EFFECT OF BRCA1 AFTER UV-B-IRRADIATION IN CELL CYCLE PROGRESSION BETWEEN HUMAN MELANOMA CELL LINES DETERMINED BY FLOW CYTOMETER.

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ABSTRACT

The aim of the current study was to evaluated the effect of BRCA1*(breast cancer 1 early-0nsent)* after UV-B-irradiation in a panel of human malignant melanoma cells using flow cytometry method. Different human melanoma cell lines (SK-MEL28,SK-MEL93, ,SK-MeL93/shBRCA1 and SK-Mel93/shplKO.1) were cultured for triplicaten experiments. Cells were exposed to UV-B ($10mJ/cm^2$)for different point time(1/2,1,3,6,9,12,and 24h). Cells at 0hr were a non-irradiation control. The cell cycle was evaluated by flow cytometry. SK-MEL28 cell line seemed to be synchronized at the G1/S boundary phase until 12h after UVB-irradiation. a decrease in the fraction of S cells and a reciprocal increase of cells in Sub-G1 phase was observed , from 12 to 24h.While, the SK-MEL93 a rapid increase (about 35%) of cells in Sub-G1 phase was observed already 6h after UVB irradiation. Moreover, we found that the SKMel93/shBRCA1 cells seemed an increase (about 15%) of cells in Sub-G1 phase already 12h after UVB irradiation compared with cells infected with the empty lentiviral vector (SK-Mel93/shplKO.1). we conclude that an important role played by BRCA1 providing new insights into the molecular mechanisms underlying UV-induced melanomagenesis.

INTRODUCTION

Melanoma probably is the most aggressive cancer in humans, and remains one of the leading causes of cancer death in developed countries [1]. The main etiological risk factor for the development of melanoma is UV radiation, although hereditary reasons play a notable role in the progression of melanoma. Pigmentary traits, such as red hair, fair complexion, and a tendency to freckle have been show as the main risk factor for the development of melanoma [2]. Clinically, melanoma is classified according to the thickness in millimeters, mitotic rate, presence of ulceration, penetration depth, location of existing metastases. Also, increasing age, male sex, and tumor location on the trunk, head, or neck also worsen prognosis [3]. The resistance of melanoma to therapy and its recurrence are related to the genetic heterogeneity and genomic instability of the tumor. For many years these genetic alterations were thought to be linked to the accumulation of random mutations in functionally differentiated cells which transform them into malignant cells that have lost their ability to differentiate and have develop drug resistance. Therefore, available treatments can induce objective tumor regression in a small percent of patient, but these responses are not always associated with improved long-term survival [4]. Germline CDKN2A mutations were identified in 25-50% of

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familial melanoma kindreds. In sporadic primary melanoma, only a few mutations (0-25%) and homozygous deletions(10%) are found in this gene [5].However, this locus was found to carry UVB signature transversion in the sporadic primary melanomas , suggesting that UVB radiation may play a role in the etiology of melanoma development [6,7]. When UV-induced mutation affect critical genes encoding protein or enzymes contributing to DNA repair, cell cycle control or apoptosis, it is likely that cumulative or subsequent DNA alteration are not sufficiently eradicated. Disrupted function of such regulative proteins are strongly connected with early stages of skin carcinogenesis [8].Thus, UV- fingerprint mutations can be abundantly detected in the well characterized and pathogenically important tumor suppressor gene p53 from squamous and basal cell carcinoma of human skin [9,10]. The aim of our study was using flow cytometer to detect the Effect of BRCA1 after UV-b-irradiation on cell cycle progression to induced apoptosis in two types of human melanoma cell lines (SK-MEL93 and SK-MEL28).

MATERIAL & METHODS

Cell Culture

Human melanoma cells, SK-MEL28, *p53 mutant*, and SKMEL93 were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin (Sigma) at 37°C in a 5% CO_2/95% air atmosphere. HEK 293T, Human Embryonic Kidney cell, was grown in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin (Sigma) at 37°C in a 5% CO_2/95% air atmosphere.

RNA Interference

The siRNA duplexes were synthesized by Sigma. The sequence targeting BRCA1 gene was :5'- ccggccctaagtttacttctctaaactcgagtttagagaagta aacttagggttttt-3'. The non-silencing siRNA

was purchased from Sigma and used as mock controls.The schematic representation of lentiviral vectors (pLK0.1/empty vector, pLK0.1/non-targetshRNA,pLK0.1/BRCA13'UTRshRN A and pLK0.1/Turbo GFP) utilized are represent in fig.1,2and 3 , and show the locations of major identifiable landmarks on DNA like restriction enzyme sites, gene of interest, plasmid name and length, etc.

