Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide. Colorectal carcinogenesis is a multistep process involving progressive disruption of epithelial-cell proliferation, apoptosis, differentiation, and survival mechanisms (1–2). About half of the individuals with locally advanced CRC can be cured by surgery and multimodal treatment. Because traditional methods do not allow precise prediction of prognosis for the patients after surgical removal of the primary tumor, there is an acute need for biomarkers capable of distinguishing patients with poor or good prognosis (3).

The homeobox genes encode a family of transcriptional factors, which are essential for morphogenesis and differentiation (4, 5). Homeobox genes are categorized into 2 large groups. Homeobox genes of class I, also called HOX genes, contain at least 39 members that are organized in 4 clusters (A, B, C, and D) located on chromosomes 7, 17, 2, and 12, respectively (6). Homeobox genes of class II, known as divergent homeobox genes (non-HOX), are scattered on different chromosomes (6). Homeobox genes of both classes play key roles in development and carcinogenesis in gastrointestinal tract (7, 8). For instance, Cdx1 and Cdx2, 2 divergent Homeobox genes, are normally expressed in the gut during development and adulthood. However, expressions of Cdx1 and Cdx2 are often lost in CRC cell lines and tissues (9). Downregulation of Cdx1 or Cdx2 expression can promote tumor development and invasiveness, whereas overexpression of Cdx2 in CRC cell
lines can inhibit cell growth and tumorigenesis (10–12). On the contrary, HOXB6, B8, C8, and C9 transcripts were upregulated at various stages of CRC (13). Other researchers found that expressions of HOXA9, HOXB7, and HOXD11 were altered in primary colon cancers and their hepatic metastases (14). In short, these findings suggest an association between deregulation of HOX gene expression and CRC progression.

It has been well documented that HOXB7, a member of class I homeobox, plays an important role in tumorigenesis. First, deregulation of HOXB7 was observed in several kinds of tumors (15–19). Overexpression of HOXB7 was closely associated with poor prognosis of breast cancer and oral cancer patients (18, 20). Second, HOXB7 facilitated transforming ability in NIH3T3 fibroblasts and in immortalized breast epithelial cells MCF10A (21, 22). Enforced expression of HOXB7 protein, as a valuable marker of CRC prognosis, plays an important role in the development and progression of human CRC.

Materials and Methods

Cell cultures

The human CRC cell lines Caco2, Colo205, LS174T, DLD-1, HT-29, HCT116, SW480, and SW620 were purchased from American Type Culture Collection. SW620 and HT29 were cultured in DMEM medium (Gibco) supplemented with 10% FBS (PAA). Caco2, Colo205, LS174T, DLD-1, HT-29, HCT116, and SW480 were cultured in RPMI 1640 medium (Gibco) with 10% FBS (PAA).

Vectors construction and retroviral infection

The HOXB7 construct was generated by subcloning PCR-amplified full-length human HOXB7 cDNA into pLncx2. For deletion of HOXB7, shRNA sequence (GCTCAG-GAACGTACCACCAAAC) was cloned into pSuper-retro-puro. Retroviral production and infection were performed as previously described (26). Stable cell lines expressing HOXB7 or shHOXB7 were selected for 10 days with 400 mg/mL G418 or 0.5 μg/mL puromycin, respectively.

Patient samples

Paraffin-embedded, archived CRC samples were obtained from 224 patients diagnosed as CRC between January 2001 and December 2003 at Nanfang Hospital, Southern Medical University. Medical records of the 224 patients provided information of age, gender, and following parameters: tumor histology, pathologic stage, Dukes stage, T stage, lymph node metastases, and distant metastasis. Survival data were available for all patients. The median follow-up time was 57.5 months (range, 2–87 months). The mean age at diagnosis was 56.4 years (range, 23–86 years). One hundred and twenty-seven patients (56.7%) were male. At the end of the follow-up period, distant metastasis was noted in 72 patients. Eight biopsies of CRC tissues and the matched adjacent noncancerous mucosa tissues were frozen and stored in liquid nitrogen until further use.

RNA extraction, RT-PCR and real-time PCR

Total RNA extraction, RT-PCR and real-time PCR were performed as previously described (27). See Supplementary Methods for details.

Western blotting

Western blotting was performed according to standard methods as described previously (27), using anti-HOXB7 (Sigma-Aldrich), anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT, anti-phospho-GSK3β, anti-GSK3β, anti-Cyclin D1, and anti-p27 antibodies ((Bioworld Technology). A mouse monoclonal anti-α-Tubulin antibody (Sigma-Aldrich) was used as inner control to confirm equal loading of proteins.

