

## EXPRESSION PROFILING OF CYCLIN B1 AND D1 IN CERVICAL CARCINOMA

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**Aim:** Cyclins are a family of regulatory proteins that play a key role in controlling the cell cycle. Abnormalities of cell cycle regulators, including cyclins and cyclin dependent kinases, have been reported in various malignant tumors. This study was undertaken to quantitatively detect cyclin B1 and D1 in cervical cancer. **Methods:** A quantitative real-time reverse transcription polymerase chain reaction and Western blot assay were used to analyze the expression of cyclin B1/D1 mRNA and proteins, respectively, in fresh invasive cervical cancer (n = 41) and normal cervical tissues (n = 10). **Results:** There was significantly greater cyclin B1 expression in invasive cervical cancer than in normal cervical tissue (P = 0.019). However, cyclin D1 expression was not significantly different. A Western blot assay yielded similar results. **Conclusion:** Our results were consistent with the concept that up-regulation of cyclin B1 expression occurred in cervical cancer and an aberrant expression of cyclin B1 might play an important role in cervical carcinogenesis.

**Key Words:** cyclin B1, cyclin D1, cervical cancer, real-time RT-PCR, Western blot.

Cervical cancer is an important public health problem. It is the second most common malignancy among women worldwide and the first in many developing countries, including Korea [1]. It is widely accepted that high-risk type human papillomavirus (HPV) infection is an obligatory factor in malignant transformation within the epithelial cells of the uterine cervix [2]. However, HPV infection alone is insufficient to cause invasive cervical cancer [3]. The role of promoting cofactors in the later stages of progression into cervical malignancy remains uncertain. Possible candidates are the cyclin proteins that are cyclically synthesized and destroyed during the cell cycle.

There are many well-known cyclins, including 11 major classes and some subclasses, labeled A, B1-2, C, D1-3, E1-2, F, G1-2, H, I, K, T1-2 to H. They share a conserved amino acid region, known as the cyclin box, through which they interact with their cyclin dependent kinases (cdks) [4, 5]. Cyclins are also grouped according to their phase of activity. The G1 cyclins include D1 and E, which are active in the G1 phase and regulate the G1–S phase transition. Cyclin A is active in the S and late G2 phase, while B1-2 are active in the G2 phase and regulate mitosis. Cell cycle dysregulation is a feature of all human cancers and there is evidence suggesting a role for cyclins in human cancer [6].

The human B-type class of cyclins consists of three closely related members, cyclins B1, B2, and B3, which control the G2–M transition through interaction with the cdk1 protein kinase.

Cyclin B1 was the first human cyclin identified and is the best characterized among the three members [7]. Cyclin B1 complexes solely with cdk1 (cdc2) to

form the mitosis-promoting factor, which regulates the G2–M transition and is the primary regulator of mitosis. The mitosis-promoting factor is located in the cytoplasm late in the S phase, but relocates to the nucleus shortly before the envelope breakdown. P53 was recently shown to prevent the G2–M transition by decreasing cyclin B levels and attenuating activity of the cyclin B promoter [5, 8, 9].

The cyclin D1 protein is a major positive regulator of the progression from the G1 to S phase. There is a homeostatic feedback loop between cyclin D1, Rb, and p27<sup>Kip1</sup> [5, 10]. Cyclin D1 is often found disrupted in the cancer cell genome by chromosome 11q13 translocation or gene amplification. Approximately 50% of mammary carcinomas have been shown to overexpress the cyclin D1 protein. Molecular aberrations accounting for increased mRNA transcripts of cyclin D1 in other cancers include gene rearrangements and amplification. Amplification has been implicated in many tumors, including bladder and ovarian cancer [11]. In contrast, our previous study suggested that there is no correlation between gene alterations and protein D1 in cervical squamous cell cancer [12].

Previously, we studied a large collection of fresh or paraffin-embedded samples from patients with cervical cancer to analyze the expression levels of cyclin D1, E, A by reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical staining methods [12–14]. We found that RT-PCR or immunohistochemical assay could detect the cyclins message in nearly all the cervical cancer specimens. However, these methods lacked specificity and only yielded a semi-quantitative result. Gene expression analysis requires sensitive, precise, and reproducible measurement. Hence, we reported the development of a quantitative real-time RT-PCR assay for cyclin B1 and D1 expression with high sensitivity and specificity for cervical cancer. Our objective was to perform a quantitative real-time RT-PCR assay in normal and cervical cancer tissues to evaluate the expression level and status of cyclin B1 and D1.

