Discussion
SECTION I

Molecular markers in oral carcinogenesis

The specific pattern of genetic alterations required for progressive transformation in oral carcinogenesis process has not been fully delineated. Nevertheless, a preliminary molecular model in HNSCC based on chromosomal alterations has been described by Califano et al. (1996). Further studies in progression models for HNSCC have identified the importance of genes Rb, cyclin D1, p16 and p53 controlling G1 to S phase progression of the cell cycle (Ralhan 1999; Califano et al., 2000). The above models, although excellent, do not take into account alterations to genes and gene products such as EGFR, Stat3, c-myc, H-ras, Ki-67 and Bcl-2, which govern cell division control elements. These biologically important markers have been studied in isolation or in dual combinations but the potential interaction of these alterations in oral carcinogenesis has not been completely understood. Further, there are no reports that have examined the cumulative abnormalities along with Stat3, cell cycle regulatory proteins (p53, cyclin D1, p16 and Rb), Ki-67 and Bcl-2 in oral carcinogenesis and have addressed their risk in malignant transformation and tumour progression. Thus, with the goal of identifying intermediate risk predicting molecular markers in the multi-step process of oral carcinogenesis, the present study, examined the protein expression of molecular markers EGFR, intracellular signal transducer Stat3, transcription factors H-ras and c-myc, cell cycle regulators p53, cyclin D1, p16 and Rb along with proliferative marker Ki-67 and anti-apoptotic marker Bcl-2 in pre-malignant and malignant oral epithelial lesions.
Data analysis revealed that abnormal expression of the studied molecular markers occurred with varying frequency in all phases of oral carcinogenesis. Moreover, the number of markers altered increased with progression of the disease. Compared to normal mucosa, hyperplasia exhibited a substantial loss of Rb protein expression with a significant increase in nuclear cyclin D1 and Ki-67. Similarly, in dysplasia compared to hyperplasia, the significantly expressed markers included EGFR, Stat3, c-myc, and p53 along with a significant loss of p16. In early stage carcinoma, significant aberrant expression was observed for c-myc, p53, cyclin D1, Rb, and Ki-67. With further progression to advanced stages, a significant increase in the frequency of EGFR, Stat3, c-myc, H-ras, p53, Ki-67, and Bcl-2 was seen. Thus, inappropriate oncoprotein expressions suggested that deregulation occurred at an early stage and are likely to contribute to the process of oral carcinogenesis.

In addition to examining molecular abnormalities, the present study evaluated the risk of molecular alterations in the transition phases of oral carcinogenesis. Relative risk evaluation revealed that the most significant molecular alteration predicting risk for hyperplasia from normal oral mucosa was Ki-67. Thus, it may represent a reliable tool for evaluating the proliferative potential of the cells to divide. Higher expression of Ki-67 in hyperplasia compared to normal mucosa has been shown by Kannan et al. (1996) and de Paula et al. (2000). Oral dysplasias are not only difficult to diagnose based on histological evaluation but also controversial in nature because of the relative degree of “differentiation”. The molecular markers that may be diagnostically useful to predict risk for dysplasia included EGFR, Stat3, p16, and c-myc. Likewise, the most significant predictor of risk for advanced stage disease progression from early stage carcinoma was EGFR followed by Stat3, H-ras, and c-myc. This suggested that dysplastic lesions already have genetic aberrations commonly found in advanced stage oral cancers and emphasized the fact that oral cancer is preceded by pre-malignant
lesions. Moreover, it is interesting to note that both, in dysplasia and advanced stage disease progression, majority of the significant markers predicting risk in multivariate analysis were of the mitogenic signaling pathway.

Comprehensive data over the past 20 years strongly support a role of EGFR and its ligands in the development and progression of HNSCC (Ford and Grandis, 2003). The increase in growth factor results in sustained expression of c-myc and alteration in the expression of other oncogenes leading to malignant transformation (Srinivasan and Jewell, 2001). We observed a significant correlation between EGFR and c-myc in advanced stage oral tumours. Besides c-myc, the other downstream targets of EGFR include Stat3 and H-ras (Kalyankrishna and Grandis, 2006).

Stat3 drives the transcription of various target genes including cyclin D1, c-myc, p53 and Bcl-2 (Turkson, 2004). Through a combined inhibition of apoptosis and activation of cell cycle progression, this protein is believed to play a critical role in cell oncogenesis (Bromberg et al., 1999; Catlett-Falcone et al., 1999; Bowmen et al., 2000; Bromberg, 2002). However, there is limited data concerning the role of Stat3 in oral carcinogenesis (Grandis et al., 2000a; Masuda et al., 2002; Nagpal et al., 2002; Klosek et al., 2004). Stat3 activation seen in 60% cases of dysplasia and in 79% cases of advanced stage disease supports our previous study (Shah et al., 2006) and strengthens the hypothesis that constitutive Stat3 expression is an early event in oral carcinogenesis and also supports a dual role of Stat3 in oral cancer - as anti-apoptotic during tumour initiation and as a critical regulatory switch governing cell cycle progression during tumour progression (Song and Grandis, 2000; Nagpal et al., 2002; Chan et al., 2004). Alternately, in malignant tumours, Stat3 might be functioning as antidote to apoptosis until other genes take over the function of cell viability. It has been reported that acquisition of genetic alterations such as mutant p53 could replace the effect of Bcl-2 (Sierra et
al., 1995). Our data showed good correlation of Stat3 with p53 in hyperplastic lesions, with Bcl-2 in early stage malignant tumours and with both, p53 and Bcl-2 in advanced stage tumours.

