

TOP1 modulation during melanoma progression and in adaptative resistance to BRAF and MEK inhibitors

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ABSTRACT

In melanomas, therapy resistance can arise due to a combination of genetic, epigenetic and phenotypic mechanisms. Due to its crucial role in DNA supercoil relaxation, TOP1 is often considered an essential chemotherapeutic target in cancer. However, how TOP1 expression and activity might differ in therapy sensitive versus resistant cell types is unknown. Here we show that TOP1 expression is increased in metastatic melanoma and correlates with an invasive gene expression signature. More specifically, TOP1 expression is highest in cells with the lowest expression of MITF, a key regulator of melanoma biology. Notably, TOP1 and DNA Single-Strand Break Repair genes are downregulated in BRAFi- and BRAFi/MEKi-resistant cells and TOP1 inhibition decreases invasion markers only in BRAFi/MEKi-resistant cells. Thus, we show three different phenotypes related to TOP1 levels: i) non-malignant cells with low TOP1 levels; ii) metastatic cells with high TOP1 levels and high invasiveness; and iii) BRAFi- and BRAFi/MEKi-resistant cells with low TOP1 levels and high invasiveness. Together, these results highlight the potential role of TOP1 in melanoma progression and resistance.

Keywords: melanoma, TOP1, resistance, Topotecan, MITF

1. INTRODUCTION

BRAF mutations occur in more than 50% of cutaneous melanomas (1-3), the most aggressive and lethal skin cancers. Although the development of BRAF inhibitors (BRAFi) revolutionized the treatment of metastatic melanoma, most patients relapse after 5–6 months (4-6). BRAFi therapy has since been replaced by the BRAF-MEK inhibitor combination, leading to improved response durations, but almost inevitably, ultimately, treatment fails (4, 7-10). Understanding the mechanism underlying resistance to BRAF inhibition is, therefore, a key issue.

Resistance to MAPK pathway inhibition occurs primarily because of a combination of genetic and non-genetic intra-tumor heterogeneity. Genetic alterations in melanoma cells play a crucial role in acquired resistance to BRAFi via reactivation of the MAPK pathway and, to a lesser extent, the PI3K–AKT pathway (11, 12). However, in many cases, the emergence of drug-resistant clones cannot be explained by genetic mechanisms (13). Phenotypic heterogeneity, arising as from stresses within the tumor microenvironment, has been observed both among genetically diverse tumors and within genetically homogeneous populations of cells and is associated with variability in tumor cell sensitivity to BRAF and MEK inhibitors (14-18). Non-genetic therapy resistance is underpinned by cell-intrinsic plasticity in the epigenetic landscape. Upon drug treatment, distinct subpopulations of drug-tolerant cells emerge with dynamic and differential gene expression profiles (17, 19).

MITF (Microphthalmia-associated transcription factor) (Goding and Arnheiter 2019) is a lineage survival oncogene (20, 21) that cooperates with BRAF in melanoma initiation (Lister et al. 2014). Furthermore, MITF expression has been associated with proliferative or differentiated melanoma subtypes, including BRAF inhibitor resistance, whereas low MITF expression is linked to an invasive phenotype and drug resistance markers (15, 16, 22).

There is, therefore, an intense effort to better understand mechanisms of resistance to BRAFi and MEKi, and develop new agents that target the resistance (23-32).

Topoisomerase1 (TOP1) is a ubiquitous enzyme essential for the relaxation of DNA supercoiling during replication, transcription and chromosomal recombination (33, 34) and has been considered a target for the treatment of human tumors. Although its functional role in melanomas remains unclear, the TOP1 gene is amplified in 54.5% of melanoma cell lines and 40% of primary melanomas (35). Moreover, many metastatic cases have higher amplification levels than primary melanomas, suggesting a possible role for TOP1 in advanced stages of the disease (36). TOP1 forms a stable protein–DNA cleavage complex (TOP1cc) through its enzymatic activity and becomes covalently bound to the catalytically generated DNA strand break. Normal cells use DDR pathways to maintain genomic stability (37) with both single-strand break (SSB) and Double-strand break (DSB) repair mechanisms repairing TOP1-induced DNA lesions. Consequently, TOP1 inhibitors are used to treat highly malignant and fast-proliferating tumor cells that depend on TOP1 function for survival. For example, the FDA-approved CPT derivatives topotecan and irinotecan are currently used to treat ovarian and colorectal cancers, respectively (38-40).

