Combined evaluation of hexokinase 2 and phosphorylated pyruvate dehydrogenase-E1α in invasive front lesions of colorectal tumors predicts cancer metabolism and patient prognosis

Atsushi Hamabe, Hirofumi Yamamoto, Masamitsu Konno, Mamoru Uemura, Junichi Nishimura, Taishi Hata, Ichiro Takemasa, Tsunekazu Mizushima, Naohiro Nishida, Koichi Kawamoto, Jun Koseki, Yuichiro Doki, Masaki Mori and Hideshi Ishii

Departments of 1Gastroenterological Surgery; 2Frontier Science for Cancer and Chemotherapy; 3Cancer Profiling Discovery, Graduate School of Medicine, Osaka University, Osaka, Japan

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Correspondence
Hideshi Ishii, Department of Frontier Science for Cancer and Chemotherapy, Graduate School of Medicine, Osaka University, Suita, Yamadaoka 2-2-2, Osaka 565-0871, Japan. Tel: +81-(0)6-6879-2641, 2640; Fax: +81-(0)6-6879-2639; E-mail: hishi@gesurg.med.osaka-u.ac.jp

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Colorectal cancer (CRC) is the second most common cancer in the world. Each year, >1.2 million individuals develop CRC, and approximately 600 000 deaths occur. Although the efficacy of treatment has been gradually improving because of advances in chemotherapy or surgical technologies, the prognosis of patients with distant metastases and recurrence has not improved much. Numerous studies have shown that the activation of tumor-promoting genes and inactivation of growth-constraint tumor suppressor genes through genetic and epigenetic alterations contribute to the activation of biological phenomena, such as cell invasion, movement, and colonization, in distant organs during the metastatic process; however, the precise molecular mechanisms involving biologically active metabolites in cancer are not completely understood.

Recent studies have indicated that deregulation in intratumor metabolism is involved in malignant behaviors of cancer cells. In glucose metabolism, lactate is preferentially produced in cancer cells, even in the presence of adequate oxygen in culture, a critical biological phenomenon termed aerobic glycolysis, how glycolysis contributes to tumor invasion, a critical phenomenon in metastasis, remains unclear. With regard to colorectal cancer (CRC), we studied two critical gate enzymes, hexokinase 2 (HK2), which is involved in glycolysis, and phosphorylated pyruvate dehydrogenase-E1α (p-PDH), which is involved in oxidative phosphorylation (OxPhos). Immunohistochemical analyses using anti-HK2 and p-PDH antibodies were performed on surgically resected CRC samples (n = 104), and the expression in invasive front lesions of tumors was assessed. Positive HK2 expression correlated with extensive tumor diameter (P = 0.0460), advanced tumor depth (P = 0.0395), and presence of lymph node metastasis (P = 0.0409). Expression of p-PDH tended to be higher in right-sided CRCs than in left-sided CRCs (P = 0.0883). In survival analysis, the combined evaluation of positive HK2 and negative p-PDH was associated with reduced recurrence-free survival (RFS) (P = 0.0169 in all stages and P = 0.0238 in Stage II and III patients, respectively). This evaluation could predict RFS more precisely than the independent evaluation. The present study indicated that high HK2 expression combined with low p-PDH expression in the invasive front lesions of CRC tumors is predictive of tumor aggressiveness and survival of CRC cases.
ubiquitously expressed, whereas HK2 is expressed in limited types of tissues, such as adipose tissues, skeletal muscles, and the heart.(12) In cancer cells, HK2 and, to a lesser extent, HK1 are expressed,(13) which suggests a preferential role of HK2 in the glucose flux of cancer cells. Recent studies have indicated that HK2 is necessary for the tumorigenicity of non-small cell lung cancer and breast cancer in humans, whereas HK2 depletion results in rapid suppression of tumor growth.(9)

