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Original Article

BRAF^{V600E} mutation promoted the growth and chemoresistance of colorectal cancer

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Abstract: BRAF mutation occurs frequently in colorectal cancer (CRC), which is associated with poor prognosis. Numerous clinical studies have indicated the undesirable effect of BRAF mutation in CRC patients; however, in vitro studies on the role and functional mechanism of BRAF mutation in CRC are limited. Here, we analyzed the association between BRAF mutation and the clinical features of CRC by using data deposited in the TCGA database. We found that BRAF mutation was closely related to the age and the pathological stage of CRC patients. Additionally, BRAF mutation also indicated poor overall survival in stage II CRC patients. Furthermore, we experimentally explored the function of BRAF mutation by generating a series of HCT116 stable cell lines expressing mutant BRAF^{V600E}, wildtype BRAF^{WT}, and vector control (NC). We found that BRAF^{V600E} mutation promoted not only the invasion of HCT116 cells through inducing epithelial-mesenchymal transition (EMT), but also cell proliferation as well as the chemoresistance to 5-Fluorouracil (5-FU) and oxaliplatin. Moreover, we confirmed our in vitro findings in mouse xenograft model, in which tumors derived from BRAF^{V600E} expressing HCT116 cells showed significantly increased growth compared with that from HCT116-BRAF^{WT} and HCT116-NC cells. Consistently, HCT116-BRAF^{V600E} tumors also showed significantly increased resistance to 5-FU compared with HCT116-BRAF^{WT} and HCT116-NC tumors. Taken together, our study revealed that BRAF mutation not only promoted the progression of CRC via enhancing EMT but also enhanced chemoresistance.

Keywords: BRAF mutation, colorectal cancer, chemoresistance, epithelial mesenchymal-transition, invasion

Introduction

BRAF, as one of the most important oncogenes in cancers, is frequently mutated in primary tumors with an incidence of about 8%. The major type of BRAF mutations is BRAF^{V600E} mutation, which mainly occurs in melanoma, colorectal cancer (CRC) and thyroid cancer [1, 2]. BRAF^{V600E} mutation simulates its phosphorylation at S598/T601 sites, leading to the constitutively active form of BRAF. As a result, its downstream mitogen-activated protein kinase (MAPK) signal transduction pathway is overactivated. As one of the most important signal pathways in cells [3, 4], the RAS/RAF/MAPK/ERK signaling pathway can promote cell growth by influencing cell cycle and lead to the transformation of normal cells [6, 7].

Compared to CRC patient harboring wildtype BRAF (BRAF^{WT}), patients with BRAF mutation are more likely to present with microsatellite instability (MSI) and show high-grade tumors as well as higher incidence of lymph node metastasis [8, 9]. Hence, BRAF mutation status is a strong predictor of survival in metastatic CRC patients. Even with adjuvant therapy, the patients with BRAF mutation have shorter disease-free survival (DFS) and worse overall survival (OS) after recurrence [10, 11]. A previous study reported the effect of BRAF and KRAS mutations in 1404 patients with stage II-III CRC and determined that the OS of patients with BRAF mutant was worse than that of patients with wildtype BRAF. After delaminated by MSI status, this difference was more obvious [12, 13]. For CRC patients with liver metastasis,

Table 1. The association between BRAF mutation and the clinical features of CRC patients

Characteristics	BRAF-WT	BRAF-MUT	Chi-square value	*P* value
Status				
Alive	359	53		
Dead	99	15	0.007	0.934
Age	65.3±12.7	69.9±13.6	NA	0.01
Gender				
Male	209	41		
Female	249	47	0.027	0.869
Race				
American Indian	1	0		
Asian	8	4		
Black	58	5		
White	240	41	5.984	0.106
pT				
T1	14	2		
T2	79	11		
T3	308	47		
T4	55	8	0.112	0.994
pN				
N0	244	49		
N1	124	13		
N2	29	6	4.106	0.128
pM				
M0	321	56		
M1	71	5	3.717	0.054
Pathological stage				
I	76	11		
II	152	37		
III	138	15		
IV	102	5	15.451	0.001

compared with BRAF^{WT} patients, the DFS of patients with BRAF mutation was less than 6 months after radical resection of metastasis, and the OS was also poor [14]. Overall, the median survival time of BRAF-mutated CRC patients after standard cytotoxic chemotherapy was only 1/2-1/3 of that of BRAF^{WT} patients [15]. Although compared to FOLFOXIRI treatment alone (5-fluoruracil, leucovorin, and irinotecan), FOLFOXIRI combined with bevacizumab prolongs the PFS of patients with BRAF-mutated metastatic CRC by 2.5 months, the survival time of BRAF-mutated CRC patients is still much shorter than that of BRAF^{WT} patients, with the median OS of 19.0 months *vs* 41.7 months, respectively [16].

Previous clinical studies have revealed the association between BRAF mutation and the clinical features of CRC patients; however, studies on the precise role of BRAF mutation in CRC cells are limited [17, 18]. Therefore, in this study, we aimed to investigate the effect of BRAF mutation on the malignancy of CRC cells. Our findings will shed light on the development of therapeutic strategy for CRC patients with BRAF mutation.

