

HOXC11 positively regulates the long non-coding RNA HOTAIR and is associated with poor prognosis in colon adenocarcinoma

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Received May 15, 2020; Accepted February 22, 2021

DOI: 10.3892/etm.2021.10745

Abstract. Colorectal cancer ranks third in terms of incidence and second in terms of mortality worldwide. The homeobox transcript antisense intergenic RNA (HOTAIR), which was found to be located on the antisense chain of the homeobox C (HOXC) gene cluster, is a long non-coding RNA involved in multiple types of tumors. The role of HOXC11 in tumors remains unclear. Reverse transcription-quantitative PCR was performed to detect the expression level of HOXC11 in colon adenocarcinoma. Cell proliferation and invasion were assessed. RNase protection assay was used to test the possibility of RNA duplex formation. The increased expression and co-expression trend of HOXC11 and HOTAIR were identified in multiple types of cancer from The Cancer Genome Atlas and the results were validated in 12 colon adenocarcinoma and paired non-tumor tissue samples. The expression of HOXC11 and HOTAIR was found to be associated with poor prognosis in colon adenocarcinoma and kidney renal clear cell carcinoma. Furthermore, HOXC11 was found to positively regulate HOTAIR by RNA duplex formation and promoted the proliferation and invasion of colon adenocarcinoma cells.

Introduction

In total, >1.8 million new colorectal cancer cases and 881,000 deaths are estimated to have occurred in 2018, accounting for ~1 in 10 cancer cases and deaths worldwide (1). Colorectal cancer ranks third in incidence and second in mortality globally, and poses a serious threat to human health, particularly in certain European countries (e.g., Hungary, Slovenia, Slovakia, The Netherlands and Norway), Australia/New Zealand, Northern America and Eastern Asia (including Japan, the Republic of Korea and Singapore) (1). The currently approved therapeutic approaches to colon adenocarcinoma include surgery, radiofrequency ablation, chemotherapy, radiation therapy, cryosurgery and targeted therapy (2).

Long non-coding RNAs (lncRNAs), a class of RNAs >200 nt long and lacking protein-coding ability, have emerged to play key roles in numerous biological processes, such as differentiation, development, cellular address codes and oncogenesis (3-5). The homeobox transcript antisense intergenic RNA (HOTAIR) is a well-known long non-coding RNA that functions as an oncogene (6,7). Abnormal expression of HOTAIR has been reported in multiple cancers, and may lead to abnormal cell differentiation and proliferation, invasion and metastasis (8-11).

Located in chromosome 12 (q13.13), homeobox C11 (HOXC11) belongs to the homeobox family of genes, which encode a highly conserved family of transcription factors that play key roles in embryonic implantation, evolution and morphogenesis in all multicellular organisms (12-14). Disorders of HOXC11 may lead to abnormal embryonal development, endometriosis and infertility (14,15). HOTAIR was reported to regulate the expression of homeobox genes and is located in the antisense chain of HOXC11, where an overlapping region between HOTAIR and HOXC11 has been identified (16). Thus, the present study was undertaken to explore the interaction between HOTAIR and HOXC11. Data from The Cancer Genome Atlas (TCGA) database were analyzed, and a co-expression trend was found between the lncRNA HOTAIR and the transcription factor HOXC11 in several types of cancer, such as colon adenocarcinoma, esophageal carcinoma, breast cancer and kidney renal clear cell carcinoma, among others. Therefore, assays with colon adenocarcinoma cells and tissues were performed to identify the interaction between HOTAIR and HOXC11 and their functions in colon adenocarcinoma.

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Key words: colon adenocarcinoma, antisense chain of the homeobox C 11, homeobox transcript antisense intergenic RNA, survival

Materials and methods

Cell cultures. The SW480 and HCT116 colon cancer cell lines were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). SW480 cells were cultured in Leibovitz's L-15 Medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. HCT116 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂.

Specimens. A total of 12 paired colon carcinoma and adjacent normal tissue samples were obtained from the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai, China) between 18th February and 6th December 2018. All patients were diagnosed with primary colon carcinoma. Ethics approval (approval no. K206-1) was obtained from the Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. Informed consent for their tissues to be used for research purposes was obtained from all patients prior to performing this study.

