MEX3A induces the development of thyroid cancer via targeting CREB1 Running title: MEX3A promotes thyroid cancer development

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Research Article

Keywords: Thyroid cancer, MEX3A, CREB1, proliferation, apoptosis

Posted Date: July 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1799226/v1

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Abstract

Thyroid cancer is a frequent endocrine cancer, and its incidence continues to rise globally. MEX3A has been revealed to be strongly expressed in many human malignant tumors including thyroid cancer and is related to patients’ prognosis. However, the molecular mechanisms underlying the tumorigenic capacities of MEX3A in thyroid cancer have not been elucidated. In this study, we verified that MEX3A is overexpressed in thyroid cancer tissues and cell lines. High levels of MEX3A positively link to AJCC stage. Moreover, MEX3A depletion attenuated abilities of proliferation and migration, ameliorated cell apoptosis as well as arrested tumor growth. Mechanistically, we found that the tumor-promoting effects of MEX3A in thyroid cancer was mediated by CREB1. Silencing CREB1 could relieve the promotion of MEX3A elevation on malignant phenotypes of thyroid cancer cells. Together, the results of this study highlight the importance of the MEX3A-CREB1 interaction for thyroid cancer cells to growth and suggest the potential of MEX3A as a therapeutic target for thyroid cancer.

Introduction

Thyroid cancer is one of the top 10 cancer types, with approximately 567,233 new cases and 41,071 deaths each year [1]. Thyroid cancer originates from follicular epithelial cells or parafollicular C cells [2]. Among them, follicular cell-derived thyroid cancers include three histological types: well-differentiated thyroid cancers (WDTCs), and poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). WDTC is subdivided into papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), which account for the majority of thyroid cancer cases [3]. Although ATC is the most aggressive histological subtype of thyroid cancer, most thyroid cancer-related deaths are from WDTC [2, 4]. Currently, surgical resection is the standard of care for patients with low-risk WDTC [1]. For advanced and metastatic thyroid cancer, although targeted therapies approved for differentiated thyroid cancer could prolong progression-free survival, these therapies are not curative [5-7]. Therefore, more therapeutic targets need to be developed.

MEX3A is one of four members (MEX3A-D) that make up the human MEX3 (muscle excess) protein family. This protein family is characterized by their domain structure, with 2 HNRNPK homology (KH) domains that mediate RNA binding and a C-terminal really interesting new gene (RING) domain, which functions as an E3 ligase [8]. Based on its domain composition, MEX3 proteins have been reported to regulate RNA fate and protein ubiquitination [9, 10]. On the other hand, MEX3 paralogs exhibit an oncofetal expression pattern, i.e. they are severely downregulated after birth but re-expressed in various malignancies [8]. In addition, strong expression of MEX3 protein in various cancers is associated with poor prognosis, indicating their oncogenic potential. For example, it has been evidenced that MEX3A regulates proliferation and migration of tumor cells in vitro and tumor growth in xenograft studies [11, 12]. More importantly, MEX3A has been revealed to be up-regulated in thyroid cancer and is related to patients’ prognosis [13]. However, the molecular mechanisms underlying the tumorigenic capacities of MEX3A have not been elucidated.
Therefore, in this study, we aimed to investigate the expression pattern and functional importance of MEX3A in thyroid cancer, and to further reveal the potential downstream mechanisms to provide novel insights into the pathogenesis of thyroid cancer.

Materials And Methods

Tissues samples and cell lines

A paraffin-embedded tissue microarray containing 41 cases of thyroid cancer and 50 cases of non-tumor samples was employed in this study, which was provided by Xi’an Alina Biological Technology Co., Ltd. This study was approved the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, and prior written informed consents were obtained from all participants. Here, normal thyroid cell line Nthy-ori 3-1 and three human thyroid cancer cell lines B-CPAP, TPC-1 and SW579 were purchased from American type culture collection (ATCC) (https://www.atcc.org/), which were grown in 1640+10%FBS and maintained in a 37°C incubator with 5% CO₂.

