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Original Article

HK2 is a radiation resistant and independent negative prognostic factor for patients with locally advanced cervical squamous cell carcinoma

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Abstract: The mechanism by which overexpression of hexokinase 2 (HK2) indicates locally advanced cervical squamous cell carcinoma (LACSCC) with radio-resistance is still unknown despite being an independent biomarker of poor prognosis. Here, we retrospectively analyzed 132 female patients receiving radiotherapy for cervical squamous cell carcinoma including 85 radiation-sensitive cases and 47 radiation-resistant cases. The expression of HK2 was examined by immunohistochemistry. The percentage of high HK2 expression in the radiation-resistant group differed from the radiation-sensitive group with statistical significance ($P < 0.001$) even if divided into three subgroups including a lower 5-year progression free survival group (PFS) for comparison ($P < 0.001$). The Kaplan Meier curve analysis showed that there were differences between the two groups ($P < 0.001$). Therefore, this study proves a close relationship between HK2 expression and radio-resistance. Multivariate Cox regression analysis implied that HK2 was an independent prognostic indicator of cervical squamous carcinoma (HR (95% CI), 2.940 (1.609, 1.609); $P = 0.002$).

Keywords: Locally advanced cervical squamous cell carcinoma, radiation resistance, glycolysis, HK2, immunohistochemistry

Introduction

Cervical cancer represents 9% of female cancer cases and is the third leading cause of cancer in women worldwide with than 529,000 new cases receiving radiotherapy of cervical squamous cell carcinoma and 275,000 deaths per year [1]. Nearly 85% of cases occur in developing countries. Radiotherapy (RT) is an important treatment for cervical cancer, and is a preoperative or postoperative adjuvant or primary treatment in most locally advanced cervical cancer. The therapeutic effects of external beam RT (EBRT) and brachytherapy (BRT) are relatively minor although RT plays a significant role in the treatment of cervical squamous cell carcinoma. Indeed, recurrence and metastasis after radiotherapy remains a major problem in the treatment of locally advanced cervical cancer. Therefore, insight into the molecules affecting radio-resistance is important to determine its underlying mechanism [2, 3].

Malignant tumors vary in their response to irradiation as a consequence of resistance mechanisms. The radiation sensitivity can be affected by lack of oxygen [4], cell cycle [5], DNA damage and repair [6], apoptosis [7], growth factors and oncogene [8], stem cell genetic and epigenetic [9], etc. In addition, glycolysis is closely related with radiation sensitivity [10]. Glycolysis is a common source of tumor energy supply and is a glycolytic energy source in a wide variety of tumor cells regardless of oxygenation [11]. Many studies have shown that glycolytic metabolism in malignancies correlates with radio-

resistance [12, 13]. In recent study, some abnormal molecular biology changes have been shown to play central roles in the progression of cervical cancer and cervical precancerous lesions. These abnormal molecules can be proposed as biomarkers that correspond to radiation insensitivity and forecast the prognosis of cervical squamous cell carcinoma (SCC) [14]. Thus, an earlier detection of the resistant cases can provide an earlier therapy to improve the prognosis.

Alterations in glucose metabolism have been demonstrated for diverse disorders ranging from heart disease to cancer. Hexokinases catalyze the essentially irreversible first step of the glycolytic pathway [15]. To explore the relationship between glycolysis and cervical squamous cell carcinoma, we here studied the critical speed limit enzyme HK2 in the glycolytic pathway. We measured its expression levels on cervical lesions with immunohistochemical methods and studied its relationship to radiation resistance with inferences to prognosis.

