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HOXA10 promotes nasopharyngeal carcinoma cell proliferation and invasion via inducing the expression of *ZIC2*

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Abstract. – OBJECTIVE: In this study, we aimed to explore the dysregulated genes in nasopharyngeal carcinoma (NPC) and to investigate the regulative effect of HOXA10 on ZIC2 expression and their involvement in NPC cell proliferation and invasion.

MATERIALS AND METHODS: Microarray data that compared the transcription profile of NPC tissues and normal tissues was searched in GEO datasets and was re-analyzed. The expression of HOXA10 and ZIC2 mRNA were retrieved in TCGA database. CNE1 and CNE2 cells were used as an in-vitro cell model. Luciferase reporters carrying truncated ZIC2 promoter sequences were generated to verify the predicted HOXA10 binding site. CCK-8 assay and transwell assay were applied to assess cell proliferation and invasion respectively.

RESULTS: HOXC6, HOXA3, and HOXA10 were upregulated in NPC tissues. Data mining in TCGA database showed that HOXA10, but not HOXC6 or HOXA3 is positively correlated to ZIC2 expression. Enforced HOXA10 expression significantly elevated ZIC2 expression at both mRNA and protein levels in both CNE1 and CNE2 cells. HOXA10 can directly bind to the promoter of ZIC2 and upregulate ZIC2 transcription. ZIC2 knockdown significantly reduced cell proliferation and invasion capability of CNE1 cells and also partly abrogated the effect of HOXA10 overexpression on enhancing cell proliferation and invasion.

CONCLUSIONS: Both HOXA10 and ZIC2 are upregulated in NPC tissues compared to the normal tissues. HOXA10 can increase ZIC2 expression via binding to the ZIC2 promoter. Functionally, the HOXA10-ZIC2 axis can enhance NPC cell proliferation and invasion.

Key Words:
HOXA10, Nasopharyngeal carcinoma, Proliferation, Invasion, ZIC2.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common type of head and neck cancer in southern China and Southeastern Asia[1,2]. The complex interactions among environmental factors, such as viral infections and other genetic factors synergistically drive the initiation and development of NPC[3,4]. Although the standard combination of radiotherapy and chemotherapy has improved survival of the patients, management of NPC in advanced stages is still difficult[5]. The 5-year survival rate of stage III/IV disease is only around 55% due to local recurrence and distant metastasis[6]. Therefore, understanding the molecular mechanisms underlying NPC is necessary for future targeting therapy.

Homeoboxes (HOX) is a family of transcription factors that were involved in developmental control, while their dysregulations were also observed in a range of malignancies[7]. Homeobox A10 (HOXA10) is a transcription factor encoded by the *HOXA10* gene and is aberrantly expressed in some cancers. Its upregulation can promote pancreatic cancer cell invasion and MMP-3 expression via TGFbeta2-mediated activation of the p38 MAPK pathway[8]. In some types of head and neck cancer, such as in oral squamous cell carcinoma, *HOXA10* upregulation drives growth, epithelial to mesenchymal transition (EMT) and invasion of the cancer cells[9,10]. However, its role in NPC is not clear.

Zinc finger protein ZIC 2 (ZIC2) is also a transcription factor encoded by the *ZIC2* gene, which has C2H2 zinc fingers and can interact with multiple DNA and proteins[11]. Recent studies reported that *ZIC2* acts as an oncogene in multiple cancers.

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Its overexpression increases the growth rate and foci formation of epithelial ovarian cancer and stimulates anchorage-independent colony formation[12]. ZIC2 can also activate transcription of *OCT4* via recruiting the nuclear remodeling factor (NURF) complex to the *OCT4* promoter in liver cancer cells and thus drives the cancer stem cell (CSC) properties[13].

In this study, we firstly reported that HOXA10 can increase *ZIC2* expression in NPC cells via binding to the *ZIC2* promoter. Functionally, the HOXA10-ZIC2 axis can enhance NPC cell proliferation and invasion.

Materials and Methods

Bioinformatic Data Mining

Microarray data that compared the transcription profile of NPC tissues and normal tissues were searched in GEO datasets. One available Affymetrix Human Genome U133 Plus 2.0 Array (accession: GDS3341) compared gene expression profile of 10 normal nasopharyngeal tissue and 31 NPC tissue samples. The raw data was downloaded and reanalyzed to identify the most upregulated genes in NPC tissues[14].