Immunohistochemistry (IHC) staining

The tissues were first repaired with 1×EDTA (Beyotime Biotechnology Co., Ltd, Shanghai, China) and blocked with 3% H₂O₂ for 5 min. Then, the tissues were incubated with MEX3A antibody (1:200, abcam, #ab79046) or Ki-67 (1:300, abcam, #ab16667) and secondary antibody (goat anti-rabbit IgG H&L (HRP):1:400, abcam, #ab97080) overnight at 4°C. After that, 3,3′-diaminobenzidine substrate (DAB, Sigma-Aldrich) and hematoxylin (Baso DiagnosticsInInc., Zhuhai, China) were used to detect immunoreactivity. Finally, the slides were sealed with neutral resin (China National Pharmaceutical Group Co., Ltd, Beijing, China) and then the images were captured and analyzed under an optical microscope. All slides were examined randomly by two independent pathologists. Staining percentage scores were classified as: 1 (1%-24%), 2 (25%-49%), 3 (50%-74%) and 4 (75%-100%). Staining intensity were scored 0 (Signalless color), 1 (light yellow), 2 (brown yellow) and 3 (dark brown). Finally, the high and moderate expression parameters were determined by the median of IHC scores of all tissues.

The Cancer Genome Atlas (TCGA) database analysis

For thyroid cancer, there are 1440 available sample data in the TCGA database, of which there are 560 RNAseq samples, including 58 normal samples and 502 tumor samples. Our expression profiling was based on RNAseq counts from 502 tumor and 58 normal samples.

Establishment of stably infected cells

The lentivirus expressing human MEX3A or CREB1 short hairpin RNA (shMEX3A or shCREB1) was designed based on RNA interference target sequence. The control was RNAi scramble sequence (TTCTCCGAACGTGTCACGT). B-CPAP and TPC-1 cell lines (2×10⁵) were infected with lentiviral particles
containing shMEX3A, shCREB1 or shCtrl (1×10^8 TU/mL) under ENI.S+Polybrene condition. Next, the cells were cultured in 1640+10%FBS for 72 h, and infection efficiency was evaluated under a microscope. A fluorescence efficiency greater than 80% was considered successful. In order to overexpress MEX3A in B-CPAP and TPC-1 cell lines, using MEX3A gene as a template, we designed primer amplification sequences to construct a MEX3A overexpression lentiviral vector.

**RNA extraction, cDNA synthesis and qRT-PCR**

After being infected lentivirus, total RNA of B-CPAP and TPC-1 cells was isolated using TRIzol reagent (Sigma, St. Louis, MO, USA) for cDNA synthesis and qRT-PCR. 2.0 μg RNA was reverse transcribed using Promega M-MLV Kit (Promega, Heidelberg, Germany) and quantitative real-time PCR (qRT-PCR) was performed with SYBR Green mastermixs Kit (Vazyme, Nanjing, Jiangsu, China) and applied Biosystems 7500 Sequence Detection system. GAPDH was as an internal normalization control. The relative expression of mRNA was evaluated based on the 2^-△△Ct method. The primers sequences (5′-3′) were listed as follows: the forward primer of MEX3A is CCGAGTGACTCTGGCTTTGAG, the reverse primer is CAGAGGAGAAGAGCACGGAGGT; the forward primer of CREB1 is ATCTTCTGATGCACCAGGAGTG, the reverse primer is AATTGGAGTTGGCACCGGTTA; the forward primer of GAPDH is TGACTTCAACAGCGACACCCA, the reverse primer is CACCCTGTTGCTGTAGCCAAA.

**Western blot assay**

After being infected lentivirus, total protein of B-CPAP and TPC-1 cells was collected and then segregated by 10% SDS-PAGE. Then, PVDF membranes were blocked at room temperature for 1 h by adding TBST solution with 5% skim milk. Subsequently, the membranes were incubated with primary antibodies and secondary antibodies at room temperature for 2 h. After that, the membranes were washed with TBST solution for three times (10 min/time). Finally, color rendering was conducted by using immobilon Western Chemiluminescent HRP Substrote kit. All antibodies used here were detailed in Table S1.