Materials and methods

Patients and clinical tissue samples

This retrospective cohort study included 132 patients receiving radical radiotherapy in the Department of Radiation Oncology, Xiangya Hospital of Central South University and Cancer Hospital of Hunan Province between January 2005 and March 2012. The inclusion criterion were (a) pathologically proven SCC of the cervix; (b) no evidence of distant metastasis at diagnosis (FIGO stage IB-IVA); (c) tissue blocks were available for the research; and (d) neither receiving other anticancer treatment before primary radiotherapy nor receiving operation after radiotherapy. The study was approved by the Research and Ethics Committee. Follow-up was up to May 2012. The PFS was defined as the period from the end of therapy to the date of the first documented evidence of recurrent or metastatic disease. The median follow-up for survivors was 45 (ranging from 2~85.5) months. The median PFS was 43.5 (ranging from 0~85.5) months. The median age was 51 (ranging from 28~80) years. All of the 132 cases were divided into two parts: the radiation-sensitive group (n = 85) and the radiation-resistant group (n = 47) [2]. The radiation-sensitive group included the patients who showed no local recurrence and distant metastasis for at least 3 years after primary treatment (PFS ≥ 36 months). The radiation-resistant group included the patients who showed that radiation did not control local recurrence or distant metastasis for less than 3 years after the primary treatment (PFS < 36 months).

The radiation-resistant group was further divided into 3 subgroups including radiation uncontrolled subgroup, local recurrence subgroup and distant metastasis subgroup. The radiation uncontrolled subgroup included the patients whose cervical tumor never disappeared until the time of death. The local recurrence subgroup included patients who showed local recurrence within 3 years after the primary treatment. The distant metastasis subgroup included patients who showed distant metastasis within 3 years after the primary treatment. Physical examination, pathological biopsy or imaging studies were used as evidence to diagnose radiation uncontrolled cases, local recurrence and distant metastasis after primary radiotherapy. Each cervical primary tumor diameter was directly measured with physical examination rather than imaging study. There were 9 cases in the radiation uncontrolled subgroup, 17 cases in the local recurrence subgroup and 22 cases in distant metastasis subgroup. One case experienced distant metastasis during radiotherapy and the tumor never disappeared until the patient died-this case belonged to both the radiation uncontrolled subgroup and the distant metastasis subgroup. Three cases experienced distant metastasis after radiotherapy, but their PFS was longer than 36 months. These 3 cases belonged to the radiation-sensitive group.

All patients were treated with external beam radiotherapy (EBRT) and high-dose rate (HDR) intracavitary brachytherapy after consultation with the radiation oncologist. The HDR brachytherapy was started at 3 to 4 weeks after the initiation of EBRT. The median total dose at point A was 90 (range 66~102) Gy. The median dose of EBRT at point A was 46 (range 30~52) Gy. The median dose of HDR brachytherapy at point A was 42 (range 20~54) Gy. Some patients also received a platinum-based chemotherapy, but the combined chemotherapy drugs were not unified and even the platinum drugs such as cisplatin, carboplatin and oxaliplatin were not unified. These differences

Table 1. Patient characteristics

Parameters	Patients n = 132	Radiation sensitivity		*P*-value
		Radiation-resistant group	Radiation-sensitive group	
		N = 47 (%)	N = 85	
Age				0.559[a]
< 50 years	49	19 (38.8)	30	
≥ 50 years	83	28 (33.7)	55	
FIGO stage				0.004[a]
I + II	70	17 (24.3)	53	
III + IV[a]	62	30 (48.4)	32	
Histopathological grade				0.014[b]
High	10	0 (0)	10	
Middle + Low	114 + 8	47 (38.5)	75	
Tumor diameter				< 0.001[a]
≤ 4 cm	79	16 (20.3)	63	
> 4 cm	53	31 (58.5)	22	
Combined chemotherapy (platinum-based)				0.426[a]
Yes	106	36 (34.0)	70	
No	26	11 (42.3)	15	
Histological type (SCC[d])	132	47 (35.6)	58	
Total dose at point A median dose (range) (Gy)		92 (67-102)	89 (66-102)	0.585[c]
EBRT dose of point A median dose (range) (Gy)		48 (30-52)	46 (36-50)	0.518[c]
Brachytherapy dose at point A median dose (range) (Gy)		46 (21-54)	42 (20-54)	0.387[c]

[a]*P* value was estimated by chi-square test; [b]*P* value was estimated by Fisher's exact test; [c]*P* value was estimated by t-test; SCC[d]: Squamous Cell Carcinoma.

decreased the degree of statistical confidence.

