THE EXPRESSION OF MISMATCH REPAIR GENES (MLH1 AND MSH2) AND TUMOR SUPPRESSOR GENE (BRCA1 AND P53) IN HUMAN MELANOMA CELL LINES AFTER UVB-IRRADIATION

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ABSTRACT

The aim of our study to investigate the susceptibility of melanoma to UVB-irradiation: by examine the expression of hMLh1,hMSH2,BRCA1 and P53 proteins. In our work, we cultured three human melanoma cell lines (Colo38,SK-MEL28 and SK-MEL93,) in college of medicine university of magna-Gracia-Italy. We first, examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis in Colo38, SK-MEL93 and SK-MEL28 cell lines. After that the 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. After exposure the expression of MLH1,MSH2,BRCA1 and p53 proteins were assessed by western Blot analysis. We found that the expression of BRCA1,hMSH2 and P53 proteins were increased at 3h after UV-B-irradiation in SK-MEL93 cells line, while, the same amount of the expression proteins in the Colo38 and SK-MEL28 cell lines were obtained . Moreover; our data detected an equal amounts of hMLH1protein in three cell lines, were involved in this study..

INTRODUCTION

Mismatch repair genes present several functions relating to genetic stabilization, such as correcting errors in DNA synthesis, ensuring fidelity of genetic recombination or participating in the initial steps of apoptotic responses to different classes of DNA damage [1]. Since the discovery of the major human genes with DNA mismatch repair function, mutations in five of them have been correlated with susceptibility to Lynch syndrome: mutS homolog 2 (MSH2); mutL homolog 1 (MLH1); mutS homolog 6 (MSH6); increased 1(PMS1) [2]. Germline abnormalities in MLH1 and MSH2 genes are found in more than 90% of HNPCC mutation carriers [3], 50% relating to hMLH1, 40% to hMSH2 and 10% distributed among the others [4]. The DNA mismatch repair (MMR) system play a critical role in maintaining genomic integrity in both prokaryotes and eukaryotes [5].However; tumor suppressor genes are normal genes that slow down cell division, repair mistakes, and tell cells when to die. When tumor suppressor genes don't work property, cells can grow out of control, which can lead to cancer. Damage to tumor suppressor genes contributes to a large number of different types of tumors .This is known as the "two-hit" model of carcinogenesis. There are a growing number of genes that have been identified as having some function as function as tumor suppressor gene (es.P53,BRCA1,BRCA2,APC and RB1) [6,7].The p53 tumor suppressor belongs to a small family of related proteins that includes two other members p63 and p73[8]. Although structurally and

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functionally related, p63 and 73 have clear role in normal development, whereas p53 seems to have evolved in higher organisms to prevent tumor development. p53 is activated in response to several malignancy-associated stress signals, resulting in the inhibition of tumor-cell growth [9,10].The BRCA1 tumor suppressor gene encodes a phosphoprotein involved in many cellular key function including DNA repair, transcription regulation, cell-cycle control and apoptosis. Germline mutations in BRCA1 are present in nearly 50% of inherited breast cancer cases, and the acquisition of a single defective allele leads to an elevated predisposition to both breast and ovarian cancer [11,12,13].Since its discovery, many studies have addressed the function of BRCA1 with a view to understanding how it contributes to the maintenance of genome stability and how defects in this process result in cancer progression .

MATERIAL & METHODS

Cell Culture

Human melanoma cells, Colo38 and 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% CO2/95% air atmosphere.

UV-B irradiation

Different human melanoma cell lines (Colo38, SK-MEL93& SK-MEL28) 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), anti-p53 (Bp-53-12), anti-MLH1 (H-300), anti- MSH2 (N-19) and γ-tubulin (Santa Cruz Biotechnology, Heidelberg, Germany).

RNA extraction and semiquantit-ative reverse transcription-PCR.

