Hexokinase 2 as an independent risk factor for worse patient survival in esophageal adenocarcinoma and as a potential therapeutic target protein: A retrospective, single-center cohort study

SU IR LYU[1], ADRIAN GEORG SIMON[1], JIN-ON JUNG[2], CAROLINE FRETTER[1], WOLFGANG SCHRÖDER[2], CHRISTIANE J. BRUNS[2], THOMAS SCHMIDT[2], ALEXANDER QUAAS[1] and KARL KNIPPER[2]

[1]Faculty of Medicine and University Hospital of Cologne, Institute of Pathology, University of Cologne, D-50937 Cologne, Germany; [2]Department of General, Visceral and Cancer Surgery, Faculty of Medicine and University Hospital of Cologne, University of Cologne, D-50937 Cologne, Germany

Received March 15, 2024; Accepted June 26, 2024

DOI: 10.3892/ol.2024.14628

Abstract. Cancer cells exhibit a distinct metabolic profile that features an upregulation of less efficient glycolysis accompanied by lactate production for energy generation, in contract to the characteristic metabolism of normal cells. Consequently, cancer research has focused on the enzymes that participate in these cancer metabolic pathways. Among them, hexokinase 2 (HK2) has an important position as the initial enzyme in the glycolytic pathway. Increased expression levels of HK2 have been correlated with an increased risk of poor patient outcomes and advanced tumor stages in a number of malignant tumors, such as gastric carcinoma. The present study aimed to investigate the specific role of HK2 in patients diagnosed with esophageal adenocarcinoma. A total of 643 patients with esophageal adenocarcinoma were included. Immunohistochemical staining and HK2 mRNA *in situ* probes were used to investigate the association of HK2 expression levels with clinical and molecular tumor characteristics. Patients who exhibited high HK2 expression levels demonstrated significantly reduced overall survival (OS) times compared with patients who exhibited low HK2 expression levels (29.6 vs. 39.9 months, respectively; P=0.027). Furthermore, high HK2 expression levels were demonstrated to be an independent risk factor for reduced patient survival (hazard ratio, 1.65; 95% CI, 1.09-2.50; P=0.018). Significantly reduced patient survival was also demonstrated in the subgroups of male patients, patients with primarily resected tumors, patients with HER2-negative tumors and patients with tumors exhibiting Y chromosome loss. Elevated expression of HK2 was identified as a risk factor for unfavorable patient survival in esophageal adenocarcinoma. This revelation suggests the potential for future diagnostic and therapeutic avenues tailored to this specific patient subset. Identifying patients with high HK2 expression may pinpoint a higher-risk cohort, paving the way for comprehensive prospective studies that could advocate for intensified monitoring and more aggressive therapeutic regimens. Furthermore, the targeted inhibition of HK2 could hold promise as a strategy to potentially enhance patient outcomes.

Correspondence to: Dr Karl Knipper, Department of General, Visceral and Cancer Surgery, Faculty of Medicine and University Hospital of Cologne, University of Cologne, 62 Kerpener Street, D-50937 Cologne, Germany
E-mail: karl.knipper@uk-koeln.de

Key words: esophageal cancer, hexokinase 2, prognosis, biomarker, treatment

Introduction

The incidence of esophageal adenocarcinoma (EAC) and adenocarcinoma of the gastroesophageal junction has gradually increased over the last three decades (Iincidence rate ratio, 2.45) (1). Despite stable incidence rates since then, the survival of these patients remains poor, which highlights the need for biomarkers for early disease detection and novel therapeutic strategies (1). A hallmark of cancer is altered cellular metabolism (2). The energy production in cancer cells is characterized by increased levels of the less efficient oxygen-independent glycolysis, followed by lactate production. While these pathways are also present in healthy cells, they are overly activated in cancer cells (3). The expression levels of distinct enzymes of the glycolysis pathway [hexokinase (HK)1 and pyruvate kinase isozyme M2] have been shown to correlate with disease progression, cancer cell invasion and poor patient survival in esophageal squamous cell cancer (ESCC) (4). The first step of glycolysis is the phosphorylation of glucose to glucose-6-phosphate by the HK enzyme; five different isozymes of HK have been reported, with, for instance, type I facilitating catabolic functions through mitochondrial interaction and utilizing intramitochondrial ATP, type II potentially serving anabolic roles, type III primarily

localized perinuclearly with less understood functions, type IV involved in carbohydrate metabolism as a glucose sensor, and type V involved in the gestational glucose regulation (5-8). These isozymes show tissue-specific expression patterns and varying affinities towards glucose. The expression of HK2, one of the five isozymes, in human adults is limited, as HK2 is only expressed in skeletal muscle and adipose tissues under physiological conditions (6). However, HK2 becomes upregulated in cancer cells (9). Additionally, HK2 expression levels are increased in esophageal cancer compared with those in normal esophageal tissues (10). Therefore, HK2 is not only of interest in metabolic pathway studies or as a sole biomarker, but also as a potential target for novel therapeutic options for patients with esophageal cancer.

The increased activation of glycolysis and the subsequent increased demand for glucose in cancer cells have become integral elements in cancer diagnostics, notably in fluorodeoxyglucose-positron emission tomography (FDG-PET) (11). Previous reports on the correlation between FDG uptake and HK2 expression vary. In colorectal cancer and cholangiocarcinoma, no correlation between FDG uptake and HK2 expression was detected. However, increased HK2 expression levels were positively correlated with increased FDG uptake in hepatocellular carcinoma (11,12). This suggests that different enzymes of glycolysis are activated depending on different tumor entities and therefore different tumor microenvironments.

In a previous expression pattern study, high HK2 expression levels were associated with poor patient outcomes, as well as higher tumor stages, the occurrence of lymph node metastases and increased tumor size in colorectal cancer, gastric cancer and hepatocellular cancer (13).

HK2 expression levels have been reported as significantly increased in ESCC compared with those in EAC (14). However, the role of HK2 and its prognostic implications in EAC are currently unclear. Therefore, the present study aimed to elucidate the impact of HK2 expression levels on the oncological outcome of patients with EAC.

Materials and methods

Patients and tumor samples. The study present was approved by the Ethics Committee of the University Hospital of Cologne (approval no. 21-1146; Cologne, Germany) and was conducted in accordance with the Declaration of Helsinki. The study was reported in line with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines (15). Patients from 1998 until 2019 at the University Hospital of Cologne (Cologne, Germany) were screened for the present retrospective, single-center cohort study. Inclusion criteria were as follows: A diagnosis of EAC, patients underwent an Ivor-Lewis esophagectomy, curative treatment intention, and sufficient tumor tissue was available for the tissue microarray. All patient data were collected prospectively and analyzed retrospectively for the present study. Written informed consent for inclusion in the database and tissue bank was obtained from each patient. The median age of all included patients was 63.1 years (range, 27.8-91.6 years). Overall survival (OS) was defined as the time from the date of surgery until death or being censored in case of loss of follow-up and was updated yearly. Patients who had experienced survival periods of <90 days postoperatively or lacked sufficient tissue for the subsequent analysis were excluded from the present study. The pathological assessment of tumor samples was conducted according to the 7th edition of the Union for International Cancer Control (16). The tumor borders of the primary tumor tissue samples were demarcated by an experienced pathologist and 1.2-mm tissue cylinders were punched out using a semi-automated precision instrument. The tissue cylinders were then transferred to a paraffin-embedded tissue microarray and were cut into 4-μm slices. Tissues were fixed in a 4% formaldehyde solution at room temperature for 24 h, followed by embedding in paraffin.

