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High expression of PKM2 as a poor prognosis indicator is associated with radiation resistance in cervical cancer

Yajie Zhao[1], Liangfang Shen[1], Xi Chen[2], Yujie Qian[1], Qin Zhou[1], Ying Wang[1], Kai Li[1], Miaomiao Liu[1], Sai Zhang[3] and Xinqiong Huang[1]

[1]Department of Oncology, Xiangya Hospital, Central South University, Hunan Province, [2]Guangzhou Centers for Disease Control and Prevention, Guangdong Province and [3]Institute of Medical Science, Xiangya Hospital, Central South University, Hunan Province, P.R. China

Summary. Our study aimed to investigate the association of Pyruvate Kinase isozyme type M2 (PKM2) with radiation resistance in locally advanced cervical squamous cell carcinoma (LACSCC). We retros-pectively reviewed 132 female patients who received primary radiation therapy to treat LACSCC at Federation Internationale of Gynecologie and Obstetrigue (FIGO) stages IB-IVA. Forty-seven patients with progression free survival (PFS) of less than 36 months were regarded to have radiation resistance. Eighty-five patients with PFS no less than 36 months were regarded as radiation sensitive. Using immuno-histochemistry, we found that the overexpression rate of PKM2 in radiation resistant and radiation sensitive patients was 87.2% and 57.6%, respectively, and the difference was statistically significant ($p < 0.001$). The 5-year progress free survival rates in patients with low and high expression of PKM2 was 80.4% and 60.5%, respectively, and the difference was statistically significant ($p=0.008$). Multivariate Cox regression analysis identified that high expression of PKM2 is an independent negative prognostic factor in cervical cancer patients [Hazard ratio (95% CI), 2.888 (1.347, 6.194) $p=0.006$]. These results demonstrate that overexpression of PKM2 contributes to radiation resistance and acts a poor prognosis indicator in patients with LACSCC.

Offprint rquests to: Dr. Xinqiong Huang Department of Oncology, Xiangya Hospital, Central South University, No. 87, Xiangya Road, Changsha, Hunan Province 410008, People's Republic of China. e-mail: wzyj7177@126.com.

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Introduction

Epidemiological studies have shown that cervical carcinoma is ranked as the third highest among malignant carcinomas in females worldwide (Jemal et al., 2011). In China, with the development of clinical treatments for cervical carcinoma, the mortality of cervical cancer has sharply decreased, and 70.9% of patients live more than 5 years (Lei et al., 2011). Radiation therapy (RT) plays an important role in the treatment of locally advanced cervical squamous cell carcinoma. However, treatment results are still not satisfactory. One of the major therapeutic problems is radiation resistance (Randall et al., 2010). Therefore, it is important to find the causes of radiation resistance in cervical carcinoma cells.

Cells utilize two sources of energy, mitochondria aerobic oxidation, and glycolysis. In normal cells, the Krebs cycle is the main way to provide ATP and the energy coming from glycolysis is very limited (Shi et al., 2009). Otto Warburg and his colleagues first demonstrated that even under aerobic conditions, glycolysis is still the main way to provide ATP in many types of tumor cells; this phenomenon is known as the Warburg effect (Warburg, 1956). With the rapid proliferation of tumor volume, peripheral vascular distribution is not established promptly and lack of oxygen and nutrients occurs more easily inside tumor

