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Pin1 overexpression is associated with poor differentiation and survival in oral squamous cell carcinoma

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Abstract. Phosphorylation on certain Ser/Thr-Pro motifs is a major oncogenic mechanism. The conformation and function of phosphorylated Ser/Thr-Pro motifs are further regulated by the prolyl isomerase Pin1. Pin1 has been shown to be prevalently overexpressed in human breast cancer cell lines and cancer tissues and to play a critical role in the transformation of mammary epithelial cells by activating multiple oncogenic pathways. Pin1 expression was found to be an excellent independent prognostic marker in prostate cancer. However, little is known about Pin1 and its downstream targets ß-catenin and cyclin D1 expressions in human oral cancers. In the present study, we quantified Pin1 expression in 74 paired normal/tumor human oral cancer samples as well as oral cancer cell lines. Pin1 was overexpressed in oral squamous cell carcinoma (OSCC) and its level correlated with ß-catenin accumulation and cyclin D1 expression. Moreover, we examined Pin1 mRNA expression in OSCC and cancer cell lines by RT-PCR analysis. The results showed that there is concordance in the relationship between the Pin1 mRNA level and Pin1 protein expression. The up-regulation of Pin1 mRNA expression in tumor part when comparing with that in non-tumor part was in agreement with that of the Pin1 protein overexpressed in OSCC. In addition, we showed that the molecular and immunohistochemical profiles of Pin1 overexpression were associated with progression of OSCC. Taken together, these results indicate that Pin1 is a regulator of cyclin D1 expression in OSCC and might play a role in oral oncogenesis. The overexpression of Pin1 can be used as an indicator for pathological diagnosis of OSCC.

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Introduction

Pin1 is a peptidyl-prolyl cis-trans isomerase that specifically recognizes phosphorylated Ser/The-Pro motifs and isomerizes only phosphorylated Ser/Thr-Pro (pS/T-P) peptide bonds (1-4). Previously, Pin1 has been demonstrated as the first PPIase that is essential for cell growth (3). It regulates mitosis progression and replication checkpoint in human and yeast cells (3,5). Structurally and functionally, Pin1 can be divided into N-terminus WW domain and C-terminus PPIase domain (6). The WW domain has been demonstrated to work as a phosphoserine/threonine-proline binding module that binds to phosphoproteins (7,8). The PPIase domain has catalytic activity that specifically isomerizes Ser/Thr-Pro peptide bonds (4). This novel post-phosphorylation isomerase regulates the conformation of a subset of phosphoproteins such as p53, Cdc25C and the microtubule-associated protein tau, thereby affecting their activity and/or protein-protein interactions (1,9-14). Pin1 has been shown to interact with a number of cancer-related phosphoproteins, which suggested Pin1 might link signal transduction to pathogenesis of cancer (1,15,16). Moreover, Pin1 overexpression in several human cancers including breast and prostate and liver cancers strongly suggested that Pin1 might play a role in tumorigenesis (16-19). Given the role of Pin1 in cell growth control and tumorigenesis, Pin1 could represent a new anti-cancer target.

Although the molecular mechanism remains uncertain in many Pin1-overexpression related cancer types, recent studies have shown that the Pin1 protein level is correlated with tumor grade and with cyclin D1 level in human breast cancers (16,20). Moreover, it has been demonstrated that Pin1 directly binds the phosphorylated Ser-Pro motif next to the APC-binding site in ß-catenin, inhibits its interaction with APC and increases its translocation into the nucleus (15,20). Thus, Pin1 is a novel regulator of ß-catenin signaling in breast cancer, in which APC or ß-catenin mutations are not common. Cyclin D1 is a proto-oncogenic protein in the cell cycle and has been implicated in the pathogenesis of several types of cancers, including SCC (21,22). Cyclin D1 gene amplification has been found in 20-50% of head and neck SCC and the protein is overexpressed in up to 80%

cases (23-25). Conversely, cyclin D1 antisense oligonucleotides suppress SCC growth *in vitro* and *in vivo*, leading to cellular apoptosis (26). Moreover, overexpression of cyclin D1 has been found associated with a more aggressive phenotype and reduced survival in patients with head and neck SCC including tongue cancer (27-31). However, in OSCC, there were no significant differences in any of the clinical or pathological parameters and overall survival (32). These results indicate that cyclin D1 might play an important role during oncogenesis in OSCC.

The purpose of this study was to investigate whether Pin1 expression is correlated with OSCC, as well as whether Pin1 expression is associated with prognosis of OSCC. In this report, we demonstrate that Pin1 is overexpressed in OSCC. Our results showed that Pin1 protein overexpression is associated with the pathological stage and survival rate of OSCC. Pin1 overexpression therefore can be used as an indicator for pathological diagnosis and prognosis in OSCC.

Materials and methods

Patients and tumor samples. Patients for immunoblotting and RT-PCR analyses: the study protocol had the approval of the ethics committee at Kaohsiung Veterans General Hospital. We examined the biopsies from 74 patients who were evaluated during 2002 and 2004 in the Department of Dentistry of Kaohsiung Veterans General Hospital, Taiwan. None had received prior chemotherapy or radiation therapy. The clinical and pathological characteristics were examined according to the standard procedure in Department of Pathology in Kaohsiung Veterans General Hospital. Histopathological diagnosis was made routinely at the Department of Pathology and tumors were classified according to the World Health Organization classification of histological differentiation.

