Transcription Factor CDX2 Upregulates Proto-oncogenic miR-744 via Promoter Activation Mechanism in Non-small-cell Lung Cancer

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Abstract

Background: The role of caudal-related Homeobox 2 (CDX2) in the pathogenesis of non-small cell lung cancer (NSCLC) is unclear. The purpose of this study was to investigate the mRNA expression CDX2 in NSCLC, and to determine its relationship with miR-744 and its potential as a biomarker of NSCLC.

Methods: Mir-744 is overexpressed in A549, H460 and H1299 cell lines. QRT-PCR was used to detect mRNA expression. CHIP essay was performed to determine CDX2 binds sites. Then we conducted luciferase reporter essay to analyze interaction between Mir-744 and 3'UTRs. Migration and Boyden chamber method were used to study cell mobility.

Results: In this study, we found that ectopic CDX2 increased the expression of miR-744, while the attenuation of CDX2 reduced the expression of miR-744 by qRT-PCR. Chromatin immunoprecipitation experiments confirmed that CDX2 directly binds to the promoter of miR-744. Luciferase reporter assay further verified the binding sites of -347 to -358bp in the most likely promoter like sequence of miR-744. MiR-744 upregulation induced by CDX2 can significantly promote the migration and invasion of non-small cell lung cancer (NSCLC) cells. While miR-744 knockdown, ectopic CDX2 is sufficient to rescue the migration and invasion capacity of these cells.

Conclusion: In summary, our results for the first time confirmed the regulatory mechanism of CDX2 on miR-744 transcription and provided a potential mechanism for CDX2 as oncogene in lung cancer.

Introduction

Lung cancer has gradually become the malignant tumor with the highest morbidity and mortality around the world with its morbidity (11.6% globally) and mortality (18.4% globally) ranking the first among malignant tumors[1]. Metastasis occurs in more than 70% patients and accounting for more than 90% of cancer-related deaths [2]. Increasing evidence supports the participation of microRNAs in the metastatic process of cancers including lung cancer by negatively or positively regulating the expression of metastasis associated genes through posttranscriptional repression, mRNA degradation, or promoter activation[3-4]

Recently,miR-744 was reported as a tumor related gene, and its dysregulation was identified in serum or specimens of patients with head and neck cancers, multiple myeloma, gastric cancer, hepatocellular carcinoma, or breast cancer [3-7]. In our previous study,we have reported that miR-744 expression was upregulated in NPC specimens and was significantly associated with NPC progression,and confirmed for the first time that miR-744 participate in the process of cancer metastasis as a proto-oncogene[8].Furthermore, we have identified miR-744 as a upregulated miRNA in non-small-cell lung cancer (NSCLC) cell line and miR-744 upregulation could significantly promote migration and invasion of nasopharyngeal carcinoma (NPC) cells and non-small cell lung cancer (NSCLC) cells [9].

There existed several regulatory mechanisms of miRNAs expression, such as defects in the miRNAs biogenesis machinery,structural genetic alterations,epigenetic changes, altered transcription factor activity and so on[10]. In our previous study ,we have identified that transcription factor c-Jun directly binds to miR-744 promoter region, resulting in elevated miR-744 expression in nasopharyngeal carcinoma (NPC) cells and non-small cell lung cancer (NSCLC) cells [9].

Besides, bioinformatics analysis (PROMO 2.0) revealed the 2-kb region upstream of miR-744 gene contains three potential binding motifs for transcription factor CDX2. Caudal-type homeobox transcription factor 2 (CDX2) is expressed in the nucleus of the intestinal epithelial cells and is essential for embryonic formation and differentiation of the intestine, whose reduced expression can result in metastatic tumors[11]. Loss of CDX2 synergizes with loss of NKX2-1 was reported to fully activate the metastatic program of lung adenocarcinoma and were consistently down-regulated in metastatic cells compared with nonmetastatic cells[12].However,the function and mechanism of CDX2 in non small cell lung cancer still remains unknown.CDX2 and hindfoot, or pituitary homologous frame gene 1 (BFT or PITX1) are members of the homologous frame familyIn addition, BFT expression plays a critical role in lung development (13). Recently, a study reported that BFT expression may be associated with the development of lung cancer[14]

The aim of this study was to investigate the mRNA expression CDX2 in NSCLC, and to detect its relationship with miR-744 and its potential as a biomarker of NSCLC.