Fluorescence in situ hybridization (FISH). FISH was conducted as described in a previous study (17). Briefly, analysis was performed for the long (green) and short (red) arm of the Y chromosome [cat. no. (long), 05J10-024; cat. no. (short), 05J27-079; Abbott]. The ready-to-use FISH pretreatment kit was utilized (Vysis IntelliFISH Universal FFPE Tissue Pretreatment Protease; cat. no. 08N85-005; Abbott), all in accordance with the manufacturer's instructions. A fully automated upright fluorescence microscope Leica DM5500 B (Leica Microsystems) was used. The imaging was performed with a JVC KY-F75 digital camera (JVCKenwood) (Fig. S1A and B). FISH data were analyzed by two experienced pathologists. The absence of green and red staining was defined as Y chromosome loss. Internal controls were performed by screening normal epithelial tissue, fibroblasts or lymphocytes on the tumor sample slide. Samples were excluded if no clear control could be obtained.

Immunohistochemistry (IHC). HK2 staining was conducted using the automatic staining system Leica BOND-MAX (Leica Biosystems). Dilutions, reagents and control tissues were used according to the manufacturer. Here, the polymer refine detection kit BOND Epitope retrieval Solution 1 (cat. no AR9961; Leica Biosystems) was used (100˚C for 5 min) to perform the automated staining according to the manufacturer's instructions. IHC staining was performed using primary antibodies for HER2 [cat. no. 4B5; Roche Diagnostics; with EDTA (BOND Epitope retrieval Solution 1; Leica Biosystems) as a buffer for the epitope retrieval; with positive control breast carcinoma cells previously confirmed to be HER2-positive] and HK2 [1:500; cat. no. ab104836; Abcam; with EDTA (BOND Epitope retrieval Solution 1, Leica Biosystems) as a buffer for the antigen retrieval; with negative control normal human esophagus epithelium cells]. HER2 and HK2 staining was analyzed by two experienced pathologists. HER2 staining was defined as either negative or positive. The staining intensity and the percentage of positive cancer cells of the HK2 staining were assessed to calculate a H-score, which was computed using these parameters as previously described (18). The patient cohort was divided into two groups using the median H-score: Low expression of HK2 (H-score <100) and high expression of HK2 (H-score ≥100).

RNAScope™ for HK2. The RNAScope™ assay was performed as described previously, following the manufacturer's instructions (19). According to the user manual of the kit, 5-μm thick tissue microarray sections were deparaffinized, pretreated,

digested, hybridized and counterstained using hematoxylin before developing the signal, using the provided RNAScope 2.5 HD Assay-RED (cat. no. 322360; Bio-Techne) and mRNA probe RNAscope Probe-Hs-HK2 (cat. no. 487731; Advanced Cell Diagnostics; Bio-Techne). Analyses of the RNAScope assay results were performed independently by two experienced pathologists using a Leica DM2500 light microscope (Leica Microsystems). For imaging, the slides were scanned with the Aperio GT 450 DX (Leica Biosystems). Signal scoring followed the manufacturer's guidelines (score 0, <1 dots/cell; score 1, 1-3 dots/cell; score 2, 4-9 dots/cell; score 3, 10-15 dots/cell; and score 4, >15 dots/cell). Positivity was defined as a score >0, which reflected the presence of detectable signals according to the specified scoring criteria.

Validation of the IHC HK2 antibody. HK2 IHC results were compared with results using the aforementioned RNAscope mRNA *in situ* hybridization probes targeting HK2 in 10 early-stage esophageal adenocarcinoma cases, which were included in the total study cohort, to validate use of the HK2 antibody. In all cases, the expression levels detected by the IHC HK2 antibody corresponded with those identified by RNAScope, demonstrating agreement between the HK2 expression levels detected using both methods. Of these, the four HK2 positive cases exhibited high HK2 expression levels using both assays, which confirmed the accuracy and reliability of the immunohistochemical HK2 antibody.

Statistical analysis. All statistical analyses were conducted using SPSS (version 29.0.1.1; IBM Corp.). Survival data are presented as Kaplan-Meier curves and were analyzed using the log-rank test. Associations between clinicopathological values and survival data were assessed using univariate and multivariate Cox regression analyses. Qualitative values were compared using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Correlation between HK2 expression and clinicopathological values. In the present study, 643 patients with EAC who underwent Ivor-Lewis esophagectomy at the University Hospital of Cologne were included (Fig. 1). A large proportion of the included patients were male (87.6%). The median OS time of the total patient cohort was 24.0 months. Neoadjuvant therapy was administered to 69.2% of patients (n=445). Lymph node metastases were diagnosed in 59.6% of the included patients (n=383) (Table I). The study cohort was stratified into two groups using the median H-score for IHC staining: i) Tumors with low HK2 expression (n=307); and ii) tumors with high HK2 expression (n=336) (Fig. 2A). IHC staining was verified through comparison with RNAScope HK2 staining (Fig. S1C and D). In all cases evaluated, the RNAScope and IHC results corresponded, which confirmed the association between HK2 mRNA and protein expression levels. The clinicopathological characteristics between these two groups were compared (Table I). High HK2 expression levels were significantly associated with patients who underwent perioperative therapy with chemoradiotherapy for oesophageal cancer followed by surgery (CROSS; P=0.033). No other significant differences between patients with low and high HK2 expression in all other variables mentioned in Table I could be detected.

High HK2 expression is associated with worse patient survival in the total cohort. Survival analyses was performed to assess the impact of HK2 expression on patient survival. High HK2 expression levels were significantly associated with reduced patient times survival in the total cohort (median OS, 29.6 vs. 39.9 months, respectively; P=0.027; Fig. 2B).