Here, we show that TOP1 expression is increased during melanoma progression and decreased in BRAFi- BRAFi/MEKi-resistant cells. TOP1 positively correlates with invasion in non-tumor cells, and in double-resistant cells, the decrease in TOP1 levels by pharmacological inhibition also impairs invasion markers.

2. MATERIAL AND METHODS

2.1 Cell culture and treatments

BRAF-mutated melanoma cell lines SKMEL28, SKMEL29, UACC62, WM164, 501 mel, and A375 were grown in DMEM or RPMI supplemented with 10% FBS 50 U/ml penicillin, and 50 µg/ml streptomycin. BRAFi- and BRAFi/MEKi- resistant melanoma cell lines were generated and validated as described before (28, 30, 32). The range of inhibitor concentration used was 3– 6µM for vemurafenib (BRAFi) and 50 nM for trametinib (MEKi). For MITF-expressing cell lines experiments, IGR37, SKMEL30, and HBL were used as high-MITF; 501 mel, SKMEL28, A375M, WM266, WM164 and WM115 were medium-MITF, and lastly, WM9, CHL1, WM793, and IGR39 were classified as low-MITF.

To analyze the presence of mutations in fifty cancer-related genes, the STR profiling was done in sensitive and resistant melanoma cell lines using the Ion Ampli Seq™ Cancer Hotspot Panel v2 (Life Technologies™)(30). All cell lines were checked for mycoplasma contaminations by PCR. All cells were maintained at 37 °C under 5% CO₂ atmosphere. Melanocytes were obtained from human foreskin samples donated to the University of São Paulo Hospital (HU-USP:943/09; CEP/FCF/USP:534). Cells were isolated and cultivated as described previously by our group (41, 42).

Lipofectamine RNAiMAX (Invitrogen, Carlsbad) was used to transfect the cells with TOP1 siRNA sequences (s14304 and s14306 – Thermo Fisher Scientific). Trypan blue exclusion was used to monitor the inhibition concentration of the cell lines upon exposure to Topotecan (TOP1 Inhibitor T2705 Sigma) for 24h. For acute treatments, we used Topotecan at 0.5µM for 48h and 20µM for 2h.

2.2 Hematoxylin and eosin and immunohistochemistry staining

Samples from melanoma patients were obtained from the Souza Pathology Laboratory tissue bank (Maringá/Paraná, Brazil) after patients signed the informed consent form. They were analyzed, and an independent pathologist confirmed the diagnosis. Samples were fixed, followed by dehydration, clarified by xylol, then paraffin included, sectioned, and stained with H&E for morphological analysis. Immunohistochemistry assay was performed using the Dako Envision Flex system and TOP1 antibody (Abcam ab109374). All images were obtained by optical microscopy (100x magnification) and analyzed by the NIS Elements software (Nikon Instruments, Melville, NY, USA). Semi-quantification was assessed by a scoring system by the pathologist Aloisio Souza Felipe da Silva.

2.3 Protein Expression in the Human Protein Atlas Database

The protein expression level of TOP1 (HPA019039 antibody, Sigma) was analyzed in tissue samples available at Human Protein Atlas (<https://www.proteinatlas.org/>). Two patients were selected as an example of low and high staining in the melanoma cohort. Low staining/weak intensity: Patient ID 2112, Male, age 41, soft tissue malignant melanoma, metastatic site; High staining/strong intensity: Patient ID 2534, Male, age 53, skin, malignant melanoma, NOS (Not Otherwise Specified).