Bioinformatics analysis. The gene expression data of colon carcinoma, esophageal carcinoma, breast cancer and kidney renal clear cell carcinoma were obtained from TCGA website (<https://cancergenome.nih.gov/>). Kaplan-Meier analysis was plotted by UALCAN (17). Overlapping region was found between HOXC11 and HOTAIR by UCSC genome browser (assembly ID, hg38) (18).

Transfection. HOXC11 knockdown lentivirus [pSLenti-U6-shRNA(HOXC11)-CMV-EGFP-F2A-Puro-WPRE] and negative control (NC) lentivirus (pSLenti-U6-CMV-EGFP-F2A-Puro-WPRE) were obtained from Shanghai OBIO Technology (Shanghai) Corp., Ltd. SW480 and HCT116 cells were transfected with lentiviruses at a multiplicity of infection (MOI) of 10 in the presence of 4 µg/ml puromycin. After 1 week of puromycin screening, SW480 and HCT116 cells were used for subsequent experimentation. Transfection of the HOXC11 plasmid [pcDNA3.1(+)] was performed according to the protocol of Lipofectamine[®] 3000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In total, 1 µg HOXC11 or negative control plasmid, 2 µl P3000[™] Reagent, and 1.5 µl Lipofectamine[™] 3000 Reagent were added to 50 µl Opti-MEM medium per 1 × 10⁵ cells. The medium was changed after 6 h and the transfection effect was detected after 2 days.

RNase protection assay. The composition of the RNase digestion mixture for RNase protection assay (RPA) was as follows: 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA (pH 7.5), and 20 µl of RNase A/T1 Mix per 1 ml of reaction mixture. RNase A/T1 can digest single-stranded RNAs, but not RNA duplexes. The RNA samples were incubated at 37°C for 60 min prior to treatment with an RNase A+T cocktail (Sigma-Aldrich; Merck KGaA). Subsequently, the samples were incubated at 37°C for 30 min after the addition of the RNase cocktail, and treated with proteinase K, as previously described (19). The RNA used in the RPA was extracted from SW480 and HCT116 cells.

RT-qPCR analysis. Total RNA from SW480 and HCT116 cells was isolated with TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Fast All-in-One RT Kit (cat. no. ES-RT001; Shanghai Yishan Biotechnology, Co., Ltd.; <https://www.esunbio.com/search-1--/u0072/u0074/u0030/u0030/u0031.html>) was used to perform the reverse transcription using the temperature protocol of 42°C for 15 min. GoTaq[®] qPCR Master Mix A6001 (cat. no. A6001; Promega Corporation) was used to perform qPCR. The thermocycling conditions included an initial denaturation step (95°C, 2 min), followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primer sequences used were as follows: HOXC11 forward, 5'-GCTACTCCTCCTGCTATGC-3' and reverse, 5'-GACGCTGTTCTTGTTGACTG-3'; HOTAIR forward, 5'-GCCAAGCACCTCTATCTC-3' and reverse, 5'-GACACTG AACGGA CTCTG-3' and GAPDH forward, 5'-AGAAGGCT GGGGCTCATTG-3' and reverse, 5'-AGGGGCCATCCACA GTCTTC-3'. The relative expression level of mRNAs was normalized to GAPDH and was calculated using the 2^{-ΔΔC_q} method (20).

MTT assay. The cells were inoculated into 96-well plates at a concentration of 2,000 cells per 100 µl at 37°C with 5% CO₂ overnight. After incubation for 4 h at 37°C with 5% CO₂, 50 µl 1X MTT reagent (Nanjing KeyGen Biotech Co., Ltd.) and 150 µl DMSO were added to well. The mixture was shaken well and the absorbance was detected at 490 nm using the SpectraMax[®] 340PC38 spectrophotometer (Molecular Devices, LLC).

Statistical analysis. Statistical analyses were performed by SPSS 20.0 (IBM Corp.). Graphs were generated by GraphPad Prism 7 (GraphPad Software, Inc.). P<0.05 from a two-tailed test was considered to indicate statistically significant difference. Unpaired student's t-test was used to evaluate the differences between two groups of independent samples. Data are presented as mean ± SD. All the results were repeated ≥three times.