Pyruvate dehydrogenase has a gate-keeper role in a branching point that links glycolysis to OxPhos in the citric acid cycle by converting pyruvate to acetyl-CoA in the mitochondria. The catalyzing activity of PDH is inhibited by phosphorylation at serine residue(s) by PDH kinase (PDK), whereas PDH is activated by PDH phosphatase.(14,15) Reportedly, the process of aerobic glycolysis is at least partially maintained by the attenuation of mitochondrial function through PDH inhibition.(16) A melanoma study showed that PDH inactivation by serine 293-phosphorylation by PDK led to high tumorigenic activity, whereas PDK depletion resulted in hypophosphorylation of PDH, regression of tumors, and further eradication of subpopulations resistant to a specific inhibitor to oncogene Braf V600E(17) which suggested an exclusive dependency on aerobic glycolysis of melanoma growth.

In the present study, we immunohistochemically analyzed the expression of HK2 and p-PDH in the invasive front lesions of clinical CRC samples and assessed their ability to predict tumor aggressiveness and survival. We identified an unexpected association of p-PDH with improved survival and additionally, in regard to HK2 antibody, the absorption test was carried out in immunohistochemical analysis (Suppl. Fig. S4). The slides were incubated overnight at 4°C at the following dilutions: anti-HK2 antibody, 1:200; anti-p-PDH, 1:500; anti-PDH-E1α, 1:200.

Sections were counterstained with hematoxylin. We rated the intensity of staining on a scale of 0 to 2: 0, negative; 1, weak; and 2, strong. We used pancreatic tissue as a positive control for HK2 according to the previous study,(18) and a case of Stage I rectal cancer, the staining of which at the deepest part was strong as a positive control of p-PDH. Phosphate buffered saline instead of the antibodies was used as a negative control. We assigned colorectal tissue stained as intense as the positive control to “intensity score 2”, while unstained colorectal tissue similar to the negative control was assigned to “score 0” (Fig. 1). The tissue stained weaker than the positive control but stronger than the negative control was categorized into “score 1” (Fig. 1b,c). The intensity at the deepest part of the tumor was recorded in each sample. The reason why the intensity at the deepest part of the tumor was assessed was that the cancer cell was stimulated to invade into surrounding tissues at this region.(19,20)

Assessment of tumor budding. Tumor budding was estimated according to the definition proposed by Ueno et al.(21,22) An isolated cancer cell or a cluster composed of fewer than five cancer cells was defined as tumor budding. The number of budings was counted in the field under a magnification of ×200 in the invasive front area.

Cell lines and culture. Human colon cancer cell line, SW480, was obtained from the ATCC (Manassas, VA, USA). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, and grown at 37°C in a humidified incubator with 5% CO2.

Induction of EMT. Cells were seeded at the concentration of 5.0 × 104 cells/mL and incubated in a humidified atmosphere (37°C and 5% CO2) in standard medium for 48 h. After 48 h incubation, the cells were treated with transforming growth factor-β1 (TGF-β1) (2.5 ng/mL) and were incubated with MEM medium supplemented with FBS free, 10 ng/mL epidermal growth

Materials and Methods

Clinical tissue samples. Colorectal tissue samples (n = 104) were collected during surgery (2007–2009) at the Department of Surgery, Osaka University. None of the patients had undergone preoperative chemotherapy or irradiation. Samples were fixed in buffered formalin at 4°C overnight, processed through graded ethanol solutions, and embedded in paraffin. The specimens were appropriately used under the approval of the ethics committee at the Graduate School of Medicine, Osaka University.

