

RESEARCH ARTICLE

# Expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (Total) and pCyclin B1<sup>Ser126</sup> in Vulvar Squamous Cell Carcinoma and Their Relations with Clinicopathological Features and Prognosis

Zhihui Wang<sup>1\*</sup>, Ana Slipicevic<sup>1</sup>, Mette Førsvund<sup>1</sup>, Claes G. Trope<sup>2</sup>, Jahn M. Nesland<sup>3</sup>, Ruth Holm<sup>1</sup>

**1** Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway, **2** Department of Obstetrics and Gynecology, The Norwegian Radium Hospital, Oslo University Hospital and University of Oslo, Oslo, Norway, **3** Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital and University of Oslo, Oslo, Norway

\* [zhihui.wang@rr-research.no](mailto:zhihui.wang@rr-research.no)



**OPEN ACCESS**

**Citation:** Wang Z, Slipicevic A, Førsvund M, Trope CG, Nesland JM, Holm R (2015) Expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (Total) and pCyclin B1<sup>Ser126</sup> in Vulvar Squamous Cell Carcinoma and Their Relations with Clinicopathological Features and Prognosis. PLoS ONE 10(4): e0121398. doi:10.1371/journal.pone.0121398

**Academic Editor:** Xin-Yuan Guan, The University of Hong Kong, CHINA

**Received:** November 17, 2014

**Accepted:** January 31, 2015

**Published:** April 7, 2015

**Copyright:** © 2015 Wang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** This study was financially supported by The Inger and John Fredriksen Foundation for Ovarian Cancer Research and The Norwegian Cancer Society (URL: <https://kreftforeningen.no/>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

Cyclin B1-CDK1 complex plays an important role in the regulation of cell cycle. Activation of Cyclin B1 and CDK1 and the formation of the complex in G2/M are under multiple regulations involving many regulators such as isoforms of 14-3-3 and CDC25 and Wee1. Abnormal expression of Cyclin B1 and CDK1 has been detected in various tumors. However, to our knowledge no previous study has investigated Cyclin B1 and CDK1 in vulvar cancer. Therefore, we evaluated the statuses of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> in 297 cases of vulvar squamous cell carcinomas by immunohistochemistry. Statistical analyses were performed to explore their clinicopathological and prognostic values. In at least 25% of tumor cases high expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> was observed, compared to the low levels in normal vulvar squamous epithelium. Elevated levels of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> were correlated with advanced tumor behaviors and aggressive features. Although CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> could not be identified as prognostic factors, combinations of (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>N</sup>), (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>ηC</sup>), (pCDK1<sup>Thr161 C+N</sup> + Wee1<sup>C</sup>) and (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>σN</sup> + 14-3-3<sup>ηC</sup> + Wee1<sup>C</sup>) were correlated with disease-specific survival ( $p = 0.036$ ,  $p = 0.029$ ,  $p = 0.042$  and  $p = 0.007$ , respectively) in univariate analysis. The independent prognostic significance of (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>σN</sup> + 14-3-3<sup>ηC</sup> + Wee1<sup>C</sup>) was confirmed by multivariate analysis. In conclusion, CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> may be involved in progression of vulvar squamous cell carcinoma. The combination of pCDK1<sup>Thr161</sup>, 14-3-3<sup>σ</sup>, 14-3-3<sup>η</sup> and Wee1 was a statistically independent prognostic factor.

**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

Vulvar carcinoma accounts for 3–5% of all female genital cancers with 27,000 new diagnosed patients worldwide each year [1,2]. Although vulvar cancer occurs most frequently in women above age of 65 [3,4], an increasing incidence has recently been observed among younger women [5–11]. Vulvar squamous cell carcinoma (VSCC) is the most common histological subtype, accounting for more than 80% of the cases [12]. For the last two decades, radical surgery has been the standard treatment for most patients but it is associated with high treatment-related morbidity. In recent years, less invasive novel treatments have been introduced; unfortunately a significant improvement in survival has not been achieved yet [6,13]. Therefore, identification of new biomarkers and potential therapeutic targets is highly warranted.

Cyclin B1-CDK1 complex plays an important role in G2/M cell cycle. Activation of CDK1 protein kinase and formation of Cyclin B1-CDK1 complex is an obligate step for entry into mitosis and hence is under extensive regulations [14,15]. Activity of CDK1 is controlled through association with Cyclin B1, reversible phosphorylation [16] and subcellular localizations [14,17]. Throughout the early phases of the cell cycle, inhibitory phosphorylation of CDK1 on Tyr<sup>15</sup> and Thr<sup>14</sup> by Wee1 and MYT1 keeps it in inactive state [15]. In late G2, CDK1 is activated by CDC25C phosphatase through dephosphorylation upon both Thr<sup>14</sup> and Tyr<sup>15</sup> residues, as an obligate step for the G2/M transition [18–21]. To reach maximum activity of CDK1, the phosphorylation of Thr<sup>161</sup> residue by CDK1 activating kinase (CAK) is a requirement [15,22]. Therefore, dephosphorylation upon Tyr<sup>15</sup> of CDK1 (CDK1<sup>Tyr15</sup>) and phosphorylation upon Thr<sup>161</sup> of CDK1 (pCDK1<sup>Thr161</sup>) are both activated forms. For Cyclin B1, the residues Ser<sup>126</sup> and Ser<sup>128</sup> on the N-terminal are among the first ones which are autophosphorylated by Cyclin B1-CDK1 complex [17], so that pCyclin B1<sup>Ser126</sup> is regarded as the equivalent form of active Cyclin B1.

Abnormal expression of Cyclin B1 and/or CDK1 has been reported in several types of tumors, such as epithelial ovarian cancer [23], non-small-cell lung cancer [24,25], tongue cancer [26], breast cancer [27], gastric cancer [28] and colorectal cancer [29]. In some of these reports the altered level of Cyclin B1 and/or CDK1 expression indicated a poor outcome of patients [25,27]. To our knowledge, no previous study has investigated CDK1 and Cyclin B1 in vulvar carcinomas. Thus, we examined the expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> proteins in a large cohort of VSCC and explored their clinicopathologic and prognostic values. Previously in the same patient population we have identified several checkpoint proteins involved in G2/M regulations, including isoforms of CDC25 [30] and 14-3-3 [31,32] and Wee1 [33]. We evaluated the relationships of these G2/M pathway regulators together and explored combinations which might help to predict the outcome of patients with VSCC.

