Estimation of serum pyruvic acid levels in oral squamous cell carcinoma

Priyanka Guduguntla, Venkateswara Rao Guttikonda

Department of Oral Pathology and Microbiology, Mamata Dental College, Khammam, Telangana, India

Abstract

Introduction: Cancer cells generally exhibit increased glycolysis for adenosine triphosphate generation (the Warburg effect) due to mitochondrial respiration injury and hypoxia, which are frequently associated with resistance to therapeutic agents. Pyruvic acid is produced as an end product of glycolysis along with lactic acid. At room temperature, lactic acid converts into pyruvic acid as it is chemically unstable.

Aim: To evaluate the serum pyruvic acid levels in patients with oral squamous cell carcinoma (OSCC).

Materials and Methods: Thirty clinically and histopathologically confirmed cases of OSCC were included in the study. The cases were staged clinically (tumor–node–metastasis staging) and graded histopathologically (modified Broder's classification). A control group of 30 age-matched individuals with no systemic diseases were selected and the serum levels of pyruvic acid were measured and the absorbance was read using a spectrophotometer at a wavelength of 540 nm. The results were evaluated statistically and discussed.

Results: Pair-wise comparison of clinical stages and histological grades of OSCC was done using Tukey's multiple post hoc procedure. The increase in mean serum pyruvic acid levels between any two groups and grades was found to be statistically significant respectively ($P < 0.05$).

Conclusion: Serum pyruvic acid levels gradually increased from individuals without OSCC to individuals with OSCC. Higher levels of serum pyruvic acid were seen with increasing clinical stage and the mean serum pyruvic acid levels were also found to be significantly increasing with advancing histopathological grades of OSCC.

Keywords: Glycolysis, oral squamous cell carcinoma, pyruvic acid

INTRODUCTION

Head-and-neck cancers (HNCs) have emerged as a leading cause of cancer-related mortality and morbidity worldwide. Oral cancer encompasses an important group of HNC, with more than 90% of them being oral squamous cell carcinomas (OSCC).[1] It is the 6th most common cancer worldwide,[2] and the Indian subcontinent accounts for about one-third of the world burden of this malignancy.[1]

Although oral cavity is frequently examined, 60% of intraoral carcinomas were in advanced stage at the time of detection.[3] The 3-year and 5-year disease-free survival rates were 65.5% and 61.0%, respectively.[4] Persistent difficult

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problems associated with oral cancer are late diagnosis, poor response of tumor to chemotherapy, lack of reliable biomarkers for early diagnosis and posttherapeutic monitoring.[5] Therefore, early detection of oral cancer is of utmost importance for improving survival rate and prognosis of patients with the disease.[1]

Recently, tumor biomarkers are receiving more attention in early detection as well as predicting the prognosis of the lesion.[3] Biomarkers are the biological molecules found in blood, other body fluids or tissues as a sign of a normal or abnormal process or of a condition of disease such as cancer.[1] They play an important role in distinguishing between the presence or absence of disease. Therefore, estimation of various biomarkers in serum may act as an adjunct to conventional biopsy and provide a cost-effective method for screening the oral cancer patients.[2]

Biomarkers include nucleic acids, proteins, peptides, enzymatic changes, antibodies, metabolites, lipids and carbohydrates.[6] Pyruvic acid ($C_3H_4O_3(CH_3COCO_2H)$) is a three-carbon ketoacid that plays an important role in biochemical processes. It is an important intermediary cellular metabolic product of fat, protein and carbohydrates.[7]

In normal physiologic process of glycolysis, pyruvic acid and lactic acids are produced as the end products. Pyruvic acid produced by glycolysis cycle will be utilized by Krebs cycle in mitochondria for further adenosine triphosphate (ATP) production. This ATP is synthesized by oxidative phosphorylation in mitochondria. However, this method of production of ATPs is not seen in cancer cells.[8] Cancer cells show changes not only in the glycolytic pathway but also in the Krebs cycle, β oxidation and anabolic metabolism. Cancer cells are reoriented to respond to the new primary function of the cell, i.e., uncontrolled proliferation by providing not only energy but also the synthesis of nucleotides, amino acids and fatty acids.[9]

Mitochondrial respiratory function is seen compromised by many factors which result in insufficient oxygen availability in the cellular microenvironment. Some of the factors include mutations in mitochondrial deoxyribonucleic acid (mtDNA), malfunction of the electron transport chain and aberrant expression of enzymes involved in energy metabolism causing insufficient oxygen availability in the cellular microenvironment. Hypoxia can also initiate the Warburg's effect as the rate of angiogenesis is comparatively less than that of the rate of growth of the tumor; this will induce depleted oxygen environment in the neoplastic area and induces alternative respiratory method for the

energy production and its survival, i.e., glycolysis pathway.[10] Hence, glycolysis is a central metabolic pathway that finely regulates cell proliferation by adapting the cancer cell metabolism to the conditions.

