A Role for Proteomics in Identifying Targets for Radiosensitizing Strategies in Melanoma: The FKBP51 Paradigm

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Abstract

The treatment of metastatic melanoma is challenging and is in the vast majority of cases unsuccessful. Melanoma cells are resistant to most standard therapeutics. We have recently demonstrated that FKBP51 regulates melanoma response to ionizing radiation (IR). To find out molecular targets for radiosensitizing strategies to apply in this neoplasm, we investigated the changes of protein profiles in irradiated melanoma depleted or not of FKBP51, by protein microarray approach. Among the multiple molecules that were found modulated in our cell model, the decrease of several pPKC isoforms in the FKBP51-depleted (IR-sensitive) melanoma appeared to us particularly interesting, because PKC is involved in radiation response. Therefore, PKC was chosen for further investigation. After validating by western blot proteomics results, we found that targeting PKC, with the pan PKC inhibitor LY317615 or enzastaurin, significantly enhanced IR-induced cell death. Most interestingly, enzastaurin combined with IR appeared to be effective in eliminating a subset of melanoma cells expressing a stemness marker. Our study highlighted a role for proteomics in finding useful targets to overcome melanoma resistance, and suggested a combination treatment, which deserves to be investigated in a clinical setting.

Keywords: Melanoma; PKC; Enzastaurin; Ionizing radiation

Introduction

Melanoma was diagnosed in about 100,000 and killed 22,000 people in Europe in 2012 [1]. Although representing about the 3% of all diagnosed skin cancer, melanoma accounts for the 65% of deaths due to skin cancer [2]. Radiation therapy (RT) is a useful component of the therapeutic armamentarium for malignant melanoma, and may be indicated in about a fourth of patients bearing the disease [3]. In fact, RT can ameliorate the local control of locally advanced surgically resected melanoma and to palliate symptoms in the metastatic patients, sometimes slowing the progression of the disease with possible prolongation of survival [4]. However, the benefits from radiotherapy and cytotoxic drugs are limited because of the inherent resistance of melanoma. The definition of the molecular mechanisms underlying the phenomenon of cellular resistance to cytotoxic agents may be of help in the process of designing more effective treatments for melanoma.

In a previous work, we provided evidence that targeting of FK506 binding protein 51 (FKBP51) with siRNA determined a significant apoptosis sensitizing effect of irradiated melanoma [5]. Our studies have highlighted a relevant role for FKBP51 in melanoma resistance to genotoxic agents [5-7]. To find out molecular targets for radiosensitizing strategies of melanoma, we investigated the changes of protein profiles in irradiated melanoma depleted or not of FKBP51, by protein microarray approach. Among the multiple molecules that were found modulated in our cell model, the decrease of several pPKC isoforms in the FKBP51-depleted (IR-sensitive) melanoma appeared to us particularly interesting. PKC isoforms are serine/threonine kinases that have been involved in multiple self-reinforcing oncogenic loops in melanoma [8]. The phosphorylation usually triggers a change in the state of activity of the target molecule, which leads to further changes in cellular functions. PKCs activate a range of kinases, including phospholipase C, the phosphatidylinositol 3-kinase, mitogen activated protein-kinases cascade and G-proteins coupled receptors [9]. PKC is one of the earliest responders to IR and inhibition of PKC activity increased the cell killing by IR [10-12].

The decrease of pPKC in IR-sensitive melanoma prompted us to investigate whether a PKC inhibiting agent, namely enzastaurin, could overcome IR resistance, in melanoma. Enzastaurin is an ATP-competitive, PKC-β selective small-molecule inhibitor, at low concentrations (nanomolar), but able to impair the activity of multiple PKC isoforms at higher concentrations that are widely reached or surpassed in clinical trials [13]. In a previous work, we showed the efficacy of this drug in sensitizing melanoma to cell death induced by genotoxic stress [14]. Using combination index analyses [14], we found that enzastaurin and doxorubicin exerted synergism or additive effect on melanoma apoptosis. On the basis of this observation, and after confirming by western blot that FKBP51 downmodulation resulted in a reduced phosphorylation of PKC, we employed enzastaurin in association with IR and measured cell death in a melanoma in vitro model. Our finding show that enzastaurin significantly increased melanoma IR-induced cell death. In addition, we provide data in support of a role for enzastaurin, combined with IR, in eliminating a subset of melanoma cells expressing a stemness marker.

