Human Voltage-Gated Proton Channel Hv1: A New Potential Biomarker for Diagnosis and Prognosis of Colorectal Cancer

Yifan Wang1,2,*, Xingye Wu3, Qiang Li1,2, Shangrong Zhang1,2, Shu Jie Li1,2*

1 Department of Biophysics, School of Physics Science, Nankai University, Tianjin, China, 2 Department of Pathology, Tonghua Center Hospital, Tonghua, China

Abstract

Solid tumors exist in a hypoxic microenvironment, and possess high-glycolytic metabolites. To avoid the acidosis, tumor cells must exhibit a dynamic cytosolic pH regulation mechanism(s). The voltage-gated proton channel Hv1 mediates NADPH oxidase function by compensating cellular loss of electrons with protons. Here, we showed for the first time, that Hv1 expression is increased in colorectal tumor tissues and cell lines, associated with poor prognosis. Immunohistochemistry showed that Hv1 is strongly expressed in adenocarcinomas but not or lowly expressed in normal colorectal or hyperplastic polyps. Hv1 expression in colorectal cancer is significantly associated with the tumor size, tumor classification, lymph node status, clinical stage and p53 status. High Hv1 expression is associated significantly with shorter overall and recurrence-free survival. Furthermore, real-time RT-PCR and immunocytochemistry showed that Hv1 is highly expressed in colorectal cancer cell lines, SW620, HT29, LS174T and Colo205, but not in SW480. Inhibitions of Hv1 expression and activity in the highly metastatic SW620 cells by small interfering RNA (siRNA) and Zn2+ respectively, markedly decrease the cell invasion and migration, restraints proton extrusion and the intracellular pH recovery. Our results suggest that Hv1 may be used as a potential biomarker for diagnosis and prognosis of colorectal carcinoma, and a potential target for anticancer drugs in colorectal cancer therapy.

Introduction

The voltage-gated proton channel Hv1 was identified using bioinformatics searches based on known cation channels, which is mainly expressed in immune cells such as macrophages, neutrophils, and eosinophils [1,2]. Hv1 in mammalian phagocytes was proposed to be responsible for the proton-transporting pathway, which regulates intracellular pH during oxygen consumption associated with phagocytosis, called “respiratory burst” [3,4]. Hv1 is activated by depolarization and intracellular acidification, whose activity maintains intracellular pH neutral to keep reactive oxygen species (ROS) generation [5,6]. Hv1 not only regulates pH in cytoplasm, but can also provide protons in the phagosome, a closed membrane compartment for killing and digestion of a pathogen [3]. Hv1 is extremely selective for H+, with no detectable porosity to other cations [7,8]. The voltage activation relationship of Hv1 depends strongly on both the intracellular pH (pHi) and extracellular pH (pHe). Increasing pHi or lowering pHe promotes H+ channel opening by shifting the activation threshold to more negative potentials [3]. Furthermore, Hv1 current is inhibited by submillimolar concentrations of Zn2+ and Cd2+ and other divalent cations [9].

Hv1 contains three predicted domains: N-terminal acid and proline-rich domain, transmembrane voltage-sensor domain (VSD), and C-terminal domain. Voltage-gated K+ channels are comprised of four subunits, each of which has a pore domain and a VSD. The four pore domains come together to form one single central pore, and four peripheral VSDs control the gate of the pore [10]. In contrast to the voltage-gated K+ channels, the Hv1 contains a VSD but lacks the pore domain. Recent studies showed that Hv1 functions as a dimer in which the intracellular C-terminal domain is responsible for the dimeric architecture of the protein, and each subunit contains its own proton-transporting pathway [11–14]. The intracellular C-terminal domain of Hv1 forms a dimer via a parallel z-helical coiled-coil and is essential for the protein localization [14].

Tumor cells often exist in a hypoxic microenvironment, and possess high-glycolytic activity and produce acidic metabolites [15,16]. To avoid the acidosis resulting from reducing in cytosolic pH, tumor cells must extrude excessive cytosolic protons to maintain cytosolic pH, which results in acidic tumor microenvironment. The hypoxic and acidic tumor microenvironment plays a key role in cancer development, progression, and metastasis [15]. Our previous work showed that Hv1 is specifically expressed in highly metastatic human breast tumor tissues and cell lines, and promotes breast cancer cell progression and metastasis, through regulating breast cancer cell intracellular pH [17,18]. In the present study, we investigated the expression of Hv1 in colorectal
tumor tissues and cell lines and its potential association with clinicopathological features and post-resectional survival. Inhibitions of Hv1 expression and activity in the highly metastatic colorectal cancer cells markedly decrease the cell invasion and migration, restraint proton extrusion. Our results suggest that Hv1 over-expression may be used as an independent biomarker for the prognosis and diagnosis of patients with colorectal cancer.

Materials and Methods

Ethics Statement

All of the procedures were done in accordance with the Declaration of Helsinki and relevant policies in China. We obtained the written informed consent from all participants involved in our study. The study obtained ethics approval for our study from the ethics committee of Tonghua Center Hospital.

Patients and samples

Colorectal cancer tissue samples were obtained from patients who underwent routine curative surgery at the Department of Surgery, Tonghua Center Hospital between 2001 and 2007. The patients were not pretreated with radiotherapy or chemotherapy prior to surgery. 139 colorectal cancer tissues and paired adjacent non-tumor colorectal tissues were fixed in 10% formalin and embedded in paraffin for immunohistochemical analysis. The clinicopathological features of these patients were shown in Table 1. In addition, to verify the expression of Hv1 in premalignant dysplastic lesions, 10 normal colorectal, 18 hyperplastic polyp and 20 adenoma tissues were also examined using immunohistochemistry. Each patient’s clinical status was classified according to the pathologic tumor grade, tumor size, lymph node status. Tumor differentiation was classified by the Edmonds grading system. This study was approved by the Ethics Committee of Tonghua Center Hospital, and informed consent was obtained from each patient.

