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Biochemical markers in oral cancer.

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Abstract

β_2 -Microglobulin is the invariant chain of the Major Histocompatibility Class I (MHC-I) molecule on the cell surface of all nucleated cells and it reflects the cell turn over. Altered glycosylation of glycoconjugates is among the important molecular changes that accompany malignant transformation. The objectives of this study were to validate the diagnostic value of serum β_2–microglobulin, Total Sialic Acid (TSA) and Lipid-Bound Sialic Acid (LSAB) and to correlate these parameters with the stages of the malignancy. Clinically and histopathologically proven 40 oral cancer patients were selected for the study. According to TNM stage of cancer, patients studied 11, 8, 10, 11 were of stages I, II, III and IV respectively. 40 healthy controls were selected for comparison. β_2 -Microglobulin was estimated by ELISA and total sialic acid and lipid-bound sialic acid determined by the spectrophotometric method of Plucinsky et al. The concentration of β_2-microglobulin, total sialic acid and lipid-bound sialic acid were significantly elevated in different stages of oral cancer patients when compared with the controls. Circulating levels of these biomarkers were also elevated significantly among different clinical stages with progressive rise from stage I to stage IV of the disease and found to reflect tumor burden. These biomarkers have good sensitivity, specificity and efficiency values for oral cancer. Thus, findings of the study suggest that the evaluation of these markers would be useful in assessing early malignant change, increasing accuracy of clinical diagnosis and also in assessing the spread and invasiveness of the cancer of the oral cavity.

Key words: Oral cancer, β_2-microglobulin, Total sialic acid, Lipid- bound sialic acid, Biomarkers.

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Introduction

Malignant neoplasms are major causes of fear, morbidity and mortality all over the world. Globally 'oral cancer' is the sixth most common cause of cancer-related death [1]. Oral cancer accounts for approximately 30-40% of all cancers in India [2]. Despite the recent advances in tumor surgery and multimodal treatment regimes, the prognosis of oral squamous cell carcinoma is still relatively poor. This may be because symptoms that indicate the presence of the carcinoma often appear when the tumor is in an advanced stage [3].

In the light of such problems, it would be very useful to find biochemical markers that allow to suspect the presence of the carcinoma at early stages. During the course of tumor development, quantitative changes have been shown to occur in a variety of substances in serum. These substances are collectively referred to as biochemical markers or tumor markers [3,4].

β_2-Microglobulin is a non-glycosylated peptide and is the invariant chain of the Major Histocompatibility Class (MHC) I molecules on the cell surface of all nucleated cells [2, 5]. Its best characterized function is to interact with and stabilize the tertiary structure of the MHC class I α-chain. Because it is non-covalently associated with the α-chain and has no direct attachment to the cell membrane, β_2-microglobulin on the cell surface can exchange with free β_2-microglobulin present in serum containing medium. Free β_2-microglobulin is found in body fluids under physiologic and pathological conditions as a result of shedding from cell surfaces or intracellular release [6]. Many properties of mammalian cells are expressed at, or mediated through the cell surface [7,8]. Immense increase in knowledge of the altered characteristics of malignant cells has shown that altered cell surface is the hallmark of malignant cells [9]. Glycoproteins and glycolipids are major constituents of cell membrane [10]. Altered glycosylation of glycolcon-

jugates is one of the important molecular changes that accompany malignant transformation [9, 11]. Studies of malignant cells have revealed alterations in cell surfaces and membranes in terms of sialic acid content of glycoproteins and glycolipids. Sialic acid, a family of acetylated derivatives of neuraminic acid is widely distribu ted in mammals [12]. They are the end moieties of the carbohydrate chains and are biologically important and essential for functions of glycoconjugates [11, 12]. Sialic acid is thought to be important in determining the surface properties of cells and has been implicated in cellular invasiveness, adhesiveness, and immunogenicity. They are released into circulation through increased turnover, secretion, and/or shedding from malignant cells [7,13]. Increased levels of β_2-microglobulin and glycoconjugates like total sialic acid and lipid-bound sialic acid have been reported in serum of patients of oral cancer [2, 5,6,7,10,14,15,16].

In oral cancer the study of tumor markers have been limited. Several tumor makers with clinical promise need further evaluation [2, 5]. Three such tumor markers are β_2-microglobulin, total sialic acid and lipid-bound sialic acid.

Considering the high prevalence of oral malignancy, the present study was undertaken to validate diagnostic value of serum β_2-microglobulin, total sialic acid and lipid-bound sialic acid and to correlate these parameters with the stages of malignancy.

Material and Methods

The study comprised of 80 subjects. 40 oral cancer patients admitted in the Shri Chhatrapati Shivaji Maharaj General Hospital and Shree Siddheshwar Cancer Hospital and Research Centre, Solapur (Maharashtra) proved by the clinical and histopathological evidence were selected for the study. According to TNM staging system of the UICC [17], out of 40 oral cancer patients studied, 11, 8, 10 and 11 were of stages I, II, III and IV respectively. 40 healthy controls were selected for comparison.