Cancer cells exhibit increased glycolysis rate for their energy needs and this increased glycolytic rate produces more of its end products such as pyruvic acid and lactic acid. As lactic acid is an unstable compound, it converts back to pyruvic acid. This excess pyruvic acid either leaches into blood or oral cavity, leading to the imbalance in the production and excretion of the pyruvic acid.

Hence, the aim of the present study is to estimate and compare the serum levels of pyruvic acid in normal individuals and in patients with OSCC.

Aim and objectives
Aim
To evaluate the serum pyruvic acid levels in patients with OSCC.

Objectives
1. To estimate the serum levels of pyruvic acid in healthy individuals and in patients with OSCC
2. To compare the serum levels of pyruvic acid with respect to the clinical staging in patients with OSCC
3. To compare the serum pyruvic levels in OSCC with respect to the histopathological grading.

MATERIALS AND METHODS

Thirty cases of clinically proven and histopathologically confirmed cases of OSCC attending the outpatient department of our hospital were included in the study. A group of 30 healthy individuals acted as controls. Exclusion criteria included patients with a history of systemic diseases such as cardiac diseases, diabetes and other carbohydrate metabolic disorders. Patients under chemotherapy and radiotherapy and patients treated previously for oral cancer were also not included in the study. The OSCC cases were staged clinically based on tumor–node–metastasis (TNM) staging[11] and were graded histopathologically based on the modified Broder's system of classification.[12]

Collection of blood and serum separation
After histological confirmation, the patients were recalled for the collection of blood. 5 ml of venous blood was collected from a large peripheral vein of the selected subjects. The samples were collected in sterile plastic tubes coated with ethylenediaminetetraacetic acid (EDTA). Blood was mixed with EDTA by shaking the tubes gently. Blood

samples were immediately transferred to deep freeze at 4°C to halt the rate of biochemical and bacterial responses.

Estimation of serum pyruvic acid

Biochemical analysis of the serum collected was analyzed based on the modified 2,4-dinitrophenylhydrazine procedure, and the absorbance was read using a spectrophotometer according to the method proposed by Landon J et al.[13]

Test procedure

I. 2 ml of heparinized blood was mixed with 4 ml of 0.6M per chloric acid and kept in an ice bath for 10 min, and then the mixture was subjected to centrifugation of 3000 rpm for 5 min and supernatant fluid was collected

II. 3 ml of supernatant fluid was mixed with 1 ml of dipotassium phosphate solution, and this solution was centrifuged at 3000 rpm for 10 min and again the supernatant fluid was collected. This supernatant fluid obtained was protein free filtrate of blood sample

III. 1 ml of dinitrophenylhydrazine was added to each solution and kept at 37°C for 10 min

IV. 10 ml of freshly prepared 0.4M sodium hydroxide was added and quantified with a spectrophotometer of wavelength of 540 nm after 10 min.

RESULTS

Thirty cases of OSCC were included in the present study and compared with equal number of controls [Table 1].

According to clinical staging, OSCC patients were clinically subdivided into the following stages as T1, T2, T3 and T4. T1 included 8 (26.66%) cases, T2 included 11 (36.66%) cases, T3 included 8 (26.66%) cases and T4 included 3 (10%) cases [Table 2].

According to histopathological grading, OSCC cases were graded as well differentiated, moderately differentiated and poorly differentiated. Well-differentiated grade included 10 (33.33%) cases, moderately differentiated included 10 (33.33%) cases and poorly differentiated included 10 (33.33%) cases [Table 3].

Serum pyruvic acid levels were evaluated in both the study group and the control group. The mean serum pyruvic acid levels with SD are calculated for the control group (0.95 ± 0.18) and OSCC (2.65 ± 0.80).