## Methods

### Patient materials

A retrospective study including 297 patients with VSCC who had undergone surgery at The Norwegian Radium Hospital between 1977 and 2006 was performed. The median age of patients at diagnosis was 74 years (range, 35–96 years). Nine patients received pre-surgery treatment, six of which were treated with radiotherapy and the other three with radiotherapy/chemotherapy. Radical vulvectomy was performed on 192 (65%) patients, whereas 105 (35%) patients were subjected to non-radical surgery. Seventy patients received postoperative treatment including 3 patients given chemotherapy, 63 treated with irradiation, and 4 received combination of irradiation and chemotherapy. All the patients were followed until death occurred

or 5 years after study inclusion. Of the 297 patients, 100 (34%) died of vulvar cancer within 5 years after inclusion.

The tumour stage examination was performed according to the 2009 International Federation of Gynaecology and the Obstetrics (FIGO) classification system [34]. The histological re-examination of all cases was performed by one of the authors (J.M.N) according to World Health Organization recommendations [35]. Two hundred and eighty (94%) tumors were keratinizing/nonkeratinizing, 13 (5%) were basaloid and 4 (1%) were verrucoid. Normal vulva samples were obtained as controls from 10 patients operated for benign gynaecological diseases.

## Ethics statement

The approval of the study was granted by The Regional Committee for Medical Research Ethics South of Norway (S-06012), The Data Inspectorate (04/01043) and The Social and Health Directorate (04/2639 and 06/1478). In this study we have used paraffin embedded tumor tissue from vulvar cancer patients diagnosed between 1977 and 2006. Many of these patients are either dead or very old. Therefore, we have not been able to obtain patient consent. Permission has been obtained from The Social and Health Directorate (04/2639) to perform this study without patient consent.

## Immunohistochemistry

Three- $\mu$ m sections made from formalin-fixed paraffin embedded tissues were immunostained using the Dako EnVision + system (K8012, Dako Cooperation, CA, USA) and DAKO Auto-stainer. Deparaffinization, rehydration and target retrieval were performed in a Dako PT-link and EnVision Flex target retrieval solution with high pH for pCDK1<sup>Thr161</sup> and low pH for pCyclin B1<sup>Ser126</sup>, Cyclin B1 (total) and CDK1<sup>Tyr15</sup>. Endogenous peroxidase was blocked using Dako blocking reagent for 5 minutes followed by incubation with primary antibodies against pCyclin B1<sup>Ser126</sup> (rabbit polyclonal antibody, AP3078a, 1:400, 62.5 $\mu$ g Ig/ml, Nordic BioSite AS, Täby, Sweden), Cyclin B1 (total) (rabbit monoclonal antibody, Clone Y106, 1:700, 0.45 $\mu$ g Ig/ml, Epitomics Inc., CA, USA), pCDK1<sup>Thr161</sup> (rabbit polyclonal antibody, sc-101654, 1:100, 1 $\mu$ g Ig/ml, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and CDK1<sup>Tyr15</sup> (rabbit monoclonal antibody, Clone: 6k113, 1:1000, 0.1 $\mu$ g Ig/ml, United States Biological Inc., MA, USA). Thereafter, the sections were incubated with Dako EnVision FLEX+ rabbit linker for 15 minutes followed by incubation with Dako EnVision FLEX/HRP for an additional 30 minutes. For visualization of staining, the sections were treated with 3'-diaminobenzidine tetra-hydrochloride (DAB), counterstained with haematoxylin and mounted in Richard-Allan Scientific Cytoseal XYL (Thermo Scientific, Waltham, MA, USA).

All of the sample series had appropriate positive controls including tonsil [pCyclin B1<sup>Ser126</sup> and Cyclin B1 (total)] and placenta (pCDK1<sup>Thr161</sup> and CDK1<sup>Tyr15</sup>). Negative controls included substitutions of (i) polyclonal anti-pCyclin B1<sup>Ser126</sup> (ii) polyclonal anti-pCDK1<sup>Thr161</sup> and (iii) monoclonal anti-Cyclin B1 (total) and CDK1<sup>Tyr15</sup> with (i) normal rabbit Ig (ii) normal rabbit IgG and (iii) normal rabbit IgG monoclonal at the same concentration as the primary antibodies.

The immunohistochemical staining was evaluated without knowledge of the patient outcome. Semi-quantitative classes were used to describe the immunostaining. The extent of staining was scored into 4 levels regarding percent of positive tumor cells (absent, 0; < 10%, 1; 10–50%, 2; > 50%, 3), while staining intensity was scored into 4 levels as well (absent, 0; weak, 1; moderate, 2; strong, 3). Immunoreactivity in cytoplasm and nucleus was calculated separately by multiplying the scores of the staining extent and intensity of each slide, and composite

scores were ranged from 0 to 9. High protein level was defined when composite score  $> 3$  and low when composite score  $\leq 3$ . The cutoff values for the immunostaining were based on staining pattern observed in normal vulvar epithelium.

## Statistical analyses

The Pearson's chi-square ( $\chi^2$ ) test was performed to evaluate the relationship between the expression of CDK1 (CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup>) and Cyclin B1 (total and pCyclin B1<sup>Ser126</sup>) and clinicopathological parameters. Survival analyses were evaluated on the whole group of 297 VSCC and on the group of 288 VSCC which excluded nine patients who have received pre-surgery treatment. Kaplan and Meier method was used to calculate the disease-specific survival from the date of diagnosis to vulvar cancer related death. Survival rate comparison was performed by the log-rank test. Univariate and multivariate evaluation of survival were calculated by using Cox proportional hazards regression. Patients were censored after 5 years. In the multivariate analysis, a backward stepwise regression with a  $p$  value of 0.05 as the inclusion criterion was used. All analyses were executed by using SPSS 18.0 statistical software package (SPSS, Chicago, IL, USA). Statistical significance was considered as  $p \leq 0.05$ .

## Results

### CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> proteins expression

In 10 cases of normal vulvar squamous epithelium, immunoreactivity of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> were detected in basal and parabasal layers (score = 3 in both cytoplasm and nucleus, Fig 1A–1D).