Apart from EGFR and Stat3, p16 was the third important risk factor implicated in transition of hyperplasia to dysplasia, while in case of progression to advanced stage carcinoma from early stage, it was H-ras. These findings indicated that Rb-cyclinD1-p16 pathway governed by EGFR-Stat3 was active in hyperplasia to dysplasia transformation, while the H-ras pathway governed by EGFR-Stat3 was active in progression to advanced stage. Also, we observed a significantly higher positivity of cyclin D1 in pre-malignant lesions (58%) compared to malignant tumours (32%). Thus, in our series, cyclin D1 holds a key position and may be an essential mediator that links multi-step signaling pathways to cell cycle regulation. Aktas et al. (1997) have shown that constitutive expression of cyclin D1 circumvents the requirement for Ras signaling in cell proliferation, indicating that regulation of cyclin D1 is a critical target of the Ras signaling cascade. In oral carcinoma, the study by Sathyan et al. (2006) indicated that EGFR-ras pathway may possibly inhibit cyclin D1 expression rather than be involved in cyclin D1 overexpression. Experimental in vitro study also supported this finding in other types of cancer cell lines (Yu et al., 2001). This raises the possibility that in malignant tumours H-ras might be functioning as an oncogene. Evidence supporting it comes from the study of Serrano et al. (1997) who demonstrated that oncogenic ras can induce transformation only in co-operation with another oncogene such as c-myc or when an onco-suppressor gene like p53 or p16 gets inactivated. In the present study, c-myc and p53 were actively involved in disease progression. A sequential increase in percentage positivity of intense staining (+3) for c-myc (20%, 32% and 67%) and p53 (5%, 43% and 47%) was seen from dysplasia to early and then advanced stage disease. Such gradual increase in p53 positivity in the order of histological progression from benign through pre-
malignant to malignant has been demonstrated by Boyle et al. (1993) and Piatelli et al. (2002).

In the current study, c-myc was also a probable risk predictor for both, dysplasia and advanced stage carcinoma. Overexpressed c-myc protein in dysplastic and tumour cells may alter cellular response to growth factors and abrogate normal growth control mechanism by stimulating quiescent non-transformed cells to traverse G1 phase and enter S phase (Heikkila et al., 1987; Eilers et al., 1989). Also, overexpression of c-myc actively participates in the p53 concert by accumulating different genetic events and maintaining the proliferative potential of cells (Waters et al., 1991). We observed a significant correlation between c-myc and p53 in early stage oral carcinoma. A similar correlation was noted in oral carcinomas by Baral et al. (1998).

With regard to p53, the present study proved it to be valid molecular marker to foresee the predisposition of oral dysplastic lesion towards malignancy, further supporting other studies (Diogene et al., 1996; Hasina and Lingen, 2004). The current and other studies establish p53 as an early event in oral carcinomas (Boyle et al., 1993; Shin et al., 1994b) and loss of p53 function renders the cell susceptible to further genetic alterations. We observed a significant correlation between p53 and cyclin D1, both, in dysplasias and early stage tumours, again emphasizing cyclin D1 as a key mediator between the mitogenic signaling pathway and the p53 pathway. It has been reported that overexpression of cyclin D1 with increased formation of CDK-4 and CDK-6 complexes results in hyperphosphorylation and hence functional inactivation of pRb (Bova et al., 1999). In the present study, loss of Rb protein expression was a significant predictor of risk for malignant transformation at step 2 in multivariate logistic analysis. This implies that dual deregulation of p53 and Rb pathways are needed for acquisition of a malignant phenotype.
To summarize, deregulation occurred at an early stage and the number of molecular alterations increased with disease progression. Cyclin D1 holds a key position in our series that links multi-step signaling pathways to cell cycle regulation. Gene products of the mitogenic signaling pathway (EGFR, Stat3, H-ras, c-myc) play an equally significant role as proteins involved in cell cycle regulatory pathways (Rb-cyclin D1-p16 and p53) in oral carcinogenesis. 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. Besides, this will possibly lead to the development of newer therapeutic possibilities for better patient management.
Clinical impact of molecular markers in OSCC