Immunohistochemistry. Tissue sections (3.5 μm thick) were prepared from paraffin-embedded blocks. After antigen retrieval treatment in 10 mM citrate buffer (pH 6.0) at 115°C for 15 min using Decloaking Chamber NxGen (Biocare Medical, Concord, CA, USA), immunostaining was performed using the Vectastain ABC Peroxidase Kit (Vector Laboratories, Burlingame, CA, USA). Antibodies used for immunohistochemistry were anti-HK2 rabbit antibody (2867; Cell Signaling Technology, Danvers, MA, USA), anti-pancyruvate dehydrogenase E1α subunit (PDH-E1α) mouse antibody (ab110330; Abcam, Cambridge, UK), and pSer293 (p-PDH), the anti-phosphorylated form of PDH-E1α rabbit antibody (AP1062; Millipore, Darmstadt, Germany). The specificity of the antibodies was confirmed by the data showing that each antibody detected single band corresponding to the targeted protein in Western blot analysis (Suppl. Figs S1–3), and additionally, in regard to HK2 antibody, the absorption test was carried out in immunohistochemical analysis (Suppl. Fig. S4). The slides were incubated overnight at 4°C at the following dilutions: anti-HK2 antibody, 1:200; anti-p-PDH, 1:500; anti-PDH-E1α, 1:200.
growth factor (Sigma-Aldrich, St Louis, MO, USA), 100 × insulin/transferrin/selenium (ITS) (Life Technologies, Carlsbad, CA, USA), and 50 nmol/L hydrocortisone (Tokyo Kasei, Tokyo, Japan) for 48–72 h.

**Biochemical assay.** Biochemical activities of SW480 were analyzed using Hexokinase Colorimetric Assay Kit (ab136957; Abcam) for hexokinase activity and Pyruvate dehydrogenase Enzyme Activity Microplate Assay Kit (ab109902; Abcam) for pyruvate dehydrogenase activity according to the manufacturer’s instructions.

**Western blot analysis.** Total protein was extracted from the cell lines in radio immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA). Aliquots of protein were electrophoresed on SDS-PAGE, Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were transferred to PVDF membranes using iBlot (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of total protein were extracted from the cultured cells using RNeasy Mini Kit and QIA shredder (Qiagen, Valencia, CA, USA). Complemetary DNA was synthesized with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Real-time quantitative polymerase chain reactions (qRT-PCR) were conducted with the LightCycler-FastStart DNA Master SYBR Green I kit.

| Hexokinase 2 | Positive (n = 61) | Negative (n = 43) | P-value |
|--------------|------------------|------------------|---------|
| Patient background | | | |
| Gender (Male/Female) | 37/24 | 28/15 | 0.6436 |
| Age (mean ± SD) | 63.2 ± 12.4 | 66.6 ± 10.6 | 0.9265 |
| BMI (kg/m²) | 22.2 (20.5, 26.1) | 22.4 (20.3, 25.2) | 0.9579 |
| CEA (ng/mL) | 3 (2, 9) | 3 (1, 5) | 0.5232 |
| CA19-9 (U/mL) | 11 (7, 21) | 13 (5, 22) | 0.8013 |
| Tumor characteristics | | | |
| Tumor diameter (mm) | 40 (25, 54) | 30 (20, 44) | 0.0460* |
| Location | C/A/T | 5/10/7 | 3/5/7 | 0.9013 |
| D/S/R | 2/13/24 | 2/7/19 | |
| Tumor type | Type 0 | 13 | 13 | 0.3008 |
| Type 1/2/3/4/5 | 3/32/12/0/1 | 3/22/0/1 | |
| Histological type | tub1/tub2/pap | 0/4/3 | 0/4/2 | 0.3118 |
| por/muc | 17/39/1 | 13/23/1 | |
| Depth | T0/T1/T2 | 5/5/13 | 3/10/12 | 0.0395* |
| T3/T4 | 31/7 | 15/3 | |
| Lymph node metastasis | N0 | 35 | 33 | 0.0409* |
| N1/N2/N3 | 16/7/3 | 7/0/3 | |
| Distant metastasis | M0 | 53 | 40 | 0.5190 |
| M1 | 8 | 3 | |
| Lymphatic duct invasion | ly0 | 15 | 19 | 0.2321 |
| ly1/ly2 | 34/12 | 21/3 | |
| Venous invasion | v0 | 48 | 36 | 0.6176 |
| v1/v2 | 9/4 | 7/0 | |
| Stage | 0/1/II | 5/13/15 | 3/20/9 | 0.0350* |
| IiIA/iIB/iIV | 13/7/8 | 6/2/3 | |

*Statistically significant. Data are presented as median (first quartile, third quartile). A, ascending colon; BMI, body mass index; C, cecum; D, descending colon; muc, mucinous carcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; R, rectum; S, sigmoid colon; T, transverse colon; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.