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**Abbreviations used:** HPV – human papillomavirus; FAM – 6-carboxyfluorescein; GAPDH – glyceraldehyde-3-phosphate-dehydrogenase; RT-PCR – reverse transcription polymerase chain reaction; TAMRA – 6-carboxytetramethylrhodamine.

## MATERIALS AND METHODS

**Patients.** Fresh cervical tissue samples were obtained from 51 patients treated at Yonsei Medical Center. These included 41 invasive cervical cancers and 10 normal cervix tissues that served as the control. Of the 41 patients with cervical cancer, 9 cases were stage I, 27 cases were stage II, and 5 cases were stage III. Dissected tissue samples were immediately frozen and stored in liquid nitrogen. The patients' ages ranged from 29 to 80 years with a mean of 53.08 years. Regarding distribution according to stage, patients with stage II cervical carcinoma were the most numerous. According to cell type, 37 contained squamous cells and 4 were adenocarcinomas. The HPV infection was detected in 41 cervical carcinoma tissues, and the HPV types included types 4, 11, 14, 16, 18, 33, 35, 45, 58, 63, 69 and others.

**RNA isolation and cDNA preparation.** For RNA preparation, samples were disrupted into small pieces and RNA was isolated from the tumor and control samples using SV total RNA isolation system (Promega). A total of 1 µg RNA of each sample was reverse transcribed using an oligo d(T) primer and RNase H<sup>-</sup> MMLV reverse transcriptase, according to the manufacturer's protocol (Fermentas). The reverse transcription reaction contained the following in a 50 µl volume: 200 units MMLV enzyme, 1× MMLV buffer, 20 units Rnasin, 0.5 µg oligo d(T), 2.5 mM dNTP mix, and DNase treated total RNA. The reaction was incubated at 70 °C for 10 min. The control and reference RNA's were reverse-transcribed alongside patient RNA for each run. The cDNA was stored at -20 °C and was used for each PCR reaction. The quality of the cDNA was confirmed by amplification of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and only samples with consistent and strong amplifications were included in the final analyses.

**Analyses of gene expression by real-time quantitative RT-PCR.** The quantitation of mRNA levels was carried out using a real-time fluorescence detection method. The cDNA was prepared as described above and amplified by PCR in the ABI prism 7700 sequence detector (PE Biosystems, USA). The reaction mixture for cyclin B1/D1 contains the following in a final volume of 50 µl: 25 µl Taqman mix, 2.5 µl cyclin B1 or D1 primer mix, 1 µg cDNA, and added free-water to 50 µl. The quantitative real-time PCR was performed as follows: an initial cycle for 10 min at 95 °C, followed by 50 bi-phasic cycles of 15 s at 95 °C, and 1 min at 60 °C.

The primer and probe sequences were published previously (Table 1) [15, 16]. All primer and probe combinations were positioned to span an exon-exon junction. The probes were labeled at the 5' end with a FAM (6-carboxyfluorescein) probe and at the 3' end with TAMRA (6-carboxytetramethylrhodamine), which served as a quencher. Initial template concentration was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction.

**Table 1.** The sequences of primer and probe of cyclin B1 and D1

Cyclin B1 :	
Forward primer:	5'–CTC CTG TCT GGT GGG AGGA–3'
Reverse primer:	5'–CTG ATC CAG AAT AAC ACC TGA–3'
Probe:	5'–FAM-AGA GTG GAG TTG TGC TGG CT-TAMRA–3'
Cyclin D1 :	
Forward primer:	5'–CTG GCC ATG AAC TAC GTG GA–3'
Forward primer:	5'–GTC ACA CTT GAT CAC TCT GG–3'
Probe:	5'–FAM-AGA AGC GTG TGA GGC GGT AGT AGG A-TAMRA–3'
GAPDH :	
Forward primer:	5'–GAA GGT GAA GGT GGG AGT C–3'
Forward primer:	5'–GAA GAT GGT GAT GGG ATT TC–3'
Probe:	5'–FAM-CAA GGT TCC CGT TCT CAG CC-TAMRA–3'

