphere formation assays indicated that FOXD1 knockdown markedly impaired the self-renewal ability of HNSCC cells (Fig. 3C, D). In addition, CCK8 assay revealed a lower proliferation ability of FOXD1 knockdown cells (Fig. 3E). And EdU assay showed there existed fewer proliferative cells among CAL27 and SCC25 cells when FOXD1 was silenced (Fig. 3F). Altogether, the findings verified that FOXD1 knockdown decreased the stemness of HNSCC.

**FOXD1 overexpression promotes EMT and cancer stem-like properties of HNSCC**

We overexpressed FOXD1 in CAL27 and SCC25 cells using lentivirus transduction system. Western blot and PCR were used to detected FOXD1 expression in transfected cells after they were screened with 2 μg/ml puromycin for 1 week (Fig. 4A, B). In contrast to FOXD1 knockdown, FOXD1 overexpression dramatically reduced the E-cadherin expression and increased N-cadherin, Vimentin, CD44, ALDH1A1, and BMI1 expression in both protein and RNA levels (Fig. 4A, B). Besides, the areas of migration were bigger and the numbers of invasive cells were larger (Fig. 4C, D). The colony formation and sphere formation abilities were stronger (Fig. 4E, F). CCK8 and EdU assays indicated FOXD1 overexpression increased the
proportion of proliferative cells and promoted cell proliferation (Fig. 4G, H). Jointly, FOXD1 overexpression promoted EMT and cell stemness of HNSCC cells.

**SNAI2 is a target of FOXD1**

SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, NANOG, SOX2, and POU5F1 are the key regulatory genes contributing to EMT process and cell stemness [17,18]. The correlation analysis acquired from GEPIA showed that the SNAI2 expression was positively correlated to FOXD1 expression (Fig. 5A), while other genes were not (Additional file 2: Fig. S1A), indicating SNAI2 might be a target of FOXD1. Then, the SNAI2 expression of FOXD1 knockdown and overexpression cells were detected, separately. In consistent with the data got from GEPIA, we found SNAI2 expression was positively correlated to FOXD1 expression in both CAL27 and SCC25 cells (Fig. 5E, F; Additional file 2: Fig. S1B, C). FOXD1 is a transcription factor that plays its role by regulating the transcription of its target genes. The results acquired from JASPAR [19] showed there existed several binding sites of FOXD1 in the promoter region of SNAI2 (Additional file 1: Table S4). To verify whether FOXD1 could directly regulate the transcriptional activity of SNAI2, dual-luciferase reporter assay was performed. The results showed markedly increased luciferase activity in FOXD1 overexpression cells, indicating FOXD1 could promote the transcription of SNAI2 (Fig. 5G). Next, we examined the role of SNAI2 in CAL27 and SCC25 cells. Three specific siRNAs were used for interfering with the expression of SNAI2. The results confirmed siSNAI2#2 was the most effective sequence for knockdown SNAI2 expression (Additional file 2: Fig. S1D). Besides, we found SNAI2 silencing substantially inhibited the EMT process and reduced the cell stemness (Fig. 5H, I), which was similar to the results caused by FOXD1 knockdown. Also, we found SNAI2 was highly expressed in HNSCC samples when compared with normal samples (Fig. 5B). Nonetheless, the expression of SNAI2 was not correlated with clinical prognosis (Fig. 5C, D). In collection, these results revealed SNAI2 was a target for FOXD1. FOXD1 promoted EMT and enhanced cell stemness by transcriptional activation of SNAI2.

**FOXD1 promotes HNSCC progression in vivo**

To further investigate the role of FOXD1 on tumorigenicity, the mouse xenografts model was applied. FOXD1 overexpression and negative control CAL27 cells were prepared in vitro. Then cells were subcutaneously injected into mice, separately. On the 45th day, we dissected and detected the tumors. As expected, FOXD1 overexpression cells possessed obviously stronger tumor formation ability (Fig. 6A). Both the volume and weight of tumors in FOXD1 overexpression group were higher than the negative control group (Fig. 6B, C). In addition, tumors of the FOXD1 overexpression group displayed increased expression of FOXD1, SNAI2, N-cadherin and CD44 (Fig. 6D, E, F, G, H). All in all, the results demonstrated FOXD1 promoted HNSCC progression in vivo by regulating EMT and cell stemness, as well.

