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Influence of PD-0332991 treatment on cell cycle regulatory genes in breast cancer stem cells

Meme kanseri kanser kök hücrelerinde PD-0332991 uygulanmasının hücre döngüsü düzenleyici genler üzerine etkisi

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Abstract

Aim: Palbociclib (PD-0332991) is an inhibitor for cyclin-dependent kinase 4/6 complex and exhibits more activity in luminal ER+ breast cancer. However, sensitivity of breast cancer stem cells (BCSCs) to PD-0332991 treatment and expression patterns of cell cycle regulatory genes after PD-0332991 treatment in BCSCs are still unclear. This study aims to determine response of BCSCs to PD-0332991 treatment.

Materials and Methods: An experimental in vitro study was designed on breast cancer cell lines. MCF-7 and BCSCs cell lines were used in this study. Water soluble tetrazolium salt-1 (WST-1) test was used for the cytotoxicity assay. Cell cycle distribution pattern and apoptosis were examined with flow cytometry according to IC50 values at 48th h. Real-Time PCR was used to detect expression profiles of CDKN1A, CHEK1, CDKN2A, CDC25A, and CCND1 genes.

Results: PD-0332991 decreased cell proliferation in both cell lines. G0/G1 arrest was detected for both cell lines. There was no apoptotic effect of PD-0332991 on MCF-7 cells and BCSCs. In MCF-7 cells, expression levels of CDKN1A, CDKN2A, and CCND1 genes were increased as 3.11, 3.21, and 1.05 folds, respectively. Expression levels of CHEK1 and CDC25A genes were decreased as 4.75 and 3.73 folds, respectively. In BCSCs, expression levels of CDKN1A, CHEK1, CDKN2A, and CCND1 were decreased as 1.15, 2.01, 1.32, and 1.68 folds, respectively. No expression of CDC25A gene was found in BCSCs group.

Conclusion: In this study, it was observed that PD-0332991 leads to different expression profiles for cell cycle regulatory genes between BCSCs and breast cancer cells.

Keywords: PD-0332991, breast neoplasms, neoplastic stem cells, G1 cell cycle arrest, cell cycle.

Öz

Amaç: *Palbociclib (PD-0332991) siklin bağımlı kinaz 4/6 kompleksi için bir inhibitördür ve luminal ER+ meme kanserlerinde daha fazla aktivite sergilemektedir. Ancak, meme kanseri kök hücrelerinin (MKKH) PD-0332991 uygulamasına olan hassasiyeti ve hücre döngüsü düzenleyici genleri üzerine PD-0332991'in etkisi MKKH'leri için açıklığa kavuşmamıştır. Bu çalışma, MKKH'lerinin PD-0332991 uygulamasına olan yanıtın belirlenmesini amaçlamaktadır.*

Gereç ve Yöntem: *Meme kanseri hücre hatları üzerinde deneysel bir in vitro çalışma tasarlanmıştır. Bu çalışmada, MCF-7 ve MKKH hücre hatları kullanıldı. Suda çözünür tetrazolyum tuzu-1 (WST-1) testi sitotoksisite deneyi için kullanıldı. Hücre döngüsü dağılımı ve apoptosis, 48. saat IC50 değerlerine göre flow sitometri ile incelendi. CDKN1A, CHEK1, CDKN2A, CDC25A ve CCND1 genlerinin ifade profillerinin belirlenmesinde Real-Time PCR kullanıldı.*

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Bulgular: *Her iki hücre hattında, PD-0332991 hücre proliferasyonunu azalttı. Her iki hücre hattı için G0/G1 tutuklanması belirlendi. PD-0332991'in apoptotik etkisi MCF-7 ve MKKH hücrelerinde bulunmadı. MCF-7 hücrelerinde, CDKN1A, CDKN2A ve CCND1 ifade düzeyleri sırasıyla 3,11; 3,21 ve 1,05 kat arttı. CHEK1 ve CDC25A ifade düzeyleri sırasıyla 4,75 ve 3,73 kat azaldı. MKKH'lerinde CDKN1A, CHEK1, CDKN2A ve CCND1 ifade düzeleri sırasıyla 1,15; 2,01; 1,32 ve 1,68 kat azaldı. MKKH'lerinde, CDC25A geni ifadesi bulunmadı.*

Sonuç: *Bu çalışmada, MKKH ve meme kanseri hücreleri arasında PD-0332991'in hücre döngüsü düzenleyici genler için farklı ifade profillerine neden olduğu gözlenmiştir.*

Anahtar Sözcükler: *PD-0332991, meme tümörleri, neoplastik kök hücreler, G1 hücre döngüsü bloğu, hücre döngüsü.*

Introduction

Breast cancer is the most common cancer type among women and it is the second leading cause of the cancer-related death after respiratory system and lung cancers (1). Approximately, estimated 231,840 new cases of breast cancer are expected to be diagnosed in the USA according to a report by Siagel et al. (1). Novel effective drugs are a requirement to provide therapies due to high incidence and mortality rates of breast cancer. Cell cycle inhibitors have been evaluated for breast cancer treatment (2,3). Palbociclib (PD-0332991) is a cyclin dependent kinase-4/6 (CDK-4/6) inhibitor, leading to G1 arrest in cancer cells (4). This inhibitor binds specifically to CDK-4/6 complex involving G1/S transition in cell cycle (5-7). PD-0332991 blocks specifically cell cycle progression in G1 phase due to no activity on other kinases (2). Thus, the cancer cells are allowed to be arrested specifically in G1 phase.

Studies demonstrated that PD-0332991 is more effective for estrogen positive (ER+) breast cancer cells (4,8-11). Finn et al. showed that ER+ breast cancer cells as well as HER-2 positive cells are sensitive to PD-0332991 treatment (11). Sensitivity of breast cancer cells to PD-0332991 treatment is associated with expression patterns of cell cycle regulatory genes such as retinoblastoma (RB1), *CDKN2A* (p16), and cyclin D1 (11). However, expression patterns of genes that determine molecular subtypes of breast cancer are not implicated in sensitivity to PD-0332991 treatment. Paternot et al. (12) identified that PD-0332991 stabilizes CDK-4/6-cyclin D3 complex devoid of p21 and p27 proteins. Depending on withdrawal of PD-0332991, stabilized cyclin D3-CDK4/6 complex *via* PD-0332991 contributes to entry S phase in cell cycle (12). Thus, it has been suggested that genes which regulate G1/S checkpoint in cell cycle may also assess sensitivity of breast cancer cells to PD-0332991 treatment (11,12).

