MAP Kinase Modulation in Squamous Cell Carcinoma of the Oral Cavity

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Abstract. *Background: Oral squamous cell carcinoma (OSCC) represents the sixth most diffused cancer in developed countries. Mitogen-activated protein kinases (MAPKs) are proteins which transduce a vast array of extracellular signals into intracellular responses. The role of MAPK signalling pathway in cancer is not completely understood. Materials and Methods: In this study, we attempted to specifically evaluate the activation state of MAPK in OSCC. MAPK expression and activation were analyzed by immunoblotting in thirty tissue samples of OSCC and their paired nonneoplastic perilesional tissues. On the same tissues, the activation and expression of MAPK JNK/SAPK were also evaluated by ELISA assay. Results: Analyzing the levels of phospho-ERK1/2$^{p44/p42}$, a statistically significant reduction was observed in tumors compared to normal tissues. No statistically significant difference between tumor and control tissue was found for p38MAPK or JNK/SAPK. Conclusion: These results suggest that a reduction in ERK1/2$^{p44/p42}$ phosphorylation is correlated with tumor growth in OSCC.*

Oral cancer is the sixth most common cancer in the world and the incidence of new cases indicates a continuing rise in developing countries (1). About 30,000 new cases of oral cancers are diagnosed annually in the United States with about 7,500 resultant deaths (1). For the last four decades, the mortality rate from oral cancer has remained high (~50%), in spite of new treatment modalities (2). Over 90% of oral malignancies are histologically characterized as squamous cell carcinomas (SCC) (3). When diagnosed at an early stage, the prognosis of this cancer is favourable, with a 5-year survival rate of 60-80% . However, unfortunately only 15%-30% of patients present with stage I or II, while 60%-80% present with locoregionally advanced disease and, therefore, the prognosis is less favourable, with a 5-year survival rate of only 20-50% depending on the therapy (4).

In this context, new studies have been carried out in an attempt to better understand the molecular mechanism of the proliferation of SCC, with the aim of identifying molecular markers useful for diagnosing a tumor at an early stage. Several genetic alterations are associated with oral squamous cell carcinoma (OSCC) and, among them, the most frequent are the mutations of the oncosuppressors *p53* and *p16* (5), Ras oncoprotein (6) and EGF receptor (7). The overexpression of EGFR or Ras can lead to the deregulation of several intracellular signaling pathways, playing a key role in the development and progression of different kinds of cancer (8).

One of the major signaling routes downstream of EGFR is the mitogen-activated protein kinase (MAPK) pathway. MAPKs are a family of protein kinases by which cells transduce a vast array of extracellular signals into intracellular responses controlling proliferation, differentiation, cell motility and apoptosis. To date, three major MAPK subfamilies have been described in detail: ERK1 and 2 (extracellular signal regulated kinase 1 and 2), p38-MAPK and JNK/SAPK (c-Jun amino-terminal kinase/stress activated protein kinase), although other MAPK members have been identified *e.g.* ERK5 and ERK7 (9). Each MAPK pathway comprises a three-layer kinase module in which each element is activated by serial phosphorylations on tyrosine and threonine residues and inactivated by dephosphorylation by specific phosphatases (10).

ERK1 and 2, also called p44^{ERK1} and p42^{ERK2}, were first identified as growth factor activated protein kinases, and the constitutive activation of this pathway has been described as

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occurring following mutations of *Ras* or *Raf* oncogenes in different types of cancer (11). The activation of ERK results in the phosphorylation and activation of multiple cytoplasmatic substrates, such as downstream protein kinases, or cytoskeletal proteins. In addition, phosphorylated ERK1/2 can translocate to the nucleus and activate several transcription factors, including Elk-1, Sp-1 and AP-1, regulating the transcription of different genes. Although the ERK pathway was originally thought to be essentially associated with cell proliferation and differentiation, more recent observations show that this pathway regulates cellular responses ranging from survival to apoptosis (12-15).

The p38 MAPK represents a group of four kinases (α, β, γ, δ), identified by their ability to respond to a wide range of extracellular stressor stimuli such as osmotic shock, hypoxia, proinflammatory cytokines and UV irradiation (14).

The JNKs, also named SAPK, are activated in response to inflammation cytokines, growth factors and environmental stress such as ionizing radiation and DNA damage. Upon activation, JNK phosphorylates several transcription factors including ATF-2, c-Jun, Elk-1 and p53 regulating both differentiation and apoptosis (14). Although activation of JNK generally results in the promotion of cell death, in particular conditions it can also favour cell survival or tumor progression (15).