HK2 expression levels are correlated with patient survival in primarily resected patients, but not in neoadjuvant-treated patients. Due to the standard use of multimodal therapy for a large portion of patients with EAC, the patient cohort was divided into two subsets: i) Patients who had received neoadjuvant treatment; and ii) patients who had undergone primary surgery without preceding adjuvant interventions. The aforementioned impact on patient survival was substantiated within the subgroup of primarily resected patients, as high HK2 expression was significantly associated with reduced patient survival time in the primary surgery cohort (median OS, 33.9 vs. 140.9 months, respectively; P=0.013; Fig. 2C). However, no significant difference was demonstrated with regard to the impact of HK2 expression levels on patient survival for those who underwent neoadjuvant therapy (median OS, 28.1 vs. 31.6 months, respectively; P=0.391; Fig. 2D). Furthermore, survival analyses were conducted for two perioperative therapy regimes, namely, CROSS and fluorouracil, leucovorin, oxaliplatin and docetaxel (FLOT). No significant survival differences associated with HK2 expression levels were demonstrated in the CROSS (median OS, 27.2 vs. 26.8 months, respectively; P=0.400; Fig. 2E) or the FLOT (median OS, 24.1 vs. 22.8 months, respectively; P=0.712; Fig. 2F) subgroups.

High HK2 expression is an independent risk factor for worse patient survival in multivariate Cox regression analysis. Cox regression analysis was performed to assess the association between clinicopathological values and patient survival. In univariate analyses, high pathological tumor status (y)pT-status, high pathological lymph node status (y)pN-status, high grading (G) and high HK2 expression were associated with reduced patient survival in the total patient cohort [(y)pT, P<0.001; (y)pN, P<0.001; G, P=0.001; HK2 expression, P=0.028; Table SI]. In multivariate analyses, high HK2 expression levels were demonstrated to be an independent risk factor, as a significant association with reduced patient OS was demonstrated (HR, 1.629; 95% CI, 1.077-2.465; P=0.021; Table II). Additionally, (y)pT-, (y)pN- and lymphatic vessel invasion (L)-status were associated with significantly reduced patient survival, thus representing independent risk factors for patients with EAC [(y)pT, P=0.009; (y)pN, P<0.001; L-status, P=0.009; Table II]. Similar findings could be found in the primarily resected subgroup. Here, higher age, higher (y)pT-, (y)pN- and G-status, and high HK2 expression were correlated with worse patient survival in the univariate analyses [age, P<0.001; (y)pT, P<0.001; (y)pN, P<0.001; G, P=0.002; HK2 expression, P=0.014; Table SI]. In the multivariate analyses, (y)pT-, (y)pN-stage and high HK2 expression were independent risk factors for reduced patient survival [(y)pT, P=0.004; (y)pN, P<0.001; HK2 expression, P=0.040; Table II].

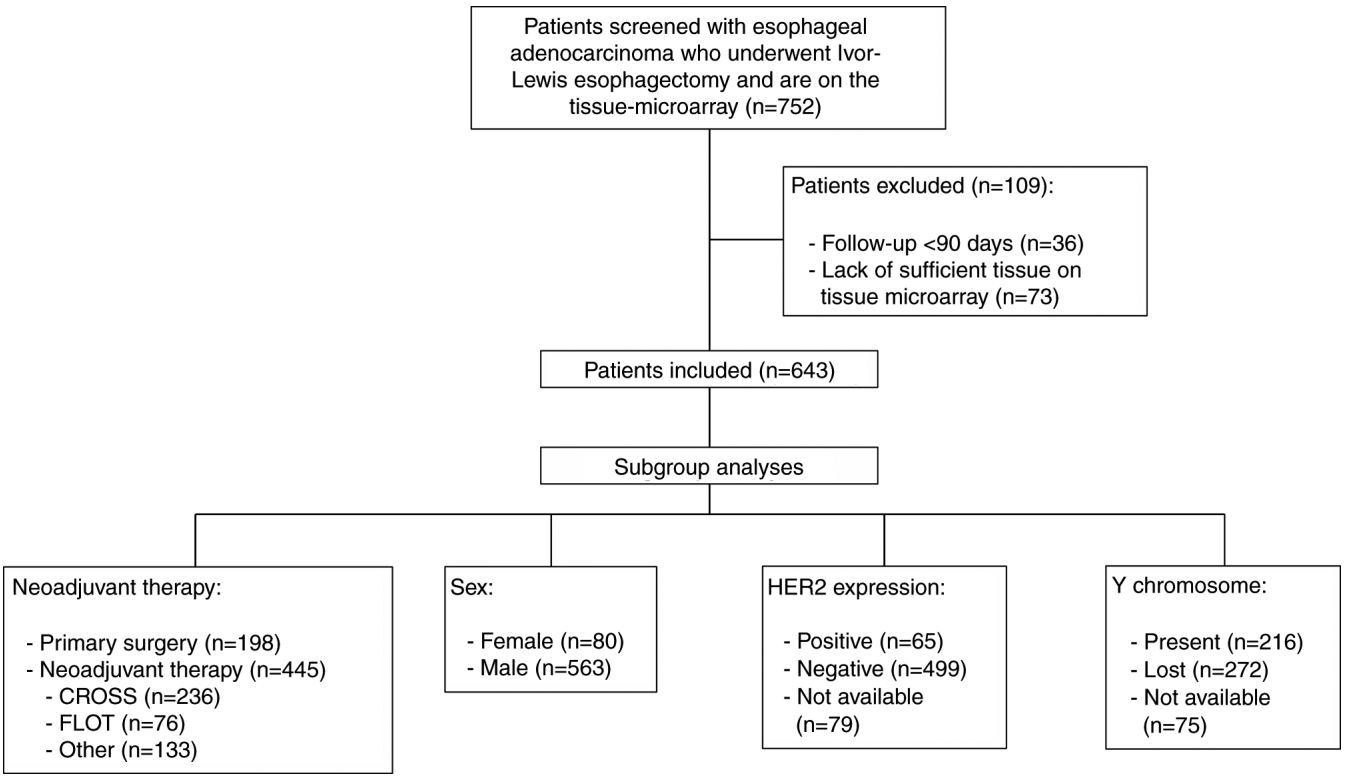

Figure 1. Flow diagram of the inclusion process and subgroup analyses. CROSS, chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study; FLOT, fluorouracil, leucovorin, oxaliplatin and docetaxel.

High HK2 expression was associated with worse patient survival in male patients, HER2-negative tumors and tumors with Y chromosome loss.

Since patient sex, HER2 expression levels and Y chromosome loss are reported factors that influence patient survival in patients with EAC (20-22), further analysis of the impact of HK2 expression on patient survival in these subgroups was performed. The patient cohort was divided into subgroups based on sex (female or male), HER2 expression (negative or positive) and the state of the Y chromosome in the tumor cells [present (positive) or lost (negative)]. Patients with high HK2 expression showed a significantly reduced OS in subgroups of male patients, HER2-negative tumors and tumors with Y chromosome loss (sex, P=0.038; HER2 status, P=0.020; Y chromosome loss, P=0.018; Fig. 3A-F).

