Motor Neuron and Pancreas Homeobox 1 (MNX1) Is Involved in Promoting Squamous Cervical Cancer Proliferation via Regulating Cyclin E

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Background:
Cervical cancer is one of the most lethal gynecologic malignancies worldwide. The objective of this study was to assess the role of MNX1 in cervical cancer and its underlying mechanisms.

Material/Methods:
The expression of motor neuron and pancreas homeobox 1 (MNX1) in immortal epithelial cervical cell line ECT, cervical cancer cell HeLa, and SiHa and cervical cancer, as well as in adjacent noncancer tissues, was detected and analyzed. CCK-8 and colony formation assays were performed to evaluate the effects of MNX1 overexpression on cervical cancer cell proliferation. Transwell assay was used to detect migration and invasion after MNX1 knockdown or overexpression. Real-time PCR and Western blotting were used to examine MNX1 and cell cycle regulator expression.

Results:
Data from our study indicated that MNX1 was upregulated both in cervical cancer cell lines and cervical cancer tissues. The high levels of MNX1 are related to advanced stages and lymph nodes metastasis. The overexpression of MNX1 promoted cervical cancer cells proliferation, migration, and invasion. Moreover, MNX1 upregulated 2 critical cell cycle regulators, CCNE1 and CCNE2.

Conclusions:
These findings reveal MNX1 as a novel oncogene of cervical cancer and indicate MNX1 is a promising therapeutic and prognostic biomarker.

MeSH Keywords: Cell Cycle • Cell Proliferation • Uterine Cervical Neoplasms

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Background

As the second most common gynecological cancer, cervical cancer is the fourth leading cause of cancer-related deaths worldwide [1,2]. Many patients, particularly those with advanced stages, undergo recurrence and metastasis in spite of improvements in surgical therapy and radiotherapy [3]. Therefore, effective therapies, especially targeted therapies, are necessary for cervical cancer.

Cyclins together with cyclin-dependent kinase (CDK) play critical roles in regulating the cell cycle [4]. The lethal characteristic of malignant tumors is known to be persistent proliferation [5]. In general, disruption of cell number homeostasis followed by uncontrolled proliferation occurs after cell cycle progression is dysregulated. It is widely recognized that the activity of CDKs increases during transition of G1-S stage. It was reported that cyclin E, consisting of cyclin E1 (CCNE1) and cyclin E2 (CCNE2), binding to CDK2 phosphorylates retinoblastoma (Rb) and promotes G1-S stage progression, leading to sustained cancer cell proliferation [6,7]. High levels of CCNE are positively related to poor prognosis of patients with bladder cancer [8,9]. As a result, numerous CDKs inhibitors have been studied and developed into targeted therapies in recent years [10].

MNX1 is located on human chromosome 7q36.3 and is a member of the EHG homeobox genes family [11,12]. As a homeodomain-containing transcription factor, MNX1 is involved in numerous types of pathological progression. It is generally accepted that Currarino syndrome is primarily caused by MNX1 deletion or mutation, which suggests that MNX1 affects human development and cell transformation [13,14]. In addition, MNX1 was reported to be overexpressed in several cancers [15,16]. It was proposed that MNX1 is upregulated in poorly differentiated liver cancer [17]. In 2016, Das reported that MNX1 is a novel prostate cancer oncogene, acting partially via promoting lipid synthesis [18]. Consistent with the above findings, Zhang et al. reported MNX1 was oncogenically upregulated in African-American patients with prostate cancer [19]. In 2016, a study published in Nature performed an integrated genomic analysis in pancreatic adenocarcinoma and found that MNX1 was overexpressed in pancreatic progenitor tumors [20]. Chen et al. detected high MNX1 expression in bladder cancer cell lines and bladder cancer tissues and confirmed that high MNX1 was correlated with shorter 5-year overall survival [21]. Recently, it was reported that MNX1 was associated with tumor size, receptor status, lymph node metastasis, and poor prognosis in breast cancer [22]. Taken together, MNX1 upregulation is involved in progression of several cancers, which indicates MNX1 probably is an oncogene. Until now, the roles of MNX1 in cervical cancer have been unclear.

Here, to investigate the role of MNX1 in cervical cancer, we detected the expression of MNX1 in normal cervical epithelial and cervical cancer cell lines. We also collected cervical cancer and adjacent nontumor tissues and compared the MNX1 expression. In addition, we constructed the overexpression and knockdown cervical cancer cell lines and investigated the effects of MNX1 on the proliferation, migration, and invasion of cervical cancer cell lines as well as the underlying mechanisms.