In a previous study, we found a deregulation of the MAPK pathway and, in particular, a selective reduction in the levels of phosphorylated forms of ERK1/2 in a set of laryngeal carcinomas (16). In this present study, we attempted to specifically evaluate the activation state of MAPKs in the OSCC in order to establish whether a derangement of the MAPK pathway occurs also in this context. We measured the levels of total and phosphorylated MAPKs in tumoral specimens in comparison with normal perilesional specimens from the same patient.

Materials and Methods

Thirty previously untreated patients were enrolled in this study, after giving written informed consent. Fresh tissue samples were obtained from surgical resection of oral cavity neoplasia at the Department of Otorhinolaryngology of the San Gerardo Hospital, Monza, Italy between March 2003 and April 2007. A histological examination classified all the tumor samples as SCC. For each case, a tumor sample excluding any possible necrotic center and a control sample corresponding to a specimen of mucosa taken from a site adjacent to the resection margins, free from neoplastic infiltration (established at a subsequent histological examination), were collected in cooperation with the Department of Pathology of the San Gerardo Hospital.

Based on the degree of differentiation of the tumor cells compared with their normal counterparts, and the number of mitoses within the tumor specimens, carcinomas were classified by grading them from 1 to 3: G1, well-differentiated; G2, moderately differentiated; and G3, poorly differentiated. Three biopsy specimens were G1, fifteen were G2 and twelve were G3. The mean age of patients was 60 years, with a range of 43-82 years; 26 were men and 4 were women.

Tissue samples were dissected, immediately frozen in liquid nitrogen and stored at –70˚C. Samples were homogenized on ice in lysis buffer [10% glycerol, 25 mM Tris-HCl (pH 7.5), 1% Triton® X-100, 5 mM EDTA, 1 mM EGTA], supplemented with freshly added proteases and phosphatase inhibitors (4 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate), and finally sonicated. Protein concentration was determined using the Bradford method. Tissue lysates were mixed with 5x Laemmli Buffer [β-mercaptoethanol 5% , SDS 10% , glycerol 50% , 400 mM Tris HCl (pH 6.8) and bromophenol blue 0.5%] and denaturated by heating to 95˚ for 5 minutes. Protein lysates (60 μg) were separated on a 13% SDS-PAGE and then electroblotted onto nitrocellulose membranes (Amhersham, Cologno Monzese, Italy).

After brief washing, the membrane was blocked with 5% nonfat milk for 1 hour at room temperature. Immunoblotting was performed with primary polyclonal antibodies anti-ERK1/2$^{p44/p42}$, anti-phospho-ERK1/2$^{p44/p42}$, anti-p38, anti-phospho-p38, anti-JNK/SAPK$^{p46/p54}$ and anti-phospho JNK/SAPK$^{p46/p54}$ (New England Biolabs Inc., Beverly, MA, USA) and anti-actin (Santa Cruz Biotecnology, CA, USA) followed by a secondary antibody (horseradish peroxidase-conjugated anti-IgG; PerkinElmer, Monza Italy). Finally, the immune complexes were visualized using the ECL chemiluminescence system digitized and quantified by densitometric analysis of nonsaturated autoluminographs. As a loading control, an actin immunoblot was performed.

Results are expressed as a relative expression (RE) considering the ratio between the optical density of the protein band under study and that of the corresponding actin band. Finally, the levels of expression of each protein in normal tissue were set to 1. The relative expression of the protein under study in each tumor sample was obtained comparing the value in the tumor sample with its paired normal tissue. In some samples, however, the immunoblot analysis did not produce bands of high enough quality to be efficiently quantified by densitometric analysis. Consequently, the number of cases analyzed for the different signaling effectors is not the same.

The level of total JNK/SAPK was also assayed using the BioSource JNK1/2 (total) kit, a solid-phase sandwich ELISA (BioSource International Inc, Camarillo, CA, USA).

The level of the activated forms of JNK/SAPK was also analyzed using the BioSource JNK1/2 [pTpY 183/185] kit, a solid-phase sandwich ELISA (BioSource International Inc, Camarillo, CA, USA).

The paired Student's *t*-test was used to analyze the differences in MAPK expression between cancer specimens and adjacent normal mucosa. For the various effectors considered, the levels of expression in each normal tissue were set to 1 and the relative change in the expression (fold increase or decrease) in each tumor was obtained in comparison with its paired normal tissue. A p-value <0.05 was considered statistically significant.