Total RNA extraction for semiquantitative reverse transcription-PCR (RT-PCR) was done from three different human melanoma cell lines (Colo38,SK-MEL93 and SK-MEL28) at 80% to 90% confluence with TRIzol reagent (Life Technologies) according to the manufacturer's protocol. A total of 5 μg DNase treated RNA was reverse transcribed into first-strand cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexanucleotide primers. cDNA (2 μL) was amplified for BRCA1, hMSH2, hMLH1 and p53 genes with the following primers:

BRCA1:
forward 5'-ggcaacttattgcagtgtg-3' and reverse 5'-tccccatcatgtgagtcatc-3'.

hMSH2:
forward 5'-gccattttggagaaaggaca-3' and reverse5'-ctcacatggcacaaaacacc-3'.

hMLH1:
forward 5'-gctgatgttaggacactacc -3'and reverse 5-aggaattggagcccaggagc -3'.

p53:
forward5'-cggacgatattgaacaatg-3'
andreverse5'-ggaactgttacacatgtag-3'.

A human glyceraldehyde 3-phosphate dehydrogenase cDNA fragment
was amplified as the internal control for the amount of cDNA in the PCR
with the following primers:

GAPDH:
forward 5'-tgatgacatcaagaaggtggtgaag-3' and reverse 5'- tccttggaggccatgtgggccat-3'
Following nested PCR amplification, the products were separated by agarose gel and stained by ethidium bromide.

RESULTS

Effect of UVB-irradiation in three human melanoma cell lines: Colo 38, SK-MEL93 and SK-MEL28.

Recently, by laboratory(*in vitro and in vivo*) investigations have shown that
the DNA repair system modulates in melanoma cells UVB-induced DNA
repair, cell cycle progression and apoptosis (14). To investigate the
susceptibility of melanoma to UVB-irradiation, we used three human melanoma cell lines (Colo38, SK-MEL93 and SK-MEL28).

We first, examined the expression of BRCA1, hMSH2, hMLH1 and p53
proteins by Western Blot analysis in Colo38, SK-MEL93 and SK-MEL28 cell lines. As shown in **Fig. 1**, we found equal amounts of BRCA1 and hMLH1proteins in the three cell lines. Conversely, hMSH2 protein was over-expressed in Colo38 cell line, while p53 protein was over-expressed in Colo38 and SKMEL28 cell lines. Moreover, BRCA1, hMSH2, hMLH1 and p53 over-expression was assessed by RT-PCR carried out on an aliquot of RNA done from Colo38, SK-MEL93 and SK-MEL28 cell lines. As shown in **Fig. 2**, two independent assays confirmed the results obtained by Western Blot analysis. Glyceraldehyde 3-phosphate dehydrogenase cDNA was used as control of quantify and quality of RNA preparation. Next, we exposed Colo38, SK-MEL93 and SK-MEL28 cells to 10mJ/cm2 UV-B-irradiation and examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis at different time (0,30-24 h). As shown in **Fig 3, 4** and 5 we found equal amounts of hMLH1 protein in the three cell lines. Similar results, with equal amounts of BRCA1, hMSH2 and p53 proteins, were observed with the cell lines Colo38 and SK-MEL28 (**Fig. 3 and 4).** Conversely, the expression of BRCA1, hMSH2 and p53 proteins, in the SKMEL93 was found increased at 3h after UV-B-irradiation **(Fig. 5**).

Discusion

Melanoma is a malignant tumor type characterized by a poor prognosis
partly due to ineffective radiotherapy and chemotherapy (15,16), 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 particular, it was suggested that the mismatch repair

system is an initial step of the damage signaling and repair cascade. Additional, increasing evidence indicate an important function of hMSH2 for other pathways that are of importance for UV-induced melanomagenesis, including cell cycle regulation and modulating the apoptotic response of cells following UV-exposure (17). Moreover, the hMSH2 gene has been identified as a possible novel p53 regulated target gene, indicating a direct involvement of p53 in repair mechanisms via DNA binding of a mismatch repair gene. In this context, BRCA1 tumor suppressor gene, known to play a central role in controlling cell progression and apoptosis, seems to be one possible candidate (18). In our work, we exposed Colo38, SK-MEL93 and SK-MEL28 cells to 10mJ/cm2 UV-B irradiation and examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis at different time (0,30-24h). The expression of hMLH1 protein in the three cell lines shows no alteration. Similar results, with equal amounts of BRCA1, hMSH2 and p53 proteins, were observed with the cell lines Colo38 and SK-MEL28. Conversely, the expression of BRCA1, hMSH2 and p53 proteins, in the SK-MEL93 was found increased at 3h after UV-Birradiation.