Generation of an anti-Hv1 polyclonal antibody

An anti-Hv1 polyclonal antibody was generated against the carboxyl terminal domain of Hv1 (residues 221-273 of Hv1, KTRSEQQLRLKQMNQVIAAIKIQHLEFSCSEKE-QEIERLKNKLKQHGILGEVN). The protein was purified to homogeneity after expression in Escherichia coli [19]. The purified protein was injected into mice and the anti-Hv1 polyclonal antibody was purified by pProtein A Sepharose (GE, Healthcare) by wetting electroblotting devices. Nonspecific protein absorption was prevented using 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 1 h. Primary antibody incubation in PBS-T was performed for 1 h at room temperature. The HRP-coupled anti-mouse secondary antibody was used at a final dilution of 1:1000. The denatured proteins were separated by 12.5% SDS-PAGE and then transferred to a PVDF membrane (GE, Healthcare) by wetting electroblotting devices. Nonspecific protein absorption was prevented using 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 1 h. Primary antibody incubation in PBS-T was performed for 1 h at room temperature. The HRP-coupled anti-mouse secondary antibody was used at a final dilution of 1:1000 in PBS-T, and HRP was revealed with a chemiluminescent detection system (Millipore).

Immunohistochemistry

Histological diagnoses of tumourous and non-tumourous formalin-fixed and paraffin-embedded tissues were confirmed in haematoxylin and eosin-stained sections. Immunohistochemistry was performed with an anti-Hv1 polyclonal antibody (1 mg/ml) as described above with a final dilution of 1:1000. The denatured proteins were separated by 12.5% SDS-PAGE and then transferred to a PVDF membrane (GE, Healthcare) by wetting electroblotting devices. Nonspecific protein absorption was prevented using 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 1 h. Primary antibody incubation in PBS-T was performed for 1 h at room temperature. The HRP-coupled anti-mouse secondary antibody was used at a final dilution of 1:10000 in PBS-T, and HRP was revealed with a chemiluminescent detection system (Millipore).

Table 1. Clinicopathological parameters.

| Characteristics          | No. of patients (%) | n = 139 |
|-------------------------|---------------------|---------|
| Gender                  |                     |         |
| Male                    | 68 (48.9)           |         |
| Female                  | 71 (51.1)           |         |
| Age (yr) median 62.4, range 36-76 |   |         |
| >62                     | 79 (56.8)           |         |
| ≤62                     | 60 (43.2)           |         |
| Primary site            |                     |         |
| Left colon              | 29 (20.9)           |         |
| Right colon             | 43 (30.9)           |         |
| Rectum                  | 67 (48.2)           |         |
| Tumor size (cm)         |                     |         |
| <5                      | 72 (51.8)           |         |
| ≥5                      | 67 (48.2)           |         |
| Tumor classification    |                     |         |
| T1                      | 5 (3.6)             |         |
| T2                      | 40 (28.8)           |         |
| T3                      | 85 (61.1)           |         |
| T4                      | 9 (6.5)             |         |
| Lymph node status       |                     |         |
| N0                      | 80 (57.5)           |         |
| N1                      | 44 (31.7)           |         |
| N2                      | 15 (10.8)           |         |
| Distance metastasis     |                     |         |
| M0                      | 132 (95.0)          |         |
| M1                      | 7 (5)               |         |
| Clinical stage          |                     |         |
| I/II                    | 78 (56.1)           |         |
| III/IV                  | 61 (43.9)           |         |
| Differentiation         |                     |         |
| Well                    | 61 (43.9)           |         |
| Moderately              | 58 (41.7)           |         |
| Poorly                  | 20 (14.4)           |         |

Western blotting

Western blotting was performed with an anti-Hv1 polyclonal antibody (1 mg/ml) as described above with a final dilution of 1:1000. The denatured proteins were separated by 12.5% SDS-PAGE and then transferred to a PVDF membrane (GE, Healthcare) by wetting electroblotting devices. Nonspecific protein absorption was prevented using 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 1 h. Primary antibody incubation in PBS-T was performed for 1 h at room temperature. The HRP-coupled anti-mouse secondary antibody was used at a final dilution of 1:1000 in PBS-T, and HRP was revealed with a chemiluminescent detection system (Millipore).

Expression vector and transfection

Hv1 cDNA was cloned into pEGFP-N1 (Clontech) to create Hv1 expression plasmid pHv1-EGFP, fused with the enhanced green fluorescent protein (EGFP) moiety attached to the carboxyl terminal of Hv1 [14]. 293 T cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium; GIBCO) with 10% fetal bovine serum plus antibiotics (100 units/ml penicillin and 100 g/ml streptomycin, GIBCO) in a 5% CO2 incubator at 37°C. Cells grown on glass coverslips at 50–70% confluence in a six-well plate were transiently transfected with pHv1-EGFP plasmid by using lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cells were used for experiments 36–48 h after transfection.

Histological diagnoses of tumourous and non-tumourous formalin-fixed and paraffin-embedded tissues were confirmed in haematoxylin and eosin-stained sections. Immunohistochemistry was performed with an anti-Hv1 polyclonal antibody. The anti-Hv1 antibody (1.0 mg/ml) was diluted 100-fold with PBS (phosphate-buffered saline containing 0.1% Tween-20) containing 1% (w/v) BSA. The paraffin-embedded sections filled with 10 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) were heated in a microwave oven for 12 min. After cooling, the sections were treated with 0.5% Triton X-100 in PBS for 10 min, exposed to 3% (v/v) hydrogen peroxide (H2O2) for 10 min to inhibit endogenous peroxidase activity. Subsequently, the sections were incubated in PBS-T containing 5% fetal bovine serum and
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2% BSA for 30 min to reduce nonspecific binding. Incubation with primary antibody was performed overnight at 4°C in a humidified chamber. HRP-coupled anti-mouse secondary antibody was used at a final dilution of 1:400 for 1 h. Finally, the visualization signal was developed with diaminobenzidine (DAB) and the slides were counterstained in hematoxylin. Negative control was performed to treat with non-immune mouse serum as the primary antibody instead of anti-Hv1 antibody.

Stained sections were evaluated in a blinded manner without prior knowledge of the clinical information using the German immunoreactive score, Immuno-Reactive-Score (IRS). Briefly, the IRS assigns sub-scores for immunoreactive distribution (0–4) and intensity (0–3), then multiplies them to yield the IRS score. The percent positivity was scored as “0” (<5%), “1” (5–25%), “2” (25–50%), “3” (50–75%), “4” (>75%). The staining intensity score was calculated from the area of Hv1-positive staining cells as “0, −” (negative, <5%), “1, +” (weakly positive, 5–25%), “2, ++” (positive, 25–50%), and “3, +++” (strongly positive, >50%). The final Hv1 expression score was calculated from the values of percent positivity score and staining intensity score, which was ranged from 0 to 12. We estimated IRS by averaging the values in eight fields at ×500 magnification for each specimen. Hv1 expression levels were defined as follows: low expression (score ≤3) and high expression (score >3). Immunohistochemical analysis and scoring were performed by two independent experienced pathologists.

