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OTX1 promotes colorectal cancer progression through epithelial-mesenchymal transition

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ABSTRACT

Orthodenticle homeobox 1 (OTX1), a transcription factor containing a bicoid-like homeodomain, plays a role in brain and sensory organ development. In this study, we report that OTX1 is overexpressed in human colorectal cancer (CRC) and OTX1 overexpression is associated with higher stage. Functional analyses reveal that overexpression of OTX1 results in accumulation of CRC cell proliferation and invasion *in vitro* and tumor growth *in vivo*, whereas ablation of OTX1 expression significantly inhibits the proliferative and invasive capability of CRC cells *in vitro*. Together, our results indicate that OTX1 is involved in human colon carcinogenesis and may serve as a potential therapeutic target for human colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy in the world and the fifth leading cause of death among cancer patients in China [1]. The critical step in CRC progression is the ability of cancer cells to disseminate into and colonize adjacent tissues, predicting a poor prognosis for patients [2]. CRC development and progression involves deregulated oncogenes and tumor suppressor genes expression. Although a growing number of genes have been reported, the search for new genes that could be related to tumorigenesis, development, diagnosis or treatment of CRC continues.

Orthodenticle homeobox 1 (OTX1) is a transcription factor containing a bicoid-like homeodomain and represents the vertebrate homologoues of the *Drosophila orthodenticle* gene. OTX1 plays a role in brain and sensory organ development [3]. OTX1 expression starts when the anterior neuroectoderm develops into prosencephalon and mesencephalon. At later embryonic stages, OTX1 is expressed in the cortical ventricular zone and the emerging cortical plate of the neocortex. It is also found in the choroid plexus of the lateral ventricles, the ventricular zone of the ganglionic eminence, the olfactory bulb, the hippocampus, and the cerebellum [4,5]. In mice, *OTX1* gene is required for specification, maintenance, and patterning of forebrain and midbrain as well as for neuronal differentiation [6–8]. Furthermore, OTX1 is also required in the visual and acoustic sense organ development, and transient control of pituitary levels of GH, FSH, and LH hormones [8]. Recently, it has been reported that OTX1 is overexpression in medulloblastomas of the nodular/desmoplastic subtype and breast cancer, suggesting that it may represent a medulloblastoma oncogene [9,10]. However, OTX1 expression was not detected in other brain tumors including astrocytomas, glioblastomas, oligodendrogliomas, meningiomas, and ependymomas [9]. To our knowledge, studies focus on the expression and function of OTX1 in human CRC are limited.

Here, we investigated the role of OTX1 in CRC development and progression. Our results demonstrate that OTX1 is overexpression in CRC tissues. Depletion of OTX1 expression suppresses CRC proliferation and invasion *in vitro*, whereas overexpression of OTX2 promotes CRC proliferation and invasion *in vitro* and tumor growth in *vivo*. Furthermore, OTX1 is linked to the epithelial-mesenchymal transition (EMT)-like phenotype. These findings suggest that OTX1 is a potential oncogene in CRC development and progression, and a viable target for CRC therapy.

2. Materials and methods

2.1. Cell culture

Human CRC-derived cell lines SW480, HT29, HCT116, and DLD1 were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA). All media were supplemented with 10% fetal bovine serum (Invitrogen), and the cell lines were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

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2.2. Clinical samples

30 paired CRC and non-cancerous colonic mucosa samples were analyzed using Real-time quantitative reverse transcription PCR (RT-qPCR). This study was approved by the institutional review board of the Third Affiliated Hospital of Kunming Medical University, and written informed consent was obtained from all the patients.