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Materials and Methods

Cell culture and reagents

The melanoma cell line SAN [5] was established from a patient’s tumour lymph nodal metastasis and was provided by Dr Gabriella Zupi (Experimental Preclinic Laboratory, Regina Elena Institute for Cancer Research, Roma, Italy). Cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Lonza), 200 μM glutamine (Lonza) and 100 U/ml penicillin–streptomycin (Lonza) at 37°C in 5% CO₂ humidified atmosphere. The melanoma cell line G361, derived from a primary tumour, was purchased from Biosciences (San Jose, CA USA).

The antibody array-arrays/725-antibody-microarray. html#sthash.F1ZTO2Zs.dpuf.

Antibody array

SAN melanoma cells were transfected as previously described [5,15], with a specific small interfering (si) RNA for FKBP51 or a non silencing (NS) RNA as a control. After 24 h from transfection, cells were irradiated with 6 MV X-ray of the linear accelerator at the dose of 4Gy. Six hours later cells were harvested for protein extraction. Lysates were extracted in RIPA modified buffer [16] and each experimental point was labeled with a different cyanine (3 or 5) and used for the XPRESS Panorama Antibody Array Kit (Sigma). This antibody array contains 725 different antibodies each spotted in duplicate on nitrocellulose-coated glass slides useful to detect a wide variety of proteins, which can be categorized in seven subgroups, including apoptotic, cell cycle, nuclear signaling, stress, calcium-associated, cytoskeleton and signal transduction based on biological functions (see more at: http://www.sigmaaldrich.com/life-science/cell-biology/protein-arrays/). Pathway analysis was used to identify differences in protein profile changes between FKBP51 silenced, or not, cells: top ten most significant pathways from GeneGo MetaC analysis following proteins selection with GeneSpring 10.1 software. The identified deregulated proteins by protein microarray were converted into gene symbols and uploaded into MetaCore version 6.7 (GeneGo, MI, USA) for biological network building. The “GeneGo Pathway Maps” algorithms were used to map the uploaded proteins. The relevant pathway maps were then prioritized based on their statistical significance with respect to the uploaded data sets.

Western blot

Whole cell lysates were homogenized in modified RIPA buffer and assayed in Western blot as described [5,15]. Primary antibodies against Phospho-PKC (pan-γ-Thr514, rabbit polyclonal; Cell Signaling, Danvers, USA), FKBP51 (F-13; goat polyclonal; Santa Cruz Biotechnology, CA, USA); PKC-PAN (rabbit polyclonal, Sigma) were used at the dilution of 1:1000. GAPDH (rabbit polyclonal; Cell Signaling, Danvers, USA) was used at the concentration of 1:3000.

Analysis of apoptosis

Analysis of apoptosis was conducted using annexin-V staining [5]. Cells were stimulated with enzastaurin at the doses of 0.6 and 2 μM and subjected or not to IR (4 Gy). After 48 h, cells were harvested, washed in PBS and treated as previously described [5]. Caspase 3 was determined using the Carboxyfluorescein Fluorochrome Inhibitor of Caspases Assay (FLICA) Kits (B-Bridge International, San Jose, USA) according to the instructions of the manufacturer. Cells are permeable to the fluorochrome inhibitor that binds covalently to the activated caspase. Briefly, 6 h after exposure to IR and/or enzastaurin, melanoma cells were harvested and resuspended in 300 μl PBS containing FLICA reagent, for 1 h at 37°C in a 5% CO₂ atmosphere, in the dark. During the incubation, cells were gently resuspended twice to ensure an optimal distribution of the FLICA reagent among all cells. Then, the cells were washed twice in wash buffer by centrifugation at 400 g for 5 min, resuspended in 300–400 μl of PBS, and analyzed by flow cytometry. The green fluorescent signal was a direct measure of the number of cells with active caspases [14].

