BACH1 promotes the progression of human colorectal cancer through BACH1/CXCR4 pathway

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ARTICLE INFO
Article history:
Received 12 February 2018
Accepted 23 February 2018
Available online 24 February 2018

Keywords:
Colorectal cancer
BACH1
Oncogene
Tumor invasion
CXCR4

ABSTRACT
The present study was to investigate clinical significance, biological functions and underlying mechanisms of BTB Domain and CNC Homolog 1 (BACH1) deregulation in human colorectal cancer (CRC). The result showed that BACH1 upregulation was significantly associated with enhanced tumor invasion \((P=0.014)\) and gender \((P=0.028)\) of CRC patients. Kaplan-Meier method results showed that the overall survival of CRC patients with high BACH1 mRNA expression was markedly shorter than those with low expression \((P=0.015)\), and multivariate analyzes results showed that BACH1 was an independent prognostic predictor for CRC patients \((P=0.049)\). In vitro studies revealed that BACH1 efficiently promoted invasion and migration of CRC cell line. In vitro studies proved that the HCT116 cell stably expressing BACH1 formed significantly larger tumor nodules and remarkably accelerated tumor xenografts growth. In addition, Immunohistochemical scores of CD31 and Vimentin were significantly higher than those of the control group. Finally, correlation analysis indicated that BACH1 expression was positively correlated with C-X-C Motif Chemokine Receptor 4 (CXCR4) in tumor tissues and cell lines. Together, BACH1 serves as an oncogene to promote CRC progression and an independent prognostic factor for survival and metastasis. BACH1 may inhibit the progression of CRC through BACH1/CXCR4 pathway.

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1. Introduction
Colorectal cancer (CRC) is the third leading causes of cancer-related death in both men and women in the Western hemisphere, approximately 93,000 colon and 39,610 rectal cancers was diagnosed in 2015 and that 49,700 deaths occurred with this disease in the United States [1,2]. In China, the 5 leading causes of cancer death among both men and women are cancers of the lung and bronchus, stomach, liver, esophagus, and colorectum, accounting for about three-quarters of all cancer deaths [3]. Similar to many other cancers, it is of a great challenge to improve treatment and diagnosis of patients due to the cellular heterogeneity in CRC. Approximately 50% of CRC cases involve recurrence and metastasis following radical surgery [4]. Epithelial to mesenchymal transition (EMT) is a key mechanism for most cancer invasion and metastasis. In this process, the cell mobility increases as a result of loss of adhesion and an increase in mesenchymal components [5]. Thus, it is important to identify the molecular mechanism and signal pathway underlying tumor development and progression of CRC, which may provide valuable information for preventing from progression and improving prognostic levels of CRC.

The transcription factor BTB and CNC homology 1 (BACH1) consists of 739 amino acid residues and forms a heterodimer with small Maf family. It may play an important role by transcriptional activities through the small Maf family proteins [6]. BACH1 is a protein-coding gene that participates in the regulation of hemoglobin cell balance, oxidative stress response, and regulation of cell
cycle progression, inhibition of HO-1 (hemeoxygenase-1) and regulation of HO-1 in cancers [6–8]. Previous studies suggest that BACH1 deficiency may protect against oxidative tissue damage in murine models of intestine, pancreas, and cardiovascular disease [9–11]. Some suggested that BACH1 is involved in cancers, especially breast cancer. Recently, it was suggested that BACH1 was the master regulator of breast cancer bone metastasis [12]. In addition, it was suggested that BACH1 correlates with CXCR4 expression [13]. However, so far, the function of BACH1 and its underlying on human CRC has not yet been elucidated.

To address the problem, BACH1 expression patterns in CRC and non-cancerous colon tissues were examined by immunohistochemistry. Associations of BACH1 expression with various clinicopathological features and prognosis were evaluated. Moreover, we determined the BACH1 functions through in vitro (cell migration and invasion assays) and in vivo (tumor formation, angiogenesis and EMT). Furthermore, we confirm the role of BACH1/CXCR4 pathway in human CRC.