Preparation of lentiviral supernatants and transduction of SK-MEL93 cells

5 x 106 293T cells were grown on 10-cm plates to 70-80% confluence and co-transfected with 10 μg siRNA lentiviral DNA (pLK0.1/empty vector, pLK0.1/BRCA13'UTRshRNA) (Sigma-Aldrich), 2 μg VSV-G plasmid DNA and 18 μg packaging viral CMV delta 8.9 plasmid, using the calcium phosphate precipitation method. After the addition of fresh culture medium 8 hr later, the cells were cultured for an additional 2 days. The medium was harvested 48 hr post-transfection, and filtered through a 0.45 μm filter. The supernatants from 293T cultures were used to cross-transduce SK-MEL93 cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich) and subsequently clones were selected by puromycin (1μg/ml) (Sigma-Aldrich) for an additional 2 days and treated with UV-B. The transduction efficiency was calculated by the green fluorescent protein (GFP) expression and was observed under a fluorescent microscope.the preparation of lentiviral supernatants and transduction of SK-MEL93 cells were summarizes schematic in (fig.5). The efficiency of RNA interference was monitored by Western blotting analysis.

UV-B irradiation

Different human melanoma cell lines (SK-MEL28,SK-MEL93, , SKMeL93/shBRCA1 and SK-Mel93/shplKO.1) were cultured for triplicate experiments. Media was removed from 70 to 80% confluent cell cultures,

cells were rinsed with phosphate-buffered saline and exposed to UV-B (230V, 50Hz) using a Vilber Lourmat, FLX-35M at indicated doses. Medium was added immediately to continue culture until designated time points. Cells at 0hr were a non-irradiation control.

Preparation of protein extracts

For preparing whole-cell extracts, cells were washed in ice-cold PBS, harvested, and re-suspended in whole-cell extract buffer (50mM Tris-HCl; pH 8, 150mM NaCl, 1mM EDTA, 1mM DDT, 1mM PMSF, proteinase inhibitor Complete; Roche, Mannheim, Germany). After sonication on ice (two times for 10 seconds) the homogenates were centrifuged (10 000 g, 10 min at 4°C), and the clear supernatants were stored at -80°C. Protein concentrations were determined using the Bradford method (Bradford, 1976). Bradford reagent (200 μl; 0.01% G240 brilliant blue (Saba), 5% ethanol, 10% H3PO4, 85% dH2O) was added to 10 μl of a 1: 10 dilution of the protein extracts. Following 15 min incubation in the dark, the absorption was measured by photometry at 595 nm.The protein concentration was determined using a calibration curve with BSA protein, taken in parallel.

Western blot analysis

Samples of 40-80 μg of protein total extracts were separated on a 6 or 8% SDS-polyacrylamide gel. Separated protein were blotted onto a nitrocellulose transfer membrane (Sigma) in a Bio-Rad blot cell for 2 h at 50Volt using buffer consisting of 25mM Tris-HCl, 192mM glycine. The membranes were blocked for 1 h at room temperature in 5% (wt/vol) milk powder in TBS (150mM HCl, 20mM Tris pH 7.6) containing 0.1% Tween 20 (TBS–Tween) and incubated overnight at 4°C with the primary antibody (1 : 200) in 5% (wt/vol) milk powder or BSA in TBS–Tween. The membranes were washed three times for 10 min in TBS–Tween each, incubated for 1 h with a horseradish-peroxidase coupled secondary antibody (dilution 1:5000-1:10000) (Santa Cruz Biotechnology) in TBS–Tween and washed again three times for 10 min in TBS– Tween. For developing the membranes, a chemiluminescence detection system (Santa Cruz Biotechnology, Heidelberg, Germany) was used. The antibodies used were anti-BRCA1 (C-20),and γ-tubulun (Santa Cruz Biotechnology, Heidelberg, Germany).

Flow cytometry

Cells, were collected by centrifugation at 200g for 10 min and fixed with 70% ethanol at + 4°C for 24h. The cell cycle was evaluated by flow cytometry using propidium iodide (50 μg/ml) staining (Sigma), after prior incubation with 13 k-units/ml RNase, (Sigma) (20 min incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson). A total of 30000 events were evaluated using the ModFit LT 3.0Program.

RESULTS

Effect of UV-B-irradiation on cell cycle progression in , SK-MEL93 and SK-MEL28 cell lines.