Cell culture

The human colorectal cancer cell lines SW620, HT29, LS174T, Colo205 and SW480 were cultivated at 37°C in an atmosphere of 95% air and 5% CO2 with DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine.

Immunocytochemistry

SW620, HT29, LS174T, Colo205 and SW480 cells grown on glass coverslips at confluence in six-well tissue culture plates were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature for 30 min, washed in PBS, treated with 0.5% Triton X-100 in PBS for 20 min, and blocked with 5% fetal bovine serum and 2% BSA in PBS for 20 min at room temperature. The blocked coverslips were incubated with the anti-Hv1 antibody (1.0 mg/ml) at a dilution of 1:200 in 2% BSA at 4°C overnight. After washing with PBS for 5 min for four times, the coverslips were further incubated for 1 h at room temperature with a FITC-conjugated goat anti-mouse IgG at a dilution of 1:400 in 2% BSA, followed by another washing as described above. Confocal images of FITC fluorescence of SW620 and SW480 cells were recorded on a Leica TCS SP5 confocal microscope (LEICA, Germany) with the FITC-filter set for Hv1 and the DAPI filter set for the nuclear DAPI dye. The images were later processed by Adobe Photoshop software.

Suppressing Hv1 mRNA expression

The sequence of the siRNA targeting the Hv1 gene was 5'-CTACAGAGAATGGGAGAAT-3', and the random sense sequence was 5'-TTCTCCGAAGCTGTCAGT-3', both of which were obtained from Ribobio (Guangzhou, China) [17]. SW620 cells grown at confluence were passed in 6-well plates at 50% confluence and incubated overnight, then transfected with the siRNA and the negative control, respectively, using lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. The final concentration of siRNA was 100 nM. Silencing was examined 48 h after transfection. The efficiency of siRNA in suppressing Hv1 expression was determined by quantitative real-time PCR, immunocytochemistry and western blotting using anti-Hv1 antibody as described above.

Migration kinetics

To examine the effect of Hv1 on the migratory ability of colorectal cancer cells, migrations of SW620, SW480, and SW620 cells down-regulated Hv1 expression and inhibited Hv1 activity by siRNA and Zn2+, respectively, were assessed in wounded monolayer model. To down-regulate Hv1 expression, SW620 cells were grown to confluence and transfected with siRNA and negative control for 24 h, respectively. To inhibit Hv1 activity, 1 M ZnCl2 solution was added into the DMEM medium to a final concentration of 100 μM [1,2]. The SW620 and SW480 cells were planted in a 24-well plate (1.5×10^5 per well) and cultured for 24h to confluence and subsequently wounded with a tip. Cell movement was observed under phase-contrast microscopy, and were captured with a digital camera every 12 h.

Invasion and migration assays

In vitro invasion and migration assays were performed to assess the effects of Hv1 on invasive and migratory abilities of SW620 and SW480 cells. SW620 and SW480 cells were cultured in six-well plate in DMEM medium with 10% FBS at confluence, transfected with the siRNA and negative control respectively for 24 h, and then trypsinized, washed and counted. For cell invasion, transwells with 8 μm pore size filters (Millipore) were covered with matrigel (Becton Dickinson) and inserted into 24-well plates. And for cell migration, the transwells were not coated with matrigel.
DMEM medium (500 μl) containing 10% FBS was added to the lower chamber, and 200 μl of a cell suspension (5×10^6 cells) was placed in the upper chamber. The plates were incubated at 37°C in humidified atmosphere containing 5% CO2 for 24 h. To inhibit Hv1 activity, 1 M ZnCl2 solution was added into the DMEM medium to a final concentration of 100 μM [1,2]. The penetrated cells were fixed by paraformaldehyde, stained with crystal violet solution, and photographed. Each experiment was conducted four times. The migration and invasion rates were calculated as [migration cell No. of test/migration cell No. of control]×100% and [invasion cell No. of test/invasion cell No. of control]×100%, respectively.

**Activity of Hv1 channel**

The activity of Hv1 in colorectal cancer cells was assessed as a change in intracellular pH (pHi) in response to membrane depolarization by BCECF fluorescence [11]. The cells were incubated with 3.0 μM of BCECF-AM (Molecular Probe) in serum-free DMEM medium for 30 min, respectively, and washed with PSS solution (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 20 mM Tris, pH 7.5) for 3 times. Cells were incubated with NH4Cl/NMDG (N-methyl D-glucosamine) solution (100 mM NMDG, 40 mM NH4Cl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 20 mM Tris, pH 7.5) for 20 min and washed with ammonium free solution (140 mM NMDG, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 20 mM Tris, pH 7.4), rapidly inducing intracellular acidification [5]. Membrane depolarization was achieved by loading high K+ solution (145 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 20 mM Tris, pH 7.5). Intracellular pH changes acid-loaded cells were detected by fluorescent probe BCECF at excitation wavelengths of 490 nm and 440 nm and an emission wavelength of 525 nm under membrane depolarizing condition using RF-5301PC Spectrofluorophotometer (Shimadzu, Japan).

**Measurements of intracellular pH**

Intracellular pH was measured using the pH sensitive fluorescent probe BCECF-AM. Cells cultured in the monolayers were incubated with 3.0 μM of BCECF-AM (Molecular Probe) in bicarbonate-free DMEM medium at 37°C for 30 min. After loading, the cells were washed three times with HEPES buffer to remove the extracellular dye, and made to remain in the identical buffer. The HEPES buffer contained 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM Na2HPO4, 5.5 mM glucose, and 20 mM HEPES, pH 7.4. The fluorescence at excitation wavelengths of 490 nm and 440 nm was recorded at an emission wavelength of 525 nm using RF-5301PC Spectrofluorophotometer (Shimadzu, Japan). Calibration of fluorescence vs pH was performed by equilibration of external and internal pH with nigerinic (10 μM) in a high K+ buffer with a range of pH from 5.5 to 8.0. The high K+ buffer contained 145 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, and 20 mM HEPES (or MES). The relative fluorescence ratio values were plotted against corresponding pHi values, which allowed determination of the unknown pHi.