Multivariate analyses confirm high HK2 expression as an independent risk factor for reduced patient survival in the subgroup of HER2-negative tumors. Univariate and multivariate Cox regression analyses were performed on the sex, HER2 expression level and Y chromosome patient subgroups. In univariate analyses, high HK2 expression levels were associated with worse patient survival in the subgroup of patients with tumors with Y chromosome loss (HR, 1.465; 95% CI, 1.066-2.014; P=0.018; Table SII) and male patients (HR, 1.262; 95% CI, 1.012-1.572; P=0.039; Table SII). However, high HK2 expression was not significantly associated with patient survival in the subgroup of patients with tumors with Y chromosome loss (HR, 1.505; 95% CI, 0.830-2.727; P=0.178; Table SIII) or male patients in multivariate analyses (HR, 1.395; 95% CI, 0.889-2.189; P=0.147; Table SIII). In HER2-negative tumors, the expression level of HK2 was significantly associated with reduced patient survival in univariate and multivariate analyses (univariate: HR, 1.332; 95% CI, 1.045-1.697, P=0.021; Table SI; Multivariate: HR, 1.687; 95% CI, 1.029-2.764, P=0.038; Table II). In addition, (y)pT- and (y)pN-status were significantly associated with reduced patient survival and thus represented risk factors for reduced patient survival [(y)pT, P=0.031; (y)pN, P=0.006; Table II].

No survival difference is observed in subgroups with specific risk behavior, such as smoking, in regard to different HK2 expression levels. The impact of reported high-risk behaviors such as nicotine and alcohol consumption on the association between HK2 expression levels and patient survival was investigated. No significant differences in the HK2 expression levels were demonstrated in these subgroups (Table I). From survival analyses, HK2 expression levels showed no significant survival differences associated with smoking and alcohol consumption status (non-smoker, P=0.239; smoker, P=0.252; no alcohol consumption, P=0.504; alcohol consumption, P=0.889; Fig. S2).

In summary, the present study demonstrated that high HK2 expression levels may potentially represent a risk factor associated with the reduced OS of patients with EAC and in patients with EAC who underwent primary resection. Moreover, high HK2 expression levels additionally represent a potential risk factor for the specific high-risk subgroups of male patients and tumors with Y chromosome loss.

Discussion

The present study aimed to evaluate the prognostic significance of HK2 in patients diagnosed with EAC. The expression levels

Table I. General clinicopathological values of the total study population (n=643) and patients with low (n=307) or high (n=336) HK2 expression levels.

Characteristic	Patients	Low HK2 expression	High HK2 expression	P-value
Total patients, n (%)	643 (100.0)	307 (100.0)	336 (100.0)	
Sex, n (%)				0.250
Male	563 (87.6)	264 (86.0)	299 (89.0)	
Female	80 (12.4)	43 (14.0)	37 (11.0)	
Age, n (%)				0.401
<65 years	365 (56.8)	169 (55.0)	196 (58.3)	
≥65 years	278 (43.2)	138 (45.0)	140 (41.7)	
Alcohol, n (%)				0.666
No	181 (28.1)	91 (29.6)	90 (26.8)	
Yes	56 (8.7)	30 (9.8)	26 (7.8)	
Unknown	406 (63.1)	186 (60.6)	220 (65.5)	
Nicotine, n (%)				0.467
No	118 (18.4)	65 (21.2)	53 (15.8)	
Active smoker	78 (12.1)	37 (12.1)	41 (12.2)	
Former smoker	106 (16.5)	51 (16.6)	55 (16.4)	
Unknown	341 (53.0)	154 (50.2)	187 (55.7)	
Median overall survival (range), months	24.0 (3.0-233.6)	25.4 (3.0-214.8)	22.9 (3.0-233.6)	
Neoadjuvant therapy, n (%)				0.269
No	198 (30.8)	101 (32.9)	97 (28.9)	
Yes	445 (69.2)	206 (67.1)	239 (71.1)	
Neoadjuvant therapy regime, n (%)				0.033
CROSS	236 (53.0)	95 (46.1)	141 (59.0)	
FLOT	76 (17.1)	41 (19.9)	35 (14.6)	
Other	133 (29.9)	70 (34.0)	63 (26.4)	
(y)pT, n (%)				0.236
1	122 (19.0)	68 (22.1)	54 (16.1)	
2	106 (16.5)	49 (16.0)	57 (17.0)	
3	394 (61.3)	179 (58.3)	215 (64.0)	
4	21 (3.3)	11 (3.6)	10 (3.0)	
(y)pN, n (%)				0.483
0	260 (40.4)	131 (42.7)	129 (38.4)	
1	182 (28.3)	86 (28.0)	96 (28.6)	
2	106 (16.5)	51 (16.6)	55 (16.4)	
3	95 (14.8)	39 (12.7)	56 (16.7)	
L, n (%)				0.682
0	292 (45.4)	142 (46.3)	150 (44.6)	
1	351 (54.6)	165 (53.7)	186 (55.4)	
V, n (%)				0.603
0	478 (74.3)	229 (74.6)	249 (74.1)	
1	68 (10.6)	29 (9.4)	39 (11.6)	
2	97 (15.1)	49 (16.0)	48 (14.3)	
Pn, n (%)				0.586
0	416 (64.7)	200 (65.1)	216 (64.3)	
1	131 (20.4)	58 (18.9)	73 (21.7)	
2	96 (14.9)	49 (16.0)	47 (14.0)	
G, n (%)				0.285
1	1 (0.2)	1 (0.3)	0 (0.0)	
2	103 (16.0)	56 (18.2)	47 (14.0)	
3/4	86 (13.4)	39 (12.7)	47 (14.0)	
Not applicable or unknown	453 (70.5)	211 (68.7)	242 (72.0)	

(y)pN, pathological lymph node status (after neoadjuvant therapy, if applicable); (y)pT, pathological tumor status (after neoadjuvant therapy, if applicable); CROSS, Chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study; FLOT, fluorouracil, leucovorin, oxaliplatin and docetaxel; G, grading, L, lymphatic vessel invasion; Pn, perineural invasion; V, venous vessel invasion.

