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Original article

Soluble MICA in biofluids as biomarker in detection of oral cancer which correlates with disease stage

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Abstract

Expression of major histocompatibility complex class I-related chain A/B (MICA/B) has been intended to play significant role in tumor immunosurveillance. Downregulation of MICA/B expression in tissue and augmented sera levels assumed to impair antitumor immune response. In this study, the potential of sMICA as a marker for oral cancer (OC) was investigated. The sMICA levels in sera, saliva and urine of OC patients differ significantly from control. The sMICA was correlated with tumor stage to evaluate the diagnostic power of MICA as marker. However, the findings indicate that the expression of MICA/B in positive control and Stage I and IV showed significant difference as per one way analysis of variance (P value < 0.0001). Serum levels of sMICA showed significant difference in positive control and Stage I and IV (P value <0.0001). Stage II and III also show significant difference with P value 0.0028and 0.0003 respectively with positive control MICA/B expression. Analysis of sMICA in serum, saliva and urine of OC patients revealed significantly (P value < 0.0001) higher levels (median 34.25± 3.57 pg/ml in pre-treatment sera, 193.93 ±1.95 pg/ml in saliva and 109.89± 1.59 pg/ml in urine) than

in control (median <1.2pg/ml).Patients with poorly differentiated tumor exhibited a smaller amount of sMICA levels and well differentiated tumor revealed higher sMICA levels in biofluids. The release of sMICA and its expressions in biofluids reflect impairment of tumorimmunity. The sMICA levels may provide useful additional information in the diagnosis, staging and prognosis of cancer.

Keywords: Oral cancer, MICA/B, sMICA/B

Introduction

The occurrence of oral cancer (OC) is about 3% of all malignancies with increasing mortality rates, worldwide. Oral cancer is the sixth most common cancer globally responsible for cancer-related deaths. Nearly one-third of the global oral cancer burden incidences found in Indian population annually. It is dependent on tobacco, betel quid, alcohol habits and smoking. In India, Well differentiated oral squamous cell carcinoma and keratinizing squamous cell carcinoma are the second most common cancer of epithelial origin accounts for 90 – 94% of oral cancers (1–4).The major histocompatibility complex class I related antigen A and B (MICA/B) proteins have highly restricted expression pattern in healthy body and found in the gastrointestinal epithelium. The MICA/B protein is highly polymorphic and expressed in response to stress such as heat shock, viral and bacterial infections and is constrained to endothelial cells, keratinocytes, monocytes and tumor types of endothelial and hematopoietic origin(5–7). It is stress-inducible surface glycoproteins broadly expressed by epithelial tumors and illustrated UL16-binding proteins(8,9). The MICA/B proteins, act as ligands for the NKG2D, which is expressed on NK cells, $CD8^+$ T cells, and $^{\delta\gamma}$ cells (10–13). NK cells are involved in antitumor immune responses and NKG2D strongly activates NK cells(14,15). NKG2D ligand expressed by tumor cells stimulate antitumor responses, leading to tumor rejection by NK, CD8 and $^{\delta\gamma}$T cells thus giving protective immunity(16–18). .The tumor cells reduce NKG2D ligands at surface levels by shedding MICA in a soluble form (sMICA) and thus tumor escape from NKG2D-mediated immunosurveillance. High levels of serum levels of sMICA are present in gastrointestinal malignant patients and cause systemic downregulation of NKG2D surface expression impairing tumor cell lysis by CD8 T cells(6,19). The presence of sMICA in sera of patients was made known for prostate cancer, hematopoietic malignant tumor entities(20–22).

OC has high incidence and unsatisfactory poor survival rates at approximately 50% with functional defects. Although advances in therapeutic interventions, OC pathogenesis is still unknown. The early diagnosis to improve survival of patients is required since the prognosis is generally poor. TNM staging is not enough to define the understanding of the factors affecting predictive implications of the metastasis(1,23).The purpose of this study is to determine the early diagnosis of OC patients in co-relation with MICA/B expressions in tissue biopsies and soluble form. Here, we implemented MICA/B expression in tissue biopsy by IHC and Western blot. MICA specific sandwich enzyme-linked immunosorbent assay (ELISA) was implemented to explore the shedding of sMICA release by tumor cells and its solubility in biofluids serum, saliva and urine. The sMICA level was correlated with tumor stage to assess the potential diagnosis markers. We analyzed sMICA levels in serum, saliva and urine of 76 OC patients and compared these values to control sera. It revealed a correlation of sMICA levels and stages of disease. There are yet no data available regarding the correlation of sMICA levels of saliva and urine of OC entities with tumor stages the serum, saliva and urine levels of sMICA could provide the potential implementation of sMICA as diagnosis markers in OC stages.

Material and methods

Study patients and sample specimens

Specimens of 76 oral cancer patients who underwent diagnostic and therapeutic resections between 2015 -2018 were included in the study from hospital. Tumors were diagnosed by histopathological criteria. The control tissues were obtained from random healthy volunteers. These activities were approved by Institutional Ethical Committee and all subjects gave provided written informed consent. The disease was staged according to the criteria of the American Joint Committee on Cancer (AJCC). They include the measurement of primary tumor size with lymph node metastasis, distant metastasis, histological tumor type, Modified Richardson bloom score, grade, TNM staging and hormone receptor status. The tumor tissue and control tissue were obtained following resection in the operation theatre, immediately snap frozen in LN2 and placed in -80°C for further use of western blot and IHC analysis. The follow-up data of the patients involved in study have been recorded for 6 month prospectively from the date of resection of the tumor. Tissue samples routinely processed, and embedded in paraffin blocks of same patients were obtained from the Department of Pathology for Immunohistochemistry (IHC).The

retrospective paraffin embedded blocks of colon cancer tissue biopsies were utilized for comparative study. The sera used in this study were obtained from OC patients at diagnosis before the implementation of any therapy and post-treatment. Serums from 50 age matched healthy donors, free from systematic abnormalities were collected as controls. Sera were stored at -80˚C for further Elisa analysis of sMICA. Unstimulated saliva and urine was collected from 76 OC patients and from 50 healthy individuals during 2015–2018. The inclusion criteria comprised cancer patients within age group 31 to 73 years unindulged in treatment. The exclusion criteria included patients with previous chemotherapy and radiotherapy.

The inclusion criteria for control involved that the subjects be within same age group and exclusion criteria included systemic conditions, pregnancy and lactation. The patients were instructed to abstain from smoking and alcohol before the collection of saliva and urine. Saliva samples were collected by spitting into a collecting container, without any stimulation. Urine samples were collected into containers. The saliva and urine samples were centrifuged at 12,000rpm for 10 min. The supernatants were collected for further analysis of MICA.