**Statistical analysis**

All statistics were performed using SPSS16.0 software. Measurement data was represented as mean ± SD. Comparison of the mean between groups was performed by t test. P values <0.05 were considered significant. Survival analysis was assessed using Kaplan-Meier method and survival rate was compared by log-rank test.

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**Results**

**Clinicopathologic findings**

Clinicopathologic profiles from the 139 cases selected for this study were reviewed in Table 1. The average age of the patients was 62.4 years (range, 36–76), including 68 males and 71 females. The locations of their cancers were 29 left-sided and 43 right-sided colon, and 47 rectum cases, respectively. Among the 139 resected cases, the primary tumor size varied as follows: <5 cm in 72 cases, and ≥5 cm in 67 cases. Twenty of 139 tumors showed poor cytological differentiation. The tumor extent was limited (T1 or T2) in 45 cases and advanced (T3 or T4) in 94 cases. Tumor metastasis to the lymph nodes was observed in 59 of 139 cases.

**Increased expression of Hv1 in colorectal cancer**

Hv1 expressed in immune cells is associated with “respiratory burst” [3,4], but its function in tumorigenesis has not been identified. To investigate Hv1 for use as a potential biomarker and therapeutic target for colorectal cancer, Hv1 expression in 139 colorectal cancer tissues and paired normal tissues, 10 normal colorectal, 20 colorectal adenoma and 18 hyperplastic polyp tissues, was detected using immunohistochemistry with an anti-Hv1 polyclonal antibody that was generated in house. To examine the specificity of the antibody, 293 T cells were transfected with pHv1-EGFP expression plasmid. And the expression of Hv1-EGFP was detected by immunocytochemistry and western blotting with the antibody, and EGFP fluorescence. The results showed that the antibody specifically recognizes Hv1 and EGFP is a marker for Hv1 expression (Fig. 1A and B).

As shown in Fig. 1C and Table 2, Hv1 staining was mainly moderate or strong positive in colorectal cancer tissues, but not in normal colorectal and hyperplastic polyp tissues. Hv1 was mainly observed in the plasma membrane of tumor cells in colorectal tissues, as shown in Fig. 1C [h, j and l] (as indicated by arrowheads). In colorectal adenoma tissues, the staining was negative or weakly positive (Fig. 1C, e and f; Table 2). Hv1 in colorectal cancer tissues was significantly expressed compared with that in normal colorectal, hyperplastic polyps and adenoma tissues, suggesting that Hv1 may be involved in colorectal tumorigenesis. Overall, 106 of the 139 (76.3%) cases showed high expression Hv1 in the tumor tissues (IRS over 3), while 33 (23.7%) of the cases showed low expression (IRS 0–3). Generally, Hv1 density was significantly higher in cancer tissues than in adenoma tissues (7.20±3.25 versus 2.20±2.12) (Table 2).

**High Hv1 expression is associated with a poor prognosis**

The correlations between Hv1 expression and clinicopathologic characteristics are summarized in Table 3. There were significant associations with the depth of tumor classification (P=0.007), age (P=0.021), tumor size (P=0.000), lymph node status (P=0.000), clinical stage (P=0.000) and p53 status (P=0.014) in patients who had high Hv1 expression compared with patients who had low Hv1 expression. There was no significant association between Hv1 expression and the other clinical features, such as gender, differentiation and Ki-67 expression. In addition, the correlations between the expression levels of p53, Ki-67, TopoII, GST-π and P-gp and clinicopathologic characteristics were showed in Table 4. p53 status related to tumor classification (P=0.011), Ki-67 to differentiation (P=0.010), TopoII to differentiation (P=0.010), and GST-π to tumor size (P=0.007) and differentiation (P=0.000). However, P-gp did not show statistical significance with clinicopathologic parameters.

Kaplan-Meier survival curves showed that patients who had high Hv1 expression were more likely to have a shorter overall...
survival ($P = 0.008$, Fig. 2A) and recurrence-free survival ($P = 0.008$, Fig. 2B) compared with patients who had low Hv1 expression, suggesting that Hv1 over-expression may be associated with a poor clinical prognosis. Patients who had high Hv1 expression had a poor recurrence-free survival ($P = 0.008$) compared with patients who had low Hv1 expression (univariate analysis) (Table 3). Overall survival examined by Cox univariate analysis also indicated that high expression of Hv1 was significantly associated with shorter survival ($P = 0.008$). Univariate Cox regression analyses showed that Hv1 expression level was significantly associated with recurrence-free and overall survival, whereas other clinical characteristics lost their predictive significance. Furthermore, multivariate Cox regression analyses revealed that high expression of Hv1 was independent risk factor for overall survival (relative risk [RR] = 0.443, $P = 0.015$) and recurrence-free survival (RR = 0.427, $P = 0.026$) (Table 6). Patients who had high expression of Hv1 were prone to have an early recurrence compared with patients who had low expression of Hv1 (37.4 ± 3.0 vs 47.2 ± 10.7, $P < 0.001$) (Table 5).

**Figure 1.** The anti-Hv1 antibody was used to detect recombinant Hv1 expressed in 293 T cells and native Hv1 protein in colorectal cancer tissues. A, 293 T cells were transfected with pHv1-EGFP plasmid and observed by a Leica TCS SP5 focal microscopy. a, observation by EGFP fluorescence. Hv1-EGFP fluorescence is represented in green. b, anti-Hv1 immunofluorescence (TRITC wavelengths) (red). c, DAPI stain to visualize the nuclei (blue). d, image merged is composite of EGFP fluorescence, anti-Hv1 immunofluorescence, and DAPI stain. B, 293 T cells were transfected with pcDNA3.1 vector and pcDNA-Hv1 plasmid, respectively. And Hv1 was detected by western blotting. C, Hv1 is expressed in colorectal cancer tissues. a (100 ×) and b (500 ×), c (100 ×) and d (500 ×), normal colorectal tissues; e (100 ×) and f (500 ×), adenoma tissues; g (100 ×) and h (500 ×), i (100 ×) and j (500 ×), k (100 ×) and l (500 ×), colorectal cancer tissues. In normal colorectal tissues, hyperplastic polyps and colorectal adenoma tissues, the staining was negative or weakly positive. In tumor tissues, intense immunoreactivity to Hv1 was observed. Hv1 is mainly observed in plasma membrane (h, j and l) (as indicated by arrowheads). The Hv1 is stained in brown, while the background nuclei are in blue.

