Homeobox B9 Mediates Resistance to Anti-VEGF Therapy in Colorectal Cancer Patients

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Abstract

Purpose: The identification of predictive biomarkers for antiangiogenic therapies remains an unmeet need. We hypothesized that the transcription factor Homeobox B9 (HOXB9) could be responsible for the tumor resistance to the anti-VEGF agent bevacizumab.

Experimental Design: HOXB9 expression and activation were measured in eight models of colorectal and pancreatic cancer with different resistance to bevacizumab. Serum levels of Angiopoietin-like Protein (Angptl)2, CXC receptor ligand (CXCL)1, IL8, and TGFβ1 in tumor-bearing mice were measured by multiplex xMAP technology. HOXB9 expression was measured by immunohistochemical analysis in 81 pretreatment specimens from metastatic colorectal cancer patients. Differences in progression-free survival (PFS) were determined using a log-rank test.

Results: HOXB9-positive tumors were resistant to bevacizumab, whereas mice bearing HOXB9-negative tumors were cured by this agent. Silencing HOXB9 in bevacizumab-resistant models significantly (P < 0.05) reduced Angptl2, CXCL1, IL8, and TGFβ1 levels, reverted their mesenchymal phenotype, reduced CD11b+ cells infiltration, and restored, in turn, sensitivity to bevacizumab. HOXB9 had no prognostic value in patients treated with a first-line chemotherapeutic regimen noncontaining bevacizumab. However, patients affected by an HOXB9-negative tumor had a significantly longer PFS compared with those with an HOXB9-positive tumor if treated with a first-line regimen containing bevacizumab (18.0 months vs. 10.4 months; HR 2.037; 95% confidence interval, 1.006–4.125; P = 0.048).

Conclusions: These findings integrate the complexity of numerous mechanisms of anti-VEGF resistance into the single transcription factor HOXB9. Silencing HOXB9 could be a promising approach to modulate this resistance. Our results candidate HOXB9 as predictive biomarker for selecting colorectal cancer patients for antiangiogenic therapy. Clin Cancer Res; 23(15): 4312–22. ©2017 AACR.

Introduction

Angiogenesis is a hallmark of cancer and its inhibition is commonly part of the therapeutic strategies for several tumor types (1, 2). Bevacizumab, a humanized monoclonal antibody targeting VEGF has been the most extensively studied antiangiogenic agent in digestive tumors, and, in particular, the targeting VEGF has been the most extensively studied antiangio-

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Translational Relevance

The identification of predictive biomarkers for antiangiogenic therapies poses one of the greatest challenges in digestive cancer research. We demonstrated HOXB9 as crucial transcription factor to sustain tumor resistance to bevacizumab, and that silencing its expression could be a promising approach to modulate this resistance. Our results candidate HOXB9 as novel biomarker for selecting patients with colorectal cancer for antiangiogenic therapy.

We recently contributed to this field by demonstrating the overexpression of a signature of proinflammatory and proangiogenic factors—including Angiopoietin-like Protein (Angptl)2, CXC receptors (CXCR)1/2 ligands such as CXCL1 and IL8, IL1α and β, and TGFβ1—in bevacizumab-resistant preclinical models if compared to their sensitive counterparts. These factors indirectly sustained angiogenesis through the recruitment of CD11b+ myeloid cells, and increased the aggressiveness of bevacizumab-resistant tumors by inducing epithelial-to-mesenchymal transition (EMT) (13). The combined inhibition of IL1, CXCR1/2, and TGFβ1 signaling pathways modulated in vivo bevacizumab-resistance by reversing EMT and inhibiting CD11b+ cells’ tumor infiltration (14).