Immunhistochemistry

For immunohistochemical detection of HK2, a 4 μm tissue section was deparaffinated in xylene followed by microwave treatment (10 min at moderate power) in 0.01 M citrate buffer (pH 6.0). After cooling for 30 min and washing with PBS, the endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min followed by incubation with PBS containing 10% normal goat serum for 30 min. Specimens were incubated overnight at 4°C with the anti-HK2 (Cell Signaling Technology Inc., Beverly, MA) antibody at a dilution of 1:600. Detection with immunostaining was performed with the ChemMate kit (Dako, Glostrup, Denmark) and 3, 3-diaminobenzidine as the chromogen. As a negative control, the primary antibody was replaced by non-immune isotype antibodies.

Evaluation of staining

The staining was viewed separately by two pathologists without knowing the clinical or clinicopathological status of the cases. The expression of HK2 on slide was evaluated by scanning the entire tissue specimen under low-power magnification (×40), and then confirmed under high-power magnification (×400). The positive or negative result was diagnosed by stereological cell counts. Samples with no positive cells were negative (-). If the proportion of positive cells was less than 25%, the diagnosis was slightly positive (±). If the proportion of positive cell was from 25% to 50%, the result of diagnosis was positive (+). When more than 50% of positive cell was observed, it was considered as the intense positive (++) [14]. According to this method of assessment, staining scores - and ± were regarded as tumors with low expression, while staining scores + and ++ were regarded as tumors with high expression.

Statistical analysis

The association between the expression of HK2 and clinical/pathological factors were analyzed with the chi-squared test and Fisher's exact test. The radiation dose difference was analyzed by the t-test. Patients who survived until the end of the observation period were censored at their last follow-up visit. Patients who died of causes other than LACSCC were censored at the time of death.

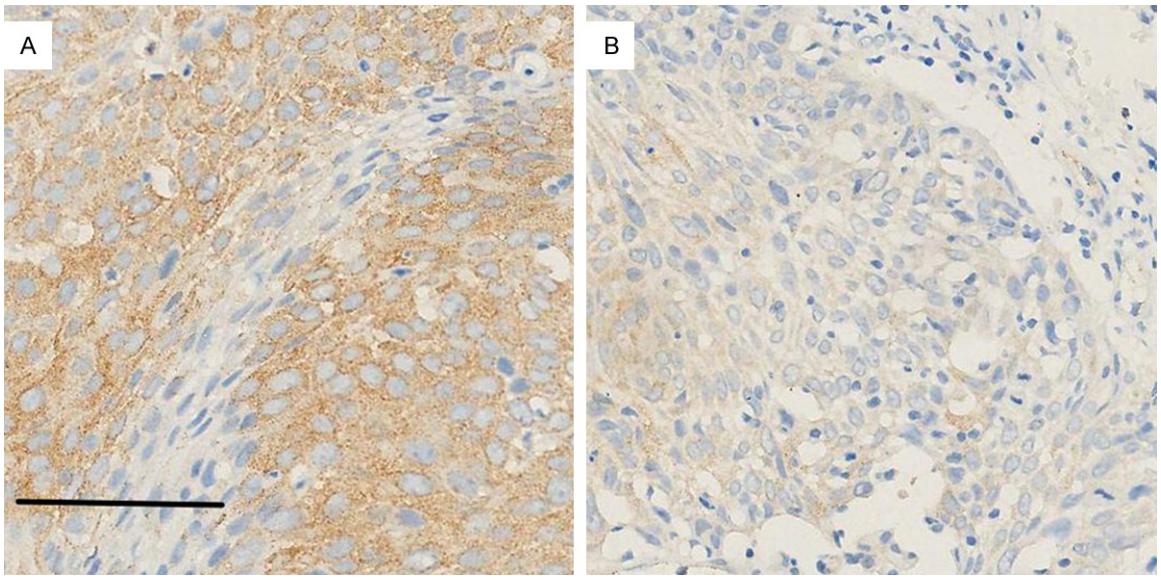

Figure 1. Examples of HK2 staining of tumors in the radiation-resistant group and radiation-sensitive group (400×). A. Strong positive staining of HK2 in the radiation-resistant group. B. Weak positive staining of HK2 in the radiation-sensitive group. The bar size is the same for all the figures and is 90 μm.