Cell Lines And Cell Culture

The human SPC-A-1 and A549 cells were purchased from Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained in RPMI-1640 medium (Corning, NY, USA) with 10% fetal bovine serum (FBS, GIBCO, South America, NY, USA). All these cells

were incubated with 5% CO₂ atmosphere at 37 °C.

Plasmid And Oligonucleotide Construction

The miR-744 mimics/inhibitor and its negative control miRNA (anti-miR-control) were designed and synthesized by Genepharma (Shanghai, China) based on miRbase database (<http://www.miRbase.org>), siRNA against CDX2 and non-specific siRNA control were also designed and synthesized by Genepharma (Shanghai, China). Plasmids expressing CDX2 and negative control pENTER were designed and synthesized by Vigene Bioscience (Shandong, China).

Transient Transfection

The SPC-A-1 and A549 cells were seeded (2×10^5 per well) in 6-well plates at 30–40% density. Transient transfection was performed with Lipofectamine 3000 reagents (Invitrogen, USA). Cells were incubated under 5% CO₂ at 37 °C and collected at 48 h after transfection.

RNA extraction and quantitative real-time PCR (qRT-PCR) analyses

Total RNA was extracted from cell lines by using TRIzol® Reagent (Invitrogen, CA, USA) according to the manufacturer's recommended protocol. For detection of miR-744 mRNA, total RNA was reverse transcribed into cDNA to perform qRT-PCR by SYBR® PrimeScript™ miRNA RT-PCR Kit (TaKaRa, Dalian, China). For detection of CDX2 mRNA, total RNA was reverse transcribed into cDNA by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). Quantitative real time PCR for CDX2 mRNA was undertaken in Mx3005P real-time PCR instrument (Stratagene, USA) with SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China). Small nuclear RNA U6 (U6 snRNA) or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for miRNA or mRNA expression, respectively.

The sequences of the primers are shown as follows: hsa-miR-744 forward: 5'-ACACTCCAGCTGGGTGCGGGGCTAGGGCTAAC-3', U6 forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3'. MiR universal reverse prime: 5'-CTCAACTGGTGTCTGGA-3', CDX2 forward: reverse: GAPDH forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAGTGGA-3'.. Relative expression of miR-744 and CDX2 was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Promoter Reporter Construction And Luciferase Assays

The upstream of Mir-744 gene was amplified by qPCR in different length regions. A mutation primer was designed for CDX2 conjugate sequences mismatched 12 bp in -347 to -358. The sequences built by these promoters are listed in supplementary table S1. The amplified products were cloned into the pGL3 base vector to produce the promoter builder of Mir-744. Cells SPC-A-1 and A549 were transfected into A 24-well plate at a density of 5×10^3 cells / . 100 ng Mir-744 promoter was constructed or empty PGL3-basic vector was transfected into these cells using Lipofectamine for 48 h. 3000 and hatched Co-fection, 100 ng Mir-744 sub-vector C or empty PGL3-basic vector and 1 ug CDX2 plasmid or its corresponding empty vector control. Double luciferase reporter gene detection system (Promega, WI, USA) was used to detect luciferase according to manufacturer's instructions.