Homeobox B9 (HOXB9) belongs to the highly conserved HOX transcription factor gene family, which is critical for embryonic development. In cancer, HOX genes expression directly drives neoplastic transformation and tumor progression through escape from apoptosis, alterations to receptor signaling, EMT, and tumor cell invasion (15). In particular, HOXB9 enhanced the competence of lung adenocarcinoma cells to form bone and the brain metastases (16). In breast cancer, HOXB9 enhanced the competence of lung metastasis and increased the expression of TGFβ (17), and induced the progression of epithelial-to-mesenchymal transition phenotype (EMT) (18). The combined inhibition of IL1, CXCR1/2, and TGFβ1 signaling through the use of siRNA silencing was modulated in vivo bevacizumab-resistance by reversing EMT and inhibiting CD11b+ cells’ tumor infiltration (14).

In this study, we hypothesized that HOXB9 could be responsible for the resistance of digestive tumors to the antiangiogenesis drug bevacizumab by coordinating the transcription of the proinflammatory and proangiogenic factors Angptl2, CXCL1, IL8, and TGFβ1. Thus, measuring the expression of HOXB9 might serve as potential biomarker to select patients with colorectal cancer more likely to benefit from antiangiogenic drugs.

Materials and Methods

Cell lines and generation of HOXB9 knockdown cell lines

All cell lines were maintained in their original culturing conditions according with supplier guidelines. Cells were ordinarily supplemented with FBS, 2mmol/L l-glutamine, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO2 air incubator. Cells were daily checked by morphology and routinely tested to be mycoplasma free by PCR assay. RFP+ HOXB9-shRNA+ cell lines were obtained by lentiviral infection as described in Supplementary Materials.

Cell-proliferation assay

On day 0, 1.0 × 10⁵ cells/well were seeded in 96-well plates. At the indicated hours, sulforhodamine B (SRB) (Sigma) assay was used to obtain relative estimates of viable cell number. Briefly, trichloroacetic acid fixed cells were stained for 30 minutes with 0.4% SRB dissolved in 1% acetic acid. SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. After being rinsed, the cultures were air dried until no standing moisture was visible. Bound dye was resuspended in Tris 10 mmol/L pH 10.5 and read with iMark Microplate Absorbance Reader spectrophotometer (Bio-Rad Lab., Inc.) at 540 nm.

Protein extraction and Western blotting

Cell lines were washed twice with cold PBS and lysed at 4°C into RIPA buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] plus protease inhibitor mix (Sigma-Aldrich). Lysates were clarified centrifugation and protein concentrations. Each lysate was separated by SDS-PAGE and probed with antibodies against E-Cadherin, HOXB9 (Abcam), Histone H3 (Cell Signaling Technology), vimentin (Dako), and γ-tubulin (Santa Cruz Biotechnology). Immunoactive proteins were detected using an enhanced-chemiluminescence reagent (ECL, Millipore) according to the manufacturer’s instructions. Images were captured by LAS4000 Digital Image Scanning System (GE Healthcare).

Electrophoretic mobility shift assay and DNA affinity precipitation assay

Nuclear extracts of pancreatic and colorectal cancer cell lines were prepared according to the method of Andrews and Fallar (20). For electrophoretic mobility shift assay (EMSA) assay, the wild-type double-stranded oligonucleotides containing the HOXB9 and control sequence site was obtained from Santa Cruz Biotechnology, Inc. and labeled with 32P to be used as probes. Instead, HOXB9-specific biotinylated oligodeoxynucleotides and control sequences were used for DNA affinity precipitation assay (DAPA) assay. The reactions were analyzed on 4% polyacrylamide gels containing 0.25X TBE buffer.

Immunofluorescence

Pancreatic cancer cell lines COLO357FG and FGCR, and colorectal cancer cell lines MDST8 and GP5D, were cultured for 24 hours on cover slips in a 24 multwell, fixed with 2.5% formalin and permeabilized with 0.1% Triton for 10 minutes at 4°C. The cells were then incubated with the primary antibody specific for HOXB9 (Abcam) for 1 hour at room temperature and with fluorophore (FITC)—conjugated as secondary antibody. Nuclei were stained with Hoechst 33342 (blue). Cover slips were mounted with pro-long antifade mountant (Invitrogen). The images were obtained with a confocal microscopy (LM5510, Zeiss).