Table 2. Correlation between HK2 expression and clinicopathological parameters for cervical cell squamous carcinoma

Parameters	Patients (n = 132)	HK2 expression		*P*-value
		low expression	high expression	
		N = 55	N = 77 (%)	
Age				0.212[a]
< 50 years	49	17	32 (65.3)	
≥ 50 years	83	38	45 (54.2)	
FIGO stage				0.175[a]
I[b] + II	70	33	37 (52.9)	
III + IV[a]	62	22	40 (64.5)	
Histopathological grade				0.318[b]
High	10	6	4 (40.0)	
Middle + Low	122	49	73 (59.8)	
Tumor diameter				0.141[a]
≤ 4 cm	79	37	42 (53.2)	
> 4 cm	53	18	35 (66.0)	

[a]*P* value was estimated by Chi-square test; [b]*P* value was estimated by Fisher's exact test.

Survival curves were calculated using Kaplan-Meier estimates, and differences between groups were tested by the log-rank test. Univariate and multivariate survival analysis was performed according to the Cox proportional hazards model. HK2 expression (high vs. low), age (≥ 50 y vs. < 50 y), FIGO stage (III + IV a vs. I b+ II), histopathological grade (middle + low vs. high) and tumor size (> 4 cm vs. ≤ 4 cm) were included in the regression model. For all statistical tests, P ≤ 0.05 was considered significant.

Results

Clinical and histopathological characteristics of the 132 LACSCC cases

Clinical and histopathological patient descriptors are given in **Table 1**. There were 132 cervical squamous cell carcinomas cases (47 in the radiation-resistant group and 85 in the radiation-sensitive group). There were 49 cases with age < 50 y and 83 cases with age ≥ 50 y. There were 70 (6 + 64) patients with FIGO stage I b + II and 62 (56 + 6) patients with FIGO stage III + IV a. Histologically, there were 10 cases, 114 cases and 8 cases diagnosed with high, middle and low grade cancer, respectively, using WHO classification for cervical SCC. The tumor diameter ≤ 4 cm were found in 79 cases and 53 cases with the diameter > 4 cm. There were significant differences in tumor diameter, FIGO stage and his-

Table 3. Relationship between HK2 expression and response to radiotherapy

Parameters	Patients (n = 132)	HK2 expression		*P*-value
		Low expression	High expression (%)	
Radiation sensitivity				< 0.001[a]
Radiation-resistant group	47	9	38 (80.9)	
Radiation-sensitive group	85	46	39 (45.9)	
RT non-response				0.030[b]
RT non-responsive subgroup	9	1	8 (88.9)	
Radiation-sensitive group	85	46	39 (45.9)	
local recurrence				0.006[a]
Local recurrence subgroup	17	3	14 (82.4)	
Radiation-sensitive group	85	46	39 (45.9)	
distant metastasis				0.009[a]
Distant metastasis subgroup	22	5	17 (77.3)	
Radiation-sensitive group	85	46	39 (45.9)	

[a]*P* value was estimated by Chi-square test; [b]*P* value was estimated by Fisher's exact test.

tological grading between the two groups ($P < 0.001$, $P = 0.004$, $P < 0.001$, respectively). There were no significant differences in patients' age, combined chemotherapy (platinum-based), total dose of point A, EBRT dose of point A and brachytherapy dose of point A between the two groups.

HK2 expression and their associations with clinical/pathological parameters

HK2 was located in the cytoplasm of cervical carcinoma cells. The staining was much stronger in the radiation-resistant group than in the radiation-sensitive group (**Figure 1A** vs. **1B**). In the 132 cervical SCC patients, there was low and high HK2 expression in 55 cases (41.7%) and 77 cases (58.3%), respectively (**Table 2**). However, no significant association was observed between HK2 expression and patient age, FIGO stage, histopathological grade or tumor diameter.

HK2 expression and response to radiotherapy

The results of the immunohistochemical expression of HK2 in the 132 cervical SCC cases are summarized in **Table 3**. In the radiation-resistant group (47 cases), there was low and high expression in 9 cases (19.1%) and 38 cases (80.9%), respectively. In the radiation-sensitive group (85 cases), there was a low and high expression in 46 cases (54.1%) and 39 cases (45.9%), respectively. The high expression proportion of HK2 was compared between the radiation-resistant group and the radiation-sensitive group-the statistical difference was significant ($P < 0.001$). The radiation-resistant group contains 3 subgroups including radiation uncontrolled subgroup, local recurrence subgroup and distant metastasis subgroup. The percentage of HK2-positive patients in the radiation uncontrolled subgroup, local recurrence subgroup and distant metastasis subgroup was 88.9% (8/9), 82.4% (14/17) and 77.3% (17/22), respectively. The HK2 expression difference was also significant (RT uncontrolled subgroup vs. radiation-sensitive group, $P = 0.030$; local recurrence subgroup vs. radiation-sensitive group, $P = 0.006$; and distant metastasis subgroup vs. radiation-sensitive group, $P = 0.009$).