cells. It has been reported that hypoxia-inducible factor can directly up-regulate glycolytic genes (Robey et al., 2005). In addition, certain oncoproteins will express Akt (Plas et al., 2001; Wieman et al., 2007), Bcr-Abl (Bentley et al., 2001; Barnes et al., 2005), and Myc (Osthus et al., 2000), which can increase the ability of tumor cells to uptake glucose by regulating the expression of the glucose transporter Glut1 (Younes et al., 1997; Haber et al., 1998; Cantuaria et al., 2001; Kawamura et al., 2001). The current study has proven that glycolysis has a close relationship with radiation resistance and that glycolysis-associated metabolic intermediates can increase tumor resistance to irradiation (Quennet et al., 2006). It is known that glycolysis is a series of enzymatic reactions and pyruvate kinase (PK) is one of the three key enzymes that participates in the last stage of glycolysis which includes four types, PKM1, PKM2, PKL, and PKR. Several studies have reported that an isoform can shift back to PKM2 during tumor formation; PKL shift to PKM2 is the late event in hepato carcinogenesis and under a glycolysis environment PKM1 can shift to PKM2 (Hacker et al., 1998; Christofk et al., 2008). Thus, PKM2 plays a critical role in the regulation of glycolysis (Mazurek et al., 2005; Vander Heiden et al., 2009; Mazurek, 2011). Some studies have shown that PKM2 is preferentially expressed in some types of tumors during tumor progression (Cortes-Cros et al., 2013; Israelsen et al., 2013); compared to matched normal tissues, PKM2 expression is increased among diverse human cancers, such as lung, breast, prostate, blood, cervix, kidney, bladder, and colon (Bluemlein et al., 2011). The level of PKM2 mRNA was more than 2-fold higher in primary gastric cancers compared to adjacent normal tissues from the same patients (Lim et al., 2012). Furthermore, PKM2 can impact the differentiation, proliferation, and overall survival rates in many tumor types. In invasive cervical cancer cells, PKM2 expression was much higher than Cervical Intraepithelial Neoplasia (CIN) (Yuan et al., 2012). Developing breast tumor cells express higher levels of PKM2 than non-developing tumor cells (Muller et al., 1988); expression of PKM2 can influence the overall survival rate of signet-ring cell cancers (Lim et al., 2012). The results from Cheng-Fu Zhou's study indicated that PKM2 regulates cell proliferation and migration in colon cancer (Zhou et al., 2012). In some experiments knockdown PKM2 related genes not only caused declines in glycolysis in a lung cancer cell line, and limited the proliferation of lung cancer cell (Christofk et al., 2008), but also increased the radiosensitivity of a NSCLC cell line by inhibiting AKT and PDK1 phosphorylation, increasing the rate of ERK1/2 and GSK3β phosphorylation, and accelerating the apoptosis and autophagy of tumor cells (Meng et al., 2015). PKM2 plays an oncogenic role in hepatocellular carcinoma (HCC) *in vitro* and *in vivo* (Liu et al., 2015). Therefore, we hypothesize that overexpression of PKM2 is an important poor prognosis signal of cervical cancer.

Our study aimed to explore the expression of PKM2 association with radiation resistance in cervical squamous cell carcinomas and to investigate the relationship between PKM2 and radiation resistance. We found that overexpression of PKM2 prompted radiation resistance and acts a poor prognosis indicator in patients with LACSCC.

Material and methods

Patients and clinical tissue samples

The population of this retrospective cohort study was composed of 132 patients who received radical radiotherapy in the Department of Radiation Oncology, Xiangya Hospital, Central South University and Cancer Hospital of Hunan Province between January 2005 and March 2012. The inclusion criteria was (a) pathologically proven squamous cell carcinoma (SCC) of the cervix, (b) no evidence of distant metastasis at diagnosis (FIGO stage IB-IVA), (c) tissue blocks available for our 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 of our institution. Follow-up was closed in May 2012. The median follow-up for survivors was 45 months (range 2-85.5 months). The median PFS was 43.5 months (range 0-85.5 months). The median age was 51 years old (range 28-80). We divided the 132 cases into two parts: the radiation-sensitive group (n=85) and the radiation-resistant group (n=47). 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) (Kim et al., 2006). The radiation-resistant group included patients who showed radiation uncontrolled, local recurrence or distant metastasis less than 3 years after primary treatment (PFS <36 months). Therefore, the radiation-resistant group was divided into 3 subgroups containing the uncontrolled radiation subgroup, local recurrence subgroup, and distant metastasis subgroup. The uncontrolled radiation subgroup comprising patients whose primary tumor persisted and did not shrink markedly after primary treatment until time of death; The local recurrence subgroup comprising patients whose primary tumor had initially disappeared but subsequently showed local recurrence at <3 years after the primary treatment; The distant metastasis subgroup comprising patients whose primary tumor had disappeared after treatment but showed distant metastasis at <3 years after primary treatment. Space PFS (progression free survival) was defined as the period from the end of therapy to the date of the first documented evidence of recurrent or metastatic disease. There must be evidence, such as clinical physical examination, pathological biopsy or imaging studies to diagnosis radiation uncontrolled, local recurrence and distant metastasis after primary radiotherapy. Each cervical primary tumor diameter was assessed by direct