Cancer stem-like cells (CSCs) are distinct and highly tumorigenic cell population in tumor tissue (13). CSCs, as a unique population in tumor tissue, express stem cell markers and they also exhibit stem cell-like features such as self-renewing (13). It is known that breast cancer stem-like cells (BCSCs) are one of the causes of resistance against radio- and chemotherapy, leading to treatment failure (13-15). Although BCSCs are found in all subtypes of breast cancer, basal-type breast cancer tumors have higher population of BCSCs than luminal type breast cancer tumors (16,17). Furthermore, the loss of p16 expression increases the percentage of BCSCs in estrogen negative breast cancer cell lines (18). Expression profiles of cell cycle regulatory genes in BCSCs may be a reason for differently observed sensitivity of PD-0332991 among breast cancer subtypes. A number of cell cycle related genes such as *CCNA1, CCND1, CCND2*, and *CDKN2A* exhibit an abnormal expression pattern in breast cancer stem cells when compared with its ordinary cancer cell line (19). Suppression of *NANOG* gene, maintaining stem cell features, in breast cancer cells reduces particularly expression of cyclin D1 protein (20). In addition, the down-regulation of *NANOG* gene increases G0/G1 arrest for breast cancer cells (20). Although cell cycle related genes, responsible for sensitivity to PD-0332991, show a different expression pattern between BCSCs and breast cancer cells, influence of expression pattern of cell cycle regulatory genes on response to PD-0332991 in BCSCs are still unclear. In this study, we therefore aimed to show expression patterns of cell cycle regulatory gene, acting together in G0/G1 arrest, in breast cancer stem cells after PD-0332991 treatment.

Materials and Methods

An experimental in vitro study was designed on breast cancer cell lines. MCF-7 breast cancer cell line and breast cancer stem cell line (CelProgen, CA, USA, Cat No: 36102-29-T25), which is positive for such stem cell markers as CD133, CD44, SSEA3/4, and aldehyde dehydrogenase, were used in the current study.

Cell culture and reagent

PD-0332991-isethionate was purchased from Sigma-Aldrich (Sigma-Aldrich, USA). MCF-7 breast cancer cell line was purchased from ATCC (ATCC, HTB-22, Wesel, Germany). RPMI 1640 medium, containing 10% FBS, was used for MCF-7 cells. Human breast cancer stem-like cell line was purchased from CelProgen (San Pedro, CA, USA). Human breast cancer stem cell

complete growth media, including all components to maintain cell culture, was used. Cells were maintained at 37^{o}C under a humidified atmosphere of 5% CO_2 in air. The passage numbers of BCSC and MCF-7 cell lines were five and seven, respectively.

WST-1 cell proliferation assay

WST-1 test (Roche Diagnostic GmbH, Mannheim, Germany) was performed to show cytotoxicity of PD-0332991 on MCF-7 and BCSC cell lines. Before the cytotoxicity assay was performed, the cell numbers per well were optimized via WST-1 test in order to eliminate a deceptive apoptotic effect due to doubling time differences between cell lines. The cells, starting with $1x10^6$ in a well and diluted in 1/2 ratio, were seeded on 96 well-plate. After 72h incubation with the media, without agent, proliferation characteristic was determined with WST-1 test for BCSCs and MCF-7 cells. Thus, in assay, MCF-7 cell line was seeded as $1x10^4$ cells per well in 96 well-plates. For BCSC cell line, $7x10^3$ cells per well were seeded in 96 well-plates. PD-0332991 treatments for both cell lines were depending on time and concentrations. Cells were treated with the logarithmic dilution of PD-0332991 ranging from 100µM to 0.0001µM for 24h, 48h, and 72h. WST-1 reagent, 10µl, was added to 100µl medium containing PD-0332991. Absorbance values at 450nm were taken with the microplate reader (Thermoscientific, Multiskan FC, Finland) after incubation periods. Absorbance value at 620nm was used as the reference wavelength. The viability of the cells was calculated according to absorbance value made up of WST-1 reduction (21, 22). The absorbance of control cells was assumed as showing 100% viability for normalization.

Cell cycle assay by flow cytometry

IC_{50} values of PD-0332991 at 48th h were used in cell cycle experiment for both cell lines. Non-treated cells were used as control. BD Cycletest Plus DNA Reagent Kit (BD Pharmigen, CA, USA) was used for cell cycle assay. Protocol was performed according to manufacturer's instructions. For the determination of cell cycle pattern by flow cytometry, BCSCs and MCF-7 cells were seeded at a density of $5x10^5$ cells per 25cm^2 cell culture flasks. For defined concentrations, depending on IC_{50} values at 48th h, 8 ml media with agent was prepared and the cells were treated. Media, 8ml without agent, was used for the control group which has the same cell number as the treatment group. After 48h incubation, cells were removed from surface of cell culture flask and cells were taken to falcon tubes. The cell suspensions were centrifuged at 300x*g* for 5 min. After removing the supernatant, the pellet was resuspended with 1ml washing buffer. Washing step was repeated twice. Washing buffer was removed without disturbing the pellet. 250µl of solution A (trypsin buffer) was added to the pellet and mixed gently. Tubes were incubated at room temperature for 10 min. After incubation, 200µl of solution B was added to tubes without removing solution A. Incubation step was repeated. 200µl of solution C (propidium iodide stain solution with RNase enzyme) kept in 4^{o}C was added to tubes. Samples were read at the flow cytometry (BD Accuri C6 flow cytometry, Becton–Dickinson, USA). The argon laser light source, emitting at 488nm wavelength, was used for excitation. A fluorescence detector equipped with the 585/40 band-pass filter was used to show the PI-DNA content (FL2-A) for the analysis.