HK2 expression and survival

When the patient cohort was stratified according to tumor expression of HK2, the 5-year progression free survival (PFS) rates in patients of low HK2 expression (n = 55) and high HK2 expression (n = 77) were 80.8% and 56.5%, respectively. The Kaplan-Meier analysis (log-rank test) revealed a significant influence between the 2 groups ($P < 0.001$, **Figure 2A**). The 5-year PFS rates for all the 132 cases were 66.8% (**Figure 2B**).

Univariate analyses showed that HK2 [HR (95% CI), 3.454 (1.764, 6.765); $P < 0.001$], FIGO stage [HR (95% CI), 2.610 (1.453, 4.689); $P = 0.001$] and tumor diameter [HR (95% CI), 3.366 (1.885, 6.012); $P < 0.001$] were prognostic predictors of PFS in patients with cervical SCC (**Table 4**). Multivariate Cox regression analysis indicated that HK2 [HR (95% CI), 2.940 (1.609, 5.790); $P = 0.002$], FIGO stage [HR (95% CI), 2.290 (1.267, 4.141); $P = 0.006$] and tumor diameter [HR (95% CI), 2.956 (1.637, 5.337); $P < 0.001$] were prognostic predictors of PFS in patients with cervical SCC (**Table 4**).

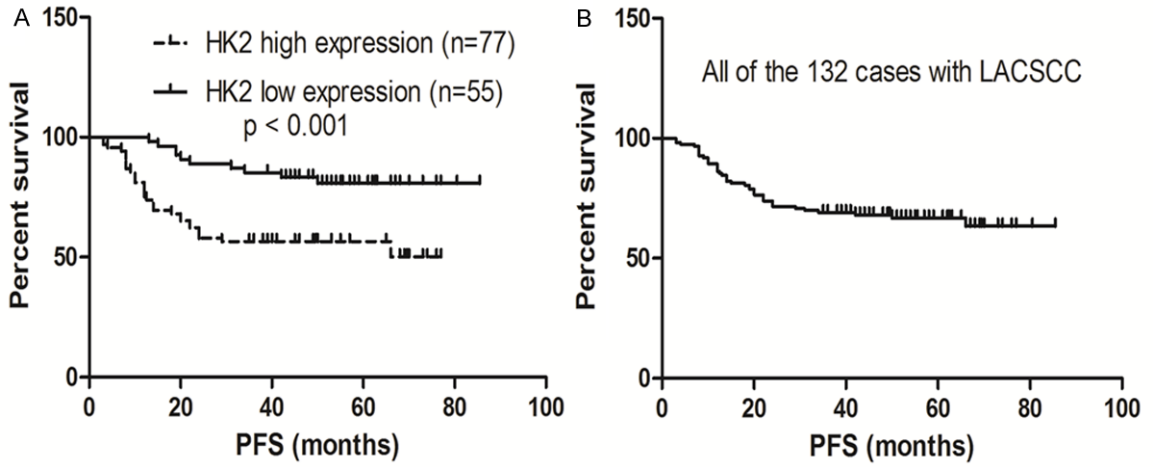

Figure 2. Kaplan-Meier curves of overall LACSCC patient survival. A. The 5-year PFS rates were 80.8% and 56.5%, respectively in patients with low HK2 expression (n = 55) and high HK2 expression (n = 77). There was a significant difference in the overall survival rate between the 2 groups ($P < 0.001$). B. The 5-year PFS rate was 66.8% in all 132 cases with LACSCC.

