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From Cell Biology to Tissue Engineering

Hexokinase 2 in colorectal cancer: a potent prognostic factor associated with glycolysis, proliferation and migration

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Summary. Background. It is well known that proliferating carcinoma cells preferentially use aerobic glycolysis rather than oxidative phosphorylation for energy production. Hexokinase 2 (HK2) plays a pivotal role in the glycolytic pathway. Previous studies have demonstrated that HK2 activity is markedly increased in various malignant neoplasms, but the clinical and biological significance of HK2 remain largely unclear in the colorectal carcinoma.

Patients and methods. We performed immunohistochemistry for HK2 in 195 colorectal carcinoma tissues. We also used HCT8 and HT29 colon carcinoma cells in *in vitro* studies.

Results. HK2 immunoreactivity was detected in 100 out of 195 (51%) colorectal carcinoma tissues, and the immunohistochemical HK2 status was significantly associated with tumor size, depth of invasion, liver metastasis and TNM stage in these cases. Moreover, the HK2 status was significantly associated with increased incidence of recurrence and overall mortality of the patients, and multivariate analyses demonstrated that HK2 status was an independent prognostic factor for both disease-free and overall survival. Subsequent *in vitro* experiments revealed that both HCT8 and HT29 colon carcinoma cells transfected with specific siRNA for HK2 significantly decreased the lactate production, proliferation activity and migration property.

Conclusion. These results suggest that HK2 plays important roles in the glycolytic, proliferation and migration properties of colorectal carcinoma and, therefore, HK2 status is a potent worse prognostic factor in colorectal cancer patients.

Key words: Colorectal cancer, Immunohistochemistry, Invasion, Prognosis, Proliferation

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Introduction

Colorectal cancer is the second most common cancer in the world (Siegel et al., 2015). The mortality of colorectal cancer has recently declined because of improved early detection and screening and therapeutic advances, such as chemotherapy and some target specific therapies (Cunningham et al., 2004; Fedewa et al., 2015; Kumar et al., 2015). However, the estimated 5-year overall survival for early-stage colorectal carcinoma is 90%, decreasing remarkably to 70% and 13% for regional and distant disease, respectively (American Cancer Society, 2014), and approximately 20% of Stage II patients still recur despite the adjuvant chemotherapy (Kumar et al., 2015). Therefore, it is very important to examine clinical and biological markers in colorectal cancer patients to predict their prognosis after surgery and to evaluate indications of additional therapies appropriately.

It is well known that proliferating carcinoma cells preferentially use aerobic glycolysis rather than oxidative phosphorylation for energy production, and the metabolic alteration is commonly referred to as the

"Warburg effect" (Warburg, 1956). Hexokinase (HK) catalyzes the conversion of glucose resulting in the production of glucose-6-phosphase, and is the first and rate-limiting step in the glycolytic pathway (Zhao et al., 2011). The HK family contains HK1-4 in mammals, and among these, HK2 is normally expressed only in the limited tissues such as adipose tissue and skeletal muscle (Robey and Hay, 2005). However, HK2 activity is markedly increased in various malignant cells and HK2 is considered to play a pivotal role in providing carcinoma cells with energy (Mathupala et al., 1995). HK2 immunolocalization in colorectal carcinoma was very recently reported by Hamabe et al. (2014), but its clinical significance remains unclear, partly due to the relatively small sample set and lack of information, including overall survival of the patients. Moreover, the biological functions of HK2 have not been clarified in colorectal carcinoma. Therefore, in this study, we examined HK2 in colorectal carcinoma by immunohistochemistry and *in vitro* studies to explore its clinical and biological significance.

Materials and methods

Patients and tissues

195 colorectal carcinoma specimens were obtained from patients who underwent surgical treatment between 2000 and 2008 in the Department of Surgery at Tohoku University Hospital, Sendai, Japan (range of age; 25-93). No patients who received irradiation or chemotherapy prior to surgery were included. Patients clinically suspected to have hereditary nonpolyposis colorectal cancer, and carcinoma associated with inflammatory bowel disease were excluded from this study. A review of the charts revealed that 72 patients received 5-fluorouracil (5-FU)-based adjuvant chemotherapy. The clinical outcome of the patients was evaluated by disease-free and overall survival, and the mean follow-up time was 58 months (range; 1-131 months). All the specimens had been fixed in 10% formalin and embedded in paraffin wax. Research protocols for the present study were approved by the Ethics Committee at Tohoku University School of Medicine (2014-1-328).

Immunohistochemistry

Rabbit monoclonal antibody for HK2 (C64G5) was purchased from Cell Signaling Technology (Danvers, MA, USA). A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin-biotin amplification method was used. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin. Human breast tissue was used as a positive control for HK2 immunostaining (Sato-Tadano et al., 2013). We skipped a step of incubation with primary antibody as a negative control in this study.

Immunoreactivity for HK2 was detected in the cytoplasm of carcinoma cells, and the cases that had more than 10% of positive carcinoma cells were considered positive for HK2 status according to the previous report (Sato-Tadano et al., 2013).