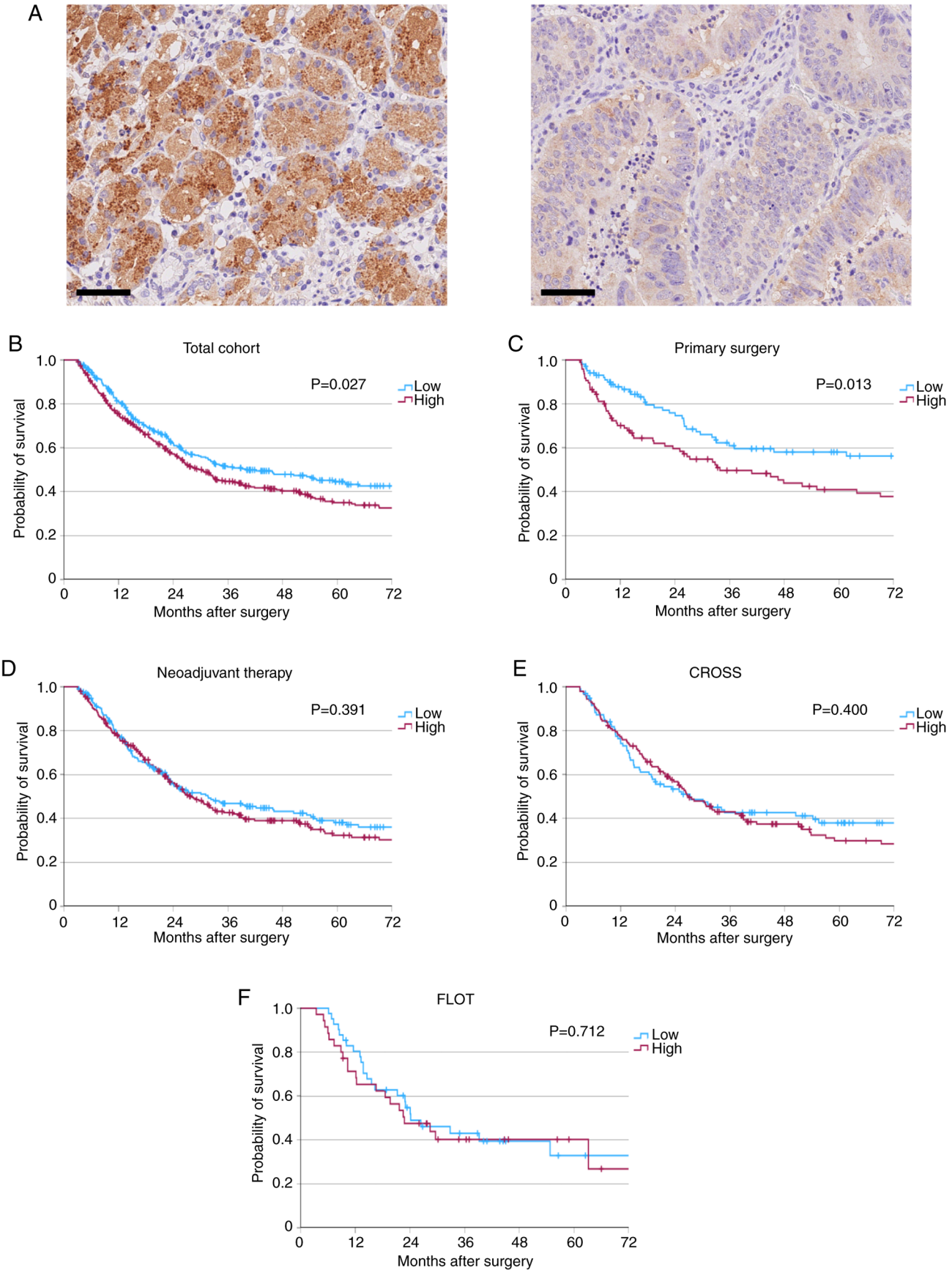

Figure 2. Representative immunohistochemical staining and survival analyses of patients with EAC. (A) Representative immunohistochemical images of tumor samples with high (left) and low (right) HK2 expression levels. Kaplan-Meier curves for overall survival classed by HK2 expression levels of (B) the total patient cohort (low, n=307; high, n=336; P=0.027) and the following subgroups: (C) Primarily resected patients (low, n=101; high, n=97; P=0.013), (D) patients following neoadjuvant therapy (low, n=206, high, n=239; P=0.391), (E) patients who received CROSS (low, n=95; high, n=141; P=0.400) and (F) patients who received FLOT as perioperative treatment (low, n=41; high, n=35; P=0.712). Scalebar, 50 μm. CROSS, Chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study; FLOT, fluorouracil, leucovorin, oxaliplatin, docetaxel.

Table II. Multivariate Cox regression analyses of the total cohort, patients following primary surgery and patients with negative HER2 expression.

		Total cohort			Primary surgery			Negative HER2 expression		
Characteristic	Borders	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age, years	≥65 vs. <65	1.394	0.863-2.252	0.174	1.445	0.895-2.331	0.132	1.590	0.909-2.779	0.104
(y)pT	≥2 vs. 1	1.512	1.107-2.066	0.009	1.568	1.153-2.133	0.004	1.462	1.036-2.064	0.031
(y)pN	≥1 vs. 0	1.497	1.185-1.891	<0.001	1.582	1.264-1.980	<0.001	1.449	1.112-1.887	0.006
L, stage	1 vs. 0	2.121	1.208-3.724	0.009	-	-	-	1.582	0.824-3.035	0.168
V, stage	≥1 vs. 0	0.692	0.501-0.954	0.025	0.882	0.671-1.161	0.371	0.763	0.519-1.122	0.170
G, stage	≥2 vs. 1	1.055	0.670-1.661	0.817	1.216	0.784-1.887	0.382	1.102	0.639-1.899	0.727
Hexokinase 2 expression	high vs. low	1.629	1.077-2.465	0.021	1.541	1.021-2.327	0.040	1.687	1.029-2.764	0.038

(y)pN, pathological lymph node status (after neoadjuvant therapy, if applicable); (y)pT, pathological tumor status (after neoadjuvant therapy, if applicable); G, grading, L, lymphatic vessel invasion; Pn, perineural invasion; V, venous vessel invasion; vs, versus; 95% CI, 95% confidence interval; -, not applicable.

of HK2 were analyzed in the tumor samples from 643 patients with EAC and survival and subgroup analyses were performed. High HK2 expression levels were significantly associated with reduced patient OS time. Furthermore, high HK2 expression levels were identified as an independent risk factor for reduced OS time of patients with EAC. To the best of our knowledge, this is the first report of the prognostic value of HK2 expression levels in EAC; however, similar effects for high HK2 expression have been reported in gastric adenocarcinoma and breast cancer (23,24). Patients with high HK2 expression levels could potentially benefit from more frequent follow-up exams but also from more aggressive neoadjuvant therapy options. In the future, HK2 expression levels could be assessed in biopsy material obtained during endoscopic evaluation before the initiation of neoadjuvant therapy, as these biopsies are routinely performed as part of the primary staging process (25). Further subgroup analyses would be necessary to confirm this hypothesis. The present study demonstrated HK2 expression levels to be an independent risk factor for patients with EAC following primary surgery. HK2 expression levels did not show prognostic value in patients who underwent esophagectomy after neoadjuvant therapy. Consistent with this observation, a previous study reported that neoadjuvant radiochemotherapy or chemotherapy induces general alterations in gene expression in EAC (26). Furthermore, HK2 expression levels differ significantly between pretreated patients and therapy-naive patients with EAC (27). Future studies could assess HK2 expression levels in pre- and post-neoadjuvant biopsies to further evaluate the prognostic value of varying HK2 expression during therapy. FDG uptake, a marker for glucose demand in cells used in staging exams, negatively correlates with HK2 expression in patients with esophageal carcinoma, and its increase is associated with worse patient survival and more frequent detection in patients with EAC (14), which could further suggest prognostic value for the assessment of HK2 expression levels. Patients with high HK2 expression levels could potentially represent a high-risk patient group, which may have otherwise been classified as low-risk based on the FDG-PET result. However, a previous clinical trial reported a correlation between HK2 expression and the accumulation of FDG (28). Therefore, further clinical studies are needed to assess the prognostic implications of the alteration in HK2 expression levels during tumor progression and therapy.