2. Materials and methods

2.1. Patients and tissue samples

A tissue microarray (TMA) including 90 CRC tissues and 90 normal colon tissues were purchased from Xi’an Ailina Biotechnology Co, Ltd (Xi’an, China; Cat No: CO1801). Patients with known chemotherapy or radiotherapy before surgery were excluded from the study. To analyze the association between BACH1 expression level and the clinical features and prognosis of CRC patients, we used the cancer genome atlas (TCGA) dataset including 192 primary CRC tissues and mRNA sequences (tcga-data.nci.nih.gov, Colorectal cancer) to check the expression of BACH1 at the mRNA level and performed survival analysis in this study. Our research was applied to and approved by the Ethics Committee of Guangzhou First People’s Hospital, Guangzhou Medical University, China.

2.2. Cell culture and transfections

Human CRC cell line HCT116 was purchased from American Type Culture Collection (ATCC, USA) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; Cellgro). All cell lines were maintained at 37 °C in a humidified chamber supplemented with 5% CO2. BACH1 suppression and overexpression plasmids were constructed. To package the construct, 293 TN cells were transfected with BACH1/NC or sh-BACH1/sh-NC by pPACKH1 Packaging Plasmid Mix, and then after 3 days, the virus particles were collected according to the packaging protocol of SBI with the Lenti-Concentrin Virus Precipitation Solution. HCT116 cell was infected with TransDux virus transduction reagent. The infected cells were isolated with a flow cytometer and cultured in 96-well plates.

2.3. Animals

In the present study, animal experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research at Sun Yat-sen University, Guangzhou, China. Eight BALB/c nude mice (4–5-week-old, males) were purchased from Experimental Animal Center of Sun Yat-sen University and were housed four per cage in wire-top cages with sawdust bedding in an isolated, clean, air-conditioned room at a temperature of 25 °C and a relative humidity of 50%, light 12 h/day.

2.4. Generation of the in vivo xenograft model

For the in vivo tumor formation assays [14], HCT116 cells with BACH1 overexpression and NC lentivectors were trypsinized and suspended in Phosphate-buffered saline (PBS). Then, the cells were subcutaneously injected into the flanks of each nude mouse. HCT116 cells were subcutaneously injected as a mixture of 1.6 × 10^6 cells and an equal volume of Matrigel, reaching a total concentration of 10 mg/ml. Once the tumor is measurable, the tumor sizes were measured every three days and the tumor volumes were calculated: \( V = \frac{1}{2} \times width^2 \times length \) (mm^2)/2. On the 25th day, the mice were sacrificed and the weight of each tumor was weighted.

2.5. Transwell invasion assay

The cells were digested and formulated to cell suspension 200 μL at a density of 6 × 10^4 cells/mL. The upper surface of the upper chamber of the transwell (CORSTOR, Cat. No. 3422) was previously coated with 20 μL matrigel (BD company) diluted 8-fold with serum-free 1640. The upper chamber was filled with 100 μL of cell suspension, and the lower chamber contained 10% fetal bovine serum as a chemical antagonist. After incubation for 24 h, cells migrated through the membrane, 0.1% crystal violet at room temperature fixed staining 30min, we selected four field of view to take pictures and counted the cell number under microscope [15].

2.6. Wound-healing assay

For the scratch wound-healing motility assay, a scratch was made with a pipette tip when the cells reach the confluence. After being cultured under standard conditions with mitomycin for 48 h, plates were washed twice with fresh medium to remove non-adherent cells and then photographed [15]. The migration distance was calculated by Imagepro-Plus software.

2.7. Western blot analysis

The proteins were extracted for Western blot analyses. Proteins (30 mg) were fractioned on SDS-PAGE and transferred onto Hybond nitrocellulose membranes (GE Healthcare). The membranes were blocked with 5% skim milk in PBS-Tween 20 and probed with anti-BACH1 (bs-4092 R, Bioss), anti-CXCR4 (bs-1011 R, Bioss) antibody. The results were visualized with the SuperSignal West PICO chemiluminescent detection system (Pierce Biotechnology). GAPDH (10494-1-AP, Proteintech) was used as an internal control.