Immunohistochemistry

Immunohistochemistry was carried out on retrospective OC tissues. Colon cancer tissue biopsies were used as positive control for comparative analysis. After standardized routine processing, sections were incubated with the mouse monoclonal IgG antibody (Santa Cruz Biotechnology, CA; USA) diluted at 1:100 (v/v) and goat anti-mouse IgG, HRP conjugated (Santa Cruz Biotechnology, CA;USA) for 2 hr in a humid chamber. After rinsing with 0.5% tween20, the standard IHC staining was conceded out using a 3,3-diaminobenzidine tetrahydrochloride (DAB) (BioGenex, Fremont CA; USA). The slides were lightly counterstained with hematoxylin (HiMedia; India) and mounted with DAKO (Agilent Technologies, US). An identical tissue sections were processed the same without adding primary antibody to serve as a negative control. The sections were evaluated in a Nikon EclipsTi microscope. Crispy coppery colored expressions of cytoplasm and membranes in the tumor tissues were considered as positive response for MICA/B. Two investigators scored MICA/B expression in a blinded fashion. The degree of immunoreactivity was scored depending upon the extent of positive expressions of MICA/B in each tissue sections. The expressions were graded as weak (+), moderate (++) and

intense (+++) MICA/B signal. Negative MICA/B expression was defined as no (-) MICA/B signal.

Western blot

Cell lysate were harvested with RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 0.5% deoxycholic acid) with protease inhibitor added (Sigma Aldrich; India) and centrifuged in cooling centrifuge at 12,000 RPM.30 µg total proteins were resolved by BoltTM 10% Bis-Tris plus precast gels (Invitrogen, India), and then transferred onto0.45micron nitrocellulose membranes (Thermo scientific, India). Membranes were blocked with in PBST (10 mmol/L Tris–HCl, pH 7.4, 150mmol/L NaCl, 0.05% Tween-20) containing non-fat milk and then incubated with primary antibodies overnight at 4°C. Primary antibodies used were mouse monoclonal anti-MIC A/B (Santa Cruz, CA; US) with 1:2000 dilution in iBindTM flex solution (500ul flex additive and 10 ml flex 5X buffer in 39.5 ml D/W; Invitrogen India). The secondary antibody was horseradish peroxidase (HRP) conjugated Goat anti-mouse(Santa Cruz, CA; US) with 1:7000 dilutions in flex solution and developed with the ECL substrate reagent (Invitrogen India).The signals were detected using Western lightning enhanced chemiluminescence. The densities of the signals were calculated by Image J software and expressed in arbitrary units.

ELISA

Serum levels of sMICA were determined using sandwich ELISA by using Duoset kit (R&D systems, MN; USA). The plates were coated with Human MICA capture antibody at 5.6ul/ml in ELISA plate coating buffer, then blocked with 1X reagent diluents for 2 h at 37˚C and washed. The standard and samples were incubated in plates for 2 h at 37˚C. After incubation, plates were washed/aspirated and incubated with the Human MICA detection antibody diluted in reagent diluents with 2% goat serum. Plates were washed and incubated with streptavidin HRP A (5ul in 1ml reagent diluents). Plates were washed again and developed using the Substrate A and B followed by incubation with stop solution. Absorbance was measured at 450 nm by using LISA Microplate Reader (Rapid Diagnostic, china) according to the manufacturer's instructions. The sMICA levels in saliva and urine of OC patients (n=76) and control (n=50) is validated according to the manufacturer's instructions as described above. Soluble MICA levels≥ 1 pg/ml were considered positive based on the detection limit of the ELISA.

Results

Immunohistochemistry

Paraffin embedded blocks of OC tissue samples from 76 patients with alveolus and gingiobuccal sulsus carcinomas, buccal mucosa carcinomas, well differentiated squamous cell carcinoma of lateral borders of tongue and tip of tongue and well differentiated squamous cell carcinoma of angle of mouth and mouth floor is analyzed for MICA/B expression by IHC staining. Immunohistochemistry was carried out with mouse monoclonal IgG antibody. Immunoreactivity scoring was based on proportions of cells exhibiting cytoplasmic strong dark shiny brown signal of MICA/B immunoreactivity. The control tissue do not showed any signal for MICA/B expression (Fig.1A). In majority of tumor, intense to moderate MICA/B immunoreactivity was detected in the tissues and was accorded the grading of MICA/B (++) or MICA/B (+++). Therefore data was categorized into groups for the analysis purpose. All tumors show MICA/B expression, with the majority of tumor cells staining positively in all the cases. Moderate MICA/B expression is present in 39 (51.31%) and low expressions is present in 37 (48.68%) of 76 OC patients. Almost all biopsies within obtained tumor samples display moderate intensity of MICA/B staining. The poorly differentiated tumor specimens showed moderate staining (figure. 1B, 1C) and well differentiated tumor specimens showed low expressions in stage III (figure. 1D) and moderate staining in stage IV for MICA/B (figure. 1E). MICA/B expression in the tumor is significantly increased in patients with poorly differentiated, low invasive with no lymphatic involvement tumors at early TNM stages (I and II) as compared to well differentiated, deeper invasion status at advanced TNM stage III. Poorly differentiated stage IV shows significant moderate MICA/B expressions. Highly considerable relationship is established between TNM stages, tumor size. Tissue expressions of MICA/B with patient age and survival rate revealed no significant correlation.

Western blot

To identify the MICA/B expression for oral cancer detection, comparison of the MICA/B expression profiles between OC-free control subjects and OC patients was performed using western blot analysis. Western blot analysis is carried out on RIPA extracted homogenized OC tissue to quantify tissue levels of MICA/B. The intensity of each protein band was quantified and analyzed using Image J software. Optical density (OD) is evaluated at ~38 kDA. A representative image is shown in Figure 2A. Visual observation revealed that MICA/B

expression is lower in non OC control tissue compared to tumor tissue and MICA/B protein levels are considerably higher in both stage I and II tumor tissue. Stage I and II tumors has higher MICA/B expression than stage III and IV. MICA/B expression for all stages of OC showed significant level of difference for one way analysis of variance (***P value 0.0001). Dunnett's multiple comparisons test show significant difference in positive control and Stage I and IV sMICA expression (****P value <0.0001). Stage II and III also show significant difference with **P value 0.0028and ***P value 0.0003, respectively with positive control MICA/B expression (Figure 2B).

