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Serum Lactate Dehydrogenase: A Possible Metabolomics Biomarker for the Early Detection of Head and Neck Cancer Lymph Node Metastasis

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ABSTRACT

Head and neck cancer (HNC) appears with one of the most recurring sites of cancer, and the percentage of metastases is also high in both localized region and distant areas. Diagnosis of metastases is correlated with poor prognosis, with a median survival of about 10 months. Therefore, early diagnosis is of utmost significance in the treatment of HNC. Metabolomics study targeting altered metabolic pathways exhibits a promising approach for discovering early novel noninvasive targets and biomarkers in HNC lymph node metastasis. An increased level of Lactate dehydrogenase (LDH) has been observed as a major diagnostic metabolomics biomarker for poor survival among HNC patients. It is also recommended as a more efficient biomarker of metastasis. For present study serum samples were collected from healthy controls (n=10) and HNC patients with and without lymph node metastasis (n=10 each). Prepared serum samples were analyzed by UV-spectrophotometer. Further, obtained data were analyzed using the one-way ANOVA test. Significant difference ($p<0.05$) was reported in the levels of serum metabolites between healthy control group and HNC lymph node metastasis ($p<0.0006$). Comprehensively, this study provides valuable insights in alteration of serum LDH enzyme level in early diagnosis and prediction of HNC. We proposed LDH could be used as an early metastasis biomarker in screening HNC lymph node metastasis patients.

Keywords: Head and neck cancer, Lymph node metastasis, Metabolomics, Lactate dehydrogenase (LDH), Serum

INTRODUCTION

Head and Neck Cancer (HNC) is the sixth most frequent cancer worldwide with approximately 890,000 cases diagnosed per year resulting 450,000 deaths annually [1]. More than 90% of HNC cases are squamous cell carcinoma and emerge from mucosal surfaces of the larynx, pharynx and oral cavity [2]. There is large geographical variation in the incidence and anatomical distribution of HNC worldwide, which is attributed to the differences in the alcohol consumption and tobacco use, the major risk factors [3]. Worldwide, 57.5% of global HNC patient exist in Asia especially in India. India accounts for one-third (30%) of the world burden of this malignancy. Almost 60 to 80% of HNC patients present in India with advanced disease as compared to 40% in developed countries. Consequently, Mortality is also high in India approximate half of incidence due to late prognosis [4].

Metastasis is a foremost cause of mortality among HNC patients. Usually, at the time of finding HNC patients mostly appear with metastasis and poor prognosis. Therefore, early detection of disease is

necessary to prevent mortality from HNC. Heretofore, a variety of biomarkers have been enrolled in the management of HNC but they have lack of specificity [5-6]. Therefore, search of novel biomarkers is an urgent need for necessary management of HNC.

Presently, among metabolomics biomarkers, increased levels of Lactate dehydrogenase (LDH) have been recognized as a more efficient marker of metastasis, for poor survival among multiple malignancies including HNC. A larger number of cancer cells are alternatively fueled by aerobic glycolysis, this process is known as "the Warburg effect" [7]. The LDH enzyme is the main player in this effect. LDH is an important glycolytic cycle enzyme composed of mainly four polypeptide subunits, involved in the reduction of pyruvate to form lactate in aerobic conditions [8] (figure-1). Studies have suggested that LDH has been associated with the activation of some proto-oncogenes, such as HIF- α and Myc [9]. LDH also has a vital role in tumor metabolism, proliferation, invasion, and metastasis [10].

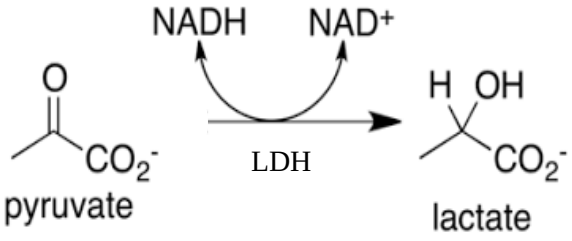

Fig.1: Reduction of pyruvate to lactate by Lactate dehydrogenase

Therefore, Inhibition of the LDH level may be used as a therapeutic marker also. Amongst all serum markers, serum LDH level is easy to quantify and its levels can be figured out through simple processes and cost efficient also to measure. The serum LDH value was found to be significant in patients with malignancy and also correlated with clinical TNM staging. Therefore, the present study was aimed to quantify LDH level in serum samples of HNC patients and to furnish more important facts of LDH as a metastatic marker.