Immunofluorescence

Melanoma cells were treated with or without enzastaurin at the dose of 2 μM and exposed, or not, to 4 Gy IR. After 72 h, cells were harvested, ABCG2 expression was measured using the mouse monoclonal antibody anti-ABCG2-PE (R&D Systems, Minneapolis, MN, USA) and analyzed by flow cytometry, as described [5,15]. Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA, USA) and from paraffinized tissues using the High Pure RNA Paraffin Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Q-PCR

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA, USA) and 1 μg of each RNA was used for cDNA synthesis with iScript Reverse Transcriptase (Bio-Rad, Hercules, CA, USA). Gene expression was quantified by quantitative (Q) PCR using SsoAdvanced Universal qPCR Supermixe (Bio-Rad) and specific QPCR primers for the relative quantitation of the transcripts, performed using co-amplified ribosomal GAPDH as an internal control for normalization:

\[
\text{hGAPDH-Fw: } 5\'-AGGTCATCCATGACAACTTTGG-3' \\
\text{hGAPDH-Rev: } 5\'-TTGTCATACCAGGAAATGAGCT-3'
\]

\[
\text{hOCT4-Fw: } 5\'-CCTGAAGCAGAAGAGGATCA-3' \\
\text{hOCT4-Rev: } 5\'-CCGCAGCTTACACATGTTCT-3'
\]

\[
\text{hNestin-Fw: } 5\'-AGAAGAGGACCAGAGTATTGTGAG-3' \\
\text{hNestin-Rev: } 5\'-TCCGTCGCTGTTGAGTCTC-3'
\]

\[
\text{hJAGGED1-Fw: } 5\'-ATCCTCGAGAGCACCAGCGAACAGAC-3' \\
\text{hJAGGED1-Rev: } 5\'-ATCGAATTCCCCCGGCTGC-TATACGAT-3'
\]

\[
\text{hHES1-Fw: } 5\'-ATCACCCACACATGCACACCC-3' \\
\text{hHES1-Rev: } 5\'-ACTAGGGGGCGCTGCAAGG-3'
\]

\[
\text{hHEY1-Fw: } 5\'-ATCACCCACACATGCACACCC-3' \\
\text{hHEY1-Rev: } 5\'-ACTAGGGGGCGCTGCAAGG-3'
\]

\[
\text{hJAGGED1-Fw: } 5\'-ATCCTCGAGAGCACCAGCGAACAGCAG-3' \\
\text{hJAGGED1-Rev: } 5\'-ATCGAATTCCCCCGGCTGC-TATACGAT-3'
\]

\[
\text{hNestin-Fw: } 5\'-AGAAGAGGACCAGAGTATTGTGAG-3' \\
\text{hNestin-Rev: } 5\'-TCCGTCGCTGTTGAGTCTC-3'
\]

\[
\text{hHSS1-Fw: } 5\'-AGGTAATCCAGTCACAAAATCGAG-3' \\
\text{hHSS1-Rev: } 5\'-TCCGTCGCTGTTGAGTCTC-3'
\]

Statistical analysis

The statistical significance of differences between means was
estimated using Student’s t-test. Values of $P \leq 0.05$ were considered statistically significant.

**Results**

**FKBP51 knockdown decreases levels of active PKC**

Melanoma cell depleted of FKBP51 is highly sensitive to IR [5]. We investigated changes in protein profiles in irradiated melanoma depleted or not of FKBP51, by protein microarray approach. Proteins differentially expressed in the FKBP51 silenced melanoma cells were primarily involved in the regulation of apoptosis ($p=1.16\times10^{-11}$), cytoskeleton remodeling ($p=4.11\times10^{-39}$) and EGFR signaling ($p=1.5\times10^{-39}$). A relevant map is shown in the Figure 1. Proteins on the map are represented by different symbols corresponding to the functional class of the protein reported in the legend. Red and blue thermometers show increased and decreased expression, respectively, in FKBP51 depleted cells. The top ten most significant pathways, which were ranked based upon $p$-value, are also shown in Figure 1. The map highlights that several pPKC isoforms are downregulated in FKBP51 depleted melanoma. To confirm the effect of FKBP51 in regulation of PKC activation, we measured by western blot the levels of phosphoPKC, using a pan PKC antibody. The results are reported in the legend. Red and blue thermometers show increased and decreased expression, respectively, in FKBP51 depleted cells.