The 100 upregulated genes and the upregulated HOX family transcription factors were loaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) database for analysis of protein-protein interaction (PPI) network. To ensure the high validity of the network, only experimentally validated interactions with a high confidence score ≥ 0.70 were included.

The expression profile of HOXA10 in head and neck cancer tissues and the normal squamous epithelial and glandular cells were reviewed in Human Protein Atlas (http://www.proteinatlas.org/).

The expression of *ZIC2* mRNA and the expression of the dysregulated HOX family transcription factors, including *HOXC6*, *HOXA3* and *HOXA10* were retrieved in TCGA database, by using the UCSC Xena (http://xena.ucsc.edu/). The correlation between *HOXA10* and *ZIC2* was also analyzed using the UCSC Xena.

The HOXA10 binding sites in the promoter region of *ZIC2* was predicted using the JASPAR Database (http://jaspar.genereg.net/).

Cell Culture and Transfection

Human NPC high differentiated cell line CNE1 and low differentiated cell line CNE2 cells were obtained from the Experiment Animal Center of Sun Yat-Sen University. HEK-293 cells were obtained from ATCC. CNE1 and CNE2 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640), while HEK-293 cells were cultured in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin in an incubator with a humidified atmosphere and 5% CO_2 at 37°C.

Human *HOXA10* cDNA ORF clone (cat. RG202939) and the empty control pCMV6-AC-GFP vector (cat. PS100010) were obtained from OriGene (Rockville, MD, USA).

CNE1 and CNE2 cells were transiently transfected with either the cDNA ORF clone or the control vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). CNE1 cells without transfection and CNE1 cells 24 h after transfection of *HOXA10* cDNA ORF clone or the empty controls were infected the ready-to-use *ZIC2* shRNA (h) lentiviral particles (sc-45881-V, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western Blotting

The protein samples extracted from cell samples were loaded and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene fluoride (PVDF) membrane. Then, the membranes were incubated with primary antibodies against HOXA10 (ab180222, Abcam, Cambridge, UK), ZIC2 (ab150404, Abcam) and anti-β-actin (ab3280, Abcam). Then, the membranes were washed and further incubated with secondary antibodies coupled to HRP. The blot signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, lL, USA).

QRT-PCR Analysis

Total RNAs in the cell samples were extracted using the Trizol Reagent (Invitrogen). Then, the RNA samples were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. The mRNA level of *HOXA10* and *ZIC2* was measured using qRT-PCR analysis with the following primers (*HOXA10*: F, 5'-CTGGGCAATTCCAAAGGTGA-3' and R, 5'-ACTCTTTGCCGTGAGCCAGT-3'; *ZIC2*: F, 5'-TCCGAGAACCTCAAGATCC-3' and R, 5'-TAGGGCTTATCGGAGGTG-3') and the SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). β-Actin was detected as the endogenous control.

Plasmid Preparation

The promoter clone of *ZIC2* (NM_007129) was obtained from GeneCopoeia (Rockuille, MD, USA; cat. HPRM19513). The detail promoter sequence was given in supplementary Table I. According to the prediction, HOXA10 has a conserved binding site located between -224 to -214 upstream the TSS site of *ZIC* promoter. To check whether HOXA10 has an effect on transcription activity of the promoter, five truncated promoter sequences, including -244 to +1013, -215 to +1013, -100 to +1013, and -50 to +1013 were PCR amplified from the promoter clone. After that, the truncated promoter sequences were inserted into the *XhoI-Hind III* site of the pGL3-basic luciferase reporter vector respectively.

Dual Luciferase Assay

HEK-293 cells were seeded into 12-well plates at a density of 2×10^5 cells per well and were further transfected with *HOXA10* cDNA ORF clone or the empty control. 24 h later, the cells were co-transfected the with 1.5 µg luciferase construct plasmid or the empty reporter vector DNA and 0.05 µg phRL-TK by using Superfectin (Qiagen, Valencia, CA, USA) and were further cultured for another 24 h. Then, the luciferase activity of the cell lysate was quantified using the dual-luciferase reporter assay system with a luminometer (Promega, Madison, WI, USA) following the manufacturer's protocol.

CCK-8 Assay

NES1 cells with *HOXA10* overexpression or *ZIC2* knockdown alone or with *HOXA10* overexpression and *ZIC2* knockdown in combination were seeded in a 96-well plate (3×10^3 cells per well) and were further cultured for 3 days. Then, cell viability was measured by WST-8 assay using Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) following the manufacturer's protocol.