### Table 2. p-PDH expression and clinicopathological features of colorectal cancer

| Phospho-PDH-E1α | Positive (n = 34) | Negative (n = 70) | P-value |
|-----------------|------------------|------------------|---------|
| Patient background | | | | |
| Gender (Male/Female) | 22/12 | 43/27 | 0.7461 |
| Age (mean ± SD) | 66.0 ± 10.4 | 63.5 ± 13.3 | 0.1669 |
| BMI (kg/m²) | 23.6 (20.6, 25.6) | 21.9 (20.3, 25.6) | 0.4316 |
| CEA (ng/mL) | 2.5 (1, 6) | 3 (2.0, 7.3) | 0.4401 |
| CA19-9 (U/mL) | 12.5 (5.0, 23.3) | 12.5 (7.0, 21.3) | 0.8538 |
| Tumor characteristics | | | |
| Tumor diameter (mm) | 37 (22, 52) | 35 (22, 52) | 0.6126 |
| Location | C/A/T | 5/6/5 | 3/9/9 | 0.0883 |
| D/S/R | 3/3/12 | 1/17/31 |
| Tumor type | Type 0 | 11 | 15 | 0.2275 |
| Type 1/2/3/4/5 | 1/16/4/0/2 | 5/38/12/0/0 | |
| Histological type | tub1/tub2/pap | 10/18/1 | 20/44/1 | 0.2893 |
| por/muc | 2/3 | 2/3 | |
| Depth | T0/T1/T2 | 3/4/7 | 5/11/8 | 0.4779 |
| T3/T4 | 16/4 | 30/6 | |
| Lymph node metastasis | N0 | 23 | 45 | 0.7354 |
| N1/N2/N3 | 5/4/2 | 18/3/4 | |
| Distant metastasis | M0 | 29 | 64 | 0.3339 |
| M1 | 5 | 6 | |
| Lymphatic duct invasion | ly0 | 12 | 22 | 0.6857 |
| ly1/ly2 | 15/7 | 40/8 | |
| Venous invasion | v0 | 26 | 58 | 0.4394 |
| v1/v2 | 7/1 | 9/3 | |
| Stage | 0/1/II | 3/9/10 | 5/24/14 | 0.7461 |
| IiIA/iIB/iIV | 4/3/5 | 15/6/6 | |

Data are presented as median (first quartile, third quartile). A, ascending colon; BMI, body mass index; C, cecum; D, descending colon; muc, mucinous carcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; R, rectum; S, sigmoid colon; T, transverse colon; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.

Phospho-PDH-E1α was used to detect phosphorylated pyruvate dehydrogenase (PDH-E1α). The antibodies used were an anti-PDH-E1α antibody (ab209924; Abcam) for PDH-E1α and an anti-E-cadherin (ab14165; Abcam) for E-cadherin as an internal control. The phosphorylation status of PDH-E1α was determined using Western blotting and chemiluminescence imaging.
assessed using the t-test. Associations between discrete variables were analyzed using Pearson’s χ² test or Fisher’s exact test as appropriate. Mean values were compared using the Mann–Whitney U-test. P-values <0.05 were considered to indicate statistical significance.

Statistical analysis. JMP pro 10.0.2 software (SAS Institute, Cary, NC, USA) was used to perform statistical analysis. The Kaplan–Meier method was used to estimate tumor recurrence and the log-rank test was used to determine the statistical significance. Associations between discrete variables were assessed using the χ² test or Fisher’s exact test as appropriate. Mean values were compared using the Mann–Whitney U-test. P-values <0.05 were considered to indicate statistical significance.