Cell lines

Two human colon carcinoma cell lines, HCT8 and HT29, were provided from the American Type Culture Collection (Manassas, VA, USA), and subjected to short tandem repeat (STR) genomic profiling for authentication by BEX Co. Ltd. (Tokyo, Japan) using the CELL ID System (Promega, Madison, WI, USA). These cells were frequently used in previous reports as representative colon carcinoma cells (Mou et al., 2013; Lee et al., 2015), and ONCOMINE, a cancer microarray database (Rhodes et al., 2004), showed that HK2 was highly expressed in HCT8 and moderately expressed in HT29. The cells were cultured at 37°C with 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) and McCoy 5A Medium (Invitrogen), respectively, containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin–streptomycin (Invitrogen).

Small interfering RNA (siRNA) transfection

siRNA oligonucleotides for HK2 were purchased from Invitrogen. The target sequences of siRNA against HK2 (siHK2) were as follows: 5'-UCGCAUCUGCUU-GCUUACUUCUUCAd(TT)-3' (sense) and 5'-UGAAGAAGUAGGCAAGCAGAUGCGAd(TT)-3' (anti-sense). Stealth RNAi Negative Control (Invitrogen) was also used as a negative control (siCTRL). Cells were transfected with siRNA (final concentration of 10 nM) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA BIO, Kusatsu, Japan). All the processes were carried out according to the manufacturer's instructions.

Real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus), ROX plus (TAKARA BIO). The primer sequences used were as follows: HK2 (NM_000189.4): forward 5'-ATTGTCCAGTGC-ATCGCGGA-3' and reverse 5'-AGGTCAAACTCC-TCTCGCCG-3', and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: NM_001289745.1) as an internal control: forward 5'-GCACCGTCAAGG-CTGAGAAC-3' and reverse 5'-TGGTGAAGACGCC-AGTGGA-3'. The relative HK2 mRNA level in each sample was calculated as the ratio of GAPDH, and the

relative HK2 mRNA level was then evaluated as the ratio of that in the cells transfected with siCTRL in this study.

Immunoblotting

The whole-cell proteins from HCT8 and HT29 cells were extracted using Radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA). The lysate proteins (20 μg) were subjected to Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA). Following SDS–PAGE, the proteins were transferred onto Sequi-Blot PVDF (polyvinylidene difluoride) Membrane (Bio-Rad). Primary antibody used was anti-HK2 antibody the same as immunohistochemistry (C64G5; Cell Signaling Technology). In addition, anti-GAPDH (14C10; Cell Signaling Technology) antibody was used as an internal control. Antibody-protein complexes on the blots were detected using Clarity Western ECL (Bio-Rad), and the protein bands were visualized using an LAS-4000 mini image analyzer (Fuji Photo Film, Tokyo, Japan).

Cell proliferation assay

HCT8 and HT29 cells were transfected with siHK2 or siCTRL in a 96-well culture plate. The cell proliferation status was measured by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) method using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) 1-5 days and 1-6 days after the transfection in HCT8 and HT29 cells, respectively.

Wound-healing assay

Wound-healing assay was performed using Culture Inserts (Ibidi GmbH, Munich, Germany). Briefly, HCT8 cells and HT29 cells were seeded into each well of culture insert ($5x10^4$ cells). After cell adherence, Culture Inserts were removed and the remaining gaps were evaluated under light microscopy and quantified using NIS Elements software v3.0 (Nikon, Tokyo, Japan). The relative migration area was then calculated as the ratio of that in the control cells transfected with siCTRL according to the previous report (Onodera et al., 2014).

Lactate assay

HCT8 and HT29 cells were seeded at $3x10^5$ cells per well in a 6-well culture plate, and transfected with siHK2 or siCTRL. The lactate concentrations in the medium during a period of 3 days were determined using the Lactate Assay Kit II (Biovision, San Francisco, USA) and adjusted by the number of cells (nmol/μl per 10^6 cells).

Statistical analysis

HK2 status and clinicopathological factors were evaluated by Student's t-test or a cross-table using the chi-squared test. Disease-free and overall survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Uni- and multivariate analyses were evaluated by a proportional hazard model (Cox). P values<0.05 and 0.05≤P values<0.10 were considered significant and borderline-significant in this study, respectively (Sato-Tadano et al., 2013). In the *in vitro* experiments, the statistical analyses were performed using Student's t-test. The JMP 10 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

Results

HK2 immunolocalization in human colorectal carcinoma

HK2 immunoreactivity was detected in the cytoplasm of colorectal carcinoma cells (Fig. 1A-D). HK2 immunoreactivity was widely detected in the colorectal carcinoma regardless of the invasive front in this study. HK2 was focally and weakly immunolocalized in the non-neoplastic epithelial cells adjacent to the carcinoma (Fig. 1E), but not in the stroma. HK2 immunoreactivity was markedly detected in the breast carcinoma cells as a positive control (Sato-Tadano et al., 2013), but no significant immunoreactivity was detected in the same areas of negative control section (Fig. 1F).