Materials and methods

Data acquisition and the screening of differentially expressed genes

We identified and downloaded the transcriptome data from The Cancer Genome Atlas (TCGA) database through the R package "TCGA-Assembler", including 88 cases of BRAF-mutated CRC patients, and 458 patients with wildtype BRAF. Additionally, the relevant clinical information was also acquired and shown in **Table 1**.

R software "Limma" package was utilized to normalize the expression of mRNA based on transcript data derived from the TCGA database. "DEGseq" package was further used to screen DEGs between the different groups. The threshold of P<0.05 and fold-change >1.5 or fold-change <-1.5 was used for the DEGs.

Gene ontology and KEGG pathway

For Gene Ontology (GO) and KEGG pathways analyses, all screened DEGs were analyzed by Database for Annotation Visualization and Integrated Discovery (DAVID, david.ncifcrf.gov/) online tool. Concreate pathways and annotations were obtained by the tool and further visualized by R software.

Cell culture and lentivirus infection

Human CRC cell line HCT116 was acquired from the University of Colorado Cancer Center Cell Bank and cultured in McCoy's 5A medium, supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmo-

Table 2. Primers for RT-qPCR

Gene	Forward primer	Reverse primer
Actin	GGGACCTGACTGACTACCTC	TCATACTCCTGCTTGCTGAT
E-cadherin	AGTCACTGACACCAACGATAAT	ATCGTTGTTCACTGGATTTGTG
Vimentin	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC
Snail	AAGGATCTCCAGGCTCGAAAG	GCTTCGGATGTGCATCTTGA
Twist	GTACATCGACTTCCTCTACCAG	CATCCTCCAGACCGAGAAG
Slug	CTGTGACAAGGAATATGTGAGC	CTAATGTGTCCTTGAAGCAACC

sphere. The constructs of human BRAF^{WT} and BRAF^{V600E} in lentiviral vector were acquired from Genomeditech Inc. (Shanghai, China). The indicated construct and lentiviral packaging plasmids (psPAX2 and PMG.2G) were co-transfected into the 293T cells to obtain lentivirus containing BRAF^{WT}, BRAF^{V600E} or empty vector as negative control (NC). Then, HCT116 cells were infected with the virus particles (multiplicity of infection, MOI=10) for 24 h and further screened by puromycin (1 ug/ml) for additionally 72 h for stable expression selection.

Western blot analysis

The transfected cells were lysed with RIPA buffer and cleared by centrifugation at 4°C for 15 minutes. The supernatants were then collected, and the total protein was quantified by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). All samples (20 μg proteins/sample) were separated by 10% SDS-PAGE gels, transferred onto PVDF membranes (0.45 mm, Merck Millipore, Billerica, MA, USA), blocked with 5% bovine albumin (BSA) for 1 h at room temperature, and then incubated with specific primary antibodies: E-cadherin, Vimentin, Snail, BRAF and BRAF^{V600E} rabbit polyclonal antibodies (1:4000, Abcam, UK) at 4°C overnight. After extensive washing, the membrane was incubated with corresponding secondary antibodies (1:4000 dilution) for 1 h at room temperature, and the protein bands were visualized by ECL reagents (Merck Millipore).

RNA extraction, reverse transcription, and quantitative PCR (RT-qPCR)

The total RNA was extracted from cells by Trizol Regent (Invitrogen) according to the manufacturer's instruction and quantified by absorbance at 260 nm. Subsequently, cDNA was obtained by reverse transcribing RNA using a reverse transcription kit (Takara Bio, Inc., Otsu, Japan) and then was used as template for RT-qPCR with SYBR Green (Takara Bio) and ABI 7900HT Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers used in this study are shown in **Table 2**. The comparative cycle threshold values (2-ΔΔCt) were adopted to analyze the final results.

Clonogenic assay

Briefly, transfected HCT116 cells (1000 cells/well) were seeded into 6-well plates in triplicate and cultured continuously until cell colonies were visible. After washing with PBS, the colonies were fixed with 4% paraformaldehyde solution, stained with 0.1% crystal violet for 30 minutes, and counted under microscope.

Cell proliferation assay

Briefly, transfected HCT116 cells (2000 cells/well) were seeded into 96 well plates in 5 replicates and cultured for 1, 2, and 3 days. To measure the cell viability at the indicated time, 10 μl CCK-8 solution was added into each well and incubated for 2 h. The absorbance values of each sample were measured at 490 nm.

Cell migration and invasion assays

Briefly, transfected HCT116 cells (10^4 cells/well in serum free medium) were seeded in the upper chamber of Transwell inserts (BD Biosciences, Bedford, MA, USA), while complete medium (with 10% FBS) was added to the wells. For invasion assay, the membrane of the upper chamber was coated with 50 μL Matrigel (1:8 dilution; BD Biosciences). After 24 h culture, the chambers were fixed by 4% paraformaldehyde for 30 minutes and then stained by 0.1% crystal violet for additionally 30 min. The migrated cells on the membrane were imaged using microscope.

