Original Article

Combined evaluation of both WEE1 and phosphorylated cyclin dependent kinase 1 expressions in oral squamous cell carcinomas predicts cancer recurrence and progression

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Abstract
Background/purpose: WEE1 is a mitotic inhibitor at G2 checkpoint of the cell cycle that negatively regulates cyclin-dependent kinase 1 (CDK1) through inhibitory phosphorylation. This study assessed whether the expressions of both WEE1 and phosphorylated CDK1 in specimens of oral squamous cell carcinoma (OSCC) might predict the OSCC recurrence and progression.

Materials and methods: This study used immunohistochemistry to examine the expressions of WEE1 and phosphorylated CDK1 proteins in 75 specimens of OSCC and 30 specimens of normal oral mucosa (NOM).

Results: The mean WEE1 labeling index (LI) was significantly lower in 75 OSCC samples than in 30 NOM samples (P < 0.001), whereas the mean phosphorylated CDK1 LI was significantly higher.
higher in 75 OSCC samples than in 30 NOM samples ($P < 0.001$). We found a significant association of low WEE1 LI ($< 21\%$) with OSCC recurrence ($P = 0.047$) and a significant association of low phosphorylated CDK1 LI ($< 10\%$) with larger tumor size ($P = 0.011$) and more advanced clinical stages ($P = 0.021$) of OSCC.

**Conclusion:** Combined evaluation of WEE1 and phosphorylated CDK1 LI in specimens of OSCC may predict the OSCC recurrence and progression.

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**Introduction**

Head and neck cancer, including oral cancer, is the sixth most common cancer in males worldwide. More than 90% of all oral cancers are squamous cell carcinomas (OSCC). In Taiwan, according to the latest statistics from the Ministry of Health and Welfare (year 2021), oral cancer is the sixth leading cause of cancer death in the whole population and the fourth leading cause of cancer death in males.

Two genetic characteristic features of OSCC are the diversity of mutational etiology and frequent mutations of tumor suppressor genes. Recent large-scale whole exome screening studies revealed that the most frequently mutated gene in OSCCs is TP53 gene, which is an important tumor suppressor gene and a key regulator of the G1 checkpoint. Therefore, an effective G1 checkpoint is often destructed in OSCC tumor cells due to loss-of-function of mutated TP53, and thus these tumor cells depend more on the G2 checkpoint when DNA damage occurs.

Wee was first proposed by Nurse in *S. pombe*. He found that mutant cells are smaller than wild-typed cells after cell division. Wee is considered to be involved in controlling cell size after mitosis, because WEE1 and cell division cycle protein 2 (cdc2) are two major proteins to monitor cell size and growth rate at the time of mitosis. Because WEE1 has been proved to be a protein kinase important for maintaining genomic integrity, more and more interests in WEE1 and its associations with cancers or even cancer therapies have been evoked in the past decades. One of its functions is to negatively regulate cyclin-dependent kinase 1 (CDK1) through inhibitory phosphorylation on the tyrosine 15 site of CDK1 (phospho Y15) in the G2 phase. Because CDK1 can synchronize with cyclin B to drive cell into mitosis, inhibition of CDK1 may delay mitosis and maintain an arrest in G2 cell cycle. Therefore, WEE1 is regarded as a gatekeeper at G2 checkpoint of the cell cycle when there is a DNA damage.

Besides, WEE1 also participates in regulating histone synthesis. It directly phosphorylates the mammalian core histone H2B at tyrosine 37 in the nucleosomes at the end of S phase to terminate histone transcription and in turn prevent overproduction. Taken together, before entry into mitosis, WEE1 kinase can phosphorylate CDK1 to prevent exit from S phase until the correct DNA replication completed. WEE1 kinase can also phosphorylate H2B in late S phase to end the histone synthesis. Therefore, WEE1 plays a crucial role in maintaining the integrity of the DNA.

Because WEE1 is essential in cell cycle regulation and chromatin synthesis, dysregulation of WEE1 kinase may lead to uncontrolled cell growth and even malignant transformation. Overexpression of WEE1 has been observed in several human malignancies including osteosarcoma, breast cancer, and glioblastoma. Moreover, high WEE1 activity is found in hepatocellular carcinoma. Its correlations with tumor differentiation, recurrence, and patients’ survival in certain cancers have been reported but the exact relation between WEE1 and malignancies is still controversial. Moreover, the correlation between the WEE1 protein expression and clinicopathological parameters of OSCCs has not yet been assessed.