Comparison of control and oral squamous cell carcinoma groups with respect to serum pyruvic acid levels (mg%) by independent t-test

In pair-wise comparison of the groups, i.e., control

and OSCC, done by independent t-test procedure, the increase in the mean serum pyruvic acid levels between different groups was found to be statistically significant with $P < 0.05$ [Table 4 and Figure 1a].

Pair-wise comparison of tumor–node–metastasis staging (I, II, III and IV) of oral squamous cell carcinoma with respect to serum pyruvic acid levels (mg%) by Tukey's multiple post hoc procedures

The mean values of different clinical stages such as T1, T2, T3 and T4 were 1.63, 2.54, 3.26 and 3.89, respectively.

Table 1: Distribution of cases and controls in the study sample

Groups	Number of samples	Percentage of samples
Controls	30	50%
OSCC	30	50%

Table 2: Distribution of OSCC patients by clinical staging

Clinical staging	Number of samples	Percentage of samples
Stage 1 (T1)	8	26.66%
Stage 2 (T2)	11	36.66%
Stage 3 (T3)	8	26.66%
Stage 4 (T4)	3	10%
Total	30	100

Table 3: Distribution of OSCC patients by histopathological grading

Histopathological grading	Number of samples	Percentage of samples
Well differentiated	10	33.33%
Moderately differentiated	10	33.33%
Poorly differentiated	10	33.33%
Total	30	100

Table 4: Comparison of mean serum pyruvic acid levels among Control and OSCC groups using independent t-test

Group	Pyruvic acid levels	
	Mean	SD
Controls	0.95	0.18
OSCC	2.65	0.80
P	0.0001*	

*$P<0.05$

Table 5: Pair wise comparison of oral squamous cell carcinoma clinical stages with respect to serum pyruvic acid levels by Tukey multiple post hoc procedures

Clinical stages	T1	T2	T3	T4
Mean	1.63	2.54	3.26	3.89
SD	0.22	0.27	0.45	0.12
T1	-			
T2	P=0.0002*	-		
T3	P=0.0002*	P=0.0008*	-	
T4	P=0.0002*	P=0.0002*	P=0.0277*	-

*$P<0.05$

In pair-wise comparison of clinical stages of OSCC done by Tukey's multiple post hoc procedure, the increase in the mean serum pyruvic acid levels between any two groups was found to be statistically significant ($P < 0.05$) [Table 5 and Figure 1b].

Pair-wise comparison of histological grading (well, moderate and poor) of oral squamous cell carcinoma with respect to serum pyruvic acid levels (mg%) by Tukey's multiple *post hoc* procedures

The mean values of well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma were 1.76, 2.63 and 3.49, respectively. In pair-wise comparison of different histological grades of OSCC, done by Tukey's multiple *post hoc* procedure, the increase in the mean serum pyruvic acid levels between any two different grades of OSCC was found to be statistically significant ($P < 0.05$) [Table 6 and Figure 1c].

DISCUSSION

OSCC is the most common cancer in the oral cavity with a 5-year mortality rate of almost 50%, which has not changed significantly in the last five decades, despite the advances in the multimodality treatment.[14]

Timely intervention can cure the cancer as well prevents from more mutilating surgery. During cancer growth, certain substances are quantitatively changed in the serum known as tumor markers or biochemical serum markers, which are receiving more attention in early diagnosis as well as predicting prognosis of the lesion.[15]

Serum biomarkers are defined as substances changing quantitatively in the serum during tumor development. They aid in early diagnosis of tumor and prediction of prognosis. These tumor markers/substances can be classified as tumor specific and tumor associated. Tumor-specific substances are considered as a direct result of oncogenesis, while tumor-associated markers are various proteins, enzymes, hormones and immunoglobulins, which occur in the blood and are mediated by the tumor itself or by the influence of the tumor on the involved tissues.