**Enzastaurin increases sensitivity of melanoma to IR-induced cell death**

Because PKC activity was found decreased in FKBP51-silenced melanoma, and because FKBP51-silenced melanoma is radiosensitive [5], we investigated whether enzastaurin, a PKC inhibitor, could increase IR cytotoxicity in melanoma. For this reason, we analyzed the effect of enzastaurin on IR-induced cell death by annexin-V staining. We found that neither IR, nor enzastaurin, as single agents, significantly enhanced melanoma cell death (Figure 3A). Differently, annexin-V positive cells were significantly increased by the combination of enzastaurin and IR, in comparison to untreated cells or cells subjected to IR alone. This suggested a sensitizing action of enzastaurin on IR cytotoxicity. Measure of caspase 3 activation, by flow cytometry, showed an increase of the active caspase in enzastaurin + IR sample, suggesting that cells died, at least in part, by apoptosis (Figure 3B).

We then investigated whether an additive effect could be measured using FKBP51 knock down and enzastaurin. Interestingly, we found that enzastaurin was able to kill melanoma cells in the absence of IR, in condition of FKBP51 depletion (Figure 4). This suggested that FKBP51-silencing inhibits further survival pathways in addition to that PKC-driven, which is consistent with previous studies [5-7]. According to these findings, enzastaurin, per se, can activate the melanoma apoptotic machinery, following FKBP51 knockdown.
Since radiocurability of a tumour implicates that the cancer stem/initiating cell is killed [17], we investigated the effect of the association IR and enzastaurin on the viability of melanoma cancer stem/initiating cell. For this experiment, we thought to use A375 melanoma cell line, that in previous studies was shown to be more aggressive than SAN [14,18]. Melanoma cells, irradiated or not, and cultivated in the absence or the presence of enzastaurin, were harvested after 72 h and stained with ABCG2 monoclonal antibody. It is noted that ABCG2 belongs to the family of ATP-binding cassette transporters, which was demonstrated to be a marker of cells capable of recapitulating melanomas [19]. The number of ABCG2+ cells was calculated by flow cytometry. A gate was placed on live cells based on FSC/SSC physical parameters, and ABCG2 count was expressed as the number of positive cells/10⁴ total cells. A result representative of four different experiments is shown in the Figure 5. Interestingly, we found that ABCG2+ cells were even increased in irradiated cultures, suggesting that IR killed ABCG2- cells, thus producing an enrichment of ABCG2+ in the remaining cells; or, as recently hypothesized in an in vivo model of glioblastoma [20], that a transition stimulus towards a stem like phenotype is induced by IR. Interestingly, the ABCG2+ subset was significantly reduced after combination treatment of enzastaurin with IR, suggesting such an association was suitable for eliminating cancer stem cells. To address this issue, we irradiated SAN and A375 melanoma cells with a 4 Gy dose and, after a 16 h and a 36 h incubation, cell was harvested, and RNA extracted. The levels of the transcript of several genes involved in cancer stemness, namely ABCG2 [15], HES1 [21], HEY [21], Jagged [22], Nestin [15], and OCT 4 [22] was measured by QPCR. Results of two different experiments are represented in Figure 6. In accordance with results of Leder et al [20], we found an increased expression of some stemness-related genes after IR, particularly, ABCG2 increased expression was consistent with the enhanced ABCG2+ population in irradiated melanoma.
shows that among 37 melanoma cell lines there is a very wide range of sensitivity. On average, melanoma cell lines are not less sensitive than other cell lines of cancers (i.e. breast cancer and lung cancer) for which RT plays a major role in the cure of disease. Furthermore, the same study indicated that, although B-Raf inhibitors potentiate the effect of RT on B-Raf mutated melanoma cells, B-Raf and N-Ras mutational status are not associated with the response to ionizing radiation [23]. Thus the definition of the molecular mechanisms involved in radioresistance may suggest new druggable targets for combination therapy aimed at ameliorating the outcome of this disease. In this frame, our proteomic study discloses the molecular alterations able to modify the response to ionizing radiation.

Figure 4: FKBP51 knock down promotes enzastaurin citotoxicity of melanoma. A: Graphic representation of cell death values (columns are means and bars standard deviations) of SAN melanoma cells. Cells were transfected with a specific siRNA for FKBP51 and a NS RNA as control, irradiated with a 4 Gy dose and incubated in the absence or the presence of 0.6 μM enzastaurin for 48 h. Cell death was measured by annexin-V staining. Data were obtained from three independent experiments, each performed in triplicate.