Apoptosis assay by flow cytometry

Both cell lines were treated with IC_{50} values of PD-0332991 at 48th h. Non-treated cells were used as control. BCSCs and MCF-7 cells were seeded at a density of $5x10^5$ cells per 25cm^2 cell culture flasks. The control group had the same cell number as treatment group. PE-Annexin V Apoptosis Detection Kit I (BD Pharmigen, San Diego, CA, USA) was used to detect apoptosis after PD-0332991 treatment in both cell lines. Protocol was performed according to manufacturer's instructions. Cells were washed twice with 1X PBS. After washing step, 1X binding buffer was added to tubes. 5µl of PE-Annexin V and 7-AAD antibodies were added to tubes and samples were incubated for 15 min at room temperature in the dark. After incubation, 400µl of binding buffer was added to samples and the prepared samples were read at the flow cytometry (BD Accuri C6 Flow Cytometer, Becton–Dickinson, USA) within 1h. The argon laser light source, emitting at 488nm wavelength, was used for excitation. The 670nm band-pass filter for 7-AAD fluorescence (FL3-A) and a fluorescence detector equipped with the 585/40 band-pass filter for FL2A were used for analysis. A total of 20.000 events were acquired for analysis with using Cell Quest Software.

Gene expression analysis

In order to detect mRNA expression of cell cycle regulatory genes, total RNA were isolated from PD-0332991 treated and non-treated cell lines. Total RNA purification Kit (Jena Bioscience, Jena, Germany) was used to isolate total RNA from cell lines according to manufacturer's instructions. Total RNA (1-5µg) was converted to cDNA with using SCRIPT cDNA Synthesis Kit (Jena Bioscience, Jena, Germany). For the detection of gene expression, 1.0 µL cDNA were mixed with 10 µl qPCR GreenMaster with UNG Mix (Jena Bioscience, Jena, Germany) and sterile bidistilled water was added to PCR mix. The expression studies of *CDKN1A*, *CHEK1, CDKN2A, CDC25A,* and *CCND1* genes were performed with LightCyler (Roche 480

LightCycler, Mannheim, Germany) according to the GreenMaster protocol (Jena Bioscience, Jena, Germany). Relative quantification method described by Livak et al. (23) was used to analyze data. *ACTB, HPRT1, 18SRNA,* and *GAPDH* genes were chosen as housekeeping genes for normalization. Ct values were obtained for both the target genes and the housekeeping genes in all cell lines. The expression data was normalized to Ct values obtained from non-treated cell lines to calculate $2^{-\Delta\Delta Ct}$. Primers for gene expression were designed with Probe Finder Software (Roche Applied Science). Primers were given in Table-1.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) was used for statistical analysis. The two-way ANOVA test was performed with Dunnett's multiple comparisons test to compare the mean differences between treatment concentrations and time in the cytotoxicity assay. The same test was also used to compare the mean differences for two independent variables, cell cycle phase and treatment, in the data analysis of cell cycle assay. A *p* value under 0.05 was accepted as significant.

Table-1. Primer Sequences Used for RT-PCR Study.

Genes	Sequences	Tm	GC%	Amplicon size
CDKN1A				
Forward	ccgaggcactcagaggag	59	67	112 bp
Reverse	agctgctcgctgtccact	59	61	
CHEK1				
Forward	tgacttccggctttctaagg	59	50	113 bp
Reverse	atgtggcaggaagccaaa	60	50	
CDKN2A				
Forward	gtggacctggctgaggag	59	67	133 bp
Reverse	tctttcaatcggggatgtct	59	45	
CDC25A				
Forward	cgtcatgagaactacaaaccttga	60	42	96 bp
Reverse	tctggtctcttcaacactgacc	59	50	
CCND1				
Forward	gccgagaagctgtgcatc	60	61	90 bp
Reverse	ccacttgagcttgttcacca	59	50	
ACTB				
Forward	attggcaatgagcggttc	59	50	79 bp
Reverse	cgtggatgccacaggact	60	61	
HPRT1				
Forward	gaccagtcaacaggggacat	59	55	95 bp
Reverse	gtgtcaattatatcttccacaatcaag	59	33	
18SRNA				
Forward	ctcaacacgggaaacctcac	60	55	110 bp
Reverse	cgctccaccaactaagaacg	60	55	
GAPDH				
Forward	ccccggtttctataaattgagc	60	45	127 bp
Reverse	caccttccccatggtgtct	60	58	

Results

PD-0332991 decreases proliferation of BCSC and MCF-7 cells

PD-0332991 decreased cell proliferation in both MCF-7 and BCSCs groups. IC_{50} values at 24^{th} h, 48^{th} h, and 72^{nd} h of treatments for MCF-7 cells were 34.37μM, 25.21 μM, and 26.9 μM, respectively (Figure-1a). IC_{50} values of PD-0332991 in BCSC cell line were 37.37 μM, 13.16 μM, and 4.12 μM at 24^{th} h, 48^{th} h and 72^{nd} h, respectively (Figure-1b). Cytotoxicity at 48^{th} h and 72^{nd} h of treatment was higher in BCSCs compared to MCF-7 cells at 10 μM concentration. BCSCs were found to be more sensitive to treatment.