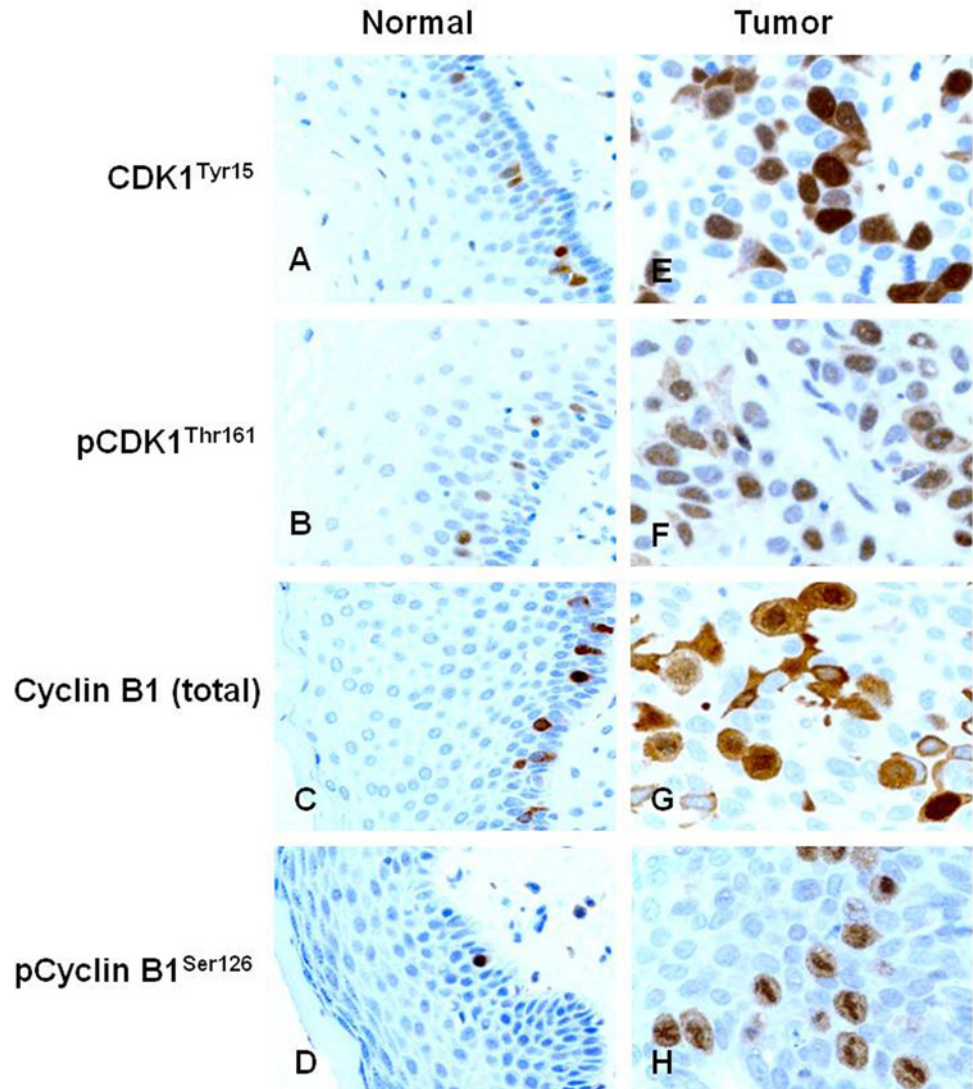
The immunostaining results in vulvar carcinomas are summarized in S1 Table. High cytoplasmic staining (score  $> 3$ ) of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> were observed in 195 (66%), 97 (33%), 208 (70%) and 73 (25%) of the cases, respectively. In the nucleus, high expression (score  $> 3$ ) of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> were detected in 78 (26%), in 231 (78%), 82 (28%) and 75 (25%) of the cases, respectively (Fig 1E–1H). No difference of the immunostaining was observed on the front of invasion and the center of the tumor.

### Association of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> proteins with clinicopathological variables

High cytoplasmic level of CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> and high expression of Cyclin B1 (total) in either cytoplasm or nucleus were correlated with large tumor diameter, poor histological differentiation and deep invasion (S2 and S3 Tables). High nuclear level of pCDK1<sup>Thr161</sup> and high expression of pCyclin B1<sup>Ser126</sup> in either cytoplasm or nucleus were associated to younger age, high FIGO substage and poor histological differentiation. High protein level of CDK1<sup>Tyr15</sup> in cytoplasm and that of pCDK1<sup>Thr161</sup> in nucleus also had correlation with presence of lymph node metastasis.

### Correlations between CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> and other G2/M cell cycle factors

Since our cohort of VSCC has previously been tested for isoforms of CDC25 [30] and 14-3-3 [31,32], Wee1 [33] and HPV [36], we have examined the relationship between CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> and these factors (S4 and S5 Tables).



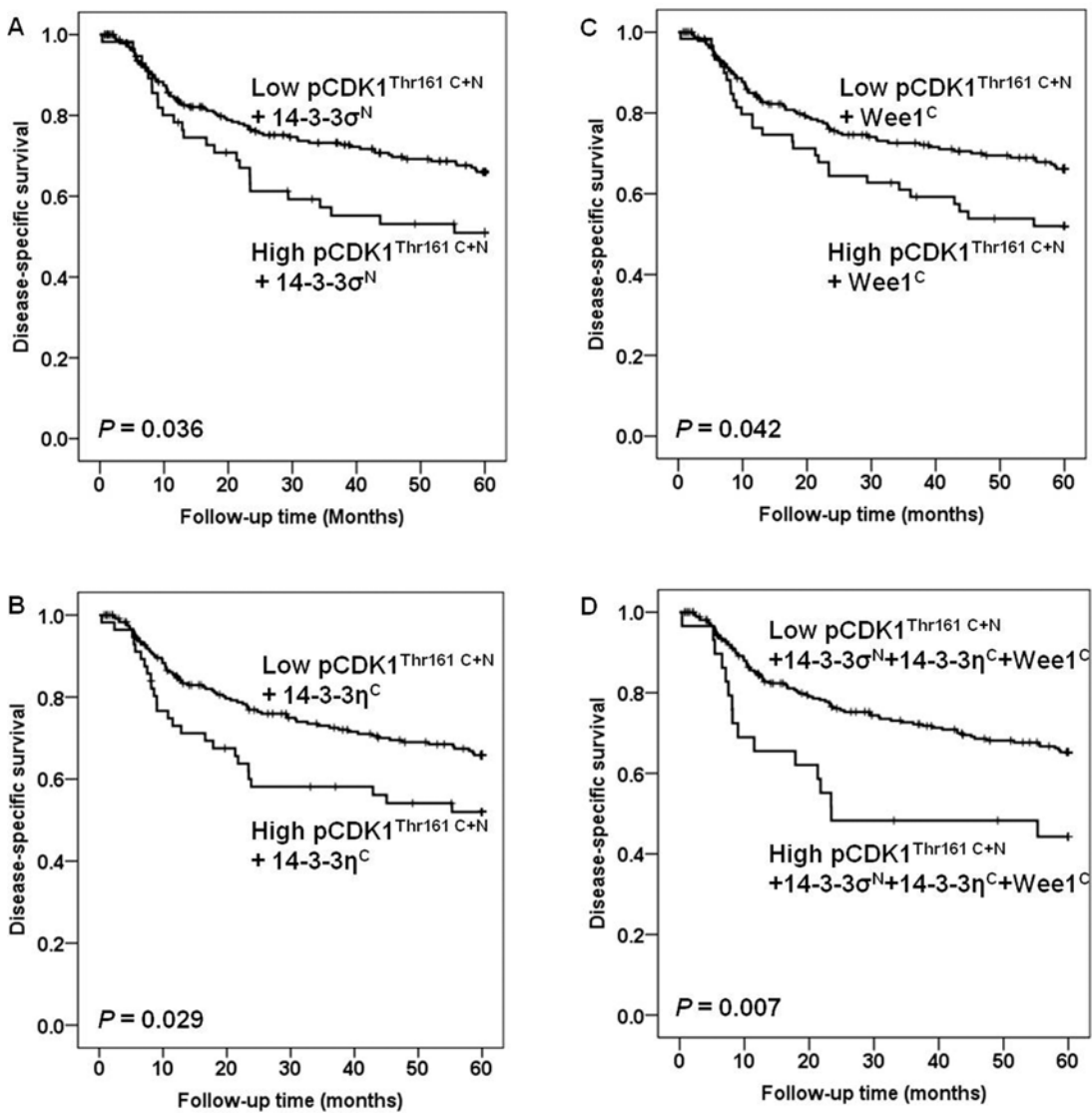
**Fig 1. Expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> in vulvar squamous epithelium.** Immunostaining of (A) CDK1<sup>Tyr15</sup>, (B) pCDK1<sup>Thr161</sup>, (C) Cyclin B1 (total) and (D) pCyclin B1<sup>Ser126</sup> in normal vulvar epithelium ( 300) and (E) CDK1<sup>Tyr15</sup>, (F) pCDK1<sup>Thr161</sup>, (G) Cyclin B1 (total) and (H) pCyclin B1<sup>Ser126</sup> in VSCC ( 600).