The aims of this study were to evaluate the expressions of WEE1 and phosphorylated CDK1 proteins in 75 OSCC and 30 normal oral mucosa samples and to evaluate the correlations between the WEE1 or phosphorylated CDK1 protein expression in OSCC samples and the clinicopathological parameters of OSCCs.

**Materials and methods**

**Patients and specimens**

Formalin-fixed, paraffin-embedded specimens were obtained from 75 patients (67 men and 8 women, mean age 52 years, range 23-82 years) with OSCC. Diagnosis of OSCC was based on histological examination of hematoxylin and eosin-stained tissue sections. All patients received total surgical excision of their OSCCs at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan during the period from 2006 to 2010. Specimens were obtained from total surgical excision of the lesions. If cervical lymph node was diagnosed as positive for OSCC, neck dissection and postoperative radiation therapy and/or chemotherapy were also included in the treatment protocol. None of the patients had received any form of tumor-specific therapy before total surgical excision of the lesion. Of the 75 cases of OSCC, 27 (36%) were tongue, 23 (31%) buccal mucosa, 17 (23%) gingiva, 4 (5%) hard palate, 2 (3%) lip, 1 (1%) floor of the mouth, and 1 (1%) alveolar mucosa cancers. Histological features of OSCC were further classified into three different types (well-, moderately-, and poorly-differentiated OSCC). Of the 75 OSCC cases, there were 68 (91%) well- and 7 (9%) moderately-differentiated OSCCs. The TNM status and clinical stages of OSCCs at initial presentation were determined according...
Comparisons of the mean WEE1 or phosphorylated CDK1 LIs between NOM and OSCC samples were performed by Student’s t-test. The relation between WEE1 and phosphorylated CDK1 protein expression analysis was analyzed by the Spearman correlation and differences were tested by two-tailed t-test. The correlation between clinical-pathological parameters (including age, gender, T status, N status, clinical staging, tumor differentiation, and recurrence) and the expression status (high or low) of WEE1 or phosphorylated CDK1 protein were analyzed by chi-square or Fisher exact test, where appropriate. The correlation between T stage, N stage or recurrence and WEE1 or phosphorylated CDK1 protein expression were also analyzed by Mann—Whitney U test. Furthermore, the relationship between single parameter (T stage, N stage or recurrence) and both WEE1 and phosphorylated CDK1 protein expressions were analyzed by binary logistic regression. The 5-year survival was compared between groups by Kaplan-Meier survival curves and log-rank test. The procedures were conducted by software SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and the P-value < 0.05 was considered statistically significant.

Results

WEE1 protein expression in oral squamous cell carcinoma (OSCC) and normal oral mucosa (NOM) samples

Expression of WEE1 protein was observed in all OSCC samples (n = 75). The WEE1 LIs in OSCC samples ranged from 2% to 65% with an average of 23 ± 12% (Table 1). The median for WEE1 LIs was 21% and this value was adopted as the cut-off value to further divide the OSCC samples into low-WEE1-expression group (LI < 21%, n = 37) and high-WEE1-expression group (LI ≥ 21%, n = 38). In the low-WEE1-expression group, WEE1-positive cells were discovered

Table 1 The mean WEE1 and phosphorylated cyclin-dependent kinase 1 (CDK1) labeling indices (LIs) in 30 normal oral mucosa (NOM) and 75 oral squamous cell carcinoma (OSCC) samples.

| Groups              | Mean LI ± SD (%) | P-value* |
|---------------------|------------------|----------|
| **WEE1**            |                  |          |
| NOM samples (n = 30) | 38 ± 9           | P < 0.001|
| OSCC samples (n = 75)| 23 ± 12          |          |
| **Phosphorylated CDK1** |                |          |
| NOM samples (n = 30) | 5 ± 1            | P < 0.001|
| OSCC samples (n = 75)| 11 ± 6           |          |

* Comparison of the mean WEE1 or phosphorylated CDK1 LIs between NOM and OSCC samples by Student’s t-test.
mainly at the peripheral one cell layer of the cancer nest (Fig. 1A). In the high-WEE1-expression group, WEE1-positive cells were detected at the peripheral 3 to 5 cell layers of the cancer nest (Fig. 1B).

The WEE1 LIs of NOM samples ranged from 24% to 58% with a mean of 38 ± 9% (Table 1). The mean WEE1 LI was significantly higher in NOM samples than in OSCC samples (P < 0.001). The range of WEE1 LI was narrower in NOM samples than in OSCC samples, indicating a more stable WEE1 protein expression in NOM samples. In NOM samples, WEE1 protein expression was found mainly in the nuclei of the basal, parabasal, and spinous normal epithelial cells (Fig. 1C).