Immunohistochemistry

Immunohistochemistry (IHC) staining and scoring were done as previously described (27). For details, please see Supplementary Methods.

Colony formation assays

Cells were trypsinized and plated on 6-well plates (200 cells/well) and cultured for 2 weeks. The colonies were stained with 1% crystal violet for 30s after fixation with 4% paraformaldehyde for 5 minutes. The number of colonies, defined as >50 cells/colony were counted. Three independent experiments were performed. The data was calculated using paired t test.
HOXB7 Accelerates Progression of Colorectal Cancer

Figure 1. Expression of HOXB7 was elevated in CRC. A, expression of HOXB7 protein in CRC cell lines. Expression levels were normalized with α-Tubulin. B, expression of HOXB7 mRNA in CRC cell lines by Real-time PCR. Expression levels were normalized for GAPDH. Error bars represent mean ± SD calculated from 3 parallel experiments. C and D, expression of HOXB7 protein (C) and mRNA (D) in each of the primary CRC (T) and adjacent noncancerous tissues (N) paired from the same patient by western blotting (C) and RT-PCR (D). E, real-time PCR analysis of HOXB7 expression in each of the T and N tissues. GAPDH was used as an internal control. Columns, mean from 3 parallel experiments; bars, SD.

Soft agar assays

Cells (1 × 10⁶) were resuspended in RPMI 1640 containing 10% fetal bovine serum with 0.3% agarose and layered on top of 0.6% agar in medium supplemented with 20% fetal bovine serum on 60-mm plates. The plates were incubated at 37°C in a humid atmosphere of 5% CO₂. After 2 to 3 weeks, cell colony numbers were counted under microscope and cell colonies were photographed at an original magnification of 100×. Only cell colonies containing more than 50 cells were counted. The experiment was performed for 3 independent times for each cell line.

MTT assays and cell cycle analysis

See Supplementary Methods for details.

Tumorigenesis in nude mice

Xenograft tumors were generated by subcutaneous injection of cells (2 × 10⁶) for SW480/Vector and SW480/HOXB7, 5 × 10⁵ for SW620/Scramble and SW620/shHOXB7, n = 5) on the hindlimbs of each 4- to 6-week-old Balb/C athymic nude mouse (nu/nu) obtained from the Animal Center of Southern Medical University, Guangzhou, China. All mice were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines and approved by the Use Committee for Animal Care. Tumor size was measured by a slide caliper and tumor volume was determined by the formula 0.44 × A × B² (A indicates tumor base diameter one direction and B the corresponding perpendicular value). The tumors were rapidly taken out and fixed in 10% neutral buffered formalin, embedded in paraffin. Sections of 4 μm were cut and stained with haematoxylin and eosin according to standard protocols. Sections were further under IHC staining using antibodies against HOXB7 and Ki-67.

Statistical analysis

All statistical analyses were performed using the SPSS 13.0 statistical software package. Comparisons between groups for statistical significance were performed with a 2-tailed paired Student’s t test. The relationships between HOXB7 expression and clinicopathologic characteristics were tested using Chi-square test. Survival curves were plotted by Kaplan–Meier method and compared by log-rank test. The significance of various survival-related variables was assessed by Cox regression model in the multivariate analysis. P < 0.05 was considered statistically significant.

Results

HOXB7 was frequently upregulated in CRC

Real-time PCR and western blotting analysis revealed that all 8 CRC cell lines, including Colo205, Caco2, Ls174t, DLD1, HT29, HCT116, SW480, and SW620, exhibited different levels of HOXB7 expression. HOXB7 expression was relatively lower in Ls174t and SW480 than that in other cell lines (Fig. 1A and B). Comparative analysis indicated that HOXB7 was significantly upregulated in 8 examined tumor samples paired with adjacent noncancerous tissues from the same patients (Fig. 1C and D). The tumor/normal (T/N) ratio of HOXB7 mRNA expression was >2-fold in all samples, and the highest ratio was up to 16.4-fold, as analysis by Real-time PCR (Fig. 1E).
HOXB7 overexpression was associated with progression and poor survival in CRC

Expression of HOXB7 protein was determined by IHC in 224 paraffin-embedded, archived CRC tissues. HOXB7 protein was detected in 179 of 224 (79.9%) cases of tissue samples (Fig. 2A, e–h), whereas there was no or weak signal in adjacent noncancerous areas in all sections detected (Fig. 2A, a–d). In addition, HOXB7 was differently expressed in tumor stroma cells (mainly in fibroblasts, Fig. 2A f, A h, C f, arrows) in some samples.