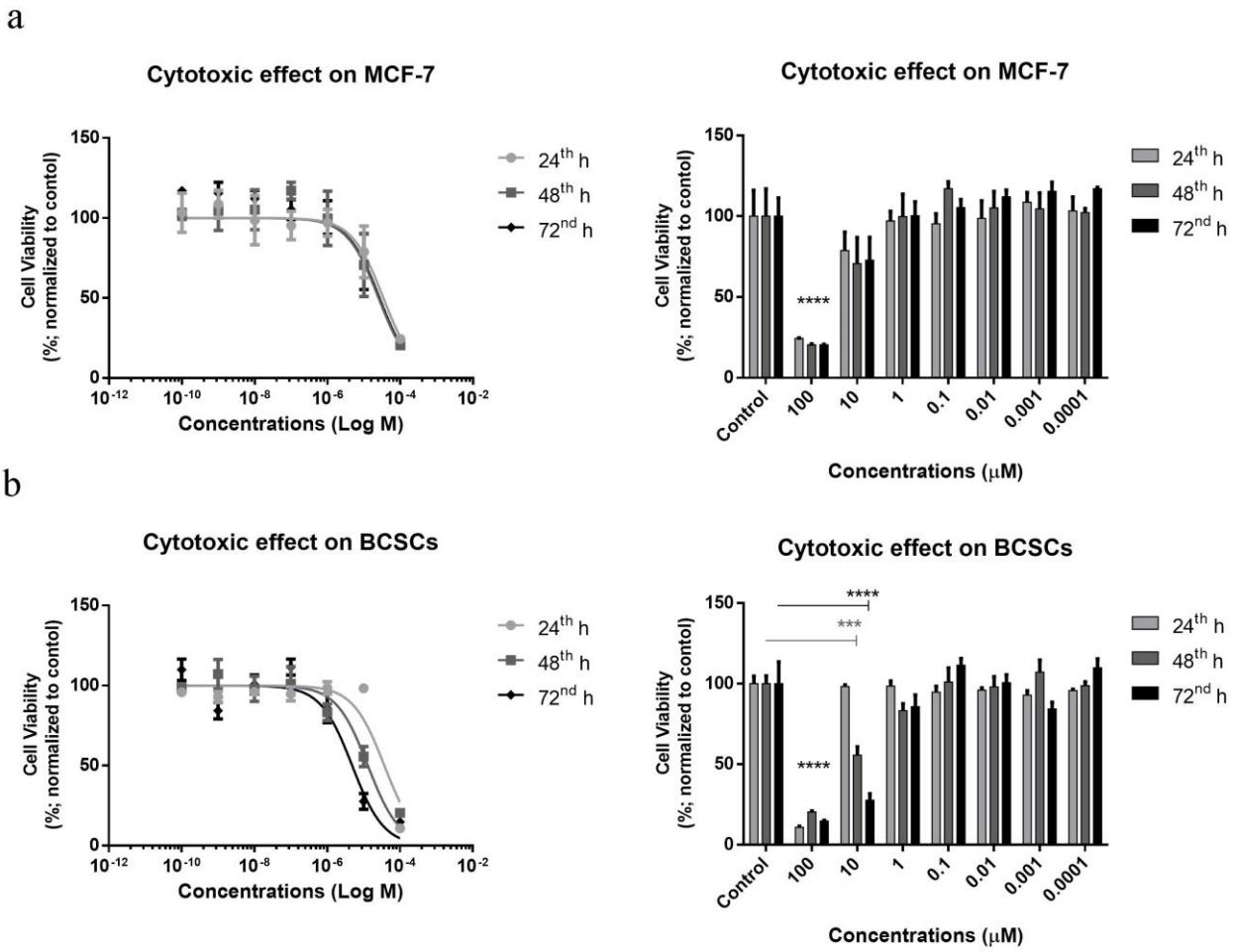

Figure-1. Results of WST-1 proliferation assay after PD-0332991 treatment. IC_{50} values at 24th h, 48th h, and 72nd h of treatment in MCF-7 cells were 34.37μM, 25.21 μM, and 26.9 μM, respectively **(a)**. IC_{50} values for BCSCs were 37.37 μM, 13.16 μM, and 4.12 μM at 24th h, 48th h and 72nd h, respectively **(b)**. **** and *** marks describe respectively *p* values under 0.0001 and 0.001 with respect to two way ANOVA analysis.

G0/G1 arrest in MCF-7 cells and BCSC cells after PD-0332991 treatment

IC_{50} values of PD-0332991 at 48th h were used in cell cycle analysis of MCF-7 and BCSC cell lines. For both cell lines, G0/G1 arrest was detected after treatment. In MCF-7 control group, the percentage of cells at G0/G1, S, G2/M were determined as 48.9%, 23.5%, and 29.6%, respectively. The percentages of cells in G0/G1, S, G2/M phases for PD-0332991 treated cells were 73.9%, 13.1%, and 12.2%, respectively (Figure-2a). In BCSC control group, the percentage of cells at G0/G1, S, G2/M were determined as 57.0%, 17.0%, and 27.6%, respectively. After treatment, in BCSC cell line, the percentages of cells in phases of G0/G1, S, G2/M were 71.3%, 11.8%, and 17.8%, respectively (Figure-2b). PD-0332991 treatment induced G1 arrest and significant increase in the percentage of cells in G0/G1 phase was detected for both cell lines.