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### Table 2. Average density of immunohistochemistry staining.

| Type of tissues | Staining density $P$ |
|-----------------|----------------------|
| Mean            | Standard deviation   | Score range |
| Normal colorectal | 0.00 | 0.00 | 0 | <0.001 |
| Hyperplastic polyps | 1.15 | 0.75 | 0–2 | <0.001* |
| Adenoma | 2.20 | 2.12 | 1–8 | <0.001** |
| Colorectal cancer | 7.20 | 3.25 | 1–12 | <0.001*** |

*, 0.001; **, 0.001; and ***, 0.001 were compared with normal colorectal.
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### Table 3. Correlation between Hv1 expression levels in colorectal cancer and clinicopathological parameters.

| Characteristics | High | Low | $P$ |
|-----------------|------|-----|-----|
| Gender Male | 56(82.4) | 12(17.6) | 0.098 |
| Female | 50(70.4) | 21(29.6) | 0.021 |
| Age (yr) $>62$ | 66(83.5) | 13(16.5) | 0.007 |
| $\leq 62$ | 40(66.7) | 20(33.3) | 0.007 |
| Primary site Left colon | 25(86.2) | 4(13.8) | 0.207 |
| Right colon | 34(79.1) | 9(20.9) | 0.007 |
| Rectum | 47(70.1) | 20(29.9) | 0.006 |
| Tumor size (cm) $<5$ | 46(63.9) | 26(36.1) | 0.007 |
| $\geq 5$ | 60(89.6) | 7(10.4) | 0.007 |
| Tumor classification T1/T2 | 28(62.2) | 17(37.8) | 0.007 |
| T3/T4 | 78(83.0) | 16(17.0) | 0.007 |
| Lymph node status Negative | 52(65.0) | 28(35.0) | 0.007 |
| Positive | 54(91.5) | 5(8.5) | 0.007 |
| Distance metastasis M0 | 99(75.0) | 33(25.0) | 0.007 |
| M1 | 7(100) | 0(0.0) | 0.007 |
| Clinical stage I/II | 50(64.1) | 28(35.9) | 0.007 |
| III/IV | 56(91.8) | 5(8.2) | 0.007 |
| Differentiation Well | 45(73.8) | 16(26.2) | 0.589 |
| Moderately | 44(75.9) | 14(24.1) | 0.589 |
| Poorly | 17(85.0) | 3(15.0) | 0.589 |
| p53 status $\leq 25\%$ | 48(67.6) | 23(32.4) | 0.014 |
| $>25\%$ | 58(85.3) | 10(14.7) | 0.014 |
| Ki-67 status $\leq 25\%$ | 51(73.9) | 18(26.1) | 0.519 |
| $>25\%$ | 55(78.6) | 15(21.4) | 0.519 |
| Topo II status $+/+$ | 81(75.7) | 26(24.3) | 0.077 |
| ++ | 25(78.1) | 7(21.9) | 0.077 |
| GST-π status $+/+$ | 14(77.8) | 4(22.2) | 0.871 |
| $+/++$ | 92(76.0) | 29(24.0) | 0.871 |
| P-gp status $+/+$ | 35(76.1) | 11(23.9) | 0.937 |
| $+/++$ | 71(76.3) | 22(23.7) | 0.937 |

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and disease-free survival than the patients with high expression.

Figure 2. Cumulative overall and disease-free survival curves of patients with high and low expression of Hv1 in colorectal cancer tissues. The patients with low expression have longer overall and disease-free survival than the patients with high expression.

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Table 4. Correlation of Hv1 expression levels in colon cancer with clinicopathological parameters, p53, Ki-67, TopoII, GST-π and P-gp expression levels.

| Characteristics | p53 | Ki67 | Topo II | GST-π | P-gp |
|-----------------|-----|------|---------|-------|------|
|                  | High| Low  | P       | High  | Low  | P    | High  | Low  | P    |
| Gender          |     |      |         |       |      |      |       |      |      |
| Male            | 30(44.1) | 38(55.9) | 0.268 | 36(52.9) | 32(47.1) | 0.551 | 12(17.6) | 56(82.4) | 0.141 | 56(82.4) | 12(17.6) | 0.106 | 44(64.7) | 24(35.3) | 0.589 |
| Female          | 38(53.5) | 33(46.5) | 0.251 | 34(47.9) | 37(52.1) | 0.271 | 18(22.8) | 61(77.2) | 0.939 | 70(88.6) | 9(11.4) | 0.530 | 53(67.1) | 26(32.9) | 0.958 |
| Age (yr)        | >62 | 42(53.2) | 27(46.8) | 72(56.0) | 36(44.6) | 0.271 | 18(22.8) | 61(77.2) | 0.939 | 70(88.6) | 9(11.4) | 0.530 | 53(67.1) | 26(32.9) | 0.958 |
| ≤62             | 26(43.3) | 34(56.7) | 27(45.0) | 33(55.0) | 14(23.3) | 46(76.7) | 51(85.0) | 9(15.0) | 40(66.7) | 20(33.3) | 0.357 |
| Primary site    | Left colon | 15(15.1) | 14(14.8) | 0.415 | 13(14.4) | 16(15.5) | 0.768 | 6(20.7) | 23(79.3) | 0.146 | 26(89.7) | 3(10.3) | 0.720 | 17(58.6) | 12(41.4) | 0.491 |
| Right colon     | 24(45.8) | 25(54.2) | 0.696 | 39(48.8) | 41(51.2) | 0.658 | 19(23.8) | 61(76.2) | 0.812 | 70(87.5) | 10(12.5) | 0.910 | 51(63.8) | 29(36.2) | 0.573 |
| Tumor size (cm) | <5  | 30(41.7) | 42(58.3) | 0.076 | 32(44.4) | 40(55.6) | 0.274 | 15(20.8) | 57(79.2) | 0.525 | 68(94.4) | 4(5.6) | 0.007 | 50(69.4) | 23(30.6) | 0.510 |
| Tumor classification | T1/T2 | 30(43.3) | 38(56.7) | 0.393 | 33(49.3) | 34(50.7) | 0.209 | 29(47.0) | 47(52.0) | 0.598 | 8(11.9) | 45(67.2) | 22(32.8) | 0.357 |
| Lymph node status | Positive | 38(47.5) | 42(52.5) | 0.696 | 39(48.8) | 41(51.2) | 0.658 | 19(23.8) | 61(76.2) | 0.812 | 70(87.5) | 10(12.5) | 0.910 | 51(63.8) | 29(36.2) | 0.573 |
| Distance metastasis | M0 | 65(49.2) | 67(50.8) | 0.742 | 66(50.0) | 66(50.0) | 0.742 | 30(22.7) | 102(77.3) | 0.720 | 116(87.9) | 16(12.1) | 0.207 | 89(66.4) | 44(33.6) | 0.753 |
| Clinical stage  | I/II | 38(48.7) | 40(51.3) | 0.957 | 38(48.7) | 40(51.3) | 0.662 | 19(24.4) | 59(75.6) | 0.672 | 68(87.2) | 10(12.8) | 0.959 | 50(64.1) | 28(35.9) | 0.427 |
| III/IV          | 30(49.2) | 31(50.8) | 0.525 | 28(47.5) | 28(52.5) | 0.547 | 11(24.4) | 34(75.6) | 0.783 | 37(82.2) | 8(17.8) | 0.241 | 29(66.4) | 16(33.6) | 0.670 |
| Differentiation | Well | 27(44.3) | 34(55.7) | 0.458 | 24(39.3) | 37(60.7) | 0.010 | 9(14.8) | 52(85.2) | 0.025 | 52(85.2) | 9(14.8) | 0.000 | 36(59.0) | 25(41.0) | 0.212 |
| Poorly          | 25(43.3) | 32(56.7) | 0.271 | 33(55.0) | 33(55.0) | 0.271 | 18(22.8) | 61(77.2) | 0.939 | 70(88.6) | 9(11.4) | 0.530 | 53(67.1) | 26(32.9) | 0.958 |