Associations between immunohistochemical HK2 status and various clinicopathological parameters in the colorectal carcinoma cases are summarized in Table 1. The number of HK2-positive cases was 100 out of 195 (51%) in this study. The HK2 status was significantly associated with tumor size (P=0.017), depth of invasion (P=0.0004), liver metastasis (P=0.0094) and TNM stage (P=0.0074), and marginally associated with histological differentiation (P=0.083) and lymph node metastasis (P=0.086). On the other hand, no significant association was detected between HK2 status and other factors examined such as patients' age, gender and tumor location.

Association between HK2 status and clinical outcome of the patients

As shown in Fig. 2A, HK2 status was significantly associated with an increased incidence of recurrence in 166 Stage 0 - III colorectal cancer patients treated by surgery (P<0.0001 by log-rank test). A similar tendency was detected between HK2 status and adverse clinical outcome of all the Stage 0 - IV colorectal cancer patients examined (n=195; P<0.0001) (Fig. 2B). Significant association between HK2 status and worse prognosis was also detected in the aggressive phenotypes, such as pT3-4 cases (P=0.0085 in disease-free survival (n=109;

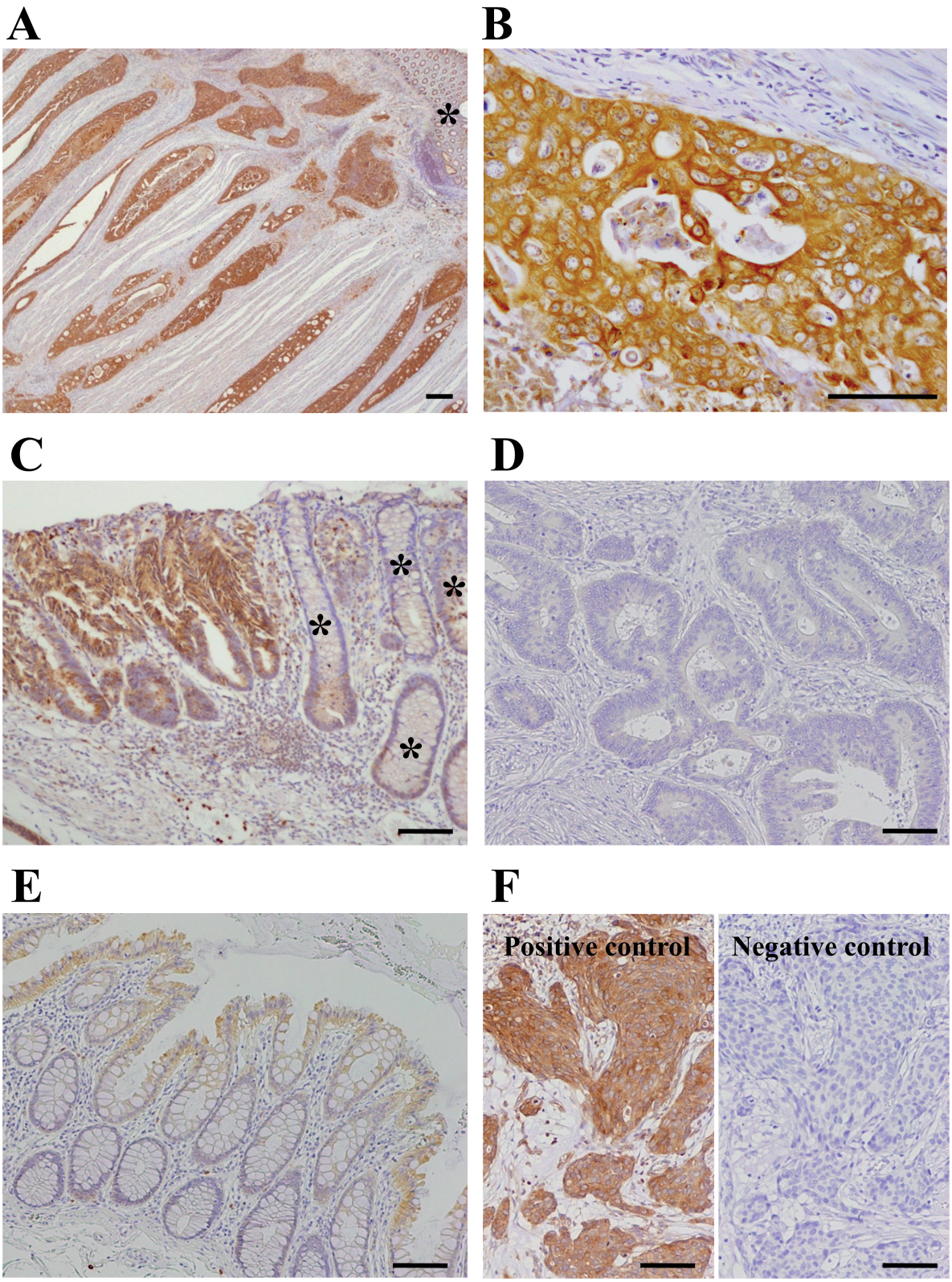

Fig. 1. Immunolocalization of HK2 in human colorectal carcinoma. **A.** HK2 immunoreactivity was widely detected in colorectal carcinoma, but not in the non-neoplastic mucosa adjacent to the carcinoma (*) (lower magnification). **B.** HK2 was immunolocalzied in the cytoplasm of carcinoma cells. **C.** HK2 was positive in the in situ lesion of colorectal carcinoma, but not in the non-neoplastic mucosal epithelium adjacent to the carcinoma (*). **D.** HK2-negative colorectal carcinoma case. **E.** HK2 was weakly detected in non-neoplastic mucosal epithelium. **F.** Positive control (left panel) and negative control (right panel) for HK2 immunohistochemistry (breast carcinoma). Same area. Scale bar: 100 μm.

Fig. 2C) and P=0.0027 in overall survival (n=136)) and cases with lymph node metastasis (P=0.0065 in disease-free survival (n=64; Fig. 2D)) and P=0.0084 in overall survival (n=84)). A similar tendency was also detected regardless of the status of adjuvant chemotherapy (cases without chemotherapy: P=0.015 in disease-free survival (n=109; Fig. 2E) and P=0.0002 in overall survival (n=123), and cases with chemotherapy: P=0.021 in disease-free survival (n=57; Fig. 2F) and P=0.088 in overall survival (n=72)).