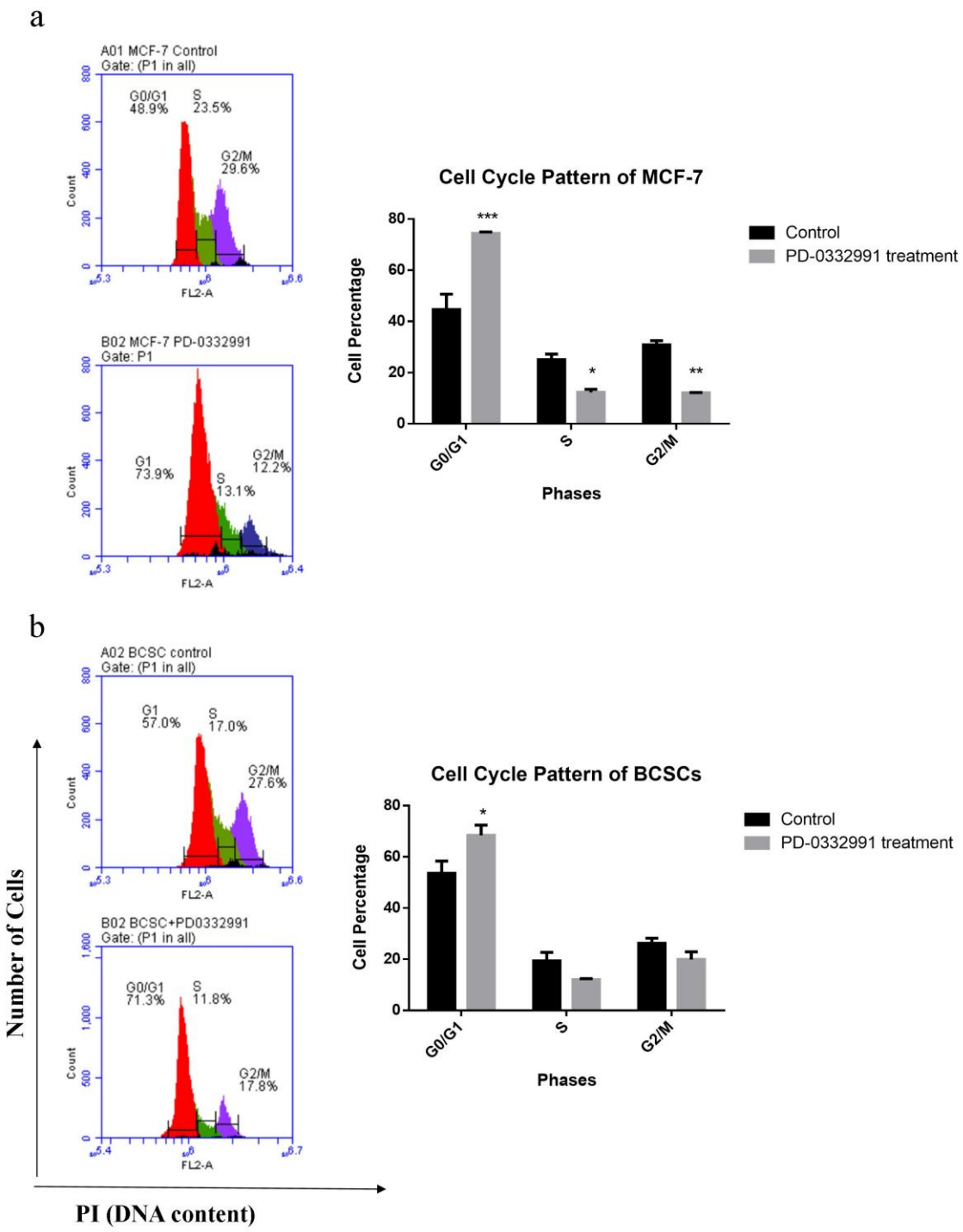

Figure-2. Cell cycle patterns of MCF-7 cells **(a)** and BCSCs **(b**) after treatment of IC_{50} values at 48th h. With PD-0332991 treatment, G0/G1 arrest was detected for both cell lines. ***, ** and * marks describe respectively *p* values under 0.001, 0.01, and 0.05 with respect to two way ANOVA analysis.

Apoptotic effect of PD-0332911 on BCSC and MCF-7 cell lines

Cells were treated with concentrations (IC_{50} values at 48th h) of PD-0332991. Non-treated cells were used as control. In MCF-7 control group, the percentage of living cells was 91.0%. The percentage of apoptotic cells in MCF-7 control group was 2.4%. After treatment, the percentages of living and apoptotic cells were 86.3% and 9.3%, respectively (Figure-3a). In BCSC control group, the percentages of living and apoptotic cells were detected as 96.1% and 2.6%, respectively. After treatment for BCSCs, distributions of the percentages of living and apoptotic cells were 96.8% and 2.1%, respectively (Figure-3b). There was no apoptotic effect of PD-0332991 on MCF-7 cells and BCSCs.

