

# Survivin phosphorylation and M-phase promoting factor in oral carcinogenesis

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**Summary.** Survivin is a recently described inhibitor of apoptosis and mitotic regulator which is selectively over-expressed in human tumors. Its expression rate is predictive of disease progression, early recurrences and resistance to therapy. Up-regulation of survivin in oral pre-malignant lesions (OPL) and in oral squamous cell carcinoma (OSCC) has already been demonstrated in previous studies. A critical step for activation of survivin has been identified in the phosphorylation on Thr<sup>34</sup> by the main mitotic kinase p34<sup>cdc2</sup>-cyclin B1. The aim of this work was to investigate the relationship between survivin, its phosphorylated active form (p-survivin) and M-phase promoting factor (MPF), p34<sup>cdc2</sup>-cyclin B1 in oral carcinogenesis. 32 OSCCs and 17 OPLs from surgical specimens were studied for cyclin B1, p-survivin, survivin, and p34<sup>cdc2</sup> expression by immunohistochemistry. All cases of OSCC expressed survivin and its expression rate was correlated to p-survivin levels (P<0.05). Cyclin B1 was positive in 80% of cases, while p-34<sup>cdc2</sup> was over-expressed in all OSCCs. All OPLs associated with OSCC expressed survivin and its levels were correlated to p-survivin levels (P<0.05). Cyclin B1 was positive in 70% of cases, while p-34<sup>cdc2</sup> was positive in all OPLs. In conclusion, this study demonstrated that MPF, survivin and p-survivin are expressed during early and late phase of oral carcinogenesis. MPF proteins, which are co-expressed on mitotic apparatus, could represent a potential target for therapies based on manipulation of survivin phosphorylation, which would induce apoptosis in cancer cells.

**Key words:** OSCC, Survivin, p34<sup>cdc2</sup>, Cyclin-B1, p-survivin, Apoptosis, MPF

## Introduction

Survivin is a recently described inhibitor of apoptosis and mitotic regulator which is selectively over-expressed in human tumors; its expression rate is predictive of disease progression, early recurrences and resistance to therapy. Up-regulation of survivin in oral pre-malignant lesions (OPL) and in oral squamous cell carcinoma (OSCC) has already been demonstrated in previous studies (Campisi and Margiotta, 2001; Lo Muzio et al., 2001, 2003a,b, 2004, 2005a,b; Chen et al., 2005; Kim et al., 2005; Lin et al., 2005). Interestingly, these studies outlined that expression of survivin is an early event during oral carcinogenesis and that survivin, which is over-expressed in more than 80% of OSCCs, might be a potential prognostic marker in these tumors (Campisi and Margiotta, 2001; Lo Muzio et al., 2001, 2003a,b, 2004, 2005 a,b; Tanaka et al., 2003; Chen et al., 2005; Kim et al., 2005; Lin et al., 2005). Furthermore, the fact that survivin is over-expressed in most human malignancies and its very low or absent expression rates in the corresponding normal tissues make survivin an excellent target for new therapies which could disrupt cell viability pathways in cancer (Ambrosini et al., 1998). Potential cancer therapies proposed and reviewed by D. Altieri and co-workers are based on inhibition of survivin activation. This is a major point to be addressed since there are few studies concerning the expression of active p-survivin in tumors and no studies about the expression and distribution of p-survivin in oral malignancies. Expression of survivin is regulated both in cell cycle-independent and dependent pathways. The cell cycle-independent pathway, which is cytokine-dependent, is restricted to CD34+ stem cells during hematopoiesis (Saito et al., 2001) and endothelial cells during neoangiogenesis. In the other cell types, the expression of survivin is regulated in a cell cycle-dependent way so that this protein is selectively

expressed at G2/M transition and prevents the cell from apoptotic death during mitoses. A critical requisite for survivin function was identified in the phosphorylation on Thr<sup>34</sup> by the main mitotic kinase p34<sup>cdc2</sup> – cyclin B1. The aim of this study was to determine the expression of p-survivin, p34<sup>cdc2</sup> and cyclin B1 and their relationship with survivin in oral carcinogenesis.

## Materials and methods

### Selection of cases

32 oral squamous cell carcinomas (OSCCs) and 17 dysplasias from areas near to OSCC were selected for this study. According to the model of common clonal origin of synchronous primary SCC, the study of dysplasia near to OSCC, together with the respective OSCC, represents a good experimental model to analyze both early and late event of oral carcinogenesis (Bedi et al., 1996; Califano et al., 1996; Carey, 1996).

All the patients received surgical treatment with curative intention. Clinical data were reviewed to record sex, age of patients and their follow-up status (Table 1). Clinical and pathological staging was determined according to the TNM classification of the International Union against Cancer (UICC) (International Union Against Cancer, 2000).

