The phosphorylation of survivin Thr34 by p34^{cdc2} in carcinogenesis of oral submucous fibrosis

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Abstract. Survivin is a crucial node molecule involved in apoptosis, cell division and drug discovery. Up-regulation of survivin in the tissues of oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) originated from OSF has already been demonstrated. Survivin Thr34 phosphorylation is involved in the inhibition of apoptosis and cell division. To determine the potential involvement of survivin Thr34 phosphorylation in carcinogenesis of OSF, 40 OSFs, 42 OSCCs originated from OSF and 10 normal tissues from surgical specimens were studied. Immunohistochemistry showed that the positive staining rate of the survivin phosphorylation on Thr34 in OSCC originated from OSF group was significantly higher than that in OSF group ($P<0.01$), and none in the normal oral mucosa specimens. Survivin phosphorylation on Thr34 is predominantly located in the nucleus, which account for its function in apoptosis at cell division. Western blotting analysis showed increasing expression of survivin Thr34 phosphorylation, cyclin B1 and p34^{cdc2} in carcinogenesis of OSF. Furthermore, p34^{cdc2}-cyclin B1 kinase was confirmed to phosphorylate survivin on Thr34 in carcinogenesis of OSF by immunoprecipitation and immunoblot. These results suggest that the phosphorylation of survivin on Thr34 critically regulate survivin and plays an important role during the malignant transformation of OSF, which will provide an indication to early diagnosis and therapy in carcinogenesis of OSF.

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Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide. Imaging system and treatment strategies in OSCC are improving substantially, however, 5-year survival statistics are still low (1). Oral carcinogenesis is a complex and multi-step process, which results from multiple environmental factors and genetic susceptibility. Most of OSCCs arise in the presence of clinical premalignant lesions or condition, including oral leukoplakias (OLK), oral lichen planus (OLP), and oral submucous fibrosis (OSF) (2,3). OSF is a chronic progressive disorder and predominantly seen in India, Bangladesh, Pakistan, Taiwan and Mainland China, such as Hunan, Hainan (4). Pindborg (3) and various groups have put forward five criteria to prove that OSF is precancerous (5). Malignant transformation rate of OSF was found in the range of 7-13% depending on the different study population mainly in India and Taiwan (6). The distribution of OSF has obvious geographic characteristics mainly owing to the habit of chewing betel quid (BQ) (containing areca nut, slaked lime or other species). With the development of society and the commerce of BQ, more and more people like chewing BQ, the incidence of OSF and the malignant transformation rate of OSF will be higher in the future (7,8). Therefore, finding the key molecule in OSF malignant transformation is very urgent, and may contribute to current knowledge on the prevention, diagnosis and therapy of this disease.

Survivin is a recently characterized member of the inhibitor of apoptosis gene family that is differentially expressed in human tumors. Survivin is widely involved in apoptosis, proliferation, embryo development, blood vessel growth and immune regulation as well as tumor metastasis, which is

especially required for tumor maintenance and adequately embodies the nodal role of survivin in cancer networks (9). Recent evidence showed that survivin is an important predictive/prognostic parameter of poor outcome in human tumors and will be a diagnostic/therapeutic target in the development of malignant tumors (10). The expression of survivin is finely regulated by transcriptional, post-transcriptional and post-translational mechanisms (11). The post-translational modifications of survivin included phosphorylation and ubiquitination events that contributed to apoptosis and cell division. The phosphorylation of survivin has been studied, three phosphorylation sites have been mapped on survivin, containing threonine 34 and 117 residues and serine 20 residues. Three types of serine/threonine kinases have been confirmed to participate in the upstream signaling leading to p53 phosphorylation ($p34^{cdc2}$, Aurora kinase B, PKA), but the precise mechanism of this signaling and its regulation in oral tumors are not well understood (12-14).

In OSCCs, increased survivin protein expression has been reported in some studies, which indicated that survivin plays an important role during oral carcinogenesis (15-17). The major purpose of this study was to examine the active function of survivin Thr34 phosphorylation and its related kinases in different OSF pathological stage and OSF transformed OSCC.