2.3. Plasmid, siRNA, and transfection

The cDNA fragment encoding human full length OTX1 was isolated by PCR using the Pfu DNA Polymerase (Thermo Scientific, Rockford, IL, USA). The PCR product was cloned into the pcDNA3.1 (Invitrogen). The construct was confirmed by sequencing. siRNAs targeting OTX1 were purchased from RiboBio (Shanghai, China). For transient transfection, 2×10^5 cells were plated into 6-well plates and kept in antibiotic-free medium for 24 h before transfection. The cells were then transfected with the siRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For generation of stable OTX1-overexpressed cells, cells were transfected with pcDNA3.1-OTX1. 2 days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St. Louis, MO, USA) for about 2 weeks.

2.4. Western blot

Cells and specimens were lysed in lysis buffer with 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). 30 μg of proteins was loaded onto 10% SDS–PAGE gel and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with anti-OTX1 (1:1000 dilutions, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:3000 dilutions, Santa Cruz Biotechnology) at 4 °C overnight. Next day, the membranes were incubated with horseradish peroxidase conjugated-secondary antibody, and the bands were visualized using ECL detection reagents (Millipore). Actin served as the loading control.

2.5. RT-qPCR

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions. 4 μg of total RNA was converted to first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions.

2.6. Proliferation assay

Both MTT and plate colony formation assays were used to evaluate the ability of cell proliferation. For MTT assay, 24 h after transfection, 5×10^3 cells were seeded in 96-well plates per well. Then 24, 48, 72 h after transfection, the cells were incubated with 10 μl MTT (0.5 mg/ml; Sigma–Aldrich) at 37 °C for 4 h. The medium was then removed, and precipitated Formosan was dissolved in 100 μl DMSO. The absorbance at 490 nm was detected using a micro-plate auto-reader (Bio-Rad, Richmond, CA, USA). To perform the plate colony formation assay, 24 h after transfection, 500–1000 cells were seeded in 6-well plates per well. After about 2 weeks, the colonies obtained were washed with PBS and fixed with 10% formalin for 10 min at room temperature and then washed with PBS followed by staining with hematoxylin. The colonies were counted and compared with control cells.

2.7. Motility assay

Cell motility was evaluated by transwell migration test. Cells in 0.2 ml RPMI 1640 without FBS were placed on the top chamber of each insert (BD, Biosciences, San Jose, CA, USA). The lower chamber was filled with 600 μl of RPMI 1640 medium with 10% FBS to act as the nutritional attractant. 24 h later, the migrant cells that had attached to the lower surface were fixed with 20% methanol and stained for 20 min with crystal violet. The membranes were then carved and embedded under cover slips with the cells on the top. Cells in three different fields of view were counted and expressed as the average number of cells per field of view.

2.8. Xenograft assay

1×10^7 cells were injected into the left or right flanks of nude mice ($n = 6$; Institute of laboratory animal sciences, CAMS, Beijing, China). The tumor volume was calculated as length $\times$ width$^2 \times 0.5$. The tumor formation assessed every 3–4 days. At the end of experiment, the mice were sacrificed and the final volumes of tumor tissues were determined.

2.9. Statistical analysis

All statistical analyses were made using Statistical Package for Social Science (SPSS; version 18.0) for Windows (SPSS Inc., Chicago, IL, USA). Data are reported as mean ± SD, and mean values were compared using the Student's t test. Results were considered statistically significant when $P < 0.05$ was obtained.

3. Results

3.1. OTX1 is significantly up-regulated in CRC

To gain insights into the role of OTX1 in colorectal tumorigenesis, we analyzed the mRNA expression levels of OTX1 in CRCs and matched normal mucosa ($n = 30$) by RT-qPCR: OTX1 mRNA was significantly up-regulated in 70% (21/30) of the cases (Fig. 1A). Next, we performed Western blot analysis to assess the OTX1 protein expression in CRCs and matched normal mucosa. The results showed that the OTX1 protein expression is also up-regulated in CRCs tissues than the normal mucosa ($n = 8$; Fig. 1B). Furthermore, the OTX1 mRNA expression levels were higher in advanced tumor stage III and IV than tumor stage I/II, as illustrated in the box plot (Fig. 1C). Taken together, our results provide evidence that OTX1 is associated with CRC progression acting as a potential oncogene.