Wound healing migration assay

Cell lines were seeded to 90% of confluence in 100 mm cell culture dishes. After 24 hours cells, a straight scratch was made using a pipette tip to simulate a wound. The cells were washed gently with cold PBS 1× and rinsed with fresh medium. Photographs at five different points at least were taken immediately and after 24 and 36 hours of culture.
Figure 1.
HOXB9 is overexpressed and activated in bevacizumab-resistant FGBR pancreatic cancer models. A, Twenty athymic nude mice bearing orthotopic luc-GFP+ bevacizumab-sensitive COLO357FG, or bevacizumab-resistant FGBR pancreatic tumors were randomly assigned to 4 groups (n = 5 per group) to receive 100 μg of either bevacizumab or saline (vehicle) intraperitoneally twice a week. Differences among survival duration of mice in each group were determined by log-rank test. B, Western blot analysis for the expression of HOXB9 in cytosolic and nuclear compartment from COLO357FG and FGBR cell lines. C, Representative images showing HOXB9 protein expression (red) by confocal microscopy, nuclei were stained with Hoechst 33342 (blue). Fluorescence intensity analysis was performed by ImageJ software, and bars represent mean ± 95% CI from analysis of eight separate high-power field images; * * * , P < 0.001, ** , P < 0.05, by two-tailed unpaired Student t test. (Continued on the following page.)
Gene expression microarray and pathway analysis

Differences in gene expression between cell lines were examined using Illumina Human 44K gene chips (Illumina). Briefly, synthesis of cDNA and biotinylated cRNA was performed using the IlluminaTotalPrep RNA Amplification Kit (Ambion), according to the manufacturer’s protocol using 500 ng of total RNA. Hybridization of cRNAs (750 ng) was carried out using Illumina Human 48k gene chips (Human HT-12 V4 BeadChip). Array washing was performed using Illumina High Temp Wash Buffer for 10' at 55°C, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences). Probe intensity data were obtained using the Illumina Genome Studio software (Genome Studio V2011.1). Raw data were Loess normalized with the Lumi R package and further processed with Excel software. Each microarray experiment was repeated twice. Differentially expressed transcripts were tested for network and functional interrelatedness using the IPA software program (Ingenuity Systems). Gene expression microarray data have been deposited in the GEO database with accession number (GSE59857).

RNA isolation and quantitative RT-PCR assay

Total RNA was obtained from cells using TRIzol reagent (Invitrogen) according to manufacturer’s instructions. Total RNA was quantified by absorbance at 260 nm. Reverse transcription was performed using the High Capacity Reverse Transcription Kit (Qiagen). The cDNA obtained was evaluated for real-time PCR performed using the High Capacity Reverse Transcription Kit and SYBR Green. QuantiTect primer and SYBR Green. QuantiTect (Qiagen) were used to quantify cDNA levels of CDH1, VIM, ANGPTL2, CXCL1, IL1A, IL1B, CXCL8, VEGFa, TGFβ2 and β-actin. Gene expression was calculated using 2^-ΔΔCT method and normalized to β-actin expression.

Xenograft model in nude mice

The orthotopic injection of luc+ / GFP+ pancreatic cancer cells and the subcutaneous heterotopic implantation of colorectal and pancreatic cancer cells was performed as described previously in (21, 22). Tumor-bearing mice were randomly assigned (n = 5 per group) to receive 100 μg of bevacizumab i.p. twice a week or saline as a control. In mice bearing heterotopic xenografts, the tumor size was measured with a caliper. Mice were euthanized using carbon dioxide inhalation when evidence of advanced bulky disease developed or a cut-off volume of 2 cm³, which was considered the day of death for the purpose of survival evaluation. Animal study was approved by the local ethics committee. Additional details of methods are provided in Supplementary Methods section.