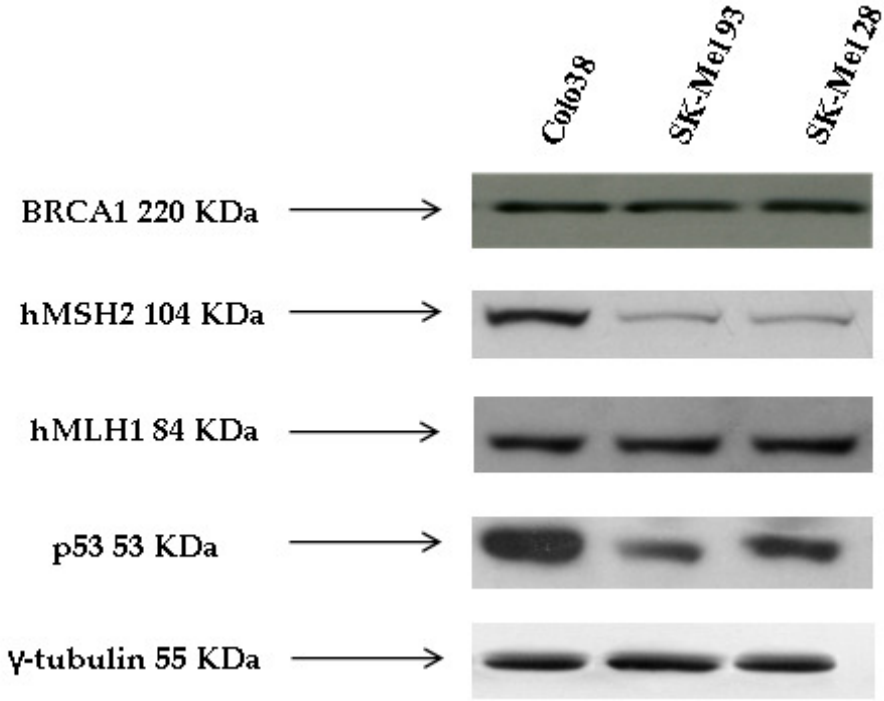

Fig.1. Western blot analysis of BRCA1, hMSH2, hMLH1 and p53 in different melanoma cell lines. γ-tubulin immunoblot analysis was performed to ensure equal levels of protein loading.