Material and Methods

Cells

HeLa and SiHa (human cervical cancer cell lines) and immortal cervical epithelial ECT were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin-streptomycin, and 5% CO₂ at 37°C.

Tissue samples

Twenty pairs of cervical cancer and adjacent nontumor tissues were collected from our hospital between July 2016 and July 2017. Tissues were stored at −80°C and used for qRT-PCR and Western blot analysis. Both cervical cancer and adjacent nontumor tissues were pathologically confirmed by independent pathologists. The inclusion criteria of our study were: squamous cell carcinoma, consistent pathology diagnosis from 2 independent pathologists, and no malignant history, including gynecological tumors. We collected patient information, including age, differentiation grade, International Federation of Gynecology and Obstetrics (FIGO) stage, and lymph node metastasis. All patients signed an informed consent form and our study was approved by the Institutional Review Board of Renmin Hospital of Wuhan University.

RNA extraction and qRT-PCR

Total RNA from tissues and cells was extracted and isolated with Trizol regent (Thermo Fisher Scientific, MA) followed the handbook provided by the manufacturer. We extracted 1 μg of RNA reversed into cDNA with PrimeScript RTMasterMix (Takara Bio, Japan). RT-PCR was performed with a SYBR Green PCR Master Mix (Takara Bio) detected by use of the CFX96 Real-Time PCR Detection System (Bio-Rad, CA). The relative mRNA levels of MNX1 and CCNE1 and CCNE2 were evaluated with GAPDH as an internal reference using the ΔΔCt method.

Western blotting assay

Cells and tissues were lysed with PIPA buffer (Beyotime) to get total protein. We determined protein concentration with
a protein assay kit (Beyotime, China). SDS-PAGE was used to separate protein, and it was then transferred to PVDF membranes purchased from Millipore (Billerica, MA). We used 5% fat-free milk to block nonspecific protein interactions in TBST buffer. The membranes loaded with proteins were incubated with primary antibodies at 4°C. After washing 3 times, membranes were then incubated at room temperature with secondary antibody (1: 5000, Abcam) conjugated with horseradish peroxide (2 h). After washing these membranes in TBST buffer, we developed the membranes using chemiluminescence to detect antibody concentration and used GAPDH as the internal control. The antibodies anti-MNX1 (1: 1000, Sigma Aldrich), anti-CCNE1 (1: 500, Proteintech), anti-CCNE2 (1: 500, Proteintech), and anti-GAPDH (1: 1000, Abcam) were used in our study.

**Cell transfection**

shRNA against MNX1 (shMNX1) and MNX1-overexpression lentivirus were obtained from Shanghai Genechem Co. Briefly, cells were cultured in 6-well plates, grown to 40% confluence, and infected with lentivirus. About 8 h later, the cell medium was changed into virus-free medium.

**Cell proliferation assay**

We seeded transfected SiHa cells into 96-well plates at a density of 5×10⁴ cells per well. Forty-eight hours later, 100 µl DMEM medium mixed with 10 µl CCK-8 solution was added to each well. The absorbance of each well was detected at 450 nm after incubation at 37°C for 30 min. The analysis was repeated at least 3 times.

**Colony formation assay**

Transfected SiHa cells were plated into 6-well plates (500 cells/well) and cultured for 10 days. Cell colonies were fixed in formaldehyde (10%) for 10 min and stained with crystal violet (1%).

**Transwell assay**

Transfected cells suspended with 200 µl serum-free medium were seeded in the upper chamber of 24-well Boyden chambers. About 600 µl of medium supplemented with 10% FBS was added to the lower chamber. Twenty-four hours later, the chambers were fixed in formaldehyde (4%) and stained with 1% crystal violet. For invasion assay, the chambers were changed into Matrigel chambers. We randomly selected 5 fields in each membrane and analyzed migrated cells.
Immunohistochemistry