3.2. Depletion of OTX1 suppresses CRC cell proliferation and migration in vitro

To investigate the role of OTX1 in CRC progression, we examined its expression in 4 different CRC cell lines by RT-qPCR and Western blot. OTX1 mean levels in normal colonic mucosa were considered as calibrator; by applying this criterion, OTX1 expression in SW480 and HT29 cells was comparable to that observed in the normal mucosa; DLD1 and HCT116 cells, instead, displayed higher levels (Fig. 2A). To examine the function of OTX1 in CRC cell biology, we used siRNAs targeting OTX1 to inhibit the OTX1 expression in HCT116 cells. The effective knockdown of OTX1 was confirmed by RT-qPCR and Western blot assays. Compared to the control cells, OTX1 siRNAs-transfected cells (HCT116-siOTX1-1 and HCT116-siOTX1-2) showed significantly reduced OTX1 expression (Fig. 2B). Next, we determined the effect of reduced OTX1 expression on cell proliferation using MTT and colony

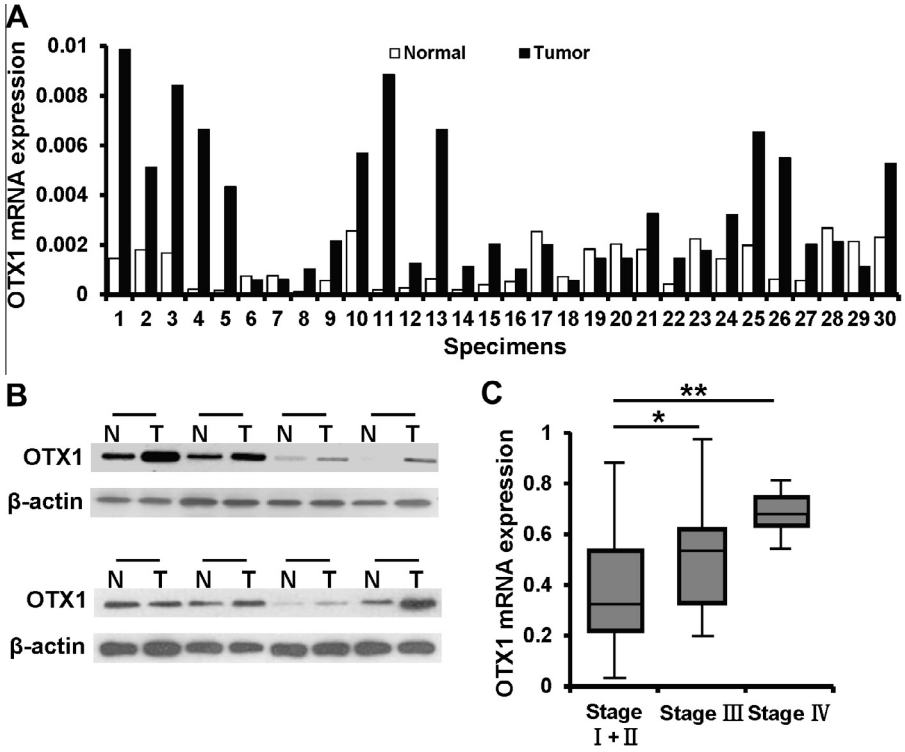

Fig. 1. OTX1 expression in CRC. (A) The OTX1 mRNA expression levels were determined using RT-qPCR for 30 paired CRC samples. (B) The OTX1 protein expression levels were determined using Western blot for 8 paired CRC samples. (C) The box plot depicts OTX1 mRNA levels assess by RT-qPCR in 30 CRC samples classified according tumor stage (Stage I + II, $n = 17$; Stage III, $n = 9$; Stage IV, $n = 4$) $^{**}P < 0.01$, $^{*}P < 0.05$.