For p53 and Ki-67, ≥25%, as a low expression; >25%, as a high expression. For TopoII, GST-π and P-gp, +/-, as a low expression; +, as a high expression. doi:10.1371/journal.pone.0070550.t004

Distribution of Hv1 in human colorectal cell lines

To examine whether Hv1 is also expressed in human colorectal cancer cell lines, the expression of Hv1 in human colorectal cancer cell lines, SW620, HT29, LS174T, Colo205 and SW480, was detected by immunocytochemistry and real time RT-PCR. As shown in Fig. 3, the expression levels of Hv1 among these colorectal cancer cell lines have significant difference. Hv1 is expressed at a high level in SW620, HT29, LS174T, and Colo205 cell lines, SW620 and HT29 cells (Fig. 3D). Suppressions of Hv1 expression and activity by siRNA (f, g, h, i and j in Fig. 4A, B and C) and Hv1 activity by siRNA and ZnCl2 (k, l, m, n and o in Fig. 4A, B and C) clearly

Inhibition of Hv1 activity decreases invasion and migration in highly metastatic colorectal cancer cells

Invasion and migration are two prominent hallmarks of tumor malignancy. To evaluate the contribution of Hv1 to invasive and migratory potential in colorectal cancer cells, we performed invasion and migration assays. First, we examined the kinetics of migration and invasion in colorectal cancer cells, we performed wound healing assays. Fig. 4A, B and C showed the migration kinetics of SW620 (Fig. 4A), SW480 (Fig. 4B) and HT29 (Fig. 4C) cells. A wounded monolayer of SW620 cells allowed for wound closure after 48 h (Fig. 4B) and HT29 (Fig. 4C) cells. A wounded monolayer of SW620, HT29 and LS174T, and Colo205 cells, but not in SW480 cells. The localization of Hv1 in SW620 cells was determined by a confocal microscopy. As shown in Fig. 3C, Hv1 is highly expressed in SW620 cells, which is localized in both intracellular sites and plasma membrane (as indicated by arrowheads), whereas Hv1 is hardly expressed in SW480 cells. The result that Hv1 expression is higher in SW620 cells than that in SW480 cells is identical with the results from immunocytochemistry (Fig. 3A) and real time RT-PCR (Fig. 3B).

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decreased the migration in SW620 and HT29 cells, but almost without affecting on SW480 cells (Fig. 4B, f, g, h, i and j for siRNA; k, l, m, n and o for 100 μM ZnCl₂). The time-dependent wound distances of SW620 (Fig. 4A), SW480 (Fig. 4B) and HT29 (Fig. 4C) were shown in right panels.

We then studied invasion and migration of SW620, SW480 and HT29 cells using transwell inserts. As shown in Fig. 4D and E, SW620 cells have markedly higher invasive and migratory abilities than SW480 cells, which is consistent with the results from migration kinetics study above. Inhibitions of Hv1 expression by siRNA and Hv1 activity by ZnCl₂ remarkably decreased the invasion and migration of SW620 cells, but almost did not influence SW480 cells (Fig. 4D and E). The data indicated that suppression of Hv1 expression and activity could inhibit the invasion and migration of the highly metastatic colorectal cancer cells in vitro, which suggested that Hv1 is involved in the invasion and migration of the metastatic human colorectal cancer cells.

| Variable          | Recurrence-Free Survival | Overall Survival |
|-------------------|--------------------------|------------------|
|                   | No. of Patients | Median (95% CI) | P  | No. of Patients | Median (95% CI) | P  |
| Hv1 expression    |              |                 |    |                |                 |    |
| High              | 106          | 37.4(33.5–41.4) | 0.008 | 106          | 40.0(35.9–44.1) | 0.008 |
| Low               | 33           | 47.2(40.8–53.5) | 0.173 | 33           | 50.7(45.0–56.5) | 0.414 |
| Gender            |              |                 |    |                |                 |    |
| Male              | 68           | 37.9 (33.2–42.7) | 0.173 | 68           | 41.2(36.3–46.2) | 0.414 |
| Female            | 71           | 41.5 (36.4–46.5) | 0.173 | 71           | 43.8(38.8–48.8) | 0.414 |
| Age (yr)          |              |                 |    |                |                 |    |
| >62               | 79           | 37.5 (32.7–42.2) | 0.302 | 79           | 38.6 (33.8–43.5) | 0.024 |
| ≤62               | 60           | 42.7 (37.7–47.6) | 0.302 | 60           | 47.8 (43.0–52.5) | 0.024 |
| Primary site      |              |                 |    |                |                 |    |
| Left colon        | 29           | 38.1(30.3–45.8) | 0.162 | 29           | 41.7(33.8–49.5) | 0.193 |
| Right colon       | 43           | 34.7(28.3–41.1) | 0.162 | 43           | 37.4(30.7–44.2) | 0.162 |
| Rectum            | 67           | 43.7(36.3–43.2) | 0.162 | 67           | 46.2(41.4–50.9) | 0.162 |
| Tumor size (cm)   |              |                 |    |                |                 |    |
| <5                | 72           | 39.0(35.0–44.5) | 0.91 | 72           | 42.8(38.0–47.6) | 0.885 |
| ≥5                | 67           | 39.7(34.7–44.7) | 0.91 | 67           | 42.3(33.7–47.4) | 0.91 |
| Differentiation   |              |                 |    |                |                 |    |
| Well              | 61           | 41.0(35.9–46.2) | 0.683 | 61           | 43.8(38.7–49.0) | 0.653 |
| Moderately        | 58           | 38.9(33.5–44.3) | 0.683 | 58           | 41.7(36.2–47.3) | 0.683 |
| Poorly            | 20           | 38.0(28.6–47.5) | 0.683 | 20           | 40.9(31.0–50.7) | 0.683 |