MATERIALS & METHODS

In the present prospective study blood samples were collected from fasting groups and provided by the Bhagwan Mahavir Cancer hospital, Jaipur. The study was validated by the Ethics Committee of the Bhagwan Mahavir Cancer hospital, Jaipur and written informed consent was obtained from all the patients. All the blood sample were taken before the start of cancer treatment as radiotherapy, surgery or chemotherapy. Patients of disease like liver disease, myocardial infarction, hypothyroidism were excluded from the study. Cancer diagnosis and staging was performed by the histopathological analysis of tissue specimens. Staging of HNC patients was done according to the standard cancer staging manual. [11] Blood sample collected from 20 HNC patients with age group 31 to 65. HNC cases were categorized into two groups: head and neck cancer patients without lymph node involvement (HNC-WON) and head and neck cancer patients with lymph node metastasis (HNC-LNM) (10 samples each). 10 healthy controls were also included in the study. The Healthy Control (HC) included persons whose gender and age were equivalent to HNC patients. Clinical characteristics of studied groups are given in Table-1.

Blood samples were collected in a neat red cap tube and immediately centrifuged at 3000 × g for 10 min at 4 ◦C. The supernatant serum was immediately shifted into a fresh tube and analyzed immediately after collection. As LDH was reported to be stable in serum for four days at 2-8 ◦C. freezing or exposure to high temperature may inactive thermolabile LDH isoenzyme in serum sample. Further, LDH level estimation was performed from these collected serum samples [12]. Principle of LDH enzyme analysis was "LDH enzyme catalyzes the reduction of pyruvate by NADH to form lactate and NAD^+. The catalytic concentration was determined from

the rate of decrease of NAD^+ measured at 340 nm".

Working solutions of reagents R_1 and R_2 included 100 mmol/L TRIS Buffer (PH 6.8), 0.07 gm/L EDTA, sodium pyruvate 1.20 mmol/L, sodium chloride 160 mmol/L and NADH 0.08 mmol/L (concentration 0.08 mmol/L). Linearity of the reagent was up to 2000 IU/L. Assay procedure conditions comprised UV-kinetic mode of reaction, decreasing reaction direction, 340 nm wavelength, 37^0C, zero setting with distilled water, factor 8109, 60 sec delay time, 4 readings, 30 sec time interval and blank absorbance limit ± 1.000Abs. Normal values at various temperatures 37^0C, 30^0C and 25^0C were found to be 240-480 IU/L, 161-322 IU/L and 120-240 IU/L respectively. Working solutions were mixed (4 R_1+1 R_2, R_1=800µl, R_2=200 µl total 1ml) at 37^0C and added in serum sample (0.02ml). After proper mixing of working solution and serum sample, the mixture was immediately transferred to the thermostat cuvette. By UV-spectrophotometer at 340 nm first reading was noted at 60^{th} second and subsequently three more readings with 30 second interval.

LDH activity was estimated as: Average change in the absorbance per minute= Δ Abs./30 sec.× 2. Activity of LDH in IU/L= Δ Abs. /min × 8109.

Further obtained data were subjected to statistical analysis using statsmodels.api python software. One-way ANOVA was used to compare the data among the three groups. Tukey post hoc test was also used to uncover specific differences between the groups.

RESULT

Patients and clinical characteristic

Clinical characteristics of HNC patients are summarized in Table 1. The age of the HNC-WON patients ranged from 31 to 61 years with a mean of 50 years. Whereas, the age range of HNC-LNM patients was from 48 to 64 years with the mean of 55.4 year and age range for HC group was 35 to 61 years with the mean of 50.8 years.

Table1: Clinical characteristics of healthy control subjects and HNC patients.