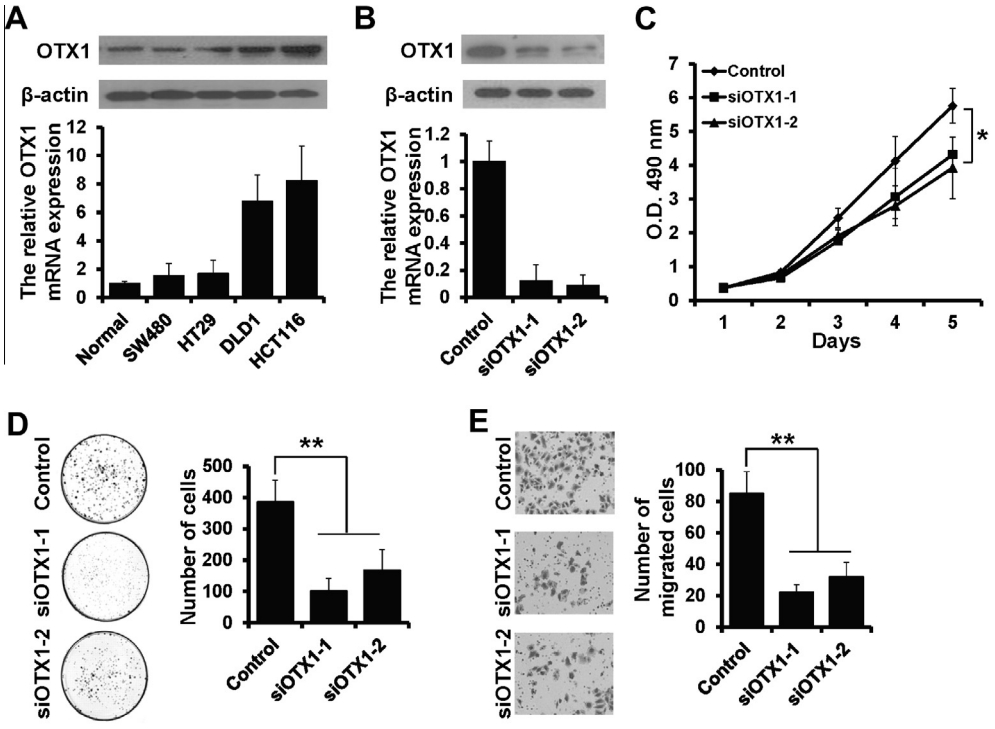

Fig. 2. Depletion of OTX1 expression suppresses CRC cell proliferation and migration. (A) OTX1 expression in the indicated cell lines by RT-qPCR and Western blot. (B) OTX1 expression in OTX1-depleted and control HCT116 cells detected by RT-qPCR and Western blot. (C and D) Cell proliferation ability of OTX1-depleted and control HCT116 cells by MTT (C) and colony formation (D) assays, respectively. (E) Cell migration ability of OTX1-depleted and control HCT116 cells by transwell assay $^{**}P < 0.01$, $^{*}P < 0.05$.

formation assays. The results showed that the OTX1-depleted HCT116 cells had significantly decreased proliferation ability than the control cells (Fig. 2C and D). To test migration, we used transwell assay. We found that both HCT116-siOTX1-1 and HCT116-siOTX-2 cells had reduced migration ability compared to the control cells (Fig. 2E). Taken together, these results demonstrated that

depletion of OTX1 expression suppresses HCT116 cell proliferation and migration *in vitro*.