Results

HK2 in CRC. We performed immunohistochemical staining of HK2 in clinical samples of CRCs. The intensities of HK2 staining rated in three stages were assigned to two groups: the HK2-negative group included scores of 0 or 1, while the HK2-positive group included score of 2 (representative cases are shown in Fig. 1b). As summarized in Table 1, HK2 expression was significantly associated with extensive tumor diameter (P = 0.0460), advanced tumor depth (P = 0.0395), and positive lymph node metastasis (P = 0.0409), suggesting that the HK2-positive group had more patients with advanced stages than the HK2-negative group. HK2 expression was not associated with the backgrounds of the patients, including serum tumor markers, tumor locations, and histological types of tumors.

p-PDH in CRCs. Immunohistochemical staining of p-PDH was performed in a manner similar to that of immunohistochemical analysis of HK2 (Fig. 1c). Correlations between p-PDH expression and clinicopathological factors are summarized in Table 2. In contrast to HK2 expression, p-PDH expression did not show any correlations with tumor depth, lymph node metastasis, or other patient backgrounds. Assessment of tumor locations indicated that p-PDH expression tended to be higher in right-sided CRCs than in left-sided CRCs, but did not reach statistical significance. We also performed immunohistochemical analysis to detect the total amount of PDH-E1α regardless of phosphorylation status in 19 colorectal cancer tissues. The result showed that total PDH-E1α-positive cases showed p-PDH positive or negative expression (Fig. 2; Figs S2 and S3), whereas the total PDH-E1α-negative cases (intensity score 0) was absent for p-PDH expression, although the low expression cases (score 1) could be p-PDH positive (Table 3; data not shown), suggesting that the phosphorylation event is independent of the protein amount, and that phosphorylation control may be critical in clinical status of tumors.

Heterogeneity of HK2 or p-PDH staining in CRC and in normal mucosa. We examined whether the heterogeneity of HK2 or p-PDH staining in CRC might be observed, and moreover, the staining intensities in normal mucosa. As shown in Table 4, positive correlation could be observed between the staining intensities in the deep part and in the superficial part of tumors regarding HK2 and p-PDH expression. In relation to the expression in normal mucosa, both expressions could scarcely be observed and any correlations with the staining in the deep part of tumor could not be observed.

Recurrence-free survival. We studied the correlations of HK2 or p-PDH expression with recurrence-free survival (RFS) in all the patients except for nine Stage IV patients who underwent non-curable resection. HK2 could separate the patients by prognosis, with positive HK2 expression being associated with

Table 3. Correlation between the immunohistochemical staining of phosphorylation status of PDH-E1α

| p-PDH | Positive | Negative | P  |
|-------|----------|----------|----|
| Total PDH-E1α | 6 | 8 | 1.0000 |
| Negative | 2 | 3 | 0.0024* |

Table 4. Assessment of the staining heterogeneity of HK2 and p-PDH in tumor superficial and deep part and in normal mucosa

| HK2 staining in deep part | HK2 staining in superficial part | P  |
|---------------------------|---------------------------------|----|
| Positive | 30 | 10 | 0.0074* |
| Negative | 31 | 33 | |
| HK2 staining in normal mucosa | 1 | 1 | 1.0000 |
| Negative | 54 | 38 | |

| p-PDH staining in deep part | p-PDH staining in superficial part | P  |
|-----------------------------|----------------------------------|----|
| Positive | 28 | 36 | 0.0024* |
| Negative | 6 | 34 | |
| p-PDH staining in normal mucosa | 0 | 0 | NA |
| Negative | 31 | 66 | |

*Statistically significant. In the assessment of staining in normal mucosa, the cases that did not contain normal mucosa in paraffin section were not included.
a poor survival rate ($P = 0.0290$) (Fig. 3a). In contrast, negative p-PDH expression tended to correlate with poor RFS, but this difference was not statistically significant ($P = 0.2572$) (Fig. 3b). We then assessed the ability of the combination of two metabolic markers to predict aggressive phenotypes of tumors and survival of patients. We classified the patients into