In the earlier part of section I, the complex interplay of molecular alterations and their significance in oral carcinogenesis was addressed. The other aspect in OSCC that needs to be addressed is the clinical relevance of these molecular markers in predicting prognosis, as the 5 year survival is only 20% to 30% and further the prognosis of patients with similar and identical staging remains different. The conventional clinicopathological features have only limited prognostic value. Hence, it is clinically important to identify new prognostic factors that can accurately reflect the biological aggressiveness of disease and provide more precise prognostic and therapeutic characteristic of individual tumour. Most published studies have used single markers (Yuen et al., 2002; Lo Muzio et al., 2003), whereas, a minority of studies (Bova et al., 1999; Carlos de Vicente et al., 2002) have used small groups of prognostic markers. It is McGuire et al. (1990) who demonstrated the importance of studying multiple markers on a single tumour and multifactorial statistical analysis of a large patient database, which may provide "a framework" from which important biological and prognostic factors could be used to make treatment decision. Hence, in this part of section I the concurrent analysis of alterations in the expression of gene products in serial tissue sections of OSCC were correlated to clinicopathological parameters and disease outcome to identify independent predictors of prognosis. The panel of molecular markers included EGFR, Stat3, c-myc, H-ras, p53, cyclin D1, p16, Rb, Ki-67 and Bcl-2. Amongst these, Stat3, identified recently, is an important transcription factor for many genes that regulate overall biological behaviour of cancer (Darnell, 1997; Takahashi et al; 2003). Although, its presence has been demonstrated in head and neck cancer, there is paucity of data concerning its association with clinical outcome. To our knowledge there are no reports that
performed multivariate analysis of 10 such molecular markers, studied by IHL, in a single tumour of the oral cavity.

The present study strongly suggested the involvement of genes in OSCC as only 3 out of 135 cases of OSCC failed to show an abnormality in any of the studied gene products. Conceptually, however, this data raises the possibility that still other mechanisms important in few OSCC cases may exist.

With regard to clinicopathological parameters, significant differences were not observed between expression of molecular markers and tobacco habit. This finding is comparable to our earlier study in tongue cancer (Vora et al., 2003) and to reports by several other investigating groups (Davidson et al., 1996; van Oijen et al., 1998; Putti et al., 2002; Schildt et al., 2003). In contrast, several authors have shown significant association between tobacco use and elevated expression (Xu et al., 1998; Pande et al., 2002; Raju et al., 2005). These conflicting results indicated that different environmental and geographic factors might interrelate in the initiation and progression of this cancer.

Exploring the correlation of oncoproteins (EGFR, Stat3, c-myc and H-ras), involved in mitogenic signaling pathways, with clinicopathological parameters, our data demonstrated a positive association with tumour size, LN status and tumour stage. Moreover, overexpression of EGFR also exhibited a correlation with lymphatic permeation and c-myc showed positive association with both, lymphatic and vascular permeation. In HNSCC, except for EGFR, there are few studies that have correlated the protein expression of Stat3, c-myc and H-ras with clinicopathological parameters. In head and neck cancer, Putti et al. (2002) have observed a positive correlation of EGFR with tumour size, LN status and tumour stage. Other studies have demonstrated a positive correlation of EGFR with tumour stage and nodal status only (Kawamoto et al., 1991; Yano et al., 1991; Kusukawa et al., 1996).
In HNSCC, Nguyen et al. (2003) have reported a significant association between elevated c-myc expression and advanced tumour stage. Apart from this, in oral cancer, Todd et al. (1997) have found its positive association with poorly differentiated tumours. Regarding H-ras in head and neck cancer, majority of the studies are on H-ras mutations (Saranath et al., 1991; Milasin et al., 1994; Kuo et al., 1994) with few reports on its protein expression (McDonald et al., 1994a; Yabrough et al., 1994; Kiaris et al., 1995). McDonald et al. (1994a) have reported that immunohistochemically detectable H-ras was most frequently associated with an increase in tumour size and later stages of disease, with no apparent correlation with LN involvement, site of occurrence, degree of differentiation, age, sex or race. In contrast, Yarbrough et al. (1994) observed no correlation with TNM staging.

Concerning Stat3 expression in HNSCC, Masuda et al. (2002) have reported significant association of phospho-Stat3 with presence of nodal metastasis and clinical stage, while Klosek et al. (2004) found a strong association with tumour stage. Even in colorectal cancer, Ma et al. (2004) have noted correlation with the existence of nodal metastasis and disease stage. It is noteworthy that although the percentage positivity of Stat3 in advanced stages was high (79%), stage wise analysis of Stat3 expression indicated that only 18% of Stat3 positive patients showed intense immunoreactivity. On the other hand, amongst the 45% early stage Stat3 positive patients, intense staining was seen in 52% of the tumours. Likewise, Nagpal et al. (2002) observed high accumulation of activated Stat3 in T1 and T2 tumours compared to T3 and T4 tumours. This finding strengthens the hypothesis that constitutive Stat3 activation is an early event in oral carcinogenesis. Further, the presence of intense staining in a portion of the advanced stage patients suggested that it might also be actively involved in a subset of late stage tumours.
Amongst the cell cycle regulatory genes (p53, cyclin D1, p16 and Rb), high incidence of p53 was positively correlated with LN metastasis, advanced tumour stage and advanced tumour size suggesting that tumour aggressiveness is influenced by p53 status. Some authors have found prevalence of p53 expression in patients with LN involvement (Langdon and Partridge, 1992; Ravi et al., 2001; Teni et al., 2002). In contrast, Cheng et al. (2004) have demonstrated inverse correlation with LN status, while Teni et al. (2002) and Ravi et al. (2001) have observed a correlation with tumour stage only.