The threshold cycles (Ct) were recorded for the target gene and reference in all the samples. The results were calculated relative to a reference standard, called a calibrator. The results of the real-time PCR were calculated by "Comparative Ct method of Quantitation" ( $\Delta\Delta Ct$ ) [17, 18]. This method was outlined in the ABI Prism Sequence Detection System User Bulletin from the following formulas:

$$\Delta Ct (\text{sample}) = Ct (\text{target gene}) - Ct (\text{GADPH}),$$

$$\Delta\Delta Ct = \Delta Ct (\text{tumor}) - \Delta Ct (\text{control})$$

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

The three targets were analyzed separately. Then, cyclin B1 and D1 were each normalized to GAPDH as an internal standard. Final relative cyclin B1/D1 expression levels of each unknown sample were obtained by division of cyclin B1/D1 copy numbers by GAPDH copy numbers. The normalization accounts for variability in the samples' reverse transcription efficiency and RNA quantity and quality. The  $\Delta\Delta Ct$  was calculated separately for cyclin B1 and cyclin D1, for each sample, using the cell line HeLa as the calibrator. HeLa was chosen as the reference calibrator because it expressed high levels of cyclin B1 or cyclin D1, and it is potentially a consistent source of calibrator template. At least two independent analyses were performed for each sample and each gene. Analyses of the gene expression data were performed without knowledge of the patients' data.

**Real-time PCR efficiency assay.** To use the  $2^{-\Delta\Delta Ct}$  calculation, the PCR efficiencies of the target and control assays need to be similar [17]. The relative efficiencies were measured by varying the input amount of RNA, measuring the Ct values, and calculating the  $\Delta Ct$  values for the various assays. Graphing the  $\Delta Ct$  values relative to input total RNA should give a line with a slope < 0.1. The range of input RNA should be from 10 ng to 1000 ng.

**Western blot analysis of cyclin B1 and D1.** The cervical tissues were all homogenized and then lysed with cell lysis buffer (Cell Signaling Technology, Inc), according to the manufacturer protocol. After incubation on ice for 10 min, tissue lysates were clarified by centrifugation at 13,000 rpm for five min and protein concentration of the supernatants was determined with a Bio-Rad DC protein assay (Bio-Rad, USA). 40 µg of total protein was loaded in each lane. The samples were subjected to 8–10% SDS-PAGE. The resolved proteins were blotted to a PVDF membrane,

which were then blocked at overnight 4 °C in TBS buffer (20 mM Tris base, 137 mM sodium chloride, 1 M hydrochloric acid, pH 7.6) containing 5% non fat milk. The membrane was then incubated with the primary antibodies: mouse-anti cyclinB1 (clone V152, Cell Signaling Technology, Inc), anti-human cyclinD1 (clone Dcs6, Cell Signaling Technology, Inc) and mouse-anti GAPDH (Cell Signaling Technology, Inc) for 1 h at room temperature. The concentration of each antibody was used as suggested by the suppliers. Peroxidase labeled anti-mouse antibodies were used as a secondary antibody, diluted in TBS with 5% (w/v) milk, and developed using the chemiluminescent ECL kit and Hyperfilm ECL (Amersham Biosciences, USA). Quantification of proteins was performed by using a laser densitometer and analysis software (IMAGE READER LAS-1000 lite, Fuji Photo Film Co., Ltd., Japan), while referring to the standard series. The densitometric integral derived from each sample band, i.e. the integral of a mean density over a measured area, was taken to calculate the amount in each sample, according to the known standard values.

**Statistical analyses.** Data were analyzed using parametric and nonparametric statistics, SPSS 11.0 (Chicago, USA). Descriptive statistics were used for quantitative experimental data and are summarized as means and standard deviations. Continuous variables were examined for a normal distribution (Kolmogorov-Smirnov test) before adopting parametric statistics. Differences between continuous variables were evaluated by an independent-sample T test. One way ANOVA and logistic regression analysis were done for normally distributed variables. The Mann-Whitney U test and the Kruskal-Wallis test were done for variables that were not normally distributed. Differences were considered significant when the probability of error was below 5% ( $P < 0.05$ ).