3.3. Overexpression of OTX1 promotes CRC cell proliferation and migration in vitro and tumor growth in vivo

Next, we established a stable OTX1-overexpressing HT29 cell line (HT29-OTX1) to confirm the effect of OTX1 expression on CRC cell biology. Both OTX1 mRNA and protein expression were significantly increased in HT29-OTX1 compared to control cell line by RT-qPCR and Western blot assays (Fig. 3A). The MTT and colony formation assays showed that the HT29-OTX1 cells had significantly increased proliferation ability than the control cells (Fig. 2B and C). The transwell assay indicated that HT29-OTX1 had increased migration ability compared to the control cells (Fig. 2D). Together, these results demonstrated that overexpression of OTX1 expression promotes HT29 cell proliferation and migration *in vitro*. Furthermore, the tumor volume was markedly larger in HT-29-OTX1 cells than in control cells (Fig. 3E). Together, these results indicated that overexpression of OTX1 promotes HT29 cell proliferation and migration *in vitro* and tumor growth *in vivo*.

3.4. Overexpression of OTX1 is linked to the EMT-like phenotype

EMT implicates loss of epithelial markers, concomitant acquisition of mesenchymal ones. Our results indicated that forced expression of OTX1 in HT29 cells led to a reduction of E-cadherin expression and an increased expression of N-cadherin and Vimentin by Western blot assay (Fig. 4A). Furthermore, the EMT-related transcription factors, Twist1, Snail, Slug, and ZEB1 were significantly up-regulated in OTX1-overexpressed HT29 cells compared to control cells by RT-qPCR (Fig. 4B) and Western blot assays (Fig. 4C). Collectively, these results indicated that OTX1 promotes CRC progression through induction of EMT.

4. Discussion

The OTX1 gene locus is located on human chromosome 2 at position p13-15 [11], which is frequently rearranged in mature B-cell

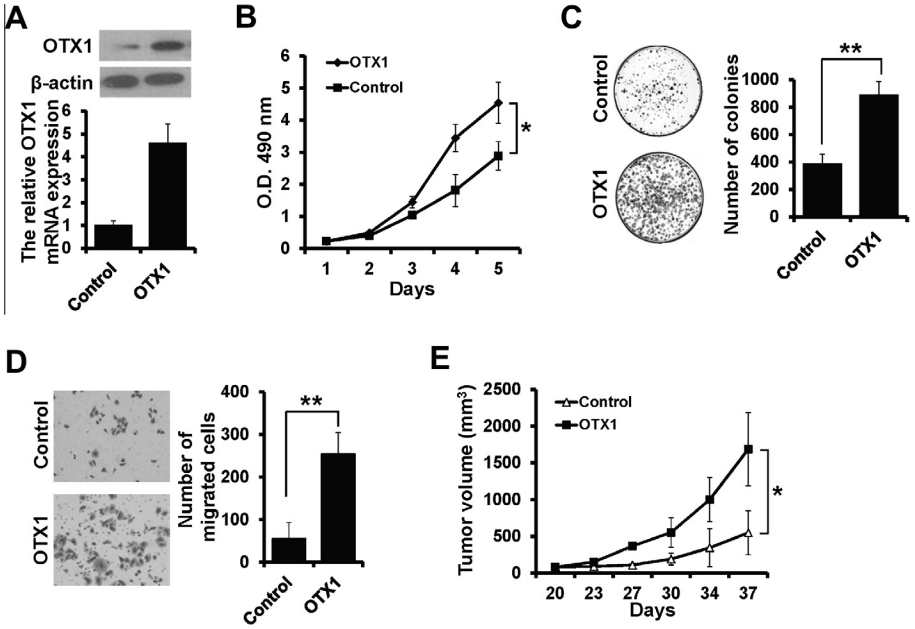

Fig. 3. Overexpression of OTX1 promotes CRC cell proliferation and migration. (A) OTX1 expression in stable OTX1-overexpressed and control HT29 cells detected by RT-qPCR and Western blot. (B and C) Cell proliferation ability of OTX1-overexpressed and control HT29 cells by MTT (B) and colony formation (C) assays, respectively. (D) Cell migration ability of OTX1-overexpressed and control HT29 cells by transwell assay. (E) 1×10^7 cells as in (A) were inoculated into the left or right flanks of nude mice and tumor growth was recorded with a caliper-like instrument. Tumor volumes were calculated according to the formula volume = length × width^2 × 0.5 $^{**}P < 0.01$, $^{*}P < 0.05$.

