Summary

Oral cancer is the sixth leading malignancy and a major public health problem, globally. Three-quarters of these cases occur in developing countries, including India. In India, majority of the cases present with locally advanced stage at the time of diagnosis. Further, despite improvements in surgery, radiotherapy and chemotherapy the current treatment protocols fail to improve prognosis, especially in node negative patients. Early detection, a key factor for effective control of this disease can best be achieved by understanding the biological behaviour of oral cancer. In this context, molecular markers were studied to determine their significance in oral carcinogenesis, to identify high-risk group of pre-malignant and malignant lesions and for early detection of micrometastasis. For this purpose, the thesis was divided into 3 sections, the summary of which is given below.

SECTION I

Molecular markers in oral carcinogenesis

The molecular markers EGFR, intracellular signal transducer Stat3 and H-ras transcription factors c-myc, cell cycle regulators p53, cyclin D1, p16 and Rb along with proliferative marker Ki-67 and anti-apoptotic marker Bcl-2 were studied in apparently looking normal mucosa (N=12), oral pre-malignant lesions (N=60) and histopathologically confirmed OSCC (N=135) which were further subgrouped into hyperplasia (N=35) and dysplasia (N=25), and into early stage (stage I/II, N=65) and advanced stage (stage III/IV, N=70), respectively. The protein expression of these markers was studied by Immunohistochemistry using the ABComplex technique. The incidence of positive markers, risk of molecular alterations at stage of
transition and correlation between markers was analyzed using SPSS statistical software. The noteworthy observations were as follows:

1. Aberrant expression of molecular markers occurred with varying frequency in all stages of oral carcinogenesis.

2. The number of molecular markers altered increased with progression of disease.

3. Deregulation of markers occurred at a very early stage in the process of oral carcinogenesis.

4. Amongst all markers, significantly high positivity of cyclin D1 in pre-malignant lesions compared to malignant lesions suggested it to be an essential mediator that links upstream signaling pathways to cell cycle regulators.

5. Ki-67 was the most significant marker predicting risk for hyperplasia and hence a reliable tool for evaluating proliferative potential of cells to divide.


7. The most significant risk predictors for early stage disease from dysplasia were p53 and Rb.

8. The significant risk predictors for advanced stage disease were EGFR, Stat3, H-ras and c-myc.

9. Majority of the significant risk predictors for advanced stage disease and for dysplastic lesions were common and of the mitogenic signaling pathway, emphasizing the fact that oral cancer is preceded by pre-malignant lesions.

10. Constitutive Stat3 activation is an early event in oral carcinogenesis.

11. Rb-cyclinD1-p16 pathway governed by EGFR-Stat3 was active in hyperplasia to dysplasia transition stage while H-ras pathway governed by EGFR-Stat3 was active in progression to advanced stage.

12. Dual deregulation of both, p53 and Rb pathways were needed for acquisition of malignant phenotype.
Clinical impact of molecular markers in OSCC

The molecular markers were studied in histologically confirmed OSCC patients (N=135) which were further subgrouped into early stage (stage I/II, N=65) and advanced stage (stage III/IV, N=70) disease. The protein expression of all the studied markers was correlated with clinicopathological parameters and with disease outcome. The significant observations were as follows:

1. In 98% of OSCC patients, at least one marker was abnormally expressed, suggesting involvement of genes in OSCC.

2. The markers that were frequently associated with either tumour size, LN status and tumour stage were EGFR, Stat3, H-ras, c-myc, p53 and Bcl-2 suggesting their contribution to the aggressive behaviour of the tumour.

3. Higher expression of cyclin D1 in tongue tumours compared to buccal mucosa suggested that different anatomic sites of the tumour influenced cyclin D1 expression.

4. Amongst all markers, p53 and nuclear Stat3 were significant independent predictors of unfavourable prognosis in total and early stage patients, respectively.