The results of univariate analysis of disease-free survival using Cox (Table 2), HK2 status, lymph node metastasis, tumor location and depth of invasion were demonstrated to be significant prognostic factors for disease-free survival. Multivariate analysis revealed that HK2 status (P=0.0047) and tumor location (P=0.036) were independent prognostic factors.

As shown in Table 3, univariate analyses for overall survival revealed HK2 status, liver metastasis, lymph node metastasis, depth of invasion and histological differentiation as significant prognostic variables in these patients, and subsequent multivariate analysis showed that liver metastasis (P=0.0006), HK2 status (P=0.0036) and lymph node metastasis (P=0.018) were independent parameters of these patients.

Biological functions of HK2 in colon carcinoma cells

In order to further evaluate biological functions of HK2 in human colon carcinoma cells, we transfected specific siRNA for HK2 in HCT8 and HT29 colon carcinoma cells. As shown in the left upper panel of Fig. 3A, levels of HK2 mRNA were markedly decreased in HCT8 cells transfected with siHK2 at 3 days after transfection compared with those in cells transfected with negative control siRNA (siCTRL) (P<0.001 and 0.066-fold). Decreased protein level of HK2 was confirmed by immunoblotting under the same conditions (Fig. 3A, left lower panels). Similarly, HK2 expression levels were also decreased in HT29 cells transfected with siHK2 compared with those transfected with siCTRL both at mRNA (Fig. 3A, right upper panel (P<0.05 and 0.61-fold)), and protein (Fig. 3A, right lower panel) levels.

As shown in Fig. 2B, lactate concentrations in the medium were significantly decreased both in HCT8

Table 1. Association between immunohistochemical HK2 status and clinicopathological parameters in 195 colorectal carcinomas.

	HK2 status		
	Positive (n=100)	Negative (n=95)	P value
Age* (years)	64.5±13.1	64.5±11.5	0.97
Gender			
Men	57	60	
Women	43	35	0.38
Tumor location			
Colon	45	44	
Rectum	55	51	0.85
Tumor size* (mm)	56.1±25.2	46.6±22.8	0.017
Histological differentiation			
tub1 + tub2	78	83	
por + muc	22	12	0.083
Depth of invasion			
pTis + pT1 + pT2	19	40	
pT3 + pT4	81	55	0.0004
Lymph node metastasis			
Negative	51	60	
Positive	49	35	0.086
Liver metastasis			
Negative	87	92	
Positive	13	3	0.0094
TNM stage			
0 + I + II	43	59	
III + IV	57	36	0.0074

*: Data are presented as mean ± SD. All other values represent the number of cases. Statistical analysis was evaluated by the Student's t test or a cross-table using the chi-square test. P value<0.05 and 0.05≤P value<0.10 were considered significant and borderline significant, and are listed in bold and italic respectively.

Table 2. Univariate and multivariate analyses of disease-free survival in Stage 0 - III colorectal cancer patients cured by surgery (n=166).