Dysplasia was classified according to WHO classification (Pindborg et al., 1997). If dysplastic modifications were observed in the basal third, in the basal two thirds or in the whole thickness of the epithelium, dysplasia was scored as mild, moderate, or severe, respectively. According to these criteria, two pathologists classified our lesions as mild (5 cases), moderate (10 cases), or severe dysplasia (2 cases). Normal tissues were used as a control group.

### Immunohistochemistry

Four µm serial sections from formalin fixed and paraffin-embedded blocks were cut and mounted on poly-L-lysine-coated glass slides. Immune-staining was performed by linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP) using specific antibodies (Abs). The Abs utilized were: 1) 1:200-diluted rabbit polyclonal anti-survivin (AB469 - Novus Biological, UK) for 120 min at 24°C; 2) 1:25-diluted mouse monoclonal anti-cyclin B1 (Novocastra Ltd, Newcastle, UK) overnight; 3) 1:300-diluted anti-p-survivin (Santa-Cruz Biotechnology, USA) for 60 min at 24°C; 4) 1:250-diluted mouse monoclonal anti-p34<sup>cdc2</sup> (Zymed) for 120 min.

Negative control slides without primary antibody were included for each staining.

### Evaluation

Immunostained sections were analyzed with a double-headed Leitz light microscope, using a 40x objective. In this analysis the expression level of the

proteins was measured by evaluation of the percentage of cells which were positive for each antibody in at least 4 random high power fields. The mean fraction of the positive cells was graded as follow: 0 (<5%), 1 (6-25%), 2 (26-50%), 3 (51-75%), 4 (76-100%). All the sections were examined without previous knowledge of the clinical and follow-up data of each case. Inter-rate reliability between the two investigators examining the immune-stained sections was assessed by the Cohen's K test, yielding K values higher than 0.70 in almost all instances.

Regarding dysplastic specimens inter-rate reliability between the two pathologists was obtained evaluating and discussing simultaneously the cases with different assessments until a final unique diagnosis was reached.

### Statistical analysis

The data were analyzed by the Stanton Glantz statistical software 3 (MS-DOS). Differences between the groups were determined using the one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. Pearson's method was used to study linear correlation. Only p values <0.05 were considered significant.

## Results

Data about expression of survivin (svv), p-survivin (p-svv), cyclin-B1, p34<sup>cdc2</sup> in oral carcinogenesis and their clinical-pathological relation are shown in table 2 and 3.

### Survivin expression

#### Premalignant lesions

Our study showed survivin immunostaining in all cases of oral pre-malignant lesions with a mean score of

**Table 1.** Patients characteristics.

| Gender  | Male | 20           | Female | 12    |      |
|---------|------|--------------|--------|-------|------|
| Age     |      | Mean (years) | SD     | Range |      |
| Male    |      | 62.2         | 8.85   | 43-72 |      |
| Female  |      | 62           | 19.1   | 20-81 |      |
| Grading |      | G1           | 3      |       |      |
|         |      | G2           | 15     |       |      |
|         |      | G3           | 14     |       |      |
|         |      | N0           | N1     | N2    | Tot. |
| T1      | 11   | 1            | 4      | 16    |      |
| T2      | 8    | 2            | 1      | 11    |      |
| T3      | 0    | 1            | 0      | 1     |      |
| T4      | 3    | 0            | 1      | 4     |      |
| Tot.    | 22   | 4            | 6      | 32    |      |

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1.44 (SD 0.49). This score was statistically significant if it is compared to normal controls ( $P<0.05$ ). Interestingly, except for one case of severe dysplasia showing score 3, different lesions showed an homogeneous low score, which demonstrated that survivin is expressed in oral lesions surrounding tumors independently from histological diagnosis of pre-malignant lesion. The intracellular localization of survivin detected by polyclonal Ab was prevalently cytoplasmic and focally nuclear.

### Oral squamous cell carcinoma

This study showed survivin immunostaining in all cases of oral SCC (100%). The expression of survivin in OSCC was heterogeneous in different cases (range 1-4), with an overall high immune-staining of the protein (mean score 2.77, SD 1.15), and significantly higher than in normal epithelium and dysplasias ( $P<0.05$ ) (Table 2). The intracellular localization of survivin in tumor cells was prevalently cytoplasmic and focally nuclear. Statistical evaluation of survivin expression revealed significantly high expression of this protein in the poorly differentiated group of tumors compared to

well and moderately differentiated ones (One way analysis of variance:  $P=0.008$ ; Student-Newman-Keuls' test:  $P<0.05$ ). Analysis of expression levels of survivin compared to other clinical-pathological findings such as age, sex, site, tumor size, lymph nodal involvement and TNM staging, did not reveal further statistically significant differences in this randomly chosen sampling of tumors.