Results

HOXC11 and HOTAIR expression is increased in multiple types of cancer. After analyzing the data of colon adenocarcinoma, esophageal carcinoma, breast cancer and kidney renal clear cell carcinoma, which were downloaded from TCGA, it was observed that both HOXC11 and HOTAIR were significantly upregulated in the tumor groups compared with the normal groups (Fig. S1). Similar expression trends were observed between HOXC11 and HOTAIR. In addition, RT-qPCR assay was performed to detect the expression of HOXC11 and HOTAIR in 12 paired colon adenocarcinoma and adjacent normal tissue samples. It was determined that HOTAIR and HOXC11 were upregulated in the majority of colon adenocarcinoma tissues, which was consistent with the results of TCGA database (Fig. 1A and B).

Co-expression of HOXC11 and HOTAIR. A co-expression trend was observed between HOXC11 and HOTAIR in colon adenocarcinoma (r=0.797), esophageal carcinoma (r=0.838), breast cancer (r=0.922) and kidney renal clear cell carcinoma (r=0.719), with statistically significant differences (supplementary Fig. S2A-D). Similar results were obtained from

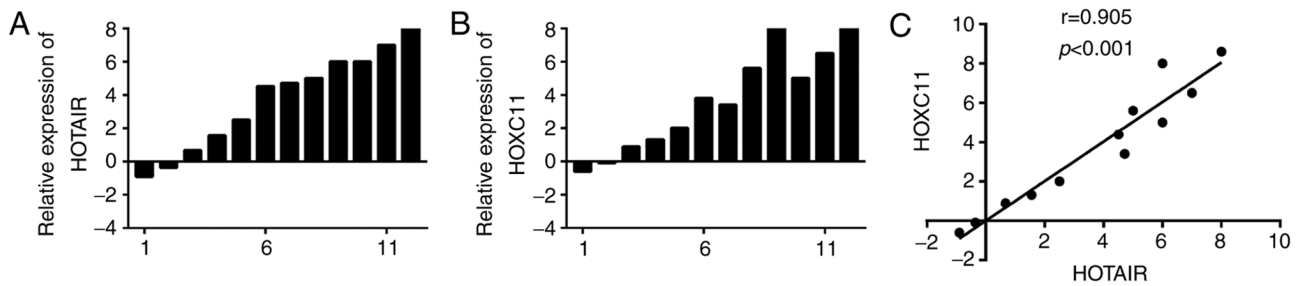


Figure 1. Increased expression of HOTAIR and HOXC11 in colon adenocarcinoma tissues. (A) Increased expression of (A) HOTAIR and (B) HOXC11 was observed in 10 of 12 colon adenocarcinoma tissues compared with the paired non-tumor tissues. (C) Co-expression of HOTAIR and HOXC11. HOTAIR, homeobox transcript antisense intergenic RNA; HOXC11, homeobox C11.