To determine the MNX1 expression level in cervical cancer and normal tissues, immunohistochemistry was performed. In brief, fixed tissues embedded in paraffin were cut into 4-µm sections and baked at 65°C for 1 h, followed by deparaffinization and antigen rehydration. Bovine serum albumin (5%) was used to block nonspecific antigen binding. Then, tissues were incubated in rabbit anti-MNX1 (1: 200, Sigma Aldrich) at 4°C overnight, and goat serum was used as a negative control. The next day, sections were washed 3 times in PBS and incubated with secondary antibody. The 3,3’-diaminobenzidine (DAB) Horseradish Peroxidase Color Development Kit (Cell Signaling Technology) was used to develop color. Sections were stained with hematoxylin, dehydrated, and mounted. Two independent pathologists blinded to study outcome evaluated the staining. Any disagreements were arbitrated by a third pathologist. The staining index was calculated according to percentage of positive cells and staining intensity score. The percentage of positive cell was scored as follows: 0, no positive staining; 1, <25%; 2, 26–50%; 3, 52–75%; 4, >75%. The staining intensity score was: 0, negative; 1, light yellow; 2, yellow brown; 3, brown. Possible staining index scores were 0, 1, 2, 3, 4, 6, 8, 9, and 12. Staining index ≥6 was considered as high expression and <6 as low expression.

Statistical analysis

SPSS 18.0 (SPSS, Chicago, USA) was used to perform statistical analysis. All data are presented as mean ±SD. The clinicopathological characteristics was analyzed using the chi-squared test. Differences between 2 groups were analyzed with the t test and all experiments were performed at least 3 times. A P < 0.05 was regarded as statistically significant.

Results

MNX1 was upregulated in cervical cancer

MNX1 mRNA and protein expression were detected in ECT cells, SiHa cells, and HeLa cells (Figure 1A, 1B) as well as in cervical cancer and adjacent nontumor tissue cells (Figure 1C, 1D). Results from PCR and Western blotting consistently showed that MNX1 was obviously upregulated in cervical cancer cells (Figure 1). Immunohistochemistry was performed to identify the correlation between MNX1 level and clinicopathological characteristics in cervical cancer cells. Consistent with the results from PCR and Western blotting, immunohistochemistry revealed high MNX1 expression in cervical cancer cells compared with adjacent nontumor tissues (Figure 2A). Furthermore, the high expression level was positively associated with lymph node metastasis.
metastasis and advanced FIGO stage (Figure 2B, 2C). The association between the expression of MNX1 and clinicopathological factors of cervical cancer patients are shown in Table 1. The recruitment process is shown in Figure 3.

MNX1 upregulation promoted cervical cancer cell proliferation

To determine the influence of MNX1 on cervical cancer proliferation, MNX1 knockdown and overexpression cells were constructed (Figure 4A, 4B). CCK-8 assay indicated that MNX1 overexpression stimulated the proliferation of cervical cancer cells and knockdown reduced its proliferation (Figure 4C). In accord with CCK-8, colony formation assay showed obviously increased colony numbers of cervical cancer cells while MNX1 knockdown showed the opposite effect (Figure 4D). These results demonstrate that MNX1 overexpression enhanced cervical cancer proliferation.

MNX1 overexpression enhanced cervical cancer cell migration and invasion

To determine whether MNX1 affects the migration and invasion of cervical cancer cells, we performed the Transwell assay. Overexpression of MNX1 promoted migration and invasion of HeLa and SiHa cells, while the knockdown of MNX1 inhibited the migration and invasion of SiHa and HeLa cells (Figure 5), which shows that MNX1 plays roles in cervical cancer cell migration and invasion.

MNX1 upregulated CCNE1 and CCNE2 in cervical cancer cells

To identify the effects of MNX1 on the cell cycle, several cell cycle regulators were detected by qRT-PCR in SiHa cells (Figure 6A). Results showed that CCNE1 and CCNE2 were significantly upregulated after MNX1 was overexpressed, but they were suppressed in MNX1 knockdown cells (Figure 6B).

Discussion

MNX1 was reported to play vital roles in development of several cancers. Researchers have constructed MNX1-knockout mice and found that MNX1 early expression was correlated with pancreatic bud formation and β cell maturation [23]. In addition, MNX1 induced β cell differentiation from endocrine progenitor cells and balanced α and β cells [24]. In recent years, Patients diagnosed with cervical cancer (N=100)

Exclusions:
• Non-squamous cell carcinoma (N=29)
• Inconsistent diagnosis from pathologists (N=5)
• Malignant tumor history (N=3)
• Reject to participate (N=39)

Consented patients (N=24)

Recruited patients finally (N=20)

Lost to follow-up (N=4)

Table 1. Correlation between MNX1 expression and clinicopathological parameters of cervical carcinoma.

| Characteristics          | Patients (n=20) | MNX1 expression | P value |
|--------------------------|----------------|-----------------|---------|
|                         | Low expression (n=6) | High expression (n=14) |         |
| Age (years)              |                |                 | 0.292   |
| <55                      | 8              | 3               | 5       |
| ≥55                      | 12             | 3               | 9       |
| Histopathological grade  |                |                 | 0.176   |
| Moderate + poor          | 7              | 3               | 4       |
| Well                     | 13             | 3               | 10      |
| Stage                    |                |                 | 0.003   |
| I+II                     | 7              | 5               | 2       |
| III+IV                   | 13             | 1               | 12      |
| Lymph node               |                |                 | 0.024   |
| (-)                      | 9              | 5               | 4       |
| (+)                      | 11             | 1               | 10      |

Figure 3. The recruitment process.

