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# Scatter Factor Receptor (c-Met) as Possible Prognostic Factor in Patients with Oral Squamous Cell Carcinoma

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**Abstract.** *This report was performed to study the biological role of c-Met in oral tumorigenesis by analyzing its expression in relation to clinicopathological features. Seventy-three cases of oral squamous cell carcinoma and 10 of normal mucosa were analysed for c-Met expression by immunohistochemistry. Normal oral squamous epithelium showed absent or low membranous positivity in the intermediate (malpighian – spinous) layer. Fifty-seven cases (78%) of carcinoma showed immunopositivity, with a prevalently membranous positivity and scattered areas also showing a cytoplasmic localization. Sixteen cases of carcinoma (22%) showed no positivity for c-Met. Among positive tumours, well-differentiated areas showed low or absent cytoplasmic positivity, while low-differentiated areas showed both membranous and cytoplasmic positivity. There was no statistically significant correlation between c-Met expression and sex, recurrence, staging or grading. The frequency of lymph node metastases was higher in c-Met-positive tumours (17/57, 29%) than in c-Met-negative ones (4/16, 25%). When analysed for prognostic significance, patients with negative/reduced c-Met expression had better survival rates than patients with high expression. The difference between survival rates was statistically significant ($p<0.05$). These data suggest that c-Met expression may be useful to identify cases of oral squamous cell carcinoma with a more aggressive and invasive phenotype.*

Squamous cell carcinoma of the oral cavity (OSCC) is a rapidly increasing malignancy which represents the most frequent malignant tumour of this region. The incidence of metastasis depends on the degree of cellular differentiation, depth of invasion and site of the primary tumour. However, the clinical behaviour of OSCC is difficult to predict considering classical histopathological parameters only. Biological markers that can help to identify the lesions with an aggressive phenotype and worse prognosis need to be identified. Since the majority of human neoplasms are characterized by the imbalance of the regulatory cell-cycle control processes, the study of the expression of proteins involved in the critical check-points of cell growth and apoptosis, as well as growth factors and their receptors, could elucidate the process of oral carcinogenesis and metastasis.

The transmembrane Met tyrosine kinase has been identified as the receptor for hepatocyte growth factor/scatter factor (HGFySF) (1). Under normal conditions, HGFySF expression is usually limited to cells of mesenchymal origin and acts predominantly on epithelial and endothelial cells expressing the Met receptor to elicit cellular responses, including cellular proliferation, differentiation, motility and invasion. In addition to its role during normal physiological processes (2), several lines of evidence suggest that aberrant HGFySF-Met signalling contributes to tumour development and progression to the malignant phenotype.

*In vivo*, the Met receptor is found primarily on melanocytes, endothelial cells and tissues of epithelial origin. It has been shown to be overexpressed in several human tumours and probably triggers a unique biological program leading to 'invasive growth'. This phenotype results from the integration of apparently independent biological responses to HGF, including not only proliferation but, above all, cell dissociation, survival, motility, invasion of surrounding extracellular matrix, proliferation and the induction of cell polarity. Under physiological conditions, the coordination between these genetic programs leads to the formation of epithelial tubular structures (the so-called 'branched

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morphogenesis'), myoblast migration and neuronal branching. Deregulated activation of the Met-driven 'invasive-growth' program confers unrestricted proliferation, invasive and metastatic properties on cancer cells (2).

Expression of mutant Met receptors that are activated by means of point mutations, or coexpression of both Met and HGFySF, produces cells that are both tumorigenic and metastatic when injected into immune compromised mice. Studies involving a variety of solid tumours have suggested that overexpression of the SF/c-Met ligand-receptor pair is associated with the acquisition of a malignant phenotype. In addition, there are numerous reports that Met and/or HGFySF are overexpressed in a variety of human tumours, often in association with high tumour grade and poor prognosis (3).

It has been shown that c-Met receptor is constitutively activated in many human tumours of epithelial origin and that it plays a critical role in conferring invasive properties on neoplastic cells. Oral squamous cell carcinoma (OSCC) is a neoplasm characterized by a high degree of local invasion and an elevated rate of metastasis to cervical lymph nodes.