measurement during the clinical physical examination rather than by an imaging study. In our research, there are 9 cases in the uncontrolled radiation subgroup, 17 cases in the local recurrence subgroup and 22 cases in the distant metastasis subgroup. One case experienced distant metastasis during radiotherapy and the tumor never disappeared, so we thought this case belonged not only to the uncontrolled radiation subgroup but also to the distant metastasis subgroup. Three cases experienced distant metastasis after radiotherapy, but their PFS was longer than 36 months; therefore, we thought these 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 a radiation oncologist. HDR brachytherapy was started 3 to 4 weeks after the initiation of EBRT. The median total dose at point A was 90 Gy (range 66-102 Gy), the median dose of EBRT at point A was 46 Gy (range 30-52 Gy), and the median dose of HDR brachytherapy at point A was 42Gy (range 20-54 Gy). Some patients received platinum-based chemotherapy; however, the combined chemotherapy drugs were not unified and these differences increased the degree of statistical difficulty.

Immunohistochemistry

For immunohistochemical detection of PKM2, a 4-μm tissue section was deparaffinized 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 in PBS, 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- PKM2 antibody (Abcam, USA) at a dilution of 1:600. Detection of immunostaining was performed using the ChemMate kit (Dako, Glostrup, Denmark) with 3,3-diamino-benzidine as the chromogenic substance. For the negative control, the primary antibody was replaced with non-immune isotypic antibodies.

Evaluation of staining

The staining was viewed separately by two pathologists blinded to the clinical or clinic-pathological status of the cases. The expression of PKM2 on the slides was evaluated by scanning the entire tissue specimen under low-power magnification (×40), and then confirmed under high-power magnification (×400). The result of positive or negative was diagnosed by the method of stereological cell counts. The absence of positive cells was diagnosed as negative (-) When the observed positive cells were less than 25 percent the diagnosis was slightly positive (±). A positive (+) diagnosis was made when the proportion of positive cells ranged from 25 to 50 percent. An intense positive (++) diagnosis was made when more than 50 percent of positive cells was observed (Yuan et al., 2012). 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

Association between the expression of PKM2 and clinic pathological factors were analyzed by using the chi-square 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 because of causes other than cervical cancer were censored at their date of death. Survival curves were calculated using Kaplan-Meier estimates, and differences between groups were tested by log-rank test. Univariate and Multivariate survival analysis was performed according to the Cox proportional hazards model. PKM2 expression (high vs. low), age (≥50 y vs. <50 y), FIGO stage (III + IVa vs.Ib + II), histo-pathological grade (moderately+ poor vs.well), and tumor diameter (>4 cm vs. ≤4 cm) were included in the regression models. For all statistical tests, $p \leq 0.05$ was considered significant.

Results

Clinical and histopathological characteristics of the 132 LACSCC cases

Clinical and histopathological patient characteristics are given in Table 1. There are 132 cervical squamous cell carcinomas (47 in the radiation-resistant group and 85 in the radiation-sensitive group). There were 49 cases with ages <50 years old and 83 cases with ages ≥50 years old. There were 70 (6 + 64) patients with FIGO stage Ib + II and 62 (56 + 6) patients with FIGO stage III + IVa. Histologically, all patients were classified as squamous cell carcinoma and 10, 114, and 8 cases were diagnosed with well, moderately, and poor differentiation according to the WHO classification, respectively. 79 cases had a tumor diameter ≤4 cm and 53 cases had diameters >4 cm. There were significant differences in tumor diameter, FIGO stage, and histological grading between the two groups ($p<0.001$, $p=0.004$ and $p<0.001$, 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.