Table 5. Results of univariate and multivariate Cox regression analysis for Stage 0–IV patients

| Variables                  | Univariate HR (95% CI) | Univariate $P$ | Multivariate HR (95% CI) | Multivariate $P$ |
|----------------------------|------------------------|---------------|--------------------------|------------------|
| Age                        | 1.017 (0.977–1.063)    | 0.4066        | 4.186 (1.365–18.220)     | 0.0120*          |
| Gender                     |                         |               |                          |                  |
| Female                     | Reference              |               |                          |                  |
| Male                       | 3.015 (1.004–12.963)   | 0.0491*       | 4.186 (1.365–18.220)     | 0.0120*          |
| Tumor diameter             | 1.014 (0.994–1.032)    | 0.1515        |                          |                  |
| Location                   |                         |               |                          |                  |
| Right-sided                | Reference              | 0.2371        |                          |                  |
| Left-sided                 | 1.806 (0.691–5.588)    |               |                          |                  |
| Tumor type                 |                         |               |                          |                  |
| Type 0                     | Reference              | 0.0062*       |                          |                  |
| Type 1/2/3/4/5             | 7.787 (1.608–140.039)  |               |                          |                  |
| Histological type          | tub1/tub2/pap          | 0.5763        |                          |                  |
| por/muc                    | 1.557 (0.247–5.434)    |               |                          |                  |
| Depth                      |                         |               |                          |                  |
| T0/T1/T2                   | Reference              | 0.0028*       |                          | 0.0162*          |
| T3/T4                      | 4.497 (1.631–15.781)   |               | 3.695 (1.257–13.500)     |                  |
| Lymph node metastasis      |                         |               |                          |                  |
| N0                         | Reference              | 0.0009*       |                          | 0.0190*          |
| N1/N2/N3                   | 4.716 (1.892–12.704)   |               | 3.156 (1.207–8.912)      |                  |
| Immunohistochemistry       |                         |               |                          |                  |
| Others                     | Reference              | 0.0198*       |                          | 0.0656           |
| HK2+ and p-PDH-            | 2.952 (1.187–7.933)    |               | 2.383 (0.946–6.475)      |                  |

*Statistically significant. CI, confidence interval; HR, hazard ratio.
two groups: the combined HK2-positive and p-PDH-negative group and the group consisting of the other cases. This immunohistochemical evaluation of combined enzyme expression showed that positive HK2 expression combined with negative p-PDH expression was associated with poor RFS rates ($P = 0.0169$), which could be considered as more sensitive prognostic factor than HK2 alone (Fig. 3a–c). In the multivariate analysis, tumor depth and lymph node metastasis were found to be independent prognostic factors, while the combined evaluation showed the statistical significance in univariate, but not in multivariate analysis (Table 5).

Furthermore, we carried out a similar analysis of RFS for the 52 patients, including 24 Stage II patients and 28 Stage III patients, who had a certain level of recurrence risk and might benefit from adequate estimation of recurrence risk in that the necessity of adjuvant therapy could be evaluated (Table 6). (23) The immunohistochemical evaluation of combined enzyme expression showed that positive HK2 expression combined with negative p-PDH expression tended to separate the patients by prognosis; however, significant differences were not observed ($P = 0.0796$ and 0.0591, respectively; Fig. 3d,e). Interestingly, the immunohistochemical evaluation of combined enzyme expression showed that positive HK2 expression combined with negative p-PDH expression significantly correlated with poor RFS rates ($P = 0.0238$) (Fig. 3f). Multivariate analysis showed the combination of positive HK2 and negative p-PDH expression was independently associated with poor prognosis ($P = 0.0389$) (Table 7).

**Budding.** To confirm the reason why the combined evaluation of both HK2 and p-PDH expression strongly correlated with RFS in colorectal cancer patients especially in Stage II and III, we analyzed the association between the combined evaluation and “budding”. As a result, positive HK2 and negative p-PDH associated with the increased number of budding ($P = 0.0199$) (Fig. 4).