ELISA

Pretreatment serum sMICA of oral cancer patients:

We analyzed the sMICA levels in serum of same 76 OC patients enrolled for study. In parallel, we analyzed control sera from 50 healthy age matched individuals. Age matched control sera revealed significant lower sMICA values close to the detection limit of the ELISA reader. Significant high levels of sMICA were detected in the patient sera in both pre and post treatment as compared to the sera of control (Table II). The sMICA levels in serum correlated with the disease stage and TNM staging. There was association between serum sMICA levels and tumor size, grade or lymph node involvement. It is not associated with Modified Richardson bloom score. Furthermore, pretreatments MICA levels were more elevated in patients with well differentiated tumors than poorly differentiated tumors. The sMICA in pretreatment illustrated significant difference as per one way analysis of variance in all stages (****P value < 0.0001) (Figure 3).The sMICA levels in patients correlated with the stages of disease. The resulting stagewise serum sMICA values demonstrate the diagnostic potential in this subgroup. Though significantly higher serum sMICA levels were detected in pretreatment; sMICA levels did not differ significantly between every month F/W. The difference range of sMICA levels in the F/W was very less.

The sMICA in saliva and urine

Total 76 Oral cancer patients with 50 ages matched Oral cancer-free control individuals are recruited in this study. The age range of patients and control is 30–70 with an average age of 50

years. Male candidates are more common in oral cancer group. No significant differences are detected in sMICA values with respect to gender distribution. Among these 76 patients, 20 (26.31%) has alveolus and gingiobuccalsulsus carcinomas, 28 (36.84%) had buccal mucosa carcinomas, 14 (18.42%) has well differentiated squamous cell carcinoma of lateral borders of tongue and tip of tongue and 14 (18.42%) patient has well differentiated squamous cell carcinoma of angle of mouth and mouth floor. Moderately differentiated keratinizing squamous cell carcinoma and well differentiated squamous cell carcinoma is the most common histopathological appearance. The patients are classified into T1 (12.24%), T2 (34.69%), T3 (38.77%) and T4 (14.28%) according to AJCC TNM staging. The saliva and urine sMICA profile are compared between control subjects and oral cancer patients using ELISA analysis (Table III). The sMICA concentration in saliva sample of OC at different stages show significant difference as per one way analysis of variance (P value<0.0001). As per Tukey's multiple comparisons test sMICA concentration in saliva show all stages are significantly different corresponding to each other with P value <0.0001 and 0.0005 respectively (Figure 4A).The sMICA concentration in urine sample of OC at different stages show significant difference as per one way analysis of variance (P value < 0.0001). As per Tukey's multiple comparisons test sMICA concentration in urine show all stages are significantly different corresponding to each other with P value <0.0001 (Figure 4B).

Discussion

Recent studies have implicated the NKG2D and MIC function in T-cell-mediated tumor immunity. However, the role of the MIC remains poorly understood in malignancies. NKG2D ligands are broadly expressed on tumors. Expression of MICA/B was documented in lung, gastric, pancreatic, renal, colon, ovarian, and oral malignancies. The shedding of MICA/B by tumor cells modulates NKG2D mediated tumor immune surveillance. Elevated levels of sMICA were detected in serum of prostate cancer, colon adenocarcinoma and pancreatic cancer patients (24).

In the present study, we performed IHC, WB and ELISA to determine the MICA/B expressions at tissue level and soluble level. We focused on correlation of MICA/B expression in tumor tissue and sMICA in serum, saliva and urine. In a study of 76 oral cancer patients, Seventy six

oral cancer specimens with a mean age of 51± 21 (median age: 51, range: 30-72 years) were analyzed for MICA/B expression by immunohistochemical staining. IHC staining (figure 1) with an anti-MICA (H-300) rabbit polyclonal antibodies (Santa Cruz Biotechnology, USA) diluted 1:200revealed the moderate dark brown signal of MICA/B expression present in poorly differentiated tumor specimens (Figure 1B, 1C) and well differentiated tumor specimens showed low expressions in stage III (fig. 1D) and moderate staining in stage IV (fig. 1E) for MICA/B but not in control cells (Figure 1A). All samples were positive for MICA/B (Table 1). Among these MICA/B-positive tumors, MICA/B expression was graded as low and moderate in 37, 39 samples, respectively. Moderate MICA/B expression is present in 39 (51.31%) and low expressions is present in 37 (48.68%) of 76 OC patients. MICA/B expression is significantly increased in poorly differentiated tumor, low invasive with no lymphatic involvement tumors at early TNM stages (I and II) as compared to well differentiated, deeper invasion status at advanced TNM stage III. Poorly differentiated stage IV shows significant moderate MICA/B expressions. Highly considerable relationship is established between TNM stages, tumor size. In contrast, clinicopathologic analysis did not reveal any significant correlation between tissue expression and gender, age and survival rate. The significant increase in the protein level of MICA/B correlated with increasing tumor size as represented by the AJCC TNM staging system ($P < 0.001$). This study analysed elevated soluble MICA in serum levels correlated significantly with cancer stage. The potential of soluble MICA level to diagnose stage and aggressiveness of all cancer has yet to be ascertained. The results of this study suggest a role for soluble MICA in the staging of OC patients. The patients diagnosed with stage IV had significantly elevated levels of soluble MICA in their serum compared with those of control individuals. Also, we found a statistically significant relationship between elevated sMICA and regional lymph node metastasis in OC patients. Furthermore, pre treatments values of sMICA were more elevated in patients with well differentiated tumors than poorly differentiated tumors. Elevated sMICA in well differentiated tumors may reveal consequence of enhanced MICA/B shedding. The sMICA levels in the follow up were very less which indicates after treatment, sMICA level in sera decreased significantly but monthly monitoring did not show remarkable variability in sMICA levels in sera of OC patients. Furthermore, we compared the sMICA in saliva and urine from OC with control group. It is the first study to conclude the sMICA in saliva and urine of OC patients. Also, significant increase in the sMICA level of saliva and urine correlated with increasing

tumor size as represented by the AJCC TNM staging system. Poorly differentiated tumor showed low levels of sMICA in saliva and urine. The sMICA level in saliva ad urine samples, it is successively increased in stage I to III. An increase in the sMICA level in saliva and urine samples correlated with increasing tumor size as represented by the TNM staging system (****P value < 0.0001).The sMICA concentration in OC urine show all stages are significantly different corresponding to each other with ****P value <0.0001 and increases 27.79 fold in stage I, 127.25 fold in stage II, 101.66 fold in stage III and 108.53 fold in stage IV. In saliva, all stages are significantly different corresponding to each other (****P value <0.0001 and ***P value 0.0005) and increases 38.95, 128.89, 189.2, 59.18 fold in stage I, II, III and IV respectively. The sMICA was a more reliable, non invasive prognostic marker compared with tissue expressions of MICA/B, as it is easier to detect. Overall, sMICA in saliva and urine will be a potential candidate as a diagnostic marker for OC, allowing for the development of diagnostic assays.