2.4 Quantitative real-time PCR for relative RNA levels

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The reverse transcription reactions were performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, New Jersey, USA). Real-time PCR analyses were performed using the ABI Prism®7500 Sequence Detection System (Applied Biosystems, New Jersey, USA). TaqMan® Probe-Based Gene Expression (Life Technologies) kits were used to perform the assays. The

target genes evaluated were: *APTX* (Hs00544364_m1), *AXL* (Hs01064444_m1), *LIG3* (Hs00242692_m1), *MITF* (Hs00243257_m1), *PARP1* (Hs00242302_m1), *PARP2* (Hs00193931_m1), *SOX10* (Hs00366918_m1), *TDP1* (Hs00217832_m1), *TOP1* (Hs00243257_m1), *XRCC1* (Hs00959834_m1). Data were normalized to beta-actin (Hs01060665_g1) levels in triplicates. Relative expression was calculated using the delta Ct method (43).

2.5 Western Blotting for protein levels

Whole-cell extracts were prepared by the direct addition of 1× Laemmli sample buffer (62.5mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.02% bromophenol blue, 5% 2-mercaptoethanol) to the cells in the culture plate. Cells were scraped with a cell scraper (TPP, Trasadingen, Switzerland), and lysates were collected then incubated at 95 °C for 5 min. Cell extracts were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using 12% polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane (Whatman, Kent, UK) by electroblotting at 150 mA for 90 min on ice in a transfer buffer comprising 25mM Tris, 192mM glycine, and 20% isopropanol. Ponceau staining was used to assess the efficacy of the transfer and to facilitate cutting the membrane when probing the same membrane with multiple antibodies. Primary antibodies (anti-TOP1 ab109374 Abcam; anti-MITF HPA003259 Cambridge Bioscience Ltd; anti-vinculin V9131 Sigma; anti-actin A4700 Sigma) were incubated with membranes overnight at 4 °C with agitation. Protein bands were detected by an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The bands' intensity was semi-quantified using the software ImageJ.

2.6 RPPA and analysis methods

Reverse Phase Protein Arrays (RPPA) were conducted as previously described (44, 45). We used parental and BRAFi/MEKi-resistant SKMEL-28 cells both untreated and treated with IC₅₀ values of TOP1i for 24h. Volcanos plots were calculated using log 2 (fold-change) plotted against -log (p-value). The biological process enrichments from the protein-protein interactions between the comparison groups were constructed by Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>)(46).

2.7 Multiplex panel for tumor microenvironment markers

Media from parental, BRAFi-resistant and BRAFi/MEKi-resistant SKMEL28 cells untreated and treated with IC₅₀ values of TOP1i for 24h were collected and prepared for analysis in a 96-well plate utilizing a custom-made Milliplex Assay for IL-1a (interleukin 1 alpha), IL-6 (interleukin 6), IL-8 (interleukin 8), MMP-1 (metalloproteinase 1), MMP-2 (metalloproteinase 2) and MMP-9 (metalloproteinase 9) (Millipore Corp., Billerica, MA) and following the kit-specific protocols provided by Millipore. Analytes were quantified using a Magpix analytical test instrument, which utilizes xMAP technology, multiple analyte profiling (Luminex Corp., Austin, TX), and xPONENT 4.2 software (Luminex). Triplicated samples were measured in duplicate, and the concentration was calculated by reference to the standard curve for each cytokine.

2.8 Survival Analysis

The OncoLnc online analysis tool was used to analyze the prognosis of melanoma cancer according to the expression of TOP1. The analysis tool can perform Kaplan–Meier prognosis analyses based on gene expression and the prognostic correlation in the TCGA database. For cutaneous skin melanoma, the database contains mRNA data from 459 patients, being 284 men and 175 women, with an average age of 58 years old (47).

2.9 Bioinformatics analysis

The Skin Cutaneous Melanoma TCGA PanCancer data was accessed by the cBioPortal (<https://www.cbioportal.org/>) to generate the data related to alterations in TOP1 in these patients. A sample window size of $n=20$ was used to generate the moving average expression of averaged signatures or individual genes of interest, and trendlines were added to the bar plots. An R-script for calculating and generating moving average plots of TCGA cancer cohorts implementing TCGA access via cBioportal was provided previously (48). The significance of the Spearman rank correlation was determined by an asymptotic Spearman correlation test using the original log2 expression values and not the moving average values.