Bio-Plex and ELISA for cytokines detection in murine plasma

Multiplex biometric ELISA-based immunoassay was performed according to the manufacturer’s instructions (Bioplex, Bio-Rad Lab., Inc.). Soluble molecules were measured in murine plasma using a commercially available kit which provides a series of combined reagents for the simultaneous measurement of human cytokines in serum, plasma, or tissue culture supernatant. Each experiment was performed in duplicate. Serum levels of all proteins were determined using a Bio-Plex array reader (Bio-Rad Lab., Inc.) that quantitates multiplex immunoassays in a 96-well format with very small fluid volumes. The analytes concentration was calculated using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software). ANGPTL2 quantification in murine plasma was performed by HANGPTL2 ELISA (Cusabio Biotech Co.) following the manufacturer’s instructions.

Patients

Eighty-one patients with metastatic colorectal cancer were included in the retrospective study. Fifty-eight patients underwent first-line bevacizumab-based therapy and 23 did not receive bevacizumab. HOXB9 protein expression in patient tissue samples was compared by immunohistochemical analyses. Informed consent was obtained from all patients. REMARK guidelines compliance is reported in Supplementary Table S2.

Statistical analysis

Progression-free survival (PFS) was calculated as the period from the first day of treatment to the date of tumor progression. PFS curves were drawn by Kaplan–Meier estimates and compared by log rank test. Univariate and multivariate analyses of PFS with stepwise variable selection were conducted by Cox’s proportional hazard regression models. Multivariate analysis was conducted using the variables that were significant in univariate analysis (P ≤ 0.05). The relationship between HOXB9 expression and clinicopathological characteristics was examined using the χ² method for linear trend. Additional details of methods are provided in Supplementary Methods section.

Results

Silencing HOXB9 modulates anti-VEGF resistance in pancreatic cancer models

To test the hypothesis that HOXB9 could be responsible for the anti-VEGF resistance in digestive tumors, we initially studied its role in the bevacizumab-resistant FGBR pancreatic cancer model established in (13). We verified that COLO357FG tumors were sensitive to bevacizumab, whereas mice bearing FGBR tumors exhibited resistance showing survival rates similar to untreated control mice (Fig. 1A). We also confirmed that the expression of the epithelial marker E-cadherin was suppressed, and that of the mesenchymal marker Vimentin was strongly upregulated in FGBR if compared with COLO357FG cells (Supplementary Fig. S1A). Although they had similar proliferation rates (Supplementary Fig.
S1B), the FGBR cells had a significantly higher migration rate than did COLO357FG cells (Supplementary Fig. S1C).

We demonstrated that FGBR cells had significantly higher nuclear expression levels of HOXB9 than did COLO357FG cells (Fig. 1, B and C). Consistently, we measured an increased HOXB9 DNA-binding activity in FGBR compared with COLO357FG cells (Fig. 1D and Supplementary Fig. S1A). When orthotopically injected in nude mice, FGBR tumors exhibited a significantly stronger expression of HOXB9 than did COLO357FG tumors (Fig. 1E). Importantly, we measured significantly higher plasma levels of human Angp12, CXCL1, IL8, and TGFβ1 in mice bearing FGBR tumors than in those bearing COLO357FG tumors (Fig. 1F).

To demonstrate the role of HOXB9 in sustaining resistance to bevacizumab, we knocked-down the expression of HOXB9 in FGBR cells by transducing them with lentiviruses expressing HOXB9-specific shRNAs or a scramble sequence as control (Fig. 1G and Supplementary Fig. S2A). Consistently, HOXB9 knockdown FGBR cells had a reduced HOXB9 DNA-binding activity compared with control and scramble cells (Fig. 1H).