Materials and methods

Tissue specimens. Tumors, OSF and healthy oral mucosa, were obtained at the time of surgical resection at Xiangya Hospital, Central South University. The patients' informed consent was obtained under a protocol reviewed and approved by the institutional review board of the Xiangya Hospital, Central South University. OSCC was diagnosed according to WHO criteria in 1997 (18), the clinical diagnosis and pathological stage of OSF is determined in terms of Pingborg criteria by the Department of Oral Pathology, Xiangya Hospital, Central South University. Ten normal specimens were obtained from healthy oral mucosa. Forty cases of OSF were incident, newly diagnosed without OSCC or neoplastic disease admitted to the same hospital for a wide spectrum of general surgical procedures from 2002-2007. OSF was classified into three grades: early stage, moderately advanced stage and advanced stage. Forty-two cases of OSCC are subsequent progressed from OSF in the surgical pathology archives (1990-2007). After careful removal of the tumor mass, or OSF tissue in buccal epithelium layer or grossly normal-looking buccal epithelium, all these samples were divided into two; one part was fixed in 4% buffered formalin solution for pathologic diagnosis and immunohistochemical staining; the second part was stored at -80˚C until protein isolation. All patients had OSCC or OSF that was confirmed histologically, H&E staining, and morphological re-examination was performed on each of the frozen blocks before further experiments.

Immunohistochemistry. Immunohistochemical staining was performed on 4 μm serial sections from 4% formalin-fixed paraffin-embedded specimens. After deparaffinisation and hydration, the slides were treated with endogenous peroxidase in 3% H_2O_2 for 20 min, after which the sections were blocked for 30 min at 37˚C with 1.5% blocking serum in TBS before reacting with p-survivin Thr34 antibody (1:200 dilution, sc-23758, Santa Cruz, CA) at 4˚C in a moist chamber overnight. Negative control slides are duplicate sections in absence of primary antibodies. For evaluating p-survivin Thr34 expression, a scoring method was used (19). A mean percentage of positive tumor cells was determined by the examination of 500 cells in at least 5 areas at magnification, x400. Cells were assigned to one of the five following categories according to percentage of positive cells (PP): a) 0, <5%; b) 1, 5-24%; c) 2, 25-49%; d) 3, 50-75%; or e) 4, >75%. The intensity of the p-survivin staining (*SI*) was scored as follows: a) 0, no -; b) 1, weak +; c) 2, moderate ++; d) 3, intense +++. The final immunoreactive score (*IRS*): IRS=SIxPP, was: a) -, 0; b) +, 1-2; c) ++, 4-6; d) +++; 8-12. Statistical significance was evaluated by χ^2 analysis, Fisher's exact test. The stained tissues were scored in a blind manner with respect to clinical patient data.

Western blotting. The tissues obtained from resected specimens were homogenized in nitrogen liquid and lysed with preparation of modified radioimmunoprecipitation buffer. Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM HEPES, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 1% Nonidet P-40, phosphatase inhibitor mixture, and protease inhibitor mixture. After centrifugation at 13,000 rpm for 10 min, the supernatant was collected and the protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Protein extract (50 μg) was electrophoresed on 12% Tris-glycine polyacrylamide gels, transferred onto NC membranes, and blocked for 1 h at room temperature in 5% skim milk. Immunoblot NC membranes were washed with 0.1% Tween-20 in TBS (TBS-T) 3 times, incubated primary antibodies recognizing human p-survivin Thr34(sc-23758, Santa Cruz), cyclin B1(sc-7393, Santa Cruz), $p34^{cdc2}$ (sc-954, Santa Cruz), p-$p34^{cdc2}$ (9114, Cell Signaling, USA), ß-actin (CA-5441, Sigma, USA) at 4˚C overnight. Secondary antibodies used were goat anti-rabbit IgG-HRP (SC-2004, Santa Cruz) and goat anti-mouse IgG-HRP (sc-2005, Santa Cruz). The bound antibody was detected using the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA). Gel imaging system application VDS was used to calculate gray value of proteins, the relative protein expression level equal to the ratio of gray value of target protein to that of internal control. Statistical significance was evaluated by Student's t-test and the Analysis of variance (Anova).

Immunoprecipitation assay. The cells from the normal epithelium, OSF tissues and OSCC originated from OSF tissues were treated as the described in Western blot section. The lysates were immunoprecipitated using IgG against $p34^{cdc2}$ or survivin antibodies and protein A/G plus agarose. The beads were washed extensively to eliminate non-specific binding, and levels of phosphorylated proteins of survivin or $p34^{cdc2}$ were selectively measured by immuno-blotting using a specific antibody and chemiluminescent detection system.