### P-survivin expression

#### Premalignant lesions

P-survivin detected by polyclonal antibody was expressed in 16/17 cases of pre-malignant lesions surrounding tumors (mean score 1.29, SD 0.29). This score was statistically significant if it is compared to normal controls ( $P<0.05$ ). Furthermore, a statistically significant linear correlation between expression of p-survivin and expression of survivin in corresponding slides was observed (Pearson's correlation:  $r=0.641$ ,  $P=0.006$ ). The p-survivin was detected both in nucleus and cytoplasm of positive cells. Mitotic cells localized in basal and para-basal layers of epithelial dysplasia

**Table 2.** Mean and standard deviation of survivin, p-survivin, cyclin-B1, p34 cdc2 scores in normal epithelium, dysplasia surrounding tumors and Oral Squamous Cell Carcinoma (OSCC).

| Tested Proteins     | Group A) Normal epithelium (n =17) |                     | Group B) Dysplasia (n=17) |                     | Group C) OSCC (n=32) |                     | Comparison among groups (A vs B vs C)<br>Statistical significance ( $P<0.05$ ) |
|---------------------|------------------------------------|---------------------|---------------------------|---------------------|----------------------|---------------------|--|
|                     | Positive cases (%)                 | Mean score $\pm$ SD | Positive cases (%)        | Mean score $\pm$ SD | Positive cases (%)   | Mean score $\pm$ SD |  |
| Survivin            | 15                                 | 0.5 $\pm$ 0.2       | 100                       | 1.44 $\pm$ 0.49     | 100                  | 2.77 $\pm$ 1.15     | Yes  |
| p-survivin          | 15                                 | 0.6 $\pm$ 0.3       | 94                        | 1.29 $\pm$ 0.29     | 97                   | 2.48 $\pm$ 1.31     | Yes  |
| cyclin-B1           | 10                                 | 0.5 $\pm$ 0.5       | 70                        | 0.71 $\pm$ 0.47     | 81                   | 0.97 $\pm$ 0.6      | No   |
| p34 <sup>cdc2</sup> | 10                                 | 0.4 $\pm$ 0.4       | 100                       | 1.88 $\pm$ 0.78     | 100                  | 3.03 $\pm$ 0.87     | Yes  |

**Table 3.** Statistical analysis of survivin, p-survivin, cyclin-B1, p34 cdc2 scores in 32 OSCC grouped according to clinical-pathologic findings.

| Groups of OSCC analysed for proteins tested  | Proteins tested      | Mean $\pm$ SD   |                 | Statistical analysis ( $P<0.05$ ) |
|--|----------------------|-----------------|-----------------|-----------------------------------|
|  |                      | Group A         | Group B         |                                   |
| A) TNM stages I and II (N. 18) vs B) TNM stages III and IV (N.14)                              | Survivin             | 2.53 $\pm$ 1.18 | 3.07 $\pm$ 1.07 | No                                |
|  | P-survivin           | 2.47 $\pm$ 1.28 | 2.50 $\pm$ 1.40 | No                                |
|  | Cyclin B1            | 1.22 $\pm$ 0.55 | 0.79 $\pm$ 0.58 | Yes                               |
|  | P-34 <sup>cdc2</sup> | 3.00 $\pm$ 0.84 | 3.07 $\pm$ 0.92 | No                                |
| A) Histological well and moderately differentiated (N. 18) vs B) Poorly differentiated (N. 14) | Survivin             | 2.29 $\pm$ 1.11 | 3.36 $\pm$ 0.93 | Yes                               |
|  | P-survivin           | 2.47 $\pm$ 1.20 | 2.50 $\pm$ 1.40 | No                                |
|  | Cyclin B1            | 0.94 $\pm$ 0.54 | 1.00 $\pm$ 0.68 | No                                |
|  | P-34 <sup>cdc2</sup> | 3.17 $\pm$ 0.71 | 2.86 $\pm$ 1.03 | No                                |
| A) Lymph node negative (N. 21) vs B) Lymph node positive (N. 11)                               | Survivin             | 2.55 $\pm$ 1.19 | 3.18 $\pm$ 0.98 | No                                |
|  | P-survivin           | 2.60 $\pm$ 1.27 | 2.27 $\pm$ 1.42 | No                                |
|  | Cyclin B1            | 0.95 $\pm$ 0.15 | 1.00 $\pm$ 0.45 | No                                |
|  | P-34 <sup>cdc2</sup> | 2.95 $\pm$ 0.92 | 0.75 $\pm$ 0.23 | No                                |