To investigate the susceptibility of melanoma to UVB-irradiation, we used two human melanoma cell line (SK-MEL93 and SK-MEL28), DNA content was serially observed after UV-B irradiation (10mJ/cm^2) by flow cytometry. As show in Fig. 6 Panel A, SK-MEL28 cells seemed to be synchronized at the G1/S boundary phase until 12h after 10mJ/cm2 UV-B-irradiation. From 12 to 24h a decrease in the fraction of S cells and a reciprocal increase of cells in Sub-G1 phase was observed. although did not seemed to be synchronized and the increase of cells in Sub-G1 phase was observed already to 9h after UVB irradiation. While, the SK-MEL93 cell line did not seemed to be synchronized but a

rapid increase (about 35%) of cells in Sub-G1 phase was observed already 6h after UVB irradiation (Fig.6 Panel B).

Transduction of BRCA1 shRNA (BRCA13'UTR siRNA) into SK-MEL93 cells results in modulation of UV-B-induced cell cycle arrest

To determine whether BRCA1 is involved in signalling UV-B-mediated effects, we used siRNA technology to inhibit its expression. We generated stable knocked down BRCA1 into SK-MEL93 cells, using shRNA lentiviral specific for BRCA1 (BRCA13'UTR shRNA) . As shown in Fig. 7, BRCA1 protein expression was not detectable in SK-Mel93/shBRCA1 cells when compared with cells infected with the empty lentiviral vector (SKMel93/ shplKO.1). To enforce the idea that BRCA1 protein may be intimately linked to pathway caused by UV-B and evaluate the effect of UV-B on the cell cycle of SK-Mel93/shBRCA1 cell line, DNA content was serially observed after UV-B irradiation (10mJ/cm2) by flow cytometry. As show in Fig. 8 Panel A, SKMel93/ shBRCA1 cells seemed an increase (about 15%) of cells in Sub-G1 phase already 12h after UVB irradiation compared with cells infected with the empty lentiviral vector (SK-Mel93/shplKO.1) (Fig.8 Panel B).