Conclusion

In OC patients with impaired immunity, MICA/B protein expressed on the tumor cell surface is released into the biofluids-sera, saliva and urine in OC patients, resulting in an impaired NKG2D-based immune evasion. We analyzed the sMICA in oral cancer patients in attempts to improve early prognosis and treatment. The sMICA could serve as prognostic marker and staging of OC patients. Our results suggest that high level of sMICA levels may help to identify disease in advanced stage or regional lymph node metastases. Further study is required to determine whether levels of sMICA in the serum, saliva and urine are of prognostic value in OC, and whether sMICA can be considered as a general tumor marker. Detection of sMICA from body fluids is also becoming increasingly important in liquid biopsy. As alterations in sMICA expression patterns correlate with disease, it can be promising prognostic biomarkers for oral cancer. These findings will suggest that sMICA can be potential biomarkers for oral cancer detection and will open a new avenue for soluble biomarker discovery for OC.

Table I. IHC result analysis of oral cancer tissue.

Variables	Tissue MICA/B Expression
Oral cancer Patients (n=76)	

Stage I (n=6)	Moderate expression (Fig.1B)
Stage II (n=26)	Moderate expression(Fig.1C)
Stage III (n=37)	Low expression in tissue(Fig.1D)
Stage IV (n=7)	Moderate expression in tissue(Fig.1E)

TABLE II: Pretreatment and post treatment levels of sMICA in sera of patients with oral cancer and control.

Stage	Pre-treatment patients sMICA (pg/ml)	Post treatment (F/W) sMICA (pg/ml) Month						sMICA (pg/ml) Control
		1st	2nd	3rd	4th	5th	6th	
I	6.83 ±1.67	5.38±0.19	5.46±0.26	5.20±0.25	5.11±0.38	6.05±0.08	7±0.09	≤ 1 pg/ml
II	28.04±5.3	25.16±1.71	23.39±0.83	20.55±0.53	25.08±1.6	26.11±1.87	25.54±2.18	
III	36.47±3.77	36.14±1.06	31.89±5.12	34.05±0.47	35.28±0.69	35.02±7.58	36.35±0.46	
IV	65.64±3.57	60.43±0.22	61.60±0.52	62.71±0.67	63.24±0.22	62.64±1.17	62.60±0.42	

Table III:sMICA concentration in saliva and urine

Stage	**Saliva sMICA (pg/ml)**	**Urine sMICA (pg/ml)**
I	66.30±1.96	33.35 ± 1.4
II	215.08 ± 3.28	152.7 ± 1.62
III	245.03 ± 0.95	122.77 ± 1.56
IV	249.32 ± 1.64	130.74 ± 1.79

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Figure 1: MICA/B expressions in different stages of oral cancer

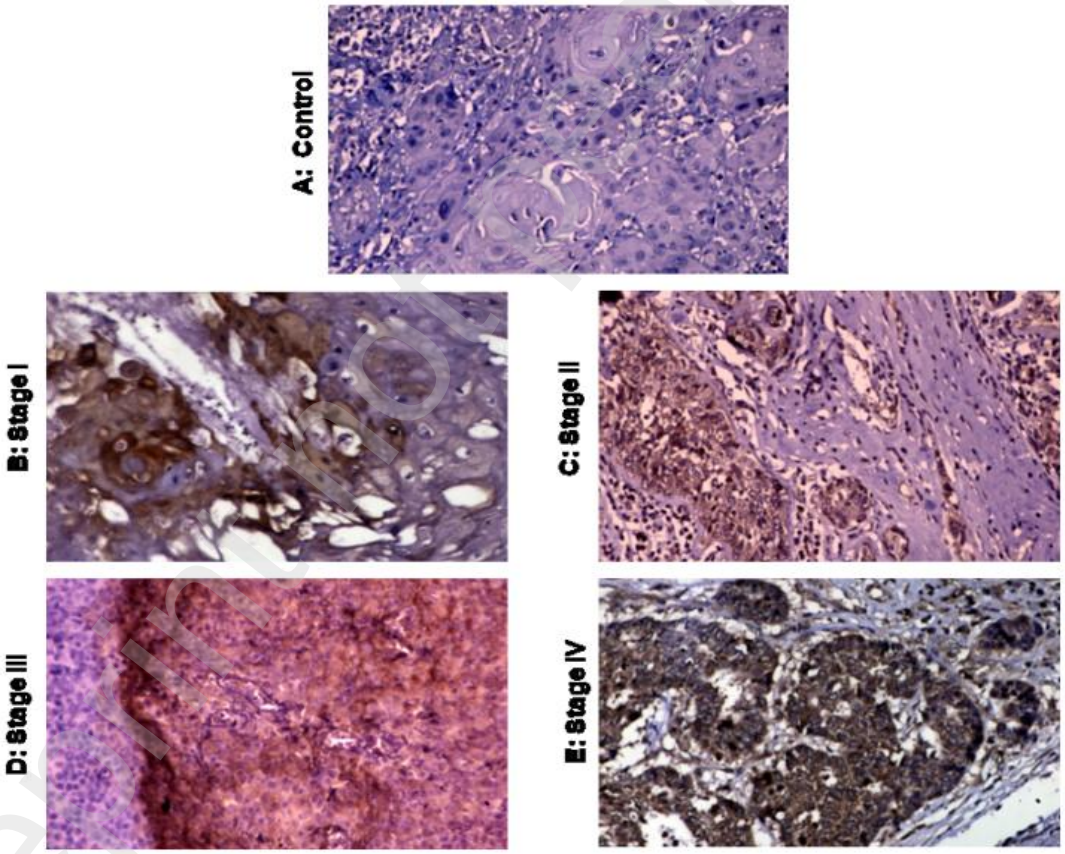


Figure 1: MICA/B expressions in different stages of oral cancer: MICA/B expression is higher in the tumor tissue than in control. (A)Representative IHC for MICA/B in control tissue sections. It

did not show expressions of MICA/B (Magnification x40). (B, C) IHC staining of poorly differentiated stage I and II tumor specimens showed intense to moderate staining for MICA/B and (D, E) well differentiated tumor specimens of stage III and IV showed moderate staining.