Patients for immunohistochemistry and survival curve analyses: we reviewed the medical records of 74 patients treated for squamous cell carcinoma of the oral cavity at the Department of Dentistry of Kaohsiung Veterans General Hospital between 1998 and 2000. Clinicopathological data were obtained by retrospective chart review. Paraffin-embedded tissue samples were obtained from the hospital archives. All tumors sections were confirmed being primary SCC by the Clinicopathology Department of the hospital. The mean age of the patients was 52 years (age range 27-77 years). The 74 OSCC patients were derived from the following sites: buccal mucosa (n=34), tongue (n=21), gingiva (n=5), lip (n=3), palate (n=4) and others (n=7). The median follow-up period was 54 months (follow-up range 0-86 months).

Immunoblot analysis. Four oral cancer cell lines, SAS, TW2.6 CA922, and Cal27 were obtained from Dr Mark Kuo, Department of Dentistry, National Taiwan University Hospital. Forty oral cancer tissue and its adjacent non-tumor tissue specimens were used to detect Pin1 expression levels by immunoblotting. The tumor chunks were homogenized and dissolved in the sample buffer [0.5 M Tris-HCl (pH 6.8), 100% glycerol, ß-Mercaptoethanol, and 20% SDS] and boiled for 10 min. The protein samples were electrophoresed by using SDS-PAGE and transferred onto an Immobilon™-P transfer Membranes (Millipore, Bedford, USA). The membrane was then soaked in the blocking solution (1X TBS, 0.05% Tween-20, 5% non-fat dried milk) for overnight, incubated with anti-Pin1 polyclonal antibody (1:1,000, Oncogene Research Products, San Diego, CA, USA) diluted with 1X TBST for 2 h at 25˚C, and washed three times with TBST (1X TBS, 0.05% Tween-20). The membrane was then incubated with the secondary antibody (biotinylated anti-rabbit IgG, 1:5,000, Perkin-Elmer Life Sciences, Boston, MA, USA) for 1 h and washed with TBST three times. The enhanced chemiluminescence (ECL) system Western blotting detection reagents (Perkin-Elmer Life Science) were used according to the manu-facturer's recommendations.

Immunohistochemical analysis. Sections (5 μm) were cut from the formalin-fixed and paraffin-embedded specimens. Briefly, the sections were deparaffinized in xylene and rehydrated in serial dilutions of ethanol (100, 95 and 75%) for 5 min each. Antigen retrieval was performed by autoclave treatment for 10 min in 0.01 M citrate buffer (pH 6.4). Endogenous peroxidase was quenched by treatment with 3% H_2O_2 in absolute methanol for 30 min at room temperature, followed by blocking of endogenous biotin activity using a biotin blocking kit (Dako Corporation, Carpinteria, CA, USA). The section were then blocked in 10% normal goat serum in Tris-buffered saline, followed by incubated overnight with Pin1 (1:100) rabbit polyclonal antibody. After 16 h of incubation at 4˚C, the slides were incubated for 30 min at room temperature with biotinylated anti-rabbit IgG antibody diluted at 1:200 with TBS containing 5% normal goat serum. After washing twice for 5 min with PBS, the standard ABC process was then followed, according to the manufacturer's instructions. Diaminobenzidine was used as a chromogen, followed by counterstained with hematoxylin.

For Pin1 protein expression levels and extent oral cancer paraffin sections were scored according to the Pin1 staining intensity in the nucleus and the extent of staining of tumor cells. Scoring was: 3 (+++), strong staining in a normal distribution; 2 (++), patchy or heterogeneous staining; 1 (+), weak and fragmented; 0 (+/-), weak or loss of staining. In addition, a score of 4 (++++) was used to indicate intense and high staining accumulation in the nuclear/cytoplasmic fraction in tumor tissues. In normal oral epithelial cells, the staining intensity in the nucleus was weak and moderate. Therefore, high level of Pin1 staining was defined as strong (score 3) and intense (score 4) immunohistochemical staining, and low level of Pin1 as weak (score 1) and moderate (score 2) staining.

Pin1/shRNA preparation and transfection. Oral Squamous SAS and Cal27 cells were cultured in Dulbecco's modified Eagle's medium (Gibco™, NY, USA) supplemented with 10% heat-inactivated fetal calf serum incubated at 37˚C with 5% CO_2. For experiments using cell lines, cells were transiently transfected with Pin1/shRNA (Expression Arrest™, AL, USA) using Arrest-In Transfection Reagent (Expression Arrest) in Opti-MEM for 6 h, and then the medium was changed back to growth medium without antibiotic for