The interesting observation about cyclin D1 was that it was the only marker to be positively associated with anatomical site, with the expression being more frequent in tongue tumours compared to buccal mucosa. Our earlier study (Vora et al., 2003) in tongue cancer also showed overexpression of cyclin D1. Several studies have described expression of cyclin D1 in various sites of the oral cavity with expression being more often detected in sites like tongue, retromolar region, palate and gingiva (Xu et al., 1998; Lam et al., 2000). This variation in expression by site may be related to racial differences and varying environmental risk factors. Further, the different cyclin D1 expression patterns in different sites in head and neck region may be one of the genetic abnormalities that have contributed to their differences in clinical behaviour.

Except for Rb exhibiting higher loss of expression in exophytic growth pattern compared to ulcerative growth pattern, neither Rb nor p16 were significantly correlated with any of the clinicopathological parameters. In contrast, some investigators have shown association of altered Rb expression with tobacco/betel quid and clinically aggressive oral cancers (Pavelic et al., 1996; Pande et al., 1998; Regezi et al., 1999) and that of p16 overexpression with advanced tumour stage, nodal metastasis and higher histological grade (Cheng et al., 2004). Despite conflicting results, Rb and p16 are important cell cycle regulatory
proteins, as noted in the earlier part of this section, the under expression of which may allow cancer cells to proliferate without control.

Higher incidence of Ki-67 was significantly correlated with presence of nodal metastasis, advanced tumour stage, higher tumour differentiation and with presence of lymphatic permeation. Several studies have shown a strong positive correlation between Ki-67 expression and histological grading only (Kannan et al., 1996; Matsumoto et al., 1999; Wamakulasuriya, 2000). A recent study has found expression of Ki-67 at the tumour-infiltrating front (TIF) of oral cancer (Dissanayake et al., 2003).

Overexpression of Bcl-2 was significantly associated with higher tumour size and advanced tumour stage. In accordance to our results, Lo Muzio et al. (2003) have observed a trend towards tumour stage. In contrast, Teni et al. (2002) have reported positive correlation with LN status and Jordan et al. (1996) observed no significant relationship with tumour size or stage.

The important aspect of this study was to evaluate the prognostic relevance of the studied molecular markers. Univariate analysis for RFS and OS in total patients indicated that amongst the panel of markers studied, expression of p53 and Stat3, and loss of p16 expression in tumours were independent predictors of unfavourable prognosis. However, when multivariate analysis was performed using Cox's forward proportional stepwise regression model, stage attained statistical significance at step1 followed by p53 at step 2, for both RFS and OS. However, in early stage OSCC patients, nuclear Stat3 was the most significant prognostic factor for RFS and OS, both, in univariate and multivariate analysis. Thus, amongst all the molecular markers studied, the results indicated highly significant association of p53 expression and nuclear Stat3 with unfavourable prognosis in total patients and early stage patients, respectively.
Concerning p53, survival data analysis revealed that patient whose tumour showed higher nuclear p53 accumulation was 2.02 times more likely to develop recurrence than a patient with absence of p53 expression. Similarly, p53 protein expression was found to be an independent marker of worse prognosis with a relative hazard ratio of 2.90, after stage. Further, the correlation of p53 protein expression with advanced stage and node positivity suggested that tumour aggressiveness is reflected by the stage of the disease and is probably influenced by p53 status. Several authors have found a close correlation between p53 expression and survival time (Langdon and Partridge, 1992; Field et al., 1993; Shimaya et al., 1993). Field et al. (1993) demonstrated a correlation between p53 overexpression and very poor prognosis for patients with end-stage disease, while McDonald et al. (1994b) reported that p53 accumulation in early OSCC might identify a subgroup of patients with a tendency for an aggressive behaviour. Still, in another study (Teni et al., 2002), higher survival was seen in patients with p53 negative oral tumours than in patients with p53 positive tumours. Similarly, Ravi et al. (2001) in Cox regression analysis showed that the risk of recurrence was double with the expression of p53 protein when compared with p53 negative cases. Hence, IHL of p53 might be a good molecular marker for predicting prognosis in oral cancer.