Results

In this study, we analyzed the level of expression and the activation status of some mitogenic signaling effectors in 30 cancer samples obtained from surgical resection of oral cancer neoplasia compared with adjacent normal mucosa. Using immunoblot analysis or ELISA assay, we evaluated the total protein levels of ERK1/2$^{p44/p42}$, p38 and

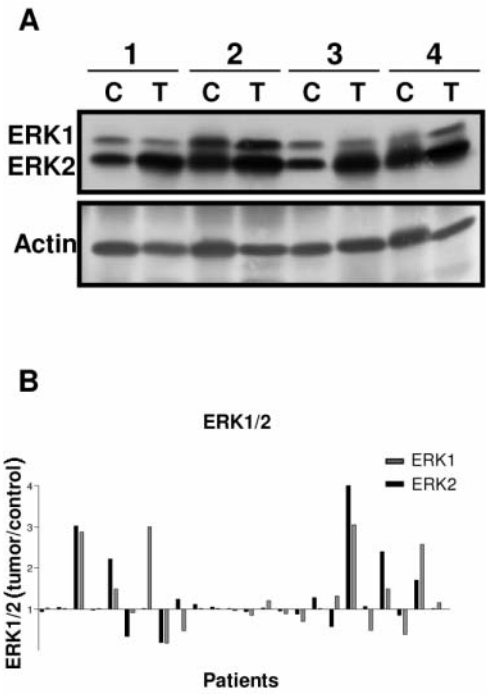

Figure 1. *Expression of the total amount of ERK1/2$^{p44/p42}$ in oral cavity tumors. Comparing tumors (T) with their paired normal tissues (C) no statistically significant difference emerges. A, Representative immunoblotting analysis showing expression of total amount of ERK1/2$^{p44/p42}$ in oral cavity tumors and paired normal tissues. B, Expression of total amount of ERK1/2$^{p44/p42}$ was quantified by densitometric analysis of autoluminographs. To control for differences in protein loading, the expression of the total amount of ERK1/2$^{p44/p42}$ was normalized to the expression of actin in each sample. The graph shows the average relative expression for each tumor/normal tissue pair.*

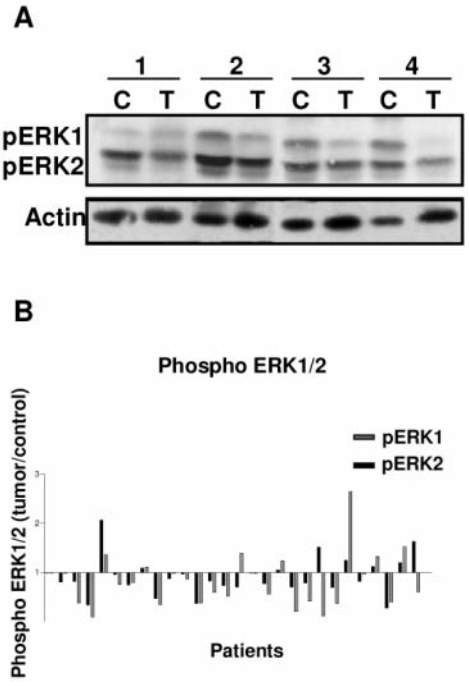

Figure 2. *The levels of phospho-ERK1/2$^{p44/p42}$ are statistically reduced in oral cavity tumors (T) compared to their paired normal tissues (C). A, Representative immunoblotting analysis showing expression of phospho-ERK1/2$^{p44/p42}$ in oral cavity tumors and paired normal tissues. B, Expression of phospho-ERK1/2$^{p44/p42}$ was quantified by densitometric analysis of autoluminographs. To control for differences in protein loading, the expression of phospho ERK1/2$^{p44/p42}$ was normalized to the expression of actin in each sample. The graph shows the average relative expression for each tumor/normal tissue pair.*

JNK/SAPK$^{p46/p54}$, as well as the levels of the activated (phosphorylated) form of the same proteins. By using immunoblotting, the different MAPK isoforms were detected in every tissue sample examined.