DISCUSSION

Melanoma is a malignant tumor type characterized by a poor prognosis partly due to ineffective radiotherapy and chemotherapy (11,12), although radiotherapy is widely applied for treatment of melanoma patients. Recently, it has been reported that several molecular factors, such as those involved in DNA repair or in the cell cycle, modulate in melanoma cells UV-B induced DNA repair, cell progression and apoptosis. In our work, we have evaluated the effect of BRCA1 after UV-B-irradiation in a panel of human malignant melanoma cells. We exposed SK-MEL28 and SK-MEL93 cells to 10mJ/cm^2 UV-B irradiation and examined the DNA content was serially observed after UV-B irradiation (10mJ/cm^2) by flow cytometry. SK-MEL28 cell line although did not seemed to be synchronized and the increase of cells in Sub-G1 phase was observed already to 9h after UVB irradiation. While, the SK-MEL93 cell line did not seemed to be synchronized but a rapid increase (about 35%) of cells in Sub-G1 phase was observed already 6h after UVB irradiation. Based on our results, we speculate that the increase of cells in Sub-G1 phase, after UVB irradiation may be intimately linked to specific human melanoma cell line (SK-MEL93). In concordance with this hypothesis, we generated stable knocked down BRCA1 into SK-MEL93 cells, using shRNA lentiviral specific for BRCA1 (BRCA13'UTR shRNA). BRCA1 protein expression was not detectable in SKMel93/ shBRCA1 cells when compared with cells infected with the empty lentiviral vector (SK-Mel93/shplKO.1). Then, SK-Mel93/shBRCA1 cells were treated with UV-B-irradiation at different time (0,3-24h). Moreover, we evaluated the effect of UV-B on the cell cycle of SKMel93/ shBRCA1 cell line and found that SK-Mel93/shBRCA1 cells seemed an increase (about 15%) of cells in Sub-G1 phase already 12h after UVB irradiation compared with cells infected with the empty lentiviral vector (SKMel93/ shplKO.1). In conclusion, our findings support the hypothesis that BRCA1 expression modulates UV-B-induced effects on cell cycle progression. Taken together, our data lend support to the general hypothesis of an important role played by BRCA1 providing new insights into the molecular mechanisms underlying UV-induced melanomagenesis, with a special focus on the cascade of events triggered in a specific human melanoma cell line (SKMEL93).
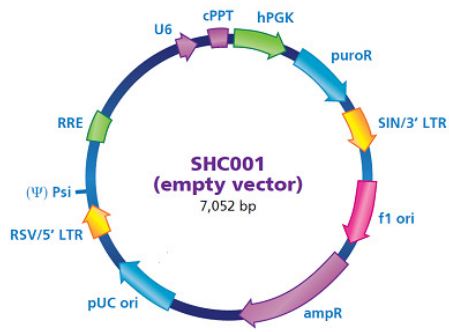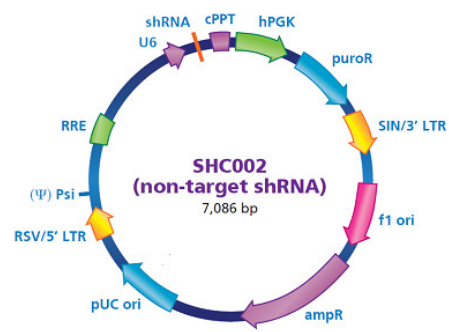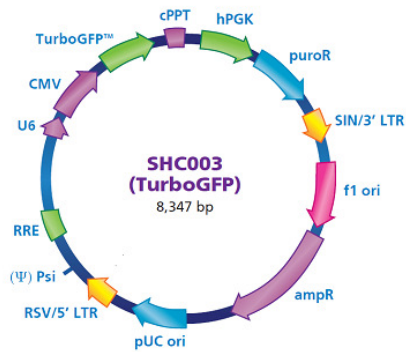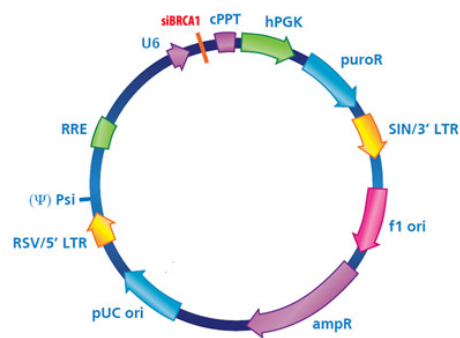
Fig.(1,2,3and4). Representation of lentiviral vectors (pLK0.1/empty vector, pLK0.1/non-targetshRNA,pLK0.1/BRCA13'UTRshRNA and pLK0.1/Turbo GFP)
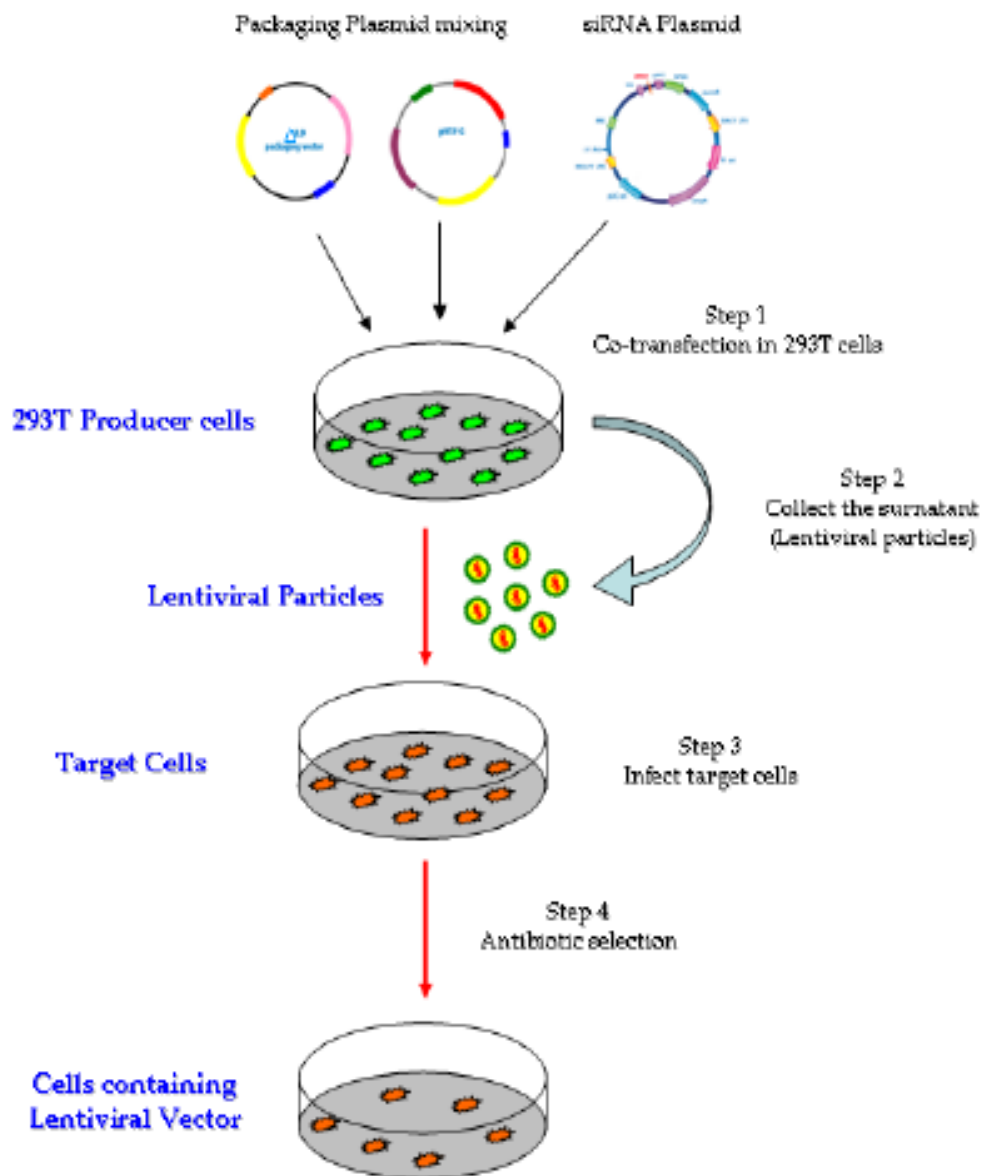
Fig. 5: summarizes schematic representation of the preparation of lentiviral supernatants and transduction of SK-MEL93 cells.