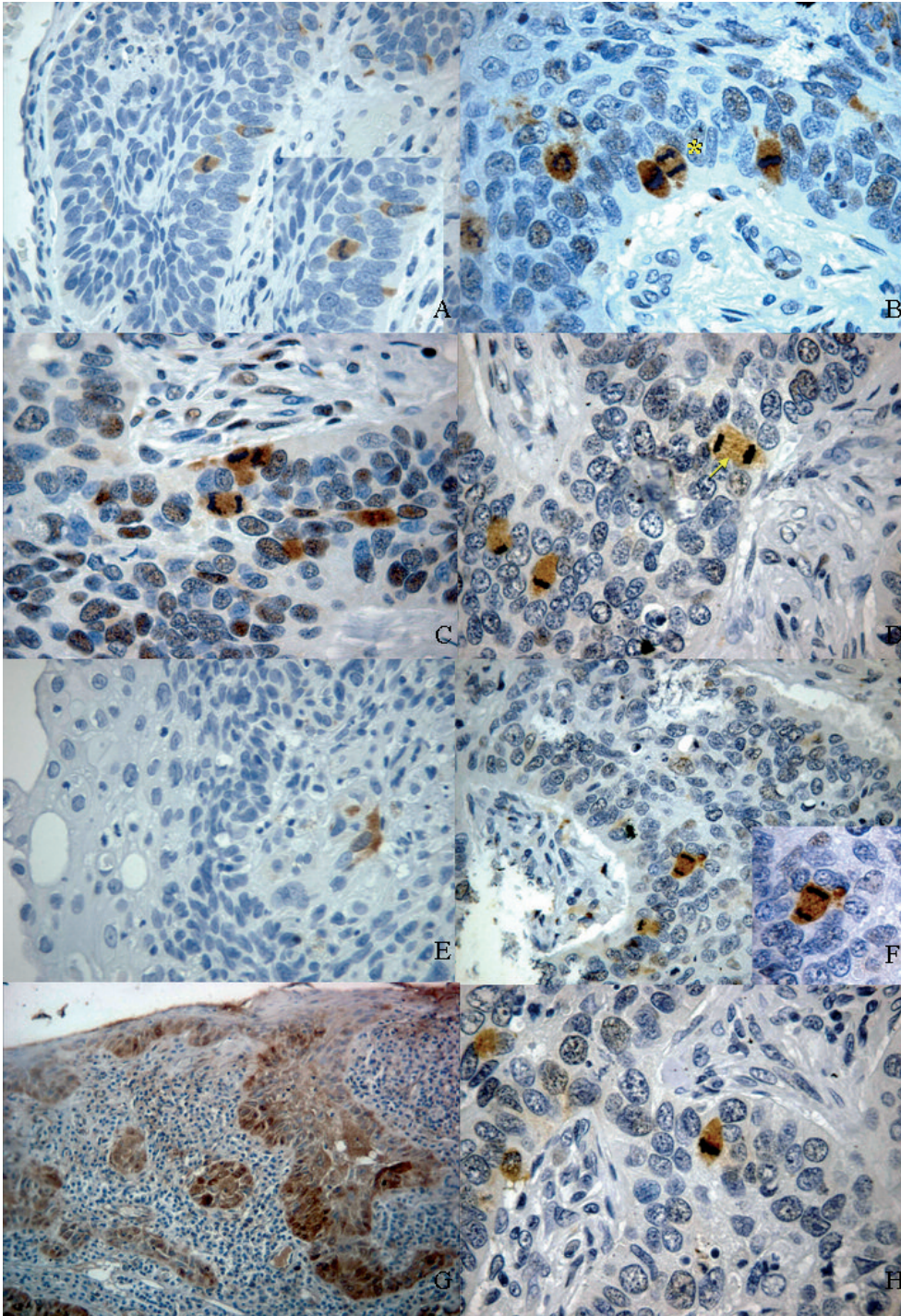


surrounding tumors showed strong cytoplasmic expression of p-survivin (Fig. 1A-B).

