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Alterations in glucose metabolism proteins responsible for the Warburg effect in esophageal squamous cell carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is the most frequent esophageal tumor in the world. ESCC presents late diagnosis, highly aggressive behavior and poor survival. Changes in tumor cell energy metabolism appear to have a prominent role in malignant transformation. Tumor cells consume glucose avidly and produce lactic acid, even under normoxia. Among the factors that may contribute to the stimulation of glycolysis in tumor cells, there are changes in the glycolytic pathway enzymes such as: pyruvate kinase M1 and M2 (PKM2 and PKM1), hexokinase II (HKII), glucose transporter isoform 1 (GLUT-1), and transcription factor induced by hypoxia (HIF1α), responsible for the transcription of proteins cited. The objective of this study is to evaluate the alterations of these proteins and their association with clinicopathological data in ESCC. We performed immunohistochemistry to determine HIF-1α, GLUT-1, PKM1, PKM2, HK2 and Ki67-expression in ESCC patients and controls. Also, we used RT-qPCR to evaluated mRNA expression of GLUT-1 in esophageal mucosa of individuals without cancer, but are alcohol drinkers and tobacco smokers. Our results showed the exclusively expression of GLUT-1 in tumors cells and dysplastic samples. We also observed a compartmentalization of the expression of PKM1 and PKM2 in relation to tumor cells and stroma associated to tumor areas. All of the proteins evaluated, excepted GLUT-1, were frequently detected in normal mucosa. No correlations between clinicopathological features and protein expressions were observed. GLUT-1 expression appears in initial tumor lesions and is maintained through ESCC evolution. We reported for the first time PKM1 staining in normal esophagus and ESCC, being mostly present in more differentiated cells.

1. Introduction

Esophageal cancer (EC) ranks eighth in incidence and is the sixth leading cause of cancer related death worldwide (Ferlay et al., 2010). Among the different EC tumor pathologies, esophageal squamous cell carcinoma (ESCC) is the most frequent one (Arnold et al., 2015). ESCC etiology is complex because different environmental and lifestyle factors have been associated with its onset (da Costa et al., 2013). The major risk factors for ESCC in western populations are alcohol consumption and tobacco smoking (Castellsagué et al., 1999). In its initial stages, ESCC is asymptomatic and the disease is usually diagnosed only in its late stages when lymph node metastasis has already taken place, which explains its high lethality rate (Gamliel and Krasna, 2005). Molecular markers that predict tumor behavior and allow early diagnosis and disease prognosis would be of major importance for tailored therapeutic decisions. To date, however, there are few molecular markers identified along the steps that lead to ESCC.

ESCC is usually detected by endoscopy followed by biopsy of suspicious areas for histopathologic diagnosis. As an aid to ESCC detection, Lugols iodine solution can be employed in conventional endoscopy. In

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this procedure the normal mucosal cells that contain glycogen stain brown whereas abnormal areas containing glycogen depleted neoplastic cells do not (Dubuc et al., 2006; Lopes and Fagundes, 2012). This high rate of glycogen utilization can be observed since the early stages of esophageal carcinogenesis (Hori et al., 2011; Wang et al., 2004). The biochemical basis for this behavior was described in the 1920s by Otto Warburg, who observed that tumor cells consume glucose avidly and produce lactic acid even under normal oxygen levels (Warburg et al., 1927). This metabolic peculiarity has since been recognized as a common occurrence in a variety of tumor types and the study of aerobic glycolysis has regained increasing interest in the last few years (Hanahan and Weinberg, 2011).

Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that mediates the adaptation of cells to low oxygen levels and directs cells to produce ATP via anaerobic glycolysis. In hypoxia, a rapid increase in HIF-1α heterodimerization with the HIF1β protein is observed, leading to increasing amounts of HIF-1 in the cytoplasm and in the nucleus (Semenza, 2013). Among other biological activities, HIF-1 upregulates the expression of proteins involved in glucose metabolism such as glucose transporter isoform 1 (GLUT-1), hexokinase isoform 2 (HK2) and pyruvate kinase isoform M (PKM) (Marín-Hernández et al., 2009).

GLUT-1 is a glucose transporter that is normally expressed in erythrocytes, endothelial cells, the perineurium of peripheral nerves and placenta (Gould et al., 1991). Hexokinase (HK2), a key enzyme that catalyzes the first step in the glycolysis pathway, whereas pyruvate kinase catalyzes the last step of the glycolytic pathway, which produces pyruvate and ATP. There are four pyruvate kinase isozymes in mammals (L, R, M1, M2) that are encoded by differential mRNA splicing of 2 different genes, *PKLR* and *PKM* (Marie et al., 1981; Noguchi et al., 1986; Tani et al., 1988). *PKM* generates PKM1, the main form in muscle, heart and brain; and PKM2, found in differentiated tissues and in most cancer cells (Mazurek, 2011). It has been shown that, in carcinogenesis, there is a decrease in the expression of tissue specific pyruvate kinases PKL and PKR, with a consequent increase of PKM2 expression (Hacker et al., 1998; Steinberg et al., 1999). However, there is little information about the relationship between PKM1 and PKM2 during neoplastic transformation in the esophagus.