Cell Migration And Invasion Assays

Cell invasion experiments were carried out in a 24-well plate by transwell chamber (Corning, NY, USA) At 48 h after transfection, each group of cells (105 cells /100 largose) were resuspended in serum-free medium, inoculated in the upper chamber with 5% matrix coated membrane (BD Bioscience, San Jose, CA), and the lower chamber was filled with fresh and complete medium of 500 largose. After incubation at 37°C with 5% CO₂ for 24 hours, the non-invasive cells were scraped from the upper surface of the filter with cotton swab. The invading cells adhering to the lower surface of the lumen were fixed with methanol and stained with hematoxylin. An inverted microscope was used to manually count the invading cells at 200 x magnification in three random fields. Similar non-matrix inserts were used for migration experiments.

Wound healing assay

Artificial wound is created with 20-ul pipette head at the time cells have grown to about 90% fusion (48 h after transfection), then we cultured the cells in a fresh medium. Images were taken at 0 hours and 36 hours to observe wound healing, respectively. Relative

percentage of the wound healing was counted as (0 h wound width – 36 h wound width)/ 0 h wound width.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of triplicates. Each experiment was repeated at least three times. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, USA). The student's t-test was used to assess differences among groups in in vitro studies. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

CDX2 is overexpressed in NSCLC cell lines

As shown in Fig. 1, the expression of CDX2 significantly increased in human NSCLC cell lines compared with normal lung epithelial cell line (HBE). Besides, the level of CDX2 was much higher in A549, H460 and H1299 in comparison with SPC-A-1 and 95D, and the capacity of cell migration and invasion is also higher in these NSCLC cell lines with high CDX2 level .which suggested that CDX2 may play a role in the migration and invasion of non-small-cell lung cancer.

Cdx2 Can Upregulated Mir-744 Expression In Nsclc Cell Lines

To identify the potential transcription factors (TFs) regulated miR-744 transcription, we used PROMO 2.0 to search for possible transcription factor binding sites (TFBS) in miR-744 3'UTR promoter region(Fig. 2). The bioinformatics analysis reported the 2-kb region upstream of miR-744 gene contains three potential binding sides for transcription factor CDX2.

To confirm the bioinformatical prediction and explore the relationship between CDX2 and miR-744, we use CDX2 siRNA, CDX2 plasmid and their controls to transiently transfect into A549 and SPC-A-1 cells. The expression of CDX2 mRNA was measured by qRT-PCR to detect the efficiency of transfection((Fig. 3)

Data showed that transfection of CDX2 siRNA led to dramatically knockdown of miR-744 expression (Fig. 3). Meanwhile, overexpression of CDX2 led to a remarkable up-regulation of miR-744 in non small cell lung cancer cell lines (Fig. 3).

To further determine whether CDX2 could affect endogenous expression of miR-744 in these cells, we performed a co-transfection of CDX2 plasmid-miR-744 mimics and inhibitor and controls in A549 and SPC-A-1 cells .The exist of CDX2 can reverse the knock down of miR-744 and intensify the effect of miR-744 mimics (Fig. 3). Taken together ,these results suggested that CDX2 plays an extremely important role of miR-744 transcription regulation.

CDX2 can be recruited to binding to promoter region of miR-744

To sum up, transcription factors can bind to either enhancer or promoter regions of DNA adjacent to the genes that they regulate [15]. We performed a chromatin immunoprecipitation (ChIP) assay coupled with qRT-PCR to determine whether CDX2 directly binds to its potential promoter region as bioinformatical predicted to upregulate the transcription of miR-744.

We design primers for ChIP assay based on the predicted CDX2 binding sites in miR-744 potential promoter region which were – 347 to -358 bp, -1186 to -1197 bp, -1232 to -1243 bp, and take the percentage of similarity between CDX2 DNA-binding domains (DBDs) into consideration.

Because the minimum amplicon size for transcription factor binding site is about 150 base pairs in qRT-PCR, it's technically difficult to design primers to amplify – 1186 to -1197 bp and – 1232 to -1243 bp regions separately. Therefore, we amplifie these two binding sites as one binding region, renamed to Site 2 in our future study. The remaining – 347 to -358 bp were named Site 2 .