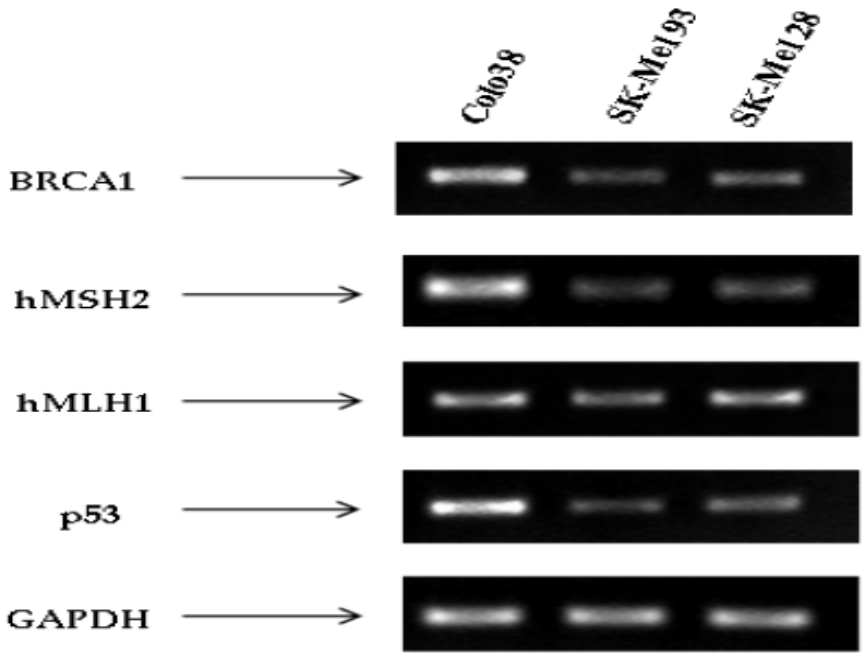

Fig. 2. Reverse transcription-PCR amplification was performed on total RNA from different melanoma cell lines. Amplification of GAPDH cDNA served as a control for quantity and quality of RNA preparations.

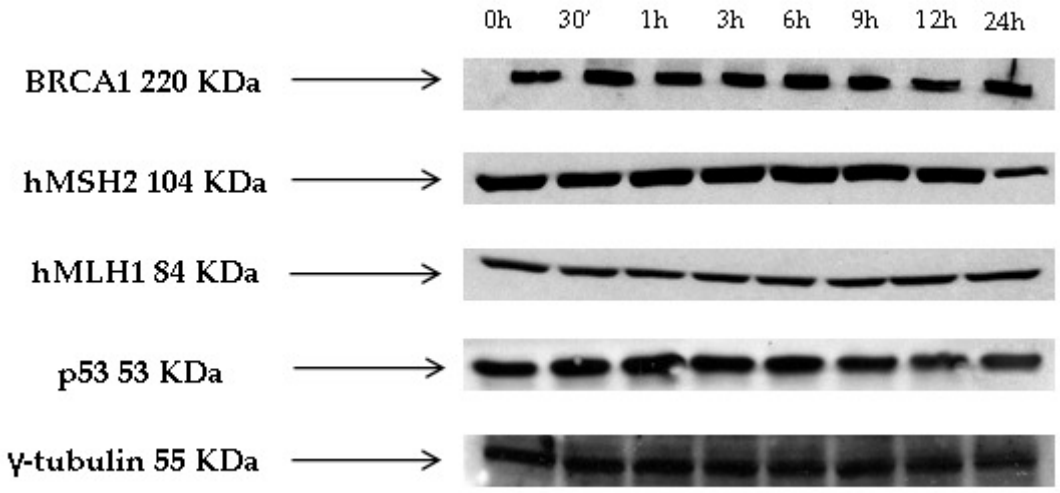

Fig 3. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in Colo38 cells after treatment with UVB 10mJ/cm2, for the indicated time periods. γ-tubulin was used as control for loading.

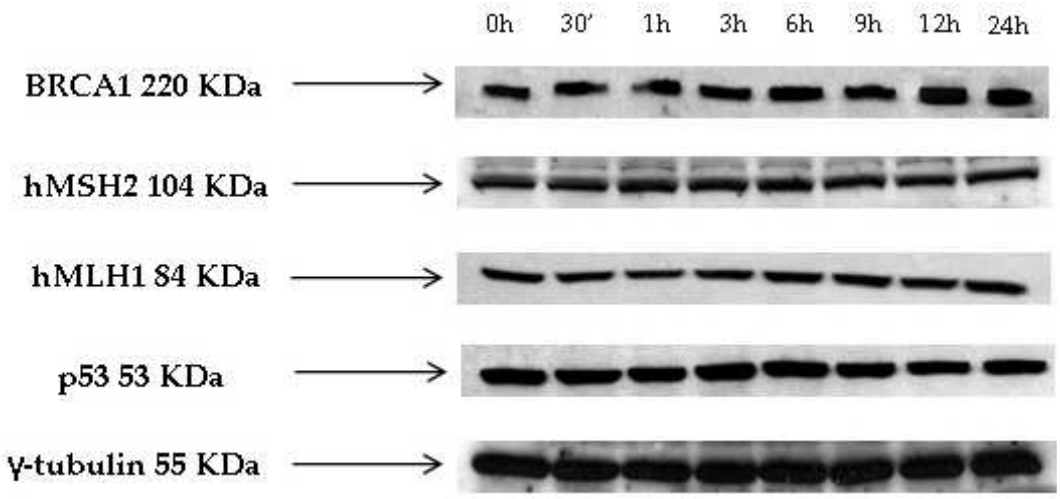

Fig 4. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in SKMel28 cells after treatment with UVB 10mJ/cm2, for the indicated time periods. γ -tubulin was used as control for loading.