*CI indicates confidence interval.

| Variable          | Recurrence-Free Survival | Overall Survival |
|-------------------|--------------------------|------------------|
|                   | No. of Patients | RR* (95% CI)* | P*  | No. of Patients | Median (95% CI)* | P*  |
| Hv1 expression    |              |                 |    |                |                 |    |
| High              | 106          | 1.000           | 0.015 | 106          | 1.000           | 0.015 |
| Low               | 33           | 0.443(0.230–0.853) | 0.015 | 33           | 0.427(0.202–0.903) | 0.015 |
| Gender            |              |                 |    |                |                 |    |
| Male              | 68           | 1.000           | 0.329 | 68           | 1.000           | 0.7  |
| Female            | 71           | 0.799(0.510–1.253) | 0.329 | 71           | 0.908(0.554–1.486) | 0.329 |
| Age (yr)          |              |                 |    |                |                 |    |
| >62               | 79           | 1.000           | 0.329 | 79           | 1.000           | 0.7  |
| ≤62               | 60           | 0.931(0.590–1.471) | 0.329 | 60           | 0.650(0.387–1.092) | 0.7  |
| Primary site      |              |                 |    |                |                 |    |
| Left colon        | 29           | 1.000           | 0.329 | 29           | 1.000           | 0.421 |
| Right colon       | 43           | 1.225(0.690–2.175) | 0.329 | 43           | 1.246(0.664–2.337) | 0.329 |
| Rectum            | 67           | 1.511(0.897–2.547) | 0.329 | 67           | 1.461(0.824–2.591) | 0.329 |
| Tumor size (cm)   |              |                 |    |                |                 |    |
| <5                | 72           | 1.000           | 0.201 | 72           | 1.000           | 0.201 |
| ≥5                | 67           | 0.730(0.451–1.182) | 0.201 | 67           | 0.745(0.441–1.258) | 0.201 |
| Differentiation   |              |                 |    |                |                 |    |
| Well              | 61           | 1.000           | 0.685 | 61           | 1.000           | 0.685 |
| Moderately        | 58           | 0.761(0.386–1.500) | 0.685 | 58           | 0.764(0.361–1.614) | 0.685 |
| Poorly            | 20           | 0.889(0.458–1.728) | 0.685 | 20           | 0.877(0.421–1.826) | 0.685 |

*RR and CI indicate relative risk and confidence interval, respectively.

P values were obtained by Cox proportional hazards analysis modeled for high and low/negative levels of Hv1 expression.

Table 6. Multivariate Cox proportional hazards analysis for recurrence-free survival and overall survival according to Hv1 expression.
Hv1 involved in regulating intracellular pH

The H+ channel activity of Hv1 in SW620 and SW480 cells was measured with a pH-sensitive probe BCECF. BCECF is a widely used pH indicator for estimating intracellular pH (pHi) [20,21]. Its fluorescence intensity at maximum emission wavelength is pH-dependent: a fall in pH with a decrease in fluorescence intensity, and to a rise in pH with an increase in fluorescence intensity. We acid-preloaded and exposed SW620 and SW480 cells to an outward-acting proton force (in high-K+ medium) that will drive an efflux of H+ ions. As shown in Fig. 5A the sharp increase on the fluorescence intensity of BCECF at membrane depolarization was observed for SW620 cells (Fig. 5A, a (nc)), indicating that an increase in pHi occurred. In contrast to SW620 cells, the fluorescence intensity of BCECF at membrane depolarization almost did not change for SW480 cells (Fig. 5A, b (nc)). Suppression of Hv1 expression obviously decreased the fluorescence intensity of BCECF at membrane depolarization in SW620 cells (Fig. 5A, a (si)), whereas did not affect on SW480 cells (Fig. 5A, b (si)). Inhibition of Hv1 activity by 100 μM ZnCl2 also inhibited outward proton extrusion in SW620 cells (Fig. 5A, a (Zn2+)). These results revealed that the pHi recovery was due to active Hv1.

To examine the effect of Hv1 on intracellular pH (pHi), we measured the pHi in SW620 and SW480 cells using BCECF. Down-regulation of Hv1 expression by siRNA and inhibition of Hv1 activity by 100 μM ZnCl2 induced a decrease in intracellular pH in the highly metastatic colorectal cancer SW620 cells, but not the poorly metastatic colorectal cancer SW480 cells (Fig. 5B). As shown in Fig. 5B, down-regulation of Hv1 expression in SW620 cells significantly increased acidity of intracellular pH from 7.5 to 7.0, while inhibition of Hv1 activity by 100 μM ZnCl2 more remarkably induced acidity of intracellular pH from 7.5 to 6.9 in SW620 cells. The finding showed that the inhibitions of Hv1 expression and activity in SW620 cells notably suppressed proton extrusion.