Expression of c-Met in human adenocarcinomas is well described, but few studies on its expression in human squamous cell carcinomas have been reported (4-9). We studied c-Met expression in oral cancers by immunohistochemistry, analyzing its expression in relation to the classical clinicopathological features, in order to evaluate its role in prognostic evaluation of these tumours.

## Materials and Methods

*Selection of cases.* Seventy-three samples from paraffined and ten from frozen specimens of primary oral squamous cell carcinomas, plus 6 from paraffined specimens of lymph node and tissutal metastases of OSCCs included in this study were used. Specimens were fixed in 10% neutral buffered formalin.

None of the patients had been treated previously. They received surgical treatment with curative intention. No case in this study concerned patients with contemporaneous multicentric lesions. Clinical data was reviewed to record sex and age of the patient and site and size of the lesion. The group consisted of 52 men and 21 women with a mean age of 63.6 years (range 20-81). There were 31 cases in stage I, 20 stage II, 8 stage III and 14 stage IV. Forty-three patients were analysed for survival rates (the follow-up time was sufficient for statistical analysis, > 3 years). Survival was calculated from the date of operation to the date of the latest follow-up visit or death due to cancer. Patients who died of postoperative complications within 30 days were excluded. The histopathological grading was assessed on paraffin H & E - stained sections. Tumour extent was classified according to the TNM system by UICC (UICC, 1987) and tumours were divided into grades 1, 2 and 3 using the WHO classification of histological differentiation.

Ten paraffined specimens of healthy oral mucosa were obtained with informed consent from patients who had undergone routine oral surgical procedures and were used as control.

*Immunohistochemistry.* Four-μm serial sections from formalin-fixed paraffin-embedded blocks were cut for each case and one section stained with haematoxylin-eosin (H.E.) was used to confirm the histopathological diagnosis. Only sections containing sufficient epithelium to assess the antibody reactivity with 1000 cells were considered for this study.

Immunohistochemistry was then performed on the remaining sections mounted on poly-L-lysine-coated glass slides. Deparaffinized and rehydrated sections were incubated for 30 minutes in 3% $H_2O_2$/methanol to quench endogenous peroxidase activity and then rinsed for 20 minutes with phosphate-buffered saline (PBS) (Bio-Optica M107, Milan, Italy). Non-specific protein binding was attenuated by incubation for 30 minutes with 5% horse serum in PBS. A polyclonal antibody (C-12) against the C-terminal peptide of the c-Met receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:100. The antibody was applied directly to the sections and the slides were incubated overnight (4°C) in a "humidified chamber". The sections were washed three times with PBS at room temperature. Immune complexes were subsequently treated with the secondary biotinylated antibody and then detected by streptavidin peroxidase, both incubated for 30 minutes at room temperature (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). After rinsing with 3 changes of PBS, the immunoreactivity was visualized by development for 2 minutes with 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide (DAB substrate kit, Vector Laboratories). Sections were counterstained with Mayer's haematoxylin, mounted with permanent mounting medium and examined by light microscopy.

Positive controls consisted of tissue specimens with known antigenic positivity and included sections of renal carcinoma(10). A negative control was performed in all cases by omitting the primary antibody and incubating the section with normal rabbit serum. Negative controls in all instances resulted in a negative immunoreactivity. To quantitate the c-MET expression, 300 cells were examined in at least five areas at x400 magnification and a mean percentage of positive tumour cells was determined assigning cases to one of the following four categories: 0, no staining or weak staining in less than 10% of tumour cells; 1, weak to moderate staining in 5±30% of cells; 2, moderate staining in 31±50% of cells; and 3, strong staining in more than 50% of tumour cells(11).