PKM2 expression and its associations with clinicopathological parameters

PKM2 was located in cervical carcinoma cell cytoplasm and the staining was much stronger in the

radiation-resistant group than the radiation-sensitive group (Fig. 1A,B). In the 132 cervical SCC patients, there were low and high expressers of PKM2 in 42 and 90 cases, respectively (Table 2). Thus, the low expression percentage of PKM2 was 31.8% (42/132) and the high expression percentage of PKM2 was 68.2% (90/132). No significant association was observed between PKM2 expression and patient age, FIGO stage, histopathological grade, and tumor diameter.

PKM2 expression and response to radiotherapy

The results of the immunohistochemical expression of PKM2 in the 132 cervical SCCs are summarized in Table 3. In the radiation-resistant group (47 cases), there were low and high PKM2 expression in 6 and 41 cases, respectively. Accordingly, the low expression percentage was 14.6% (6/47) and high expression percentage was 87.2% (41/47). In the radiation-sensitive group (85 cases), there were low and high expression of PKM2 in 36 and 49 cases, respectively. Hence, the low expression percentage was 42.4% (36/85) and high expression percentage was 57.6% (49/85). We compared the high expression proportion of PKM2 between the radiation-resistant and radiation-sensitive groups and the statistical difference was significant ($p<0.001$). According to the patients' clinical information, the radiation-resistant group contains 3 subgroups, including uncontrolled radiation subgroup, local recurrence subgroup, and distant metastasis subgroup. The proportion of high PKM2 expression in uncontrolled radiation subgroup, local recurrence subgroup, and distant metastasis subgroup was 100% (9/9), 88.2% (15/17), and 81.8% (18/22), respectively. The PKM2 expression difference was significant (uncontrolled radiation subgroup vs. radiation-sensitive group, $p=0.012$; local recurrence subgroup vs. radiation-sensitive group, $p=0.026$; and distant metastasis subgroup vs. radiation-sensitive group, $p=0.048$).

Table 1. Patient characteristics.

Parameters	Patients	Radiation sensitivity		p-value
	n=132	radiation-resistant group n=47 (%)	radiation-sensitive group 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]
Ib + II	70	17 (24.3)	53	
III + IVa	62	30 (48.4)	32	
Histopathological grade				0.014[b]
Well	10	0 (0)	10	
Moderately+Poor	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 (SCCd)	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.

Table 2. Correlation between PKM2 expression and clinicopathological parameters for cervical squamous cell carcinoma.

Parameters	Patients	PKM2 expression		p-value
	(n=132)	low expression n=42	high expression n=90 (%)	
Age				0.165[a]
<50 years	49	12	37 (75.5)	
≥50 years	83	30	53 (63.9)	
FIGO stage				0.163[a]
Ib + II	70	26	44 (62.9)	
III + IVa	62	16	46 (74.2)	
Histopathological grade				0.228[b]
Well	10	5	5(50.0)	
Moderately+Poor	122	37	85 (69.7)	
Tumor diameter				0.064[a]
≤4 cm	79	30	49 (62.0)	
>4 cm	53	12	41 (77.4)	

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

Table 3. Relationship between PKM2 expression and response to radiotherapy.

Parameters	Patients	PKM2 expression		p-value
	(n=132)	low expression	high expression (%)	
Radiation sensitivity				<0.001[a]
radiation-resistant group	47	6	41 (87.2)	
radiation-sensitive group	85	36	49 (57.6)	
RT non-response				0.012[b]
RT non-responsive subgroup	9	0	9 (100)	
radiation-sensitive group	85	36	49 (57.6)	
local recurrence				0.026[b]
local recurrence subgroup	17	2	15(88.2)	
radiation-sensitive group	85	36	49 (57.6)	
distant metastasis				0.048[b]
distant metastasis subgroup	22	4	18 (81.8)	
radiation-sensitive group	85	36	49 (57.6)	

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

PKM2 expression and survival

When the patient cohort was stratified according to PKM2 tumor expression, the 5-year progression free survival (PFS) rates in patients with low PKM2 expression (n=42) and high PKM2 expression (n=90) were 80.4% and 60.5%, respectively. Kaplan-Meier analysis (log-rank test) revealed a significant influence