2.8. Immunohistochemistry

Expression pattern and subcellular localization of BACH1 protein in clinical CRC tissues, and those of CD31, Vimentin and CXCR4 proteins in subcutaneous tumor xenografts of nude mice were detected by immunohistochemistry. Tumor specimens were scored in a semiquantitative manner [14,16,17] due to the heterogeneity of the staining of the target proteins. The percentage was grouped as follows: 0–25% = 1, 25–50% = 2, 50–75% = 3 and 75–100% = 4. The staining intensity was categorized as follows: none = 0, weak = 1, moderate = 2, and strong = 3. A final immunoreactivity scores (IRS) was obtained for each case by products of the percentages and the intensity scores, and the expression was divided into high level (IRS > 6) and low level (IRS <6). Vasculature density in tumor xenografts was determined by the number of CD31 positive vessels.
2.9. Statistical analysis

Statistical analyzes were performed using SPSS 20.0 (SPSS Inc, IL, USA) statistical software. Continuous variables were expressed as mean ± SD. Count data were analyzed by Pearson chi-square test. The correlation between the BACH1 gene expression level and the clinical features of colorectal cancer patients was analyzed by two independent samples t-test and One-way ANOVA test in TCGA dataset. The clinical survival of patients with colorectal cancer was analyzed by Kaplan-Meier method and log-rank test. Cox regression model Univariate and multivariate analysis was used to evaluate whether BACH1 can be an independent predictor of colorectal cancer prognosis. Differences were considered statistically significant when the p value was less than 0.05.

3. Results

3.1. Increased expression of BACH1 protein associates with the aggressive progression of human CRC

Sub-cellular localization and expression levels of BACH1 protein in CRC and benign colon tissues were examined by immunohistochemistry analysis. As a result, BACH1 was moderate or weak expression in cellular nucleus and cytoplasmic in CRC tissues but weak or even negative in benign colon tissues (Fig. 1A). Statistically, the IRS value of BACH1 protein in CRC tissues was significantly higher than that in Benign colon tissues (IRS, CRC, 8.39 ± 2.09 vs. Benign, 5.48 ± 1.79, P < 0.05, Fig. 1B).

The patients with CRC were divided into BACH1 relatively higher expression (moderate) group and BACH1 relatively lower expression (weak) group according to the immunoreactive scores of BACH1 protein in CRC tissues. We found that high BACH1 expression was significantly associated with enhanced tumor invasion (P = 0.014) and Gender (P = 0.028) interesting (Table 1). Since the TMA data did not include more clinicopathological characteristics such as CEA level, Tumor status and MSI Status, we used the TCGA dataset (consisting of 192 CRC tissues) to analyze the association between BACH1 expression level and the clinical features with CRC. It was found that BACH1 expression level was significantly correlated with age (P = 0.025), CEA level (P = 0.015) and MSI Status (P = 0.006). No significant correlation was found between BACH1 expression level and gender, clinical stage, tumor invasion, lymph node metastasis and distant metastasis.

3.2. Increased expression of BACH1 protein predicts poor prognosis in human CRC

The Kaplan-Meier method was performed to evaluate the
that BACH1 was a signiﬁcant prognostic factor (HR, 3.008; 95% CI, 1.809–4.453) compared with those of control cells, whereas the opposite effects were showed in BACH1 downregulated HCT116 cell line (Fig. 2B). Similar results were also obtained in the Wound healing assays, overexpression of BACH1 markedly enhanced the migratory activities of HCT116 cells expressing or knocking down BACH1 in HCT116 cells (Fig. 2A).