The aim of this study was to evaluate the expression of HIF1α, HK2, PKM1, PKM2, and GLUT-1 in normal esophageal mucosa and in ESCC and assess whether there is an association with etiologic and clinicopathological factors.

2. Methods

2.1. Patients and tissue samples

Tissue samples were collected from 44 patients with a histologically confirmed diagnosis of ESCC that were recruited between 1997 and 2008 from two Brazilian hospitals: Instituto Nacional de Câncer (INCA) and Hospital das Clínicas de Porto Alegre (HC-UFRGS). Tumors were obtained as formalin fixed paraffin embedded (FFPE) samples. At the time of sample collection patients had not undergone any chemo or radiotherapy treatments. Data was collected regarding clinical intervention, tumor stage and differentiation, esophageal localization, presence of a second primary head and neck tumor and, whenever possible, overall survival time after diagnosis. Another set of esophageal mucosa FFPE samples from 18 healthy individuals were used as controls for immunohistochemistry (IHC). In addition, esophageal mucosa tissue biopsies from 45 healthy individuals who were submitted to endoscopic routine examination but were free from cancer or esophageal disorders were also included in this study to evaluate GLUT-1 mRNA expression (see below). These individuals, some of whom were smokers and/or alcohol drinkers, were attended at Hospital Universitário Pedro Ernesto (HUPE-UERJ). All individuals who took part in this study signed an informed consent form and personal information was obtained by a standardized questionnaire. Personal information consisted of data on tobacco smoking, concerning the number of cigarettes smoked and the duration of the habit (expressed as pack·year, defined as the number of packs smoked per day multiplied by the number of smoking years) and individuals were classified as never or ever-smokers (defined as smoking at least one cigarette per day and persisting for more than 1 year). Information was also obtained regarding alcohol intake and individuals were classified as never or ever-drinkers (defined as drinking alcoholic beverages at least twice a week and persisting for more than 1 year). The study proposal and all ethical proceedings were approved by the Ethic Committees of the hospitals involved.

2.2. Immunohistochemistry

The expression of HIF-1α, GLUT-1, PKM1, PKM2, HK2 and Ki67 was assessed by immunohistochemical staining using a Polymer detection system (Novocastra Novolink Polymer). Histologic sections, 3 μm in thickness, of FFPE tissues were treated with xylene and rehydrated with an ethanol series. Antigen retrieval was carried out as described in Table 1. After washing in Tris-buffered saline, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 40 min. Then, the sections were washed with Tris-buffered saline and nonspecific binding was blocked using Protein Block (Novolink Polymer Detection System, Leica Biosystem®) for 40 min. The primary antibody was diluted as described in Table 1 and the sections were incubated overnight at 4 °C. In negative controls, the incubation step with the primary antibody was omitted.

Subsequent steps were performed according to the instructions of the manufacturer's of the Polymer detection system (Novocastra Novolink Polymer). To detect antigen-antibody binding, 3,3,-diaminobenzidine (DAB)/hydrogen peroxide (Novolink Polymer Detection System, Leica Biosystem®) was used and sections were counterstained with haematoxylin.

Scoring was performed in a double-blind manner by two pathologists without knowledge of the clinical data. Any disagreement was resolved by a third evaluation of another pathologist. Because of the diversity of staining patterns found and the absence of optimized and standardized score, there were differences in the immunoscore used in markers. So, for Ki67 only the percentage of positive cells was considered and was categorized as weak when ≤40%, and strong when >40% of

Table 1
Summary of the immunohistochemical methods used, as well as antibody description and dilution.

Antigen	Source[a]	Reference[a]	Solution[b]	pH[b]	Method[b]	Time[c]	Dilution
HIF-1α	Abcam	Ab2185	Tris-EDTA	9	Pressure cooker	3	1:1400
GLUT-1	Abcam	Ab40084	Dako Target Retrieval Solution	9	Water bath (98 °C)	40	1:400
PKM1	Proteintech	15821–1-AP	Dako Target Retrieval Solution	9	Water bath (98 °C)	40	1:100
PKM2	Cell Signalling	#4053	Dako Target Retrieval Solution	6	Water bath (98 °C)	40	1:8000
HK2	Cell Signalling	#2867	EDTA	8	Water bath (98 °C)	40	1:1000
Ki67	DAKO	M7240	Citrate	6	Pressure cooker	3	1:1200

[a] Antibody company catalog reference.
[b] Antigen retrieval method.
[c] Time for antigen retrieval in minutes.