**Discussion**

Surgery, chemotherapy, and radiotherapy are traditional therapeutic strategies for HNSCC. However, due to their existing limitations, the 5-year survival rate of HNSCC is less than 50% [3,20]. Therefore, finding
genes that are crucial for tumor progression may provide new therapeutic targets for HNSCC. Recent studies demonstrated that upregulated FOXD1 was related to the metastasis status and adverse clinical outcomes of oral squamous cell carcinoma [10]. Besides, FOXD1-AS1 could enhance the proliferation and decrease the apoptosis of nasopharyngeal carcinoma by upregulating FOXD1 expression [21]. These results indicate that FOXD1 can behave as an oncogene in cancer. In our research, FOXD1 was upregulated in HNSCC and related to poor clinical outcomes. Silencing FOXD1 inhibited the EMT process and decreased cell stemness. Conversely, overexpression of FOXD1 promoted EMT and cell stemness. Notably, FOXD1 overexpressed cells showed stronger tumorigenic ability. Besides, we verified SNAI2, which was closely related to EMT and cell stemness, was the target for FOXD1. FOXD1 could bind to the promoter region and activate the transcription of SNAI2 (Fig. 6I).

EMT is a process during which cancer cells lose their polarity and convert into spindle-like mesenchymal morphology [11]. Parikh et al. found that partial EMT population in oral cancer was closely correlated with lymph node metastasis, perineural invasion, and tumor grade [22]. Zhang et al. revealed that CD100 motivated EMT process, thus leading to higher metastasis possibility of HNSCC [23]. EMT plays important role in cancer initiation, progression, recurrence, and metastasis [24]. Here, we found upregulated FOXD1 was related to adverse prognosis of HNSCC. Knockdown of FOXD1 significantly decreased the migration and invasion abilities and inhibited EMT process of HNSCC. On the contrary, overexpression of FOXD1 promoted EMT and increased migration and invasion abilities. These results are consistent with the previous studies [25], indicating FOXD1 can affect the prognosis of HNSCC through regulating the EMT process.

Cancer stem cells, a minority cluster of cells in tumors, possess the abilities of self-renewal and initiating tumor formation from very few cells [26]. Leticia et al. found treatment with JQ1 could reduce the stemness, and result in less invasive and more chemo-sensitive breast cancer [27]. Muhammad et al. revealed that c-Fos overexpression promoted EMT, cell stemness, and tumor growth when compared with control cells [28]. Cancer cells with increased stemness have stronger abilities of self-renewal and tumorigenesis, and contribute to adverse clinical outcomes [29]. Previous studies illustrated that FOXD1 could regulate the stemness of mesenchymal glioma stem cells [9]. However, the relationship between FOXD1 and the cell stemness of HNSCC is unclear at present. Here, we found the cell stemness was decreased when FOXD1 was silenced in HNSCC. Conversely, overexpression of FOXD1 enhanced the self-renewal, proliferation, and tumorigenesis. Our findings indicated that FOXD1 involves in the progression of HNSCC by regulating cell stemness.

Snai2, a transcription factor closely related to EMT and stemness [17,30], is positively correlated with the expression of FOXD1 in our research. Fan et al. found SNAI2 could induce EMT by suppressing the transcriptional activity of miR-222-3p and upregulating the expression of PDCD10 [31]. Tian et al. found that SNAI2 promoted cell stemness of prostate cancer potentially via modulating GSK-3β/β-CATENIN pathway [32]. Here, we found FOXD1 bound to the promoter region of SNAI2 and promoted its transcription. Silencing SNAI2 inhibited EMT and decreased cell stemness. For the first time, we identified SNAI2 as the downstream target gene for FOXD1. And we revealed FOXD1 could promote EMT and cell
stemness of HNSCC by transcriptional activation of SNAI2. Interestingly, although SNAI2 was the target for FOXD1, SNAI2 itself was not a good prognostic marker for HNSCC.

**Conclusion**

We find FOXD1 is upregulated in HNSCC and correlated with adverse clinical outcomes. And we verify FOXD1 can promote EMT and cell stemness by transcriptional activation of SNAI2. Our studies expand the current understanding of FOXD1 in tumor biology and provide a promising molecular target for the diagnosis and treatment of HNSCC.

**Abbreviations**

EMT: Epithelial-mesenchymal transition; HNSCC: Head and neck squamous cell carcinoma; FOXD1: Forkhead box D1; shRNAs: Short hairpin RNAs; siRNAs: Small interference RNAs; IHC: Immunohistochemistry; GEPIA: Gene Expression Profiling Interactive Analysis.