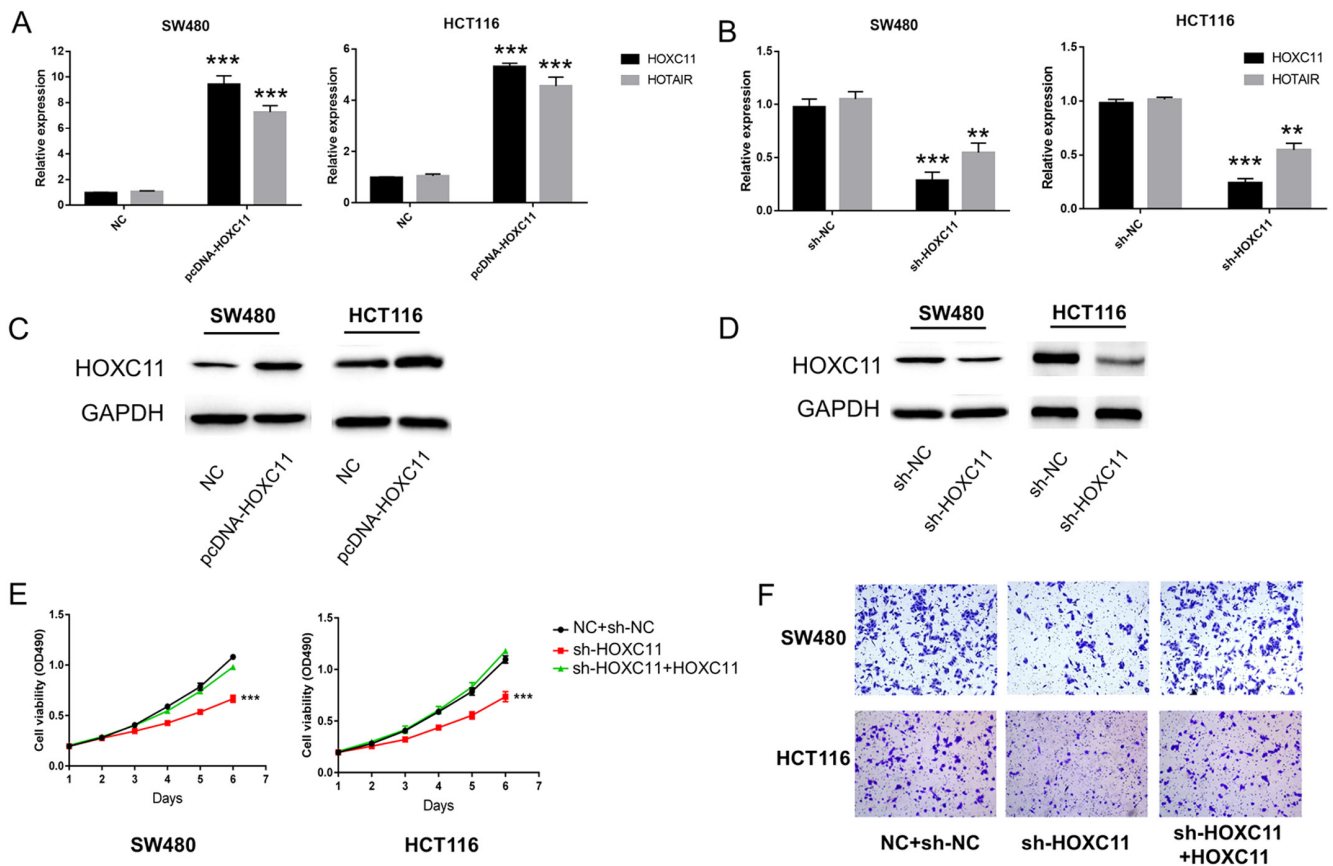


Figure 2. Reduced expression of HOXC11 inhibits the proliferation and invasion ability of colon adenocarcinoma SW480 and HCT116 cells. (A) HOXC11 overexpression was shown to upregulate the expression of HOTAIR. (B) Knocking down HOXC11 inhibited the expression of HOTAIR. (C and D) Protein levels of HOXC11 after overexpression and knockdown. (E) Cell proliferation was assessed following HOXC11 knockdown and overexpression in SW480 and HCT116 cells. (F) Invasion ability was inhibited in cells with HOXC11 knockdown and was rescued after overexpressing HOXC11 (magnification, x100). *** $P<0.001$, ** $P<0.01$. HOTAIR, homeobox transcript antisense intergenic RNA; HOXC11, homeobox C11; NC, group transfected with negative control plasmid; sh-NC, group infected with negative control lentivirus.

colon adenocarcinoma and adjacent normal tissues ($r=0.905$, $P<0.001$; Fig. 1C).

HOXC11 and HOTAIR expression is associated with poor prognosis. Higher HOXC11 expression was associated with poorer prognosis in colon adenocarcinoma ($P=0.02$) and kidney renal clear cell carcinoma ($P=0.006$), but not in esophageal carcinoma ($P=0.26$) and breast cancer ($P=0.28$; supplementary Fig. S3A-D). Higher expression of HOTAIR was associated with poorer prognosis in colon adenocarcinoma ($P=0.021$), breast cancer ($P=0.047$) and kidney renal clear cell carcinoma

($P<0.001$), but there was no statistically significant difference in the prognosis of esophageal carcinoma by HOTAIR expression ($P=0.11$; supplementary Fig. S3E-H).