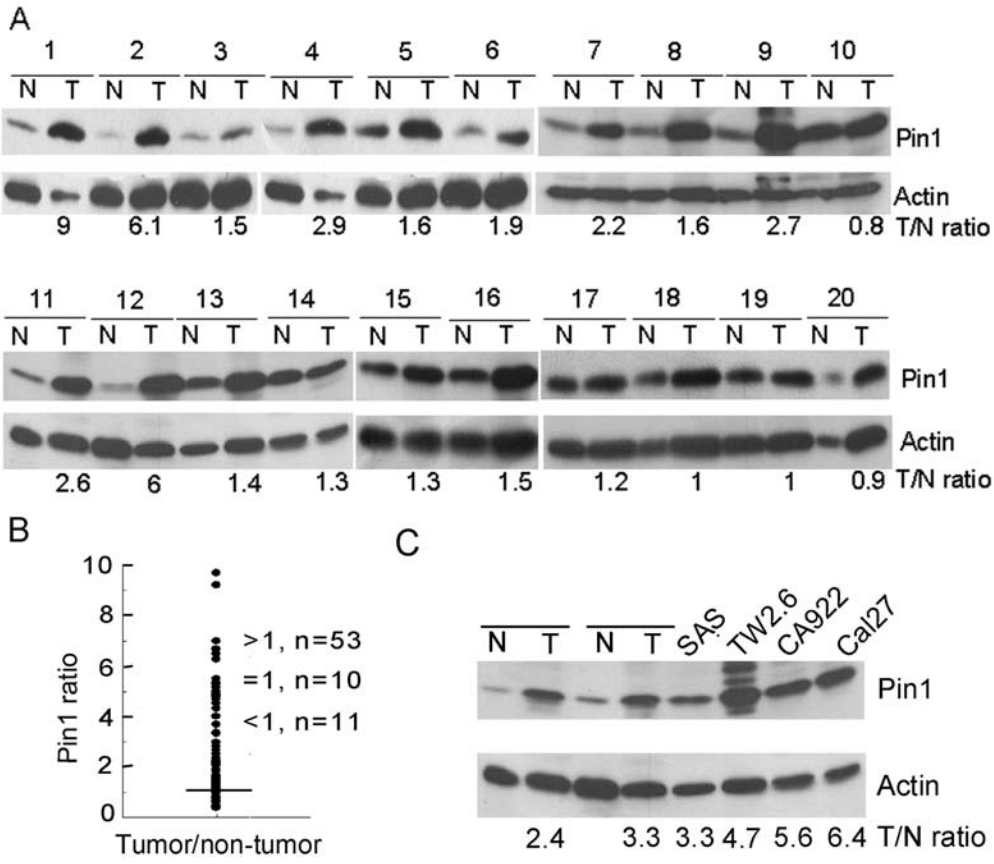

Figure 1. Examination of the Pin 1 protein expression levels in oral cancer tissues. (A) Immunoblot analysis for detecting PIN1 expression patterns in oral cancer tissue using the polyclonal rabbit anti-PIN1 and monoclonal mouse anti-actin served as an internal control. Twenty pairs of oral caner tissues (N/T pairs) were chosen and represented for total 74 pairs examined. The number underneath the tumor Pin1 in the immunoblot membrane indicates the T/N ratio of Pin level when compared the Pin1: actin ratio of tumor part (T) and that of its non-tumor counterpart (N). (B) Summary of the fold increase of Pin levels in OSCC. (C) Immunoblot analysis for detecting PIN1 expression patterns in four oral cancer cell lines.

additional incubation. Drug selections in SAS, Cal27 cells were performed with 3 μg/ml puromycin. For Western blots, time course and puromycin-selected cells were lysed in 2X sample buffer. Protein lysate was separated on 15% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon™-P transfer Membranes (PVDF, Millipore). Membranes were blocked by 5% skin milk in TBST buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween-20) at 4˚C overnight, incubated for 2 h at room temperature with following primary antibodies: monoclonal anti-ß-catenin (Santa Cruz Biotechnology, CA, USA), anti-actin (Santa Cruz Biotechnology), anti-cyclin D1 (Zymed Laboratories Inc., CA, USA), and anti-Pin1. Immunoreactive proteins were detected using Anti-Rabbit IgG, HRP-conjugated secondary antibodies (Perkin-Elmer Life Sciences) and by using the ECL system.

Statistical analysis. The relationship between Pin1, cyclin D1 and ß-catenin protein expression were determined using the χ^2 test after the expression level for each variable was dichotomized as described above. The correlation of the level of Pin1 and the degree of cell differentiation was evaluated by spearman correlation. We used the Kaplan-Meier method to estimate survival rates and the curves. The difference between the survival curves was examined using the log-rank test. Overall survival was calculated from the time of surgery to the date of death, or the date of last follow-up. All statistical analysis were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Pin1 is overexpressed in primary oral cancers. To examine the role of Pin1 in OSCC, we analyzed the expression profile of Pin1 protein using 74 pairs of human non-tumor oral tissues (N) and their tumor counterparts (T) by immunoblotting analysis. Non-tumor or oral squamous cell carcinoma tissues were pulverized freshly in liquid nitrogen and the lysates were directly subjected to immunoblotting analysis with anti-Pin1 antibodies, followed by semi-quantification of protein levels using NIH image, as described (12,33). By using anti-Pin1 antibodies and immunoblotting, all normal human oral tissues and their tumor parts showed immuno-reactivity. The results of 20 representative pairs of total 74 pairs of primary oral tissues examined are shown in Fig. 1A. Pin1 was detected as an 18 kDa protein in immunoblots, especially in tumor tissues where Pin1 was overexpressed. To compare the levels of Pin1 in each N/T pair, actin was used as an internal control and Pin1 protein expression level was expressed as Pin1: actin ratio in each examined sample. The Pin1 over-expression was defined as ≥50% increase when compared the Pin1: actin ratio of tumor part with that of its non-