#### Oral squamous cell carcinoma.

An overall high level of expression of p-survivin was observed in 30/31 of tested cases (96.77%) (mean

score 2.48, SD 1.31). Therefore, compared to normal controls and dysplasias the over-expression of p-survivin in OSCC was statistically significant ( $P < 0.05$ ). Furthermore, a statistically significant linear correlation between expression of p-survivin and expression of survivin in corresponding slides was observed (Pearson's correlation:  $r = 0.474$ ,  $P = 0.007$ ). Strong immune-staining



**Fig. 1.** Expression of p-survivin in oral dysplasia and OSCC **A-D**. The over-expression of p-survivin is an early event during oral carcinogenesis since it has been observed in oral dysplasia. Single stained mitotic cells localized in basal and parabasal layers of epithelial dysplasia surrounding tumors showed strong cytoplasmic expression of p-survivin. Interestingly p-survivin is highly expressed both in normal (\*) appearing and aberrant mitoses in oral dysplasia (**A**, x 250; **B**, x 400) and in OSCC (**C**, x 400; **D**, x 400). Note the high expression in late mitosis, particularly at metaphase and anaphase. A filamentous pattern corresponding to spindle microtubules may be seen (arrow). Expression of cyclin B1 in oral dysplasia (**E**) and OSCC (**F**). The subcellular localization was prevalently cytoplasmic with focal nuclear positivity. The tissue staining pattern was prevalently basal and parabasal in dysplasias (**E**, x 250). Cyclin B1 positive cells in proliferating tumor tissue (**F**, x 250) correspond to M-phase activation and hence increased proliferation of the tumor cells. Expression of p34<sup>-cdc2</sup> in OSCC **G, H**. The p34<sup>-cdc2</sup> positive cells were mainly localized at the infiltrating edge of the tumor (**G**, x100). P34<sup>-cdc2</sup> was present both in the cytoplasm and in the nucleus of neoplastic cells, with an overall strong intensity of staining. In mitotic cells p34<sup>-cdc2</sup> shows elevated level of expression (**H**, x 400), which in turn give rise to survivin phosphorylation on Thr-34, during spindle checkpoint, resulting in increased survivin expression and cancer cell viability (LSAB-HRP, nuclear counterstaining with haematoxylin. Further magnifications are in inset).



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for p-survivin was detected both in nuclei and cytoplasm of positive tumor cells. Nuclear expression was observed in inter-phase, while mitotic cells of tumors showed strong cytoplasmic expression of p-survivin during prophase, metaphase, and anaphase (Fig. 1C,D). At high power magnification this immunostaining was localized on the mitotic spindle of neoplastic cells and clearly detectable during anaphase (Fig. 2).