Phosphorylated cyclin-dependent kinase 1 (CDK1) protein expression in OSCC and NOM samples

Phosphorylated CDK1 protein expression was also found in all OSCC samples (n = 75). The LIs of phosphorylated CDK1 protein in OSCC samples ranged from 1% to 27% with an average of 11 ± 6% (Table 1). The median for phosphorylated CDK1 LIs was 10% and this value was adopted as a cut-off value to divide the OSCC samples into the low-phosphorylated CDK1-expression group (LI < 10%, n = 36) and high-phosphorylated CDK1-expression group (LI ≥ 10%, n = 39). The mean LI of phosphorylated CDK1 protein in

Figure 1  Representative immunohistochemical staining of WEE1 and phosphorylated cyclin-dependent kinase 1 (CDK1) proteins in oral squamous cell carcinoma (OSCC) and normal oral mucosa (NOM) samples. (A) A well-differentiated OSCC in the low-WEE1-expression group showing WEE1-positive nuclear-staining cells mainly at the most peripheral cell layer of the cancer nest. (B) A well-differentiated OSCC in the high-WEE1-expression group exhibiting WEE1-positive nuclear-staining cells at the peripheral 3 to 5 cell layers of the cancer nest. (C) A normal oral epithelial section demonstrating WEE1-positive nuclear-staining cells at the basal, parabasal, and spinous cell layers of the normal oral epithelium. (D) A well-differentiated OSCC in the low-phosphorylated CDK1-expression group showing phosphorylated CDK1-positive nuclear-staining cells mainly at the most peripheral cell layer of the cancer nest. (E) A well-differentiated OSCC in the high-phosphorylated CDK1-expression group exhibiting phosphorylated CDK1-positive nuclear-staining cells at the peripheral 3 to 5 cell layers of the cancer nest. (F) A normal oral epithelial section demonstrating phosphorylated CDK1-positive nuclear-staining cells mainly at the parabasal cell layers of the normal oral epithelium. (Original magnification, A, B, C, D, E and F; 20×.)
NOM samples (5 ± 1%) was significantly lower than that (11 ± 6%) in OSCC samples (Table 1).

The phosphorylated CDK1 LI was approximately 10% lower than the WEE1 LI for each particular OSCC sample. In addition, the range of the phosphorylated CDK1 LI was narrower than that of the WEE1 LI (Table 1). The immunostaining pattern of phosphorylated CDK1 protein was somewhat similar to that of WEE1. In the low-phosphorylated CDK1-expression group, phosphorylated CDK1-positive cells were mainly located at the most peripheral layer of the cancer nest (Fig. 1D). In the high-phosphorylated CDK1-expression group, phosphorylated CDK1-positive cells were found in the peripheral 3 to 5 cell layers of the cancer nest (Fig. 1E). In NOM samples, phosphorylated CDK1 protein expression was demonstrated mainly in the nuclei of the parabasal normal epithelial cells (Fig. 1F).

**Correlation between WEE1 and phosphorylated CDK1 protein expressions**

The correlation between WEE1 and phosphorylated CDK1 protein expressions was evaluated by the Spearman rank correlation. The Spearman rank correlation coefficient was 0.526, indicating a moderately positive correlation between WEE1 and phosphorylated CDK1 protein expressions in OSCC samples ($P < 0.001$).

**Association of WEE1 protein expression with OSCC patients’ clinicopathological parameters**

The recurrence rate was significantly higher in the low-WEE1-expression (LI < 21%) group than in the high-WEE1-expression (LI ≥ 21%) group ($P = 0.047$) (Table 2). However, there was no significant association of the WEE1 protein expression in OSCC samples with the patients’ age and gender, T status, N status, clinical staging, and tumor differentiation. The Mann-Whitney U test also showed a significant correlation between the WEE1 protein expression and cancer recurrence ($P = 0.031$). However, no significant association of the WEE1 protein expression with T status and N status was found (Table 3). Although the 5-year survival rate was lower in OSCC patients with lower WEE1 expression than in OSCC patients with the higher WEE1 expression, the difference was not significant (Fig. 2).