**HK2 and PDH activity analysis.** In the process of invading into stroma, cancer cells acquire the ability to detach from the epithelial lining and migrate, which is regulated by the mechanism of EMT, in a manner similar to the developmental program of an embryo. A set of pleiotropically acting genes orchestrates the EMT process by evoking loss of adherent junctions, conversion to spindly shapes, increased motility, and resistance to apoptosis in invasive front lesions of tumors. (24)

To study the underlined mechanism in the present observation, we induced EMT to colon cancer cell line SW480, as a model of invading colorectal cancer cells, according to the previously described procedure followed by the analyses of HK and PDH activity. (25–28) In response to EMT stimulation, cell morphology changed from epithelial to fibroblastic-like spindle shape (Fig. 5a) and expression of E-cadherin was decreased and Vimentin was increased (Fig. 5b). By acquiring mesenchymal phenotype, HK2 expression and phosphorylation level of PDH were up-regulated (Fig. 5b). Corresponding to these shifts in the expression of the two enzymes, HK activity and PDH activity were augmented in biochemical analyses (Fig. 5c,d). Activity of PDH is regulated by two key PDH-modifying enzymes; PDK1 phosphorylates PDH to suppress the function, whereas PDP2 dephosphorylates PDH to stimulate it. (17) We found that PDP2 expression was increased in EMT condition, but PDK1 expression was stable, which might explain why p-PDH was down-regulated (Fig. 5e).

### Table 6. Results of immunohistochemistry for Stage II and III cases based on HK2 and p-PDH expression

| HK2 expression | Positive | Negative |
|----------------|----------|----------|
| p-PDH expression |          |          |
| Positive | 10 | 6 |
| Negative | 25 | 11 |

### Table 7. Results of univariate and multivariate Cox regression analysis for Stage II and III patients

| Variables | Univariate | Multivariate |
|-----------|------------|--------------|
| Age       |            |              |
| Gender    |            |              |
| Female    | Reference  |              |
| Male      | 4.657 (1.284–29.806) | 0.0166* |
| Tumor diameter | 1.011 (0.989–1.030) | 0.3140 |
| Location  |            |              |
| Right-sided | Reference |              |
| Left-sided | 2.496 (0.852–9.011) | 0.0974 |
| Histological type |            |              |
| tub1/tub2/pap | Reference |              |
| por/muc    | 1.193 (0.186–4.321) | 0.8206 |
| Depth     |            |              |
| T0/T1/T2  | Reference  |              |
| T3/T4     | 2.390 (0.480–43.287) | 0.3387 |
| Lymph node metastasis |            |              |
| N0        | Reference  |              |
| N1/N2/N3  | 2.616 (0.892–9.458) | 0.0813 |
| Immunohistochemistry |            |              |
| Others    | Reference  |              |
| HK2+ and p-PDH | 3.451 (1.179–12.463) | 0.0230* |

*Statistically significant. CI, confidence interval; HR, hazard ratio.
Discussion

An increasing amount of evidence has shown that cancer-specific alterations in metabolism, that is, enhanced glucose uptake and successive preferential conversion to lactate, are important factors in cancer metabolism (the Warburg effect)\(^{(6,29)}\) and constitute tumor growth. This system is beneficial for the biosynthetic and bioenergetic demands of proliferation by diverting glycolytic intermediates to an alternative biosynthetic, the pentose phosphate pathway. Moreover, these metabolic systems are attractive targets for possible therapeutic interventions and currently research is ongoing to demonstrate the definite mechanism of cancer metabolism.\(^{(30)}\) Although aerobic glycolysis is intimately linked to tumor growth and cancer cell proliferation, how glycolysis is involved in cellular invasion remains unclear. Invasion and metastasis are hallmarks of cancer and are closely associated with the development of pathological stages of cancer arising from precancerous lesions in epithelial tissues.\(^{(24)}\) Distant metastasis becomes clinically evident as a consequence of multistep cascades that initially occur in local invasions.\(^{(31,32)}\) Thus, the study of the effect of cancer metabolism in invasion may be beneficial for elucidating the novel mechanism of invasion and metastasis. To the best of our knowledge, this is the first study to demonstrate a significant association between the expression of the biomarkers HK2 and p-PDH and patient survival.