**Cell proliferation detection**

After being infected lentivirus, B-CPAP and TPC-1 cells were trypsinized and seeded into a 96-well plate (2500 cells/well). Next day, 20 μL of 5 mg/mL MTT was added. 4 h later, culture medium was aspirated, and then 100 μL DMSO was added to dissolve formazan particles. Finally, the plate was shaked for 2-5 min, and OD value was obtained with a microplate reader at 490/570 nm.

Another method for detecting cell proliferation was Celigo cell counting assay. B-CPAP and TPC-1 cells were trypsinized and resuspended. 100 μL cell suspension was added into each well of a 96-well plate. Cell images were taken by Celigo image cytometer (Nexcelom Bioscience, Lawrence, MA, USA) and a continuous 5-day cell proliferation curve was drawn.

**Colony forming assay**
The indicated cells were plated in a 6-well plate (500 cells per well) and cultured for 8 days. The colonies were washed with PBS, fixed with 1 mL 4% paraformaldehyde and stained using 500 μL Giemsa (Dingguo, Shanghai, China). Visible clones were recorded by fluorescence microscope (Olympus, Tokyo, Japan).

**Wound healing assay**

B-CPAP and TPC-1 cells with indicated lentivirus were cultured in a 96-well plate at the density of 5×10^4 cells/well. On the next day, low-concentration serum medium was supplemented, then a scratch tester was used to align the center of the 96-well plate and gently upward push to form scratches. The cells were washed with serum-free medium, and 0.5% FBS was subsequently added. Finally, the cells were cultured in an incubator with 5% CO₂ at 37°C. The plate was scanned at an appropriate time, and migration area was analyzed with Cellomics (Thermo, USA).

**Transwell assay**

The indicated cells (5×10^4) in 100 μl medium without FBS were seeded on a fibronectin-coated polycarbonate membrane inserted in a Transwell apparatus (Costar, MA). In the lower chamber, 500 μl medium with 10% FBS was added as a chemoattractant. After being cultured for an appropriate time, the cells adhering to the lower surface were fixed, stained with 1% crystal violet solution for 1 min and counted under a microscope in three random fields.

**Detection of cell apoptosis by fluorescence activated Cells Sorting (FACS)**

B-CPAP and TPC-1 cells with indicated lentivirus were cultured in a 6-well plate at the volume of 2 mL/well. When the cell confluence reached 85%, cell suspension was centrifuged at 1,300 rpm, and the supernatant was discarded. Then, the cells were washed with D-Hanks (4°C, pH=7.2~7.4) and stained in the dark by adding 10 μL Annexin V-APC (eBioscience, San Diego, CA, USA). The FACSCalibur (BD Biosciences, San Jose, CA, USA) was exploited to evaluate cell apoptosis level.

**Human Phospho-Kinase Array-Membrane**

The expression of 39 phospho-kinases in TPC-1 cells following lentivirus infection was detected by the Human Phospho-Kinase Array-Membrane. After the cells were lysed, the Handling Array membranes were blocked in 2 mL 1×Wash Buffer II and incubated with cell lysates and 1×Biotin-conjugated Anti-Cytokines overnight at 4°C. Finally, the signals of membranes were tracked by chemiluminescence imaging system.

**The construction of nude mouse tumor formation model**

All *in vivo* experiments were approved by the Ethics Committee of the of Animal Experiments of Beijing Tiantan Hospital and performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Ethics Committee of Animal Experiments, Beijing Tiantan Hospital. Twenty 4-week-old female BALB/c nude mice used in this study were purchased from Jiangsu Jicui Yaokang Biotechnology
Co., Ltd., and kept them in captivity under the following conditions: 5 mice per cage; temperature, 22-25˚C; humidity, 50-60%; 12 h light/dark cycle. Adequate water and food supplies ensured that mice could get them freely. Xenograft models were constructed by subcutaneously injecting shMEX3A or shCtrl TPC-1 cells (1 x 10^7 cells/each) into the right axilla of nude mouse (10 mice/group). The length and width of the tumor were measured to calculate the tumor volume according to the formula (tumor volume=π/6×L×W×W) during 17 days of feeding. After the mice were euthanized, the tumors were removed, weighed and frozen in liquid nitrogen and stored at −80°C.