Transwell Assay

In Brief, 1×10^5 NES1 cells with *HOXA10* overexpression or *ZIC2* knockdown alone or with *HOXA10* overexpression and *ZIC2* knockdown in combination were suspended in 200 µL serum-free RPMI-1640 medium and then plated into the upper chamber. The lower chamber was filled with RPMI-1640 supplemented with 20% FBS to create a chemoattractant environment. Then, the plates were cultured in a cell incubator for 24 h. After that, cells on the top surface of the insert were removed. The cells on the bottom surface were fixed with 4% polyoxymethylene and the number of invading cells were counted after staining with 0.1% crystal violet.

Supplementary Table I. The ZIC2 promoter sequence.

>HPRM19513 NM_007129; name=ZIC2; Entrez_ID=7546; Genome=hg18; chr13+:99432076-99433333; TSS=99432320; Upstream=244, Downstream=1013; Length=1257;
GAGAGGAAAAGGAGGAGACA**AAAAATAAAAA**ATAAAAGGCTGCCGCTGCAGCGTTGGCGG
CGCCCATCGAAATCAACGGAGGCGGTGGCGAACGCAGCCCACCGCAGCCGAGACCTGGGA
GCCCGCCTGGGCCTCACACTCCCTCGGGTCGCGGACTGCGCTGGGTCCACGCGGCGCGGT
CACTAGTTCCGGGCCCAGCGCCCAGGCCCGACCGGCGGGAGGGAGGAGGCGAGCGAGAGA
TCACTTTTTTATTGTTGTTATTGTTTTTCAGCCCAGCCTCCCCCTCCTCCCCTCCCCCTG
CTCGCTTTCTCCCCTCCCACATCCCCCTCCCCCCTACTCCCCCGCCTCCTCCTCCGGCAC
AACTTAAAGAAAGGGGGAGCGGCGCGGCTGCTGCCTTCATCTGGGGAAATTCGTGGCCAC
TGCAAGTTTACTACGCGAGGCGCAGCCAATGCCAAGCGCCGAGGCCGAGGAGGGCTAAAC
ACTGCGGCCGCGGCTCCGAACAATAACCGCCGCGCGCGGGGCGGCGCGAGTAGGGCCGCG
GGGGAGGGAGCTGTCGCCGCAGAGCGCCGCGGAGAGGACGCCTGGACTCCGCCTGCCGCC
CCGGCGCCCCCGCCGCGGTCAGGTGGAGCCGCTGGTGCGCTGGCCCCGGGTGCCGAGCGC
GGAGCTCGCCTCGGTCCTCTCCTCGCCCGTCTGCCTGGCGTGCGCACGGCCGCCGCGGTT
GTGACTGCATTCTACCGGCGCTGCTCGGTGCGGCCGGGCTCCGGGTCCGCTGGGCGTGCG
AGTGAGTGTGTGCGTGCGCGCGCGGGTGCGCGCAGGGGTGGGGGCTGCGGCGCGGCGCTC
GCCCCCCGGTAGCCCCCCTCTCCTCTCGGCTCCCCACACCTCCCTCCGGCTCCCTCCTCC
CGCCCGCCCTCCCCTGCCCTGCCCGCCCGCCCCCGCCGCAGCTCCTTTAATACACTTTGG
TTCTCCGCCTGGCTTTGGACTCTTCTCCTCCTCCACCTCCTCCTCCTCCTCCCGCGCCGC
CGCCTCCTCCTCCTCTTCCTCTCCGCGCCTTCGCTACGCGCCCGGCCGCCCGAGGCAGAT
CCAGGCGGCGGCGGAGGCGGCGGGCGCAGGAGAGCGGCTCCCAGGGCTGAAGTGGCCGCC
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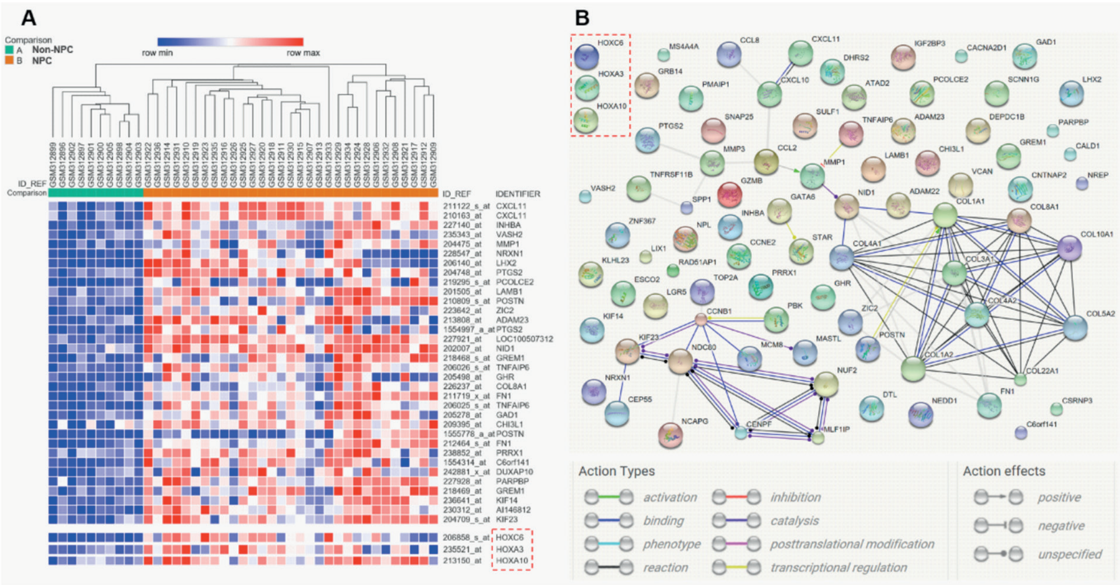