**Discussion**

In the present study, our data revealed that Hv1 expression was markedly higher in colorectal cancer tissues than in normal colorectal tissues, colorectal adenoma tissues and colorectal hyperplastic polyp tissues. We observed that Hv1 expression in colorectal cancer was significantly associated with tumor recurrence and metastasis. Patients who had high expression of Hv1 were remarkably poor recurrence-free and overall survival compared with patients who had low expression of Hv1. Multivariate analysis demonstrated that Hv1 expression level was an independent prognostic factor for recurrence-free and overall survival in patients with colorectal cancer. Our results clearly demonstrated that high Hv1 expression is associated with...
Figure 4. Hv1 increases colorectal cell migration and invasion. A, B and C, migration kinetics of SW620 (A), SW480 (B) and HT29 (C) assayed by wounded monolayer model. nc, cells transfected with the negative control (a, b, c, d and e in left panels); si, cells transfected with siRNA (f, g, h, i and j in left panels); and Zn$^{2+}$, cells cultured at a final concentration of 100 μM ZnCl$_2$ (k, l, m, n and o in left panels). Right panels in A, B and C show the time-dependent wound distances of SW620 (A), SW480 (B) and HT29 (C). Values are means ± SD (n = 5). Migration of SW620 cells is faster than SW480 cells in wounded monolayers. Down-regulation Hv1 expression by siRNA or inhibition of Hv1 activity by 100 μM ZnCl$_2$ clearly decrease the migratory ability of the highly metastatic SW620 cells. D and E, migration (D) and invasion (E) of SW620, SW480 and HT29 cells. Migration and invasion of the highly metastatic SW620 cells are significantly suppressed by down-regulation of Hv1 expression by siRNA targeting Hv1 and inhibition of Hv1 activity by 100 μM ZnCl$_2$. Values are means ± SD (n = 3). P<0.05, compared with SW620 cells transfected with negative control.

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poor prognosis and unfavorable clinical outcome of colorectal cancer.

To elucidate the mechanism that the effect of Hv1 on colorectal cancer development and metastasis, the expression of Hv1 in colorectal cancer cell lines was also detected, and the role of Hv1 in migration and invasion of colorectal cancer cells has been assessed. We found that Hv1 is highly expressed in highly metastatic colorectal cell lines, but lowly in poorly metastatic colorectal cell lines. Down-regulation of Hv1 expression or inhibition of Hv1 activity notably decreases the migratory and invasive abilities of the highly metastatic colorectal cancer cells. Suppression of Hv1 expression by siRNA and activity by 100 μM ZnCl2 notably induces a decrease in intracellular pH in the highly metastatic SW620 cells.

Maintenance of cytosolic pH is vital for all biological processes in cells. Solid tumor cells often exist in a hypoxic microenvironment with an acidic extracellular pH (pHo) value than that of surrounding normal cells [22–24]. The high glycolytic activity and acidic metabolites in cancer cells result in the excessive production of intracellular acidity. To overcome the hypoxic microenvironment and prevent the intracellular accumulation of the increased acidic metabolites, tumor cells must be enhanced by the ability to dispose of the increased intracellular protons. Several pHi regulatory mechanisms in tumor cells have been described, such as proton pumps, Na+/H+ exchangers, bicarbonate (HCO3-) transporters, and proton-lactate symporters, which have been shown to be involved in cancer progression and as promisingly therapeutic targets for future anticancer therapy [15,25–31]. Recent researches have highlighted the fundamental role of the tumour’s extracellular metabolic microenvironment in malignant invasion. This tumour cell microenvironment is acidified primarily by vacuolar H+-ATPases (V-ATPases) [15], Na+/H+ exchanger NHE1 [29], carbonic anhydrase [30] and H+/lactate cotransporter [31], which are activated in some cancer cells.

The importance of V-ATPases in cancer malignancy has been repeatedly demonstrated in several human cancer tumors and cell lines such as hepatocellular carcinoma [15,32]. Inhibition of V-ATPase function via knockdown of the protein subunit ATP6L expression using RNA interfering technology can effectively retard the growth and metastasis of human hepatocellular carcinoma xenografts [15,32]. Proton pump inhibitors (PPI), associated with the inhibition of V-ATPase activity and increasing in both extracellular pH and the pH of lysosomal organelles, trigger a rapid cancer cell apoptosis in human melanomas, adenocarcinomas, lymphomas and B cells, as a result of intracellular acidification, caspase activation and early accumulation of reactive oxygen species in tumour cells [33–36]. Research groups in all over the world have recently started an International Society of Proton Dynamics in Cancer (ispdc) in January 2010 to investigate various aspects of proton dynamics in cancer cells [28]. The newly formed society contributes to stimulate interdisciplinary collaboration for the development of more specific and less toxic therapeutic strategies based on proton dynamics in tumor cell biology.
In our previous work, we showed that Hv1 function relates to breast tumor growth and metastasis through proton extrusion [17]. Hv1 is also expressed in airway epitheilia, which mediates pH-regulated acid extrusion and acidify an alkaline airway surface liquid [37]. In the present work, the close relationship between Hv1 expression and clinicopathological features in colorectal cancer also predicted that Hv1 might boost carcinogenesis and tumor progression through regulating intracellular pH. Therefore, the voltage-gated proton channel Hv1 is a new candidate for some tumor cell intracellular and extracellular pH regulation.

The glycolysis and proton secretion in tumor cells are proposed to contribute to the proliferation and invasion of cancer cells during the process of tumorigenesis and metastasis [16,39]. The cytosolic pH value is extremely important for tumor cells, inasmuch as a decrease of cytosolic pH possibly stops tumor cell metabolism and induces cell death [15,33]. An alkaline cytosolic pH and an acidic extracellular pH resulting in high glycolytic activity and acidic metabolites are characteristics of tumor cells. The aberrant pH gradient between the alkaline cytosol and the acidic extracellular environment is involved in tumor progression and malignancy, which might be maintained by up-regulated activity of Hv1 that extrude protons outside the cell and acidify intracellular vesicles in colorectal cancer cells.

In conclusion, we demonstrated here that Hv1 is over-expressed in patients with colorectal cancer and high Hv1 expression correlated with the disease progression and poor clinical outcome in colorectal cancer. Furthermore, Hv1 was proved to be a risk factor for tumor recurrence and an independent molecular marker of prognosis for colorectal cancer and may become a novel molecular target in the strategies for the prediction of tumor recurrence and prognosis or treatment of colorectal cancer.

Author Contributions
Conceived and designed the experiments: YFW XYW SJL. Performed the experiments: YFW XYW QL SRZ. Analyzed the data: YFW XYW. Contributed reagents/materials/analysis tools: YFW XYW SJL. Wrote the paper: YFW XYW.

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