We measured a significantly reduced expression of ANGPTL2, CXCL1, IL8, and TGFβ1 genes in HOXB9 knockdown FGBR cells (Supplementary Fig. S2B). As a consequence, silencing HOXB9 modulated EMT in bevacizumab-resistant FGBR cells by inducing a re-expression of E-cadherin at levels comparable with that of the bevacizumab-sensitive COLO357FG cells, and reducing the expression of Vimentin (Fig. 1I). Although they had similar proliferation rates (Supplementary Fig. S2C), HOXB9 knockdown FGBR cells exhibited a significantly reduced migration rate compared with control cells (Fig. 1J).

Moreover, we inhibited HOXB9 transcriptional activity by treating FGBR cells with an HOXB9 decoy oligodeoxynucleotide (ODN) or a mutant ODN as control. Although no significant difference was observed in the proliferation rate upon treatment with either the HOXB9 decoy ODN or mutant ODN, HOXB9 decoy ODN induced a statistically significant reduction of migration rate respect to mutant ODN or untreated cells (Supplementary Fig. S3).

To confirm the role of HOXB9 in resistance to bevacizumab in vivo, 20 mice were orthotopically injected with HOXB9 knockdown or scramble FGBR cells and randomly assigned to be treated with bevacizumab or its intraperitoneal vehicle as control (n = 5). Initially, we measured significantly lower plasma levels of human Angp12, CXCL1, IL8, and TGFβ1 in mice bearing HOXB9 knockdown FGBR tumors than in those bearing scramble FGBR tumors as control at baseline (Fig. 2A). HOXB9 knockdown FGBR tumors had a lower expression level of HOXB9, higher expression of E-cadherin, and reduced expression of Vimentin than did scramble FGBR tumors (Fig. 2B). At necropsy, the gross pathology of scramble FGBR control tumors revealed a highly vascularized structure, whereas HOXB9 knockdown FGBR tumors appeared clearly hypovascular (Fig. 2C). Consistently, HOXB9 knockdown FGBR tumors demonstrated significantly lower infiltration by CD11b+ proangiogenic myeloid cells than did scramble FGBR tumors (Supplementary Fig. S4).

Most importantly, only mice bearing HOXB9 knockdown FGBR tumors treated with bevacizumab experienced a statistically significant reduction in tumor growth, and demonstrated a statistically significantly prolonged median survival duration (P < 0.001) if compared with vehicle-treated controls. Mice bearing scramble FGBR tumors treated with bevacizumab had tumor growth (Fig. 2D) and survival rates comparable with those in vehicle-treated control mice (Fig. 2E).

**HOXB9 sustains resistance to anti-VEGF therapy in colorectal cancer models**

We set to extend our observations to colorectal cancer, a tumor type in which bevacizumab is widely applied in the clinic. To this end, we selected three HOXB9-positive—LOVO, MDST8, and LIM2099—and three HOXB9-negative—CCK81, GP5D, and SNUC4 colorectal cancer cell lines (Fig. 3A and B and Supplementary Fig. S5A and S5B) among a large collection of 151 colorectal cancer cell lines (23). We initially measured a significantly higher expression of a number of proinflammatory and proangiogenic factors including ANGPTL2, CXCL1, IL1A, IL1B, IL8, and TGFβ1 in HOXB9-positive LOVO, MDST8, and LIM2099 cell lines than in HOXB9-negative CCK81, GP5D, and SNUC4 colorectal cancer cell lines (Fig. 3C). Thus, HOXB9-positive cell lines demonstrated a more pronounced mesenchymal phenotype if compared with HOXB9-negative cell lines, with low expression levels of E-cadherin, high expression levels of Vimentin (Fig. 3D), and a significantly higher migration rate (Supplementary Fig. S5C).