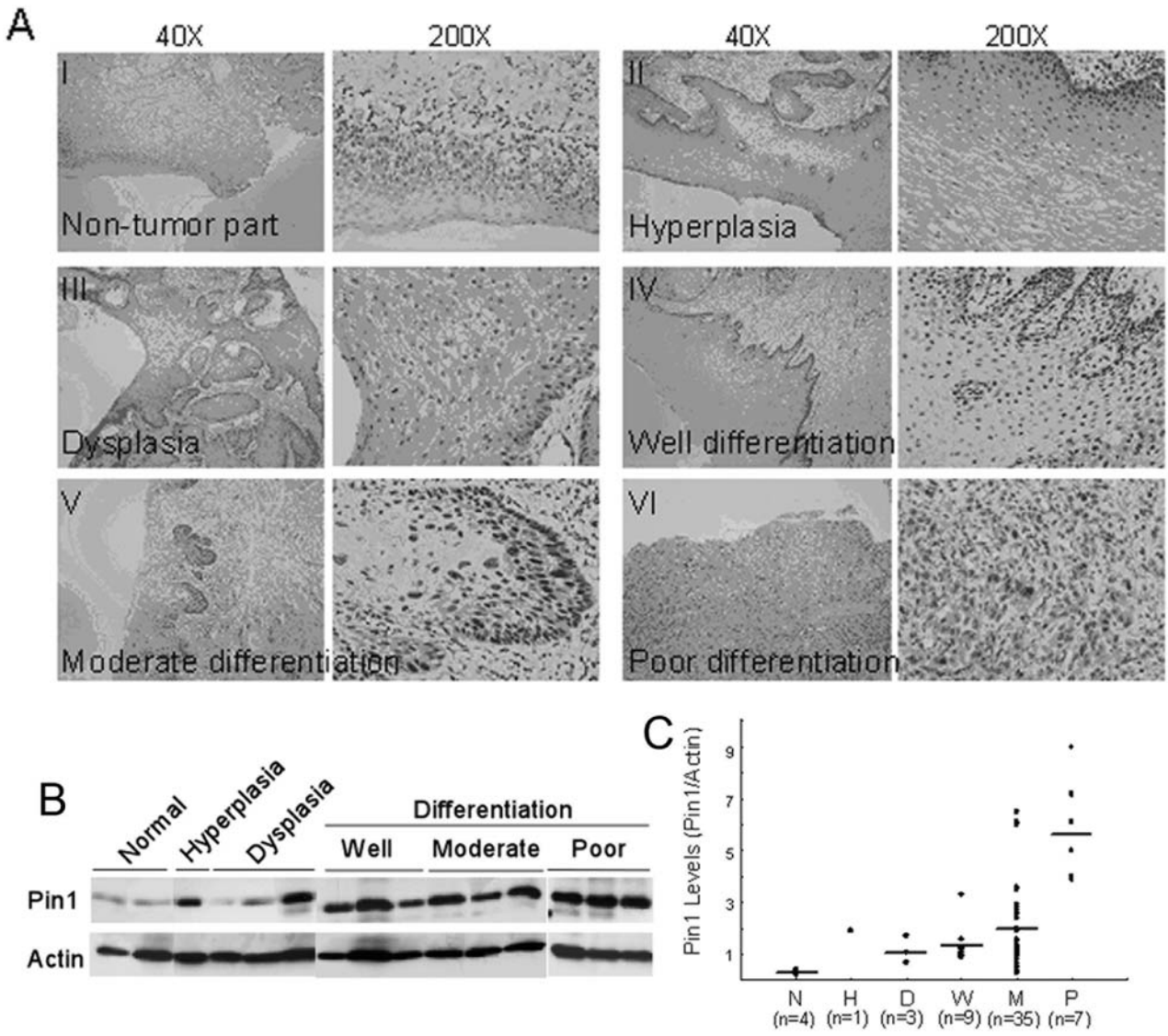

Figure 2. Pin1 protein expression level correlates with the cell differentiation status in oral squamous cell carcinoma (A-C). A (I-VI) Immunohistochemical staining patterns of Pin1 expression in oral cancer tissues. I, normal oral mucosal epithelium treated with anti-Pin1 antibody show weak, but detectable staining; II, hyperplasia; III, dysplasia; IV, well differentiation shows strong Pin1 staining exclusively in the nucleus; V, moderate differentiation shows high level of Pin1 staining in both nucleus and cytoplasm; VI, poor differentiation shows very intense Pin1 staining in both nucleus and cytoplasm. (B) Immunoblotting analysis of Pin1 expression in oral cancer tissues classified by cell differentiation. Antibodies specifically against Pin1 and actin were used for detecting the Pin1 and actin protein level, respectively. (C) Pin1 level analysis using the Pin1: actin ratios obtained form immunoblotting analyses.

tumor counterpart. In the total 74 pairs of oral tissues examined, 53 pairs (72%) of oral tissues showed Pin1 overexpression in the tumor part. The increase of Pin1 levels in 53 oral cancer pairs ranged from 0.5- to 8.0-fold (Fig. 1B). Whereas, 21 out of 74 (28%) oral cancer pairs did not show significant Pin1 level elevation (n=10, 13%) or even a decrease of Pin1 protein expression (n=11, 15%) (Fig. 1A, the right bottom panel; Fig. 1B). In order to confirm the higher Pin1 level in oral cancer cells, four oral cancer cell lines derived from human oral squamous cell carcinoma were used for Pin1 immunoblotting examination. Our results showed striking elevation from 2.3- to 5.4-fold increase of the Pin1 protein levels in these four OSCC cell lines when compared with that in non-tumor parts (Fig. 1C). Taken together, these results indicated that Pin1 is overexpressed in human oral cancer tissue and cell lines.