doi:10.1371/journal.pone.0121398.g001

Protein levels of pCDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> in either cytoplasm or nucleus were positive correlated to each other. The main findings when comparing these proteins with isoforms of 14-3-3 and CDC25 and Wee1 were that i) cytoplasmic expression of CDK1<sup>Tyr15</sup> and pCyclin B1<sup>Ser126</sup> were positive correlated to 14-3-3η, while in nucleus high expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> were correlated to high level of 14-3-3ε, ii) high protein levels of cytoplasmic CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> all related to high level of cytoplasmic pCDC25C<sup>Ser216</sup> and iii) high levels of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> in cytoplasm and nucleus were all associated with high expression of Wee1 in cytoplasm and nucleus, respectively.

### Survival

With all 297 VSCC included, univariate analysis revealed no association between protein levels of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> in either cytoplasm or nucleus and disease-specific survival. Elevated pCDK1<sup>Thr161</sup> in both cytoplasm and nucleus (pCDK1<sup>Thr161 C+N</sup>) showed a trend to poor disease-specific survival ( $p = 0.078$ ). Combinations of high expression of pCDK1<sup>Thr161 C+N</sup> with high levels of nuclear 14-3-3 $\sigma$  (14-3-3 $\sigma^N$ ), cytoplasmic 14-3-3 $\eta$  (14-3-3 $\eta^C$ ) or cytoplasmic Wee1 (Wee1<sup>C</sup>), were correlated with poor disease-specific survival ( $p = 0.036$ ,  $p = 0.029$  and  $p = 0.042$ , respectively) (Fig 2A–2C). Such correlation also existed between the combination of (pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\sigma^N$  + 14-3-3 $\eta^C$  + Wee1<sup>C</sup>) and survival ( $p = 0.007$ ) (Fig 2D). In multivariate analysis, lymph node metastases, tumor diameter, infiltration of vessel, age and combinations of (pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\sigma^N$ ),



**Fig 2. Survival curves using the Kaplan-Meier method (all 297 VSCC included).** The Kaplan-Meier curves of disease-specific survival in relation to combinations of (A) (pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\sigma^N$ ), (B) (pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\eta^C$ ), (C) (pCDK1<sup>Thr161 C+N</sup> + Wee1<sup>C</sup>) and (D) (pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\sigma^N$  + 14-3-3 $\eta^C$  + Wee1<sup>C</sup>). The  $p$ -values differ slightly from those in Table 1 due to the use of the log-rank test as opposed to the Cox-regression analysis.

doi:10.1371/journal.pone.0121398.g002

**Table 1. Relative risk (RR) of dying from vulvar cancer (all 297 VSCC included).**

Variables	Univariate analysis			Multivariate analysis		
	RR	95% CI <sup>a</sup>	p	RR	95% CI <sup>a</sup>	p
Lymph node metastases	2.55	1.98–3.28	<0.001	2.26	1.70–3.01	<0.001
Age	1.54	1.16–2.06	0.003	1.54	1.11–2.15	0.011
Tumor diameter	1.92	1.47–2.51	<0.001	1.35	1.00–1.82	0.050
Infiltration of vessel	2.29	1.51–3.47	<0.001	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>σ<sup>N</sup></sup>	1.61	1.03–2.52	0.037	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>η<sup>C</sup></sup>	1.64	1.05–2.56	0.030	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + Wee1 <sup>C</sup>	1.57	1.01–2.43	0.044	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>σ<sup>N</sup></sup> + 14-3-3 <sup>η<sup>C</sup></sup> + Wee1 <sup>C</sup>	2.05	1.20–3.50	0.009	1.93	1.09–3.43	0.024

<sup>a</sup> 95% confidence interval

<sup>b</sup> High vs low expression

C = Cytoplasm

N = Nucleus

doi:10.1371/journal.pone.0121398.t001

(pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>η<sup>C</sup></sup>), (pCDK1<sup>Thr161 C+N</sup> + Wee1<sup>C</sup>) and (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>σ<sup>N</sup></sup> + 14-3-3<sup>η<sup>C</sup></sup> + Wee1<sup>C</sup>) were included as important parameters. Lymph node metastases, age, tumor diameter and the combination of (pCDK1<sup>Thr161 C+N</sup> + 14-3-3<sup>σ<sup>N</sup></sup> + 14-3-3<sup>η<sup>C</sup></sup> + Wee1<sup>C</sup>) retained independent prognostic significance for patients with VSCC (Table 1). Survival analyses among the group of 288 VSCC which excluded the nine pre-surgery cases revealed similar results (Table 2 and Fig 3A–3D).