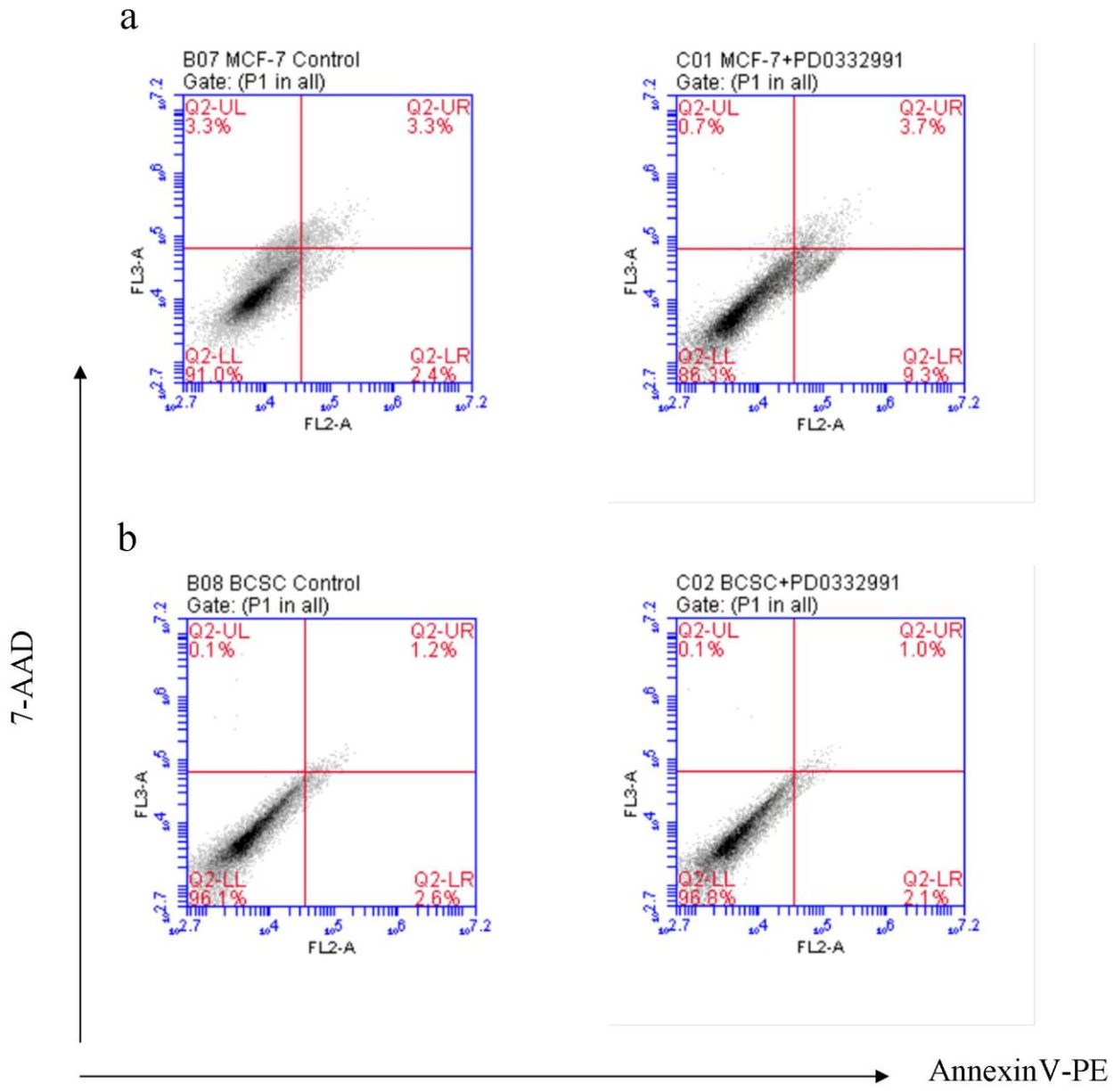

Figure-3. Apoptosis assay for MCF-7 **(a)** and BCSCs **(b)** cell lines after treatment. Non-treated cells were used as control. PD-0332991 did not induce apoptosis in BCSCs and MCF-7 cell lines.

Gene expression profiles of G1 regulatory genes after PD-0332991 treatment

After 48h PD-0332991 treatment in MCF-7 cells, expression levels of *CDKN1A, CDKN2A,* and *CCND1* genes were increased 3.11, 3.21, and 1.05 folds, respectively (Figure-4a). Expression levels of *CHEK1* and *CDC25A* genes were decreased as 4.75 and 3.73 folds, respectively. In BCSCs, expression levels of *CDKN1A*, *CHEK1, CDKN2A,* and *CCND1* genes were decreased as 1.15, 2.01, 1.32, and 1.68 folds, respectively (Figure-4b). No expression of *CDC25A* gene was found in BCSCs group.

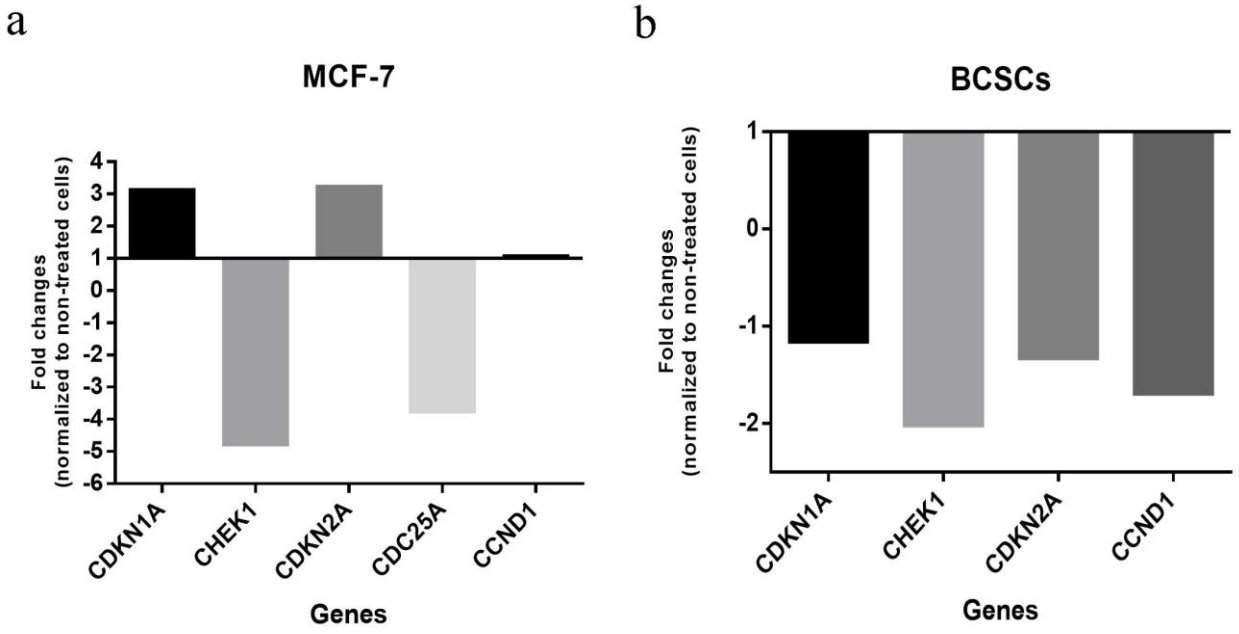

Figure-4. Expression profiles of *CDKN1A*, *CHEK1, CDKN2A, CDC25A,* and *CCND1* genes in MCF-7 **(a)** and BCSCs cell lines **(b)**. mRNA expression of *CDC25A* gene was not observed in BCSCs group.

Discussion

Effect of PD-0332991 treatment on BCSCs was identified in this study. BCSCs were more sensitive to PD-0332991 treatment with respect to MCF-7 cells. In addition, expression patterns of cell cycle regulatory genes between BCSCs and MCF-7 cell lines were dissimilar in response to treatment.

It was found that PD-0332991 decreased proliferation of MCF-7 cells which is reported by other studies (6,11). Finn et al. found that IC_{50} values of PD-0332991 for MCF-7 are at nanomolar concentration levels (11). However, in this study, IC_{50} values for MCF-7 cells were not at nanomolar levels. Different methods utilized for determining of cell proliferation and treatment period may cause differently observed IC_{50} values for MCF-7 in the literature (11,24). Robinson et al. demonstrated that IC_{50} value is not observed at 10μM concentration of PD-0332991 for 72h treatment in MCF-7 cells when MTT assay is evaluated for measuring cell proliferation (24). In this study, it was also found that PD-0332991 decreased cell proliferation of BCSCs. In addition, IC_{50} concentrations of treatment at 48^{th} h and 72^{nd} h were lower in BCSCs compared to MCF-7 cells. BCSCs were more sensitive to treatment than MCF-7 cells.