The prognostic value of HK2 in subgroups defined by the expression or loss of previously described biomarkers (for instance, Y chromosome loss and HER2-negative tumors) for patients with EAC were assessed in the present study. High HK2 expression was associated with worse patient survival in the subgroups of male patients and tumors with Y chromosome loss, which have previously described as high-risk groups (20,21). Furthermore, high HK2 expression levels were identified as an independent risk factor for reduced survival in patients with HER2-negative tumors, which is a subgroup that has previously been associated with improved patient survival (22).

HK2 could also be targeted as a potential therapeutic option for patients with EAC and hypothetically other cancer types. For instance, in head and neck squamous cell carcinoma, HK2 knockdown showed decreased cell growth *in vitro* and inhibited tumor progression *in vivo* (29). Increased HK2 expression levels are associated with decreased patient survival in nasopharyngeal carcinoma, and inhibition of HK2 using the glycolysis inhibitor 3-bromo-2-oxopropionate-1-propyl-ester reduces cell proliferation and invasion while increasing cell apoptosis *in vitro* (30). Capsaicin, which naturally occurs in red hot peppers, is recognized for its anticancer properties (31). Mao *et al* (32) reported that use of capsaicin resulted in reduced HK2 expression levels in ESCC cells. Glucose consumption and lactate production of these cells were reduced by capsaicin in the study. High lactate levels are reported to correlate with poorer patient survival (33). Diclofenac, a widely used analgesic, is also described as an anticancer drug due to its ability to reduce lactate levels in *in vivo* glioma models, which alters the immune microenvironment and could help to overcome

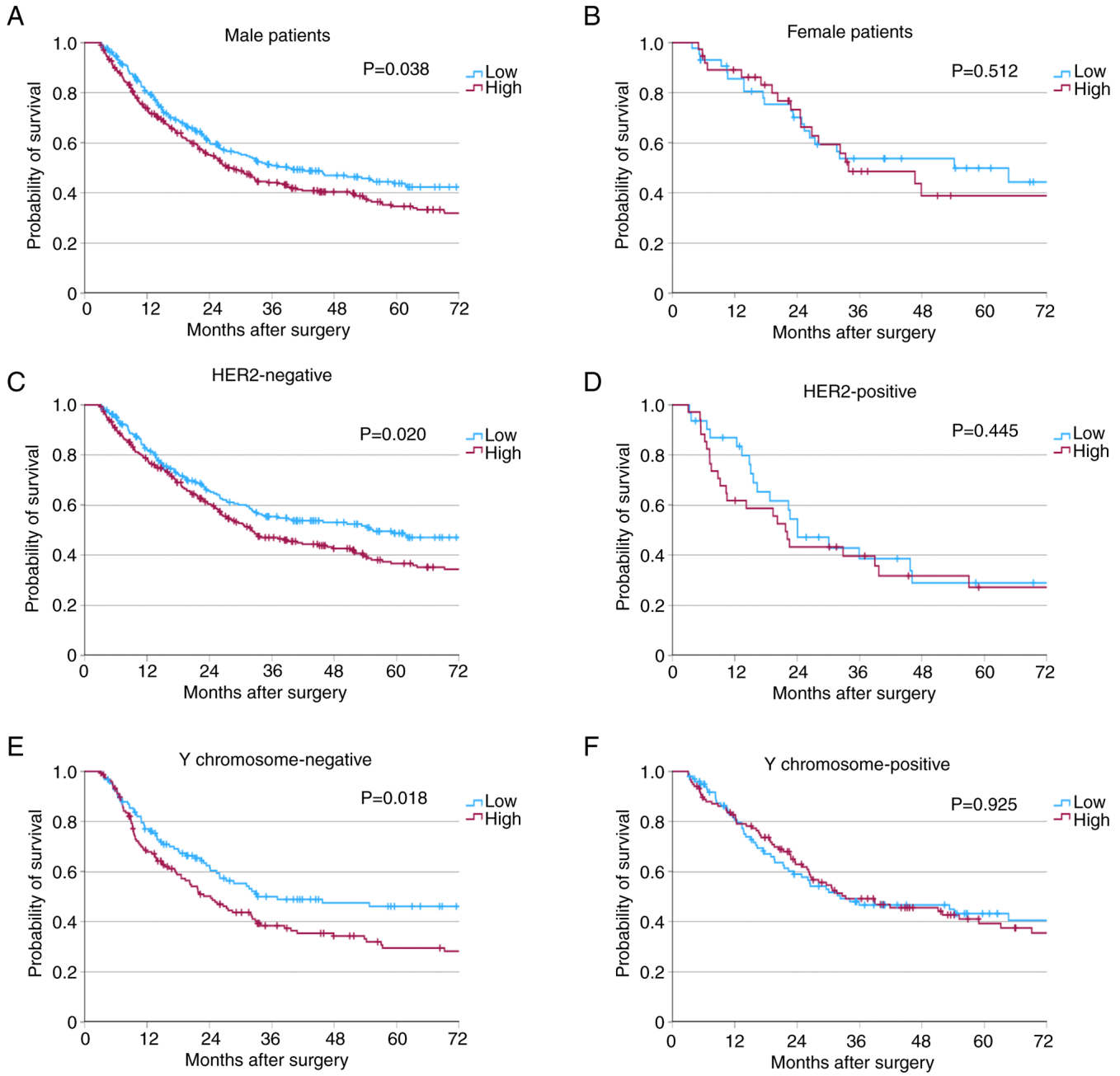

Figure 3. Survival analyses of subgroups. Kaplan-Meier curves for overall survival classed by HK2 expression levels of (A) male (low, n=264; high, n=299; P=0.038) and (B) female patients (low, n=43; high, n=37; P=0.512). The impact of HK2 expression levels on patient survival in subgroups of patients with (C) negative (low, n=234; high, n=265; P=0.020) and (D) positive (low, n=31; high, n=34; P=0.445) HER2 expression, and (E) negative (low, n=124; high, n=148; P=0.018) and (F) positive (low, n=99; high, n=117; P=0.925) Y chromosome expression.

immune escape mechanisms (34,35). HK2 expression levels were also related to altered immune cell infiltrates (36). Therefore, in the future, manipulation of HK2 expression levels via inhibitors could potentially help to overcome tumor resistance caused by immune escape. Nonetheless, the targeting of HK2 expression in patients with cancer should be further investigated, as HK2 also participates for in a number of physiological mechanisms of the glucose metabolism, including phosphorylation of glucose, anabolic functions, and subcellular localization and mitochondrial binding (6).