## Discussion

In the current study, at least 25% of VSCC showed high expression of Cyclin B1<sup>Ser126</sup>, CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> in nucleus, compared to the low levels of these proteins in normal vulvar squamous epithelium. Interestingly, the high levels of these activated types of Cyclin B1 and CDK1 did not exclusively exist in nucleus; in fact there were at least 25% of VSCC with

**Table 2. Relative risk (RR) of dying from vulvar cancer (288 VSCC without neoadjuvant treatment cases).**

Variables	Univariate analysis			Multivariate analysis		
	RR	95% CI <sup>a</sup>	p	RR	95% CI <sup>a</sup>	p
Lymph node metastases	2.58	1.99–3.54	<0.001	2.40	1.80–3.19	<0.001
Age	1.57	1.17–2.11	0.003	1.54	1.11–2.15	0.011
Tumor diameter	1.95	1.48–2.56	<0.001	1.47	1.09–1.98	0.011
Infiltration of vessel	2.40	1.56–3.67	<0.001	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>σ<sup>N</sup></sup>	1.70	1.08–2.67	0.021	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>η<sup>C</sup></sup>	1.68	1.06–2.66	0.026	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + Wee1 <sup>C</sup>	1.67	1.07–2.59	0.024	-	-	-
pCDK1 <sup>Thr161 C+N, b</sup> + 14-3-3 <sup>σ<sup>N</sup></sup> + 14-3-3 <sup>η<sup>C</sup></sup> + Wee1 <sup>C</sup>	2.15	1.26–3.68	0.005	1.91	1.08–3.39	0.026

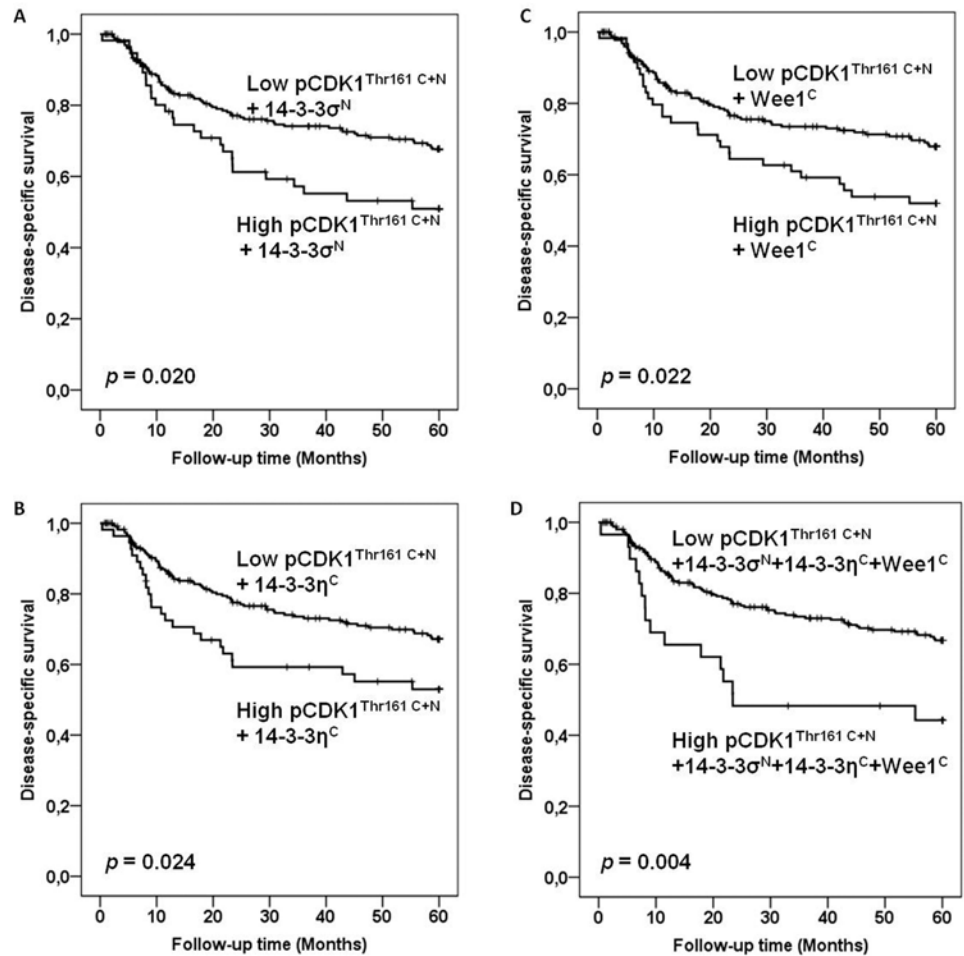
<sup>a</sup> 95% confidence interval

<sup>b</sup> High vs low expression

C = Cytoplasm

N = Nucleus

doi:10.1371/journal.pone.0121398.t002



**Fig 3. Survival curves using the Kaplan-Meier method (288 VSCC without pre-surgery treatment cases).** The Kaplan-Meier curves of disease-specific survival in relation to combinations of (A) (pCDK1<sup>Thr161</sup> C+N + 14-3-3<sup>σN</sup>), (B) (pCDK1<sup>Thr161</sup> C+N + 14-3-3<sup>ηC</sup>), (C) (pCDK1<sup>Thr161</sup> C+N + Wee1<sup>C</sup>) and (D) (pCDK1<sup>Thr161</sup> C+N + 14-3-3<sup>σN</sup> + 14-3-3<sup>ηC</sup> + Wee1<sup>C</sup>). The *p*-values differ slightly from those in Table 2 due to the use of the log-rank test as opposed to the Cox-regression analysis.

doi:10.1371/journal.pone.0121398.g003

high expression of pCyclin B1<sup>Ser126</sup>, CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> in cytoplasm as well. In addition, high protein levels of pCyclin B1<sup>Ser126</sup>, CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> in either cytoplasm or nucleus were positive correlated to each other. These data suggests that in VSCC activated complex of pCyclin B1<sup>Ser126</sup>-CDK1<sup>Tyr15</sup>/pCDK1<sup>Thr161</sup> can be formed in both cytoplasm and nucleus. This observation is supported by a recent study of Gavet et al. showing that as soon as Cyclin B1-CDK1 is activated in Hela cells, the complex rapidly accumulates in the nucleus, while a substantial amount of Cyclin B1-CDK1 still remains in the cytoplasm, thus mitotic events could synchronize from both cytoplasm and nucleus [17]. Taken together, our findings that high levels of pCyclin B1<sup>Ser126</sup>, CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> are observed together in at least 25% of our cases suggest that these proteins may contribute to tumorigenesis of a subset of VSCC.