	Univariate	Multivariate	
Variable	P value	P value	Relative risk (95% CI)
HK2 status (Positive vs Negative)	0.0009	0.0047	2.5 (1.3 - 4.9)
Lymph node metastasis (Positive vs Negative)	0.0036	0.11	1.7 (0.9 -3 .3)
Tumor location (rectum vs colon)	0.042	0.036	2.0 (1.0 -4 .0)
Depth of invasion (pT3 - pT4 vs pTis - pT2)	0.048	0.23	1.6 (0.8 - 3.6)
Age† (years) (≥64 vs <64)	0.19		
Gender (women vs men)	0.22		
Histological differentiation (muc + por vs tub1 + tub2)	0.58		
Tumor size* (mm) (≥50 vs <50)	0.82		

Statistical analysis was evaluated by a proportional hazard model (Cox). Data considered significant (P<0.05) in the univariate analyses were described as boldface, and these were examined in the multivariate analyses. *: The parameter was categorized into two groups according to the median value. CI: confidence interval.

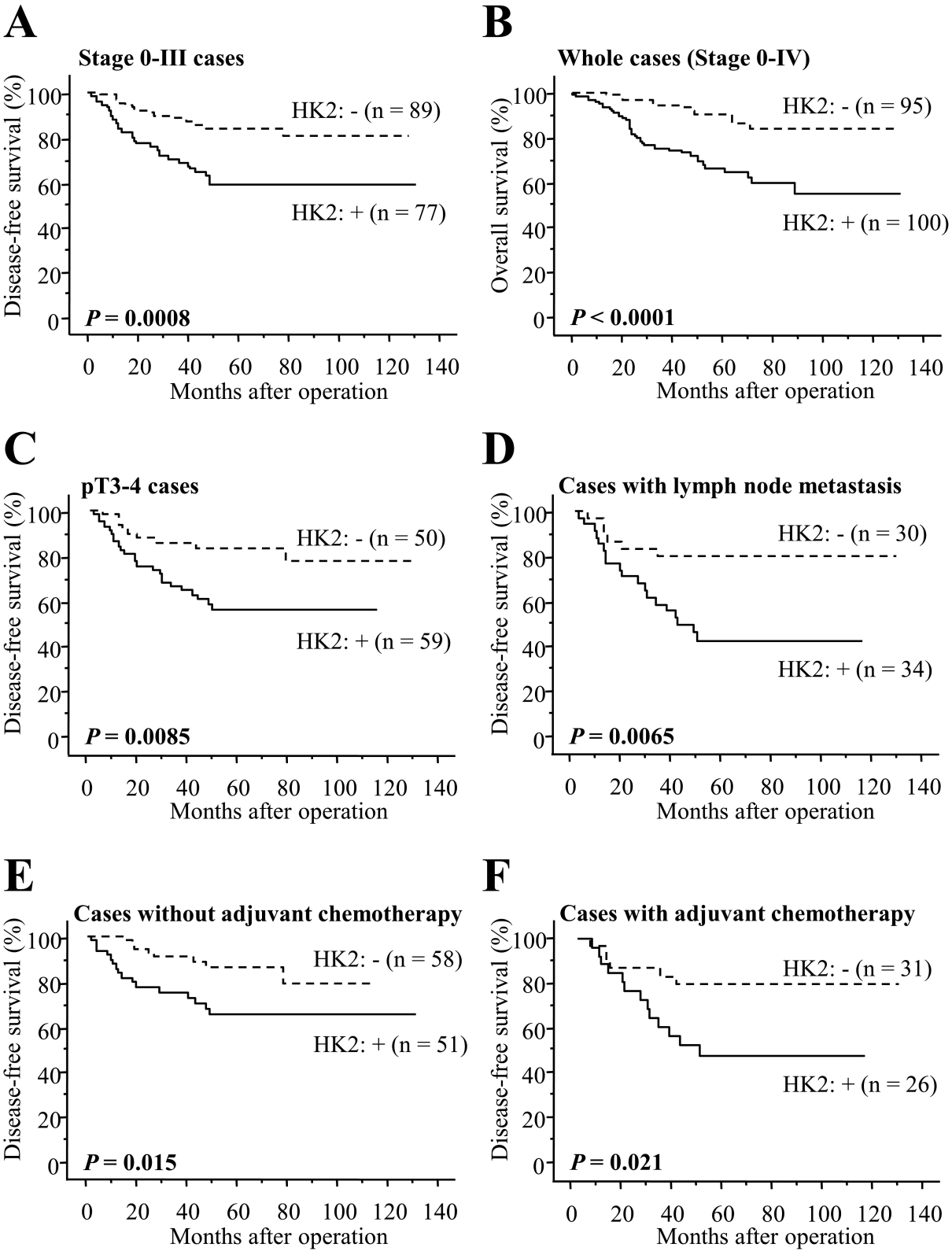

Fig. 2. Disease-free **(A, C-F)** and overall survival **(B)** of colorectal cancer patients according to HK2 status by the Kaplan-Meier method. **A.** Stage 0-III cases treated by surgery (n=166). **B.** All the Stage I-IV cases (n=195). **C.** pT3-4 cases (n=109). **D.** cases positive with lymph node metastasis (n=64). **E.** cases without adjuvant chemotherapy (n=109). **F.** cases with adjuvant chemotherapy (n=57). The solid line shows HK2-positive cases and the dashed line shows HK2-negative cases. Statistical analysis was performed using the log-rank test. P-values<0.05 were considered significant and are shown in bold.