Chi-square test showed that the levels of HOXB7 protein significantly correlated with Dukes stage ($P < 0.001$), T stage ($P = 0.012$), distant metastasis ($P = 0.007$, Supplementary Table S2). Kaplan–Meier survival analysis indicated that patients who had low HOXB7 expression levels had a better outcome (Fig. 2B). Multivariate survival analysis indicated that the HOXB7 expression level, T stage and Pathologic stage were 3 independent prognostic factors for outcomes in patients with CRC (Supplementary Table S3). Chi-square test also indicated a significant correlation between the Ki-67 labeling index and HOXB7 expression in CRC ($P = 0.007$, Supplementary Table S2). Samples that had lower level of HOXB7 expression also had a lower Ki-67 labeling index (Fig. 2C, a–d), whereas samples that had higher level of HOXB7 expression had a higher Ki-67 labeling index (Fig. 2C, e–h).

HOXB7 promoted human CRC cell growth and proliferation

To evaluate the possible role of HOXB7 in the proliferation of human CRC cells, stable HOXB7 expressed cell lines SW480/HOXB7 and Ls174t/HOXB7 were made (Fig. 3A). MTT assay showed that HOXB7 overexpression increased the proliferation of SW480 as compared with the control cells (Fig. 3B; $P < 0.05$). The population doubling time cells of SW480/HOXB7 are significantly longer as compared with control. This observation was further confirmed by a cell growth assay (data not shown). Colony formation assay revealed that SW480/HOXB7 cells formed much more and bigger colonies than that of control cells (Fig. 3C; $P < 0.01$). Similar results were observed in Ls174t cells (Fig. 3B and C; $P < 0.01$). To further investigate the impact of HOXB7 on CRC proliferation, we knockdown endogenous HOXB7 in SW620 and HCT116 CRC cells by expressing short hairpin RNAs (shRNA; Fig. 3D). MTT assay and colony formation assay (Fig. 3E and F; $P < 0.05$), and cell growth assay (data not shown) showed that knockdown of HOXB7 expression caused evident compromised viability in SW620 and DLD1 cells.

Figure 2. HOXB7 expression in paraffin-embedded CRC specimens. A, representative images of HOXB7 expression in normal intestinal epithelium and CRC specimens examined by IHC. HOXB7 was only negatively (a, b) or weakly (c, d) detected in normal intestinal epithelial cells, whereas it was positively detected in CRC cells (e and f display weak signal, whereas g and h display strong signals). B, Kaplan–Meier analysis of survival in patients with CRC. Green, patients with high HOXB7 expression ($n = 121$, 5-year survival rate 51.1%, median survival 57 months); blue, patients with low expression of HOXB7 ($n = 103$, 5-year survival rate 70.6%, median survival 64 months; $P = 0.005$, log-rank test). C, HOXB7 expression in CRC tissues was positively associated with the expression of Ki-67. IHC staining for HOXB7 and Ki-67 in a series of sections from paraffin blocks of CRC reveals weak expression of HOXB7 (a, b), weak expression of Ki-67 (c, d), strong expression of HOXB7 (e, f), and strong expression of Ki-67 (g, h). The arrows indicate the stroma cells positively stained with HOXB7.
HOXB7 promoted tumorigenesis of CRC in vitro and in vivo

We next examined the effect of HOXB7 on the tumorigenic activity of CRC cells using an anchorage-independent growth assay. The results showed that overexpression of HOXB7 in SW480 caused significant promotion of its anchorage-independent growth, as indicated by increasing in colony number and colony size on soft agar (Fig. 4A; \( P < 0.01 \)). Although depletion of endogenous HOXB7 in SW620 cells caused significant reduction in colony number and colony size on soft agar (Fig. 4B; \( P < 0.01 \)). Therefore, we determined that HOXB7 is essential for tumorigenesis of CRC cells.