## RESULTS

### **Analysis of cyclin expression in normal and cervical cancer tissues with real-time RT-PCR.**

To determine cyclin B1 and D1 transcript level in fresh human cervical carcinoma tissues, we used a real-time quantitative RT-PCR assay based on TaqMan methodology. GAPDH mRNA was measured, as a reference, to normalize cyclin B1 and D1 mRNA levels. Primers and probes were selected to avoid amplification from genomic DNA and target sequences were kept small to ensure the detection of fragmented and partially degraded RNA. The PCR efficiencies of the target and control assays were performed. The slope for cyclin B1 was 0.088 and the slope for cyclin D1 was 0.094, relative to GAPDH for the range of input cDNA of 10–1000 ng.

In the study, we compared cyclin B1 and D1 expression in cervical cancer and normal tissues. These analyses revealed that cyclin B1 transcripts had a higher expression in cervical cancer ( $P = 0.019$ ) and there was a decreased trend from stage I ( $0.76 \pm 0.68$ ) to stage III ( $0.23 \pm 0.39$ ) in cervical cancer cases (Table 2).

Although we found a high level of cyclin D1 expression in some cervical cancer cases, no significant statistical differences were observed. In addition, we also found an increase in cyclin D1 mRNA expression from stage I ( $2.17 \pm 2.33$ ) to III ( $6.66 \pm 10.48$ ) in cervical cancer cases. Moreover, no significant differences in terms of cyclin expression were found between the different stages of cervical carcinoma.

**Table 2.** Expression of cyclin B1 and D1 in the uterine cervix

Tissue	Cyclin B1	Cyclin D1
	$2^{-\Delta\Delta Ct}$ value (mean $\pm$ SD)	$2^{-\Delta\Delta Ct}$ value (mean $\pm$ SD)
Control (n = 10)	$0.03 \pm 0.05$	$3.22 \pm 2.29$
Cervical cancer (n = 41)	$0.59 \pm 0.72^a$	$3.29 \pm 4.63$
Stage I (n = 9)	$0.76 \pm 0.68$	$2.17 \pm 2.33$
Stage II (n = 27)	$0.57 \pm 0.76$	$3.24 \pm 4.32$
Stage III (n = 5)	$0.23 \pm 0.39$	$6.66 \pm 10.48$

<sup>a</sup> $P = 0.019$  (cervical cancer vs. control), Students *t*-test.

### **Western blotting of cyclin B1 and D1 in cervical carcinoma.**

The mRNA expression level of cyclin B1 and D1 were analyzed with real-time RT-PCR. We wanted to directly detect cyclin B1 and D1 protein expression. So, we selected 29 cases of cervical cancer and 6 cases of normal cervical tissues in which mRNA expression was analyzed by real-time RT-PCR. In western blotting, cyclin B1 protein was detected as a 60 KDa and cyclin D1 protein was 36 KDa band (Figure). Then, we compared the expression levels of mRNA with protein expression levels of cyclinB1 and D1 in cervix tissue with and without cervical cancer. Our study revealed a statistically significant increase in expression of cyclin B1 in patient with cervical compared to normal cervix tissue ( $P = 0.031$ ) (Table 3). These results agreed with the results of the mRNA analysis by real-time PCR. There was no statistical difference in expression of cyclin D1 between cervical cancer and normal tissues, and no correlation between the expression of mRNA and protein.