Table I. The phosphorylation of survivin Thr34 in carcinogenesis of OSF.

Groups	No.	Survivin Thr34				Survivin Thr34 expression	Mean survivin Thr34 score
		-	+	++	+++		
Normal	10	10	0	0	0	0	0
OSF	40	20	10	9	1	50.0%	0.811±0.192
E	10	7	2	1	0	30.0%	0.231±0.169
M	15	8	5	2	0	46.7%	0.515±0.102
A	15	5	3	6	1	66.7%	1.687±0.304
OSCC	42	1	2	18	21	97.6%	5.192±0.458

E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF.

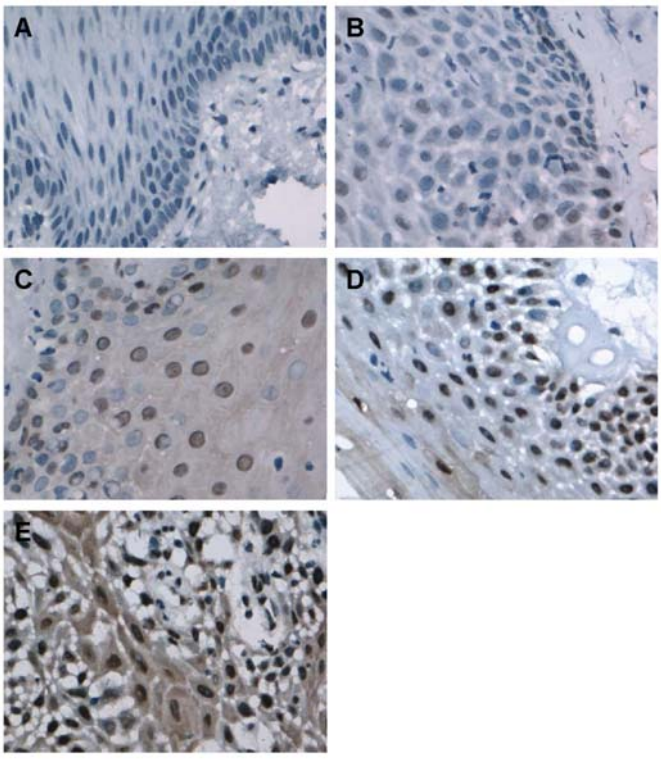

Figure 1. Immunohistochemical staining of survivin Thr34 phosphorylation in normal oral mucosa, OSF and OSCC tissue. (A) Normal oral tissue exhibited negative survivin Thr34 phosphorylation expression. Original magnification, x400. (B) In early stage of OSF and (C) moderately advanced stage of OSF weak phosphorylational level of survivin Thr34 was detected. Original magnification, x400. (D) In advanced stage of OSF moderate surviving Thr34 phosphorylation was detected. Original magnification, x400. (E) OSCC originated from OSF showed strong immunoreaction for survivin Thr34 phosphorylation. Original magnification, x400.

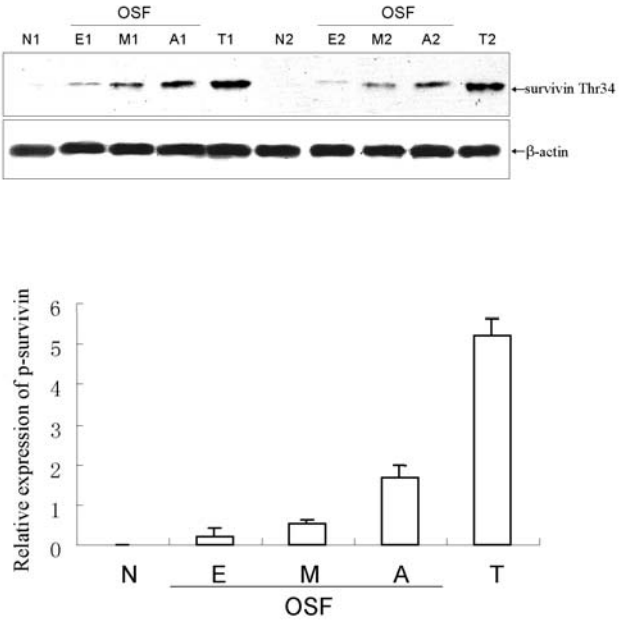

Figure 2. The phosphorylation of survivin Thr34 in selected samples of normal oral mucosa, different stages of OSF and OSCC tissues. (A) Lysates were prepared from these tissues and immunoprecipitated. Western blotting was performed with the indicated antibody of p-survivin Thr34. ß-actin was used as a normalization control. E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF. (B) The relative amount of survivin expression levels in different stages of OSF and OSCC were calculated using densitometry. Data are expressed as means ± SD.