As shown in Fig. 4A, CDX2 could bind to Site 1, Site 2 sequences in the miR-744 potential promoter region in A549 cells and validated by quantitative ChIP assays (Fig. 4B). An isotype-matched IgG was used as a negative control. These results demonstrated that CDX2 could be recruited to the three binding sites – 347 to -358 bp, -1186 to -1197 bp, -1232 to -1243 bp in miR-744 potential promoter region.

CDX2 response element existed in key promoter region of miR-744

In our previous study, we have found a most potential promoter like sequence of miR-744 existed in -500 to -1 bp region[9]. In order to verify the presence of CDX2 response elements in the key promoter region of miR-744, we conducted co-transfection studies. Luciferase activity was detected 48 h after transfection with miR-744 promoter(-500 to -1 bp) or empty PGL3-Basic vector and CDX2 plasmid or corresponding empty vector in 5-8F, HONE1, SPC-A-1 and A549 cells (Fig. 5). Consistent, miR-744 promoter construction with only -500 bp resulted in a significant increase in 5-8F relative luciferase activity in SPC-A-1 and A549 cells, which was further strengthened 2–18 times, suggesting the upstream of -500 bp CDX2 miR-744 gene in the functional promoter activation region.

MiR-744 mediates CDX2 to promote lung cancer cell migration and invasion

We have previously reported that overexpression of miR-744 can regulate up- the migration and invasion of non-small-cell lung cancer cells [9]. To investigate whether the cell functions induced by CDX2 could be mediated by miR-744, CDX2 plasmids and miR-744 inhibitor were co-transfected into SPC-A-1 and A549 cells. QPCR results showed forced expression of CDX2 and overexpression of CDX2 led to significantly increased expression of miR-744 compared with the control group (Fig. 6A). Respectively, miR-744 inhibitor reduced miR-744 expression was even rescued by CDX2 to normal levels (Fig. 6A).

Then we studied whether the CDX2 for the promotion of cancer cell migration and invasion could be offset by miR-744 inhibitor. In scratch and transwell migration and invasion experiments, it was observed that CDX2 overexpression could significantly promote the migration and invasion of SPC-A-1 and A549 cells (Fig. 6B and 6C), while the positive effects of CDX2 on migration and invasion were almost completely reduced by miR-744 inhibitor. These data showed that CDX2 increased the expression of miR-744 and participated in the promotion of CDX2 on migration and invasion.

Discussion

MicroRNAs (miRNAs) are highly conserved non-coding, small RNA molecules that can play a dual role in tumor cell apoptosis, proliferation angiogenesis, tumor invasion and metastasis[13]. miR-744 has been proved to participate in tumor invasion and migration as a tumor promoter or suppressor. In our previous miRNA microarray analysis, we found miR-744 be remarkably decreased after MTA1 gene knockdown [14], and we confirmed miR744 function as proto-oncogene in human NPC progression and metastasis[9]. Recently, we reported the oncogenic functions of miR-744 and promote invasion and migration in NPC and NSCLC. Furthermore, in that study, we found C-Jun-induced upregulation of miR-744 mediates the effects of c-Jun signaling in NPC and NSCLC, provides a new insight into miR-744 oncogenic functions.

Caudal-type homeobox 2 (CDX2) is an intestine-specific transcription factor with potential prognostic and predictive effect, and hindfoot, or pituitary homologous frame gene 1 (BFT or PITX1) are members of the homologous frame family, and nuclear transcription factors play an important role in regulating intestinal epithelial cell proliferation and differentiation in fetal and adult tissues [15–17]. CDX2 has been proposed as a potential molecular marker for several malignancies, including gastric, colon and esophageal cancers (18–20). In addition, CDX2 expression has been studied as a reliable marker of colorectal origin for the determination of pulmonary metastasis (21,22). Recent reports indicated that CDX2 expression is not limited to metastases of colorectal origin, but also can be detected in primary lung adenocarcinoma by real-time PCR and immunohistochemistry[24, 25]. In this study, CDX2 expression was consistent in primary lung adenocarcinoma,