**Declarations**

**Ethics approval and consent to participate**

Our research was approved by the Ethics Committee of the Hospital of Stomatology at Wuhan University.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors have no conflicts of interest.

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**Authors’ contributions**

Study concept and design: ZJS and KL; Data acquisition and analysis: YC and WLL; Manuscript preparation and review: ZJS and KL. All authors read and approved the final manuscript.
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Figures
Figure 1

FOXD1 is upregulated in HNSCC and correlated with poor clinical outcomes. (A) The expression of FOXD1 in HNSCC and normal samples acquired from GEPIA. (B) Overall survival rates according to FOXD1 expression in HNSCC. (C) Disease-free survival rates according to FOXD1 expression in HNSCC. (D) Representative IHC images of FOXD1 in clinical specimens. (E) FOXD1 expression in HNSCC of
different clinical stages and relapse status. n = 60. (F) The protein expression of FOXD1 in different cell lines. (G) Relative mRNA expression of FOXD1 in different cell lines.

Figure 2

FOXD1 knockdown inhibits EMT of HNSCC. (A) The protein expression of FOXD1 in HNSCC cells transduced with FOXD1 shRNAs. (B) Relative mRNA expression of FOXD1 in HNSCC cells transfected with FOXD1 shRNAs. (C) The migration results of HNSCC cells transduced with FOXD1 shRNAs. (D) The
FOXD1 knockdown decreases cell stemness of HNSCC. (A) The protein expression of CSCs-related markers in HNSCC cells transduced with FOXD1 shRNAs. (B) Relative mRNA expression of CSCs-related markers in HNSCC cells transduced with FOXD1 shRNAs.
markers in HNSCC cells transduced with FOXD1 shRNAs. (C) The sphere formation results of HNSCC cells transduced with FOXD1 shRNAs. (D) The colony formation results of HNSCC cells transduced with FOXD1 shRNAs. (E) The proliferative ability of HNSCC cells transduced with FOXD1 shRNAs. (F) The proliferative cells of HNSCC cells transduced with FOXD1 shRNAs.

Figure 4
FOXD1 overexpression promotes EMT and cancer stem-like properties of HNSCC. (A) The protein level of EMT and CSCs related markers in FOXD1 overexpression HNSCC cells. (B) Relative mRNA level of EMT and CSCs related markers in FOXD1 overexpression HNSCC cells. (C) The migration results of FOXD1 overexpression HNSCC cells. (D) The invasion results of FOXD1 overexpression HNSCC cells. (E) The colony formation results of FOXD1 overexpression HNSCC cells. (F) The sphere formation results of FOXD1 overexpression HNSCC cells. (G) The proliferation ability of FOXD1 overexpression HNSCC cells. (F) The proliferative cells of FOXD1 overexpression HNSCC cells.

Figure 5

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Figure 5

SNAI2 is a target of FOXD1. (A) The correlation analysis of FOXD1 and SNAI2 acquired from GEPIA. (B) The expression of SNAI2 in HNSCC and normal samples acquired from GEPIA. (C) Overall survival rates according to SNAI2 expression in HNSCC. (D) Disease-free survival rates according to SNAI2 expression in HNSCC. (E) Relative protein expression of SNAI2 in FOXD1 overexpression HNSCC cells. (F) Relative mRNA expression of SNAI2 in FOXD1 overexpression HNSCC cells. (G) Relative transcriptional activity of SNAI2 in FOXD1 overexpression HNSCC cells. (H) The protein expression of EMT and CSCs related markers in SNAI2 knockdown HNSCC cells. (I) Relative mRNA expression of EMT and CSCs related markers in SNAI2 knockdown HNSCC cells.
Figure 6

FOXD1 promotes HNSCC progression in vivo. (A) Xenograft tumors of negative control (NC) and FOXD1 groups. (B) Tumor volume NC of and FOXD1 groups. (C) Tumor weight of NC and FOXD1 groups. (D) Representative IHC images of FOXD1, SNAI2, E-cadherin, and CD44 in NC and FOXD1 groups in xenograft tumors. (E) Relative IHC score of FOXD1. (F) Relative IHC score of SNAI2. (G) Relative IHC score of N-cadherin. (H) Relative IHC score of CD44. (I) Schematic diagram of the roles of FOXD1 in HNSCC.
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- Additionalfile2.docx