Subcutaneous xenografts of nude mice

Male Balb/c-nu mice (5 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd. All experimental procedures were approved by the Institutional Animal Care and Utilization Committee of Fudan University Pudong Animal Experimental Center. A total of 36 mice were randomly divided into 3

groups (n=12/group), and mice in each group were subcutaneously injected in the axilla with 3×10^6/100 μl PBS/mouse of HCT116-NC, HCT116-BRAF^{WT} and HCT116-BRAF^{V600E} cells, respectively. One week after injection, mice in each group were divided into 2 subgroups (n=6/subgroup) as saline-treated control subgroup and 5-FU-treated subgroup (3 mg/kg, i.p.). The tumor growth was monitored every 3 days by caliper, and the tumor volume was calculated as: tumor volume = L*S^2/2, where L and S were the long and short diameter of the tumors. All mice were sacrificed 4 weeks after cell implantation, and the xenograft tumors were dissected for further analysis. To sacrifice the mice, the mice were euthanized first by injection of excessive 2% sodium pentobarbital, followed by a rapid cervical vertebra dislocation. During the tumor growth, we monitored the health, food intake and weight of mice carefully. When we observed a 10% weight loss a severe decrease in food/water intake, other disease development, or tumor size exceeding 20 mm, the experiments would be ended.

Immunohistochemistry (IHC) staining

Standard IHC protocol was followed. Briefly, the paraffin sections of tumor samples were deparaffinized, hydrated with decreasing concentrations of ethanol, and antigen retrieved in sodium citrate buffer. Then, the sections were blocked in 5% BSA and incubated with anti-Ki67 rabbit polyclonal antibody (1:100; Abclonal, Wuhan, China), followed by incubation with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (1:200; ProteinTech Group, Inc., Wuhan, China) for 60 minutes at room temperature. 3,3'-diaminobenzidine staining (DAB Substrate Chromogen System; Dako, Denmark) and hematoxylin staining were performed for signal development. Lastly, the sections were mounted, and the images were captured under microscope (Olympus IX71, Japan).

Statistical analysis

IBM SPSS 19.0 software was used for the statistical analysis of all experimental data, and all data were expressed as mean ± sd. Graphpad Prism version 7.0 software was used to visualize the statistical analysis results. T-test was used for comparison between the two group, while one-way ANOVA analysis was used for comparison between the multiple groups. LSD-t test was used for pairwise comparison within the group. $P<0.05$ was considered statistically significant.

Results

The association between BRAF mutation and the clinical features of CRC patients

To understand the functional significance of BRAF mutation in CRC, we first investigated the association of BRAF mutation with the survival of CRC patients and did not found an association with OS, progression-free survival (PFS), or disease-free survival (DFS) (**Figure 1A**). Subsequently, we performed subgroup analysis for OS according to the pathological stages. Interestingly, BRAF mutation was closely associated with worse OS of patients with stage II CRC (**Figure 1B**). Additionally, we analyzed the association of BRAF mutation with other clinical features and found that BRAF mutation was associated with age and pathological stage. Patients with BRAF mutation was older than those without BRAF mutation. Furthermore, BRAF mutation occurred in early pathological stages (**Table 1**), suggesting a correction between BRAF mutation and the progression of CRC.

Differentially expressed genes in patients with BRAF mutation

To investigate the molecular characteristics of BRAF-mutated CRC, we screened DEGs between the BRAF-mutated patients and the patients with wildtype BRAF and identified 207 upregulated as well as 158 downregulated DEGs in BRAF-mutated patients (**Figure 2A**, **2B**). Furthermore, we assessed the biological functions of these DEGs by GO and KEGG pathway analysis and observed that these DEGs were closely related to important signaling pathways or annotations that were involved in cancer progression such as Wnt pathway, Hippo pathway, Vitamin digestion, and cytokine receptor interaction (**Figure 2C**).