Subjects	HNC-WON	HNC-LNM	HC
No. of sample	10	10	10
Age (year)	31-61	48-64	35-61
Mean(age)	50	55.4	50.8
Gender ratio (m/f)	9/1	9/1	9/1
TNM stage			
I A	2		
IIA	3		
IIB	4	1	
IIIA	1	4	
IIIC		4	
IVA		1	

LDH value in serum sample

The normal value of LDH level ranged from 240 to 480 U/L. LDH values of HC, HNC-WON and HNC-LNM shown by boxplot in figure 2.

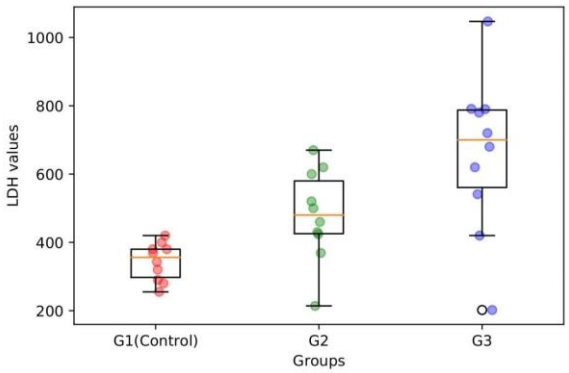

Fig. 2: Boxplot between LDH value and three groups. Pink dot represents the LDH value of the HC group, similarly green dot HNC-WON and blue dot HNC-LNM.

One-way ANOVA was used to compare the data among the three groups. The variation of overall mean LDH level among the three groups was found to have a significant *P* value < 0.0006. It means the chance of type 1 error (rejecting a correct H_0) is small (0.06%). Significant p value = 0.0006 indicate level of LDH significantly altered in HNC-LNM. In HNC-WON, about 40% cases had elevated serum LDH and the mean LDH level in this group was higher (480.7±138.84 U/L) than in HC group (343.7±55.42 U/L), with a mean difference of 137 U/L, here we did not found significant p value (p<0.147). Furthermore, 60% cases in the HNC-LNM group had elevated LDH, and the mean LDH level was

also significantly higher (659.1 ± 232.22 U/L) than in HC group, with a mean difference of 315.4 U/L (P<0.0003). Between HNC-WON and HNC-LNM group 60 % cases in the HNC-LNM group had elevated LDH, and the mean LDH level was significantly higher than in HNC-WON group, with a mean difference of 178.4U/L (P<0.004) [Table 2, figure -3]. Here, we found ANOVA F test significant (f-statistic value =10.0168).

Turkey post hoc test between the groups was also done to find specific differences between the groups. By comparing difference to critical mean we found that group 1 (HC) to group 3 (HNC-LNM) more significantly altered as compared to other groups [Table 2, figure 4].

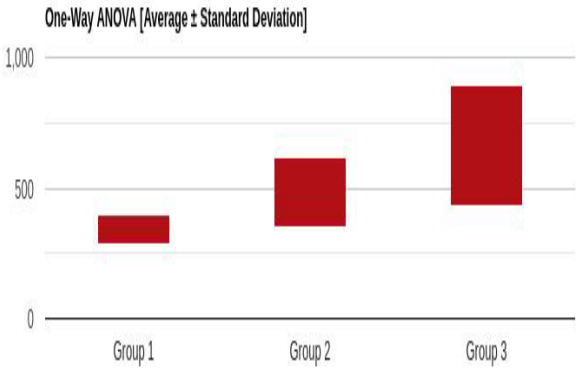

Fig. 3: Intergroup comparison of mean± standard deviation of serum LDH level among the groups. (Group1=Healthy control, Group2= HNC-WON and Group3=HNC-LNM)

Table 2: Turkey post hoc test between the groups

Pair	difference	SE	Q	Lower CI	Upper CI	p-value
x1-x2	137	49.9701	2.7416	312.2165	175.2165	0.1473
x2-x3	315.4	49.9701	6.3118	490.6165	175.2165	0.0003681
x1-x3	178.4	49.9701	3.5701	353.6165	175.2165	0.04536