*Statistical analysis.* Data were analysed using Prism (ver 3.0 for Windows) and Stanton A. Glantz (ver 3.0 for Dos) softwares. Significant differences ($p<0.05$) between groups were determined using one-way analysis of variance and the Student-Newman-Keuls-test. Survival analysis was computed comparing negative (score 0) and low expressing (score 1) with medium (score 2) and high (score 3) expressing c-Met. Survival curves were analyzed according to the method of Kaplan-Meier and for differences between curves the $p$ value was calculated by the log-rank test. A $p$ value of less than 0.05 was accepted as statistically significant.

## Results

*c-Met expression in normal oral mucosa.* Normal oral squamous epithelium of the control cases showed absent or low membranous positivity up to the intermediate (spinous) layer. The normal oral epithelium adjacent to invasive carcinomas showed a homogeneous c-Met cytoplasmic staining, with weak intensity, restricted to the parabasal layer.

Table I. *Statistical analysis of c-Met expression and associated clinicopathological findings in oral SCCs.*

| Variables | No. | Score 0 n. (%) | Score 1 n. (%) | Score 2 n. (%) | Score 3 n. (%) | Mean | Standard deviation | Standard error | $p < 0.05$ | Statistical data | Mean Diff. | $p$ value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cases | 73 | 16 (22) | 10 (14) | 28 (38) | 19 (26) | | | | | | | |
| Age | | | | | | | | | | | | |
| < 65 years | 36 | 9 | 7 | 13 | 7 | 1.500 | 1.082 | 0.180 | No° | $p=0.154$ | | |
| > 65 years | 37 | 7 | 3 | 15 | 12 | 1.865 | 1.084 | 0.178 | | | | |
| Sex | | | | | | | | | | | | |
| Male | 52 | 10 | 8 | 21 | 13 | 1.712 | 1.054 | 0.146 | No° | $p=0.756$ | | |
| Female | 21 | 6 | 2 | 7 | 6 | 1.619 | 1.203 | 0.262 | | | | |
| Grading | | | | | | | | | | | | |
| G1 | 17 | 3 | 2 | 8 | 4 | 1.764 | 1.033 | 0.250 | | | | |
| G2 | 37 | 8 | 7 | 14 | 8 | 1.594 | 1.066 | 0.175 | No* | grade 1 *vs* grade 2 | 0.171 | >0.05 |
| G3 | 19 | 5 | 1 | 6 | 7 | 1.789 | 1.228 | 0.281 | | grade 1 *vs* grade 3 | -0.024 | >0.05 |
| | | | | | | | | | | grade 2 *vs* grade 3 | -0.194 | >0.05 |
| Size | | | | | | | | | | | | |
| < 1.5 cm | 24 | 7 | 5 | 10 | 2 | 1.292 | 0.999 | 0.203 | Yes° | $p=0.0302$ | | |
| > 1.5 cm | 49 | 9 | 5 | 18 | 17 | 1.878 | 1.092 | 0.156 | | | | |
| Lymph node metastasis | | | | | | | | | | | | |
| Negative | 52 | 12 | 6 | 22 | 12 | 1.654 | 1.083 | 0.150 | No° | $p=0.704$ | | |
| Positive | 21 | 4 | 4 | 6 | 7 | 1.762 | 1.136 | 0.247 | | | | |
| Staging | | | | | | | | | | | | |
| | | | | | | | | | | | Mean Diff. | $p$ value |
| I | 31 | 7 | 5 | 14 | 5 | 1.548 | 1.028 | 0.184 | | stage 1 *vs* stage 2 | -0.2016 | >0.05 |
| II | 20 | 5 | 1 | 8 | 6 | 1.75 | 1.164 | 0.260 | No* | stage 1 *vs* stage 3 | -0.4516 | >0.05 |
| III | 8 | 1 | 2 | 1 | 4 | 2 | 1.195 | 0.422 | | stage 1 *vs* stage 4 | -0.1659 | >0.05 |
| IV | 14 | 3 | 2 | 5 | 4 | 1.714 | 1.139 | 0.304 | | stage 2 *vs* stage 3 | -0.2500 | >0.05 |
| | | | | | | | | | | stage 2 *vs* stage 4 | 0.03571 | >0.05 |
| | | | | | | | | | | stage 3 *vs* stage 4 | 0.2857 | >0.05 |
| Recurrence | | | | | | | | | | | | |
| Yes | 18 | 6 (33) | 1 (5) | 5 (29) | 6 (33) | 1.611 | 1.290 | 0.304 | No° | $p=0.743$ | | |
| No | 55 | 10 (18) | 9 (16) | 23 (42) | 13 (24) | 1.709 | 1.031 | 0.139 | | | | |