Thus, serum biomarkers not only help in monitoring tumor progression, metastasis but also helps in assessing response to treatment.[16]

Pyruvic acid is a key molecule in carbohydrate metabolism. In the physiologic process of glycolysis, pyruvic acid and lactic acid were produced as the end product.[10] This yields 2 ATPs of energy from a single glucose molecule. This energy production cascade continues by utilizing the end product (pyruvate) of glycolysis into Krebs cycle in mitochondria by oxidative phosphorylation. Cancer cell shows changes not only in the glycolytic pathway but also the Krebs cycle, β-oxidation and anabolic metabolism which are reoriented to respond to the new primary function of uncontrolled proliferation of cells by providing not only energy, but also the synthesis of nucleotides and amino and fatty acids.[17]

The reason for increased production of pyruvic acid is based on the fact that most of the cancer cells produce energy by glycolysis rather than oxidative phosphorylation via the tricarboxylic acid cycle, even in the presence of an adequate oxygen supply, which is known by the phenomenon "Warburg effect."[18] Cancer cells in contrary to normal cells mainly use glycolysis in their cytoplasm to generate ATP to provide cells with energy required for proliferation. This phenomenon of so-called aerobic glycolysis relates to the Warburg effect, which is a hallmark of cancer cell metabolism.[8] Upregulation of glycolysis in tumor cells is accompanied by mitochondrial dysfunction and decreased oxidative phosphorylation.[19] Many studies have cited a strong correlation between tumor progression and increased mtDNA mutations.[20] Excessive glycation of mitochondrial proteins, lipids and mtDNA due to mitochondria-associated carbonyl stress has been shown to contribute to mitochondrial dysfunction and mutations.[21] Thus, upregulation of glycolysis in tumor cells may contribute to increased mitochondrial damage, thereby establishing a vicious cycle that enhances the Warburg effect. Since enhanced glycolysis results in increased concentration of glycolytic intermediates, the levels of pyruvic acid are also thought to be increased in established malignancies.[22]

Table 6: Pair wise comparison of oral squamous cell carcinoma histological grading with respect to serum pyruvic acid levels by Tukey multiple *post hoc* procedures

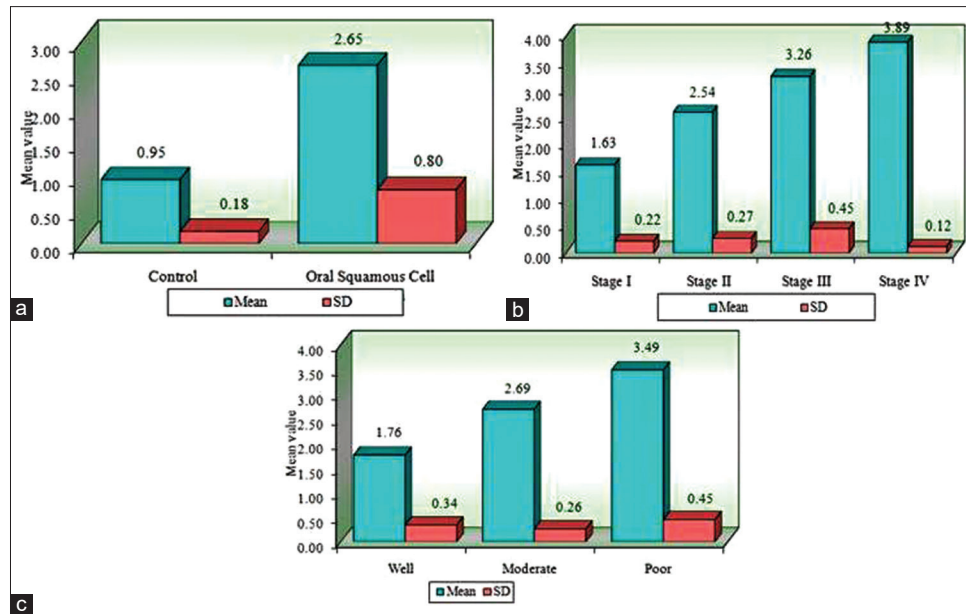| HISTOLOGICAL GRADING	WELL DIFFERENTIATED OSCC	MODERATELY DIFFERENTIATED OSCC	POOR DIFFERENTIATED OSCC
Mean	1.76	2.69	3.49
SD	0.34	0.26	0.45
Well differentiated	-		
Moderately differentiated	P=0.0001	-	
Poor differentiated	P=0.0001	P=0.0002	-

*$P < 0.05$

Figure 1: (a) Graph showing comparison of control and oral squamous cell carcinoma groups with respect to serum pyruvic acid levels (mg%); (b) Graph showing comparison of tumor-node-metastasis staging (I, II, III, IV) of oral squamous cell carcinoma with respect to serum pyruvic acid levels (mg%). (c) Graph showing comparison of histological grading (well, moderate and poor) of oral squamous cell carcinoma with respect to serum pyruvic acid levels (mg%)