3.3. BACH1 promotes invasion and migration of CRC cells in vitro

In order to study the oncogenic roles of BACH1 in human CRC, we detected the cell invasion and migration abilities by over-expressing or knocking down BACH1 in HCT116 cells (Fig. 2A). Transwell assays clearly revealed that enforced expression of BACH1 signiﬁcantly increased the invasive activities of HCT116 cells compared with those of control cells, whereas the opposite effects were showed in BACH1 downregulated HCT116 cell line (Fig. 2B). Prognostic implication of BACH1 mRNA expression in CRC patients (Average survival time: 8.01 months) of the TCGA dataset. The overall survival of CRC patients with high BACH1 mRNA expression was markedly shorter than those with low expression (Fig. 1C, P = 0.015). Finally, the Cox proportional hazards model was used to evaluate whether BACH1 is a valuable predictor for the survival of CRC patients from the TCGA dataset. Univariate analysis indicated that BACH1 was an independent prognostic factor (HR: 16.658; 95% CI: 1.011–274.562; P = 0.049) (Table 2).
abilities of HCT116 cells, while the knock down of BACH1 dramatically reduced the cell invasive activity (Fig. 2C).

### 3.4. BACH1 promotes CRC subcutaneous xenograft growth in athymic mouse

In addition to the functional experiments in vitro, we also evaluate the biological functions of BACH1 in vivo. The BACH1-overexpressed HCT116 cells and the control were subcutaneous injected into the contralateral flanks of the athymic mouse. As shown in Fig. 3 A, the BACH1-overexpressed HCT116 cells formed significantly larger tumor nodules and remarkably accelerated tumor xenografts growth compared with the controls ($P<0.01$). Moreover, the transplanted tumor tissue was immunohistochemistry stained with pan-endothelial marker CD31 protein and mesenchymal marker Vimentin [18] to evaluate the angiogenesis and invasive tendency of the tumor xenografts. The results in Fig. 3B indicated that the expression level of CD31 and Vimentin protein in the BACH1 overexpressing xenografts was remarkably higher than those in the control, suggesting that BACH1 could significantly promote tumor growth, angiogenesis and invasion in vivo.

### 3.5. The expression of BACH1 has a positive effect on CXCR4 expression

Based on the TCGA dataset, the Pearson correlation analysis clearly showed a positive correlation between BACH1 and CXCR4 mRNA expression in CRC tissues ($Pearson \ r = 0.219, P = 0.002$; Fig. 4 A). To further validate the regulatory relationship between BACH1 and CXCR4 in CRC, we conducted western blot analysis to detect CXCR4 protein expression in BACH1 overexpressing HCT116 cell. Moreover, the tumor tissue transplanted with BACH1 overexpressing HCT116 cells lines was immunized with CXCR4
Fig. 3. BACH1 overexpression promotes tumor growth in vivo. (A) The HCT116 cells stably expressing BACH1 formed significantly larger tumor nodules and remarkably accelerated tumor xenografts growth compared with the controls. (B) The expression level of CD31 and Vimentin protein in the tumor xenografts established by HCT116 cells stably expressing BACH1 was remarkably higher than that in the xenografts established by cells transfected with control vectors. Representative photographs of immunohistochemistry analysis are presented (magnification ×100 and ×400). Data are expressed as mean ± SD. *P < 0.05. **P < 0.01 compared with control.

Fig. 4. Positive correlation between expression of BACH1 and CXCR4. (A) Based on the TCGA dataset, Pearson's correlation analysis clearly showed a positive correlation between BACH1 and CXCR4 mRNA expression in CRC tissues (Pearson r = 0.219, P = 0.002); (B) Western blot analysis showed that there was a positive correlation between BACH1 and CXCR4 protein expression; (C) The result of immunohistochemistry shows that CXCR4 immunohistochemical score was significantly higher in the tumor tissue transplanted with BACH1 over-expressing HCT116 cells lines than that in the control group. Data are expressed as mean ± SD. **P < 0.01 compared with control.
CXCR4 was closely associated with cancer metastasis and BACH1. The invasion and metastasis of breast cancer by regulating vital suppressive microRNA Let-7 [19]. Liang et al. indicated that BACH1 plays an important role in breast cancer metastasis. Yun et al. showed that BACH1 can acts as an oncogene to promote CRC progression and an independent prognostic factor for survival.