°Student-Newman-Keuls Test
*One-way Analysis of Variance (ANOVA) and Student-Newman-Keuls Multiple Comparisons Test

*c-Met expression in oral squamous cell carcinoma.* Definite c-Met positivity was present in 47 out of 73 (78%) oral squamous cell cancers (Table I). In the cases with lymphonodal metastasis, c-Met positivity was observed in both the primary site and the metastatic lymph nodes in all the 4 cases enclosed in the study (100%).

The staining pattern of metastasizing samples was prominently cytoplasmatic. For that concerning non-metastasizing tumours, a membranous positivity was observed in well-differentiated carcinomas (Figure 1A), while low-differentiated tumours often showed both membranous and cytoplasmic positivity (Figures 1B, 1C).

c-Met was also expressed in neoplastic stromal compartment, in fibroblasts and in vascular endothelial cells adjacent to the tumours (Figure 1C) and in cells of minor salivary glands (Figure 1D).

The frequency of lymph node metastasis was higher in c-Met-positive tumours (17/57, 29%) than in c-Met-negative (4/16, 25%). In the lymph node metastasis of Met-positive tumours, receptor expression was maintained and sometimes increased with respect to primary tumours. Overexpression of the Met/HGF receptor was found at all tumour stages but was more significant in those associated with enlarged or multiple (N2-N3) lymph node metastases.