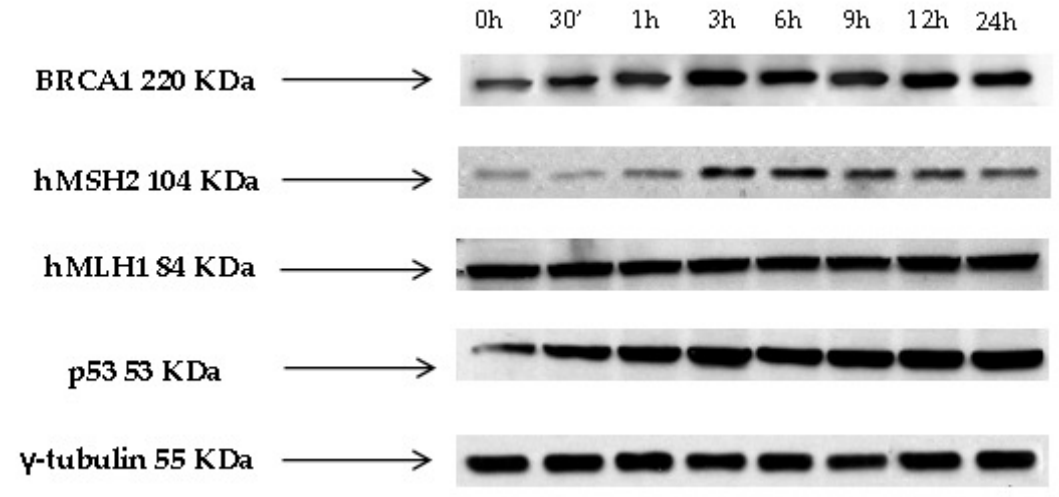

Fig 5. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in SKMel93 cells after treatment with UVB 10mJ/cm2, for the indicated time periods. γ-tubulin was used as control for loading.

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التعبير الجيني للبروتينات المسؤولة عن تصحيح العطب الوراثي والجينات المثبطة للأورام لخلايا الورم ألقتامي للإنسان بعد التعرض للأشعة فوق البنفسجية.

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الخلاصة

تهدف الدراسة الحالية إلى فحص سهولة تأثير الورم ألقتامي (ميلانوما) للإنسان بالأشعة فوف البنفسجية بواسطة حساب التعبير الجيني للبروتينات المسوؤلة عن تصحيح العطب الوراثي(MLH1&MSH2) والجينات المثبطة للسرطان (BRCA1 &P53) لسلالات الورم القتامي للإنسان(ميلانوما) بعد التعرض لأشعة فوق البنفسجية(ب).خلال عملنا قمنا بزرع ثلاث خطوط من خلايا الورم ألقتامي للإنسان (ميلانوما)

(Colo38,SK-ML28; SK-MEL9) في كلية الطب جامعة مانا كريشيا في ايطاليا. في البدأ قمنا بفحص التعبير الجيني للبروتينات المذكورة في خطوط الخلايا السرطانية الثلاث بعد ذلك 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.التعبير الجيني للبروتينات (hMSH2,P53& BRCA1) ازداد بعد ثلاث ساعات من التعرض للأشعة فوق البنفسجية للسلالة الخلوية (SK-MEL93) بينما وجدنا نفس الكمية من التعبير الجيني للسلالات الخلوية(Colo38,SKMEL28).الأكثر من ذلك وجدنا انه التعبير الجيني للبروتين (MLH1) هو نفسه لخطوط الورم ألقتامي (الميلانوما) الثلاث التي استخدمت في هذه الدراسة.

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