#### *Cyclin B1 expression*

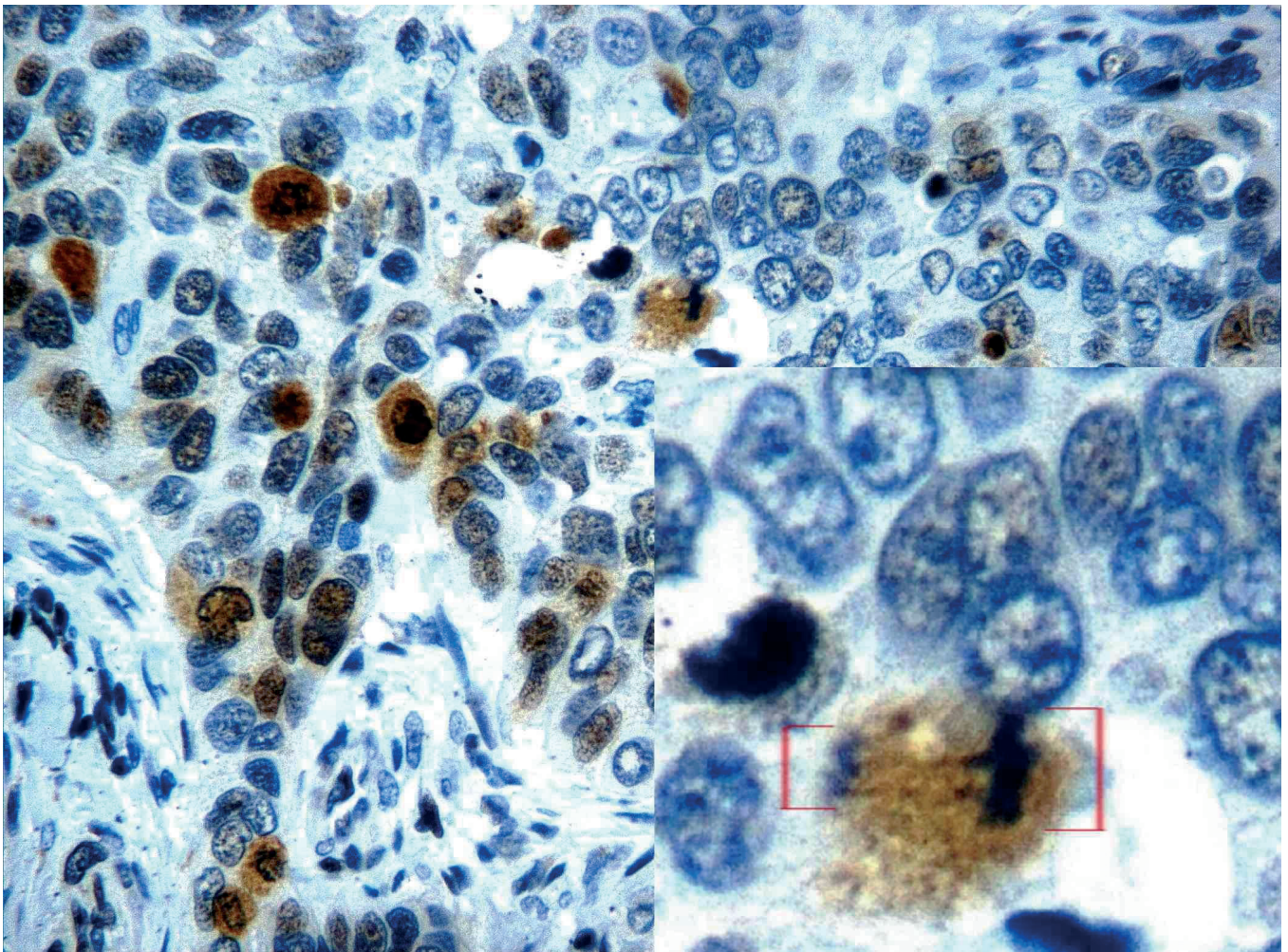
##### Premalignant lesions

Monoclonal Ab detected cyclin B1 positive cells in 12/17 cases (70%) of pre-malignant lesions surrounding tumors (mean score 0.71, SD 0.47) (Fig. 1 E). The sub-

cellular localization was prevalently cytoplasmic with focal nuclear positivity. The tissue staining pattern was prevalently basal and para-basal in slight dysplasias, with more superficial layers stained only in moderate and severe dysplasias.

##### Oral squamous cell carcinoma

The specific monoclonal antibody against cyclin B1 detected positive cells in 26/32 cases (81.25%) of malignant lesions. The mean score value for cyclin-B1 in OSCC was lower than that of the other tested proteins (mean score 0.97, SD 0.6) (Fig. 1F.) Statistical analysis showed no significant difference from dysplasia, probably because of the small number of cases tested. However, these values are remarkable because the expression of cyclin-B1 is restricted to the mitotic phase



**Fig. 2.** P-survivin and cell aneuploidy. P-survivin is highly expressed in neoplastic cells of OSCC contributing to genomic instability. Note that phospho-survivin immune-staining of aberrant mitoses show a widely unequal segregation of DNA in the future daughter cells. Magnification in the low right highlights the role of p-survivin in the maintenance and amplification of DNA aneuploidy in cancer cell. This mitosis, by-passing cell check-points and apoptosis, may generate new aneuploid clones of cancer cells endowed with aberrant karyotype and avoiding programmed cell death (LSAB-HRP, x 600; DNA counterstaining with haematoxylin).

of the cell-cycle. Therefore, cyclin B1 positive cells in proliferating tumour tissue represent a subset of Ki-67 positive cells. There was a heterogeneous distribution of cyclin-B1 immunostaining: cells located in peripheral regions of the cancer nests showed a higher expression than the ones located in a central position. The sub-cellular localization was prevalently cytoplasmic with focal nuclear staining. Statistical evaluation of cyclin-B1 expression revealed significantly low expression of this protein in the advanced Stages III and IV group of tumours compared to Stages I and II ones (One way analysis of variance:  $P=0.04$ ; Student-Newman-Keuls' test:  $P<0.05$ ).

#### *p34<sup>cdc2</sup>* expression

##### Premalignant lesions

Immunohistochemical staining for p34<sup>cdc2</sup> was detected in all cases of tested OPLs (mean score 1.88, SD 0.78). The percentage of stained cells was significantly higher than in normal epithelium ( $P<0.05$ ). However, the tissue staining pattern in OPL was quite similar to that observed in normal epithelium, where p34<sup>cdc2</sup> was localized in the cytoplasm of cells of basal and parabasal layers. Furthermore, intermediate and superficial layers were stained only in moderate and severe dysplasias. Statistical analysis of survivin, p-survivin, cyclin-B1, and p34<sup>cdc2</sup> in this group of OPL did not show significant differences if compared to histopathological grading of dysplasia (data not shown).

##### Oral squamous cell carcinoma

The main mitotic kinase p34<sup>cdc2</sup> was up-regulated in all the cases of OSCC (mean score 3.03, SD 0.87), with heterogeneous expression in different cases (score range 1-4) (Fig. 1G-H). Comparing to normal controls and dysplasia this score was statistically significant ( $P<0.05$ ). The p34<sup>cdc2</sup> positive cells were mainly localized at the infiltrating edge of the tumor. p34<sup>cdc2</sup> was present both in the cytoplasm and in the nucleus of neoplastic cells, with an overall strong intensity of staining. In mitotic cells p34<sup>cdc2</sup> was localized in the cytoplasm and was co-expressed together with p-survivin and cyclin B1.