Table 4. Univariate and multivariate COX regression analyses of the relationships between clinicopathological outcomes of 132 local cervical squamous carcinoma patients

Variable	Subset	Hazard radio (95% CI)	*P*-value
univariate analyses (n = 132)			
HK2	high vs. low	3.454 (1.764, 6.765)	< 0.001
Age	≥ 50 y vs. < 50 y		0.872
FIGO stage	III + IVa vs. I + II	2.610 (1.453, 4.689)	0.001
Histopathological grade	Low + Middle vs. high		0.067
Tumor diameter	> 4 cm vs. ≤ 4 cm	3.366 (1.885, 6.012)	< 0.001
Combined chemotherapy (platinum-based)	Yes vs. No		0.446
multivariate analyses (n = 132)			
HK2	high vs. low	2.940 (1.609, 5.790)	0.002
Age	≥ 50 y vs. < 50 y		0.254
FIGO stage	III + IVa vs. I + II	2.290 (1.267, 4.141)	0.006
Histopathological grade	Low + Middle vs. high		0.082
Tumor diameter	> 4 cm vs. ≤ 4 cm	2.956 (1.637, 5.337)	< 0.001
Combined chemotherapy (platinum-based)	Yes vs. No		0.228

Discussion

We found no consistent correlation of HK2 with clinical parameters (patient age, FIGO stage, histopathological grading and tumor diameter). The HK2 high expression was related to the radiosensitivity even when the outcome was divided into three radiation resistance subgroups. Furthermore, PFS of the patient with low expression of HK2 was longer than that with high expression of HK2. HK2 overexpression was a negative prognosis factor in SCC survival in both single factor analysis and Multivariate Cox regression. Therefore, we considered HK2 overexpression to be one of the independent prognostic risk factors, which was also associated with radiation resistance in cervical squamous carcinoma.

Recently, we studied the effects of the energy mechanism of glycolysis for the LACSCC. HK2, as a significant biomarker, reflects the neoplasm tissue transformation at the early stage of cervical cancer [14]. The same result was also reported in glioblastoma [16], and HK2 overexpression in hepatocellular carcinoma

(HCC) was confirmed positively and was related to drug resistance and survival rate [17]. In ovarian cancer, HK2 overexpression was associated with tumor recurrence [18]. Lyshchik et al. showed similar findings in pancreatic cancer [19]. Based on these findings, we concluded that a high accumulation of HK2 in cervical tumors may not only reflect a highly malignant phenotype as a general intrinsic property of the cancer cells, but may also indicate a pronounced radiation resistance.

Most malignant tumor cells prefer to metabolize glucose by glycolysis even in the presence of sufficient oxygen. This is as aerobic glycolysis or the "Warburg effect" [13, 20]. Glycolysis can generate ATP at a higher rate than oxidative phosphorylation and provides a biosynthetic benefit for tumor cells, thus allowing effective shunting of carbon to key biosynthetic pathways [21]. Tumor cells are characterized by a high rate of glycolysis that serves as their primary energy-generating pathway [22-24]. Increased glycolysis provides tumor cells with three benefits: rapid energy production, macromolecular biosynthesis for growth and proliferation, and an anti-apoptotic phenotype [25]. Hexokinases (HKs) are the rate-limiting enzymes in the first reaction of the glycolytic pathway. In mammals, there are four coenzymes-HK1, HK2, HK3 and HK4. HK2 is the major isoenzyme overexpressed (> 100-fold) and is normally found in muscle and adipose tissue in low amounts [22]. Our observation and other research have confirmed that HK2 expression was significantly higher in a variety of malignant tumors, including glioblastoma, hepatoma, colon, breast, laryngeal and renal cell carcinoma [16, 26-30]. HK2 catalyzes the first step in glucose metabolism and prevents glucose from entering the cell, which plays a critical role in ATP production [31].

It was originally believed that the mitochondrially-bound HK would have preferential access to this highly localized concentration of ATP [22]. Under aerobic condition, as much as half of the ATP produced in some tumor cells may be derived from glycolysis. However, during hypoxia, glycolysis generates more ATP than mitochondrial oxidative phosphorylation and assures the tumor cell not only survives, but may increase malignancy [22]. Studies strongly indicate that highly malignant cancer cells employ HK2 not only to assure their survival during abrupt changes in metabolic state, but are also available to glycolytic products that influence mitochondrion-initiated apoptosis [22, 32, 33].

