EPILOGUE

Present investigation evaluated bio-molecular alterations involved in the pathogenesis of oral cancer. 100 healthy individuals and 120 oral cancer patients were enrolled for the study. Oral cancer patients were followed up after initiation of anticancer treatment and 53 post-treatment follow-up samples were collected. The follow-up samples were grouped into complete-responders (CR) and non-responders (NR) based on their clinical status at the time of follow-up sample collection. Following bio-molecular markers were estimated from the tissue, serum and plasma samples using highly specific and sensitive methods:

i. Tissue levels of NF-κB p65, iNOS, Hsp-70 and apoptotic proteins (Bcl-2, Bax), latent, active, total and activation ratio of gelatinases (MMP-2, MMP-9) in malignant and adjacent normal oral tissues.
ii. Serum levels of p53 autoantibodies, IL-8 and glycoprotein conjugates, plasma levels of MMP-2, MMP-9, TIMP-1, TIMP-2, GST, GR and thiol as well as erythrocyte levels of GST, GR, SOD and Catalase were estimated from controls and oral cancer patients.
iii. Comparison of the biomarkers was carried out between subjects with tobacco habit (WHT) and no tobacco habit (NHT) in oral cancer patients.
iv. Comparison of the biomarkers with clinico-pathological parameters of the cancer patients to evaluate their clinical usefulness.
v. Alterations in biomarkers during post-treatment follow ups of oral cancer patients to evaluate their role in treatment monitoring.

SUMMARY

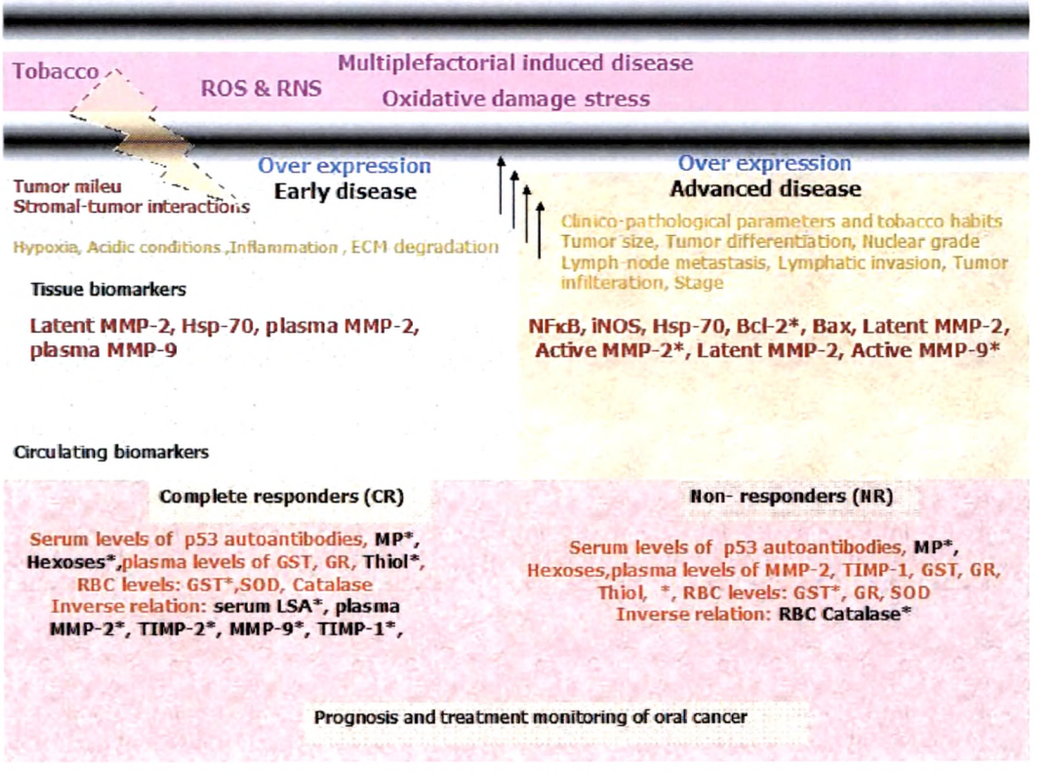
The key results of study are depicted in **Figure-6.1**