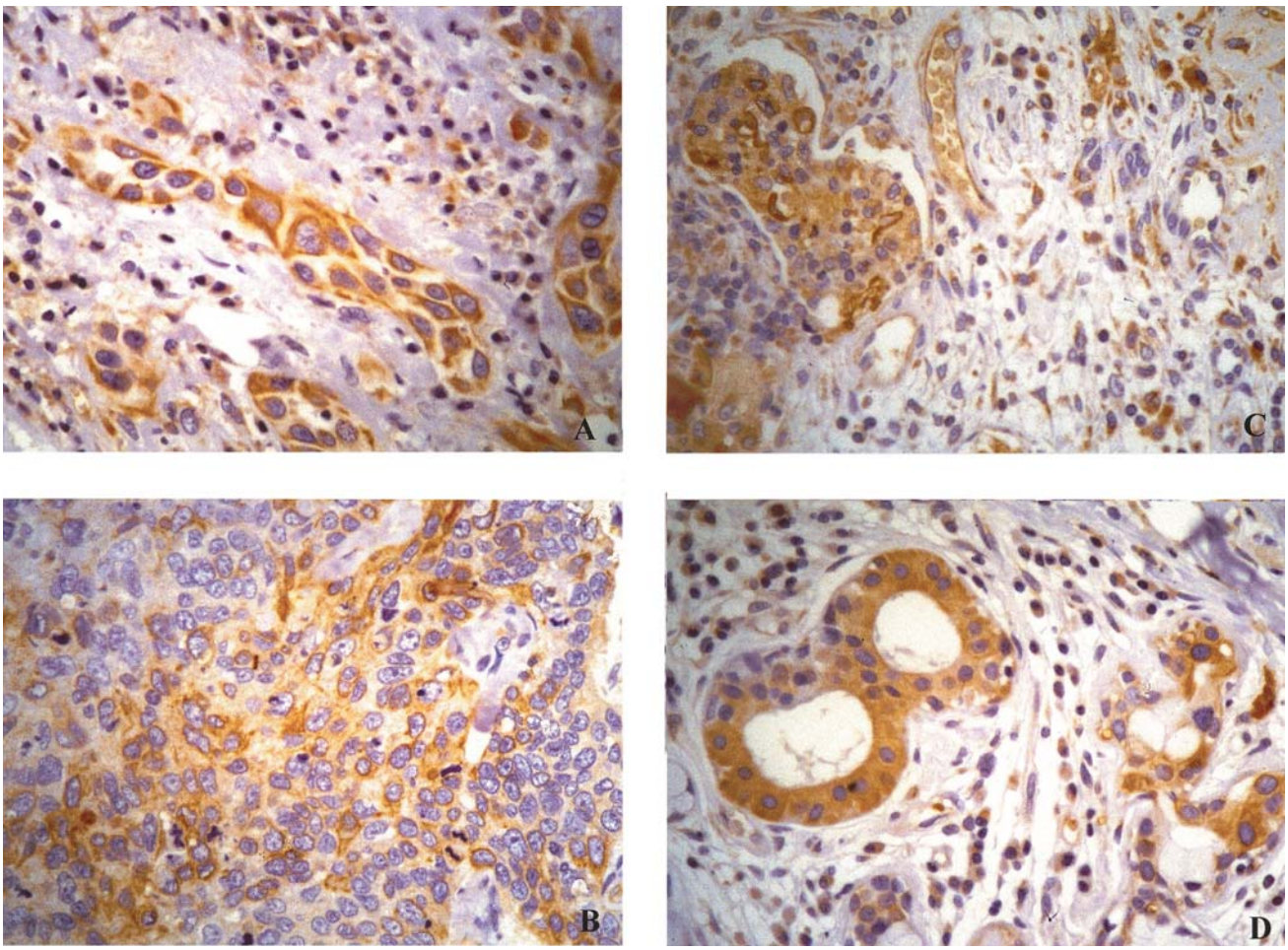


Figure 1A. *A case of oral SCC with high grade of differentiation showing membranous positivity for c-Met (200X).*
Figure 1B. *A case of oral SCC with low grade of differentiation showing intense cytoplasmatic positivity for c-Met (200X).*
Figure 1C. *A neoplastic embolus from an oral SCC with a low grade of differentiation showing an intense cytoplasmatic positivity for c-Met. Fibroblastic and endothelial cells in the stroma show a moderate positivity for c-Met (200X).*
Figure 1D. *Ductal cells of a salivary gland in the stroma adjacent to the tumor showing high cytoplasmatic expression of c-Met (200X).*

Lymph node-positive tumours showed a c-Met positivity in 17/21 (81%) cases, while lymph node-negative tumours showed a c-Met positivity in 40/52 (77%). These data show that expression of the Met/HGF receptor may be involved in the progression of OSCC towards the metastatic phase.

There was no statistically significant correlation between c-Met expression and age, sex, recurrences, staging or grading (Table I). Regarding size, small tumours (< 1.5 cm) had a lower score than large tumours (> 1.5 cm) and the differences were statistically significant ($p < 0.05$). Patients > 65 years had a higher score for c-Met.

When analysed for prognostic significance, patients with negative/low c-Met expression (score 0, 1, 2) had better survival rates than the group with high c-Met expression (Figure 2). This difference of survival rates was statistically significant ($p<0.05$).

## Discussion

The MET gene encodes the receptor for hepatocyte growth factor/scatter factor (HGFySF), which is known to stimulate the invasive growth of epithelial cells cultured *in vitro*. The Met/HGF receptor is a heterodimeric transmembrane tyrosine kinase, which is a prototype for a new family of growth factor receptors. The MET gene is a potentially harmful oncogene overexpressed in a significant fraction of human cancers. Studies on various carcinoma cell lines have shown that overexpression and structural alteration of the receptor result in its activation and confer tumorigenesis.