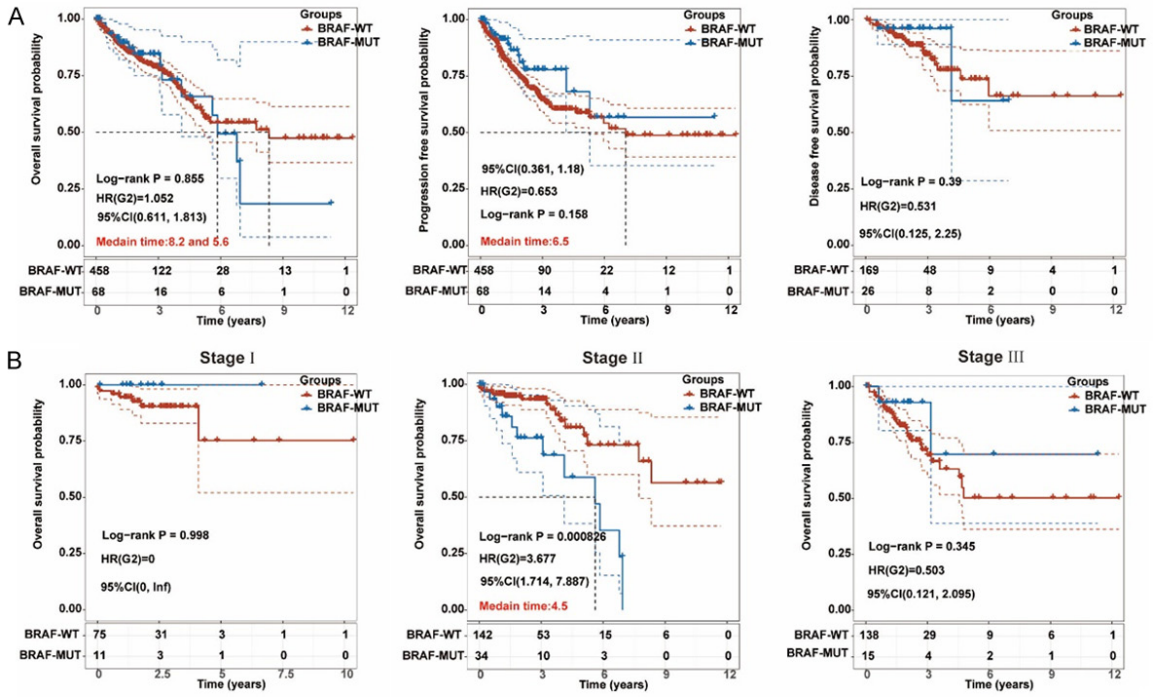

Figure 1. The association of BRAF mutation with the clinical features of CRC patients. A. The association of BRAF mutation with the survival of CRC patients analyzed by Kaplan-Meier method. OS: overall survival; PFS: progression-free survival; DFS: disease-free survival. B. Subgroup analysis for OS according to the pathological stages. BRAF mutation was associated with OS of patients with stage II CRC.

BRAF mutation promoted the mobility of CRC cells in vitro

To investigate the role of BRAF mutation on CRC cells in vitro, we transfected either wild-type BRAF or BRAF^{V600E} mutant into HCT116 cells (CRC cells harboring wildtype BRAF) and examined the mobility of these cells (**Figure 3A**). Empty vector was also transfected as negative control (HCT116-NC). We found that both the invasion and migration of HCT116-BRAF^{V600E} cells were significantly increased compared with both HCT116-BRAF^{wt} and HCT116-NC cells (**Figure 3B**). As epithelial-mesenchymal transition (EMT) is one of the most important process for cells to acquire invasive ability, we further determined the expression of major EMT makers in these transfected HCT116 cells [19]. The results demonstrated that while the mRNA level of E-cadherin was downregulated, the expression of mesenchymal markers including Vimentin, Snail, Twist and Slug were all upregulated in both HCT116-BRAF^{wt} and HCT116-BRAF^{V600E} cells compared with that in HCT116-NC cells (**Figure 3C**). Similarly, the protein level of E-cadherin was downregulated, but the protein levels of Vimentin and Snail were upregulated in both HCT116-BRAF^{wt} and HCT116-BRAF^{V600E} cells compared with that in HCT116-NC cells, as determined by western blot analysis (**Figure 3D**).

BRAF mutation promoted the proliferation and chemoresistance of CRC cells in vitro

To investigate the effect of BRAF^{V600E} mutation on the proliferation of CRC cells, CCK-8 assay for cell viability was performed. Compared with both HCT116-BRAF^{wt} and HCT116-NC cells, the proliferation of HCT116-BRAF^{V600E} cells was significantly increased (**Figure 4A**). Furthermore, we assessed the drug sensitivity of these cells to chemotherapeutic reagents, 5-FU (5 ug/ml, 24 h) and oxaliplatin (50 uM, 24 h). HCT116-BRAF^{V600E} cells showed significant resistance to both 5-FU and oxaliplatin treatments (**Figure 4B**). Meanwhile, we also performed clonogenic assay to evaluate the colony formation potential of these cells. In line with the effect on cell proliferation, the colony formation ability of HCT116-BRAF^{V600E} cells was significantly increased compared with both HCT116-BRAF^{wt} and HCT116-NC cells (**Figure 4C**).