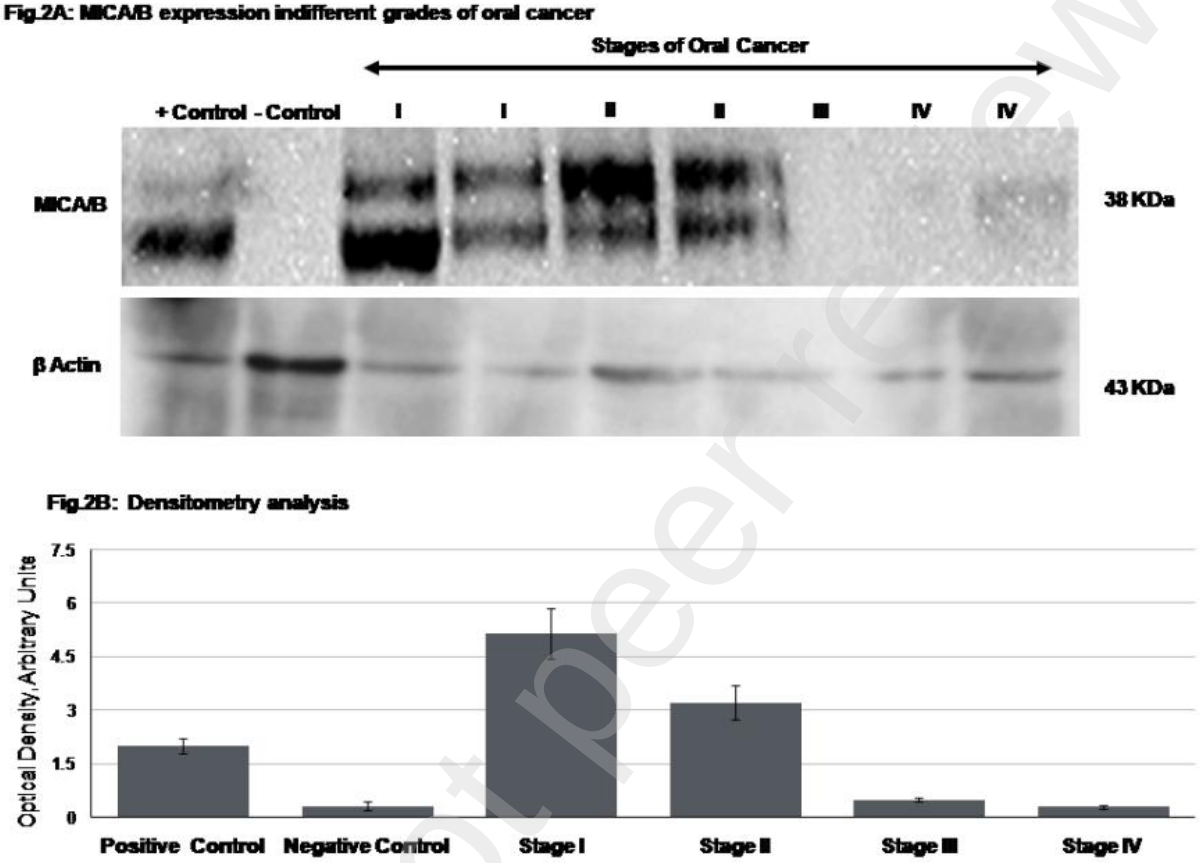


Figure 2: Western blot analysis of different stages of oral cancer-:(2A)MICA/B protein in cell lysates was separated by SDS-PAGE and detected by Western blot using mouse monoclonal anti-MICA/B and HRP conjugated Goat anti-mouse. Optical density was evaluated for the band at ~38 kDA. MICA/B expression is lower in control. MICA/B protein levels were considerably higher in stage I, II tumor tissue of as compared to control ($p<0.001$ for both). The band of a stained gel was subjected to $_{\beta}$-actin housekeeping gene analysis to verify MICA/B specificity. The MICA/B expression showed significant difference as per one way analysis of variance (****P value < 0.0001). (2B) Dunnett's multiple comparisons test show significant difference in positive control and Stage I and IV sMICA expression (****P value <0.0001). Stage II and III also show significant difference with **P value 0.0028and ***P value 0.0003, respectively with positive control MICA/B expression.

Figure 3: Pretreatment and post treatment levels of sMICA (µg/ml) in sera of patients with oral cancer

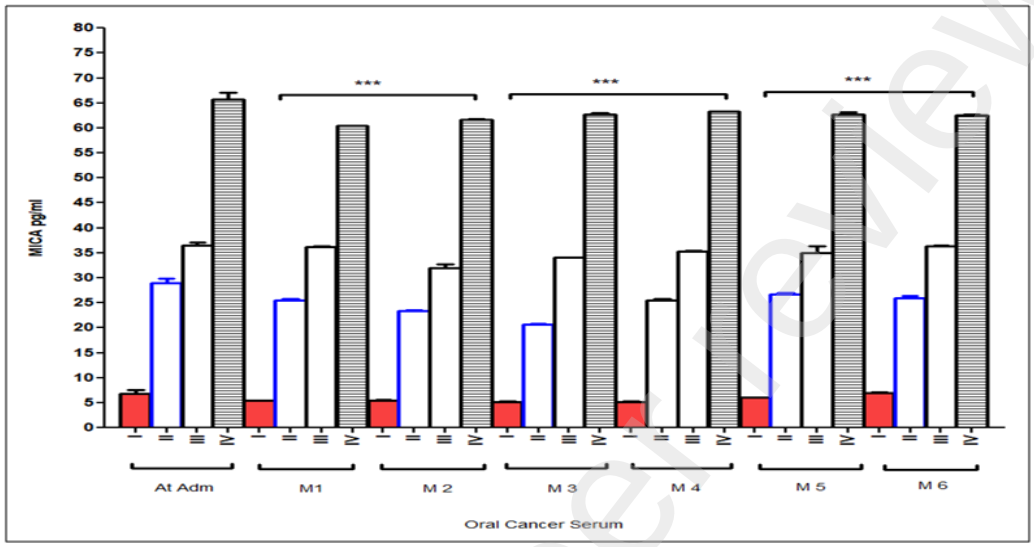
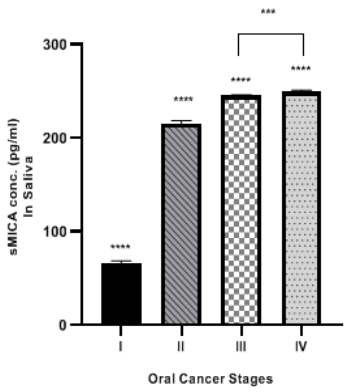


Figure 3: Pretreatment and post treatment levels of sMICA (µg/ml) in sera of patients with oral cancer- The sMICA in pretreatment illustrated significant difference as per one way analysis of variance in all stages (****P value < 0.0001). As per Tukey's multiple comparisons test, sera concentration of sMICA show all stages are significantly different corresponding to each other with ****P value <0.0001 in all stages.