When we analyzed the levels of phospho-ERK1/2, a statistically significant difference was noted in tumors compared to normal tissues. In fact, while the levels of the total protein for both ERK1^{p44} and ERK2^{p42} were not statistically different between tumor and control specimens (Figure 1), a remarkable reduction in the levels of the phosphorylated proteins was observed in tumor samples with respect to controls (Figure 2). The average (±S.D.) phospho-ERK1 expression in cancer and normal tissues was respectively 0.5675±0.3567 and 0.8036±0.4591, (p<0.05), while the average (±S.D.) phospho-ERK2 expression level was 1.186±0.7791 and 1.455±1.072 in cancer and normal tissue, respectively (p<0.05). Figure 2B shows the ratio between the levels of phosphorylated ERK1/2 in tumors and control samples. The modulation of both isoforms appears to be closely related; in fact, only 3 patients show an inverse modulation between the two isoforms. A marked reduction in the phosphorylation levels of both ERK1 and ERK2 was observed in the majority of patients, whereas only three patients showed an increase in phospho-ERK1 and phospho-ERK2 levels.

On the contrary, no statistically significant differences between cancer specimens and paired controls were evident for p38-MAPK, neither in the levels of total protein (Figure 3), nor in the levels of the phosphorylated form (Figure 4). Samples analyzed showed a great variability, particularly in the levels of the activated form. In fact, a remarkable increase was observed in four tumor samples, whereas a reduction was noted in six other samples; the values of the remaining samples did not show any significant variance between tumors and controls (Figure 4B).

Immunoblot analysis of the activated form of both isoforms of JNK/SAPK (p46/p54) did not provide satisfactory results for analysis, so we performed an ELISA assay to quantify the activation and the total protein levels. The results obtained did not show any

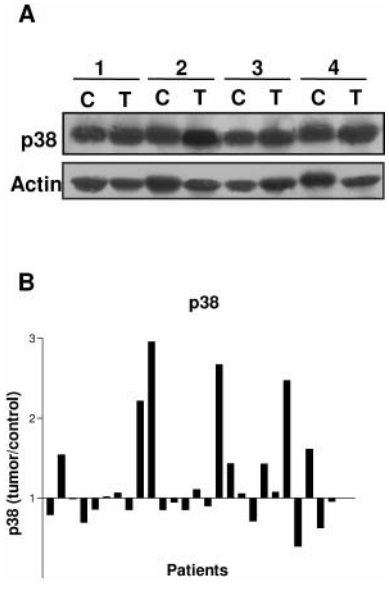

Figure 3. *Western blot analysis of the total amount of p38 in oral cavity tumors. Comparing tumors (T) with their paired normal tissues (C), no statistically significant difference emerges. A, Representative immunoblotting analysis showing expression of the total amount of p38 in oral cavity tumors and paired normal tissues. B, Expression of the total amount of p38 was quantified by densitometric analysis of autoluminographs. Expression of the total amount of p38 was normalized to to the expression of actin in each sample to avoid differences in protein loading. The graph shows the average relative expression for each tumor/normal tissue pair.*

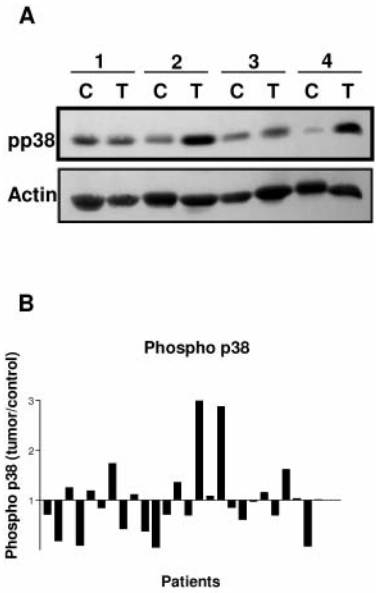

Figure 4. *Western blot analysis of phospho-p38 in oral cavity tumors. Comparing tumors (T) with their paired normal tissues (C), no statistically significant difference emerges. A, Representative immunoblotting analysis showing the expression of phospho-p38 in oral cavity tumors and paired normal tissues. B, The expression of phospho-p38 was quantified by densitometric analysis of autoluminographs. To control for differences in protein loading, the expression of phospho-p38 was normalized to the expression of actin in each sample. The graph shows the average relative expression for each tumor/normal tissue pair.*

statistically significant difference. In fact, the level of expression of phospho-JNK/SAPK was 0.6484±0.7587 (mean OD±S.D.) in controls and 0.7817±0.9912 (mean OD±S.D.) in tumors.

Discussion

The aim of this study was to characterize the modulation of MAPK in OSCC and so we evaluated the levels of total proteins and the levels of their activated forms in tumor specimens and in corresponding control tissue.