Figure 1. Bioinformatic analysis of the upregulated genes in NPC tissues compared to normal controls. ***A,*** Heat map of the upregulated genes in NPC tissues compared to normal controls. Data was obtained by re-analysis of the raw data of one available Affymetrix Human Genome U133 Plus 2.0 Array in GEO datasets (accession: GDS3341). The red frame indicates three upregulated HOX family transcription factor. ***B,*** PPI analysis of the interactions among the three HOX family transcription factors and the top 100 upregulated genes in NPC tissues by using the STRING database.

Statistical Analysis

Data were presented as means ± SD. All analyses were performed with SPSS software package (Version 19.0, SPSS Inc., Chicago, IL, USA). Data were analyzed for statistical significance by two-tailed Student's *t*-test or ANOVA with Student-Newman-Keuls test as a post hoc test. $p < 0.05$ was considered statistically significant.

Results

Bioinformatic Analysis of the Upregulated Genes in NPC tissues Compared to Normal controls

By analyzing the raw data of the microarray (GDS3341), we identified the most upregulated genes in 31 NPC tissue samples compared to 10 normal nasopharyngeal tissue (Figure 1A). Among the upregulated genes, we also observed three HOX family transcription factors, including HOXC6, HOXA3 and HOXA10 (Figure 1A, red frame). Then, we performed PPI analysis to check whether there is any known interactions among the three transcription factors and the top 100 upregulated genes in NPC tissues by using the STRING database. However, by limiting the evidence with high confidence score, we failed to identify any direct interaction (Figure 1B).

ZIC2 Expression is Positively Correlated to HOXA10 Expression in head and Neck Cancer

High *HOXA10* expression is associated more aggressive phenotypes of some cancer cells, including some head and neck cancers[9,15]. By reviewing the data in Human Protein Atlas, we also confirmed that HOXA10 expression is significantly higher in head and neck cancer tissues compared to the normal tissues (Figure 2A). Immunohistochemistry (IHC) staining of typically head and neck cancer tissue samples in the database showed moderate HOXA10 staining mainly in the nucleus of the cancer cells (Figure 2B), suggesting that this transcription factor may exert regulative effect on gene expression. Then, using the TCGA database that includes 566 head and neck cancer patients, we compared the expression profiles of H*OXC6, HOXA3* and *HOXA10* and the top upregulated genes. Interestingly, we observed that *HOXA10*, but not *HOXC6* or *HOXA3* is positively correlated to *ZIC2* expression (Pearson's r=0.5033) (Figure 2C-D).