To avoid false positive results, care was taken to exclude subjects with renal, hepatobiliary disorders, lupus erythematosus, lymphoproliferative disorders, and cardiovascular disorders as well as other malignancies [5].

Under aseptic precautions venous blood was drawn and serum was separated.

β_2-microglobulin assay:
The serum was analyzed for β_2-microglobulin by Enzyme linked immunosorbent assay (ELISA) (β_2-microglobulin EIA Kit, Orgentec, Germany).

Measurement of Lipid-bound sialic acid (LBSA) Serum lipid-bound sialic acid was determined by the Spectrophotometric method of Plucinsky et al. [18]. Briefly, 50 µl of serum were mixed with 150µl of distilled water and extracted with 3ml cold chloroform / methanol (2:1) (v/v). The lipid extract was partitioned with 0.5ml cold distilled water. After centrifugation 1ml of aqueous layer containing the sialolipid fraction was precipitated with 50 µl phosphotungstic acid solution (1gm/ml). After centrifugation supernatant fluid was discarded and the precipitate was resuspended in 1ml of distilled water. 1ml of resorcinol reagent was added and the tubes were boiled for 15 min and then cooled in ice bath for 10 min. The blue chromophore was extracted with 2ml butyl acetate n-butanol (85:15) (v/v) and determined spectropho tometrically at 580 nm. The sialic acid concentration was calculated by the use of a standard curve of N-acetyl neuraminic acid (NANA).

Measurement of Total sialic acid (TSA)
Serum total sialic acid level was determined by the method of Plucinsky et al. [18]. 20µl of serum was diluted with 980 µl distilled water. After treatment with resorcinol reagent the blue chromophore was extracted by butyl acetate / n-butanol (85:15) (v/v) and determined spectrophotometrically at 580nm and sialic acid was determined by the use of standard curve of N-acetyl neuraminic acid.

Statistical analysis
The data is expressed as mean ± SD. The statistical significance of the results was analyzed using a non-parametric Tukey's test (for unequal sample size). Values of $P<0.001$ were considered significant.

Results

The mean values of serum β_2-microglobulin, total sialic acid and lipid-bound sialic acid in various stages of oral carcinoma patients and healthy controls are given in Table 1. The levels of serum β_2-microglobulin, total sialic acid and lipid-bound sialic acid elevated significantly and progressively in various stages from stage I to stage IV of oral cancer patients compared with healthy controls ($P<0.001$) (Table 1). The average β_2-microglobulin, TSA and LBSA levels in stage II was significantly higher than that in stage I ($P<0.01$). Also, the average levels of these biomarkers in stage III was significantly higher than that in stage II ($P<0.01$) as well as stage IV patients with metastasis showed a significant rise ($P<0.01$) when compared with stage III patients (Table 1). The positive correlation was found between the circulating levels of lipid-bound sialic acid and total sialic acid in different stages of oral cancer patients (stage I $r=0.97$, stage II $r=0.84$, stage III $r=0.82$, stage IV $r=0.78$).

Out of 40 oral cancer patients only 33 had elevated levels of lipid-bound sialic acid and thus sensitivity of 82.5%. In case of total sialic acid and β_2-microglobulin the sensitivity was 90% and 100% respectively. The test showed an abnormal result in only 1 out of 40 healthy controls for LBSA and thus the specificity was of 97.5% and only 5 out of 40 healthy controls for TSA had abnormal results and thus the specificity was of 87.5%. In case of β_2-microglobulin the specificity was of 100%. For LBSA the predictive value of a positive test was 97%, predictive value of negative test was 84% and efficiency of test was 90%. For TSA, the predictive value of positive test was 87%, predictive value of negative test was 89% and efficiency of test was 88.7%. In case of β_2-microglobulin predictive value of positive test was 100%, predictive value of negative test was 100% and efficiency of the test was 100%.

***Table 1**: Levels of β_2-microglobulin, total sialic acid (TSA) and lipid-bound sialic acid (LBSA) in healthy controls and various stages of oral cancer patients.*

Clinical Condition	No. of Patients	β_2-microglobulin mg/L	TSA mg/dl	LBSA mg/dl
Healthy Controls	40	2.06 ± 0.36	66.92 ± 7.11	9.73 ± 2.73
Oral Cancer Patients				
Stage I	11	2.77 ± 0.09*	77.45 ± 8.94*	12.96 ± 1.69*
Stage II	8	3.02 ± 0.04*a	98.87 ± 5.27*a	16.25 ± 0.98*a
Stage III	10	3.48 ± 0.30*b	110.0 ± 3.09*b	21.58 ± 1.52*b
Stage IV	11	4.32 ± 0.35*c	127.90 ± 8.88*c	24.81 ± 2.35*c

** P<0.001 – Significant when compared to control*
a P<0.01 - Significant when compared to stage I
b P<0.01 - Significant when compared to stage II
c P<0.01 - Significant when compared to stage III

Discussion

The β_2-microglobulin which is synthesized and secreted by lymphocytes as well as most other nucleated cells is an intrinsic part of histocompatibility antigen. It has a low molecular weight and rapid turnover [5]. In the present study the β_2-microglobulin values were increased in different stages of oral cancer, when compared with healthy controls, and this is statistically significant (P<0.001) (Table 1). Also there was observed significant increase (P<0.01) among different clinical stages. The increased levels were correlated well with tumor burden. The increased levels of β_2-microglobulin might be due to increased production or impaired excretion. However, as the patients in this present study did not have any disorder of renal function or other systemic ailments and other malignancies, the increase in serum β_2-microglobulin appears to be a true phenomenon due to the malignant process involving oral cancer. The fact that β_2-microglobulin levels were elevated in the serum of subjects with oral carcinoma is in agreement with previous study reports [2,5,6,14].