Characteristics

Patients diagnosed with cervical cancer (N=100)

Exclusions:
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Consented patients (N=24)

Recruited patients finally (N=20)

Lost to follow-up (N=4)
MNX1 has been studied in many kinds of malignant tumors. In infant acute myeloid leukemia, MNX1 was reported to fuse to the ETV6 gene and to be involved in the progression of leukemia [25]. MNX1 was significantly overexpressed in bladder cancer and was reported to be associated with poor survival [21]. In gliomas, MNX1 bonds to the upstream region of tyrosine kinase receptor B (TrkB) and activates TrkB, followed by increased metastasis [26]. However, the biological and clinical roles of MNX1 in cervical cancer have not been reported. We collected cervical cancer specimens and adjacent nontumor tissues from our hospital and analyzed the expression of MNX1 and its association with patients’ clinical features. Results from this study revealed that MNX1 was obviously upregulated in SiHa and HeLa cells and in cervical cancer tissues, which was positively correlated with advanced stage and lymph nodes metastasis. CCK-8 and colony formation assays indicated that the overexpression of MNX1 obviously stimulated the proliferation and colony formation of cervical cancer cell lines. Transwell assay showed that MNX1 knockdown effectively inhibited cervical cancer cell migration and invasion, while MNX1 overexpression promoted cancer cell migration and invasion. Accordingly, overexpression of MNX1 stimulated cervical cancer proliferation, migration, and invasion. The human G1-phase cyclins are important regulators of cell cycle progression that interact with various cyclin-dependent kinases and facilitate entry into S-phase. In 2006, researchers confirmed the localization of the human cyclin C (CCNC) gene to chromosome 6q21 and of human cyclin E (CCNE) to 19q12 [27]. In the present study, we found that MNX1 overexpression upregulated CCNE1 and CCNE2, while knockdown of MNX1 inhibited the expression of CCNE1 and CCNE2. These findings suggest that MNX1 acts as an oncogene in cervical cancer and is a potential prognostic marker. Our study has some limitations that should be considered. First, the patient sample was too small. Second, we did not study the role of MNX1 in HPV, which is a confirmed etiology for cervical cancer. Third, the mechanism of MNX1 in promoting cervical cancer progression was not fully explored and future studies should explore the underlying mechanisms of MNX1 in cervical cancer progression more deeply. In addition, only women with squamous cell cervix cancer were included, so the result cannot be generalized to all types of cervical cancer.

Despite development of HPV vaccines and improved screening, cervical cancer remains a major female health problem, especially in developing countries [28,29]. One of the fatal characteristics of malignancy is uncontrolled proliferation. Cell cycle regulator dysregulation like CCNE1 and CCNE2 is involved in several tumors leading to G1-S stage transition disruption [30,31]. Here, we found that upregulation of MNX1 stimulated the expression of CCNE1 and CCNE2 and promoted proliferation of cervical cancer cells.
Figure 5. (A, B) Knockdown of MNX1 inhibited cervical cancer cell migration and invasion. Transwell assay was performed to detect migration and invasion potentials in SiHa and HeLa cells after MNX1 knockdown or overexpression. The results shown are the mean ±SD. *** p<0.001.
**Conclusions**

We found that overexpression of MNX1 has a role in the progression of cervical cancer, partially through upregulating cell cycle regulators CCNE1 and CCNE2. Based on the present results, we propose MNX1 as a novel cervical cancer oncogene and a promising therapeutic target.

**Conflict of interest**

None.

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**Figure 6.** MNX1 upregulated CCNE1 and CCNE2 in cervical cancer cells. (A) Real-time PCR of cell cycle-related gene mRNA expression. Gene expression levels were normalized to GAPDH. (B) Expression of CCNE1 and CCNE2 was detected by Western blotting after MNX1 knockdown or overexpression. Expression levels were normalized to GAPDH. Values are mean ± standard deviation. *** p<0.001.
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