| Groups | Case number | P-value | Case number | P-value |
|--------|-------------|---------|-------------|---------|
|        | WEE1 expression |         | Phosphorylated CDK1 |         |
|        | High LI ≥ 21% | Low LI < 21% | High LI ≥ 10% | Low LI < 10% |
| Age (year) |            |         |               |         |
| ≥ 50 (n = 42) | 25 | 17 | 24 | 18 |
| < 50 (n = 33) | 13 | 20 | 15 | 18 |
| Gender |            |         |               |         |
| Men (n = 67) | 34 | 33 | 34 | 33 |
| Women (n = 8) | 4 | 4 | 5 | 3 |
| Cancer location |            |         |               |         |
| Tongue (n = 27) | 13 | 14 | 13 | 14 |
| Buccal mucosa (n = 23) | 13 | 10 | 16 | 7 |
| Other oral mucosal sites (n = 25) | 12 | 13 | 10 | 15 |
| T status |            |         |               |         |
| T1 + T2 (n = 43) | 24 | 19 | 28 | 15 |
| T3 + T4 (n = 32) | 14 | 18 | 11 | 21 |
| N status |            |         |               |         |
| N0 (n = 59) | 27 | 32 | 29 | 30 |
| N1 + N2 + N3 (n = 16) | 11 | 5 | 10 | 6 |
| Clinical staging |            |         |               |         |
| Stage 1 + 2 (n = 36) | 20 | 16 | 24 | 12 |
| Stage 3 + 4 (n = 39) | 18 | 21 | 15 | 24 |
| Recurrence |            |         |               |         |
| Without (n = 65) | 36 | 29 | 34 | 31 |
| With (n = 10) | 2 | 8 | 5 | 5 |
| OSCC differentiation |            |         |               |         |
| Well-differentiated (n = 68) | 34 | 34 | 35 | 33 |
| Moderately-differentiated (n = 7) | 4 | 3 | 4 | 3 |

LI = labeling index.
* Comparison between two groups or among three groups by chi-square or Fisher’s exact test, where appropriate.
Association of phosphorylated CDK1 protein expression with OSCC patients’ clinicopathological parameters

Lower phosphorylated CDK1 expression in OSCC samples was significantly associated with larger tumor size (T3 + T4) (P = 0.011) and more advanced clinical stage (stage 3 + 4) (P = 0.021). However, no significant correlation was found between phosphorylated CDK1 protein expression and patients’ age and gender, N status, tumor differentiation, and cancer recurrence (Table 2). The Mann-Whitney U test also showed a significant association of phosphorylated CDK1 protein expression with the tumor size (P = 0.013). However, no significant correlation was found between phosphorylated CDK1 protein expression and N status or cancer recurrence was demonstrated (Table 3). Although the 5-year survival rate was lower in OSCC patients with lower WEE1 protein expression than in OSCC patients with the higher WEE1 protein expression (labeling index < 21%) than in 38 OSCC patients with the higher WEE1 protein expression (labeling index ≥ 21%), but the difference was not significant (P = 0.377, log-rank test).

Correlation between both WEE1 and phosphorylated CDK1 protein expressions and clinical parameters

When both WEE1 and phosphorylated CDK1 protein expressions were correlated with T status, N status or cancer recurrence by binary logistic regression, the results showed a significant association of lower WEE1 expression with higher cancer recurrence rate (P = 0.018, odds ratio = 10.09) and a significant correlation of lower phosphorylated CDK1 expression with larger tumor size (P = 0.016, odds ratio = 4.76). However, both WEE1 and phosphorylated CDK1 protein expressions were not associated with lymph node metastasis (Table 3).

Discussion

This study found the expression of WEE1 and phosphorylated CDK1 proteins in both cytoplasm and nuclei of OSCC cancer cells. The WEE1 and phosphorylated CDK1 proteins may have various subcellular locations during different phases of cell cycle. During the interphase, WEE1 is mainly located in the nucleus to prevent early entry into mitosis and then partially translocated to the cytoplasm in the prophase, preparing for mitosis. CDK1 may be located in the nucleus, cytoplasm and mitochondria. It is situated in the cytoplasm during the interphase and translocated from cytoplasm to the nucleus when the cell enters into mitotic phase. The WEE1 protein staining was generally found in the nuclei of normal epithelial cells, indicating that the majority of the normal epithelial cells are in the interphase rather than in the mitotic phase. Because the mitotic activity is higher in cancer cells than in normal oral epithelial cells, this can explain why the mean WEE1 LI is higher in normal epithelial cells than in OSCC cells and why the mean phosphorylated CDK1 LI is higher in OSCC cells than in normal oral epithelial cells.