Studies indicate that MCF-7 cell line is more sensitive to PD-0332991 treatment compared to MDA-MB-231 cell line which is non-luminal and HER-2 negative cell line (11). Interestingly, it is known that MDA-MB-231 cell line has higher $CD44^{+}/CD24^{-}$ cell population than luminal type breast cancer cell lines such as MCF-7 (16,17). The different activity of PD-0332991 among breast cancer subtypes may be associated with variable percentage of BCSCs population in tumor tissue. In the current study, cell cycle pattern and apoptosis were examined after treatment according to IC_{50} values at 48h. As expected for MCF-7 cells, G0/G1 arrest was detected after treatment (6,11). In addition, PD-0332991 did not induce apoptosis in MCF-7 which is in line with other studies (4,9,11). G0/G1 arrest and no apoptotic effect were also observed for BCSCs after treatment.

Studies indicated that response of breast cancer cells to PD-0332991 is associated with expression pattern of CDK4/6–pRb–E2F pathway mediated genes such as Rb1, *CDKN2A*, and cyclin D1 (25-27). In cell cycle, mitogenic signals induce cyclin D1 gene (*CCND1*) expression to initiate G1–S transition. Cyclin D1 is assembled spontaneously with CDK4 or CDK6 proteins to form active kinase complexes. Active CDK4 and CDK6 complexes phosphorylate Rb1 protein which contributes to release of the E2F transcription factors. Mediators such as CCNB1, CDK2 and CDK1 which promote transition to further steps in cell cycle are expressed by released E2F transcription factors (25-27). CDC25A activates CDK2–cyclin E and CDK2–cyclin A complexes during G1-S transition to entry S phase (28-30). In addition, Cdc25A activates also Cdk4-cyclin D complex in G1 phase (31). Chk1 kinase (CHEK1) is a negative regulatory factor for CDC25A action (32). The phosphorylation of regulatory region of CDC25A protein

due to active Chk1 leads to ubiquitin mediated proteolysis (32,33). Chk1 can also be a target of the CDK4/6–pRb–E2F pathway due to the fact that Chk1 has E2F-binding sites (34).

In this study, expression profiles of *CDKN1A*, *CHEK1, CDKN2A, CDC25A,* and *CCND1* genes were examined in both cell lines after PD-0332991 treatment. Defined genes were chosen due to be direct target of CDK4/6–pRb–E2F pathway which is responsible for activity of PD-0332991. *CCND1* expression was increased in MCF-7 cell line after treatment (11). In addition, elevated expressions of *CDKN1A* and *CDKN2A* were detected. For MCF-7 cells, decreased expression levels of *CHEK1* and *CDC25A* were also observed. On the contrary, expression levels of *CDKN1A*, *CHEK1, CDKN2A,* and *CCND1* were decreased in BCSCs after treatment. Expression of *CDC25A* was not found in BCSCs group. *CDC25A* is expressed in early G1 phase of cell cycle and its activity is highly observed from the G0/G1 checkpoint to the M phase (29,35). Upregulation of *CDC25A* in human embryonic stem cells shortens G1-S transition time depending on Rb-E2F pathway (29). Absence of *CDC25A* may be associated with why BCSCs was more sensitive to PD-0332991 treatment. It was found that expression patterns of cell cycle regulatory genes involved in early and late G1 phase were different between MCF-7 and BCSCs cell lines after treatment. This difference may determine biological response of breast cancer cells to PD-0332991.

Breast cancer cells, overexpressing *CDKN2A* and *CDKN1A*, exhibit senescence mediated features such as hypertrophic appearance and high β-galactosidase activity (36). Furthermore, induced expression of *CDKN2A* and *CDKN1A* triggers senescence in G1 arrested cells (36,37). After treatment, MCF-7 cells may be drifted to senescence. Contrary to MCF-7 cells, expression levels of *CCND1, CDKN1A,* and *CDKN2A* were decreased in BCSCs after treatment. Decreased expression of CDK inhibitors (CKIs) and *CCND1* in BCSCs may be an adaptation to treatment for maintaining stem cell-like features. Inhibition of *CCND1* expression by siRNA increases migration features and ALDH expression of estrogen negative breast cancer cells (38). Suppressed *CCND1* expression in ER- cell lines induces also mammosphere formation. Similar results for induced cancer stem cell-like features are observed when CDK4/6 is inhibited by siRNA or PD-0332991 (38). Expression pattern of CKIs may support for maintaining stem cell-like features. Depletion of p16 by RNA interference increases the percentage of BCSCs in estrogen negative breast cancer cell lines (18). In addition, suppression of *CDKN2A* or *CDKN1A* increases efficiency of generating induced pluripotent stem cells (iPS) in order not to be observed p21- and p16-mediated senescence (39-41).

Conclusion

In this study, the effect of PD-0332991 on expression changes of cell cycle regulatory genes in breast cancer stem cells was shown for the first time. In the current study, it was observed that PD-0332991 leads to different expression profiles for cell cycle regulatory genes between BCSCs and breast cancer cells. It was suggested that expression profile of CDK4/6–pRb–E2F pathway related genes after PD-0332991 treatment may determine divergent response due to maintenance of cancer stem cell features.