## Discussion

Several studies have demonstrated that survivin is markedly over-expressed in most common types of cancer. Survivin has been described as playing a pivotal role in the mechanisms of cell death and cell proliferation.

Survivin is located on the mitotic spindle and is able to block the apoptotic signal regulated by Fas, as well as the death pathway initiated by Bax-induced release of cytochrome c from the mitochondria. In the cytoplasm, survivin inhibits apoptosis by interaction with caspase-9

in the presence of the HBXIP cofactor (Marusawa et al., 2003), by binding to Smac (Song et al., 2003) or association to XIAP (Kinoshita et al., 1996). However, mechanisms involved in aberrant expression of survivin in cancer are still little known.

Expression of survivin is regulated both in cell cycle-independent and dependent pathways. The cell cycle-independent pathway, which is cytokine-dependent, is restricted to CD34+ stem cells during hematopoiesis and endothelial cells during angiogenesis (O'Connor et al., 2000b). In the other cell types, the expression of survivin is regulated in a cell cycle-dependent way so that this protein is selectively expressed at G2/M transition (Lo Muzio et al., 2001). Transcriptional and post-transcriptional mechanisms which control expression of survivin involve repressor elements in the survivin promoter and processes of phosphorylation of the protein, which increases its stability.

Although the survivin gene is usually expressed only during mitosis, according to the cell cycle-dependent mechanism of regulation (Li et al., 1998), expression of survivin in cancer is seen in virtually all neoplastic cells, not only in the mitotic fraction, suggesting that malignant transformation is associated with global deregulation of the expression of the survivin gene (Lo Muzio et al., 2001). The aim of this work was to investigate the relationship between survivin, its phosphorylated active form and M-phase promoting factor (MPF) in oral carcinogenesis.

O'Connor et al. firstly described in 2000 that survivin is phosphorylated by p34<sup>cdc2</sup>-cyclin B1 and that phosphorylation of the protein on Thr-34 is required to preserve cell viability to mitosis (O'Connor et al., 2000a). Furthermore, they demonstrated that cell survival at the spindle checkpoint depends on elevated activity of p34<sup>cdc2</sup> kinase and increased expression of survivin (O'Connor et al., 2002).

It is well known that checkpoints act as mechanisms of control to ensure correct progression and proper timing of the cell cycle. The formation of a bipolar spindle during mitotic phase is crucial to preserve a genetic fidelity between daughter cells; this process is monitored by a checkpoint which controls defects in assembly and stability of the spindle microtubules. Activation of this spindle checkpoint may lead to apoptosis. It has been reported that elevated activity of p34<sup>cdc2</sup> kinase, which in turn gives rise to survivin phosphorylation on Thr-34, during spindle checkpoint, results in increased survivin expression and viability to cell division in cancer cells (O'Connor et al., 2002).

Furthermore, the G2/M DNA damage checkpoint prevents the cell from entering mitosis (M-phase) if the genome is damaged (O'Connell and Cimprich, 2005).

The cdc2-cyclin B kinase is pivotal in regulating the transition G2/M (Park et al., 2001; Altnauer et al., 2004). DNA damage activates the DNA-PK/ATM/ATR kinases, initiating two parallel cascades that inactivate cdc2-cyclin B. The first cascade rapidly inhibits



progression into mitosis: the Chk kinases phosphorylate and inactivate cdc25, which can no longer activate cdc2. The second cascade is slower. Phosphorylation of p53 dissociates it from MDM2, activating its DNA-binding activity. The genes that are turned on by p53 constitute effectors of this second cascade. Cdc2 is inhibited simultaneously by three transcriptional targets of p53, Gadd45, p21, and 14-3-3 sigma (Forrest and Gabrielli, 2001; Jin et al., 2002; Saxena et al., 2005). Interestingly, recent evidence indicates that survivin is one of the genes repressed by wild-type p53, suggesting that a loss of p53 may result in de-repression of survivin gene transcription (Mirza et al., 1990; Hoffman et al., 2002; Zhou et al., 2002). Increased cellular levels of cyclin B1 induce its physical association to p34<sup>cdc2</sup> kinase (CDK1). The complex cyclin B1-p34<sup>cdc2</sup>, termed MPF (M-phase promoting factor), may be activated or inhibited by various protein kinase and phosphatase having p34<sup>cdc2</sup> as a substrate (Jessus and Ozon, 1995; Kumagai and Dunphy, 1999).