HOXA10 induces ZIC2 expression in NPC cells via binding to the promoter

Then, we investigated whether HOXA10 has direct regulative effect on *ZIC2* expres-

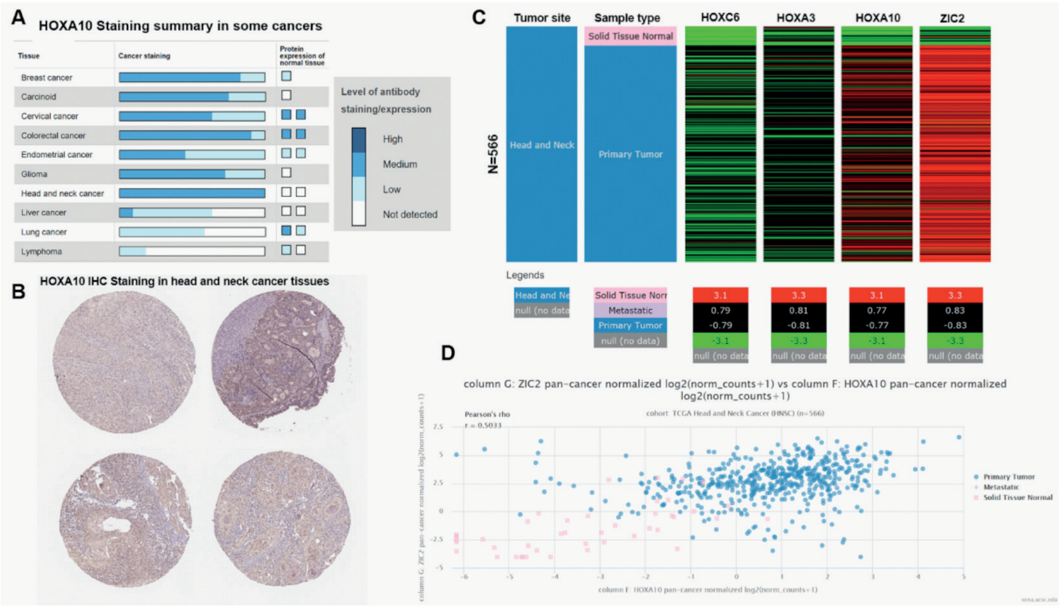

Figure 2. ZIC2 expression is positively correlated to HOXA10 expression in head and neck cancer. ***A,*** The expression profile of HOXA10 in some cancers and the corresponding normal controls. Data was obtained from Human Protein Atlas. ***B,*** Typical IHC staining of HOXA10 in head and neck cancer tissues. Data was obtained from Human Protein Atlas. ***C,*** The heat map of HOXC6, HOXA3, HOXA10 and ZIC2 mRNA expression in 566 cases of head and neck cancer tissues. Data was obtained by analysis of head and neck cancer cohort of TCGA database using the UCSC Xena. ***D,*** Regression analysis of the expression between HOXA10 and ZIC2 based on the data in figure C.

sion in NPC cells. Both CNE1 and CNE2 cells were transfected with pCMV-HOXA10 expression vector for overexpression (Figure 3A-B). Following analysis showed that enforced *HOXA10* expression significantly elevated *ZIC2* expression at both mRNA and protein levels in both cell lines (Figure 3C-F). By performing bioinformatic prediction, we observed a HOXA10 binding site in the promoter region of *ZIC2* (Figure 3G-H). To further verify the possible binding, luciferase reporter constructs carrying the truncated *ZIC2* promoter sequences were generated. The following dual luciferase assay showed that the reporter plasmid carrying integrate HOXA10 binding site had significantly higher luciferase expression than other plasmids, while truncation of the predicted HOXA10 binding site significantly decreased the transcription activity (Figure 3I).

HOXA10 Promotes NPC Cancer cell Proliferation and Invasion via Inducing ZIC2 Expression

ZIC2 has been reported as an oncogene with regulative effect on cancer stem cell properties, cancer cell growth and invasion in some cancers[12,13]. To demonstrate the functional role of HOXA10 and ZIC2 in NPC cells, CNE1 cells were transfected with pCMV-*HOXA10* or *ZIC2* shRNA alone or with pCMV-*HOXA10* and *ZIC2* shRNA in combination (Figure 4A). CCK-8 assay showed that *HOXA10* overexpression substantially increased CNE1 cell proliferation, while *ZIC2* knockdown significantly inhibited the proliferation. In addition, *ZIC2* knockdown partly counteracted the effect of *HOXA10* overexpression on facilitating cell proliferation (Figure 4B). Transwell assay also showed that *ZIC2* knockdown significantly reduced the invasion capability of CNE1 cells and also partly abrogated the effect of *HOXA10* overexpression on enhancing cell invasion (Figure 4C).