Relationship between expression pattern of Pin1 and poor differentiation in OSCC patients. To further verify the Pin1 overexpression in oral tumor cells but not in normal adjacent cells, immunohistochemistry analysis was performed to examine the Pin1 distribution and expression levels in 74 human oral cancer tissues. Normal oral squamous cells showed weak but detectable Pin1 stained primarily in the nucleus (Fig. 2AI). In contrast, strong positive Pin1 nuclear stains were found in tumor cells (Fig. 2A), while weak Pin1 stains were found in surrounding normal connective tissue. It is worth mentioning that the majority of cases with medium and poor differentiation exhibited both nuclear and cytoplasmic accumulation of Pin1 protein (Fig. 2AV and VI). In their respective non-tumor counterparts, absent to very weak cytoplasmic expression of Pin1 was observed. Interestingly, the Pin1 level from low to high was correlated

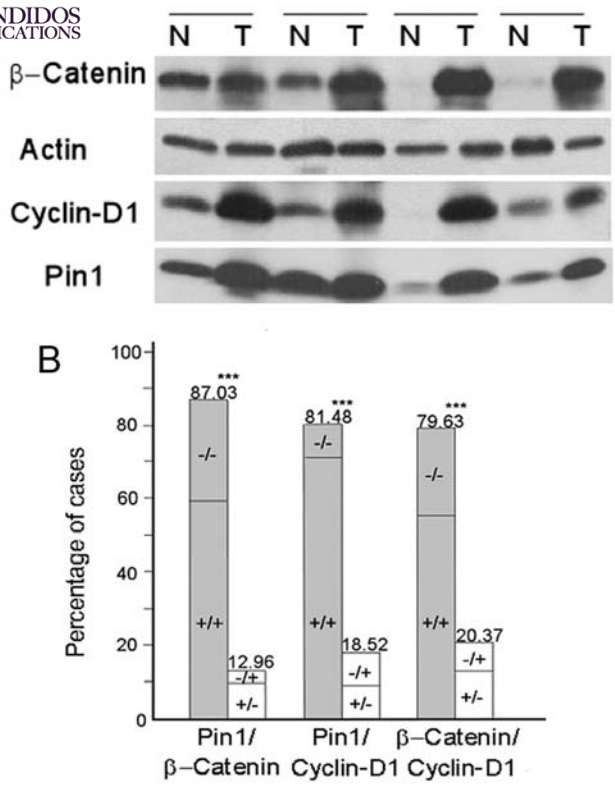

Figure 3. Expression of Pin1, ß-catenin and cyclin D1 in oral cancer tissues. (A) Immunoblot analysis for detecting Pin1, ß-catenin and cyclin D1 expression patterns in Pin1 overexpressing oral cancer tissue using the following antibodies- polyclonal rabbit anti-Pin1, monoclonal mouse anti-ß-catenin or monoclonal mouse anti-cyclin D1. Monoclonal mouse anti-actin served as an internal control. (B) Concordance analysis between protein expression of Pin1, ß-catenin and cyclin D1. The percentage of cases is indicated on the y-axis, whereas the type of comparison is shown on the x-axis. + indicates positive protein expression (Pin1, ß-catenin and cyclin D1). Numbers above the bars indicate percentages of the total concordant group (gray sections) and non-concordant group (white sections).

with the order of pathological classification in cancer tissues from well, moderate to poor differentiation (Fig. 2AIV-VI). Moreover, we found that the Pin1 level elevation can even be detected in hyperplasia and dysplasia stages (Fig. 2AIII).

In order to confirm these results, immunoblotting was performed to examine the Pin1 protein level in oral cancer tissues. Non-tumor tissues were used as references and Pin1 level was expressed as Pin1: actin ratio as described above (16). Fig. 2B shows Pin1 overexpressed in hyperplasia, dysplasia, and cancer tissues with well, moderate, and poor differentiation status when compared with Pin1 expression level of normal oral tissue. The mean value of Pin1 protein levels (shown in Fig. 2C), for dysplasia, well differentiation, moderate differentiation, and poor differentiation, were 1.4, 1.8, 2.2 and 5.4, respectively. Statistical analysis showed that the degree of cell differentiation was correlated with the level of Pin1 (correlation coefficient = 0.252, $P<0.05$). The conclusion drawn from the results of immunoblotting was consistent with immunohistochemistry investigation that the Pin1 protein levels correlated with the pathological poor differentiation in OSCC. These results also indicated that Pin1 level may be used as a pathological marker for oral cancer diagnosis.