5. The dual role of Stat3 in oral cancer was observed – as anti-apoptotic during tumour initiation and as a critical regulatory switch governing tumour progression.
SECTION II

Molecular analysis of Stat3

Stat3 mRNA expression was studied by RT-PCR in oral pre-malignant lesions (N=35), malignant tumours (N=70), and corresponding ANM of tumours (N=70). The intensity of the RT-PCR amplified products run on 2% agarose gels were measured and integrated on Gel Documentation System, using the Molecular Analyst Software, in the unit of counts/mm$^2$. Stat3 mRNA expression in pre-malignant, malignant and ANM of tumours was correlated with each other. Further, Stat3 mRNA expression in OSCC and ANM was correlated with clinicopathological parameters of the primary tumours and its usefulness as a prognosticator was investigated. The significant findings noted were as follows:

1. Stat3 mRNA expression progressively increased from pre-malignant lesions to ANM of tumours to malignant tumours suggesting up regulation of Stat3 mRNA.
2. Stat3 mRNA expression in pre-malignant lesions confirmed Stat3 activation to be an early event in oral carcinogenesis.
3. Stat3 mRNA expression in ANM of tumours suggested that ANM of tumours although histologically normal is not normal but has molecular alterations.
4. Stat3 mRNA expression in ANM of tumours was an independent predictor of worse prognosis suggesting it to be an important marker of prognostication.
Circulating p53 antibodies
Circulating p53 antibodies were estimated in the sera of healthy controls (N=35), pre-malignant (N=60) and malignant (N=80) patients, using ELISA, and the levels were correlated with clinicopathological parameters and disease outcome. The important observations were as follows:

1. Circulating p53 antibodies were significantly higher in dysplasia compared to hyperplasia and in advanced stage disease compared to early stage disease suggesting it to be an early event in oral carcinogenesis.

2. Presence of circulating p53 antibodies in healthy controls and pre-malignant lesions suggested it to be a useful non-invasive approach for screening and early detection of individuals at high risk of developing oral cancer.

3. Significant correlation of circulating p53 antibodies with tumour size, nodal status and tumour stage, all indicators of aggressive tumour behaviour suggested it to be an indicator of aggressive disease and also a useful marker for further post-operative management of node negative patients.
SECTION III

Detection of micrometastasis

SCCAg mRNA was studied in HNLNs (N=55) and PPBs (N=28) by nested RT-PCR. The second round RT-PCR products run on 1.5% agarose gels were semi-quantitated on Gel Scan Densitometer. SCCAg mRNA was studied to assess its usefulness as a marker for detecting micrometastasis and as a predictor of disease outcome. Further, the expression in HNLNs and PPBs was correlated with clinicopathological parameters of primary tumours.

1. Absence of SCCAg mRNA expression in metastatic LNs from breast and colorectal cancer patients and in blood samples from healthy individuals, and its presence in primary OSCC tissues confirmed its specificity as marker for detecting micrometastasis in oral cancer.

2. SCCAg mRNA expression seen in 27% and 29% of HNLNs and PPBs, respectively, suggested it to be a useful molecular marker for detecting micrometastasis in oral cancer.

3. Significant correlation of SCCAg mRNA expression in HNLNs and PPBs with tumour size and lymphatic permeation suggested its contribution to the aggressiveness of the tumour.

4. SCCAg mRNA expression was a significant predictor of poor prognosis in node negative patients.
Conclusion

In oral carcinogenesis, deregulation of molecular markers occurred at a very early stage. Further gene products of the mitogenic signaling pathway (EGFR, Stat3, H-ras, c-myc) played an equally significant role as proteins involved in cell cycle regulatory pathways (Rb-cyclin D1-p16 and p53) with cyclin D1 serving as a link between the two pathways. Thus, the molecular approach used to understand the hyperplasia-dysplasia-early-advanced-carcinoma sequence may provide a reference panel of markers for use in defining pre-malignant lesions and predicting risk of malignant transformation and tumour progression. Further, the current study provided evidence for the critical role of Stat3 in OSCC, as it may enhance tumour progression inclusively by affecting the expression of various genes related to cell survival and cell cycle. Thus, it may represent an attractive target for development of effective therapies due to its central regulatory role in signaling and cell cycle pathways.

Apart from this, constitutive Stat3 activation also represents a potential risk factor for poor prognosis in early stage patients with p53 contributing to tumour aggressiveness in OSCC patients. Thus in OSCC, Stat3 with p53 alterations might therefore allow for a more precise identification of patients with an aggressive phenotype, and thus, offer the possibility of more effective treatment. Circulating p53 antibodies may also serve as a useful marker, especially for post-operative management of node negative patients, in combination with other panel of prognostic markers.

Besides identifying significant prognostic molecular markers, SCCAg may serve as a useful target gene to detect micrometastasis in OSCC patients, with special emphasizes to its incorporation in examination of HNLNs for redefining staging and for further clinical management of the disease.