Regarding Stat3, our data indicated that early stage patient whose tumour showed nuclear Stat3 immunoreactivity was 3.23 times more likely to develop recurrence when compared to an early stage patient with absence of Stat3 immunoreactivity. This is in accordance with the findings of Masuda et al. (2002) who demonstrated that increased phospho-Stat3 correlated with lower disease-free survival rates in head and neck cancer, the only study that has evaluated the prognostic value of Stat3 in HNSCC. Contrary results were obtained in nasopharyngeal carcinoma, where Stat3 alone could not predict prognosis but along with Stat5 predicted better disease-free and OS (Hsiao et al., 2003). Even
in breast cancer, Stat3 did not have any significant predictive value for predicting outcome (Widschwendter et al., 2002). However, in node negative breast cancer, both nuclear Stat3 and phospho-Stat3 were predictive of significantly favourable clinical outcome (Dolled-Filhart et al., 2003). Contradictory findings in the above malignancies once again emphasized variable biological behaviour of the different tumour types. Despite conflicting results, the superiority of Stat3 over other markers may underlie the fact that prognosis is directly related to tumour biology manifested by its multiple aspect. The other markers may predict one or the other aspect of cancer biology and not the overall biological behaviour, whereas Stat3 might most likely do so, because it is an important transcription factor for many genes that may regulate all aspects of cancer biology (Darnell, 1997; Takahashi et al., 2003). Thus, 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. Besides, being a useful molecular marker for selecting early stage patients with poor prognosis, it may represent an attractive target for development of effective therapies due to its central regulatory role in signaling and cell cycle pathways.

In conclusion, immunostaining of p53 and Stat3 might be potential adjuncts to clinical stage in the pathological evaluation of oral specimens. This approach might help to precisely identify patients with an aggressive phenotype and thus offer the possibility of more effectively tailored treatment programs, in addition to the accurate prediction of risk for relapse.
SECTION II

Molecular analysis of Stat3

To further validate our earlier observations of Stat3 protein expression in OSCC and to better understand the role of Stat3 in OSCC tumourigenesis, the mRNA expression of Stat3 in pre-malignant lesions, histologically ANM to tumours and OSCC were examined. This is probably the first report correlating Stat3 mRNA expression with clinicopathological parameters and disease outcome in OSCC. A progressive increase in Stat3 mRNA expression was observed from pre-malignant lesions to histologically ANM to malignant tumours suggesting up-regulation of mRNA expression in oral carcinogenesis. Moreover, Stat3 mRNA expression in pre-malignant lesions supported our earlier findings of Stat3 protein expression, further confirming it to be an early event in oral carcinogenesis. However, a significant correlation was not seen between Stat3 mRNA and its protein expression suggesting that the results from the two methods are not comparable. The sensitivity of RT-PCR method is much higher than IHL of proteins, indicating that some proteins may be below the threshold of detection by immunohistochemistry. Alternatively, some transcripts can be untranslated due to incompletely processed or aberrantly processed mRNA species due to intron retention (Woodman et al., 1996). Inclusion of introns in mRNA has also been reported to lead to alterations in the resulting protein giving the protein that is targeted to a different compartment (Altieri, 1994; van Leusden et al., 1997) or a truncated protein with altered or no function (Ebihara et al., 1996).

Reports of Stat3 mRNA expression are very few and these include, non small cell lung cell lines (Ikuta et al., 2005), colorectal cancer (Zhang et al., 2005) and cervical warts (Arany et al., 2000). In colorectal cancer tissues, Stat3 mRNA level was 1.97 times that of the ANM which is in accordance to the present study. Two
possible explanations for expression of Stat3 mRNA in ANM cannot be excluded. The tissue adjacent to the tumour though histologically normal is not normal but has molecular alterations. Thus, the expression of Stat3 mRNA in histologically ANM to tumour reinforced the "field cancerization" hypothesis, initially proposed by Slaughter et al. (1953). The other possibility is that the tumours secrete some transforming growth factors that could activate Stat3 expression and the presence of Stat3 mRNA expression in ANM may be the result of a paracrine effect of Stat3 expression due to factors released by the tumours. Indeed, Stat3 has been shown to be closely linked to EGFR signaling (Grandis et al., 1998c; 2000a; 2000b). Several reports have shown increased expression of EGFR in ANM of tumours (Shin et al., 1994a; Grandis et al., 1996; van Oijen et al., 1998) with one study showing increased EGFR mRNA in tumour adjacent mucosa (Grandis and Tweardy, 1993). Besides EGFR, the mRNA levels of TGF-α, a ligand of EGFR, showed a 5-fold increase in histologically ANM compared to mRNA levels in control normal mucosa (Grandis and Tweardy, 1993). Even, Sriuranpong et al. (2003) demonstrated that HNSCC cells with or without active EGFR, use an autocrine/paracrine Stat3 activation mechanism mediated through the gp130 family of cytokine receptors.