HK2 expression levels have been reported to impact the therapeutic response of a number of types of tumors. Patients with ovarian cancer who exhibited high HK2 expression levels demonstrated a significantly increased frequency of chemoresistance (tumor recurrence within 6 months after the termination of first-line chemotherapy) (37). In addition, HK2 knockdown in head and neck squamous cell carcinoma cells showed an increased sensitivity to cisplatin and 5-fluorouracil, which may be of significant importance for patients with esophageal carcinoma (29). According to the ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up, patients with locally advanced esophageal carcinoma are eligible for perioperative radio-chemotherapy (25). Here, patients receive either chemotherapy with 5-fluorouracil, oxaliplatin and docetaxel (FLOT protocol) or radiochemotherapy with carboplatin and paclitaxel (CROSS

protocol) (38,39). From the aforementioned results of the present study and previous studies discussed, it could be suggested that patients characterized by low HK2 expression levels may theoretically have improved benefits from perioperative therapy.

The present study had a number of limitations. The inclusion of disease-free survival data could add more clinically significant information, although it was not evaluated in the present study. However, as a number of patients at the University Hospital of Cologne were referred nationally and internationally, reliable disease-free survival data was not available. Additionally, as the present study was conducted retrospectively, future prospective studies to assess the value of HK2 as a biomarker are warranted. Finally, as a single-center cohort study was conducted, potential selection biases may have been introduced, such as the ethnicity of patients, as predominantly Caucasian patients are treated at the University Hospital of Cologne. Therefore, further multi-center studies are required to confirm the generalizability of the present study.

In spite of the aforementioned limitations, the present study demonstrated that HK2 expression levels represent an independent risk factor for the reduced survival of patients with EAC. Through future research, HK2 expression status may potentially be used in daily clinical decision-making. The evaluation of pre-neoadjuvant HK2 has potential to streamline perioperative therapy selection. Moreover, the investigation of HK2 and glycolysis as potential therapeutic targets could encompass mechanisms relating to the induction of apoptosis, the inhibition of cell proliferation and addressing immune escape pathways.

The present study assessed the prognostic significance of the IHC expression levels of HK2 in 643 patients diagnosed with EAC. An association between HK2 expression and unfavorable patient outcomes, particularly in primary resections, male patients, cases with Y chromosome loss and tumors negative for HER2 was demonstrated. This suggested that the incorporation of HK2 as a biomarker has potential to identify a high-risk subgroup of patients in the future. This high-risk subgroup may benefit from more frequent follow-up examinations or even alternate therapeutic interventions. Moreover, patients with low HK2 expression levels could potentially derive benefits from multimodal treatment protocols, such as perioperative FLOT therapy. In the future, HK2 may be considered as a therapeutic target to improve treatment outcomes of patients with EAC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SIL, AQ, KK, WS, CJB and TS contributed to the study's conception and design. Material preparation and data collection were performed by SIL, JOJ, CF and KK. WS and AQ confirm the authenticity of all the raw data. Analysis was performed by KK. SIL, AGS and AQ analyzed pathology data. The first draft of the manuscript was written by SL and KK. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Cologne (Cologne, Germany; approval no. 21-1146). Informed consent for inclusion in the database and tissue bank was obtained from all individual participants included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Agarwal S, Bell MG, Dhaliwal L, Codipilly DC, Dierkhising RA, Lansing R, Gibbons EE, Leggett CL, Kisiel JB and Iyer PG: Population based time trends in the epidemiology and mortality of gastroesophageal junction and esophageal adenocarcinoma. Dig Dis Sci 69: 246-253, 2024.
2. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
3. Ganapathy-Kanniappan S and Geschwind JF: Tumor glycolysis as a target for cancer therapy: Progress and prospects. Mol Cancer 12: 152, 2013.
4. Li W, Xu Z, Hong J and Xu Y: Expression patterns of three regulation enzymes in glycolysis in esophageal squamous cell carcinoma: association with survival. Med Oncol 31: 118, 2014.
5. Irwin DM and Tan H: Molecular evolution of the vertebrate hexokinase gene family: Identification of a conserved fifth vertebrate hexokinase gene. Comp Biochem Physiol Part D Genomics Proteomics 3: 96-107, 2008.
6. Wilson JE: Isozymes of mammalian hexokinase: Structure, subcellular localization and metabolic function. J Exp Biol 206: 2049-2057, 2003.
7. Van Schaftingen E: Hexokinase/glucokinase. In: Encyclopedia of Biological Chemistry. Academic Press, London, pp543-547, 2013.
8. Guo C, Ludvik AE, Arlotto ME, Hayes MG, Armstrong LL, Scholtens DM, Brown CD, Newgard CB, Becker TC, Layden BT, *et al*: Coordinated regulatory variation associated with gestational hyperglycaemia regulates expression of the novel hexokinase HKDC1. Nat Commun 6: 6069, 2015.
9. Shinohara Y, Yamamoto K, Kogure K, Ichihara J and Terada H: Steady state transcript levels of the type II hexokinase and type 1 glucose transporter in human tumor cell lines. Cancer Lett 82: 27-32, 1994.
10. Li R, Mei S, Ding Q, Wang Q, Yu L and Zi F: A pan-cancer analysis of the role of hexokinase II (HK2) in human tumors. Sci Rep 12: 18807, 2022.
11. Izuishi K, Yamamoto Y, Sano T, Takebayashi R, Nishiyama Y, Mori H, Masaki T, Morishita A and Suzuki Y: Molecular mechanism underlying the detection of colorectal cancer by 18F-2-fluoro-2-deoxy-D-glucose positron emission tomography. J Gastrointest Surg 16: 394-400, 2012.