Results

The phosphorylation of survivin Thr34 in carcinogenesis of OSF. A total of 82 patients with OSF or OSCC were identified for whom there was adequate histologic material available for immunohistochemical analysis. All normal oral mucosa specimens showed no survivin Thr34 phosphorylation (Fig. 1). About 20 of 40 (50%) OSF cases showed phosphorylation of survivin Thr34. Three/10 (30%) of early stage (mean score 0.231, SD 0.169) and 7/15 (46.7%) of moderately advanced stage of OSF were found to exhibit weak survivin Thr34 phosphorylation (mean score 0.515, SD 0.102). Out of the 15 advanced stage of OSF cases, 10 (66.7%) showed moderate survivin Thr34 phosphorylation level (mean score 1.687, SD 0.304). No statistically significant correlation was found between the survivin Thr34 phosphorylation and the pathological stages of OSF (P>0.05). The phosphorylation of survivin Thr34 in OSF were

Table II. The expression of cyclin B1, $p34^{cdc2}$, $p\text{-}p34^{cdc2}$ in carcinogenesis of OSF.

Protein	Normal	OSF			OSCC
		E	M	A	
cyclin B1	0.120±0.008	0.443±0.018	0.567±0.017	3.072±0.218	10.593±0.359
$p34^{cdc2}$	0	0.335±0.814	0.802±0.108	4.069±0.084	10.978±0.344
$p\text{-}p34^{cdc2}$	0	0.174±0.021	0.400±0.076	2.463±0.513	5.141±0.158

E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF.