Decreased expression of HOXC11 inhibits the proliferation and invasion of colon adenocarcinoma cells. After knocking down and overexpressing HOXC11 in SW480 cells and HCT116 cells, it was found that the expression level of HOTAIR was modified with the changes in the expression of HOXC11 (Fig. 2A-D). Furthermore, decreased expression of HOXC11 inhibited the ability of proliferation and invasion

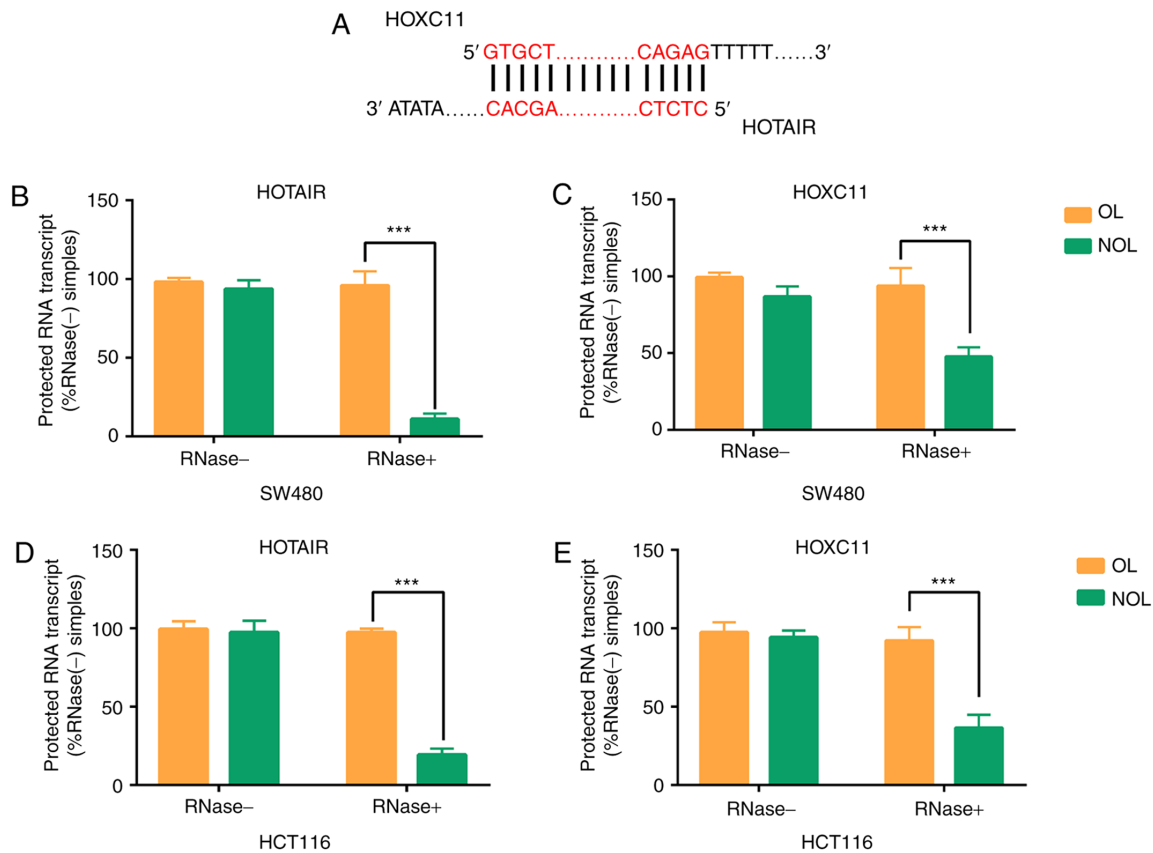


Figure 3. Overlapping region of HOXC11 and HOTAIR transcript is protected from degradation by RNase A+T, suggesting the formation of an RNA duplex. (A) Overlapping region between HOXC11 and HOTAIR. (B and C) Results of the RPA showed that non-overlapping regions of HOTAIR and HOXC11 were degraded by RNase A+T in SW480 cells. (D and E) Results of the RPA showed that non-overlapping regions of HOTAIR and HOXC11 were degraded by RNase A+T in HCT116 cells. ***P<0.001. OL, overlapping. NOL, non-overlapping; HOTAIR, homeobox transcript antisense intergenic RNA; HOXC11, homeobox C11; RPA, RNase protection assay.

of colon adenocarcinoma cells, whereas these effects were rescued by HOXC11 overexpression (Fig. 2E and F).