The fibroblast-derived activity responsible for increased invasion is the hepatocyte growth factor/scatter factor (HGFySF), a ligand for the c-Met receptor. HGFySF

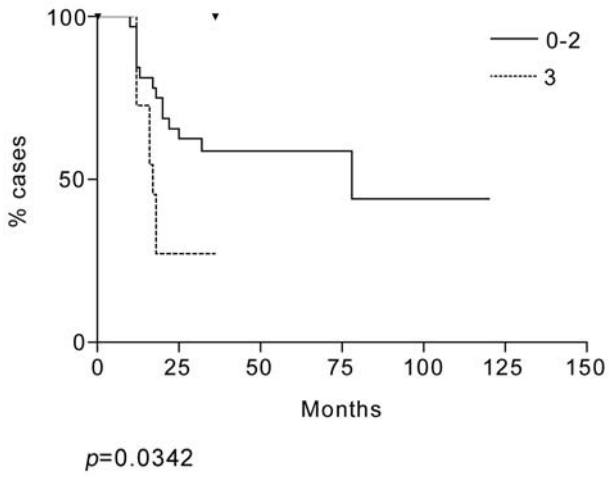


Figure 2. *Survival analysis was computed comparing negative (score 0)/reduced (score 1, 2) and positive (score 3) carcinomas for c-Met staining. Patients with negative/reduced score had better survival rates than patients with positive staining. This difference of survival rates was statistically significant ($p<0.05$).*

stimulated migration of the cells on various extracellular matrix substrates but did not alter their adhesion efficiency or integrin expression. HGFySF stimulated motility in a two-step process: initially cells spread rapidly and formed focal adhesions and then they disassembled these condensations, which was followed by increased cell locomotion(12). The focal adhesions contained vinculin, p125FAK, beta 1 integrin and phosphotyrosine. Within minutes after exposure of cells to HGFySF, proteins of 125 and 145 kDa showed elevated tyrosine phosphorylation and were identified as p125FAK and c-Met, respectively(12). Gradual loss of tyrosine phosphorylation coincided with disruption of focal adhesions and conversion to a motile phenotype.

Several studies in cell lines derived from squamous cell carcinomas and other tumours showed that HGF-induced activation of Met resulted in the acquisition of invasive properties.

Both Met and HGFySF are expressed in numerous tissues, although their expression is confined predominantly to cells of epithelial and mesenchymal origin, respectively(13). Signalling *via* this receptor-ligand complex has been shown to affect a wide range of biological activities, including cellular motility and growth, angiogenesis (14), invasion (15) and morphogenic differentiation (16,17). Met-HGFySF signalling also has been shown to be essential for normal murine embryological development (18-20) and is believed to play a role in tissue regeneration, wound healing (21) and the development of various organs. Whereas Met-HGFySF signalling clearly mediates a variety of normal cellular processes, this receptor-ligand pair also has been implicated in the generation and spread of tumours.

Met is highly expressed (22,23) and capable of inducing growth and morphogenic alterations in several normal and tumour tissues (16). For example, overexpression of MET protein was more often seen in well/moderately-differentiated than in poorly-differentiated squamous cell carcinoma of esophagus (5) compared with normal oesophageal epithelium, and this overexpression correlated with tumour differentiation in this tumour.

A recent study showed c-Met overexpression in oral squamous cancer(24), even if direct sequencing of Met mRNAs failed to find any activating mutation in its intracellular domain (24). Most frequently, Met activation is due to receptor overexpression, but also point mutations in the tyrosine kinase domain can lead to deregulated activation. In this study this receptor was overexpressed in all the primary tumours examined(24). These data confirmed early studies on c-Met overexpression in oral cancer *in vivo* (8) and *in vitro*(25).

Another study identified two somatic mutations in head neck squamous cell carcinoma, the Y1230C, known as a MET germline mutation which predisposes to hereditary renal cell carcinoma, and the Y1235D that is novel and changes a critical tyrosine known to regulate MET kinase activity (26). The mutated MET receptors are constitutively active and confer an invasive phenotype on transfected cells. Interestingly, cells carrying the MET mutations are selected during metastatic spread: transcripts of the mutant alleles are highly represented in metastases, but barely detectable in primary tumours. These data indicate that cells expressing mutant MET undergo clonal expansion during HNSCC progression and suggest that MET might be one of the long sought oncogenes controlling progression of primary cancers to metastasis (26).