To confirm this effect in vivo, we performed tumorigenesis assays in nude mice. SW480/Vector, SW480/HOXB7, SW620/Scramble, and SW620/shHOXB7 cells were inoculated in nude mice. All mice developed Xenograft tumors at the injection site. As shown in Figure 4C, tumor growth in the SW480/HOXB7 group was significantly more rapid than that in the SW480/Vector group. The volumes of tumors formed by the SW480/HOXB7 cells were significantly larger than those of vector-control cells-formed tumors (\( n = 5; P < 0.05 \)). The average final tumor volume in SW480/HOXB7 group was 712.7 ± 123.4 mm³, compared with 363.6 ± 203.5 mm³ in the SW480/Vector inoculating group (\( t \)-test, \( P < 0.05 \)). In addition to the difference of tumor volume, we also found that the tumors formed by SW480/HOXB7 cells displayed much stronger HOXB7 staining and higher Ki-67 index than those in tumors formed by SW480/Vector cells, as detected by IHC analysis of HOXB7 and Ki-67 (Fig. 4D). However, depletion of endogenous HOXB7 in SW620 cells caused significant inhibition of tumor growth in terms of tumor volume (Fig. 4E; \( n = 5; P < 0.01 \)). The average final tumor volume in control group was 115.6 ± 40.1 mm³, whereas it was only 41.5 ± 20.1 mm³ in the SW620/shHOXB7 inoculating group. Difference in the final tumor volume between these 2 groups was significant (\( t \)-test, \( P < 0.05 \)). IHC staining showed that the tumors of control group displayed much stronger HOXB7 staining and higher Ki-67 index than that in SW620/shHOXB7 group (Fig. 4F).

HOXB7 accelerated cell cycle progression

We further measured the cell cycle distribution by flow cytometry to explore the possible mechanism of HOXB7 in controlling CRC cell proliferation. As shown in Figure 5,
decrease in the cell number at the G1–G0 phase and increase in the cell number at S phase was observed in HOXB7 overexpressed cells. The percentage of cells reentering into S phase after serum starvation in SW480/HOXB7 (37.83% ± 3.42) was significantly higher than that in SW480/Vector cells (26.89% ± 0.12; Fig. 5A a and B a, *P < 0.01). The percentage of S-phase cells in Ls174t/HOXB7 was 31.96% ± 2.15, whereas it was only 22.66% ± 2.11 in control cells (Fig. 5A b and B b, *P < 0.01). On the contrary, increase in the cell number at the G1–G0 phase and decrease in the cell number at S phase was observed after endogenous HOXB7 was knocking down. The percentage of S-phase cells in HCT116/shHOXB7 (30.7% ± 1.95) and SW620/shHOXB7 (27.21% ± 4.47) were significantly less than in HCT116/Scramble (38.86% ± 3.65) and SW620/shHOXB7 (40.77% ± 2.17) cells, respectively (Fig. 5Ac-d and B c-d, *P < 0.01). These results indicated that overexpression of HOXB7 accelerated the G1 to S-phase transition in these tumor cells.
transition in CRC cell lines, which contributes to the growth promotion properties of HOXB7.

**HOXB7 regulated cell cycle factors cyclin D1 and p27Kip1: MAPK and PI3K/Akt activations in CRC cells were involved**

To better understand the mechanisms that facilitate the G₁ to S-cell cycle transition mediated by HOXB7, the expression levels of some of the cell cycle regulators, including CDK4, CDK2, cyclin D1, cyclin E, p21Cip1/WAF1, and p27Kip1 were detected. Overexpression of HOXB7 did not affect the expression of CDK4, CDK2, cyclin E, and p21Cip1/WAF1 (data not shown), whereas the expression cyclin D1 was upregulated and p27Kip1 was downregulated (Fig. 6A). In contrast, significant increases in the expression of p27Kip1 and decreases of cyclin D1 were shown in HOXB7 knockdown CRC cells (Fig. 6A). Moreover, the modulation of p27Kip1 and cyclin D1 by HOXB7 were regulated at the transcriptional level (Fig. 6B). Further more, phosphorylated levels of ERK, AKT and GSK3β were increased in HOXB7-overexpressed CRC cells, whereas they were significantly decreased in HOXB7-knockdown CRC cells than in control cells (Fig. 6A).