Figure 4. The levels of sMICA (pg/ml) in saliva and urine of oral cancer patients

A: sMICA concentration in saliva



B: sMICA concentration in urine

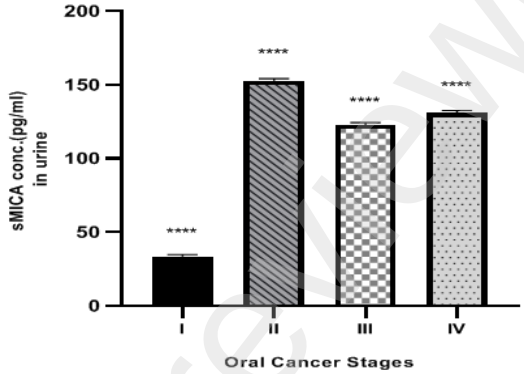


Figure 4: the levels of sMICA (pg/ml) in saliva and urine of oral cancer patients

The sMICA in saliva and urine of OC were determined by sandwich ELISA by using Human MICA detection antibody and streptavidin HRP A. The means of three replicates are shown. (A)The sMICA concentration in saliva showed significant difference as per one way analysis of variance (****P value < 0.0001). As per Tukey's multiple comparisons test sMICA concentration in saliva corresponding to each other with ****P value <0.0001. (B) The sMICA concentration in urine sample shows significant difference as per one way analysis of variance (****P value < 0.0001) and as per Tukey's multiple comparisons test, significant difference corresponding to each other with ****P value <0.0001 except stage I and IV as they are non-significant.

Figure

Figure 1: MICA/B expressions in different stages of oral cancer

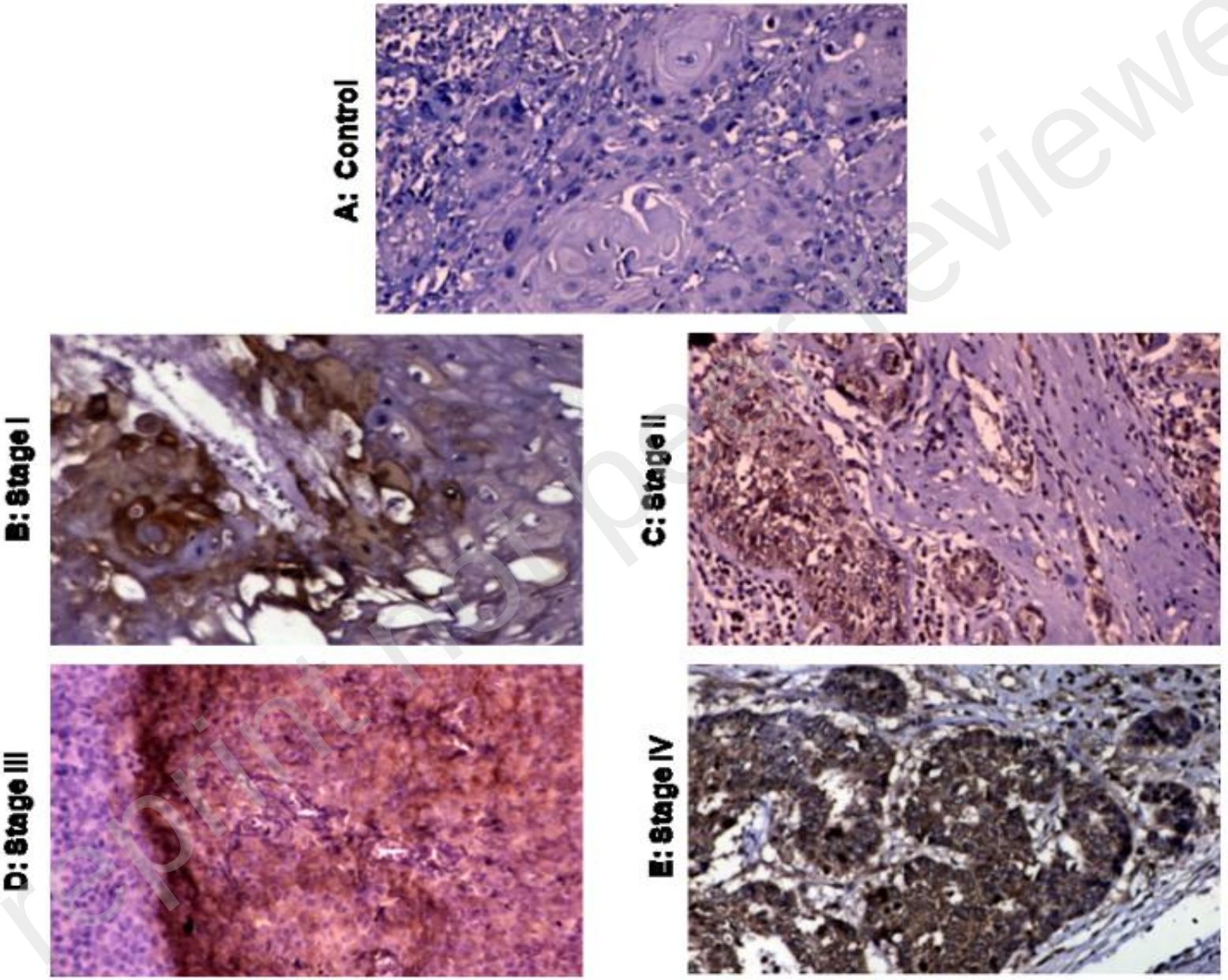
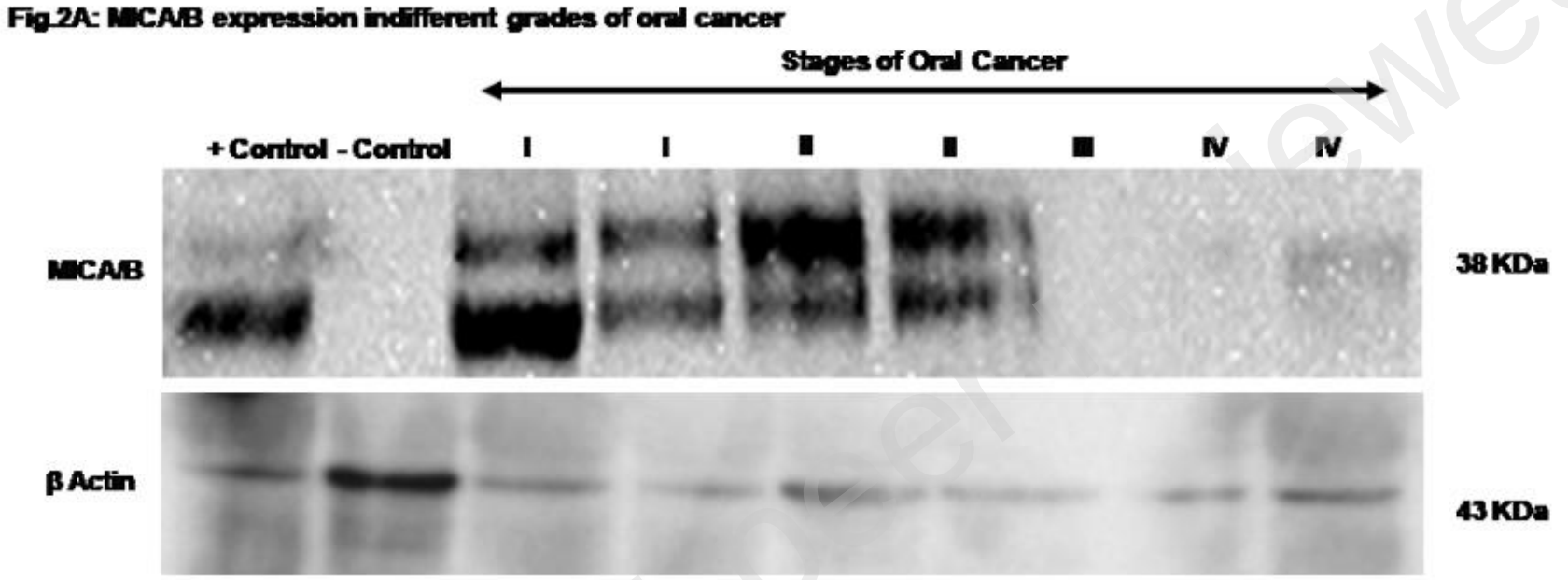