Molecular signaling of Pin1 overexpression related tumorigenesis in OSCC. Previously, Pin1 overexpression in breast cancers was found to stabilize ß-catenin proteins and consequentially caused elevation of cyclin D1 (16,20). Therefore, the expression profile of ß-catenin and cyclin D1 expression were analyzed to assess their correlation with Pin1 in OSCC. Protein levels of ß-catenin and cyclin D1 in 54 OSCC N/T paired specimens containing 39 pairs of Pin1-overexpressed and 15 pairs of Pin1 level down-regulated or without change were determined by immunoblotting to establish the relationship between Pin1, cyclin D1 and ß-catenin protein expression in OSCC. Actin level was used as the internal control to normalize the protein amount for loading. Fig. 3A shows that Pin1, cyclin D1 and ß-catenin protein levels were elevated in oral cancer tissues when compared with those in their normal counterparts. Specifically, ß-catenin protein accumulation in tumor part was detected in 35 N/T pairs accounted for 65% of total 54 pairs of OSCC specimens examined; cyclin D1 levels increased in 37 N/T pairs accounted for 69% of total 54 pairs of OSCC specimens examined. The data of Pin1, cyclin D1 and ß-catenin protein levels were then cross examined to investigate the correlation between these three parameters in OSCC. The results of statistical analysis (Fig. 3B) showed that Pin1 protein level were significantly associated with ß-catenin level (concordance group 87.0%; $P<0.001$, by χ^2 test) or cyclin D1 protein level (concordance group 81.5%; $P<0.001$, by χ^2 test), respectively. The results in Fig. 3B also indicate a statistically significant correlation between ß-catenin level and Cyclin D1 protein level (concordance group 79.6%; $P<0.001$, by χ^2 test). Furthermore, our results showed 32 of 37 (86%) cases with Pin1-overexpression had concomitant accumulation of ß-catenin protein; 32 of 37 (86%) had concomitant accumulation of cyclin D1 protein; and 30 of 37 (81%) had both ß-catenin protein and cyclin D1 protein overexpression.

In addition, the protein levels of ß-catenin and cyclin D1 in four oral cancer cell lines were also examined by immunoblotting and the results are shown in Fig. 4A. All oral cancer cell lines showed the accumulation of ß-catenin and up-regulation of cyclin D1 accompanying with Pin1 overexpression when compared with normal oral tissue (Fig. 4A). Altogether, these data suggested that Pin1 overexpression might directly lead to an increase in ß-catenin and cyclin D1 levels in OSCC.

To directly demonstrate that this molecular mechanism also plays a role in OSCC tumorigenesis, a Pin1 cDNA expression construct, Pin1-pCMV, was transiently transfected into four human oral squamous cell lines, SAS, TW2.6, CA922 and Cal27. The results from immunoblot analysis confirmed a significant increase in ß-catenin and cyclin D1 levels when Pin1 overexpressed in three cell lines SAS, TW2.6 and Cal27 (Fig. 4A). The levels of Pin1 in the transiently transfected clones positively correlated with both ß-catenin and cyclin D1 levels. In addition to Pin1-pCMV, OSCC cells transfected with Pin1 in various plasmids including pEGFP, pcDNA, pDsRed, also showed similar results (data not shown). These results suggested Pin1 up-regulation could cause ß-catenin accumulation and cyclin D1 level elevation in OSCC. This conclusion was further

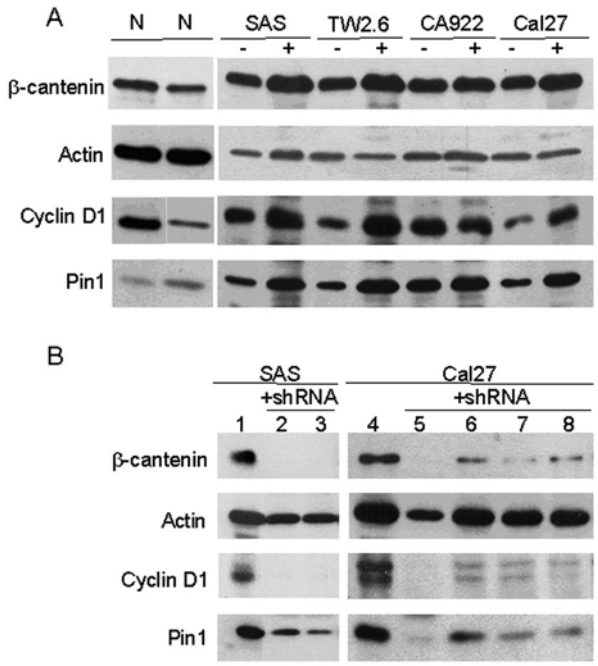

Figure 4. Expression of Pin1, ß-catenin and cyclin D1 in Pin1-transfected and Pin1/shRNA transfected OSCC cells lines. Assessment of Pin1 ß-catenin and cyclin D1 in four oral cancer cell lines. Increase in cellular ß-catenin and cyclin D1 protein by Pin1. Four oral cancer cell lines were transfected or non-transfected with Pin1, followed by immunoblotting analysis of the cell lysates with antibodies against Pin1 ß-catenin and cyclin D1, with actin as a control. Cells were transient transfected with 4 μg of Pin1/shRNA according to the manufacturer's protocol. At 2 days post-transfection, the medium was replaced with fresh growth medium without antibiotic containing puromycin at 3 μg/ml for clonal selection. Selection was maintained in a medium containing puromycin at 2 μg/ml to ensure a stable transfection.