Previously, conflict findings regarding connection between Cyclin B1 and malignant features have been reported. In some tumors like gastric cancer [28] and colorectal cancer [29],



overexpression of Cyclin B1 was associated with less aggressive tumour behaviour. In contrast, in other tumors including oesophageal [37], gastric [38], tongue [26], breast [27] and non-small cell lung cancer [24,25], high level of Cyclin B1 was associated with aggressive tumor behavior. We found that in VSCC high expression of Cyclin B1 (total) was significantly associated with malignant features, including large tumor diameter, poor histological differentiation and deep invasion. These results indicate that the role of Cyclin B1 is cancer specific. For the first time, we have found that pCyclin B1<sup>Ser126</sup>, pCDK1<sup>Thr161</sup> and CDK1<sup>Tyr15</sup> all significantly correlated to tumor malignancy and aggressiveness of VSCC. Cytoplasmic overexpression of CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> shared the connections to large tumor diameter, poor histological differentiation and deep invasion, while nuclear overexpression of both pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> were associated with younger age, high FIGO substage and poor histological differentiation. In summary, our findings indicate that CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> may play a role in the progression of VSCC.

Comparing the activated types of CDK1 and Cyclin B1 with other G2/M cell cycle regulators we found that cytoplasmic expression of CDK1<sup>Tyr15</sup> and pCyclin B1<sup>Ser126</sup> were positive correlated to cytoplasmic 14-3-3 $\eta$ . This is in line with previous findings where 14-3-3 has been reported to sequester Cyclin B1-CDK1 complex in cytoplasm and prevent its entry into nucleus, resulting in G2/M cell cycle arrest [39,40]. Interestingly, high expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> in the nucleus were correlated to high nuclear level of 14-3-3 $\epsilon$ . One hypothesis is that 14-3-3 $\epsilon$ , for unknown reason in VSCC, is unable to bind to pCyclin B1<sup>Ser126</sup>-CDK1<sup>Tyr15</sup>/pCDK1<sup>Thr161</sup> complex and transport it out of the nucleus, thus the complex will then stay in the nucleus and trigger G2/M transition [14,15]. We also discovered that the high protein levels of cytoplasmic CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> all correlated with high level of cytoplasmic pCDC25C<sup>Ser216</sup>. This is in agreement with the theory that the main function of CDC25C is to activate CDK1 by removing the inhibitory phosphate groups from residues Thr<sup>14</sup> and Tyr<sup>15</sup>, thus the active complex of pCyclin B1<sup>Ser126</sup>-CDK1<sup>Tyr15</sup>/pCDK1<sup>Thr161</sup> could be formed [18–21]. Furthermore, our results showed surprisingly positive relations between Wee1 and the activated CDK1 and Cyclin B1. We found that high levels of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup> and pCyclin B1<sup>Ser126</sup> in cytoplasm and nucleus were all associated with high expression of Wee1 in cytoplasm and nucleus, respectively. Although this observation is not in agreement with previous findings in other tumor types, in those Wee1 acts as a negative regulator of CDK1 in G2/M cell cycle [25,41,42], it is consistent with our previous study [33], where the high protein level of Wee1 is correlated to tumor malignancy and aggressive phenotype in VSCC. Those data suggests that in VSCC the activation of CDK1 and Cyclin B1 is accompanied by high Wee1 expression. In our previous analysis of the same cohort of VSCC, 70% of the cases had high expression of pCDC25C<sup>Ser216</sup> in nucleus. Therefore it is possible that this high activation of pCDC25C<sup>Ser216</sup> is opposing inhibitory Wee1 effect, resulting in activation of CDK1 and Cyclin B1. However, reasons why Wee1 loses its inhibitory function and even rather positively relates to CDK1 in VSCC deserve further investigation.

Considering bias might be raised when survival rates were evaluated in patients with heterogeneous treatment, we performed survival analyses on the group which excluded the nine patients who have received neoadjuvant treatment, in addition to the survival analyses on the whole group of 297 VSCC. The similarity of the data from the two groups suggests that the numbers of the excluded cases are low and therefore will not change the overall survival. Our results showed that neither cytoplasmic nor nuclear expression of CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> was associated with disease-specific survival in patients with VSCC. However, high expression of pCDK1<sup>Thr161 C+N</sup> showed a trend to poor disease-specific survival. In esophageal cancer [43], gastric cancer [38], lymph node-negative breast cancer [44] and non-small cell lung cancer [25], overexpression of Cyclin B1 have been found

to be an useful prognostic parameter. In gastrointestinal stromal tumor [45,46], CDK1 is associated with a shorter period of disease-free survival. Since the outcome of patients most likely is the result of multiple regulations of cell cycle, we performed survival analysis by combining CDK1<sup>Thr161 C+N</sup> and other cell cycle regulators including 14-3-3s, CDC25s and/or Wee1. Interestingly, in univariate but not in multivariate analysis, high expression of pCDK1<sup>Thr161 C+N</sup> combined with high expression of 14-3-3 $\sigma^N$  or 14-3-3 $\eta^C$  or Wee1<sup>C</sup> showed a significantly correlation with disease-specific survival. Furthermore, when combining high expression of pCDK1<sup>Thr161 C+N</sup> + 14-3-3 $\sigma^N$  + 14-3-3 $\eta^C$  + Wee1<sup>C</sup>, a significant correlation to survival was found in univariate as well as in multivariate analysis. This is in line with previous studies in ovarian cancer [23], malignant peripheral nerve sheath tumors [47] and VSCC [48], where significant correlations have been observed between some combinations of cell cycle factors and survival. In summary, our results indicate that it is important to co-analysis G2/M proteins in VSCC. We have uncovered certain combinations of G2/M regulators whose status can predict outcome of VSCC.

## Conclusion

In conclusion, the abnormal expression of pCDK1<sup>Thr161</sup>, CDK1<sup>Tyr15</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> and their associations with malignancy and aggressive phenotypes suggest that they are involved in tumorigenesis and progression of VSCC. The combinations of G2/M cell cycle regulators including pCDK1<sup>Thr161</sup>, 14-3-3 $\sigma^N$ , 14-3-3 $\eta^C$  and Wee1<sup>C</sup> may predict the survival of patients with VSCC.