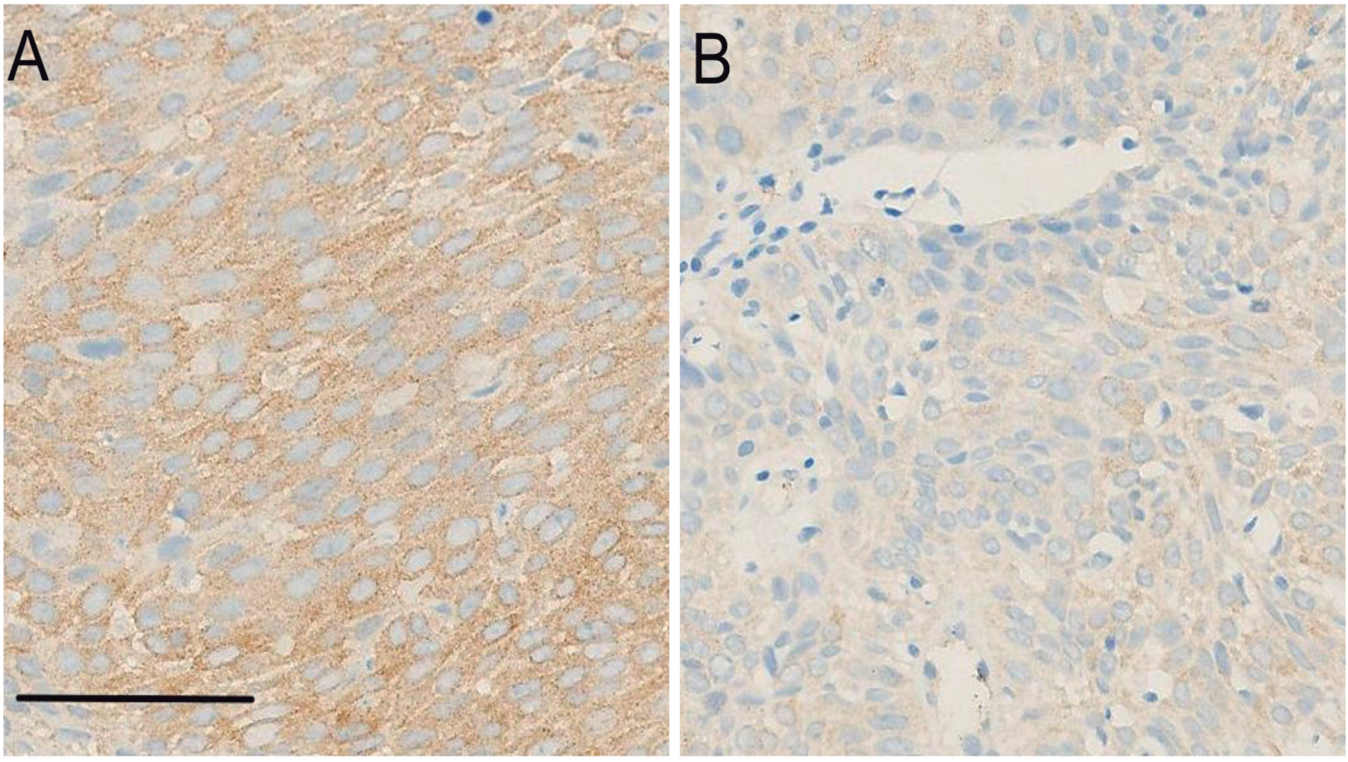

Fig. 1. Representative examples of PKM2 staining of tumors in the radiation-resistant group and radiation-sensitive group. **A.** Strong positive staining of PKM2 in the radiation-resistant group. **B.** Weak positive staining of PKM2 in the radiation-sensitive group. The bar size is the same for all figures. Scale bar: 90 μM.