Discussion

Dysregulated transcription factors play important roles in pathological development of NPC via many different pathways. For example, *SOX4* overexpression was correlated with clinical stages, lymph node metastasis, and Ki-67 expression in NPC[16]. Functionally, SOX4 can promote EMT of CNE2 cells and decrease the cisplatin sensitivity[16]. *SOX1* is downregulated in NPC due

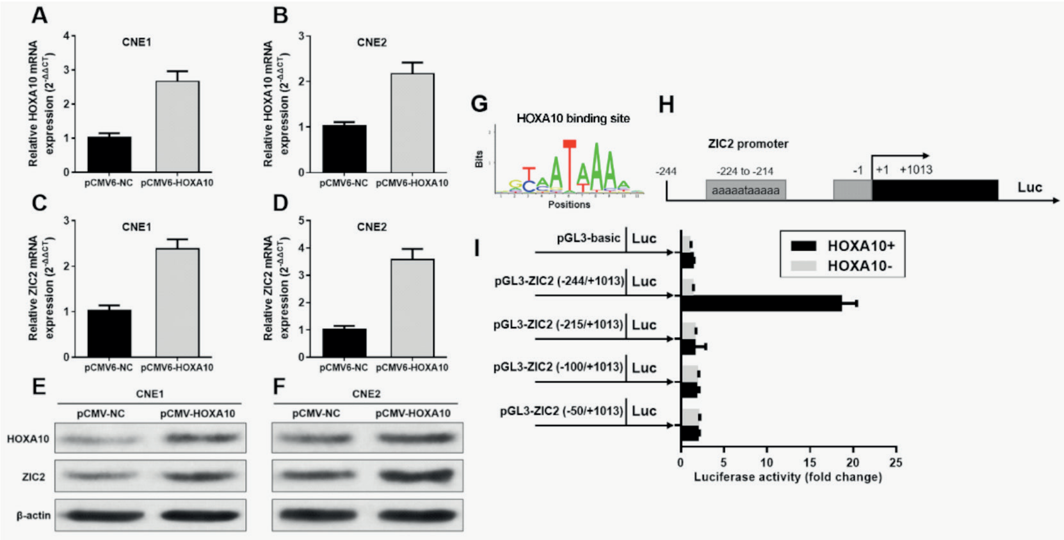

Figure 3. HOXA10 induces ZIC2 expression in NPC cells via binding to the promoter. ***A-D.*** QRT-PCR analysis of HOXA10 ***(A-B)*** and ZIC2 ***(C-D)*** mRNA expression CNE1 (A and C) and CNE2 (B and D) cells 24 h after transfection of pCMV-HOXA10 expression vector or the empty control. ***E-F.*** Western blot analysis of HOXA10 and ZIC2 protein expression in CNE1 and CNE2 cells 48 h after transfection of pCMV-HOXA10 expression vector or the empty control. G-H. HOXA10 binding positions (G) and the predicted HOXA10 binding site in the ZIC2 promoter (H). ***I,*** The luciferase reporter constructs carrying truncated ZIC2 promoter sequence were introduced into HEK-293 cells transfected with pCMV-HOXA10 expression vector or the empty control. Luciferase activity was measured 24 h post-transfection. **, *p*<0.01.