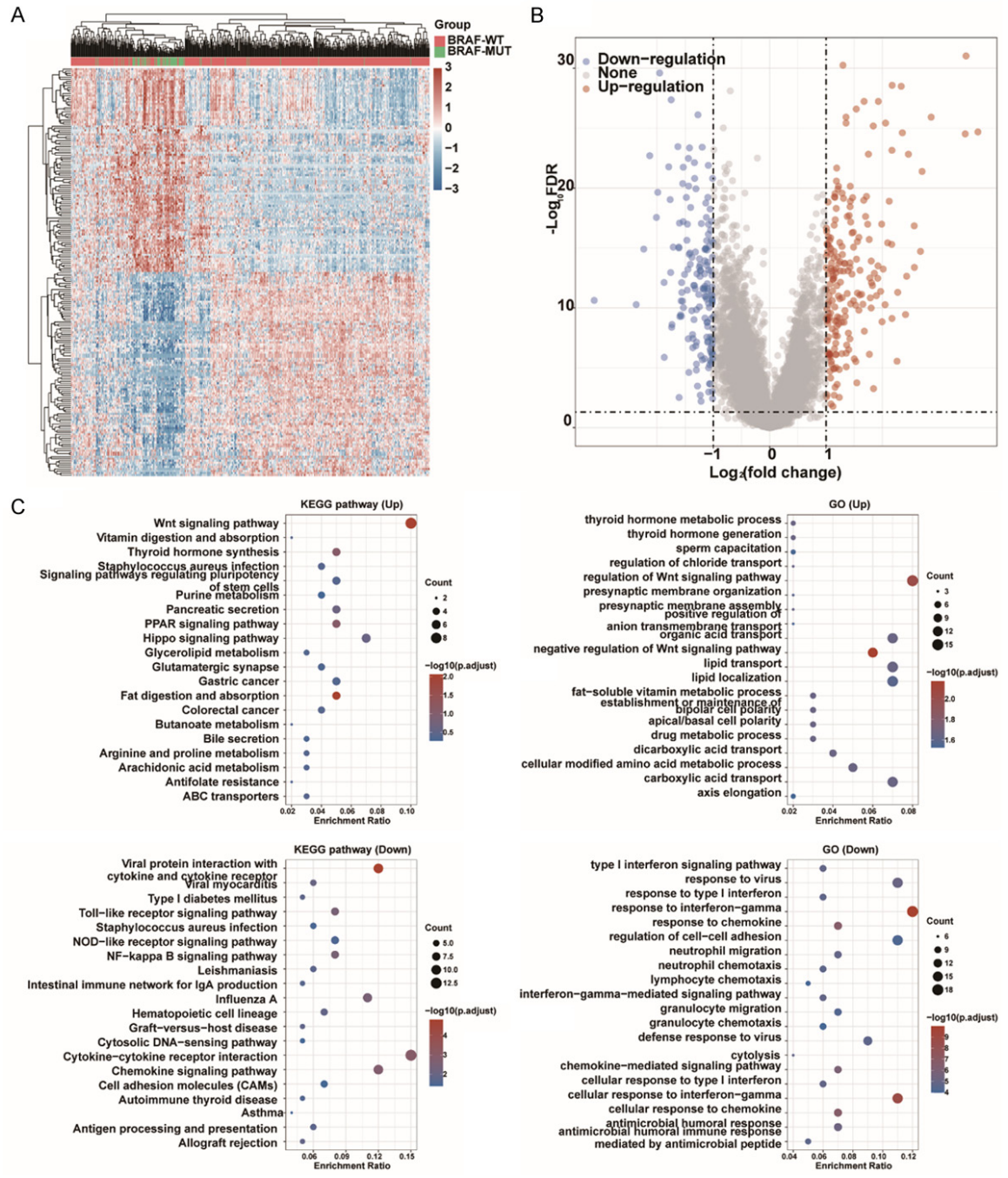

Figure 2. DEGs in patients with BRAF mutation. A. The heatmap of transcript expression in patients with wildtype and mutant BRAF derived from TCGA database. B. The volcano map of screened DEGs. The threshold of P<0.05 and Fold-change >1.5 or Fold-change <-1.5 was used for the DEGs. C. KEGG pathway and GO analysis of screened DEGs.

BRAF mutation promoted the growth and chemoresistance of subcutaneous xenografts in nude mice

We further investigated the physiological effect of BRAF^{V600E} mutation by using subcutaneous xenograft tumor model in nude mice. Consistent with the findings from our in vitro assays, tumors derived from implanted HCT116-BRAF^{V600E} cells showed significantly increased growth compared with tumors derived from HCT116-BRAF^{wt} and HCT116-NC cells. Im-