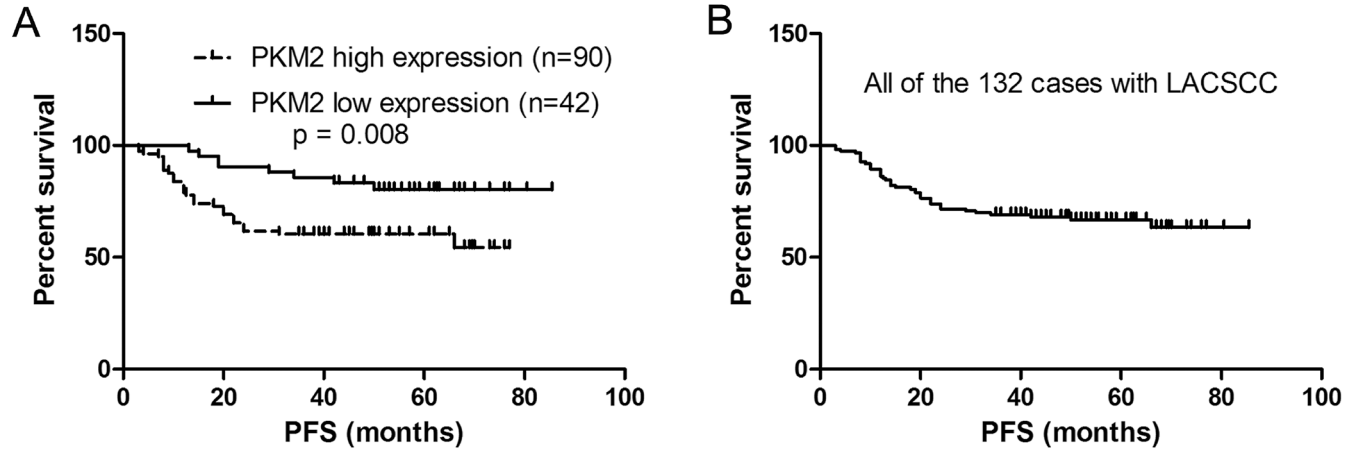

Fig. 2. Kaplan-Meier curves of overall LACSCC patient survival. **A.** The 5-year PFS rates were 80.4% and 60.5%, respectively, in patients with low PKM2 expression (n=42) and high PKM2 expression (n=90). There was a significant difference in the overall survival rate between the 2 groups (p =0.008). **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 ratio(95%CI)	p -value
univariate analyses(n=132)			
PKM2	high vs low	3.278 (1.535, 7.000)	0.002
Age	≥50y VS <50y		0.872
FIGO stage	III + IVa VS Ib + II	2.610 (1.453, 4.689)	0.001
Histopathological grade	Poor+ Moderately vs Well		0.067
Tumor diameter	>4cm vs ≤4cm	3.366 (1.885, 6.012)	<0.001
Combined chemotherapy (platinum-based)	Yes vs No		0.446
multivariate analyses(n=132)			
PKM2	high vs low	2.888(1.347，6.194)	0.006
Age	≥50y VS <50y		0.547
FIGO stage	III + IVa VS I + II	2.120(1.173，3.834)	0.013
Histopathological grade	Poor+ Moderately vs Well		0.06
Tumor diameter	>4cm vs ≤4cm	2.845(1.577，5.132)	0.001
Combined chemotherapy (platinum-based)	Yes vs No		0.254

between the 2 groups (p=0.008, Fig. 2A). The 5-year progression free survival rates for all 132 cases was 66.8% (Fig. 2B). Univariate analyses showed that PKM2 expression [Hazard ratio (95% CI), 3.278 (1.535, 7.000); p=0.002], FIGO stage [Hazard ratio (95% CI), 2.610 (1.453, 4.689); p=0.001], and tumor diameter [Hazard ratio (95% CI), 3.366 (1.885, 6.012); p<0.001] were prognostic predictors of progression free survival in patients with cervical SCC (Table 4). Multivariate Cox regression analysis indicated that PKM2 expression [Hazard ratio (95% CI), 2.888(1.347, 6.194); p=0.006], FIGO stage [Hazard ratio (95% CI), 2.120 (1.173, 3.834); p=0.013], and tumor diameter [Hazard ratio (95% CI), 2.845(1.577, 5.132); p=0.001] were prognostic predictors of progress free survival in patients with cervical SCC (Table 4).