To confirm the role of HOXB9 in resistance to bevacizumab of colorectal cancer models in vivo, 40 mice were injected with HOXB9-positive MDST8 and LIM2099 or HOXB9-negative GP5D and CCK81 colorectal cancer cell lines and randomly assigned to be treated with intraperitoneal bevacizumab or its vehicle as control (n = 5). Interestingly, we measured lower plasma levels of human Angp12, CXCL1, IL8, and TGFβ1 in mice bearing MDST8 or LIM2099 tumors than in those bearing GP5D or CCK81 tumors at baseline, with the exception of the high plasma levels of human Angp12 in CCK81 tumor-bearing mice (Fig. 3E). MDST8 and LIM2099 tumors had a higher expression level of HOXB9, lower expression of E-cadherin, and increased expression of Vimentin than did GP5D or CCK81 tumors (Fig. 3F). Moreover, tumors with higher expression of HOXB9 had a greater infiltration by CD11b+ proangiogenic myeloid cells than did HOXB9 negative tumors (Supplementary Fig. S6). Most importantly, mice bearing HOXB9-positive LOVO or MDST8 were completely resistant to bevacizumab, whereas mice bearing HOXB9-negative GP5D or CCK81 colorectal cancer tumors were cured by the treatment with this anti-VEGF agent (Fig. 3G).

To further demonstrate the role of HOXB9 in sustaining resistance to bevacizumab in colorectal cancer, we knocked-down HOXB9 expression in HOXB9-positive MDST8 cells by transducing them with lentiviruses expressing HOXB9-specific shRNAs or a scramble sequence as control (Fig. 4A and Supplementary Fig. S7A). Consistently, HOXB9 knockdown MDST8 cells had a reduced HOXB9 DNA-binding activity compared with control and scramble cells (Supplementary Fig. S7B). As a consequence, we measured a significantly reduced expression of ANGPTL2, CXCL1, IL8, and TGFβ1 in HOXB9 knockdown MDST8 cells if compared with control cells (Supplementary Fig. S7C). Although we could only measure a minimal reduction in the expression of the mesenchymal marker Vimentin (Supplementary Fig. S7D and S7E), HOXB9 knockdown MDST8 cells exhibited a significantly reduced migration rate compared with control cells (Fig. 4B). Moreover, also HOXB9 transcriptional activity inhibition by decoy ODN induced a statistically significant reduction of migration rate respect to mutant ODN or untreated cells (Supplementary Fig. S8).
To confirm the role of HOXB9 in resistance to bevacizumab in colorectal cancer models in vivo, 20 mice were orthotopically injected with HOXB9 knockdown or scramble MDST8 cells and randomly assigned to be treated with bevacizumab or its intraperitoneal vehicle as control (n=5). We measured significantly lower plasma levels of human Angptl2, CXCL1, and IL8 in mice bearing HOXB9 knockdown MDST8 tumors then in those bearing scramble MDST8 tumors as control at baseline (Fig. 4C). HOXB9 knockdown MDST8 tumors demonstrated a complete suppression of the expression of HOXB9, higher expression of E-cadherin, and reduced expression of Vimentin than did scramble MDST8 tumors (Fig. 4D). At necropsy, the gross pathology of HOXB9 knockdown MDST8 tumors appeared clearly hypovascular if compared with scramble MDST8 control tumors (Fig. 4E). Consistently, HOXB9 knockdown MDST8 tumors demonstrated significantly lower infiltration by CD11b+ proangiogenic myeloid cells than did scramble MDST8 tumors (Supplementary Fig. S9).

Most importantly, mice bearing scramble MDST8 tumors treated with bevacizumab had tumor growth and survival rates comparable with those in vehicle-treated control mice. Conversely, mice bearing HOXB9 knockdown MDST8 tumors treated with bevacizumab experienced a statistically significant reduction in tumor growth (Fig. 4F), and demonstrated a statistically significantly prolongation of their median survival duration if compared with vehicle-treated controls (scramble MDST8 vs. HOXB9 knockdown MDST8, median survival 60 days vs. 79.5 days, P=0.0025; Fig. 4G).