Warburg's effect can also be initiated by hypoxia as the rate of angiogenesis is comparatively less than that of the rate of growth of tumor; this will induce depleted oxygen environment in neoplastic area and induces alternative respiratory method for the energy production and its survival, i.e., glycolysis pathway. Hence, glycolysis is a central metabolic pathway that finely regulates cell proliferation by adapting the cancer cell's metabolism to the conditions of its current selective situation.[10]

Another reason for higher rate of glycolysis in malignant cells is based on the evidence obtained by several immunohistochemical studies that showed a higher expression of glucose transporter proteins (GLUT) like GLUT 1 in increasing grade of tumors, indicating a higher amount of glucose utilization by the malignant cells.[23] It has also been shown that epigenetic and/or mutagenic changes in cancer cells can induce: (1) overexpression of type 2 hexokinase[24] (2) activation of normally insulin-regulated glucose membrane receptors, especially GLUT1, GLUT3 and GLUT5 leading extracellular glucose to penetrate easily into cancer cells,[23] and (3) overexpression of all glycolytic enzymes in aerobic and anaerobic conditions.[25]

The quantitative measurement of metabolic products from glycolytic pathway yields more sensitive markers than enzymes in cancer patients as it is the central metabolic pathway in cancer cells. Therefore, the current study was undertaken to assess the levels of serum pyruvic acid, which is an end product of glycolysis, in patients with OSCC and to compare these values with the healthy individuals.

Various studies have been put forward by several researchers,in which they reported that elevation of serum pyruvic acid above the normal levels reflect biological abnormalities in cancer tissues.

Studies done by Diers et al.[26] and Thangaraju et al.[27] in breast cancer and colon cancer, respectively, showed a significant rise in the serum pyruvic acid levels. Hur et al.[28] have also done a study to measure the levels of organic acids in patients with gastric cancer. They concluded that there was a significant increase in the levels of organic acids such as pyruvic acid, lactate, succinic acid, malic acid and α-ketoglutaric acid in cancer patients when compared to the normal. Similarly, Bhat et al.[29] estimated the serum pyruvic acid levels in healthy and potentially malignant disorder (PMD) subjects and concluded that there was a significant rise in serum pyruvic acid levels in PMD subjects compared to healthy individuals, indicating that estimation of serum pyruvic acid can be a screening tool for PMD and malignant diseases.

In the present study, there is a significant increase in the levels of serum pyruvic acid in subjects with OSCC compared to controls ($P > 0.001$). These results are in accordance with the study done by Bhat et al.,[30] where an increase in serum pyruvic acid levels was noticed in patients with OSCC.

In our study, the serum pyruvic acid levels were also compared with respect to clinical staging and histopathological grading using Tukey's multiple *post hoc* test. The results showed a statistically significant increase in levels as the clinical stage of OSCC advances ($P < 0.005$). Similarly, as the histopathological grade of OSCC advances from well to poor, there was a statistically significant rise in serum pyruvic acid levels ($P < 0.005$). This may be due to the tumor differentiation and increased shedding of the malignant cells into the circulation as a result of metastasis. This study was first of its kind to compare the pyruvic acid levels in OSCC cases based on the clinical staging and histopathological grading.

The present study reveals that serum pyruvic acid levels can be used as a reliable biomarker for prognostic evaluation. This is a simple and a cost-effective method of estimating serum pyruvic acid levels and therefore can be used as a screening marker in identifying individuals with malignant changes. This marker also aids in increasing the accuracy of clinical diagnosis and assessing the spread and invasiveness of the cancer of the oral cavity, suggesting its use as a prognostic indicator. However, research should be carried out in a larger sample size to support the findings of the current study.

CONCLUSION

The present study with limited number of samples showed that the serum pyruvic acid levels gradually increased from normal individuals to patients with OSCC. This indicates that serum pyruvic acid levels may be used as a biochemical marker to detect changes, leading to malignancy. In OSCC, higher levels of serum pyruvic acid were seen with increasing clinical stage, indicating that as the stage advances, there is an increase in the tumor burden and increase in actively dividing cells. The mean serum pyruvic acid levels were also seen significantly increasing as the histopathological grade of OSCC advances, indicating the progression of the cancer. Therefore, pyruvic acid levels may be considered as a marker for diagnostic as well as prognostic evaluation.

Conflicts of interest
There are no conflicts of interest.

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