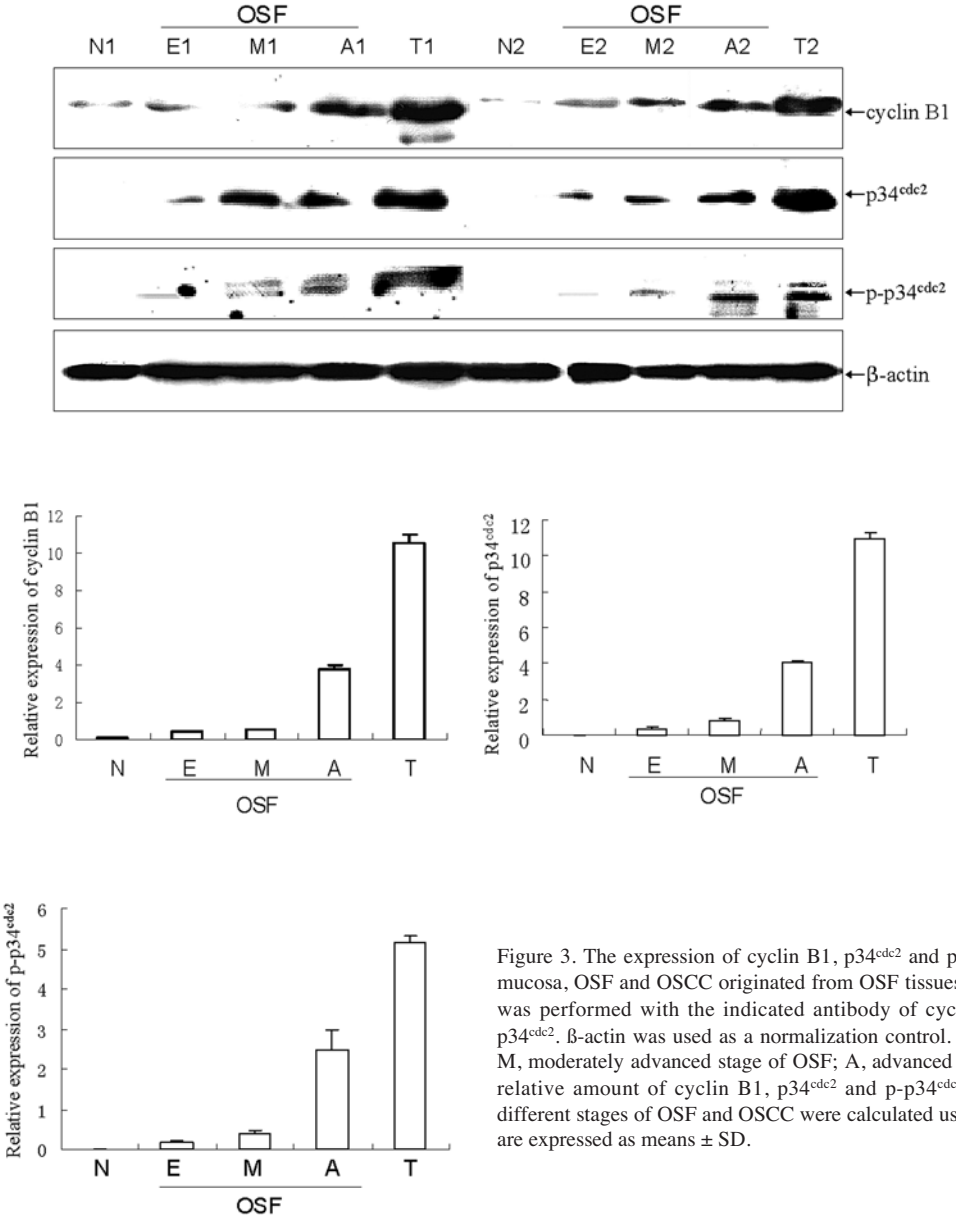

Figure 3. The expression of cyclin B1, $p34^{cdc2}$ and $p\text{-}p34^{cdc2}$ in normal oral mucosa, OSF and OSCC originated from OSF tissues. (A) Western blotting was performed with the indicated antibody of cyclin B1, $p34^{cdc2}$ and $p\text{-}p34^{cdc2}$. ß-actin was used as a normalization control. E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF. (B) The relative amount of cyclin B1, $p34^{cdc2}$ and $p\text{-}p34^{cdc2}$ expression levels in different stages of OSF and OSCC were calculated using densitometry. Data are expressed as means ± SD.

significantly higher than those in normal oral tissues (P<0.01). A total of 42 patients with OSCC transformed from OSF were examined. Forty-one of 42 cases (97.6%) of OSCC were defined as survivin Thr34 phosphorylation strongly positive (mean score 5.192, SD 0.458), which was higher than that of OSF cases (mean score 0.811, SD 0.192) (P<0.01) (Table I).

In OSF and OSCC originated from OSF tissues, the phosphorylation of survivin Thr34 is mainly localized in the nucleus with different brown granules. Normal oral mucosal

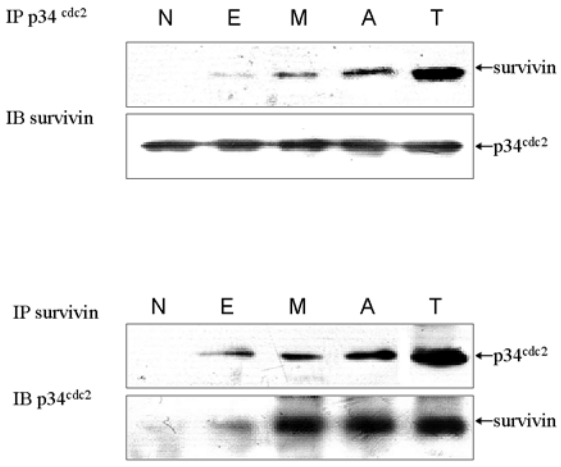

Figure 4. Physical association between survivin and p34^{cdc2} in carcinogenesis of OSF. Equal protein lysates (200 μg) were prepared from the above tissues and immunoprecipitated using antibody against survivin or p34^{cdc2}. Survivin or p34^{cdc2} immunoprecipitates were immunodetected with p34^{cdc2} or survivin antibody. Negative controls included beads only and immunoprecipitation with an unrelated antibody (not shown).