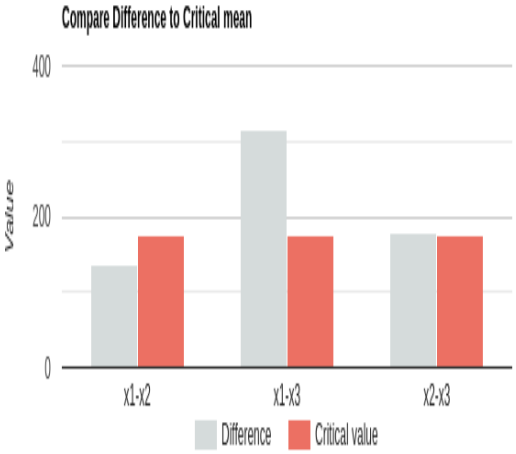

Fig. 4: Comparison of difference to critical value between the groups.
(1= HC group, 2= HNC-WON, 3=HNC-LNM)

DISCUSSION

Present work observed a relation between serum LDH level and HNC patient group. HNC-LNM patients found a significantly increased serum LDH level compared to healthy control group and HNC-WON involvement group (figure-3, 4). Furthermore, increased LDH level was dependent with higher HNC stage, suggesting an important role of LDH as a metastatic stage biomarker.

LDH level also found to be enhanced in disease like, liver disease, myocardial infarction and hypothyroidism [13,14]. As patients with all these diseases were excluded in the study, we assume that increased LDH level was due to HNC disease only. This exclusion increased the weightage of our study, exhibiting that LDH might be a potentially biomarker to assist in early prognosis of tumor burden and metastasis of HNC disease. However, in this study LDH iso-enzymes were not being quantified which could have been more beneficial to understand the specificity of HNC-LNM.

Previous study on total serum LDH level in HNSCC proposed that there was a significant correlation between serum LDH level and grades of HNSCC also [15]. Another important study on HNC also proposed that elevated tumor lactate concentration associated with the development of nodal or distant metastases predicted an increased risk of metastases. Hence, these studies allow for the confirmation of the specificity of increasing serum LDH level in HNC-LNM [16].

A study by Mohajertehran et.al (2019) also confirms the increasing role of LDH enzyme as a diagnostic marker in both tumor tissue and saliva of HNSCC patients [17]. Kallalli BN et.al (2016) studied salivary LDH level in three subjects with OSMF, oral cancer, and controls and they also found significant difference between the mean values of the salivary LDH levels [18]. K. Lokesh et.al (2016) obtained statistically significant p-value in OSCC patients as compared to control group which proved significantly higher LDH in OSCC with the histopathological grade of the tumor [19].

These approaches allow us to confirm the specificity of serum LDH level in metastasis of head and cancer. Also, in the present study we found a significant difference in serum LDH level among three group healthy controls, HNC-WON involvement group and HNC-LNM group ($p>0.0006$). Along with, we found serum LDH level more significantly elevated in HNC-LNM ($p>0.0003$). Furthermore, it was also observed a significant correlation of the enzyme levels with the differentiation of the tumor size, thus predicting prognosis and the overall treatment outcome. Therefore, in future serum LDH level could be used as a metabolomics marker of HNC-LNM.

The best part of this study is that compared to other tests, serum LDH tests are easily available in the clinics. Serum LDH test is economical and easy to estimate. It does not require sophisticated centers or any latest technology and can be performed even at our rural centers also. Larger HNC patient groups and long term studies are required to culminate the usefulness of serum LDH as a HNC-LNM prognostic and therapeutic biomarker.

CONCLUSION

This study suggests that increased serum LDH level is linked with HNC-LNM. Serum LDH level estimation is easily available in clinics as routine test at economical rate. Therefore, LDH can be used as a serum biomarker in early prognosis of metastasis of HNC. However, due to lack of sensitivity and specificity of serum biomarkers, additional markers should be used in clinical practice to evaluate the HNC-LNM.

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Ethical Approval: Approved

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