Figure 5: Enzastaurin reduced number of ABCG2+ cells in irradiated cultures. Graphic representation of ABCG2+ cell counts in A375 melanoma cultures, irradiated or not with a 4 Gy dose and incubated in the absence or the presence of 2 μM enzastaurin. After 72 h, cells were harvested, stained with anti-ABCG2-PE and acquired with a flow cytometer. A gate was placed on live cells on the basis of the physical parameters FSC/SSC; the number of ABCG2+ cells was calculated and expressed as number of positive cells/10^4 total cells. Data were obtained from four different experiments, each performed in triplicate.

Discussion

Therapy of advanced stage melanoma constitutes a difficult task for the oncologists. Although recent advances in bio- and immunotherapy have substantially improved the outcome of a significant portion of such patient population, most of the patients experience a rapid evolution of the disease and a short survival time.

Radiation therapy is one of the tools usefully employed in the treatment of melanoma patients, despite melanoma is considered one of the less radiation sensitive neoplasms. The limitations of RT, as well as of the conventional cytotoxic drugs, in melanoma, are due to an inherent resistance to genotoxic agents of a portion of the cellular population. An interesting report by Sambade et al. [23], indeed,
in radioresistance of melanoma, we focussed our attention on the molecular changes caused by the silencing of this gene in our melanoma model. Array based proteomics are routinely employed for differential proteomics studies and quantification and validation of regulated proteins, biomarkers and targets. By this approach we obtained results that are in line with previous studies, which underlines the reliability of this technology. A signal transduction map constructed with data of protein profiles, supported previous findings that FKBP51 regulated pathways related to apoptosis [5-7,24], TGF-β signaling [15,18], immune response [25], NF-κB activation [5-7,26-28], neuronal death and survival [29-31] and epithelial to mesenchymal transition [15,18]. The finding of reduced levels of several pPKC isoforms in FKBP51-depleted IR sensitive melanoma is consistent with the notion that PKC is one of the earliest responders to IR. PKC has been shown to be involved in radiation resistance in different cellular systems [32,33], and its inhibition increases the cell killing by IR [10-12]. Enzastaurin,

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**Figure 6:** Effect of ionizing radiation on expression of stemness genes in melanoma. Graphic representation of relative normalized expression rates (means and standard deviations) of IR-regulated stemness genes. A375 (upper panel) and SAN (lower panel) melanoma cells, were irradiated or not with a 4 Gy dose, and harvested after 16 h and 36 h. Unirradiated sample was arbitrarily used as reference sample (expression=1). GAPDH was used as internal control of amplification.
or LY317615, is a synthetic bisindolylmaleimide drug developed by Eli Lilly. It has been shown to be effective especially in combination with genotoxic drugs, including melphalan and doxorubicin [33,34]. Previous studies support the efficacy of this agent in malignant melanoma [14,35]. Consistent with previous findings [5,36,37], ionizing radiation produces only a slight effect on melanoma cell death. Treatment of melanoma with enzastaurin produced a significant increase in IR-induced cytotoxicity, which was accompanied by caspase 3 activation, suggesting the involvement of apoptosis machinery in the increased response to IR. Most interestingly, the cooperative effect between enzastaurin and IR appeared to be effective in reducing the counts of melanoma cells expressing the stem cell marker ABCG2. For radiotherapy treated cancers, as well as glioma and melanoma, it has been hypothesized that the tumour consists of two separate populations of cells: the largely radioresistant stem-like cells and the radiosensitive differentiated cells. This model hypothesizes that, after exposure to radiation, a fraction of the radiosensitive cells could rapidly revert to the radioresistant state, becoming capable of repopulation during the treatment period and recovery from radiation damage [20]. In this context, the finding that enzastaurin is able to reduce the number of ABCG2+ melanoma cells, with cancer stem cell features [15] could be of particular interest for the therapy of this neoplasm. In conclusion, our study supports the hypothesis that enzastaurin is a promising melanoma radiosensitizing agent that deserves to be investigated in a clinical setting.

References