HOXB9 expression is an independent predictive factor for PFS in metastatic colorectal cancer patients treated with bevacizumab

To confirm the clinical relevance of HOXB9 as predictive biomarker for bevacizumab treatment in colorectal cancer, we conducted an exploratory retrospective analysis of 81 metastatic colorectal cancer patients treated with bevacizumab. The analysis revealed that HOXB9 expression was significantly associated with worse PFS (P=0.0006). This finding suggests that HOXB9 expression could serve as a potential biomarker for predicting treatment response to bevacizumab in metastatic colorectal cancer patients.
patients who received first-line standard chemotherapeutic regimens in combination (n = 58) or not (n = 23) with bevacizumab. Patients’ clinical characteristics and compliance with REMARK guidelines are reported in Supplementary Tables S1 and S2, respectively.

Specimens obtained from patients at treatment baseline were evaluated for HOXB9 expression levels by immunohistochemical analysis, and tumor samples entirely or partially stained were classified as HOXB9-positive. Of the 81 specimens, 19 (23.46%) scored negatively and 62 (76.54%) scored positively.

In the cohort of patients treated with a first-line chemotherapeutic regimen noncontaining bevacizumab, HOXB9 expression could not distinguish patients with different PFS duration [HOXB9-positive median PFS = 10.1 months vs HOXB9-negative median PFS = 7.9 months; HR 2.004; 95% confidence interval (CI), 0.576–6.967; P = 0.27; Fig. 5A]. Conversely, in the cohort of patients treated with a bevacizumab-containing first-line chemotherapeutic regimen, we demonstrated that patients with HOXB9-negative tumors had a significantly longer PFS duration than did those with HOXB9-positive tumors (HOXB9-positive median PFS = 10.4 months vs. HOXB9-negative median PFS = 18.0 months; HR 2.037; 95% CI, 1.006–4.125; P = 0.048; Fig. 5B). Univariate analysis revealed that primary tumor size, ECOG performance status, KRAS/NRAS/BRAF mutational status, and HOXB9 expression correlate with PFS in patients treated with a bevacizumab-containing chemotherapeutic regimen. Multivariate analysis indicated that HOXB9 expression was the most significant independent predictive factor for PFS in this patient’s cohort (HR 2.736; 95% CI, 1.281–5.846; P = 0.009; Table 1). We did not demonstrate any correlation between HOXB9 expression and other clinicopathologic variables in patients treated with a bevacizumab-containing chemotherapeutic regimen (Supplementary Table S3).