Fig. 6. Panel A. Cell cycle analysis of SK-Mel28 cell line after exposure to UVB damage (10mJ/cm2). Data were analyzed with ModFit LT 3.0 software
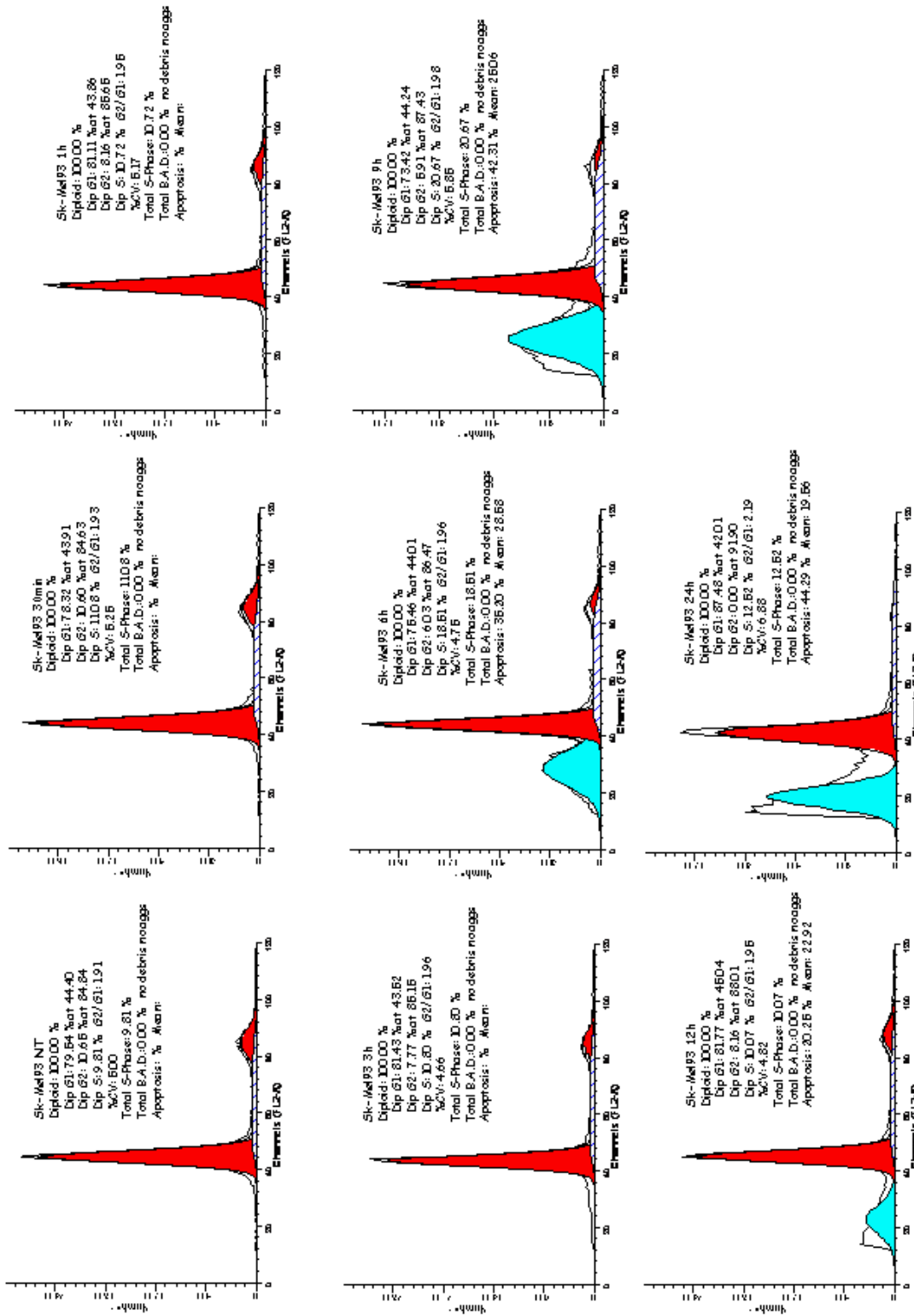
Fig. 6 Panel B Cell cycle analysis of SK-Mel93 cell line after exposure to UVB damage (10mJ/cm2). Data were analyzed with ModFit LT 3.0 software.