**Table 3.** The protein analysis of cyclin B1 and D1 by Western blot

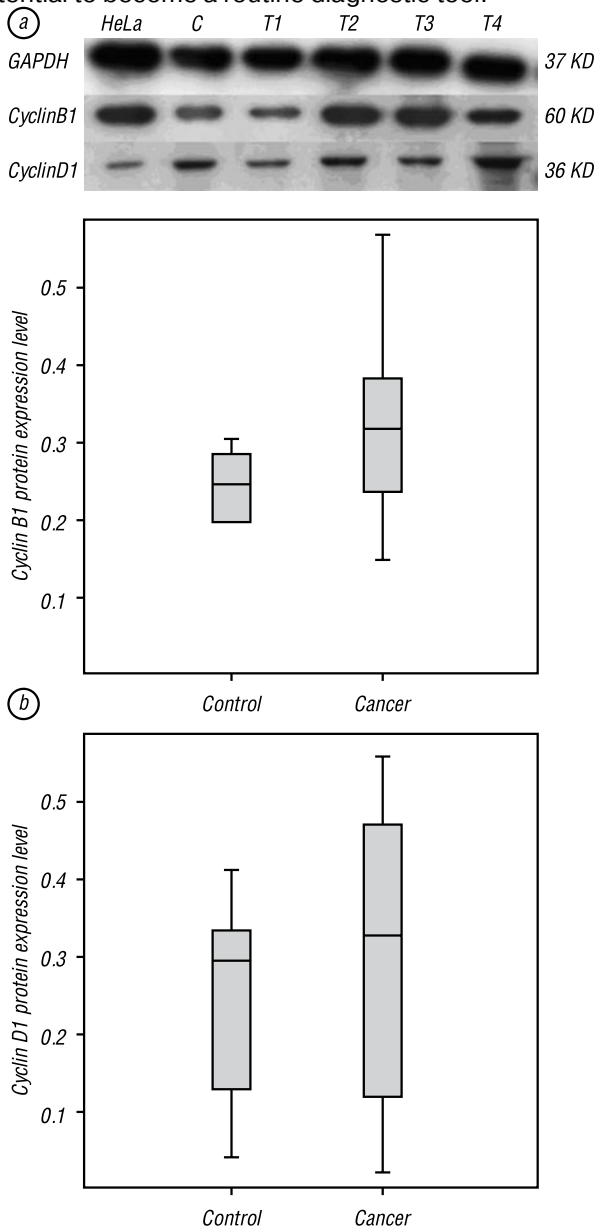
Tissue	Cyclin B1 (mean $\pm$ SD)	Cyclin D1 (mean $\pm$ SD)
Control	$0.21 \pm 0.11$	$0.27 \pm 0.16$
Cervical cancer	$0.33 \pm 0.11^a$	$0.32 \pm 0.20$

<sup>a</sup> $P = 0.031$  (cancer vs. control), Students *t*-test.

## DISCUSSION

Exact quantification of cyclin B1/D1 expression levels might elucidate the causes and consequences of cyclin B1 or D1 deregulation in cervical cancer. The quantitative real-time RT-PCR assay described in this report represents a major improvement over other assays designed to quantify cyclin B1/D1 expression in tissue specimens. This assay can detect the PCR product following each cycle of the reaction, during the linear range of amplification, then eliminates the need for post PCR analysis. Real-time RT-PCR assay is an ideal tool for the detection and quantification of cyclins in cervical carcinoma because it is reliable, rapid, sensitive, and specific. Another advantage is its applicability to the degraded nucleic acids obtained from fixed and embedded cervical tissue specimens, which neither Southern nor Northern blotting of tissue RNA can be performed successfully [17, 18]. In brief, real-time RT-PCR combined the speed and ease of a

PCR-based system with an accurate and reproducible quantification methodology and therefore has the potential to become a routine diagnostic tool.



**Figure.** Western blot of cyclin B1 and D1 (a) and boxplot graphs demonstrating the cyclin B1 and cyclin D1 protein expression in normal cervical and cervical carcinoma tissues. (a): Western blot of cyclin B1 and D1 in normal and cancer cervical tissues. Protein extracts were separated by 10% SDS-PAGE, transferred from the gel to PVDF membranes, and immunoblotted with cyclin B1 and D1 monoclonal antibodies (HeLa: HeLa cell line; C: control tissue; T1-4: tumor samples). (b): Boxplot graphs demonstrating cyclin B1 and cyclin D1 protein expression in normal cervix and cervical carcinoma (Western blot). The lines across the boxes represent the medians and the whiskers extend to the highest and lowest values, excluding outliers. The small circles and asterisks identify outliers and extreme values.

In this study, results were calculated by the comparative Ct method of quantification ( $2^{-\Delta\Delta C_t}$  method). The relative expression of cyclin and GAPDH genes could be estimated from the difference in  $C_t$  values ( $\Delta C_t$ ). The  $\Delta C_t$  value would be normalized to a control by subtracting the  $\Delta C_t$  value obtained with the calibrator to yield a  $\Delta\Delta C_t$ .  $C_t$  values for cyclin B1/D1 and GAPDH in the test tissues were compared with those obtained

with the HeLa cell line. The greater the  $\Delta\Delta C_t$  value, the lower the expression of cyclin B1/D1 in the specimens. The  $2^{-\Delta\Delta C_t}$  method is a convenient method to analyze the relative changes in gene expression from real-time quantitative RT-PCR experiments.