**Statistical analysis**

All assays were independently performed in triplicate. All data were analyzed by using GraphPad Prism 6 (San Diego, CA, USA). The data were presented in the form of the mean ± standard deviation (SD). Statistical differences were evaluated using the unpaired t-test and the value of \( P \) less than 0.05 was considered to be significantly different.

**Results**

**MEX3A is frequently upregulated in thyroid cancer**

In order to elucidate the roles of MEX3A in the pathogenesis of thyroid cancer, we first set out to examine its protein expression in thyroid cancer specimen from a tissue microarray in comparison to para-carcinoma ones. Consistent with previous findings, we found that the level of MEX3A in thyroid cancer tissues was significantly higher than that in para-carcinoma tissues (\( P < 0.001 \), Fig. 1A and Table 1). Besides, we performed analyzed MEX3A mRNA level using RNA-seq datasets from TCGA database, suggesting that MEX3A was dramatically upregulated in tumor tissues of thyroid cancer patients compared to the normal tissues of healthy volunteers (Fig. 1B). Furthermore, abundant mRNA levels of MEX3A in thyroid cancer cell lines relative to normal thyroid cell line Nthy-ori 3-1 were shown in Fig. 1C. Next, we wondered whether MEX3A expression is associated with clinicopathological parameters of patients with thyroid cancer. Notably, upregulation of MEX3A correlates with higher AJCC stage (Table 2 and Table 3). From these results, we concluded that MEX3A might function as a potential tumor-promoting factor in the development of thyroid cancer.

**Depletion of MEX3A results in attenuated cell proliferation and migration in vitro**

In this section, we aimed to investigate the consequences of loss of MEX3A expression, and we established thyroid cancer cell lines (B-CPAP and TPC-1 cells) with MEX3A knockdown. Efficient MEX3A downregulation was confirmed by qRT-PCR and western blot experiments (Fig. 1D and 1E). In the following functional assays, we found that compared with cells from shCtrl group, MEX3A silenced cells harbored significantly mitigated abilities of proliferation (Fig. 2A). Additionally, the results of colony forming assay performed in B-CPAP and TPC-1 cells further confirmed that loss of MEX3A expression diminished cell viability and proliferation (Fig. 2B). At the same time, MEX3A-deficient cells are less motile: The wound-healing experiment revealed an attenuated migration ability upon knocking down
MEX3A (Fig. 2C). Similar results were obtained in a transwell assay (Fig. 2D). Moreover, MEX3A-deficient cells displayed a more than threefold enhanced apoptosis potential (Fig. 2E). Collectively, these results showed that depletion of MEX3A caused diminished cell proliferation and migration, as well as enhanced cell apoptosis in vitro.

**Depletion of MEX3A results in impaired tumor growth in vivo**

In order to address whether MEX3A capacity in vitro results in an enhanced growth of tumor cells in vivo, we subcutaneously implanted shCtrl and shMEX3A TPC-1 cells into BALB/c nude mice and monitored tumor growth (Fig. 3A). The findings revealed that the tumors derived from MEX3A-deficient cells grew smaller than those of shCtrl TPC-1 cells, which is in line with in vitro proliferation assays showing a higher plateau of shMEX3A TPC-1 cells (Fig. 3B-3E). Moreover, IHC analysis of tumor tissues from the xenograft mice injected shMEX3A TPC-1 cells showed substantially decreased Ki67 (Fig. 3F). Together, these data confirmed that inhibition of MEX3A could significantly delay the growth of xenografted tumors.