The relationship between CDX2 expression and cancer occurrence has been less studied. For the first time, our team reported miR-744 as a upregulated miRNA in non-small-cell lung cancer (NSCLC) cell line can be upregulated via a promoter activation mechanism[9]. Recently, Chen and his colleagues for the first time confirmed changes in BFT expression in primary lung cancer, suggesting that BFT may be associated with the occurrence and development of lung cancer[26]

For the first time in this study, we proved that the transcription factor CDX2 in the transcription of miR - 744 plays an important role. miR-744 can be directly induced by tumor promoter CDX2 and is partly involved in the invasive phenotype induced by this oncogene. We also identified the upstream 347 to -358 of miR-744 gene as a directly effective CDX2 binding site in the miR-744 promoter region. In addition, our data reveal for the first time the role of CDX2 in promoting migration and invasion in NSCLC.

In summary, the data presented in this paper provide insights into the mechanism of CDX2 dysregulation for the first time, and establish a direct link between CDX2 signals and miRNAs during dysregulation. Advances in the mechanism of upregulation of CDX2 expression in cancer have laid a theoretical foundation for determining when and how targeted anti-CDX2 and miR-744 therapy are feasible. The current mechanisms provide new insights into the carcinogenic functions of CDX2 and a better understanding of the upregulation of

CDX2 in cancer. CDX2-induced miR-744 upregulation mediates the role of CDX2 signaling in NSCLC. Further studies on the role and mechanism of CDX2 in NSCLC will be summarized in our next study.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors

Availability of data and materials

Available

Competing interests

None

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Authors' contributions

(I) Conception and design: Zhou Sha, Song Hao, Xiaofeng Pei; (II) Administrative support: Zhou sha ; (III) Provision of study materials or patients: Xiaofeng Pei, Jianzhong Xian (IV) Collection and assembly of data: Song Hao, Yajun Wei, Weize Lv; (V) Data analysis and interpretation: Zhou Sha, Song Hao; (VI) Manuscript writing: Zhou Sha; (VII)

Final approval of manuscript: All authors.

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Available

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Supplementary Table

Supplementary Table S1 was not provided with this version of the manuscript.

Figures

Figure 1

CDX2 activated miR-744 transcription. The expression of CDX2-mediated miR744 was quantified by qRT-PCR analysis in A549 and SPC-A-1 cells. GAPDH or U6 was used as internal control. *P < 0.05; **P < 0.01; ***P < 0.001 compared to controls.

Figure 1

CDX2 can be recruited to binding to promoter region of miR-744. (A) In A549 cells transfected with a vector expressing CDX2, chromatin immunoprecipitation experiments were performed to identify the CDX2 binding sites on the miR-744 promoter. An isotype-matched IgG matched with the same type was used as negative control. (B) Two binding sites in the miR-744 promoter were analyzed by qRT-PCR using specific primers.

A

B

C

Figure 1

MiR-744 mediates CDX2 to promote lung cancer cell migration and invasion (A)qRT - PCR was used to analysis miR - 744 inhibitor or its vector control and CDX2 plasmid transfection of SPC- A-1 and A549 cells .(B) CDX2 promoted the migration and invasion ofSPC-A-1 and A549 cells. miR - 744 inhibitor almost completely diminished CDX2 of the positive role.Transwell, Boyden, and (C) wound healing methods were used to

Figure 1

The bioinformatics analysis by PROMO 2.0 for possible transcription factor binding sites (TFBS) in miR-744 3'UTR promoter region.

Figure 1

CDX2 response element existed in key promoter region of miR-744 Luciferase activity of miR-744 promoter reporter construct and empty PGL3-based vector were measured in SPC-A-1 and A549 cells in the presence or control of CDX2 plasmid. *P < 0.05; **P < 0.01; ***P < 0.001 compared to controls. N.S. non-significant.