The mechanism of increase in β_2-microglobulin levels in malignancies is not known but various possible hypotheses for the increased serum levels have been put forward. The β_2-microglobulin is a cell membrane constituent along with the HLA –chain (Human leukocyte antigen), so an accelerated membrane turnover or accelerated cell division could increase the shedding of β_2-microglobulin [2, 5]. The ability of the carcinoma cells to produce a higher concentration of β_2-microglobulin than the non-neoplastic cells may be due to either active synthesis or increased cell breakdown or both [2, 5]. In many neoplasms, especially those of epithelial origin, a decrease or lack of expression of HLA-I particles is found. Despite weakened expression of HLA-I complex on the neoplastic cells, the level of β_2-microglobulin in the blood serum could be increased. Recent studies explained this phenomenon as due to an imbalance of light chain and heavy chain of HLA-I complex and relative over -production of β_2-microglobulin [6]. Most frequently quoted hypothesis for high levels of β_2-microglobulin in neoplastic diseases explains this phenomenon with mono or polyclonal activation of lymphocytes, destruction of MHC I particles, and increased cellular transformation into neoplastic cells which could lead to higher concentration of protein β_2-microglobulin. Certain studies proposed that the systemic immunosuppression observed in oral cancer patients is due to the decreased functional activity of peripheral blood monocytes which is reflected by way of decreased phagocytic process [19]. The latest studies showed that a high concentration of free β_2-microglobulin

may have a negative influence on the immunological system by decreasing the expression of MHC-I (Major histocompatibility complex) particles and indirectly by increasing the levels of cytokines: IL-6, IL-10, which accelerate the development of neoplasms [6, 20].

Sialic acids are major constituent of the carbohydrate chains of cell membrane glycoproteins and glycolipids. Sialic acid concentrations of the tumor cell surfaces were shown to be related to malignant potential and changes in immunogenicity. The carbohydrate moiety may influence differentiation, growth and cell-to-cell interactions, and thus may be important in malignant transformation [7, 13]. The serum total sialic acid and lipid-bound sialic acid levels were increased in oral cancer patients when compared with healthy controls and this is statistically significant ($P<0.001$). Also there was observed significant increase ($P<0.01$) among different stages of oral cancer patients. The increased concentration of total sialic acid and lipid- bound sialic acid were found to correlate with the presence and extent of malignant disease. Our results correlated well with those of others regarding alteration in sialic acid content in oral cancer [3, 7,10,15,16].

Malignant transformation of oral epithelium is associated with atypical glycosylation of cell surface carbohydrates [13]. Neoplasms often have increased concentrations of sialic acid on tumor cell surface and sialoglycoproteins are shed or secreted by these cells increasing their concentration in blood [13]. The elevations found in sialic acid levels in cancer patients might also be due to selective increase in existing specific sialylated sequence or a tumor associated *de novo* synthesis of specific sialylated sequence [9]. A good correlation was found between circulating levels of TSA and LBSA in different stages of oral cancer patients suggesting that tissue turnover rates of both membrane glycolipid and glycoprotein were to the extent of malignant transformation. Similar findings have been reported by Rao et al. [7].

The results also revealed that the levels of β_2-microglobulin TSA and LBSA elevated significantly in Stage I of oral cancer patients and thus can give early indication of malignant change and therefore malignancy can be detected at an early and treatable stage. Furthermore, because there is progressive rise in levels of these biomarkers, even the extent of malignant disease can be ascertained. In order to assess the reliability of tumor markers, we compared the sensitivity, specificity, predictive value of positive test, predictive value of negative test and efficiency of these three biomarkers. These results suggest β_2-microglobulin to be a very accurate tumor marker. However it lacks specificity for oral carcinoma as an individual marker because it is elevated in other diseases also [5]. Regarding the cost of the measurements, the total sialic acid was the cheapest among the three tumor markers. All the three biomarkers tested showed good sensitivity values, specificity values, predictive values of positive test, predictive values of negative test and efficiency values in oral carcinoma.

Thus, our results suggest that the concentrations of β_2-microglobulin, total sialic acid and lipid-bound sialic acid significantly increase in different stages of oral cancer patients and correlates well with the progression of clinical symptoms. These biomarkers have good sensitivity, specificity and efficiency for oral cancer. Therefore, the evaluation of these markers, with TSA being the cheapest among three in terms of cost and effectiveness, would be of immense help in assessing early malignant change, in increasing the accuracy of clinical diagnosis and also in assessing the spread and invasiveness of oral squamous cell carcinoma.

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