**MEX3A moderates thyroid cancer via acting on CREB1**

Finally, we investigate the mechanisms by which MEX3A mediates the malignant phenotypes of thyroid cancer cells. We first analyzed 39 phosphorylated protein levels using Human Phospho-Kinase Array-Membrane. The results showed that MEX3A knockdown downregulated the levels of CREB (S133), ERK1/2(T202/Y204,T185/Y187), GSK-3β(S9), Hsp27(S78/S82), p53(S15), p53(S392), p70S6K(T421/S424), STAT1(Y701) and STAT6(Y641) (Fig. 4A). At the molecular level, we further detected the decrease of CREB1, p-HSP27 and p-STAT1 in response MEX3A depletion (Fig. 4B). It is reported that CCDC6 serves as an important player in the dynamics of cAMP signaling by regulating CREB1 transcriptional activity in normal and transformed thyroid cells [14]. More interestingly, CCDC6 knock-in mice develop thyroid hyperplasia associated with enhanced CREB1 activity [15]. Based on the above findings, we speculated that MEX3A may target CREB1 and thereby regulate thyroid cancer development. To address this, CREB1 levels were measured in thyroid cancer cell lines and normal thyroid cell line. qPCR experiment confirmed the elevation of CREB1 in thyroid cancer cell lines in comparison to Nthy-ori 3-1 cells (Fig. 4C). Next, we built up B-CPAP and TPC-1 cell models, with merely MEX3A overexpression, merely CREB1 downregulation, as well as simultaneously MEX3A overexpression plus CREB1 downregulation, which were employed in cell function detection. Upon overexpressing MEX3A, the abilities of B-CPAP and TPC-1 cell proliferation and migration were enhanced. Conversely, silencing CREB1 impaired cell malignant development and partially abrogated the promotion by MEX3A overexpression (Fig. 4D and 4E). Taken together, we concluded that MEX3A is able to target CREB1 and regulate its expression level, thereby promoting the development of thyroid cancer.

**Discussion**

The identification of more and more new biomarkers for thyroid cancer has deepened the public understanding of the molecular pathogenesis of thyroid cancer, thus developing more personalized
treatments for thyroid cancer patients [16]. Currently, most targeted therapies inhibit known oncogenic mechanisms in thyroid carcinogenesis and progression, such as the MAPK pathway [17-20], PI3K/Akt-mTOR pathway [21, 22], or VEGF [23-25]. Despite the discoveries of new and promising therapeutics, there is still a lack of effective treatments for advanced and metastatic thyroid cancer. Therefore, more new drug targets and therapeutic agents are urgently needed to improve the treatment outcomes of patients at high risk of distant metastasis.

It is well-known that MEX3A is an oncogene in various types of malignant tumors including thyroid cancer, and MEX3A upregulation is related to poor prognosis [11-13]. Consistent with their data, in this study we found that MEX3A is highly expressed in thyroid cancer. Additionally, elevated MEX3A implied a higher AJCC stage. Subsequently, we knocked down MEX3A in both thyroid cancer cell lines B-CPAP and TPC-1. Consequently, MEX3A depletion attenuated abilities of proliferation and migration, ameliorated cell apoptosis as well as arrested tumor growth. Furthermore, we investigated the downstream mechanism behind MEX3A regulating thyroid cancer and identified CREB1 as a possible target gene.

CREB1 is a well-characterized transcription factor of the basic leucine zipper family. Due to various external stimuli, intracellular cAMP or Ca\(^{2+}\) levels increase, and cells respond to this by activating CREB1 through phosphorylation at Ser133 [26]. Moreover, CREB1 has been evidenced to be abnormally highly expressed in breast cancer and positively correlated with VASP expression, and the promoter region of VASP contained two CRE elements, and overexpression or activation of CREB1 could significantly promote the expression of VASP, which suggested that CREB1 could act as a transcription factor to activate the expression of VASP, thereby promoting the proliferation and migration of breast cancer cells [27]. Mechanistic exploration in this study showed that MEX3A depletion was accompanied with CREB1 downregulation. More importantly, published studies have reported that CREB1 participates in thyroid cancer occurrence and development [14, 15]. Thus, we inferred that MEX3A might interact with CREB1 to play a role in thyroid cancer and validated the hypothesis by \textit{in vitro} experiments. MEX3A elevation accelerated thyroid cancer cell proliferation and migration. The collective results in cell models in which CREB1 and MEX3A were silenced and overexpressed, respectively, illustrated that silencing CREB1 reversed the promotion of MEX3A elevation on malignant phenotypes of thyroid cancer cells. Collectively, the tumor-promoting effects of MEX3A in thyroid cancer was mediated by CREB1.