The cyclin B proteins act as regulatory subunits of p34<sup>cdc2</sup> (cdk1) affecting the G2 to M-phase transition. Therefore, the cyclin B expression is restricted to a specific short period of the cell cycle. Cyclin B1-positive cells correspond to increased proliferative status of neoplastic cells. Pines and Hunter studied the sub-cellular localization of cyclins in epithelial tumor cells; they found that cyclin B1 accumulates in the cytoplasm of interphase cells and enters the nucleus only at the beginning of mitosis, before nuclear lamina breaks down (Pines and Hunter, 1991). In mitotic cells, cyclin B1 is associated with condensed chromosomes in prophase and metaphase and to the mitotic apparatus (Pines and Hunter, 1991). Cyclin B1 is the regulatory subunit of M-phase promoting factor, and proper regulation of cyclin B1 is essential for the initiation of mitosis (Clute and Pines, 1999). Increasing evidence indicates that deregulation of cyclin B1 is involved in neoplastic transformation, suggesting that suppression of cyclin B1 could be an attractive strategy for anti-proliferative therapies (Yuan et al., 2004).

MPF is the key initiator of mitosis, but when and where its activation occurs has not been precisely determined in mammalian cells. Activation may occur in the nucleus or in the cytoplasm, just before the nuclear envelope breaks down. It has been shown that cyclin B1 is initially phosphorylated on centrosomes in prophase and that Polo like kinase 1 (Plk1) phosphorylates cyclin B1. Furthermore, it has been shown that cyclin B1-Cdk1 is first activated in the cytoplasm and that centrosomes may function as sites of integration for proteins triggering mitosis (Jackman et al., 2003). In contrast to cyclins, which function as differentially-expressed Cdk regulators, the Cdks are constitutively expressed throughout the cell cycle. The prototype member of this family, p34<sup>cdc2</sup>, functions late in the cell-cycle.

It is well known that dysplasia and neoplasia contain an increased number of 'normal-appearing' and/or aberrant mitoses. Interestingly, our study showed that p-

survivin is highly expressed in both 'normal' and aberrant mitoses in squamous cell carcinoma. The over-expression of p-survivin is an early event during oral carcinogenesis, since it has been observed in oral dysplasia. Furthermore, p-survivin in OSCC cells has been localized in the cytoplasm of prophase, metaphase, anaphase and telophase cells. The immune-localization of p-survivin during mitosis in oral SCC is in agreement with previous studies in HeLa cells showing its localization on microtubules of the mitotic spindle at prophase and metaphase and on mid-bodies during telophase (O'Connor et al., 2000a). Furthermore, in this study, individual immune-staining for p34<sup>cdc2</sup> and cyclin B1 in serial sections showed a pattern of distribution which looked very similar to the one of p-survivin. Therefore, our results are in agreement with other studies showing that survivin is physically associated with p34<sup>cdc2</sup>, complexed with cyclin B1 (O'Connor et al., 2000a), and that survivin is mostly bound to microtubules. About this subject Fortugno et al. reported that the phosphorylation of survivin at G2/M takes place exclusively on spindle microtubules (Fortugno et al., 2002).

Activation of survivin on the mitotic spindle by p34<sup>cdc2</sup>/cyclin B1 has a finalistic role. This process links the regulation of mitotic switch G2/M by the major kinases to the regulation of cell death/survival by the crucial apoptotic inhibitor survivin.

Pharmacologic, genetic and molecular approaches have been used (by O'Connor and co-workers) to probe the central role of up-regulation of p34<sup>cdc2</sup> in the activation of survivin at the spindle checkpoint: phosphorylation of survivin by p34<sup>cdc2</sup> increases survivin stability (O'Connor et al., 2000a).

Previous studies have reported a relationship between expression of p34<sup>cdc2</sup> and clinical-pathologic parameters in oral carcinomas: the expression of this protein increases along with the progression of tumor stage and is positively correlated with malignant degree of oral carcinoma. Our study confirms the finding of a general up-regulation of p-34<sup>cdc2</sup>-cyclin B1 both in OLP and, at a higher level, in OSCC and correlates this over-expression to the activation of p-survivin.

Our study led to the conclusion that new and interesting therapeutic strategies, based on inhibition of the phosphorylation of survivin, might be used in oral squamous cell carcinoma.

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*Acknowledgements.* We thank A. Coppola, F. Stumpo for technical assistance and dr S. Di Lieto for translations. This work was supported in part by grants from MIUR - PRIN 2004, Università degli Studi di Foggia, and Fondazione Cassa di Risparmio di Verona, Vicenza, Belluno e Ancona 2004 - Italy.

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Accepted May 21, 2007