Many oncogenic proteins are known to participate in the cytoplasmic signaling cascade. The MAPK pathway regulates all the critical phases of cell growth, including proliferation, differentiation and apoptosis, and a deregulation of this pathway has been reported in several types of tumor. We demonstrated a significant reduction in the levels of the activated (phosphorylated) forms of ERK1/2 in tumor specimens compared with control perilesional tissues, while no significant difference in either expression or activation of the other MAPKs was noted.

In previous studies, carried out on a set of SCC specimens of the larynx, we found the same down-regulation of the activated forms of ERK1/2 (16). Even though the results of our study are in disagreement with several published studies reporting the activation of the ERK pathway in different types of cancer, including melanoma, prostate, colorectal and gastric carcinoma (17-20), they are in complete agreement with the report of Tosi and colleagues who, in a heterogeneous set of HNSCC, found a down-regulation of the levels of the activated forms of MAPKs in pathological samples (21). Our results are also consistent with Paweletz *et al.* who reported a reduction in ERK1/2 activation in clinical samples of prostate cancer isolated by laser microdissection (22). These discrepancies in reported results may depend on a different modulation of MAPK according to tumor type and their anatomical localization. However, the overexpression of ERK1/2 has also been reported in OSCC (23). Moreover, Albanell *et al.* found the activation of ERK1/2 in pathological samples of HNSCC, although this study was probably limited by the lack of a comparison with normal tissue (24). Wang *et al.* reported a stronger activation of ERK1/2 in SCC of the tongue than in normal mucosa, although the fact that the comparison was not carried out on paired controls should be taken into consideration (25).

These differences in results may also be explained by differences in the methods employed by the different groups to evaluate MAPK expression and activation, mainly based on immunohistochemistry (23-25) and on more quantitative assays for the other groups (16, 21, 22).

Furthermore, it is possible that MAPK and, in particular, ERK activation may be modulated according to the differentiation stage of the tumor. Consistent with this hypothesis, a decrease in ERK phosphorylation has been reported in advanced poorly differentiated prostate cancer, compared with specimens at an earlier stage (26, 27).

It is well known that differences in the temporal activation of MAPK may result in different and sometimes opposite cellular outcomes. It is, therefore, possible that transient activation of ERK may, for instance, trigger a proliferative signal, while sustained ERK phosphorylation may result in differentiation (28). In fact, even if ERK activation is required for G_0/G_1 phase transition, playing a central role in the cell cycle entry, there is considerable evidence indicating that sustained ERK activation can trigger cell cycle inhibition and a pro-differentiation signal (29), also in cells of epithelial origin (30). Therefore, it is possible, as suggested by Paweletz *et al*., that the loss of sustained ERK phosphorylation may favour cell proliferation during cancer progression (22).

The activation of ERK during cancer growth has usually been related to a poor outcome. In addition, some reports have demonstrated that ERK pathway activation can favour cell motility and migration of neoplastic cells (31, 32). However, the role of ERK signaling in the prognosis of tumors is not clearly understood (33). For instance, a recent study has demonstrated that ERK activation in endometrial cancer is associated with a favourable prognosis (34), while Matsuura *et al*. found that there was no correlation between ERK activation in a set of uterine cervical carcinoma and Ki-67 expression, a marker of cell proliferation (35). This suggests that ERK may play a role in tumor growth beside proliferation and they also reported a significant down-regulation of the levels of phosphorylated ERK1/2 in invasive carcinoma (35).

Recent studies have even demonstrated the ability of the ERK pathway to transduce a pro-apoptotic signal by phosphorylating and activating bax and caspase-3 (36, 37). It has recently been reported that ERK phosphorylation and PARP and caspase-3 activation were significantly down-regulated in uterine cervical carcinoma, compared with normal tissue (26). In the same way, Schweyer *et al*. found a down-regulation of the activated forms of ERK1/2 and an up-regulation of their specific phosphatase PP2A in malignant testicular germ cell tumors (12). They also demonstrated that phosphorylation of ERK is required for the induction of caspase-3-mediated apoptosis in two malignant cell lines (12). It is possible, therefore, that the reduction in ERK activation found in OSCC may be related to an inhibition of apoptosis in cancer cells.

In conclusion, the results of this study, confirming our previous published findings in SCC of the larynx, indicate an alteration of MAPK activation also takes place in OSCC suggesting that the level of activation of ERK1/2 may be a valuable marker in the prognosis of OSCC. However, further investigations are required to find a functional interpretation of these results.

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