Conflict of interest

Authors declare that there is no conflict of interest.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015;65(1):5-29.
2. Dukelow T, Kishan D, Khasraw M, Murphy CG. CDK4/6 inhibitors in breast cancer. Anti-cancer drugs 2015;26(8):797-806.
3. Tamura K. Development of cell-cycle checkpoint therapy for solid tumors. Jpn J Clin Oncol 2015;45(12):1097-102.
4. Dhillon S. Palbociclib: first global approval. Drugs 2015;75(5):543-51.
5. Rocca A, Farolfi A, Bravaccini S, Schirone A, Amadori D. Palbociclib (PD 0332991) : targeting the cell cycle machinery in breast cancer. Expert Opin Pharmacother 2014;15(3):407-20.
6. Fry DW, Harvey PJ, Keller PR, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther 2004;3(11):1427-38.
7. Toogood PL, Harvey PJ, Repine JT, et al. Discovery of a potent and selective inhibitor of cyclin-dependent kinase 4/6. J Med Chem 2005;48(7):2388-406.
8. Beaver JA, Amiri-Kordestani L, Charlab R, et al. FDA Approval: Palbociclib for the treatment of postmenopausal patients with estrogen receptor-positive, HER2-negative metastatic breast cancer. Clin Cancer Res 2015;21(21):4760-66.
9. O'Sullivan CC. Overcoming endocrine resistance in hormone-receptor positive advanced breast cancer-the emerging role of CDK4/6 inhibitors. Int J Cancer Clin Res 2015;2(4):029.
10. Finn RS, Aleshin A, Slamon DJ. Targeting the cyclin-dependent kinases (CDK) 4/6 in estrogen receptor-positive breast cancers. Breast Cancer Res 2016;18(1):17.

11. Finn RS, Dering J, Conklin D, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. Breast Cancer Res 2009;11(5):R77.
12. Paternot S, Colleoni B, Bisteau X, Roger PP. The CDK4/CDK6 inhibitor PD0332991 paradoxically stabilizes activated cyclin D3-CDK4/6 complexes. Cell Cycle 2014;13(18):2879-88.
13. Dawood S, Austin L, Cristofanilli M. Cancer stem cells: Implications for cancer therapy. Oncology 2014;28(12):1101-07.
14. Bensimon J, Altmeyer-Morel S, Benjelloun H, Chevillard S, Lebeau J. CD24(-/low) stem-like breast cancer marker defines the radiation-resistant cells involved in memorization and transmission of radiation-induced genomic instability. Oncogene 2013;32(2):251-8.
15. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 2008;10(2):R25.
16. Sheridan C, Kishimoto H, Fuchs RK, et al. CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: An early step necessary for metastasis. Breast Cancer Res 2006;8(5):R59.
17. Fillmore C, Kuperwasser C. Human breast cancer stem cell markers CD44 and CD24: Enriching for cells with functional properties in mice or in man? Breast Cancer Res 2007;9(3):303.
18. Arima Y, Hayashi N, Hayashi H, et al. Loss of p16 expression is associated with the stem cell characteristics of surface markers and therapeutic resistance in estrogen receptor-negative breast cancer. Int J Cancer 2012;130(11):2568-79.
19. Murat Dogan S, Pinar Ercetin A, Altun Z, Dursun D, Aktas S. Gene expression characteristics of breast cancer stem cells. J BUON 2015; 20(5):1304-13.
20. Han J, Zhang F, Yu M, et al. RNA interference-mediated silencing of NANOG reduces cell proliferation and induces G0/G1 cell cycle arrest in breast cancer cells. Cancer Lett 2012;321(1):80-8.
21. Cook JA, Mitchell JB. Viability measurements in mammalian cell systems. Anal Biochem 1989; 179(1):1-7.
22. Ishiyama M, Sasamoto K, Shiga M, et al. Novel disulfonated tetrazolium salt that can be reduced to a water-soluble formazan and its application to the assay of lactate dehydrogenase. Analyst 1995;120(1):113-6.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402-8.
24. Robinson TJ, Liu JC, Vizeacoumar F, et al. RB1 status in triple negative breast cancer cells dictates response to radiation treatment and selective therapeutic drugs. PloS one 2013;8(11):e78641.
25. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov 2015;14(2):130-46.
26. Johnson J, Thijssen B, McDermott U, Garnett M, Wessels L, Bernards R. Targeting the RB-E2F pathway in breast cancer. Oncogene 2016; 35(37):4829-35.
27. Lim S, Kaldis P. Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. Development 2013;140(15):3079-93.
28. Boutros R, Lobjois V, Ducommun B. CDC25 phosphatases in cancer cells: key players? Good targets?. Nat Rev Cancer 2007;7(7):495-507.
29. Neganova I, Lako M. G1 to S phase cell cycle transition in somatic and embryonic stem cells. J Anat 2008; 213(1): 30-44.
30. Donjerkovic D, Scott D W. Regulation of the G1 phase of the mammalian cell cycle. Cell Research 2000;10(1):1-16.
31. Blomberg I, Hoffmann I. Ectopic expression of Cdc25A accelerates the G(1)/S transition and leads to premature activation of cyclin E- and cyclin A-dependent kinases. Mol Cell Biol 1999;19(9):6183-94.
32. Chen MS, Ryan CE, Piwnica-Worms H. Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. Mol Cell Biol 2003;23(21):7488-97.
33. Bartek J, Lukas J. Pathways governing G1/S transition and their response to DNA damage. FEBS letters 2001;490(3):117-22.
34. Semizarov D, Kroeger P, Fesik S. siRNA-mediated gene silencing: A global genome view. Nucleic Acids Res 2004;32(13):3836-45.
35. Jinno S, Suto K, Nagata A, et al. Cdc25A is a novel phosphatase functioning early in the cell cycle. EMBO J 1994;13(7):1549-56.
36. Capparelli C, Chiavarina B, Whitaker-Menezes D, et al. CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. Cell cycle 2012;11(19):3599-610.
37. Blagosklonny MV. Geroconversion: Irreversibl step to cellular senescence. Cell Cycle 2014;13(23):3628-35.
38. Lamb R, Lehn S, Rogerson L, Clarke RB, Landberg G. Cell cycle regulators cyclin D1 and CDK4/6 have estrogen receptor-dependent divergent functions in breast cancer migration and stem cell-like activity. Cell Cycle 2013;12(15):2384-94.
39. Li H, Collado M, Villasante A, et al. The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 2009;460(7259):1136-39.
40. Hong H, Takahashi K, Ichisaka T, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 2009;460(7259):1132-35.
41. Nishi M, Akutsu H, Kudoh A, et al. Induced cancer stem-like cells as a model for biological screening and discovery of agents targeting phenotypic traits of cancer stem cell. Oncotarget 2014;5(18):8665-80.