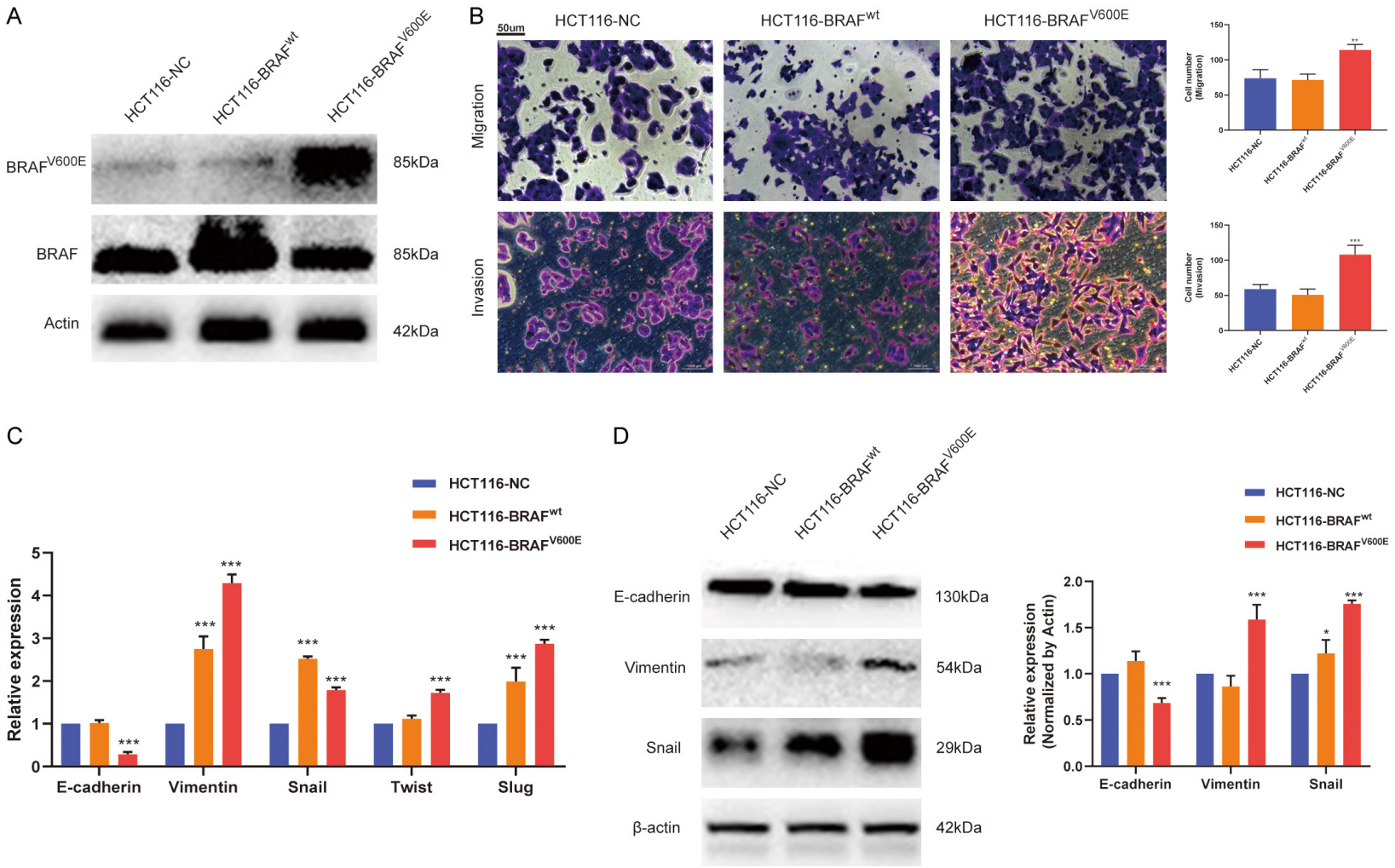

Figure 3. BRAF mutation promoted the migration and invasion of CRC cells in vitro. A. The ectopic expression of wildtype BRAF and BRAF^{V600E} in HCT116 cells were confirmed by western blot analysis. B. The migration and invasion of the indicated HCT116 cells were determined by Transwell assay (magnification: 200×). C. The mRNA expression of EMT markers measured by RT-qPCR. D. The protein expression of EMT markers measured by western blot (*P<0.05, ***P<0.001).