Comparison of molecular markers between malignant and adjacent normal oral tissues in cancer patients

The activation of NFκB p65 and expression of iNOS, Hsp-70, Bcl-2 and Bax was observed in 50%, 72%, 87.5%, 71.43% and 68.82% of malignant tissues, respectively. These biomarkers were also detected in adjacent normal tissues with variable expression. NF-κB p65 activation and iNOS, Hsp-70, Bcl-2 and Bax expression was observed in 27.78%, 60%, 81.5%, 14.29%, 80%

and 68.82% of adjacent normal tissues, respectively. Thus all the molecular markers were over-expressed in malignant tissues as compared to adjacent normal tissues. The comparison of the mean levels of these markers revealed that the expression of NF-κB p65 activation, iNOS, Hsp-70, Bcl-2, Bax, Bcl-2/ Bax and different forms of MMP-2 and MMP-9 were higher in malignant than the adjacent normal tissues.

Figure-6.1: Summary of diagnostic and prognostic implications of biomarkers in oral cancer



*= 'p' value <0.05

Among these biomarkers, bcl-2 was significantly higher and latent MMP-9 was lower in malignant oral tissues than adjacent normal tissues. Further, it was found that in malignant and adjacent normal tissues, the expression of latent, active, total and activation ratio of MMP-2 were significantly higher as compared to all the forms of MMP-9 expression. The percentage activity of these gelatinases were also compared in respective tissues; it revealed that percentage activity of latent forms of MMP-2, -9 was significantly higher in adjacent normal tissues while active forms of MMP-2, -9 were significantly

higher in malignant tissues. ROC analysis documented that all the tissue biomarkers had higher efficacy to discriminate between the malignant and adjacent normal tissues reflecting their potential to be employed as diagnostic markers.

Comparison of tissue biomarkers with clinico-pathological parameters in oral cancer patients

The alterations in the biomarkers were also assessed with clinico-pathological parameters including tumor size, infiltration, nodal involvement, lymphatic response and histological type to assess their role in prognostication of oral cancer. The activation of NF-κB p65 was also observed in 37.5% and 62.5% of patients with well and moderately differentiated tumors respectively. Moreover, 87.5 % of the patients with higher nuclear grade II and 37.5% of the patients with lymph-node metastasis showed NF-κB p65 activation. In adjacent normal tissues, expression of iNOS was found to be significantly higher in male oral cancer patients as compared to female cancer patients. Active MMP-2 expression was also found to be significantly higher in oral cancer patients with larger tumor size (T1+T2) as compared to those with smaller tumor size (T3+T4). Active MMP-9 in adjacent normal tissues was found to correlate significantly with tumor size. In malignant tissues, expression of iNOS was found to be significantly higher in well-differentiated tissues, and also higher in patients having lymphatic response and tumor infiltration. Hsp-70 expression was found to be higher in patients with larger tumor size, and moderately differentiated tumors. Expression of Bcl-2 was found to be significantly higher in oral cancer patients with larger tumor size and also higher in old age male patients with moderately differentiated tumors and lymph-node metastasis. The mean values for active MMP-2 were found to correlate significantly with lymphatic response and tumor size. Expression of active MMP-9, total and activation ratio for MMP-9 in malignant tissues were significantly higher in oral cancer patients with larger tumor size as compared to those with smaller size.

All the tissue biomarkers were also compared between early (stage I+ II) and advanced (stage III + IV) stages and also with tobacco habits in oral cancer patients. NF-κB p65 activation was found in 87.5% of patients with WHT, 37.5% patients in advanced stage. Majority of cancer patients diagnosed with advanced disease expressed NF-κB p65 activation. Tissues biomarkers including NF-κB, iNOS, Bcl-2, Bax, different forms of MMP-2 and MMP-9 were higher in advanced stage as compared to early stage of the disease. While mean levels of Hsp-70 were lower in advanced stage of patients as compared to early stage patients. Tissue levels of MMP-9 were significantly higher in patients with advanced disease showing their importance in tumor progression. The expression of biomarkers was also compared in cancer patients with or without tobacco history usage. Majority of patients with tobacco habits (WHT) had presence or higher levels of biomarkers as compared to those without tobacco habits (NHT). NF-κB p65 activation was found to be higher and also present in 87.5% of patients with WHT suggesting tobacco induced activation of NF-κB. Expression of iNOS, Hsp-70 was found to be higher in NHT as compared to WHT. While expression of NF-κB p65, Bcl-2, Bax, Bcl-2/Bax ratio, latent forms of MMP-2 and MMP-9 were found to be significantly higher in patients with WHT as compared to NHT.