In conclusion, this study revealed an important role of MEX3A in the development of thyroid cancer, implying that MEX3A may be a potential therapeutic target for thyroid cancer.

\textbf{Declarations}

\textbf{Funding}

Not applicable.

\textbf{Conflicts of interest}
The authors declare that they have no conflict of interest.

**Data availability**

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

**Code availability**

Not applicable.

**Authors' contributions**

GF and YW designed this research. GF, P W and YX operated the cell and animal experiments. GF, PW, HZ and SC conducted the data procession and analysis. GF, PW and YX completed the manuscript which was reviewed by GF, PW, HZ and YW. All the authors have confirmed the submission of this manuscript.

**Ethics approval and consent to participate**

All in vivo experiments were approved by the Ethics Committee of the of Animal Experiments of Beijing Tiantan Hospital and performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Ethics Committee of Animal Experiments, Beijing Tiantan Hospital.

**References**


Tables

Table 1. Expression patterns of MEX3A in thyroid cancer tissues and para-carcinoma tissues revealed in immunohistochemistry analysis.
Table 2. Relationship between MEX3A expression and tumor characteristics in patients with thyroid cancer.

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Table 3. Relationship between MEX3A expression and tumor characteristics in patients with thyroid cancer.
**Figures**

**Figure 1**

**MEX3A was up-regulated in thyroid cancer and MEX3A knockdown cell models were constructed.** (A) The protein expression levels of MEX3A in thyroid cancer tumor tissues and para-carcinoma tissues were determined by immunohistochemical staining. (B) MEX3A mRNA levels were analyzed using RNA-seq datasets from TCGA database. (C) The MEX3A mRNA expression in thyroid cancer cell lines and normal...
thyroid cell line were detected by qRT-PCR. (D, E) The knockdown efficiencies of MEX3A were detected by qRT-PCR (D) and western blotting (E). Results were presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

**Figure 2**

**MEX3A knockdown inhibited thyroid cancer cell proliferation and migration as well as enhanced cell apoptosis.** (A, B) After lentiviral infection, B-CPAP and TPC-1 cell proliferation was evaluated by MTT assay (A) and Colony forming assay (B). (C, D) After lentiviral infection, the changes of B-CPAP and TPC-1 cell migration were detected by wound-healing assay (C) and transwell assay (D). (E) The effects of MEX3A knockdown on B-CPAP and TPC-1 cell apoptosis were examined by fluorescence activated Cells Sorting (FACS). Results were presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$. 
**Figure 3**

**MEX3A knockdown suppressed thyroid cancer tumor growth *in vivo***. (A) A nude mice model of MEX3A knockdown was constructed. (B) The fluorescence intensity was obtained through injection of D-Luciferase before sacrificing the mice. (C) The volume of tumors was tested from feeding to sacrifice. (D) The weight of tumors was measured after sacrificing mice. (E) The photograph of tumors was taken after removing tumors. (F) The value of Ki-67 was detected by IHC in tumor sections. Results were presented as mean ± SD. ***$P< 0.001$. 
Figure 4

The exploration of molecular mechanism behind MEX3A regulating thyroid cancer. (A) 39 phosphorylated protein levels in TPC-1 cells with MEX3A depletion were measured using Human Phospho-Kinase Array-Membrane. (B) The expression of CREB1, HSP27, p-HSP27, STAT1 and p-STAT1 was detected in TPC-1 cells with MEX3A depletion by western blot. (C) The CREB1 mRNA expression in thyroid cancer cell lines and normal thyroid cell line were detected by qRT-PCR. (D, E) The changes in cell proliferation and migration of B-CPAP and TPC-1, in which MEX3A was overexpressed, CREB1 was silenced or MEX3A was overexpressed and CREB1 was silenced, were assessed by Celigo cell counting assay (D) and transwell assay (E). Results were presented as mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001.

Supplementary Files

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- SupplementaryTable1.docx