Concerning clinicopathological parameters and disease outcome, Stat3 mRNA expression in tumours showed a significant correlation with tumour size only and was unrelated to RFS and OS. On the contrary, Nagpal et al. (2002) demonstrated higher Stat3 mRNA expression in T1, T2 and T4 OSCC tumours and moderate expression in T3 tumours but did not examine its prognostic value. In colorectal cancer, Stat3 mRNA expression levels significantly correlated with LN status and tumour differentiation (Zhang et al., 2005). However, when Stat3 mRNA status of histologically ANM was compared with clinicopathological parameters of the tumour, it showed significant association with lymphatic invasion only. Moreover, it was a significant predictor of RFS and OS. For RFS,
multivariate Cox forward stepwise regression analysis using Stat3 mRNA and clinicopathological parameters demonstrated that Stat3 mRNA expression was a significant predictor of recurrence at step 1 with a relative hazard ratio of 4.81, followed by stage. Similarly, when multivariate analysis was performed for OS, Stat3 mRNA expression was found to be an independent marker of worse prognosis at step 2, with a relative hazard ratio of 4.78, after tumour stage. In this regard, Stat3 mRNA expression in histologically ANM may be regarded as a marker of early undetectable carcinogenesis rather than a marker of neoplastic progression, and may be an important tool with regard to prognostic evaluation.

To our knowledge, the prognostic value of Stat3 mRNA in histologically ANM and in OSCC has not been evaluated previously. In colorectal tumour ANM, Stat3 mRNA expression has been studied but its prognostic value has not been evaluated (Zhang et al., 2005). However, in colorectal cancer, Visca et al. (1999) have shown c-myc and p21-ras staining in adjacent-to-tumour non-neoplastic mucosa to correlate with worse prognosis.

To summarize, the present data indicated for the first time that Stat3 mRNA expressed in histologically ANM is an important prognosticator of RFS and OS, in OSCC. Increased Stat3 mRNA expression in ANM of tumours suggested that ANM of tumours although histologically normal is not normal but has molecular alterations. Thus, Stat3 mRNA expression in histologically ANM may indicate more aggressive treatment with regular follow-up.
Circulating p53 antibodies

In the earlier part of section I, it was shown that alterations in protein p53 is an early event in oral carcinogenesis and the most significant marker for determining risk for malignant transformation from dysplasia. Further, it was also a significant predictor of RFS and OS in OSCC patients. These significant observations prompted us to assess the ability of circulating p53 antibodies as a marker for early detection of high-risk group of patients with pre-malignant and malignant lesions and also as a prognosticator in OSCC. Therefore, in this part of the section, circulating p53 antibodies were evaluated in pre-malignant and malignant lesions and further associated with its protein expression in matched patient samples, and with clinicopathological parameters and survival in OSCC patients.

Our data indicated that the mean levels of circulating p53 antibodies in patients with pre-malignant and malignant lesions were significantly higher compared to healthy controls and is in agreement with the observations of Ralhan et al. (1998). Even, the pre-malignant lesions when subgrouped into hyperplasia and dysplasia showed a significant difference in the mean levels of circulating p53 antibodies. A similar difference in the mean levels of circulating p53 antibodies was noted when the OSCC patients were subgrouped into early and advanced stage disease. Together, these observations indicated an increase in circulating p53 antibodies as the disease progressed from hyperplasia to dysplasia and from early stage to advanced stage, thereby suggesting it to be an early event in oral carcinogenesis. These findings corroborated with the observations of Ralhan et al. (1998) and Cawley et al. (1998) in oral and esophageal cancer, respectively. Although, circulating p53 antibodies and p53 protein expression, studied in section I, showed an increase from hyperplasia to dysplasia and from early stage to advanced stage, there was lack of correlation between the two. This could be due to several factors including tumour lysis and shredding of the p53 protein into
Discussion

the circulation, stabilization of mutant p53 protein by other protein-protein interactions, etc (Hagiwari et al., 2000).

Circulating p53 antibodies were detected in 9% healthy individuals with and without habit of tobacco, 18% patients with pre-malignant lesions and 65% OSCC patients. The presence of p53 antibodies in healthy individuals, although at a lower incidence compared to pre-malignant and malignant lesions is comparable to other studies (Ralhan et al., 1998; Chow et al., 2001). Furthermore, in a subpopulation at high-risk of lung cancer due to exposure to vinyl chloride, p53 antibodies were detected in the serum before clinical detection of cancer (Trivers et al., 1995). Hence, presence of circulating p53 antibodies in healthy controls and in patients with pre-malignant and malignant lesions may suggest it to be a useful non-invasive approach for screening and early detection of individuals at a high-risk of developing oral cancer, especially in developing countries where the use of low cost assays could be in public health benefit. These patients need be closely monitored at regular time intervals.