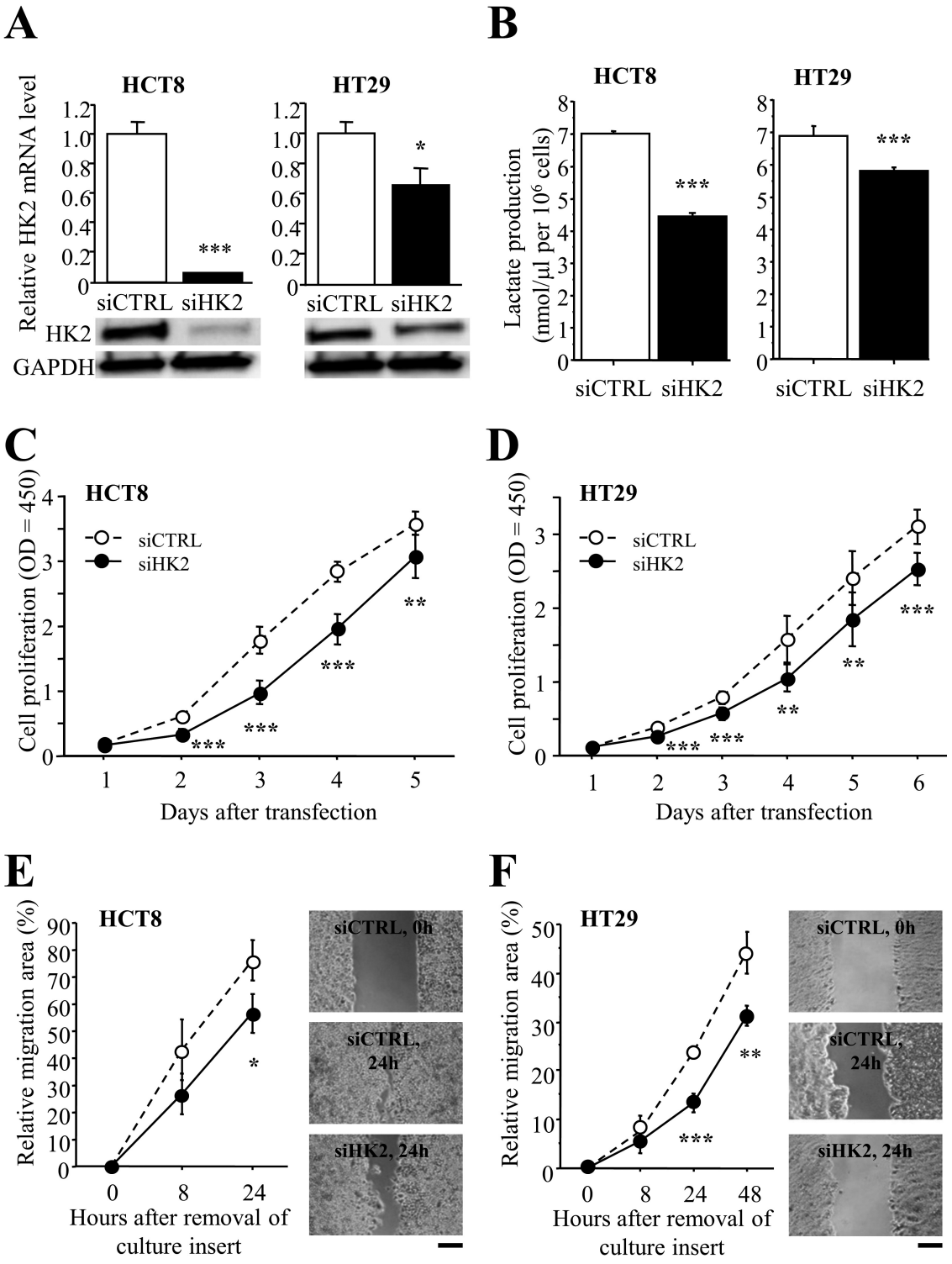

Fig. 3. Effects of HK2 on lactate production, cell proliferation and migration properties in colon carcinoma cells. **A.** HK2 expression levels in HCT8 (left panels) and HT29 (right panel) cells transfected with HK2-specific siRNA (siHK2). Upper panels summarize HK2 mRNA levels evaluated by real-time PCR. HCT8 and HT29 cells were transfected with siHK2 (closed bar) or negative control siRNA (siCTRL; open bar). Lower panels show HK2 protein levels in HCT8 and HT29 cells by immunoblotting under the same treatment. GAPDH immunoreactivity was shown as the internal control. **B.** Lactate production in HCT8 (left panel) and HT29 (right panel) cells transfected with siHK2 (closed bar) or siCTRL (open bar). **C, D.** Cell proliferation activity of HCT8 **(C)** and HT29 **(D)** cells transfected with siHK2 (closed circle) or siCTRL (open circle). **E, F.** Wound-healing assays in HCT8 **(E)** and HT29 **(F)** cells. Closed circle; siHK2 and open circle; siCTRL. Right panels show representative microphotographs of the results under the indicated conditions. Bar=100 μm. In all figures, data are presented as the mean ± SD (n=3), and the statistical analyses were performed using Student's t-test. *; P<0.05, **; P<0.01 and ***; P<0.001.