Discussion

Generally speaking, even though cervical cancer is only moderately sensitive to radiotherapy, it plays a significant role in the treatment of locally advanced cervical cancer, and radiation resistance has been considered as the main reason for treatment failure (Randall et al., 2010). In our study, we collected 132 cases of LACSCC patients. Although all of the patients received radical radiotherapy, there were still 47 patients whose progression free survival (PFS) was less than 36 months. We also found that patients with 5 years PFS had PKM2 high expression which was obviously lower than the low expression group in cervical cancer patients, and the high expression of PKM2 can prompt radiation resistance, which might lead to local uncontrolled, local recurrence or distant metastasis.

The mechanism of radiation resistance is very complex, and the underlying mechanism of the direct association between PKM2 expression and radiation resistance is still unknown. However, several investigations have demonstrated that hypoxia can lead to radiation resistance in tumor cells (Nordsmark et al., 1996; Brizel et al., 1999; Sattler et al., 2010). Ionizing radiation can cause damage to the DNA double-strand; the damaged DNA can directly lead to cell death in aerobic cells, but, hypoxic conditions can restore the damaged DNA to its original form. Therefore, DNA damage is decreased in the absence of oxygen (Brown and Wilson, 2004). Under hypoxic conditions, the radiation dose must be increased to obtain the same surviving fraction as in aerobic cells; this research demonstrated that hypoxic tumors are radiation resistant, especially those cells with a median PO2 less than 10 mmHg (Brown and Wilson, 2004). Another study hypothesis is that tissue lactate content has a relationship with radiation resistance in solid human tumors; the tumor control dose 50% (TCD50) values were positively correlated with tumor lactate levels. Transient inhibition of glycolysis during treatment might cause sensitization of tumors to irradiation (Quennet et al., 2006), They also found that lactate (Lim et al., 2012), especially pyruvate, is a potent radical scavenger to cells, because it can counteract the metabolite of ionizing radiation which can generate lots of free radicals, such as reactive oxygen species (ROS). Furthermore, another research hypothesis is that the content of lactate in primary lesions can predict a high probability of metastasis and a low survival rate (Walenta and Mueller-Klieser, 2004; Walenta et al., 2004). Therefore, the activity of glycolysis can generate large amounts of lactate and pyruvate, which may not only reflect a highly malignant phenotype as a general intrinsic property of the tumor cells, but also indicate a pronounced therapeutic resistance, in particular to radiation.

PKM2 is one of the most important isoenzymes of PK; it catalyzes the dephosphorylation of phosphoenol-pyruvate (PEP) to pyruvate, produces ATP under hypoxic conditions (Vander Heiden et al., 2009) and reprograms the glycolytic flux to satisfy the demands of proliferating tumor cells (Iqbal et al., 2014). The first

sign of PKM2's importance is during embryogenesis for proliferating cells, and is progressively replaced by other tissue specific isoenzymes during tumorigenesis (Mazurek et al., 2005). For example, PKL in the liver or PKM1 in the brain disappear and the PKM2 isoenzyme is expressed (Hacker et al., 1998; Steinberg et al., 1999). In FTO2B rat hepatoma cells, glucose was found to increase the amount of dephosphorylated transcription factor SP1, which results in a higher DNA binding activity of the transcription factor while causing an increase in PKM2 expression (Schafer et al., 1997). Recently, a research study found that via the Akt/PI3K /mTOR pathway, the expression of PKM2 is up-regulated through HIF1α/c-Myc to promote a Warburg effect and tumor growth (Sun et al., 2011). Moreover, hypoxia is a common characteristic in most tumors; it can up-regulate the expression of the PKM gene via stabilization of transcription factor HIF1α (Kress et al., 1998). Hypoxic conditions can induce high expression of PKM2 in tumor cells and might cause an increase in the resistance of hypoxic tumor cells to chemo and radiotherapy (Dewhirst et al., 2008; Lara et al., 2009). Therefore, we concluded that the high expression of PKM2 in local advanced cervical squamous cell carcinomas was associated with radiation resistance and acted as an independent negative prognostic marker for patients with cervical cancer. This will provide a new strategy to explore radiotherapy resistance mechanisms at the molecular level. For example, a decrease in the levels of lactate and pyruvate, or via knockdown of the PKM2 gene might lead to a better therapy response.

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