The 65% frequency of circulating antibodies in OSCC in this study is the highest reported in HNSCC patients compared to 44% reported by Lavielle et al. (1998) or between 17% to 34% reported by other investigators (Cough et al., 1994; Bourhis et al., 1996; Ralhan et al., 1998; Chow et al., 2001). One explanation for the high prevalence of pre-operative circulating p53 antibodies in the present series may reside in the high (~65%) incidence of advanced disease (stage III/IV) seen in this study. Moreover, when correlated with clinicopathological parameters, pre-operative circulating p53 antibodies were significantly associated with T3/T4 tumours, nodal metastasis, advanced tumour stage and presence of lymphatic invasion, factors indicative of poor prognosis. However, it showed an inverse correlation with age. Similar observations with tumour size (Ralhan et al., 1998) and LN status (Bourhis et al., 1996; Ralhan et al., 1998) in head and neck
Discussion

cancers have been reported. Angelopoulou et al. (1996) demonstrated a significant association with older age among ovarian cancer patients showing p53 antibodies, which was contrary to the present finding. In OSCC, few studies have demonstrated an association between circulating p53 antibodies and poor survival (Bourhis et al., 1996; Werner et al., 1997).

The present study using Kaplan-Meier survival function analysis indicated preoperative circulating p53 antibodies to be a predictor of worse OS. Lack of correlation with RFS in early stage patients may be due to small patient size (N=23). However, amongst all these findings, the association of circulating p53 antibodies with nodal metastasis is an important observation because it has been reported that the presence of circulating p53 antibodies in patients with nodal metastasis may reflect the lymphocyte's reaction to p53 protein in nodal metastatic cancer cells (Bourhis et al., 1996). Nodal recurrence is common after treatment of clinically node negative head and neck cancer (Yuen et al., 1997; 1999). Moreover, it is still controversial whether prophylactic treatment with neck dissection or radiotherapy of clinically node negative neck may be considered in the treatment of these patients to reduce the nodal failure rate. Still the search for the best possible panel of prognostic markers for OSCC is on going. Circulating p53 antibodies may be one of the useful prognostic markers in combination with other panel of prognostic markers for the post-operative management of node negative patients with OSCC.
In addition to identifying useful prognostic markers, section III aimed at determining the clinical relevance of SCCAg mRNA as a marker for detecting micrometastasis. The presence of metastatic squamous cells in the cervical LNs of patients with OSCC reflects disease progression and implies that the disease is no longer localized. Further, in these patients the chance of long-term control is decreased by 50% when compared to patients who have similar primary tumours without nodal metastasis (Shah and Andersen, 1995). Hence, in OSCC, the status of cervical LNs is an extremely important prognostic factor used in the clinical management of the disease. Routine histological examination may fail to detect low number of tumour cells, which is however, possible by molecular diagnosis. Given this, early detection of micrometastasis in node negative OSCC patients is expected to assist in treatment planning and thereby improve survival.

Several studies have revealed presence of micrometastatic tumour cells in the LNs after staining with epithelial specific antibodies (van den Brekel et al., 1996; Ambrosch and Brinck, 1996; Enepekides et al., 1999; Hamakawa et al., 1999) and molecular analysis (Brennan et al., 1995; McDonald et al., 1998; Hamakawa et al., 1999; Cortesina et al., 2000; Zen et al., 2003) in head and neck cancers. However, using molecular analysis, minimal data exists on the prognostic relevance of micrometastasis in HNLNs or PPBs of OSCC or their association with clinicopathological features of the primary tumour (Nieuwenhuis et al., 2003a; 2003b; Onishi et al., 2004) but there are no reports that have evaluated the prognostic value of SCCAg mRNA or correlated the expression of SCCAg mRNA with clinicopathological parameters in OSCC. Our data provided important insight...
into the possible clinical application of micrometastasis using SCCAg mRNA expressed exclusively by squamous cell tissues.