Table 3. Univariate and multivariate analyses of overall survival in Stage 0-IV colorectal cancer patients examined (n=195).

Variable	Univariate	Multivariate	
	P value	P value	Relative risk (95% CI)
HK2 status (Positive vs Negative)	**<0.0001**	**0.0036**	**2.7 (1.4 - 5.6)**
Liver metastasis (Positive vs Negative)	**<0.0001**	**0.0006**	**4.1 (1.9 - 8.1)**
Lymph node metastasis (Positive vs Negative)	**0.0016**	**0.018**	**2.1 (1.1 - 4.0)**
Depth of invasion (pT3 - pT4 vs pTis - pT2)	**0.011**	0.49	1.3 (0.6 - 3.4)
Histological differentiation (muc + por vs tub1 + tub2)	**0.011**	**0.042**	**2.1 (1.0 - 4.0)**
Tumor location (rectum vs colon)	0.066		
Gender (women vs men)	0.14		
Age† (years) (≥64 vs <64)	0.50		
Tumor size* (mm) (≥50 vs <50)	0.65		

Statistical analysis was evaluated by a proportional hazard model (Cox). Data considered significant (P<0.05) in the univariate analyses were described as boldface, and these were examined in the multivariate analyses. 0.05≤P value<0.10 was considered borderline significant, and is listed in italic. *: The parameter was categorized into two groups according to the median value. CI: confidence interval.

(P<0.001 and 0.63-fold) and HT29 (P<0.001 and 0.84-fold) cells transfected with siHK2 compared with those transfected with siCTRL. The effects of HK2 expression on cell proliferation in colon carcinoma cells are summarized in Fig. 3C,D. As shown in Fig. 3C, cell proliferation activity was significantly suppressed in HCT8 cells transfected with siHK2 from 2 to 5 days after the transfection compared with those transfected with siCTRL (P<0.001 to P<0.01 and 0.47- to 0.75-fold from Day 2 to Day 5). Similarly, cell proliferation activity was significantly suppressed in HT29 cells transfected with siHK2 from 2 to 6 days after the transfection compared with those transfected with siCTRL (P<0.001 to P<0.01 and 0.61- to 0.83-fold from Day 2 to Day 6 (Fig. 3D)).

Moreover, relative migration areas in HCT8 and HT29 cells transfected with siHK2 were significantly decreased compared with their controls (HCT8; P<0.05 and 0.75-fold after 24 hours (Fig. 3E) and HT29; P<0.001 and 0.56-fold after 24 hours and P<0.01 and 0.70-fod after 48 hours (Fig. 3F)).

Discussion

This is the first study which demonstrates HK2 as an independent worse prognostic factor of colorectal carcinoma, to the best of our knowledge. In this study, HK2 immunoreactivity was detected in 51% of colorectal carcinoma cases, but was negligible in non-neoplastic epithelium. Previously, Izuishi et al. showed that HK2 mRNA level was significantly higher in colorectal carcinoma tissues than the surrounding normal mucosa, which is in good agreement with our present findings (Izuishi et al., 2012). HK2 immunoreactivity was detected in various carcinomas, such as gastric (Qiu et al., 2011), hepatocellular (Gong et al., 2012), breast (Brown et al., 2002; Sato-Tadano et al., 2013), ovarian (Jin et al., 2014; Suh et al., 2014), laryngeal and pancreatic carcinomas (Chen et al., 2014; Ogawa et al., 2015). Moreover, Hamabe et al. reported that HK2 immunoreactivity was detected in the invasive front lesions of 59% of colorectal carcinomas (Hamabe et al., 2014). Taken together with these reports and our present results, it is suggested that HK2 is overexpressed in a subset of colorectal carcinomas. HK2 is a target gene of MYC oncogene (Dang, 2007), and it is also induced by mutated KRAS oncogene in colorectal cancer cells (Iwamoto et al., 2014). HK2 plays an essential role in Kras-driven lung cancer and ErbB2-driven breast cancer *in vivo* (Patra et al., 2013), and it is also upregulated by EGFR or combined loss of Pten (phosphatase and tensin homolog deleted on chromosome 10) and p53 (Wang et al., 2014; Liu et al., 2015). Considering that HK2 immuoreactivity was widely detected in the colorectal carcinoma regardless of the invasive front in our study, HK2 expression may be upregulated in the process of colorectal carcinogenesis mainly by various oncogenes.