2.10 Statistical analysis

All data were expressed as mean \pm standard deviation (SD). Statistical analyses and graphical representations were generated using GraphPad Version 8.2.1 (GraphPad Software, La Jolla, CA, USA). One-way and Two-way analysis of variance (ANOVA) was used for comparison, and significant differences in the means were determined using Sidak's multiple comparisons test at a significance level of $p < 0.05$.

3. RESULTS

3.1 TOP1 is overexpressed in metastatic melanoma and correlates with an invasiveness signature

To determine the role of TOP1 in the context of melanoma progression in clinical samples, we first analyzed *TOP1* gene expression in melanoma samples using data from The Cancer Genome Atlas (TCGA) (**Figure 1A**). We observed that TOP1 expression is increased in 26% of the patients within this dataset.

To support this finding, we next examined TOP1 expression in patient tissue. For this, seven melanoma samples (3 nevi, 2 primaries tumor, and 2 metastases) were investigated by TOP1 immunohistochemistry and analyzed by an independent pathologist using Hematoxylin/Eosin stain and morphological analysis. The results suggest that TOP1 expression increases during melanoma progression, with higher staining rates for TOP1 in metastatic samples (**Figure 1B**). Semi-quantification shows a significant difference between nevi and metastatic melanomas, but not between primary and metastatic (**Figure 1C**).

To determine if there is a correlation between DNA/RNA levels observed in cBioPortal for Cancer Genomics and its protein level, we analyzed the protein expression of TOP1 in The Human Protein Atlas data. The Human Protein Atlas contains clinical tissue from a variety of tumors and proteomics data for various proteins (immunohistochemistry on tissue microarrays) (49). The results obtained from immunohistochemistry of melanoma tissues showed that TOP1 staining is very heterogeneous within samples (**Figure 1D**), but also at a single-cell level where strongly and weakly stained cells can be observed in the same tumours sample.

Melanoma patient survival data was then analyzed using OncoLnc (<http://www.oncolnc.org/>) with a lower and upper percentile of 25% as a cutoff. The analysis revealed that elevated TOP1 levels were associated with a better overall survival when compared to decreased levels in melanoma patients (**Figure 1E**).

Melanoma cells can adopt different phenotypic states that broadly represent proliferation versus invasion, each characterized by a distinct gene expression profile. We therefore asked whether TOP1 expression correlated with the Verfaillie et al. (50) invasive (**Figure 1F**) or proliferative (**Figure 1G**) gene expression signatures using the TCGA melanoma cohort RNA-seq data. The results revealed that TOP1 strongly correlates with

the invasive signature and negatively correlates with the proliferative one, supporting the hypothesis that only a subset of melanoma cells expresses high levels of TOP1.