**Discussion**

Although altered HOXB7 mRNA level was observed in CRC (14), this study was the first that reported the upregulation of both HOXB7 protein and mRNA in this disease. The current study has revealed that expression of HOXB7 is significantly correlated with the invasive and aggressive characteristics of human CRC (high Dukes stage, T stage,
distant metastasis-positive tumors, and high proliferation index) and with poor survival of patients as well. These implicate that overexpression of HOXB7 protein may be a common feature in CRC and can serve as an independent prognostic marker to identify patients with poor clinical outcome. Similar to our results, HOXB7 overexpression was reported in association with the clinical progression and poor outcome of patients with breast cancer and oral cancer (17, 18, 20). Upregulation of HOXB7 mRNA and/or protein level was also observed in melanoma, ovarian cancer, and esophageal squamous cell carcinoma (15, 16, 19). However, whether HOXB7 can be used as a universal biomarker or prognostic predictor for neoplasm needs further investigation.

There were 2 opposite functions of HOXB7 that were documented in different cellular contexts. Majority studies supported that HOXB7 might play a role in promotion of multistep process of tumor formation and progression, including transformation, proliferation, angiogenesis, invasion, and metastasis (17, 18, 20–25). On the other hand, some researchers observed a promoting role of HOXB7 in differentiation in hematopoietic stem cells and multipotent mesenchymal cells (24, 28). Our results suggested that elevated HOXB7 might be associated with higher proliferation activity in CRC cells, which contributed to malignant transforming and tumorigenesis. Thus, our data mainly support the tumorigenesis promotion function of HOXB7. Interestingly, we found that HOXB7 was not only expressed in cancerous epithelial cells, but also differently expressed in tumor stroma cells (mainly in fibroblasts, Fig. 2). Similarly, it has been reported that HOXB7 is expressed in fibroblasts (29, 30). These observations suggested that HOXB7 may be involved in a stroma-specific signaling pathway that promotes initiation and progression of CRC. Thus, the expression and function of HOXB7 might be tissue specific or individual specific.

Although HOXB7 has been linked to regulation of proliferation (and thus transformation), the molecular mechanisms remain poorly identified. Majority researches only reported that bFGF, one of direct targets of HOXB7, contributed to HOXB7-induced cellular proliferation and transformation (15, 16, 18, 25). Here, more specifically, we showed the molecular mechanisms might be the acceleration of G1–S transition, upregulation of
cyclin D1 and downregulation of p27Kip1 under enforced expression of HOXB7. Previous studies revealed that PI3K/akt and MAPK signal transduction cascades, required for cell cycle progression through G1 phase, were frequently involved in proliferation (31). In addition, activation of RAS and PI3K/akt decreased the cellular levels of p27Kip1 and induced cyclin D1 mRNA and protein, thereby promoting cell proliferation (32). Moreover, HOXB7 was shown to activate the Ras-RAF-MAPK pathway in breast cancer cell lines (17). Here, we showed that PI3K/akt and MAPK pathways were activated because p-ERK, p-AKT and p-GSK3-β were upregulated by HOXB7. Therefore, the regulation of p27Kip1 and cyclin D1 by HOXB7 probably resulted from enhanced PI3K/akt and MAPK pathway activity. This thus explains the accelerating G1–S transition induced by HOXB7. Taken together, our observations link HOXB7 to the basic cell cycle regulation, which helps provide evidence for diverse molecular mechanisms by which HOXB7 promote cell growth and proliferation.

Both MAPK and PI3K/akt pathways directly regulate p27Kip1 and cyclin D1 (31). The regulation occurs not only at transcriptional level but also at posttranslational level via ubiquitin-proteasome proteolysis (33–35). p27Kip1 promoter activity could be regulated by FOXO proteins (FOXO4, FOXO3a, and FOXO1) which, in turn, could be modulated by PI3K/akt and MAPK pathways (32, 36). In this study we showed that HOXB7 overexpression could downregulate p27Kip1 expression both at mRNA and protein levels, with little difference between the 2 levels in the change fold of p27Kip1. Therefore, the effect of HOXB7 on p27Kip1 in CRC cells may be mediated via PI3K/akt and MAPK pathways at transcriptional level.

Similarly, cyclin D1 was regulated at both transcript and protein levels by HOXB7 in this study. Thus, the effect of HOXB7 on cyclin D1 in CRC cells may also be regulated mainly at transcriptional level. However, whether HOXB7 could directly promote transcription of cyclin D1 need further investigation.

In conclusion, our findings suggest that upregulation of HOXB7 might be a valuable prognostic marker of CRC progression. Altered expression of HOXB7 genes could be important for tumorigenesis and progression of CRC. Modulation of the tumor proliferation effect through inhibiting PI3K/akt or MAPK activation mediated by HOXB7 overexpression might be used as a potential target for CRC prevention and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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