Figure 2: Western blot analysis of different stages of oral cancer



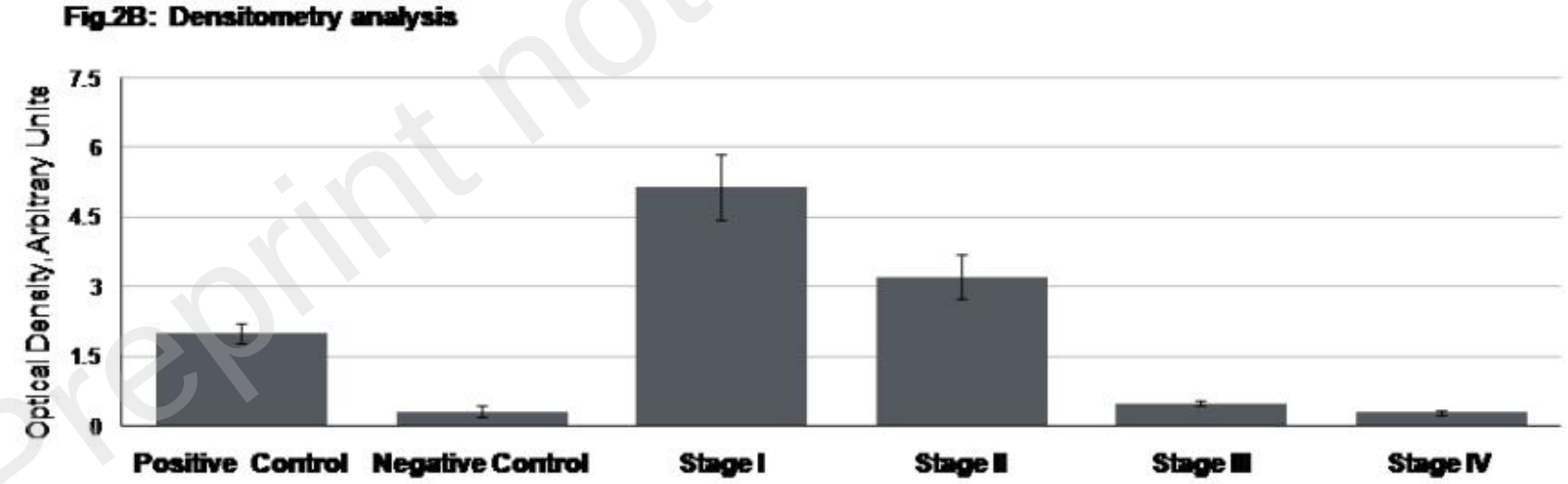


Figure 3: **Pretreatment and post treatment levels of sMICA (µg/ml) in sera of patients with oral cancer**