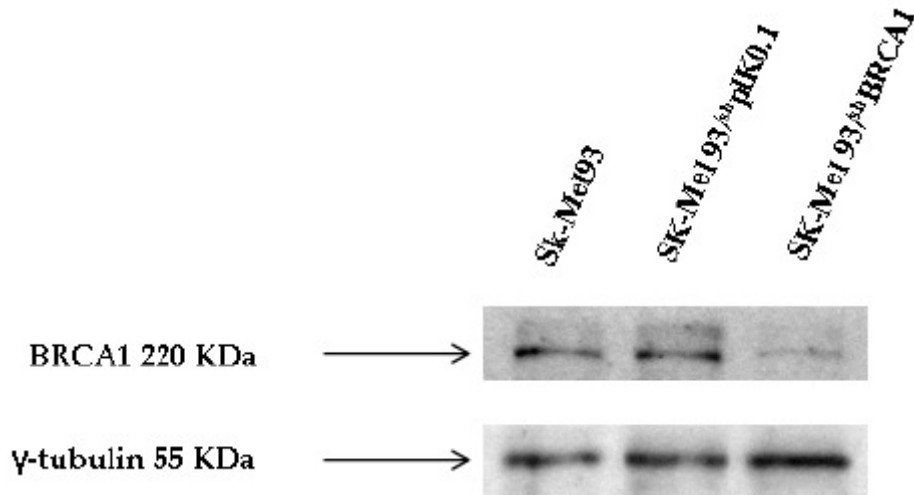
Fig. 7. Western blotting analysis of BRCA1 protein level after infection of SK-Mel93 cells with pLK0.1/shRNA (SK-Mel93/shRNA), or pLK0.1/shBRCA1 (SK-Mel93/shBRCA1). M-
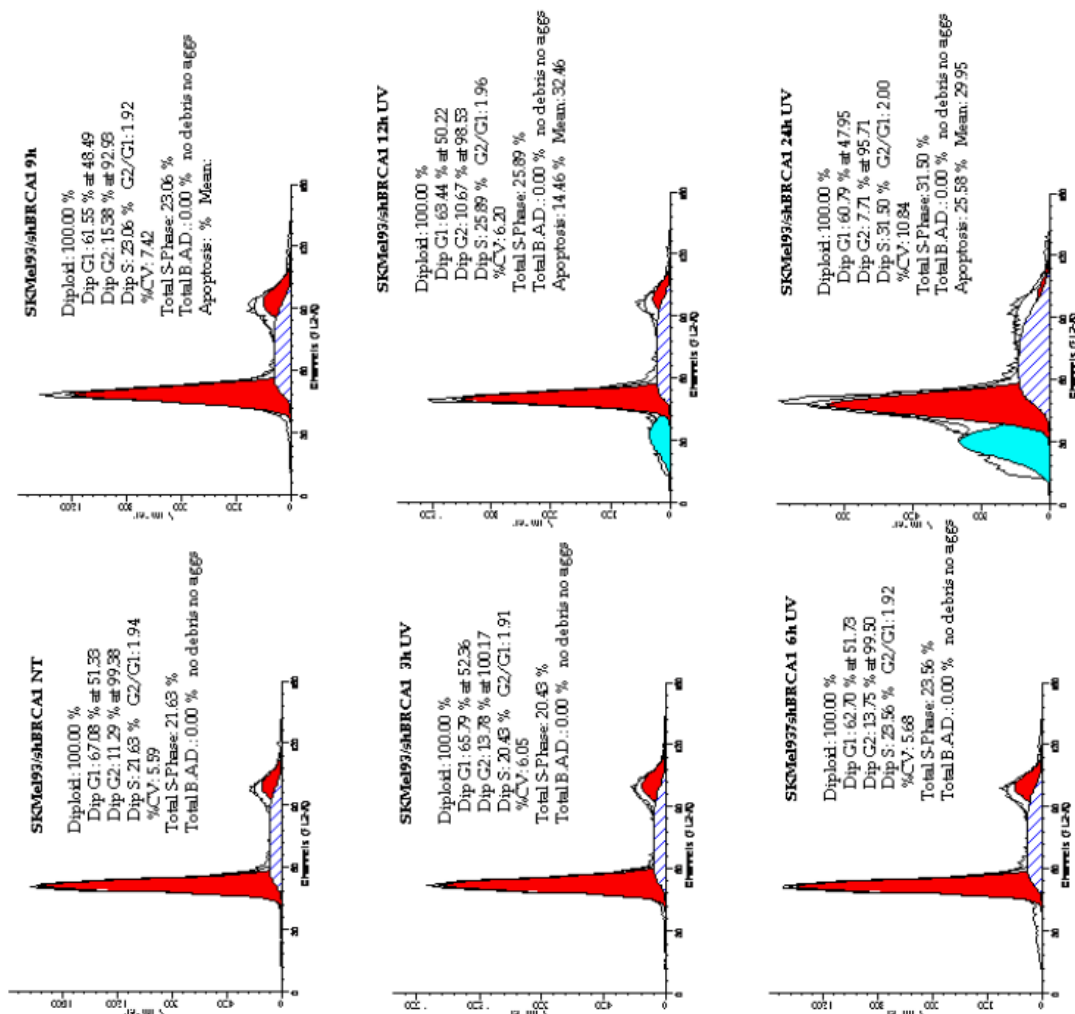tubulin was used as control for loading.