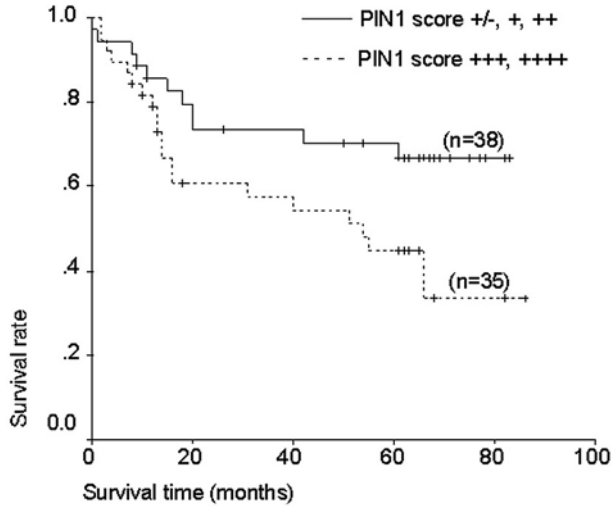

Figure 5. The relationship between disease-free survival and the Pin1 IHC score of non-tumor part. The dot-plot line represents overexpression of Pin1. Kaplan-Meier survival curves of patients with oral squamous cell carcinoma according to expression pattern of Pin1. The survival curves were analyzed by the log-rank test. Overall survival was calculated from the time of surgery to date of death, as the event of interest, or the date of last follow-up. All statistical analysis was performed using SPSS software.

confirmed by Pin1 depletion experiment by shRNA described below. The shRNA of Pin1 was used to deplete the endogenous Pin1 level in two OSCC cell lines, SAS and Cal27. As shown in Fig. 4B, the results showed that inhibition of both ß-catenin accumulation and down-regulation of cyclin D1 levels accompany the Pin1 suppression in either stable clone or transient transfection experiment. Taken together, our results demonstrated over-expression of Pin1 up-regulated ß-catenin and cyclin D1 levels in a direct manner and this might play an important role in OSCC tumorigenesis.

Relationships between expression pattern of Pin1 and pathological cell differentiation and prognosis in OSCC. We finally compared Pin1 expression pattern with clinico-pathological parameters in 73 OSCC patients as described in detail in Materials and methods. We were particularly interested in whether the Pin1 expression level can be used as a prognostic marker for OSCC. By immunohistochemistry examination, Pin1 protein expression levels and extent in oral cancer paraffin sections were scored according to the Pin1 staining intensity in the nucleus and the extent of staining of tumor cells (17). We defined the Pin1 overexpression based on the Pin1 staining score ranking 3 (+++) or above, whereas the non-Pin1 overexpression was of the score ranking from 0 (+/-) to 2 (++). As shown in Fig. 5, the results from Kaplan-Meier curves analysis revealed a significant difference among the groups overexpression and non-overexpression of Pin1. The patients who showed higher scores in the expression of Pin1 had significantly poorer survival than those who showed the lower score of Pin1 expression ($P<0.0001$ by log-rank test; Fig. 5). These results strongly suggested that Pin1 expression level might be a good prognosis marker for OSCC.

Discussion

Previously, Bao *et al*, showed Pin1 overexpression in 38 types of human cancers with elevated Pin1 levels relative to the corresponding normal tissue including most common human cancers such as prostate, cervical, brain, lung, breast, liver cancer, and melanoma (17). However, they did not determine the levels of Pin1 expression in either head and neck cancers or oral cancers. In this study, we demonstrated that Pin1 was overexpressed in oral squamous cell carcinoma (OSCC) by immunoblotting and immunohistochemistry analysis (Figs. 1 and 2). These results indicated that Pin1 overexpression is prevalent and specific in a large subset of human cancers including OSCC.

Pin1 overexpression can activate multiple oncogenic pathways and contribute to cell transformation. Recent studies have demonstrated that Pin1 increased the transcription level of several oncogenes including cyclin D1 and c-myc as well as conferred anchorage-independent cell growth to normal mammary epithelial cells (16,34). Breast cancer for example, Pin1 protein overexpression can lead to cyclin D1 elevation and transformation of breast epithelial cells (16,34). Interestingly, a number of evidence has demonstrated that Pin1 might play an essential role in regulating the expression and turnover of cyclin D1 by multiple mechanisms. Pin1 cooperates with c-Jun N-terminal kinase (JNK) or Ha-Ras increasing transcriptional activity of phosphorylated c-Jun to activate the cyclin D1 promoter (16,34). In addition, Pin1

to phosphorylated ß-catenin that consequentially the transcriptional activity towards its target gene, cyclin D1 (2,15). Moreover, the interaction of Pin1 and phosphorylated p65/RelA subunit of NFκB prevents NFκB from binding to IκB that increases its transcriptional activity and consequentially elevate the cyclin D1 level (36). Besides up-regulation of the cyclin D1 gene expression, Pin1 has been demonstrated to directly bind to pT286-Pro motif of cyclin D1 and resulted in increasing its protein stability (37). This evidence demonstrates that Pin1 overexpression can cause cyclin D1 protein elevation though at least four distinct mechanisms.