epithelial tissue was negative. In the early and moderately advanced stage of OSF tissues, the positive staining of survivin Thr34 phosphorylation was weakly expressed in basal layer and prickle layer, with spike-visible scattered in the cytoplasm and nuclei. In the advanced OSF tissues, the positive staining of survivin Thr34 phosphorylation was stronger in some basal layer, prickle layer and granulose layer cells, the nucleus and cytoplasm mainly showed positive nuclei, which were brown, dense and uniform granules. In OSCC originated from OSF tissues, survivin Thr34 phosphorylation positive cells formed strong spots, and the majority of tumor and epithelial cells were dying in nucleus and cytoplasm, while mesenchymal cells showed no stain. These data suggest that survivin Thr34 phosphorylation is localized in the nucleus and cytoplasm, and mainly in the nucleus.

To validate the immunohistochemical data, Western blotting was performed on the protein samples isolated from normal oral mucosa, premalignant OSF condition and cancer regions of survivin Thr34 phosphorylation-positive group. The strong survivin Thr34 phosphorylation band was detected in OSCC group, whereas, obviously depressed signal was showed in the moderately advanced stages and advanced stage of OSF. As showed in Fig. 2, there was a faint signal in the group of the early stages of OSF, and no signal in the normal oral group. These data suggest that increasing phosphorylation level of survivin Thr34 in carcinogenesis of OSF.

Expression of cyclin B1 and p34^{cdc2} in carcinogenesis of OSF. Cyclin B1 and p34^{cdc2} are important molecules in transition from cell cycle G2 phase to M phase. Cyclin B1 can form the active kinase complex mitosis-promoting factor (MPF) via binding activated p34^{cdc2} (p-p34^{cdc2}), which is involved in the regulation of entry into mitosis. In addition, cyclin B1/p34^{cdc2} kinase complex phosphorylated some proteins and regulate their activity and distribution, which participated in the formation of spindle and chromosome separation.

The expression of cyclin B1, p34^{cdc2} and p-p34^{cdc2} were examined by Western blot assay in 40 OSF cases, 42 OSCC originated from OSF cases and 10 normal tissues. The data showed that the expression of cyclin B1 is very weak in normal oral mucosal epithelial, but p34^{cdc2} and p-p34^{cdc2} expression was not detected. The expression of cyclin B1, p34^{cdc2} and p-p34^{cdc2} gradually strengthened in the early stage, moderately advanced stage and advanced stages of OSF (Fig. 3, Table II). Furthermore, a statistical significance was observed between OSF group and normal group ($P<0.05$). In OSCC originated from OSF tissues, the expression of cyclin B1, p34^{cdc2} and p-p34^{cdc2} were strongest compared to OSF tissues ($P<0.05$) and normal tissues ($P<0.05$) (Fig. 3, Table II). There was no statistical significance among OSF early, moderately advanced and advanced stages ($P>0.05$). These data suggested that cyclin B1 and p34^{cdc2} will be activated and accumulated in carcinogenesis of OSF.

The binding of p34^{cdc2} and survivin in carcinogenesis of OSF. Mitosis kinase p34^{cdc2}-cyclin B1 phosphorylated survivin on Thr34 via binding survivin. Whether survivin and p34^{cdc2} interact in carcinogenesis of OSF needs to be confirmed. Immunoprecipitated p34^{cdc2} in OSF tissues and OSCC originated from OSF tissues contained survivin expression by Western blotting, and its expression gradually increased with the malignant progression of OSF. Using survivin antibody immunoprecipitated tissues, the increasing expression of p34^{cdc2} was examined. No suvivin or p34^{cdc2} bands were seen from normal oral mucous tissues (Fig. 4). These suggested that the combination of survivin and p34^{cdc2} is gradually increased in carcinogenesis of OSF.

Discussion

Survivin possesses multiple facets via mediated in multiple cellular networks, more importantly, it is an appropriate target for drug discovery due to orchestrating extensive and potentially tumor-specific, signaling networks.