Fig. 8. Panel A. Cell cycle analysis of SK-Mel93/shBRCA1 cell line after exposure to UVB damage (10mJ/cm2). Data were analyzed with ModFit LT 3.0 software.
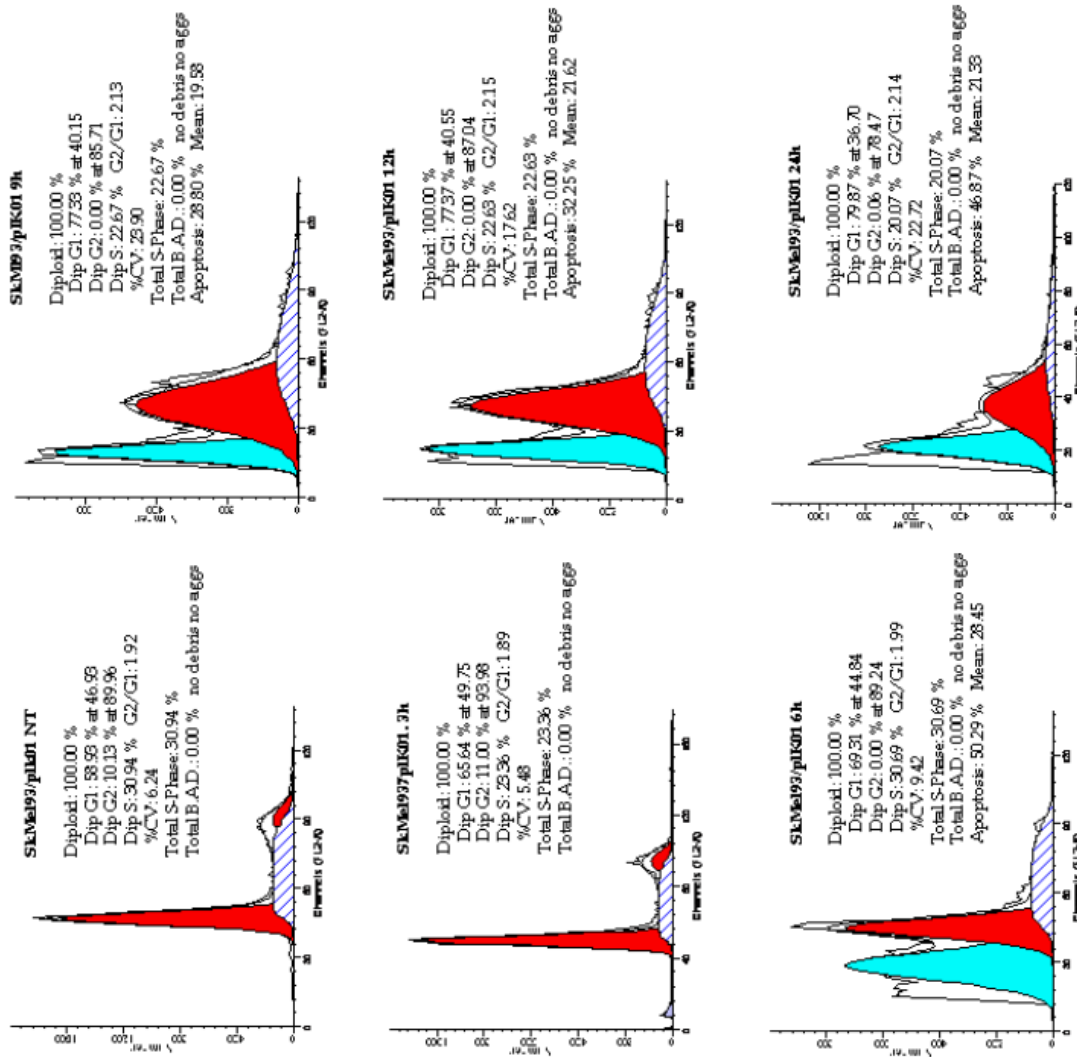
Fig. 8. Panel B. Cell cycle analysis of SK-Mel93/shplK0.1 cell line after exposure to UVB damage (10mJ/cm2). Data were analyzed with ModFit LT 3.0 software.