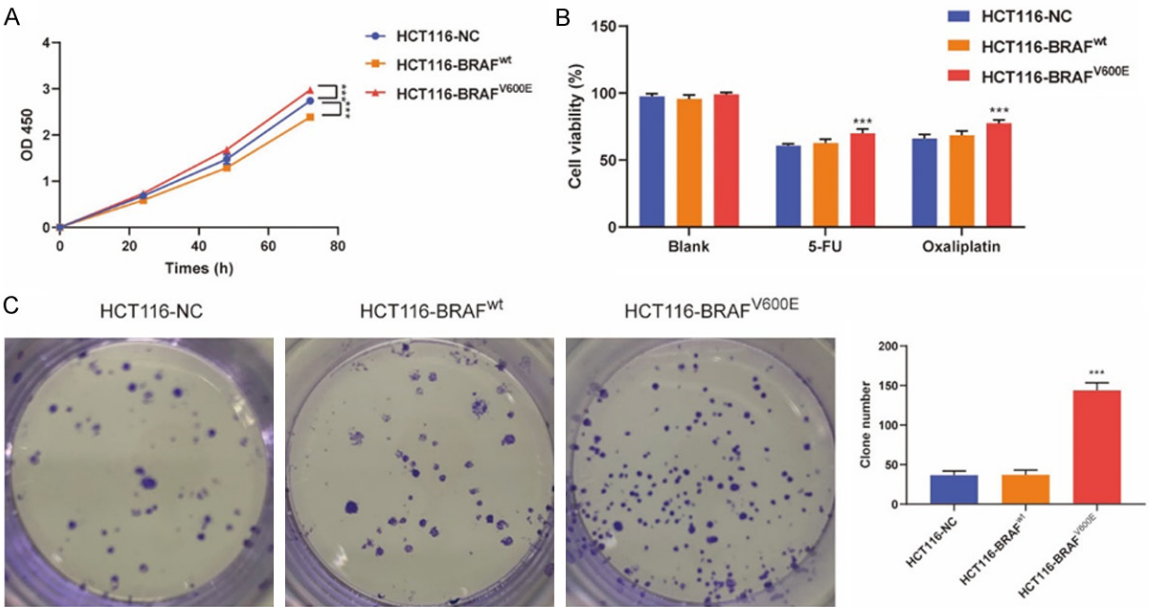

Figure 4. BRAF mutation promoted the proliferation and chemoresistance of HCT116 cells. A. The proliferation of HCT116 cells ectopically expressing wildtype or mutant BRAF. Vector transfected HCT116 cells (NC) were also assayed. B. The chemosensitivity of HCT116 cells to the 24 h treatment of chemotherapeutic agents 5-FU (5 ug/ml) and oxaliplatin (50 uM). C. The colony formation of HCT116 cells as determined by clonogenic assay (magnification: 1×) (***P<0.001).

portantly, the difference was more significant after treatment with 5-FU (3 mg/kg). HCT116-BRAF^{wt} and HCT116-NC xenografts showed satisfactory response to 5-FU, whereas HCT-116-BRAF^{V600E} xenografts exhibited strong resistance to 5-FU (**Figure 5A**). We further measured the proliferating index Ki67 in the tumors from each group by IHC, and the results supported the findings on tumor growth (**Figure 5B**).

Discussion

The prognosis of CRC patients with BRAF mutation is poor, as the efficacy of existing treatments is suboptimal. Therefore, the detection and the treatment of patients with BRAF mutation present a great challenge in clinic, and it is urgent to understand the pathogenesis and metastasis pathway as well as identify new therapeutic targets [20, 21].

To determine the association between BRAF mutation and the survival of CRC patients, we extracted clinical information from the TCGA database. Although BRAF mutation showed no significant association with the survival of all CRC patient population, further delaminating the patients based on the pathological stage indicated that BRAF mutation was significant associated with worse OS of stage II CRC patients, which was consistent with previous studies and suggested that BRAF mutation might promote the progression of CRC [12].

To further explore the molecular characteristics of BRAF-mutated CRC, we screened DEGs between BRAF-mutated and BRAF^{WT} CRC and identified 207 upregulated as well as 158 downregulated DEGs, some of which were involved in Wnt pathway, Hippo pathway, Vitamin digestion, and cytokine receptor interaction. It has been well documented that these signaling pathways play important role in the development of CRC. When Wnt signal is inactivated, adenomatous polyposis gene (APC) complex phosphorylates β-catenin, leading to the degradation of β-catenin, which prevents the deposition of β-catenin in the nucleus as well as the activation of the transcription factor (TCF), resulting in the differentiation and the homeostasis of colon epithelial cells [22]. Similarly, Hippo pathway coordinates with Wnt pathway to reprogram cancer stem cells, thus regulating the growth and metastasis of CRC [23]. On the other hand, high vitamin D receptor expression in tumor stromal fibroblasts was associated with better OS and RFS in CRC, independently of its expression in carcinoma