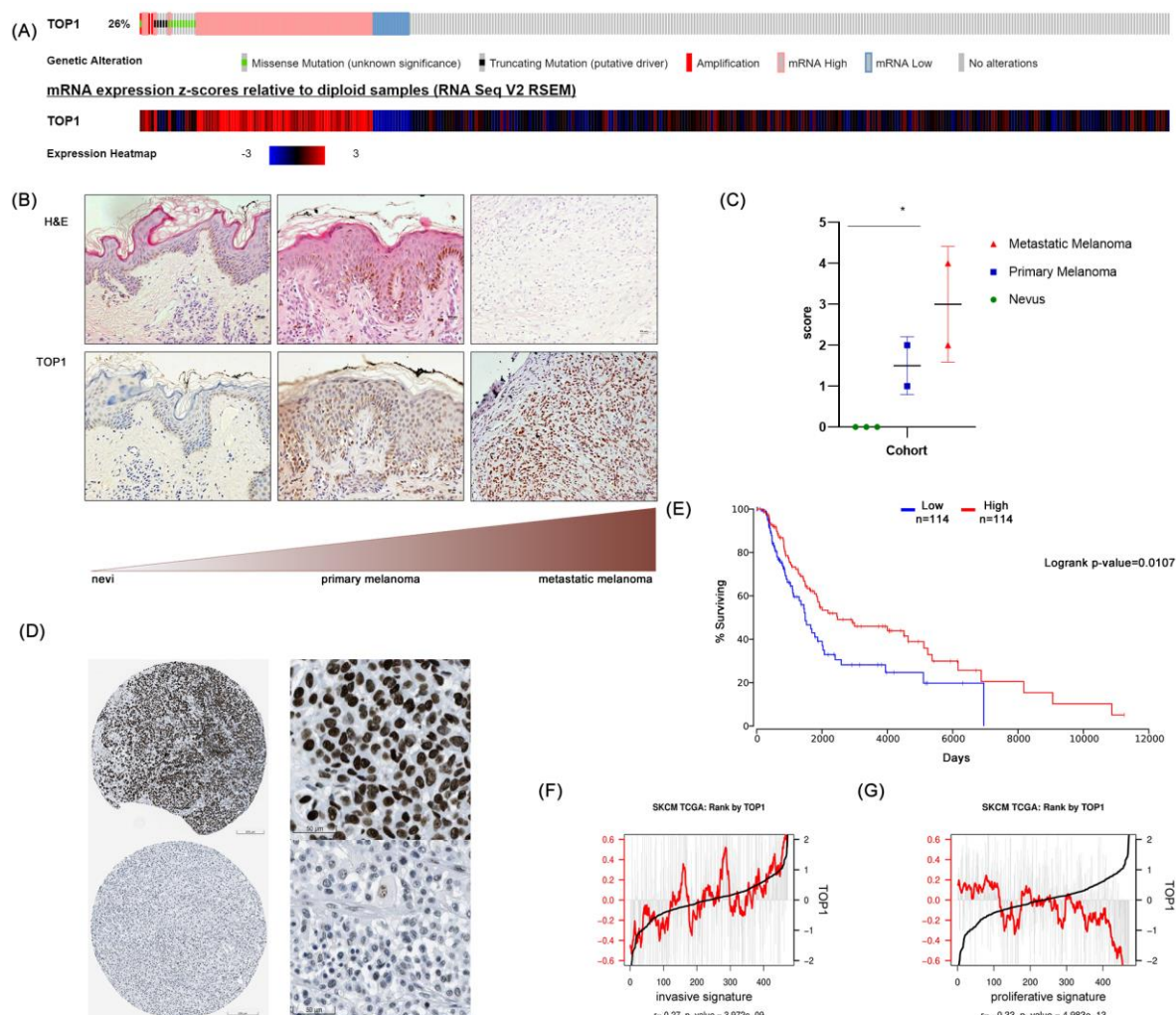


Figure 1 – TOP1 expression in melanoma patient samples. (A) Genetic Alterations and mRNA expression from The Skin Cutaneous Melanoma TCGA PanCancer data was accessed by the cBioPortal; **(B)** Hematoxylin and eosin staining, and TOP1 immunostaining in melanoma patient samples (x10 magnification; bar 100μM); **(C)** Semi-quantification of the immunohistochemistry for TOP1; **(D)** TOP1 immunostaining in melanoma patient samples available at Protein Atlas; **(E)** Melanoma patient survival data using OncoLnc with a lower and upper percentile of 25% as a cutoff. Logrank p-value=0.0107. **(F)** Positive correlation between TOP1 and a previously described invasive signature (50). The TCGA melanoma cohort were ranked by TOP1 expression (black line) and red lines the moving average of the invasiveness signature; **(G)** Negative correlation as in (F) between TOP1 and a previously described proliferative signature.

3.2 MITF and TOP1 interaction

Given the importance of MITF in controlling the switching of melanoma between the proliferative and invasive phenotypes and its links to therapy response, we next asked if TOP1 correlates with MITF using the TCGA melanoma cohort (**Figure 2A**). At low levels of MITF, a strong negative correlation between TOP1 and MITF is observed, but as MITF expression starts to increase, a positive correlation with TOP1 can be seen. This observation suggests that MITF itself is unlikely to be the main driver regulating TOP1 expression in melanomas.