Univariate and multivariate analysis were used to compare the expression of biomarkers with the clinico-pathological parameters to assess their association and clinical value for oral cancer. No significant association was observed for NF-κB p65 activation, Hsp-70, Bcl-2, Bax, and different forms of MMP-2 with clinico-pathological parameters, suggesting their role as independent variables in oral carcinogenesis. While iNOS expression showed significant association with age and sex in adjacent normal, and with tumor differentiation in malignant oral tissues. Expression of Hsp-70 also showed significant association with stage of the disease in adjacent normal tissues. Active, total and activation ratio of MMP-9 levels showed significant association with early and advanced disease in malignant tissues.

Comparison of circulating levels of the biomarkers in controls and oral cancer patients

It was observed that serum levels of p53 autoantibodies, IL-8, TSA, LSA, MP, hexoses, plasma GST, erythrocyte GST and GR were significantly higher in oral cancer patients as compared to the controls. While plasma levels of MMP-2, MMP-9, TIMP-1, TIMP-2, GR and Thiol as well as erythrocyte SOD and catalase values were lower in cancer patients as compared to the controls. Moreover, the controls showed absence and 19.23% oral cancer patients showed the presence of serum p53 autoantibodies. ROC curve analysis suggested that serum levels of p53 antibodies, IL-8, TSA, LSA, MP, hexoses and plasma levels of TIMP-2/MMP-2, MMP-9/TIMP-1, GST, GR, Thiol, erythrocyte GR, SOD, and Catalase had good efficacy to discriminate between controls and oral cancer patients.

Comparison of the circulating biomarkers with tobacco habits and clinico-pathological parameters in oral cancer patients

The circulating biomarkers were also compared with clinico-pathological parameters to assess their utility as prognosticators for oral cancer. Significantly higher levels of serum p53 autoantibodies and LSA were observed in patients with lymph-node metastasis. Serum IL-8 values were significantly higher in patients with larger tumor size, lymphatic response, tumor infiltration and advanced disease. Serum hexoses levels were significantly higher in patients with higher nuclear grade II and larger tumor size.

Univariate and multivariate analysis for circulating biomarkers with clinico-pathological parameters revealed significant association of biomarkers. Serum levels of p53 autoantibodies was associated significantly with lymph-node metastasis. Serum IL-8 was associated significantly with tumor size. Serum LSA was associated significantly with tumor infiltration. Serum MP was associated significantly with stage of the disease. While serum hexose was associated significantly with tumor size and nuclear grade of oral cancer patients. However, the alterations in plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 levels could not correlate with any of the clinico-pathological

parameters. All the circulating biomarkers were also compared between early and advanced stages and also tobacco habits in oral cancer patients. Mean levels of circulating biomarkers including serum p53 autoantibodies, IL-8, TSA, LSA, MP, Hexoses, plasma MMP-2, TIMP-2, MMP-9, TIMP-1, and erythrocyte GST, GR, SOD were higher in advanced stage patients as compared to early stage patients. Plasma levels of GST, GR, and Thiol were lower in advanced stage of patients as compared to early stage patients. Serum IL-8 was significantly higher in patients with advanced disease showing their importance in tumor progression. 21.8% of oral cancer patients showed presence of p53 autoantibodies and were all tobacco users. Mean levels of serum p53 autoantibodies, IL-8, TSA, LSA MP and hexoses and plasma levels of MMP-2, TIMP-1, TIMP-1/ MMP-9 and MMP-2/TIMP-2 ratio were significantly higher in WHT as compared to NHT. However, mean levels of plasma MMP-9, TIMP-2 MMP-9/TIMP-1 and TIMP-2/MMP-2 ratio were lower in oral cancer patients with WHT as compared to NHT.