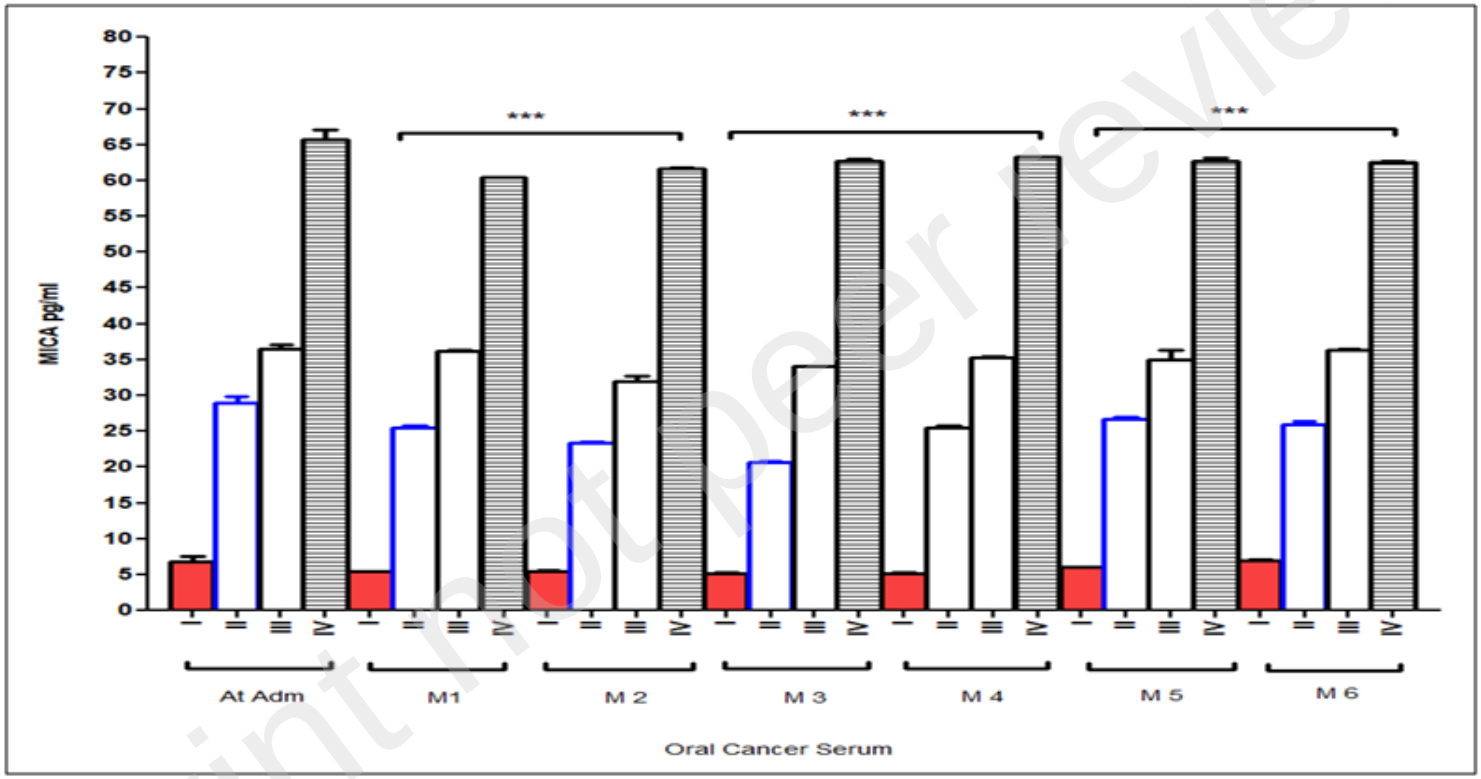
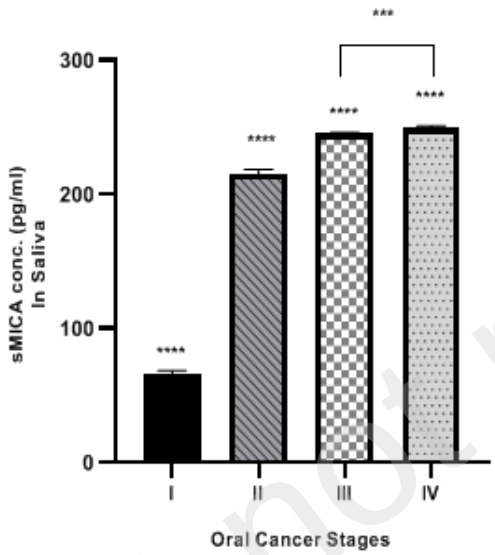


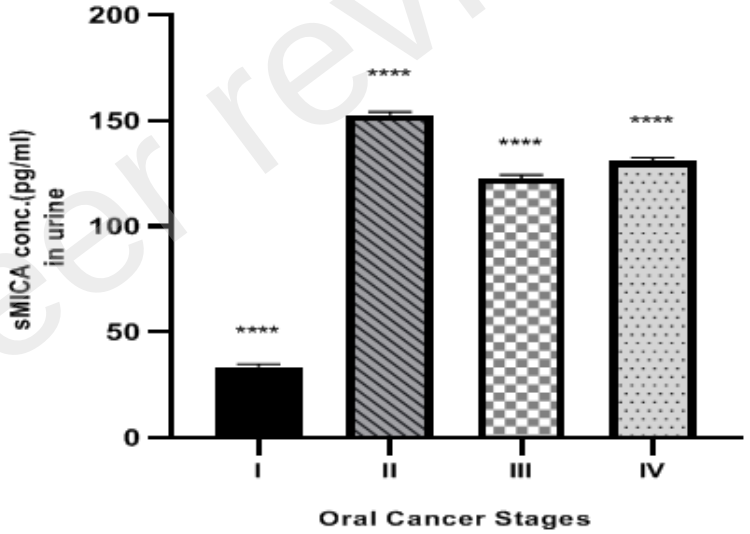
Figure 4: The levels of sMICA (pg/ml) in saliva and urine of oral cancer patients

Figure 4. The levels of sMICA (pg/ml) in saliva and urine of oral cancer patients

A: sMICA concentration in saliva



B: sMICA concentration in urine



Necessary Additional Data

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Table

Table I. IHC result analysis of oral cancer tissue.

Variables	Tissue MICA/B Expression
Oral cancer Patients (n=76)	
Stage I (n=6)	Moderate expression (Fig.1B)
Stage II (n=26)	Moderate expression(Fig.1C)
Stage III (n=37)	Low expression in tissue(Fig.1D)
Stage IV (n=7)	Moderate expression in tissue(Fig.1E)

TABLE II: Pretreatment and post treatment levels of sMICA in sera of patients with oral cancer and control.

Stage	Pre-treatment patients sMICA (pg/ml)	Post treatment (F/W) sMICA (pg/ml) Month						sMICA (pg/ml) Control
		1st	2nd	3rd	4th	5th	6th	
I	6.83 ±1.67	5.38±0.19	5.46±0.26	5.20±0.25	5.11±0.38	6.05±0.08	7±0.09	≤ 1 pg/ml
II	28.04±5.3	25.16±1.71	23.39±0.83	20.55±0.53	25.08±1.6	26.11±1.87	25.54±2.18	
III	36.47±3.77	36.14±1.06	31.89±5.12	34.05±0.47	35.28±0.69	35.02±7.58	36.35±0.46	
IV	65.64±3.57	60.43±0.22	61.60±0.52	62.71±0.67	63.24±0.22	62.64±1.17	62.60±0.42	

Table III:sMICA concentration in saliva and urine

Stage	**Saliva sMICA (pg/ml)**	**Urine sMICA (pg/ml)**
I	66.30±1.96	33.35 ± 1.4
II	215.08 ± 3.28	152.7 ± 1.62
III	245.03 ± 0.95	122.77 ± 1.56
IV	249.32 ± 1.64	130.74 ± 1.79