Since the TCGA melanoma samples include non-melanoma cells such as infiltrating immune cells and fibroblasts that can contribute to the gene expression profile obtained, we next examined a panel of 12 melanoma cell lines classified by low, medium, and high MITF mRNA levels. The results revealed an overall anti-correlation between MITF and TOP1 expression (**Figure 2B**). Most of the high-MITF (4 out of 5) melanomas present lower expression of TOP1, whereas the low MITF samples tended to express higher levels of TOP1. This result agrees with the low MITF melanomas in the TCGA cohort, also expressing high levels of TOP1.

Next, we transfected SKMEL28 cells with two different siRNA sequences targeting TOP1 and checked the mRNA and the protein expression of TOP1 and MITF in silenced cells and after treatment with Topotecan (TPT). Interestingly, the TOP1 transient inhibition significantly increases the levels of MITF in the same cells, but not after the pharmacological inhibition both in gene and protein expressions. (**Figure 2C and 2D; Supplementary Figure 1 and 2**).

To understand if the level of MITF would interfere with Topotecan mechanism of action, we investigated Single-Strand Break repair genes (*APTX*, *LIG3*, *PARP1*, *PARP2*, *XRCC1* and *TDP1*) after TOP1 inhibition in a panel of MITF high/medium/low cells. We show that

Topotecan treatment decreases the expression of SSB genes in MITF low and medium cells, although in high-MITF cells, there is no change (Figure 2E).