Some authors proposed the use of the c-MET oncogene product as a marker of tumour cells in lymph node metastases to stage head and neck squamous cell carcinoma (6,9). All together these data suggest that the MET oncogene is involved in progression of squamous cell carcinoma toward an invasive-metastatic behaviour.

In this study the pattern of c-Met protein in normal oral mucosa by immunohistochemistry revealed its expression to be localized to the suprabasal layers and in cells of minor salivary glands (Figure 1D). In oral squamous cell cancers, definite c-Met positivity was present in 57 out of 73 (78%) cases and in 6 out of 6 (100%) lymph node metastases. In lymph node metastases, c-Met positivity was observed in both the primary site and the metastatic foci in the lymph nodes in 4 out of 4 cases (100%). There was a statistically significant positive correlation between c-Met expression and patient survival. When analysed for prognostic significance, patients with negative/reduced c-Met expression had better survival rates than the group with high c-Met expression and this difference of survival rates was statistically significant ($p<0.05$).

Regarding other clinopathological data, there was no statistically significant correlation between c-Met expression and sex, staging, grading or the presence of lymph node metastases, even if tumour without lymph node metastases had scored lower for c-Met than tumour with metastases. The frequency of lymph node metastases was higher in c-Met-positive tumours (17/57, 29%) than in c-Met-negative (4/16, 25%). This study showed that more than half of the oral squamous cell carcinoma showed an overexpression of c-Met, whereas normal oral epithelium showed absent or low/moderate c-Met positivity.

Weak to moderate c-Met staining was observed in vascular endothelial cells adjacent to the tumour. A study supposed a role of c-Met to promote neoangiogenesis in head and neck squamous cell carcinoma (27). The proangiogenic activity of hepatocyte growth factor (HGF)/scatter factor has been closely associated with its ability to stimulate endothelial cell chemotaxis, migration, proliferation and capillary formation. However, the potential of HGF as a paracrine factor in regulating the expression of angiogenesis factors by tumour cells is not widely appreciated. HGF induced a significant dose-dependent increase in IL-8 and/or VEGF cytokine production in eight HNSCC lines tested, which was not observed in normal keratinocytes (27). In addition, HGF increased mRNA expression of IL-8 in 3 out of 6 and VEGF in 5 out of 6 HNSCC lines (27). The increase in induction of these factors by HGF corresponded to an increase in phosphorylation of c-Met in HNSCC (27).

So it seems that there is a relationship between c-Met expression and some clinical-histological findings of oral cancer (see survival rates). Clearly these results support the need for a larger study to verify the possible role of c-Met as a prognostic factor able to identify patients at risk of more aggressive and disseminated disease. This may be potentially relevant for the implementation of closer follow-up protocols and/or alternative therapeutic regimens. Signalling *via* the Met-HGF complex seems to promote a unique biological program leading to "invasive growth." This phenotype results from the integration of distinct biological activities including cell proliferation, motility, extracellular matrix invasion and protection from apoptosis (28). Met-mediated invasive growth requires concomitant activation of multiple signalling pathways (29). In several cases HGFySF–Met overexpression has been correlated with tumour progression and/or metastasis(10, 30-32). Together, these studies and these data suggest that the creation of HGFySF–Met autocrine loops can be intimately associated with neoplastic transformation.

Our study also showed overexpression of c-Met in stromal fibroblastic cells, endothelial cells, minor salivary gland cells and in normal epithelium adjacent to the tumour. These findings support early reports providing evidence for up-regulation of c-Met receptor expression in both epithelial and stromal cells *via* autocrine and/or paracrine mechanisms(7,33,34). This activated molecular signalling may involve both neoplastic and adjacent non-neoplastic epithelial and stromal cells.

In conclusion these data suggest that c-Met expression may be useful in identifying cases of oral squamous cell carcinoma with a more aggressive and invasive phenotype. Since nodal status is often used as an index of aggressive clinical behaviour in squamous cell carcinomas (7), c-Met expression may provide an additional novel diagnostic and prognostic information on the survival of oral cancer patients.

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