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تأثيرا لجين المسؤول عن سرطان الثدي بعد التعرض لأشعة فوق البنفسجية(ب) في تعاقب دورة حياة الخلية لخلايا الورم ألقتامي لاانسان قدرت بطريقة الفلوسايتومتري.

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الخلاصة

تهدف الدراسة الحالية الى تقيم تأثير الجين المسؤول عن سرطان الثدي بعد التعرض لأشعة فوق البنفسجية(ب)في تعاقب دورة انقسام الخلية لسلالات خلوية للورم القتامي للإنسان(ميلانو ما) بواسطة استخدام طريقة الفلوسايتومتري.

السلالات الخلوية المختلفة للورم ألقتامي للإنسان (SK-ML28; SK-MEL93;SK-MEL93/shBRCA) زرعت لتكرار التجربة ثلاث مرات. عرضت الخلايا للأشعة فوق البنفسجية(ب)(١٠ملي جول/سم٢ ولازمان مختلفة (٢/١، ١، ٣، ٦، ٩، ١٢، ٢٤ ساعة) واعتبار الخلايا في الزمن صفر كخلايا سيطرة غير معرضة للإشعاع. دورة الحياة لخلايا الورم ألقتامي للإنسان كانت قد قدرت بجهاز الفلوسايتومتري وبعدما تم استخلاص البروتين الكلي لها. السلالة الخلوية (SK-MEL28) ظهر انها تزامنت بالطور الخلوي (G1/S) حتى ١٢ ساعة بعد التعرض للأشعة فوق البنفسجية. قلت الخلايا في الطور (S) وازدادت بالتبادل مع الطور (Sub-G1) في الوقت ١٢و٢٤ساعة من التعرض.بينما السلالة الخلوية (SKL-MEL93)ازدادت بسرعة (حوالي ٣٥%) في الطور (Sub-G1)في الوقت ٦ ساعة من التعرض. كذلك الخلايا المطفرة بحذف ألجين المسؤول عن سرطان الثدي (SK-MEL193/shBRCA1) لوحظ أنها تزداد حوالي ١٥%في الطور(Sub-G1) بعد ١٢ ساعة من التعرض مقارنة بالخلايا السيطرة(SK-MEL93/shPIKO.1). نحن نستنتج إن الدور المهم الذي يلعبه ألجين المسؤول عن سرطان الثدي(BRCA1) يضيف روئية جديدة للآليات الجزيئية التي تسبب توليد الورم ألقتامي للإنسان بعد التعرض للأشعة فوق البنفسجية.

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