CDK1 can bind to cyclin B to form a complex and trigger the cell cycle from G2 to mitotic phase in eukaryotic cells. However, CDK1 is inactivated by inhibitory phosphorylation via two kinases, WEE1 and MYT1. Firstly, WEE1 phosphorylates CDK1 at Y15 site and MYT1 phosphorylates CDK1 at threonine site (T14). On the contrary, Cdc25 phosphatase can dephosphorylate Y15 site and T14 site and in turn activate CDK1, ensuring cell cycle progressing into the mitotic phase. Our immunostaining results showed colocalization of phosphorylated CDK1 and WEE1 proteins as well as a moderately positive correlation between phosphorylated CDK1 and WEE1 protein expressions. The relation between phosphorylated CDK1 and WEE1 protein expressions is consistent with the concept

| Groups            | WEE1 expression | Phosphorylated CDK1 expression |
|-------------------|----------------|-------------------------------|
| Mann-Whitney U test |                |                               |
| T status          | 0.069          | 0.013                         |
| N status          | 0.106          | 0.456                         |
| Cancer recurrence | 0.031          | 0.755                         |
| Binary logistic regression |     |                               |
| T status          | 0.474          | 0.016                         |
| N status          | 0.188          | 0.968                         |
| Cancer recurrence | 0.018          | 0.165                         |
predict cancer progression. Similarly, in melanomas, high WEE1 protein expression may be used as a biomarker to and tumor cell differentiation, implicating that the high WEE1 expression is associated with lymph node metastasis contrary, in vulvar squamous cell carcinomas, the higher WEE1 expression in OSCCs may be due to decreased synthesis or increased degradation or even both of them. Further studies are needed to explore the exact mechanisms that cause the decrease of WEE1 expression in OSCC samples.

that CDK1 is the substrate of WEE1 and that its activity can be regulated by WEE1. However, WEE1 is just one of the inhibitors of CDK1. In addition to WEE1, there are several other proteins capable of regulating the activity of CDK1.

WEE1 serves as a mitotic inhibitor in the G2 checkpoint. When there is DNA damage, WEE1 maintains G2-cell-cycle checkpoint arrest for DNA repair before mitosis. The relatively higher WEE1 expression in normal oral epithelial cells than in OSCC cells indicates at least the partial loss of WEE1-controlled checkpoint function in OSCCs and this may promote cancer cell proliferation. The causes of diminished WEE1 expression in OSCCs may be due to decreased synthesis or increased degradation or even both of them. Further studies are needed to explore the exact mechanisms that cause the decrease of WEE1 expression in OSCC samples.

Finally, downregulation of WEE1 kinase may lead to uncontrolled cell proliferation and probably cancer initiation. Recently, the association of WEE1 expressions with cancer progression and patients’ prognosis has gained a great interest. Yoshida et al. has reported that in non-small cell lung cancers, tumor cells lacking WEE1 kinase may have a higher recurrence rate and a lower patients’ survival rate. On the contrary, in vulvar squamous cell carcinomas, the higher WEE1 expression is associated with lymph node metastasis and tumor cell differentiation, implicating that the high WEE1 protein expression may be used as a biomarker to predict cancer progression. Similarly, in melanomas, high WEE1 expression is positively related to invasion depth, tumor size, and surface ulceration as well as negatively correlated with the disease-free survival. Thus, the high expression of WEE1 is strongly associated with tumor progression and poor prognosis in melanomas. In OSCCs, we found that the lower WEE1 expression was significantly associated with higher cancer recurrence rate, suggesting that a decrease or loss of WEE1 expression may worsen the patients’ prognosis. Moreover, this finding also confirms the role of WEE1 as a "G2-cell-cycle checkpoint".

The significant association of lower WEE1 protein expression in OSCC samples with cancer recurrence indicates that the WEE1 protein may serve as a cancer recurrence or prognosis predictor. Moreover, the significant correlation of lower phosphorylated CDK1 expression with larger tumor size and more advanced clinical stage suggests that the phosphorylated CDK1 may be used as a biomarker to predict OSCC progression. Hence, combined evaluation of both WEE1 and phosphorylated CDK1 expressions in OSCCs may predict the OSCC recurrence and progression. However, it still needs further investigations of the potential molecular mechanisms of WEE1 in OSCCs, particularly the association of WEE1 with other G2-M checkpoint regulators, such as CHK1 and MYT1.

Figure 3 Kaplan-Meier survival curve showing relation between phosphorylated cyclin-dependent kinase 1 (CDK1) protein expression in OSCC samples and survival of 75 OSCC patients. The duration of survival was measured from the beginning of treatment to the time of death (complete) or the last follow-up (censored). The 5-year survival rate was lower in 36 OSCC patients with lower phosphorylated CDK1 protein expression (labeling index < 10%) than in 39 OSCC patients with the higher phosphorylated CDK1 protein expression (labeling index ≥ 10%), but the difference was not significant (p = 0.188, log-rank test).

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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