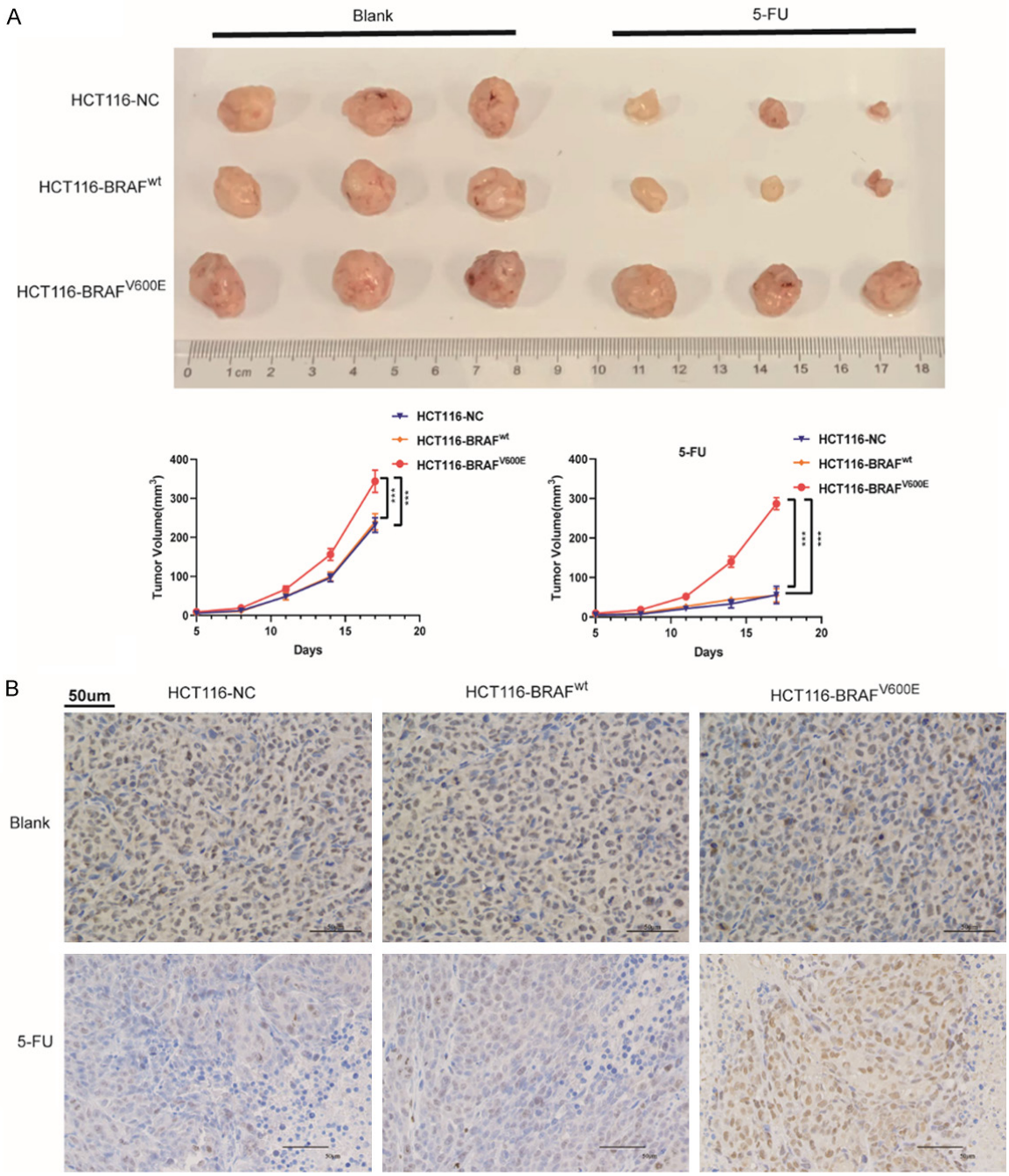

Figure 5. BRAF mutation promoted the growth and chemoresistance of subcutaneous xenografts in nude mice. A. The growth of xenograft tumor in nude mice with or without 5-FU (3 mg/kg, i.p.) treatment. B. The expression of Ki67 in the tumor samples of each group (magnification: 100×) (***P<0.001).

cells [24]. The secretion of a series of cytokines and the activation of their receptors regulate the infiltration of immune cells and tumor immune escape, thereby leading to the progression of tumor [25]. Clearly, various cancer promoting pathways and molecules are activated by BRAF mutation.

To further reveal the effect of BRAF mutation on CRC cells in vitro, we overexpressed wild-type BRAF and BRAF^{V600E} mutant in CRC HCT116 cells and investigated their influence on the migration and invasion of HCT116 cells. The BRAF^{V600E} mutant significantly promoted the mobility of HCT116 cells. As we know, EMT

refers to the biological process that epithelial cells transform into cells with mesenchymal phenotype through specific processes. EMT plays an important role in cancer metastasis and progression and is characterized by the decrease in the expression of cell adhesion molecules (E-cadherin), the transformation of cytokeratin cytoskeleton into Vimentin dominated cytoskeleton, and the morphological changes into mesenchymal cells [26]. Since we speculated that BRAF^{V600E} mutation might promoted the cell invasion through inducing EMT, we measured the expression of EMT markers: E-cadherin, Vimentin, Snail, Slug, and Twist. As expected, the BRAF^{V600E} mutation decreased the expression of E-cadherin, but increased the expression of Vimentin, Snail, Slug and Twist, supporting the notion that BRAF^{V600E} mutation promoted the cancer invasion through inducing EMT. Interestingly, although we found that overexpression of wildtype BRAF elevated the mRNA level of several key EMT transcriptional factors (Snail, Slug, etc.) as well as Snail protein level, the other two main EMT markers E-cadherin and Vimentin was not affected, suggesting that wildtype BRAF also potentially promoted some transcriptional factors of EMT, but only mutant BRAF (BRAF^{V600E}) eventually lead to the process of EMT.

Furthermore, we investigated the effect of BRAF^{V600E} mutation on the proliferation and the chemosensitivity of HCT116 cells. The BRAF^{V600E} mutation not only significantly promoted the proliferation of HCT116 cells, but also significantly caused the chemoresistance to 5-FU and oxaliplatin, the two main chemotherapeutic agents for CRC treatment. This BRAF^{V600E} mutation-associated chemoresistance was supported by a previously reported clinical study [16]. Significantly, we further validated our in vitro findings in mouse xenograft tumor model and revealed that BRAF^{V600E} mutation significantly promoted the tumor growth as well as the chemoresistance to 5-FU in vivo.

Conclusions

BRAF^{V600E} mutation influences the malignancy of CRC both in vitro and in vivo. BRAF^{V600E} mutation not only promoted the migration and invasion of CRC cells through activating EMT but also enhanced the growth and chemoresistance of CRC. Our findings may help determine therapeutic strategies for the treatment of CRC patients with of BRAF mutation.

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Disclosure of conflict of interest

None.

Abbreviations

CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; 5-FU, 5-Fluorouracil; DFS, disease-free survival; OS, overall survival; PFS, progression-free survival.

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