## Supporting Information

**S1 Table. Immunostaining results for CDK1<sup>Tyr15</sup>, pCDK1<sup>Thr161</sup>, Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup>.**

(DOCX)

**S2 Table. CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> expression in relation to clinicopathological variables.**

(DOCX)

**S3 Table. Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> expression in relation to clinicopathological variables.**

(DOCX)

**S4 Table. CDK1<sup>Tyr15</sup> and pCDK1<sup>Thr161</sup> expression in relation to cell cycle proteins and HPV.**

(DOCX)

**S5 Table. Cyclin B1 (total) and pCyclin B1<sup>Ser126</sup> expression in relation to cell cycle proteins and HPV.**

(DOCX)

## Author Contributions

Conceived and designed the experiments: ZW RH CGT JMN. Performed the experiments: MF. Analyzed the data: ZW AS RH JMN. Contributed reagents/materials/analysis tools: CGT RH. Wrote the paper: ZW AS RH.

## References

1. Judson PL, Habermann EB, Baxter NN, Durham SB, Virnig BA. Trends in the incidence of invasive and in situ vulvar carcinoma. *Obstet Gynecol.* 2006; 107: 1018–1022. PMID: [16648405](#)
2. Sankaranarayanan R, Ferlay J. Worldwide burden of gynaecological cancer: the size of the problem. *Best Pract Res Clin Obstet Gynaecol.* 2006; 20: 207–225. PMID: [16359925](#)
3. Canavan TP, Cohen D. Vulvar cancer. *Am Fam Physician.* 2002; 66: 1269–1274. PMID: [12387439](#)
4. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1998. *CA Cancer J Clin.* 1998; 48: 6–29. PMID: [9449931](#)
5. Dittmer C, Katalinic A, Mundhenke C, Thill M, Fischer D. Epidemiology of vulvar and vaginal cancer in Germany. *Arch Gynecol Obstet.* 2011; 284: 169–174. doi: [10.1007/s00404-011-1850-9](#) PMID: [21340687](#)
6. Schuurman MS, van den Einden LC, Massuger LF, Kiemeneys LA, van der Aa MA, de Hullu JA. Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma. *Eur J Cancer.* 2013; 49: 3872–3880. doi: [10.1016/j.ejca.2013.08.003](#) PMID: [24011936](#)
7. Joura EA, Losch A, Haider-Angeler MG, Breitenecker G, Leodolter S. Trends in vulvar neoplasia. Increasing incidence of vulvar intraepithelial neoplasia and squamous cell carcinoma of the vulva in young women. *J Reprod Med.* 2000; 45: 613–615. PMID: [10986677](#)
8. Jones RW, Baranyai J, Stables S. Trends in squamous cell carcinoma of the vulva: the influence of vulvar intraepithelial neoplasia. *Obstet Gynecol.* 1997; 90: 448–452. PMID: [9277660](#)
9. Baandrup L, Varbo A, Munk C, Johansen C, Frisch M, Kjaer SK. In situ and invasive squamous cell carcinoma of the vulva in Denmark 1978–2007—a nationwide population-based study. *Gynecol Oncol.* 2011; 122: 45–49. doi: [10.1016/j.ygyno.2011.03.016](#) PMID: [21474166](#)
10. Watson M, Saraiya M, Wu X. Update of HPV-associated female genital cancers in the United States, 1999–2004. *J Womens Health (Larchmt).* 2009; 18: 1731–1738. doi: [10.1089/jwh.2009.1570](#) PMID: [19951205](#)
11. Iversen T, Tretli S. Intraepithelial and invasive squamous cell neoplasia of the vulva: trends in incidence, recurrence, and survival rate in Norway. *Obstet Gynecol.* 1998; 91: 969–972. PMID: [9611006](#)
12. Schottenfeld D, Fraumeni J. *Cancer epidemiology and prevention.* 3rd ed. 2006.
13. Magrina JF, Gonzalez-Bosquet J, Weaver AL, Gaffey TA, Webb MJ, Podratz KC, et al. Primary squamous cell cancer of the vulva: radical versus modified radical vulvar surgery. *Gynecol Oncol.* 1998; 71: 116–121. PMID: [9784331](#)
14. Gavet O, Pines J. Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Dev Cell.* 2010; 18: 533–543. doi: [10.1016/j.devcel.2010.02.013](#) PMID: [20412769](#)
15. Rothblum-Oviatt CJ, Ryan CE, Piwnicka-Worms H. 14-3-3 binding regulates catalytic activity of human Wee1 kinase. *Cell Growth Differ.* 2001; 12: 581–589. PMID: [11751453](#)
16. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol.* 1997; 13: 261–291. PMID: [9442875](#)
17. Gavet O, Pines J. Activation of cyclin B1-Cdk1 synchronizes events in the nucleus and the cytoplasm at mitosis. *J Cell Biol.* 2010; 189: 247–259. doi: [10.1083/jcb.200909144](#) PMID: [20404109](#)
18. Lee MS, Ogg S, Xu M, Parker LL, Donoghue DJ, Maller JL, et al. cdc25+ encodes a protein phosphatase that dephosphorylates p34cdc2. *Mol Biol Cell.* 1992; 3: 73–84. PMID: [1312880](#)
19. Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW. cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell.* 1991; 67: 197–211. PMID: [1913817](#)
20. Dunphy WG, Kumagai A. The cdc25 protein contains an intrinsic phosphatase activity. *Cell.* 1991; 67: 189–196. PMID: [1655274](#)
21. Sebastian B, Kakizuka A, Hunter T. Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proc Natl Acad Sci U S A.* 1993; 90: 3521–3524. PMID: [8475101](#)
22. Connell-Crowley L, Solomon MJ, Wei N, Harper JW. Phosphorylation independent activation of human cyclin-dependent kinase 2 by cyclin A in vitro. *Mol Biol Cell.* 1993; 4: 79–92. PMID: [8443411](#)
23. Hashiguchi Y, Tsuda H, Inoue T, Nishimura S, Suzuki T, Kawamura N. Alteration of cell cycle regulators correlates with survival in epithelial ovarian cancer patients. *Hum Pathol.* 2004; 35: 165–175. PMID: [14991533](#)
24. Soria JC, Jang SJ, Khuri FR, Hassan K, Liu D, Hong WK, et al. Overexpression of cyclin B1 in early-stage non-small cell lung cancer and its clinical implication. *Cancer Res.* 2000; 60: 4000–4004. PMID: [10945597](#)