In the present study, the nested RT-PCR results showed absence of SCCAg mRNA expression in the LNs and peripheral blood samples of the control group, indicating a very high rate of specificity. Similar specificity for SCCAg mRNA expression was observed by Hamakawa et al. (1999) in head and neck cancer, and by Kano et al. (2000) and Kaganoi et al. (2004) in a esophageal cancer. Concerning sensitivity, various investigating groups have evaluated the detection threshold of SCCAg mRNA in LNs and PPBs (Hamakawa et al., 1999; Kano et al., 2000; Zen et al., 2003; Kaganoi et al., 2004). Our data demonstrated that SCCAg mRNA could detect micrometastasis in 27% and 29% of HNLNs and PPBs, respectively, using the sensitive nested RT-PCR assay and therefore may serve as a useful molecular marker for detecting micrometastasis. Studies demonstrating the efficacy of molecular markers to detect micrometastasis in HNLNs and PPBs in various malignancies have already been reported (Pantel et al., 1999). In HNSCC, using conventional RT-PCR, Hamakawa et al. (1999) and Onishi et al. (2004) reported SCCAg mRNA in 18.7% and 7.4% of HNLNs, respectively. With quantitative RT-PCR, 17.6% HNLNs expressed SCCAg mRNA (Onishi et al., 2004). Based on cytokeratin 5 and 13 gene expression, McDonald et al. (1998) and Hamakawa et al. (2000) were able to detect micrometastasis in 40% and 14.4% of HNLNs, respectively. Compared to these studies examining several LNs from a single patient, our study examined only one LN per individual patient. These results emphasize the importance of verifying the HNLNs for micrometastasis. With regard to detection of circulating tumour cells, there are very few reports in HNSCC that have examined the importance of molecular markers for detecting micrometastatic cells in circulation. In head and neck cancer, Brakenhoff et al. (1999) applying E48 was able to detect micrometastasis in 10% of the blood samples. In OSCC, Zen et al. (2003) showed the presence of
SCCAg mRNA and EGFR mRNA in the circulation using both conventional and quantitative RT-PCR. In other malignancies, SCCAg mRNA detected circulating tumour cells in 40% and 33% of cervical and esophageal cancer, respectively (Stenman et al., 1997; Kaganoi et al., 2004). Thus, the detection of SCCAg mRNA positive cells in PPBs in oral cancer patients is an indication of dissemination of the tumour cells. Together, these results are in agreement with the clinical data indicating a 50% decrease in survival in node negative HNSCC patients (Snow et al., 1982; Zatterstrom et al., 1991; Leemans et al., 1993) due to loco-regional recurrence or distant metastasis, further suggesting that these patients have micrometastasis and were under staged. The under staging is a well known phenomenon as demonstrated by serial sectioning of routine HNLNs and by immunohistochemistry for different epithelial molecules as well as the molecular techniques for detection of mRNA of specific molecular markers (Enepekides et al., 1999; Brennan et al., 1995; Ambrosch and Brink, 1996; Hamakawa et al., 1999; Ferlito et al., 2001).

SCCAg is identified to be a member of ova-albumin family of serine protease inhibitor (Suminami et al., 1991) and shares high amino acid sequence homology with plasminogen activator type 2 (Takeshima et al., 1992). Since plasminogen activators have been reported to play a crucial role in malignant behaviour (Burtin et al., 1987; Oka et al., 1991; Takai et al., 1991), it is quite likely that SCCAg may take some part in the invasion or metastasis of tumour cells. Hence, it would be interesting to note if SCCAg showed any association with clinicopathological features of the primary tumour. In this series, SCCAg mRNA expression in HNLNs and PPBs when correlated with clinicopathological parameters of the primary tumour, showed a significant positive correlation with tumour size and lymphatic invasion, suggesting that SCCAg mRNA may be associated with progression of primary oral tumours that have a more aggressive clinical course. Further, although, HNSCC is thought to metastasize initially via the lymph as
judged from clinical disease progression, we observed no correlation between SCCAg mRNA analyzed in the HNLNs and PPBs and even when HNLNs and PPBs were assessed from the same set of node negative OSCC patients (N=15). One of the probable reasons could be the existence of several different pathways and anastomosis of lymphatics in the neck (van den Brekel and Snow, 1994), and in this study, only one LN per patient was analyzed. To our knowledge there are no reports that have correlated SCCAg mRNA with clinicopathological prognosticators in oral cancer. However, these results are consistent with the findings of Kaganoi et al. (2004) demonstrating a correlation with tumour size and venous invasion in esophageal cancer, further confirming the association of SCCAg mRNA with the biological behaviour of the tumour.

Since node negative patients who undergo successful radical resection often encounter recurrence or distant metastasis, it is important to assess whether genetic diagnosis for micrometastasis is associated with prognosis. Multivariate statistical analysis of RFS in relation to SCCAg mRNA in HNLNs and PPBs revealed a relative risk of 10.27 and 9.64, respectively, for developing metastasis in patients with SCCAg mRNA expression compared to those without SCCAg mRNA expression. Moreover, SCCAg mRNA was also able to predict OS. In line with this, Kaganoi et al. (2004) reported SCCAg mRNA to be a significant predictor of prognosis in esophageal cancer. Even, in various carcinomas, it has been demonstrated that detection of micrometastasis has a significant impact on patient survival (Jauch et al., 1995; McGuckin et al., 1996; Bostick et al., 1999). In HNSCC, Nieuwenhuis et al. (2003a) in pathological No group demonstrated that the presence of E48 positive LNs was significantly associated with a distinctly poor cause specific survival as compared to those with E48 negative LNs. Hence, SCCAg mRNA may also be regarded as a clinically relevant prognostic factor for early detection of micrometastasis in OSCC.
In conclusion, SCCAg mRNA analyzed by nested RT-PCR may serve as an important marker for assessment of micrometastasis that contributes to poor prognosis in node negative OSCC patients. Further, it may represent a useful tool for more accurate staging, which could improve disease management and help to obtain maximal therapeutic benefit from adjuvant therapies.