The main purpose of this study was to evaluate the potential link between the expression of cyclin B1/D1 and cervical tumorigenesis. As a key cell cycle modulator of the G2-M transition, cyclin B1 is considered to play an important role in various human tumors. However, to the best of our knowledge, this is the first report to demonstrate the up-regulation of cyclin B1 expression in patients with cervical carcinoma using quantitative real-time RT-PCR assay. This finding was in agreement with the previously published study by Kanai et al. They reported elevated cyclin B1 levels in invasive carcinomas and concomitantly elevated levels of cdk1 in the immunohistochemical study [19].

On the other hand, no significant difference in the expression of cyclin B1 was found among various clinicopathological parameters including histology, histological subtype, tumor lesion, and patient's age. Furthermore, we found that there was a trend of decrease in the mRNA expression of cyclin B1 with each advancing stage. Other studies have also shown that in high-grade cervical precancerous lesions, cyclin B1 expression was up-regulated and persists into the upper epithelial layers [4]. This suggests that cyclin B1 could play a crucial role in the early phase of cervical carcinogenesis.

Because it is not clearly understood, there are controversial results on the role of cyclin D1 in cervical carcinogenesis and clinical outcome [20, 21]. The hypophosphorylated form of Rb, complexed with E2F, serves as activator of cyclin D1 transcription by binding to its promoter. This drives cyclin D1 in the early and mid G1 phase of the cell cycle. Since D type cyclins and HPV E7 possess similar binding regions for pRb and pRb-related pocket proteins, inactivation of pRb either by the cyclin/cdk complexes in G1 or by interaction with the high-risk HPV oncoprotein E7 may result in a decreased expression of cyclin D1 [6, 22]. In this study, we found no significant difference in cyclin D1 mRNA expression between cervical cancer and normal cervical tissues. This finding was slightly different from previous studies, which described significantly lower in HPV-positive cervical lesions compared to HPV-negative cases and normal cervical epithelium [22]. Different detection methods and analysis standards may have caused the conflicting observations because semi-quantitative assays, including immunohistochemical assay, RT-PCR, and Western analysis, were used to detect expression levels of cyclins and other molecules [23]. Further study of this phenomenon is warranted.

In summary, this study demonstrated that cyclin B1 mRNA expression was significantly increased in invasive cervical carcinomas compared with normal cervix. This change may play a role in uncontrolled proliferation and malignant transformation of the uterine cervix. However, additional molecular studies to identify the amplification of the cyclin B1 and D1

genes or the source of cyclin protein overaccumulation will be necessary to clarify these findings.

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## ОСОБЕННОСТИ ЭКСПРЕССИИ ЦИКЛИНОВ В1 И D1 ПРИ РАКЕ ШЕЙКИ МАТКИ

**Цель:** циклины представляют собой семейство регуляторных белков, контролирующих клеточный цикл. Наличие функциональных и структурных нарушений регуляторов клеточного цикла (циклинов и циклинзависимых киназ) было отмечено в клетках различных злокачественных новообразований. Целью данного исследования было проведение количественного определения циклинов В1 и D1 в клетках рака шейки матки. **Методы:** определение уровня экспрессии циклинов В1/D1 (mRNA и белков соответственно) в свежеполученных клетках инвазивного рака шейки матки (n = 41) и нормальной ткани шейки матки (n = 10) проводили методами RT-PCR в режиме реального времени и Вестерн-блот анализа. **Результаты:** отмечен более высокий уровень экспрессии гена циклина В1 в клетках инвазивного рака шейки матки, чем в клетках нормальной ткани (P = 0,019). Не выявлены значительные различия в уровне экспрессии гена циклина D1. При Вестерн-блот анализе получены аналогичные результаты. **Выводы:** результаты исследования подтверждают концепцию об активации экспрессии циклина В1 при раке шейки матки. Аберрантная экспрессия циклина В1 может играть важную роль при злокачественной трансформации эпителия шейки матки.

**Ключевые слова:** циклин В1, циклин D1, рак шейки матки, RT-PCR в режиме реального времени, Вестерн-блоттинг.