Although tumor heterogeneity is largely a common feature for the generation of biological plasticity, genetic diversification,
and intractability of tumors in advanced stages, the presence of subpopulations with a high invasive potential (termed "budding") characterizes tumor heterogeneity in CRCs.\(^{(19,20)}\) Budding is defined as detachment from tumor tissues into single or up to five cancer cell clusters at invasive front lesions of CRCs.\(^{(19,20)}\) Previous reports, including our own, indicate that tumor budding undergoes EMT.\(^{34–36}\) The clinical guidelines of the European Society for Medical Oncology\(^{(37)}\) and the Japanese Society for Cancer of the Colon and Rectum\(^{(38)}\) include tumor budding. Based on these backgrounds, we aimed to examine the glycolytic characteristics of the deepest part of tumor and those of the cancer cells undergoing EMT in this study.

According to the previous studies, the association between HK2 and RFS has not been clear and consistent results could not be acquired yet.\(^{39–43}\) A possible explanation for this controversy is that the samples analyzed in these studies might be obtained from the superficial tissues of tumor. In considering the role of HK2 in aerobic glycolysis in the invasive front lesions of CRCs, where the cancer cells are usually located in the deep parts of tumors and are stimulated to invade and metastasize, the present study focused on samples from the invasive front lesions of CRCs. The present study showed that enhanced glucose uptake and glycolysis in the deeper parts of the tumors was associated with tumor growth and invasion, as shown by the data of lymph node metastasis samples. Thus, in invasive fronts, our results suggest that cancer metabolism may be reprogrammed and dominantly shifted toward active glycolysis.

We also studied the expression of p-PDH. Considering that p-PDH is involved in the OxPhos inhibition in the mitochondria and contributes to the establishment of aerobic glycolysis, it is possible that high p-PDH is associated with poor prognosis. Contrary to this original expectation, the present analysis of the invasive front lesions of CRCs showed that low p-PDH was associated with poor prognosis, and evaluation of combined expression of p-PDH and HK2 demonstrated a clear association with patient prognosis. The results suggest that p-PDH plays a unique role in the malignant behavior of CRCs. Interestingly, recent studies have identified two subpopulations of cancer cells that are distinct in their energy-generating pathways.\(^{44–46}\) One subpopulation depends on anaerobic glycolysis and secretes massive lactate. The other subpopulation can use lactate from upstream glycolysis in individual cells as well as from surrounding cells, and therefore the metabolites produced by the abovementioned subpopulation. The latter subpopulation can use lactate as the energy source by employing OxPhos in the mitochondria.\(^{46}\) Also as suggested by our in vitro experiments, we speculate that CRC cells perform OxPhos in invasive front lesions. This hypothesis may be further supported by the observations that the citric cycle generates reactive oxygen species by OxPhos, which promote EMT and further cancer invasion.\(^{47}\) Assessment of combined expression of HK2 and p-PDH may be useful for detecting highly malignant CRC cells. Further investigation should be performed via more detailed mechanistic studies of cancer metabolism associated with invasion and budding to identify more accurate predictors of patient prognoses and to regulate cancer invasion and metastasis.

In conclusion, combined expression of HK2 and p-PDH, as a novel cancer metabolomics-associated biomarker measure, may be clinically useful for predicting tumor aggressiveness and survival in CRC.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Western blot analysis of extracts from HEK293, HeLa cells and three clinical samples of colorectal cancer using Hexokinase 2 antibody.

Fig. S2. Western blot analysis of extracts from HEK293 cell and three clinical samples of colorectal cancer using p-PDH antibody.

Fig. S3. Western blot analysis of extracts from HEK293 cell and three clinical samples of colorectal cancer using PDH-E1α antibody.

Fig. S4. Absorption test of HK2 antibody on pancreatic cancer (A) and colorectal cancer tissues (B–D). Scale bar, 100 μm.