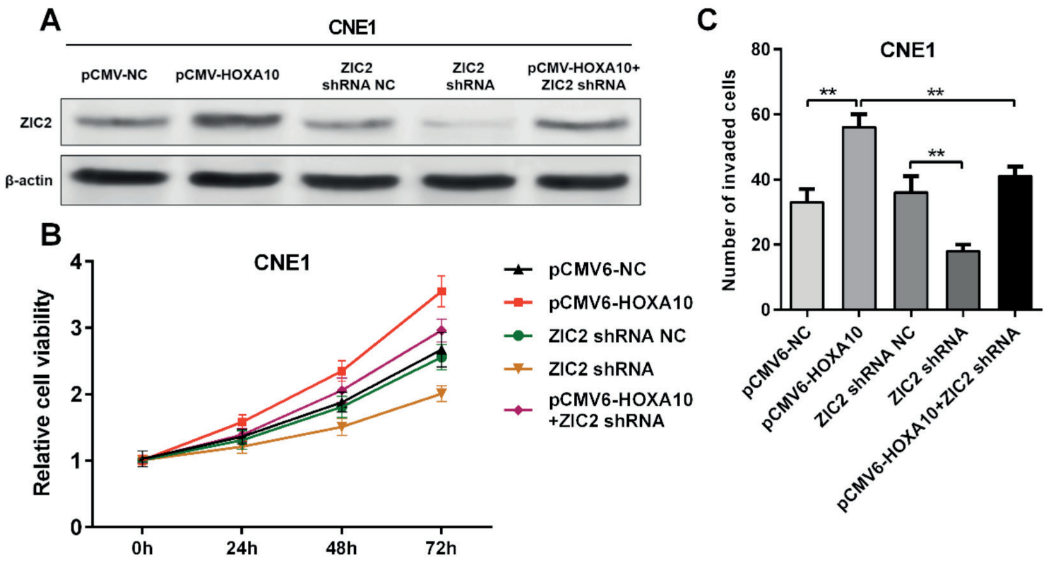

Figure 4. HOXA10 promotes NPC cancer cell proliferation and invasion via inducing ZIC2 expression. ***A,*** Western blot analysis ZIC2 expression in NES1 cells transfected with ZIC2 shRNA alone or were transfected with pCMV-HOXA10 or/and ZIC2 shRNA. ***B-C,*** CCK-8 assay of cell viability (B) and transwell assay of cell invasion (C) of NES1 cells transfected with pCMV-HOXA10 or/and ZIC2 shRNA. **, *p*<0.01.

to promoter hypermethylation[17]. Restoration of *SOX1* expression in NPC cells can suppress colony formation, proliferation, migration and EMT *in vitro* and impair tumor growth in nude mice[17]. The role of HOX family in NPC and other types of head and neck cancer has also been gradually revealed. The EBV product latent membrane protein 1 (LMP1) can silence *HOXC8* in NPC[18].

Enforced *HOXC8* expression can inhibit NPC cell proliferation both *in vitro* and *in vivo* and can also modulate genes related to glycolysis and the expression of tricarboxylic acid (TCA) cycle[18]. *HOXD10* overexpression can decrease head and neck cancer cell invasion but increased proliferation, adhesion and migration[19]. De Barros et al[20] observed that *HOXC8, HOXD10,* and *HOXD11* genes might be critical for cell colony proliferation and cell migration of laryngeal squamous cell carcinoma.

In this report, by reviewing one publicly available microarray, we found that *HOXA10* is significantly upregulated in NPC tissues compared to normal controls. The IHC staining data in Human Protein Atlas also confirmed significantly higher *HOXA10* expression in head and neck cancer tissues than in normal tissues. However, the PPI analysis failed to identify any connections between HOXA10 and the most upregulated genes. By data mining in head and neck cancer patient cohort in TCGA database, we observed that *ZIC2* expression is positively correlated to *HOXA10* expression. Since we identified a positive correlation between *HOXA10* and *ZIC2*, we decided to further investigate whether *HOXA10* has direct regulative effect on *ZIC2* expression. Our findings showed that HOXA10 can directly bind to the promoter of *ZIC2* and upregulate *ZIC2* transcription.

ZIC2 has been demonstrated as an oncogene in multiple cancers. *ZIC2* overexpression can increase the growth rate and foci formation of epithelial ovarian cancer cells, while high *ZIC2* expression is associated with worse overall survival in stage I epithelial ovarian cancer[12]. *ZIC2* can upregulate the expression of fibroblast growth factor receptor 3 (FGFR3) and Annexin A8 (ANXA8) in pancreatic cancer cells, leading to enhanced cellular proliferation and reduced cell apoptosis[21]. In liver cancer, *ZIC2* can drive the CSC properties via upregulating the expression of *OCT4*[13]. Since the role of *ZIC2* in NPC is not clear, we also investigated whether ZIC2 has regulative effect on proliferation and invasion of NPC cells. Our data suggest that *ZIC2* knockdown significantly reduced cell proliferation and invasion capability of CNE1 cells and also partly abrogated the effect of *HOXA10* overexpression on enhancing cell proliferation and invasion.

Conclusions

Both *HOXA10* and *ZIC2* are upregulated in NPC tissues compared to the normal tissues. *HOXA10* can increase *ZIC2* expression via binding to the *ZIC2* promoter. Functionally, the HOXA10-ZIC2 axis can enhance NPC cell proliferation and invasion.

Conflict of interest

The authors declare no conflicts of interest.

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