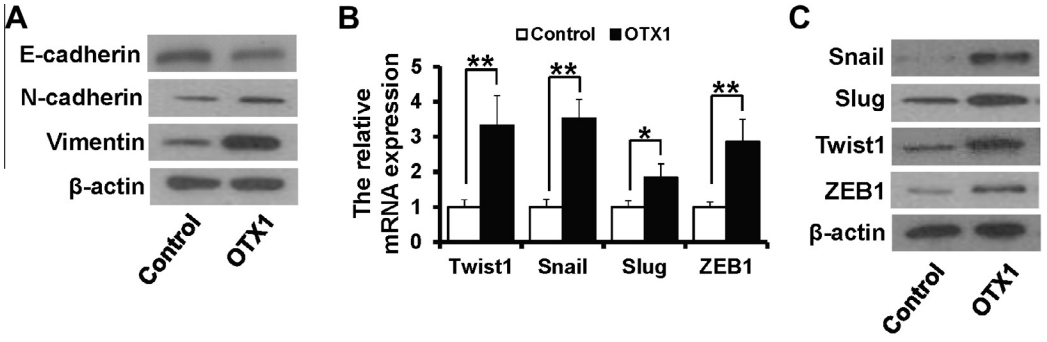

Fig. 4. OTX1 expression is linked to the EMT-like phenotype. (A) Western analysis for the indicated EMT markers in OTX1-overexpressed and control HT29 cells. (B) RT-qPCR analysis of some EMT-related transcription factors in the OTX1-overexpressed and control HT29 cells. (C) Western analysis of some EMT-related transcription factors in the OTX1-overexpressed and control HT29 cells $^{**}P < 0.01$, $^{*}P < 0.05$.

lymphomas. It has been described as a critical molecule for axon refinement in developing cerebral cortex, and the developmental role of the OTX family genes have been well established in murine brain [4,12]. Here, we provide the first evidence that OTX1 plays an important role in colon carcinogenesis. It is, in fact, frequently up-regulated in CRC, is expressed at higher in advanced tumor stages (III and IV). In agreement with our work, the overexpression of OTX1 is also observed in diffuse large B-cell lymphoma (DLBCL) and breast cancer [4,8].

We determined the potential role of OTX1 in tumor formation and progression. Our results reveal that depletion of OTX1 expression by RNA RNAi in human CRC cells inhibits cell proliferation and invasion *in vitro*. Furthermore, we show that overexpression of OTX1 promotes human CRC cells proliferation and invasion *in vitro* and tumor formation *in vivo*. These results indicated that OTX1 as a potential oncogene in colon carcinogenesis.

EMT occurs frequently during normal development in processes such as mesoderm and neural crest cell formation. During tumor progression, EMT is also crucial for loss of cell polarity of epithelial cells, thus facilitating migratory and invasive behavior [13,14]. Our results indicated that overexpression of OTX1 induces EMT programs of human CRC cells, including up-regulation of mesenchymal markers (N-cadherin and Vimentin) and EMT related transcription factors (Twist1, Snail, Slug, and ZEB1), down-regulation of epithelial marker (E-cadherin), and a decrease in cell migration and invasion *in vitro*. These findings and the analysis of CRC cell lines demonstrated that OTX1 is not only linked to cell proliferation but promotes EMT by activating essential effectors of the process such as Twist1, Snail, Slug, and ZEB1. The future study should clarify its transcriptional targeted genes in carcinogenesis.

In conclusion, OTX1 is markedly overexpressed in CRC and enhances tumorigenicity and tumor growth both *in vitro* and *in vivo*. We have elucidated a biological function of OTX1, which induces EMT in CRC. Our finding support OTX1 as an oncogene in CRC tumorigenesis and progression.

Conflict of interest

The authors declare no conflict of interest.

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