Discussion

In this study, we demonstrated for the first time that the transcription factor HOXB9 modulates the resistance of pancreatic and colorectal cancer to the anti-VEGF monoclonal antibody bevacizumab by orchestrating a complex network of alternative proinflammatory and proangiogenic secreted factors. Most importantly, to our knowledge this is the first study to candidate HOXB9 as potential biomarker for selecting patients with colorectal cancer for angiogenic therapy. HOXB9 is potently emerging as one of the master regulator of angiogenesis. In a recent systematic analysis, miR192 was identified as the most relevant miRNA for the control of angiogenesis. In the first time that the expression of this biomarker in a more relevant series of samples largely obtained at the diagnosis of metastatic disease. HOXB9 expression was correlated with the median PFS, a clinical endpoint that could be not corrupted by the effect of subsequent lines of therapy. Thus, no significant prognostic value was found for HOXB9 in the subcohort of patients treated with a first-line chemotherapeutic regimen noncontaining bevacizumab. However, patients affected by an HOXB9-negative tumor had a significantly longer median PFS compared with those with an HOXB9-positive tumor in the subcohort of patients treated with a first-line chemotherapeutic regimen containing bevacizumab, suggesting for the first time a potential predictive value of this biomarker. This study, however, had some limitations. In human-murine hybrid xenograft models, bevacizumab could only block human tumor-derived VEGF with no effect on murine VEGF derived from host cells. However, this represent a common problem also for the different models in which bevacizumab has been developed in recent years in a large number of published preclinical studies leading, thus, to its clinical development and approval.
Figure 4.
Silencing HOXB9 modulates anti-VEGF resistance in colorectal cancer models. A, Western blot analysis for the expression of HOXB9 in MDST8 control cells and in the same cells transduced with two lentiviruses expressing HOXB9-specific small hairpin RNA (shHOXB9 and shHOXB9a) or with scramble sequence as control; g-tubulin was used as loading control. B, Levels of cancer cell migration in MDST8 and MDST8 expressing shHOXB9 or a scramble sequence as control. Results are presented as percentages of the total distances between the wound edges enclosed by cancer cells. The mean values and 95% CIs from three independent experiments performed in quadruplicate are shown. Photographs of the wound area were taken using phase contrast microscopy immediately and 36 hours after the incision. C, Multiplex bead-based human cytokine assay for serum detection of circulating factors in colorectal cancer tumor bearing mice. Mean and 95% CI in ng/mL are shown. **, $P < 0.001$, *** $P < 0.05$, by two-tailed unpaired Student t test. D, Hematoxylin–eosin staining and immunohistochemistry analysis for the expression of HOXB9 and E-cadherin and Vimentin in nude mice bearing MDST8 cells expressing shHOXB9 or a scramble sequence as control. E, Representative images and relative vasculature in MDST8 expressing shHOXB9 or a scramble sequence as control tumor-bearing mice. F, Twenty athymic nude mice bearing subcutaneous MDST8 HOXB9 stably silenced or scramble cells were randomly assigned (n = 5 per group) to receive 100 μg of either bevacizumab or saline (vehicle) intraperitoneally twice a week. Mean tumor volume and 95% CI in mm$^3$ are shown, $P < 0.001$ by Student t test. G, Differences among survival duration of mice in each group were determined by log-rank test.
acknowledge that the recruitment of a stroma that produces murine VEGF is a possible mechanism of resistance to bevacizumab in tumor models. In this regard, the significance of our findings could be, indeed, an underestimation of what could actually happen in a fully human system. Moreover, the relevance of our analysis about the prognostic and predictive value of HOXB9 is limited by its retrospective design and the relatively small sample size. In this regard, a prospective randomized clinical trial about effectiveness of HOXB9 expression in selecting patients with colorectal cancer more likely to benefit from anti-angiogenic drugs is warranted.

After more than 10 years since the approval of bevacizumab for the treatment of colorectal cancer, the identification of predictive biomarker for anti-VEGF therapies remains an unmeet need in clinical oncology. Our results reduce the complexity of numerous molecular mechanisms of resistance to the inhibition of VEGF by integrating them into the expression of a single transcription factor. The usefulness of HOXB9 as an anti-VEGF treatment biomarker could be given also by the simple assessment and interpretation of its expression.

In conclusion, our study demonstrates for the first time HOXB9 as crucial transcription factor to sustain tumor resistance to bevacizumab in metastatic colorectal cancer patients treated with bevacizumab. Kaplan–Meier plots of PFS by HOXB9 expression in no-bevacizumab-treated (A) and bevacizumab-treated (B) patients. All PValues were calculated by the log rank test; (C) paraffin-embedded tumor sections stained immunohistochemically with antibodies against HOXB9. Statistical analyses are shown in Table 1.
bevacizumab. Silencing HOXB9 expression could be a promising approach to modulate this resistance. Our results candidate HOXB9 as novel biomarker to select patients with colorectal cancer more likely to benefit from antiangiogenic drugs.

**Disclosure of Potential Conflicts of Interest**
F. Loupakis is a consultant/advisory board member for Amgen, Bayer, Lilly, MerckSerono, and Roche. No potential conflicts of interest were disclosed by the other authors.

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**References**


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