25. Yoshida T, Tanaka S, Mogi A, Shitara Y, Kuwano H. The clinical significance of Cyclin B1 and Wee1 expression in non-small-cell lung cancer. *Ann Oncol*. 2004; 15: 252–256. PMID: [14760118](#)
26. Hassan KA, El-Naggar AK, Soria JC, Liu D, Hong WK, Mao L. Clinical significance of cyclin B1 protein expression in squamous cell carcinoma of the tongue. *Clin Cancer Res*. 2001; 7: 2458–2462. PMID: [11489826](#)
27. Suzuki T, Urano T, Miki Y, Moriya T, Akahira J, Ishida T, et al. Nuclear cyclin B1 in human breast carcinoma as a potent prognostic factor. *Cancer Sci*. 2007; 98: 644–651. PMID: [17359284](#)
28. Yasuda M, Takesue F, Inutsuka S, Honda M, Nozoe T, Korenaga D. Overexpression of cyclin B1 in gastric cancer and its clinicopathological significance: an immunohistological study. *J Cancer Res Clin Oncol*. 2002; 128: 412–416. PMID: [12200597](#)
29. Korenaga D, Takesue F, Yasuda M, Honda M, Nozoe T, Inutsuka S. The relationship between cyclin B1 overexpression and lymph node metastasis in human colorectal cancer. *Surgery*. 2002; 131: S114–S120. PMID: [11821797](#)
30. Wang Z, Trope CG, Florenes VA, Suo Z, Nesland JM, Holm R. Overexpression of CDC25B, CDC25C and phospho-CDC25C (Ser216) in vulvar squamous cell carcinomas are associated with malignant features and aggressive cancer phenotypes. *BMC Cancer*. 2010; 10: 233. doi: [10.1186/1471-2407-10-233](#) PMID: [20500813](#)
31. Wang Z, Nesland JM, Suo Z, Trope CG, Holm R. The prognostic value of 14-3-3 isoforms in vulvar squamous cell carcinoma cases: 14-3-3beta and epsilon are independent prognostic factors for these tumors. *PLoS One*. 2011; 6: e24843. doi: [10.1371/journal.pone.0024843](#) PMID: [21935479](#)
32. Wang Z, Trope CG, Suo Z, Troen G, Yang G, Nesland JM, et al. The clinicopathological and prognostic impact of 14-3-3 sigma expression on vulvar squamous cell carcinomas. *BMC Cancer*. 2008; 8: 308. doi: [10.1186/1471-2407-8-308](#) PMID: [18950492](#)
33. Magnussen GI, Hellesylt E, Nesland JM, Trope CG, Florenes VA, Holm R. High expression of wee1 is associated with malignancy in vulvar squamous cell carcinoma patients. *BMC Cancer*. 2013; 13: 288. doi: [10.1186/1471-2407-13-288](#) PMID: [23767999](#)
34. Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *Int J Gynaecol Obstet*. 2009; 105: 103–104. PMID: [19367689](#)
35. Kleihues P. Pathology and genetics of tumors of the breast and female genital organs. *World Health Organization of Tumors*. 2003. pp. 316–321.
36. Knopp S, Nesland JM, Trope C, Holm R. p14ARF, a prognostic predictor in HPV-negative vulvar carcinoma. *Am J Clin Pathol*. 2006; 126: 266–276. PMID: [16891203](#)
37. Takeno S, Noguchi T, Kikuchi R, Uchida Y, Yokoyama S, Muller W. Prognostic value of cyclin B1 in patients with esophageal squamous cell carcinoma. *Cancer*. 2002; 94: 2874–2881. PMID: [12115375](#)
38. Begnami MD, Fregnani JH, Nonogaki S, Soares FA. Evaluation of cell cycle protein expression in gastric cancer: cyclin B1 expression and its prognostic implication. *Hum Pathol*. 2010; 41: 1120–1127. doi: [10.1016/j.humpath.2010.01.007](#) PMID: [20334896](#)
39. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, et al. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell*. 1997; 1: 3–11. PMID: [9659898](#)
40. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. *Oncogene*. 2001; 20: 1803–1815. PMID: [11313928](#)
41. Eom M, Han A, Lee MJ, Park KH. Expressional Difference of RHEB, HDAC1, and WEE1 Proteins in the Stromal Tumors of the Breast and Their Significance in Tumorigenesis. *Korean J Pathol*. 2012; 46: 324–330. doi: [10.4132/KoreanJPathol.2012.46.4.324](#) PMID: [23110024](#)
42. Bhattacharya A, Schmitz U, Wolkenhauer O, Schonherr M, Raatz Y, Kunz M. Regulation of cell cycle checkpoint kinase WEE1 by miR-195 in malignant melanoma. *Oncogene*. 2013; 32: 3175–3183. doi: [10.1038/onc.2012.324](#) PMID: [22847610](#)
43. Nozoe T, Korenaga D, Kabashima A, Ohga T, Saeki H, Sugimachi K. Significance of cyclin B1 expression as an independent prognostic indicator of patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res*. 2002; 8: 817–822. PMID: [11895914](#)
44. Kuhling H, Alm P, Olsson H, Ferno M, Baldetorp B, Parwaresch R, et al. Expression of cyclins E, A, and B, and prognosis in lymph node-negative breast cancer. *J Pathol*. 2003; 199: 424–431. PMID: [12635132](#)
45. Nakamura N, Yamamoto H, Yao T, Oda Y, Nishiyama K, Imamura M, et al. Prognostic significance of expressions of cell-cycle regulatory proteins in gastrointestinal stromal tumor and the relevance of the risk grade. *Hum Pathol*. 2005; 36: 828–837. PMID: [16084954](#)
46. Fujita A, Yamamoto H, Imamura M, Nakamura N, Maehara Y, Tsuneyoshi M, et al. Expression level of the mitotic checkpoint protein and G2-M cell cycle regulators and prognosis in gastrointestinal stromal

tumors in the stomach. *Virchows Arch.* 2012; 460: 163–169. doi: [10.1007/s00428-011-1181-z](https://doi.org/10.1007/s00428-011-1181-z) PMID: [22190007](https://pubmed.ncbi.nlm.nih.gov/22190007/)

47. Endo M, Kobayashi C, Setsu N, Takahashi Y, Kohashi K, Yamamoto H, et al. Prognostic significance of p14ARF, p15INK4b, and p16INK4a inactivation in malignant peripheral nerve sheath tumors. *Clin Cancer Res.* 2011; 17: 3771–3782. doi: [10.1158/1078-0432.CCR-10-2393](https://doi.org/10.1158/1078-0432.CCR-10-2393) PMID: [21262917](https://pubmed.ncbi.nlm.nih.gov/21262917/)
48. Holm R, Forsund M, Nguyen MT, Nesland JM, Trope CG. Expression of p15INK(4)b and p57KIP(2) and relationship with clinicopathological features and prognosis in patients with vulvar squamous cell carcinoma. *PLoS One.* 2013; 8: e61273. doi: [10.1371/journal.pone.0061273](https://doi.org/10.1371/journal.pone.0061273) PMID: [23580324](https://pubmed.ncbi.nlm.nih.gov/23580324/)