12. Paudyal B, Oriuchi N, Paudyal P, Tsushima Y, Higuchi T, Miyakubo M, Ishikita T, Nakajima T and Endo K: Clinicopathological presentation of varying 18F-FDG uptake and expression of glucose transporter 1 and hexokinase II in cases of hepatocellular carcinoma and cholangiocellular carcinoma. Ann Nucl Med 22: 83-86, 2008.
13. Wu J, Hu L, Wu F, Zou L and He T: Poor prognosis of hexokinase 2 overexpression in solid tumors of digestive system: A meta-analysis. Oncotarget 8: 32332-32344, 2017.
14. Schreurs LMA, Smit JK, Pavlov K, Pultrum BB, Pruim J, Groen H, Hollema H and Plukker JT: Prognostic impact of clinicopathological features and expression of biomarkers related to (18)F-FDG uptake in esophageal cancer. Ann Surg Oncol 21: 3751-3757, 2014.
15. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC and Vandenbroucke JP: The strengthening the reporting of observational studies in epidemiology (STROBE) statement: Guidelines for reporting observational studies. Lancet 370: 1453-1457, 2007.
16. Rous B, Asamura H, Van Eycken E and Brierley JD (eds): TNM atlas. 7th edition. Wiley-Blackwell, Hoboken, NJ, 2021.
17. Loeser H, Waldschmidt D, Kuetting F, Heydt C, Zander T, Plum P, Alakus H, Buettner R and Quaas A: Copy-number variation and protein expression of DOT1L in pancreatic adenocarcinoma as a potential drug target. Mol Clin Oncol 6: 639-642, 2017.
18. Mazières J, Brugger W, Cappuzzo F, Middel P, Frosch A, Bara I, Klingelschmitt G and Klughammer B: Evaluation of EGFR protein expression by immunohistochemistry using H-score and the magnification rule: Re-analysis of the SATURN study. Lung Cancer 82: 231-237, 2013.
19. Schiffmann LM, Loeser H, Jacob AS, Maus M, Fuchs H, Zhao Y, Tharun L, Essakly A, Iannos Damanakis A, Zander T, *et al*: Dickkopf-2 (DKK2) as context dependent factor in patients with esophageal adenocarcinoma. Cancers (Basel) 12: 451, 2020.
20. Knipper K, Damanakis AI, Lyu SI, Simon AG, Wahler I, Bruns CJ, Schröder W, Schmidt T and Quaas A: High NANOG expression correlates with worse patients' survival in esophageal adenocarcinoma. BMC Cancer 23: 669, 2023.
21. Loeser H, Wölwer CB, Alakus H, Chon SH, Zander T, Buettner R, Hillmer AM, Bruns CJ, Schroeder W, Gebauer F and Quaas A: Y chromosome loss is a frequent event in Barrett's adenocarcinoma and associated with poor outcome. Cancers (Basel) 12: 1743, 2020.
22. Prins MJD, Ruurda JP, van Diest PJ, van Hillegersberg R and Ten Kate FJW: The significance of the HER-2 status in esophageal adenocarcinoma for survival: An immunohistochemical and an in situ hybridization study. Ann Oncol 24: 1290-1297, 2013.
23. Qiu MZ, Han B, Luo HY, Zhou ZW, Wang ZQ, Wang FH, Li YH and Xu RH: Expressions of hypoxia-inducible factor-1α and hexokinase-II in gastric adenocarcinoma: The impact on prognosis and correlation to clinicopathologic features. Tumour Biol 32: 159-166, 2011.
24. Sato-Tadano A, Suzuki T, Amari M, Takagi K, Miki Y, Tamaki K, Watanabe M, Ishida T, Sasano H and Ohuchi N: Hexokinase II in breast carcinoma: A potent prognostic factor associated with hypoxia-inducible factor-1α and Ki-67. Cancer Sci 104: 1380-1388, 2013.
25. Obermannová R, Alsina M, Cervantes A, Leong T, Lordick F, Nilsson M, van Grieken NCT, Vogel A and Smyth EC; ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org: Oesophageal cancer: ESMO clinical practice guideline for diagnosis, treatment and follow-up. Ann Oncol 33: 992-1004, 2022.
26. Wagener-Ryczek S, Schoemmel M, Kraemer M, Bruns C, Schroeder W, Zander T, Gebauer F, Alakus H, Merkelbach-Bruse S, Buettner R, *et al*: Immune profile and immunosurveillance in treatment-naive and neoadjuvantly treated esophageal adenocarcinoma. Cancer Immunol Immunother 69: 523-533, 2020.
27. Fonteyne P, Casneuf V, Pauwels P, Van Damme N, Peeters M, Dierckx R and Van de Wiele C: Expression of hexokinases and glucose transporters in treated and untreated oesophageal adenocarcinoma. Histol Histopathol 24: 971-977, 2009.
28. Tohma T, Okazumi S, Makino H, Cho A, Mochiduki R, Shuto K, Kudo H, Matsubara K, Gunji H and Ochiai T: Relationship between glucose transporter, hexokinase and FDG-PET in esophageal cancer. Hepatogastroenterology 52: 486-490, 2005.
29. Li WC, Huang CH, Hsieh YT, Chen TY, Cheng LH, Chen CY, Liu CJ, Chen HM, Huang CL, Lo JF and Chang KW: Regulatory role of hexokinase 2 in modulating head and neck tumorigenesis. Front Oncol 10: 176, 2020.
30. Zhang MX, Hua YJ, Wang HY, Zhou L, Mai HQ, Guo X, Zhao C, Huang WL, Hong MH and Chen MY: Long-term prognostic implications and therapeutic target role of hexokinase II in patients with nasopharyngeal carcinoma. Oncotarget 7: 21287-21297, 2016.
31. Clark R and Lee SH: Anticancer properties of capsaicin against human cancer. Anticancer Res 36: 837-843, 2016.
32. Mao X, Zhu H, Luo D, Ye L, Yin H, Zhang J, Zhang Y and Zhang Y: Capsaicin inhibits glycolysis in esophageal squamous cell carcinoma by regulating hexokinase-2 expression. Mol Med Rep 17: 6116-6121, 2018.
33. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK and Mueller-Klieser W: High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res 60: 916-921, 2000.
34. Pantziarka P, Sukhatme V, Bouche G, Meheus L and Sukhatme VP: Repurposing drugs in oncology (ReDO)-diclofenac as an anti-cancer agent. Ecancermedicalscience 10: 610, 2016.
35. Chirasani SR, Leukel P, Gottfried E, Hochrein J, Stadler K, Neumann B, Oefner PJ, Gronwald W, Bogdahn U, Hau P, *et al*: Diclofenac inhibits lactate formation and efficiently counteracts local immune suppression in a murine glioma model. Int J Cancer 132: 843-853, 2013.
36. Liu XS, Liu JM, Chen YJ, Li FY, Wu RM, Tan F, Zeng DB, Li W, Zhou H, Gao Y and Pei ZJ: Comprehensive analysis of hexokinase 2 immune infiltrates and m6A related genes in human esophageal carcinoma. Front Cell Dev Biol 9: 715883, 2021.
37. Suh DH, Kim MA, Kim H, Kim MK, Kim HS, Chung HH, Kim YB and Song YS: Association of overexpression of hexokinase II with chemoresistance in epithelial ovarian cancer. Clin Exp Med 14: 345-353, 2014.
38. Al-Batran SE, Hartmann JT, Hofheinz R, Homann N, Rethwisch V, Probst S, Stoehlmacher J, Clemens MR, Mahlberg R, Fritz M, *et al*: Biweekly fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT) for patients with metastatic adenocarcinoma of the stomach or esophagogastric junction: A phase II trial of the arbeitsgemeinschaft internistische onkologie. Ann Oncol 19: 1882-1887, 2008.
39. Eyck BM, van Lanschot JJB, Hulshof MCCM, van der Wilk BJ, Shapiro J, van Hagen P, van Berge Henegouwen MI, Wijnhoven BPL, van Laarhoven HWM, Nieuwenhuijzen GAP, *et al*: Ten-year outcome of neoadjuvant chemoradiotherapy plus surgery for esophageal cancer: The randomized controlled CROSS trial. J Clin Oncol 39: 1995-2004, 2021.