Our results showed that Pin1 is overexpressed in oral squamous cell carcinoma (OSCC) and statistic analysis revealed that its level correlated with cyclin D1 expression (Figs. 1 and 3). Together with the results from a previous study that mice lacking Pin1 has phenotypes remarkably similar to those in cyclin D1-deficient mice (37), these results indicated that Pin1 is a regulator of cyclin D1 expression in OSCC and might play a role in OSCC. Indeed, evidence as described below has showed that cyclin D1 elevation is tightly associated with OSCC. First, cyclin D1 protein is overexpressed in up to 80% of head and neck SCC (23-25). Second, suppression of cyclin D1 has been shown to inhibit the SCC growth *in vitro* and *in vivo* that leads to cellular apoptosis (26). Third, overexpression of cyclin D1 has been found associated with a more aggressive phenotype and reduced survival in patients with head and neck SCC including tongue cancer (27-31). The above evidence together with our results in this study suggest that cyclin D1 might play an important role during tumorigenesis of OSCC.

For the pathological mechanism of how Pin1 elevated cyclin D1 expression in OSCC, we have examined the plausibility of Pin1-ß-catenin-cyclin D1 signaling. Compelling evidence shows that Pin1 mediated ß-catenin accumulation and enhanced its transcriptional activity in both *in vitro* and *in vivo* conditions. By using an antisense approach, Ryo *et al*, showed that down-regulation of Pin1 in HeLa cells led to a decrease in ß-catenin levels (15). In Pin1 knockout mice, lower ß-catenin levels were found in all tissues compared to those of wild-type mice (15,34). In addition, Pin1 has been demonstrated to bind directly to phosphorylated ß-catenin at pT246P motif *in vitro* and *in vivo* and block the interaction between ß-catenin and APC (15,34). Moreover, Pin1-ß-catenin signaling has been shown to regulate cyclin D1 expression in human breast and liver cancers when Pin1 was overexpressed (16-20). These studies suggested a mechanism that overexpression of Pin1 contributes to the ß-catenin accumulation and increases the nuclear fraction of ß-catenin by preventing its interaction with APC in tumors that consequently elevates the cyclin D1 gene expression. It is noteworthy that both APC and ß-catenin mutations are not common in breast and liver cancers. In head and neck SCC, ß-catenin mutations or deletions are not common suggesting that other undefined mechanisms are involved in the ß-catenin stabilization and accumulation (40). Our study showed that Pin1 protein up-regulation was correlated with both ß-catenin and cyclin D1 expression levels in clinical primary tumors and four oral cancer cell lines (Figs. 3 and 4A). Directly, we also demonstrated that overexpression of Pin1 by transfection of Pin1 gene in OSCC cells can significantly elevate the protein levels of ß-catenin and cyclin D1 in oral cancer cell lines (Fig. 4A). Furthermore, the results of cancer cells transfected with shRNA of Pin1 caused significant suppression of Pin1 expression, reduction of ß-catenin accumulation as well as cyclin D1 level reduction (Fig. 4B). Taking these data together, we believed that Pin1-ß-catenin-cyclin D1 signaling played a role in OSCC tumorigenesis.

It has been reported that the pathological molecular mechanism that overexpression of Pin1-ß-catenin-cyclin D1 overexpression can be used as a marker of poor prognosis in human cancers including breast, prostate and liver (16,19). Therefore, Pin1 overexpression might also be used as an indicator for pathological diagnosis or prognosis in OSCC. We analyzed the association of Pin1 protein level with clinicopathological parameters and our results showed Pin1 overexpression was correlated with pathological cell differentiation status in OSCC (Fig. 2). Together with Kaplan-Meier curves analysis revealed a significant difference between Pin1 overexpression and non-Pin1 overexpression OSCC patients (Fig. 5). These results strongly suggested that Pin1 protein level might be a good diagnosis and prognosis marker for OSCC.

The prevalent overexpression of Pin1 in human cancers and correlation of overexpression with poor clinical outcomes in oral cancer patients are consistent with the previous findings that Pin1 overexpression plays specific roles in oncogenesis (1,17). Furthermore, several lines of evidence including ours strongly suggested that inhibition of Pin1 could suppress oncogenesis that provides a new avenue for anti-cancer therapy. Pin1 is an enzyme with high specificity that can potentiate the function of many oncogenic pathways (1-4). Pin1 overexpression can confer transforming properties on mammary epithelial cells that is associated with tumorigenesis (34). In addition, anti-sense Pin1 or dominant-negative Pin1 causes Pin1 depletion and apoptosis in cancer cells that indicates inhibition of Pin1 activity might kill cancer cells (33,34). Furthermore, the strong relationship between the Pin1 level and the clinical outcome of oral (Fig. 5) and prostate cancers, suggests that Pin1 could become a potential therapeutic target in patients with biologically aggressive tumors (18). Finally, structurally and functionally, Pin1 can be divided into two domains, PPIase and WW domain, which can serve as chemical intervention targets (6-8). It is reasonable to assume that chemical intervention on either PPIase activity or the phosphoprotein binding activity of WW domain (for example: phosphorylated cyclin D1, -ß-catenin, -NFκB and -c-Jun) might block the oncogenic signaling (1,2).

In conclusion, our data suggest that the level of Pin1 expression may be clinically useful for assessing prognosis, particularly in primary oral carcinoma specimens, warranting prospective confirmation. Furthermore, our findings suggest that Pin1 might be a useful target for new drug development.

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