Protein phosphorylation/de-phosphorylation is the most important and common form in post-translational modification. It is implicated in the regulation of cell proliferation, differentiation, apoptosis, cytoprotection and cell cycle transitions. In three survivin phosphorylated residues, the study of phosphorylation of survivin Thr34 is intensive. Since survivin Thr34 phosphorylation embodied its anti-apoptosis and cell division function, the only kinase is p34^{cdc2}-cyclin B1 kinase complex. The studies showed that non-phosphorylatable survivin Thr34→Ala could reduce survivin expression and enhance chemotherapy-induced apoptosis and anti-tumor activity *in vivo* (20,21). In bladder cancer, treated survivin Thr34 mutated transgenic animals with carcinogenic N-butyl-N-(4- hydroxybutyl) nitrosamine (OH-BBN) did not show malignant phenotype, and no changes were found in gene expression (22). Survivin Thr34 phosphorylation mutation enhanced the treatment efficiency of anti-androgen drug flutamide, and promoted its drug sensitivity in prostate cancer (23). These data suggest survivin Thr34 phosphorylation plays a key role in tumor development and therapy resistance.

In OSCC, survivin is known as a potential predictor of malignant progression, angiogenesis, metastasis and prognosis,

and is an early event in oral epithelial dysplasia such as OLK. Hunan province in China is OSF high incidence area, the malignant transformation rate of OSF is from 1.19% to 2.04% (7,8). Our previous study found that the expression of survivin is up-regulated in carcinogenesis of OSF (unpublished data). The present study further examined the phosphorylation of survivin Thr34 in the malignant transformation of OSF. Our data showed that survivin Thr34 phosphorylation exhibited significant difference between OSF and OSCC. During the carcinogenesis of OSF, the phosphorylation of survivin Thr34 increased gradually, while absence of survivin Thr34 phosphorylation was observed in normal tissue. There is no relationship between the degree of survivin Thr34 phosphorylation and the stages of OSF. Survivin is localized in the cytoplasm, the nucleus and mitotic components in cells, by Crm l-dependent nuclear export signal (24). The relationship between the subcellular localization of survivin in tumors and the relevance of clinical stage is still controversial (25). In OSCC, survivin is mainly localized in the cytoplasm, and is closely related to chemotherapy or radiotherapy resistance, poor prognosis and tumor recurrence (26). Our previous data comfirmed that survivin mainly localized in the cytoplasm in carcinogenesis of OSF and correlated with OSF malignant progression. The present data found that the phosphorylation of survivin Thr34 is mainly localized in the nucleus. There results indicate the increased phosphorylation level of survivin Thr34 in carcinogenesis of OSF.

$p34^{cdc2}$ and cyclin B1 is very important in the development of OSCC. The expression of $p34^{cdc2}$ is associated with histologic grade, lymph node metastasis, tumor recurrence and lower 5-year survival rate in tongue cancer (27). Multivariate analysis indicated that overexpression of cyclin B1 in tongue cancer is an independent predictor, which correlated with the invasion and radiation tolerance (28). Moreover, previous studies showed that $p34^{cdc2}$ and cyclin B1 participate in oral carcinogenesis, which were associated with survivin phosphorylation (29). Whether $p34^{cdc2}$ and cyclin B1 participate in carcinogenesis of OSF is not yet reported. Our data indicated that the expression of cyclin B1, $p34^{cdc2}$ and p-$p34^{cdc2}$ are gradually increased with a significant differences in carcinogenesis of OSF ($P<0.05$). The emergence of $p34^{cdc2}$ reflected the split density of cell population. In normal oral mucosa, weak expression of cyclin B1 and no expression of $p34^{cdc2}$ indicated most of normal cells are in the quiescent phase. Increased expression of cyclin B1, $p34^{cdc2}$, p-$p34^{cdc2}$ suggested acceleration of cell proliferation in carcinogenesis of OSF. Furthermore, $p34^{cdc2}$ and cyclin B1 formed the activated kinase and phosphorylate survivin on Thr34. The data showed that survivin can be combined with $p34^{cdc2}$ and transfer ATP to survivin Thr34 in carcinogenesis of OSF. Phosphorylation of survivin on Thr34 mediated the formation of survivin-caspase-9 complex, resulting in the inhibition of caspase 9-dependent apoptosis in mitotic phase (12). Phosphorylated survivin regulates microtubule stability and spindle assembly in cell division, which results in cell malignant proliferation (30).

In conclusion, survivin is not only involved in the inhibition of apoptosis, but also in the process of mitosis in carcinogenesis of OSF. The disorder of G2/M phase and the phosphorylation of survivin are the important molecular events, which confirmed that survivin must be the key indicator for malignant progression, early diagnosis and target therapy in carcinogenesis of the OSF.

Acknowledgements

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