The overexpression of HK2 also protected against oxidant-induced cell death in tissue culture [23, 34]. The mechanisms by which HK2 protects against cell death are unclear [30]. The HK2 levels are markedly elevated in many cancer cells suggesting that their overexpression could play a role in cancer growth. Ahmad et al. expressed HK2 in lung epithelial cells and showed that the expression of this protein also protects against oxidant-induced cell death [34]. In addition to this, we show here that HK2 plays an important role in glycolysis.

We observed a tight relationship between HK2 expression and radio-resistance in patients with LACSCC. However, no such similar finding has been established at the clinical level. Several studies have shown that up-regulated glycolytic activity in tumors is associated with a malignancy, resistance to chemo-and radiotherapy, and ultimately with poor prognosis [10, 18]. These data also imply that inhibition of glycolysis during treatment might possibly sensitize tumors to irradiation [12, 13]. As we mentioned above, HK2, is a rate-limiting enzyme that controls the first reaction in the glycolytic pathway and catalyzes the first step in glucose metabolism for ATP [30]. Therefore, we speculate that HK2 correlates with radio-resistance. This may be through three mechanisms.

First, HK2 can increase the energy production for high energy-demanding cancer cells. Mitochondrial-bound HK2 has direct access to mitochondrial sources of ATP and greater affinity for Mg-ATP supplied by mitochondria [35]. The efficient coupling of ATP, which is from oxidative phosphorylation to the rate-limiting step of glycolysis, is involved in the Warburg effect, and tumor cells use glycolysis even in aerobic environments [35]. Also, inhibition of HK by Glu-6-P would reduce energy production for high energy-demanding cancer cells [23, 36]. Moreover, high levels of G-6-P not only inhibit HK2 catalytic activity, but also promote PTP opening and thus apoptosis [15, 36].

A second mechanism is tumor hypoxia and the regulation of ROS. Hypoxia-mediated resistance to radiation therapy is multifactorial

involving a variety of mechanisms. Free radicals are produced when radiation is directly absorbed in tissues. Both free radicals and ROS break double-stranded DNA leading to cell death [37]. Indirectly, hypoxia-induced factors such as HIF-1 and proteomic changes may have a substantial effect on radiation resistance by altering proliferation kinetics, cell-cycle position, inhibiting apoptosis, regulation of angiogenesis and changing cellular metabolism by increasing anaerobic glycolysis. Thus results in resistance to radiation [38, 39]. Apart from the hypoxia, this increased glycolysis promotes acidosis of the tumor microenvironment and produces products of glucose metabolism such as lactic acid, which effectively scavenges free radicals and ROS and in turn to induce cancer resistance to radiotherapy [40, 41].

Third, the overexpression of HK2 also protects against oxidant-induced cancer cell death. A characteristic feature of human cancers is the inability to mount a proper apoptotic response during tumor progression or upon treatment [42]. Anti-apoptosis frequently occurs in several human cancers and is a main cause of primary or acquired treatment resistance, which also applies to the resistance of cancers to radiotherapy [43]. Mitochondrial binding of HK2 to the outer mitochondrial membrane has been shown to protect HeLa and human embryonic kidney cells from entering apoptosis [27], which was related to the blockade of the interaction of the pro-apoptotic proteins Bax and VDAC [27]. By inhibiting the interaction of VDAC with Bax and Bak, mitochondrial permeabilization was prevented and apoptosis was inhibited by blocking the release of intermembrane space proteins such as cytochromec [44-46]. However, the mechanism controlling pore permeability and the associated depolarization or hyperpolarization of mitochondria and the release of cytochromec are not well understood [15, 33, 47, 48]. Other recent studies suggest that both survival mechanisms mentioned above may be related to growth factor-induced signaling pathways dependent on the serine/threonine kinase Akt/PKB-a major downstream effector of growth factor-mediated cell survival [32]. In conclusion, we can see that HK2 is responsible for radio-resistance.

In conclusion, our data showed that there was a significant relationship between HK2 expression and radioresistance. HK2 is an independent negative prognostic factor for LACSCC that facilitates glycolysis of malignant tumor cells and inhibits cell apoptosis. HK2 is an ideal therapeutic target and has good clinical prospects worthy of further study.

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Disclosure of conflict of interest

None.

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