Comparison of biomarkers between untreated oral cancer patients CR and NR

The follow-up samples were categorized into CR and NR based on the clinical status of the patients at the time of follow-up sample collection. Lower mean levels of serum p53 autoantibodies, MP, hexoses, plasma and erythrocyte GST, plasma GR were observed in CR as compared to the untreated patients. Among these biomarkers serum MP, Hexoses, plasma GST and erythrocyte GST were significantly decreased in CR as compared to PT. Serum LSA, plasma MMP-2, MMP-9, TIMP-1, TIMP-2 and Thiol were significantly higher in CR as compared to PT.

In case of NR, circulating levels of p53 autoantibodies, MP, Hexoses, and plasma levels of MMP-2, TIMP-1, erythrocytes GR, Catalase were higher in NR than PT. While serum levels of IL-8, TSA, plasma levels of MMP-9, TIMP-2, GST, GR, Thiol, erythrocyte GST and SOD were remained lower in NR as compared to PT. Mean serum p53 autoantibody, IL-8, TSA, Hexoses, plasma

MMP-2, MMP-9, TIMP-1, TIMP-2, Thiol, GR, GST, erythrocyte SOD, GST, GR levels were comparable between NR and PT.

CONCLUSION

The process of carcinogenesis involves a complex array of genetic and epigenetic alterations, which contribute to cancer pathogenesis leading ultimately to a unique cancer tissue within a unique molecular milieu and this may change dramatically the context and clinical behavior of cancer. The present study identified a large number of biomolecular events that are associated with the occurrence, the progression and the prognosis of oral squamous cell carcinoma, one of the most deadly diseases in Indian subcontinent. The variety of markers sheds light on the complexity of oral carcinogenesis as a multistep process that requires destabilization of several control systems that otherwise account for coordinated behaviour of cells in an organ or region specific manner. Particularly, the invasive front of the tumor appears to be of great importance for prognostication and the fact that stroma/tumoral interactions are indicative for survival, necessitates the evaluation of more tumor tissue simultaneously along with non-invasive approach. Interplay of different biomarkers involved in oral carcinogenesis was evaluated and important results with tobacco habits and clinico-pathological parameters were observed. In conclusion, besides tobacco induced oxidative stress, tumor microenvironment inclusive of active proteases, chronic inflammation exists within the oral tissues. The alterations in iNOS, Hsp-70, Bcl-2, MMP-2 and MMP-9 were closely relevant to tumor differentiation, tobacco habit, tumor size, lymphatic response, advanced stage of oral carcinoma and may act as valuable index of oral cancer prognosis. The rate of expression of NF-kB p65, iNOS, Hsp-70, Bcl-2, Bax, active MMP-2, active MMP-9 in oral carcinoma were also higher in malignant tissues than those in adjacent normal tissues and are related to tumor infiltration, nuclear grade, stage of the disease, lymphatic response respectively suggesting they reflect the biological behaviour and aggressiveness of oral cancer which are relevant to its prognosis.

Moreover, present study also indicates a profound role for monitoring serum levels of (p53 autoantibodies, IL-8, MP, Hexoses), plasma levels of MMP-2, MMP-9, TIMP-1, TIMP-2, GST, GR, Thiol and RBC (GST, SOD, Catalase) in many aspects of tumor progression and response to therapy. Alterations in the levels of biomarkers need insight into the biological behaviour of disease and clinical course of the oral cancer patients. The results on NF-κB p65 thus opened interesting options for targeted research and therapy of oral cancer. Although at the prognostic and diagnostic levels, all the assessed tissue and circulating expression of above markers are not significant, but they are effective biomarkers for carcinogenesis in some tissues and signal the degree of differentiation and aggressiveness of oral cancers serving as cancer signature opening interesting options for targeted research and therapy in oral cancer. The study has reflected the association between molecular events and ill effects of tobacco in human studies fundamental to impel anti-tobacco drive and also bridging the translational research from bench to bedside strengthening further the targeted therapy for oral cancer.