Recently, several researches indicated that BACH1 play an important role in breast cancer metastasis. Yun et al. showed that BACH1 is a pro-metastatic gene and a direct target of the tumor suppressive microRNA Let-7 [19]. Liang et al. indicated that BACH1 plays a role in breast cancer metastasis to bone [12]. Aletaha M. et al. revealed that by knockdown BACH1 gene in breast cancer metastatic cell line there is a significant decrease in metastasis and breast cancer invasion [20]. However, so far, the function of BACH1 and its underlying in human CRC has not yet been elucidated. Therefore, further research is required to study the effects of BACH1 expression on human CRC. In the current study, we performed experiments in vitro by overexpressing and knocking down of BACH1 in colorectal cancer cell line, revealing that BACH1 has a promotion effect on migration and invasion of colorectal cancer cell line. Further in vivo studies of the BACH1 are needed to verify function of BACH1 in colorectal tumors. The growth rate of tumor xenografts with BACH1 overexpression HCT116 cells lines was significantly faster than that in the xenografts established by cells transfected with control vectors. In order to get a better understanding of the molecular biological function of BACH1 on CRC cells in vivo and to obtain more evidence of BACH1 concerning effect on colorectal cancer cell invasion, we performed immunohistochemical analysis of the tumor xenografts with CD31 and Vimentin antibody, the results showed that BACH1 overexpression increased the expression levels of CD31 and Vimentin proteins in the tumor xenografts established by HCT116 cells over-expressing BACH1. All of these results provide more conclusive evidence to prove that BACH1 was an oncogene and can promote the invasion of CRC cells in vitro and in vivo, which was consistent with the results of previous studies in breast cancer.

CXCR4 belongs to the CXC chemokine receptor subfamily and is classically expressed by T cells and natural killer cells [21]. The role of CXCR4 in breast cancer metastasis was confirmed by Müller A et al., for the first time in 2001 [22]. After then studies in this field increased and all of them revealed that increase in the CXCR4 receptor is closely associated with increase of cancer metastasis. Yagi et al. [23], indicated that CXCR4 is necessary for breast cancer's cellular migration. Some studies revealed the role of CXCR4 in CRC that serum CXCR4 levels may be a possible prognostic marker in metastatic/recurrent CRC [24] and mirR-125 b enhanced the expression of CXCR4, which forms a positive feedback loop in triggering tumor invasion and progression of CRC [25]. In addition, Mohammadzadeh K's studies suggested that BACH1 was involved in the invasion and metastasis of breast cancer by regulating vital metastatic genes CXCR4 [13]. Those study demonstrated that CXCR4 was closely associated with cancer metastasis and BACH1 was closely correlated with CXCR4. Therefore, CXCR4 protein expression level in BACH1 overexpressing HCT116 cells and transplanted tumor tissue was detected by western blot analysis and immunohistochemistry to investigate the regulatory relationship between BACH1 and CXCR4 in CRC. We found that the expression of BACH1 was positively correlated with CXCR4, which revealed that BACH1 may promote the progression of human CRC through BACH1/CXCR4 pathway.

In conclusion, our study suggested that BACH1 can serve as an oncogene to promote CRC progression and an independent prognostic factor for survival and metastasis. The expression of BACH1 was positively correlated with CXCR4 in CRC and BACH1 may inhibit the progression of human CRC through BACH1/CXCR4 pathway. This newly discovered aspect of BACH1 gene provides new insight into CRC progression study and stands on its master regulator role in metastasis process, raising the possibility of considering it as a potential target for CRC therapy.

Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgements
This work was supported by Guangdong Planned Project of Science and Technology (2014A020221001) and Guangzhou Planned Project of Science and Technology (201704020105).

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