HOXC11 formed RNA duplex and increased HOTAIR expression. To explore the interaction between HOXC11 and HOTAIR, HOXC11 was overexpressed and knocked down in SW480 and HCT116 cells. It was found that HOTAIR expression changed significantly with the changes in HOXC11 expression. According to UCSC genome browser, an overlapping region was found between HOXC11 and HOTAIR (Fig. 3A). The results of the RPA showed that non-overlapping regions were degraded after adding RNase A+T, which can digest single-stranded RNAs but not RNA duplexes (Fig. 3B-E). Accordingly, RNA duplex formation between HOXC11 and HOTAIR was observed.

Discussion

In the present study, it was observed that HOXC11 expression is increased in colon adenocarcinoma and may positively regulate HOTAIR by forming an RNA duplex. The expression of HOXC11 and HOTAIR has been associated with poor prognosis in multiple types of cancer (9,21,22).

HOXC11 belongs to the homeobox gene family, which encodes a highly conserved family of transcription factors that play key roles in embryo implantation, evolution and

morphogenesis in all multicellular organisms (23-26). In recent years, there has been an increasing number of studies indicating that HOX family genes are involved in the occurrence and progression of several malignancies, such as gastric cancer, Wilms' tumor and acute myeloid leukemia (27,28). HOXC11 is reported to be involved in gene fusion, which could promote abnormal gene expression and drive leukemogenesis (29,30). HOXC11 was considered to play an important role in early intestinal development during embryonic growth (31). Consistent with the present study, it was found that the expression of HOXC11 was significantly increased in multiple cancers, such as colon adenocarcinoma, esophageal carcinoma, breast cancer and kidney renal clear cell carcinoma. Furthermore, increased HOXC11 was associated with poor outcome in colon adenocarcinoma and kidney renal clear cell carcinoma, indicating that HOXC11 may participate in the development of these cancers.

In recent years, lncRNAs have been reported to play important roles in numerous biological processes, such as development, differentiation, immunity, Alzheimer's disease, cardiovascular disease and tumors (32,33). HOTAIR has been extensively investigated and was reported to participate in multiple tumors. Serum HOTAIR was considered to be a diagnostic biomarker for esophageal squamous cell carcinoma (34). HOTAIR may also promote the occurrence and development of renal cell carcinoma (7). Özeş *et al* (35) observed that HOTAIR

was implicated in the DNA damage response, cellular senescence and chemoresistance. Xue *et al* (36) reported that HOTAIR enhances estrogen receptor signaling and confers tamoxifen resistance in breast cancer. After analyzing the data of multiple cancers from TCGA, the authors of the present study found that HOTAIR was upregulated in colon adenocarcinoma, esophageal carcinoma, breast cancer and kidney renal clear cell carcinoma, and was associated with poor prognostic in most types of cancer. Although HOTAIR was reported to regulate HOX gene expression, there has been no reported research, to the authors' knowledge, on HOX genes regulating HOTAIR expression (37). The present study was the first to identify that HOXC11 could positively regulate HOTAIR by forming an RNA duplex within the overlapping region.

In summary, it was herein found and validated that the transcription factor HOXC11 and the lncRNA HOTAIR were upregulated in colon carcinoma. In addition, the decreased expression of HOXC11 may inhibit the proliferation and invasion ability of colon adenocarcinoma cells. Furthermore, HOXC11 may positively regulate HOTAIR by forming the RNA duplex. The findings of the present study may expand our current understanding of the functions of HOX genes and HOTAIR. Moreover, the expression of HOXC11 and HOTAIR may be considered as a potential biomarker and therapeutic target against colon carcinoma. However, as the main aim of the present study was to study the association between HOXC11 and HOTAIR, experiments *in vivo* were not conducted. Therefore, the effects of HOXC11 on survival should be validated in *in vivo* experiments in the future.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of materials and data

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

Authors' contributions

NG participated in manuscript preparation and performed the experiments. FL and YNW performed literature search and the collection of gene expression and survival data from TCGA and UALCAN. WQW, ZZL and TTG performed data analysis. HTC and XJM participated in research design and performed manuscript review. NG and HTC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval (approval no. K206-1) was obtained from the Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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