In our present immunohistochemical analysis, HK2 immunoreactivity was significantly associated with tumor size and depth of invasion in colorectal carcinoma, which is consistent with previous findings by Hamabe et al. (2014). Moreover, our subsequent *in vitro* experiments demonstrated that both HCT8 and HT29 colon carcinoma cells transfected with siRNA against HK2 significantly decreased lactate production, cell proliferation and migration properties. HK2 is crucial for glycolysis in carcinoma cells (Zhao et al., 2011). The glycolytic pathway provides cells with not only energy but also precursors for biomolecules necessary for proliferation, and increased glycolytic character provides tumor protection and enhance invasion (Pedersen, 2007; Lopez-Lazaro, 2008). Previous studies have demonstrated that HK2 promoted cell proliferation activity in laryngeal carcinoma and glioblastoma multiforme (Wolf et al., 2011; Chen et al., 2014). HK2 inhibits apoptosis by preventing the release of cytochrome c (Pastorino and Hoek, 2003), and promotes mitochondrial stability in colon cancer cells (Peng et al., 2008). HK2 is also reported to play a critical role in tumor proliferation by both the PI3K-dependent and the

PI3K-independent Akt signal pathways in hepatocellular carcinoma cell lines (Ahn et al., 2009). Moreover, Wang et al. reported that depletion of HK2 in Pten-/p53-deficient mouse embryonic fibroblasts significantly reduced glucose consumption, lactate production, attenuated anchorage-independent cell growth and impaired tumor growth in a xenograft model (Wang et al., 2014). Our present results were consistent with these reports, and suggest that HK2 mediates aerobic glycolysis, known as Warburg effect, and promotes cell proliferation and invasion in the colorectal carcinoma.

HK2 status was significantly associated with liver metastasis and higher TNM stage, and marginally associated with lymph node metastasis. HK2 expression was frequently detected in higher TNM stage in gastric (Qiu et al., 2011), laryngeal and serous ovarian carcinomas (Chen et al., 2014; Jin et al., 2014), which is consistent with our present findings. Hamabe et al. showed that epithelial-mesenchymal transition (EMT) accompanied HK2-upregulation in SW480 colon carcinoma cells (Hamabe et al., 2014), and Palmieri et al. reported that HK2 overexpression was detected in 77% of brain metastasis tissues of breast carcinoma (Palmieri et al., 2009). Therefore, it is suggested that HK2 is also involved in the metastatic process of colorectal carcinomas in addition to cell proliferation and invasion.

In the present study, HK2 status was significantly associated with recurrence and worse prognosis in colorectal cancer patients, and results of multivariate analyses demonstrated that HK2 status was an independent prognostic factor for both disease-free and overall survival. Previous studies demonstrated that HK2 expression was an independent worse prognostic factor of gastric (Qiu et al., 2011), breast and ovarian carcinoma patients (Sato-Tadano et al., 2013; Suh et al., 2014). In addition, HK2 expression was associated with shorter survival of hepatocellular and pancreatic carcinoma patients (Gong et al., 2012; Ogawa et al., 2015), and Hamabe et al. showed that invasive front lesions of HK2 immunoreactivity was associated with shorter disease-free survival of colorectal carcinoma patients (Hamabe et al., 2014). We also demonstrated that association between HK2 status and worse prognosis was also detected in the aggressive phenotypes, such as pT3-4 cases and cases with lymph node metastasis, and a similar tendency was detected regardless of the status of adjuvant chemotherapy. Limited information is available about association between HK2 and resistance to adjuvant therapy, but Peng et al. showed that downregulation of HK2 sensitized 5-fluorouracil (5-FU), which is widely used in the treatment of colorectal cancer, in LoVo colon cancer cells (Peng et al., 2008). On the other hand, Nakano et al. reported that a HK2 inhibitor (3-bromopyruvate) diminished ATP-binding cassette (ABC) transporter activity to restore drug retention in multiple myeloma cells (Nakano et al., 2012). Therefore, HK2 may play an important role in chemoresistence of colorectal carcinoma, and further examinations are required to clarify the molecular mechanisms. Since HK2 possibly involves a variety of biological functions of colorectal carcinoma cells as described in this section, residual carcinoma cells following surgical treatment in HK2-positive colorectal carcinomas could still have the potential to rapidly recur despite the adjuvant therapies.

In summary, HK2 immunoreactivity was detected in 51% of colorectal carcinomas, and HK2 status was significantly associated with tumor size, depth of invasion, liver metastasis and TNM stage. Moreover, HK2 status was significantly associated with worse prognosis of the patients and it was shown to be an independent prognostic factor. Subsequent *in vitro* experiments demonstrated that HK2 significantly promoted lactate production, proliferation activity and migration property of both HCT8 and HT29 cells. These results suggest that HK2 plays important roles in the glycolytic, proliferation and migration properties of colorectal carcinoma and HK2 status is a potent prognostic factor in these patients.

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Declaration of interest. We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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