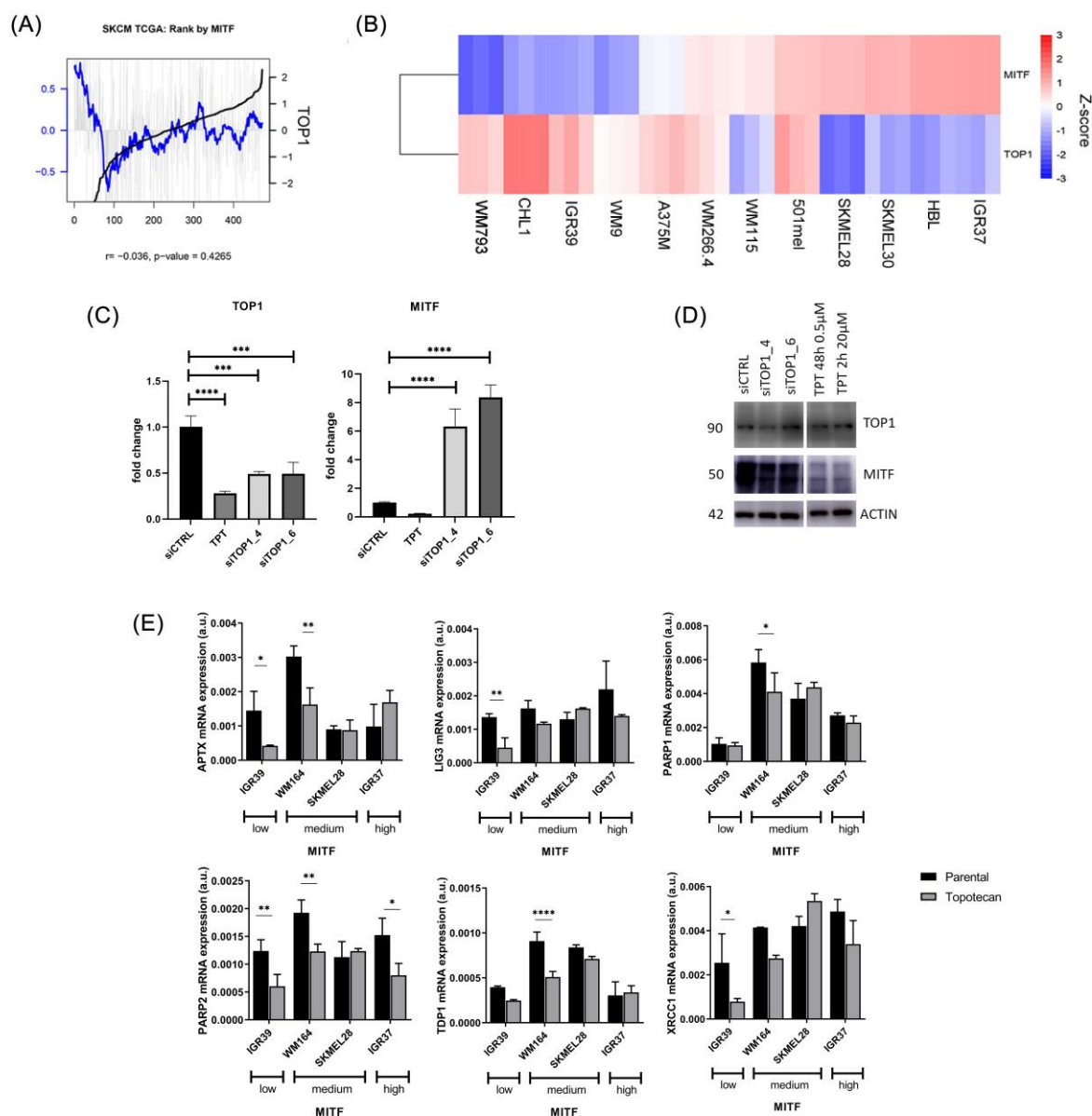


Figure 2 – The interaction of MITF and TOP1. (A) Correlation between TOP1 and MITF using TCGA melanoma cohort were ranked by MITF expression (black line) and blue lines the moving average of the TOP1 expression; (B) Heatmap showing Z-score of correlation of MITF and TOP1 expression in indicated melanoma cell lines expressing high, medium and low levels of MITF. Results show results of biological triplicate RNA-seq. (C) Relative mRNA expression of TOP1 and MITF in SKMEL28 cells after transfection with siRNA targeting TOP1 and after treatment with 20μM of Topotecan (TPT) for 2h. Beta-actin was used as housekeeping gene; (D) Protein expression of TOP1 and MITF in SKMEL28 cells after transfection with siRNA targeting TOP1 and after treatment with 20μM of Topotecan (TPT) for 2h or 0.5 μM for 48h. Actin was used as loading control; (E) Relative mRNA

expression of Single-Strand Break marker genes in melanoma cells with different levels of MITF after treatment with 20 μ M of Topotecan (TPT) for 2h. Beta-actin was used as the housekeeping gene. Error bars correspond to technical and experimental triplicates. Values are expressed as Mean \pm SD. Significance is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Abbreviations: siCTRL: siRNA control; siTOP1_4: siRNA using the s14304 sequence; siTOP1_6: siRNA using the s14306 sequence; TPT: Topotecan.

3.3 TOP1 and the DNA Single-Strand Break Repair (SSBR) genes are downregulated in resistant cells

Resistance to MAPK pathway inhibition can arise primarily because of the high degree of genetic and phenotypic intra-tumor heterogeneity. We next investigated if differences in TOP1 expression were associated with the development of resistance, especially since invasive cells tend to exhibit phenotypic drug resistance (16). In a panel of 6 BRAF-mutated melanoma cell lines and derived BRAFi-resistant clones, and for 2 BRAFi/MEKi-resistant counterparts, expression of TOP1 is decreased in the resistant cells at the mRNA (**Figure 3A**) and protein (**Figure 3B-C; Supplementary Figure 3**) levels when compared to the parental cell lines. Relative mRNA expression of TOP1 was normalized to melanocytes represented by a dotted line in **Figure 3A**. Both genetic and protein expressions of parental and resistant tumors were higher than melanocytes.

We also noted that parental SKMEL28 melanoma cells had significantly higher expression of several genes implicated in SSB repair including *APTX*, *LIG3*, *PARP1*, *PARP2*, *XRCC1* and *TDP1*, when compared to BRAFi- and BRAFi/MEKi-resistant cells (**Figure 3D**). These data suggest that the response to SSBs may be reduced in resistant and double-resistant cells.

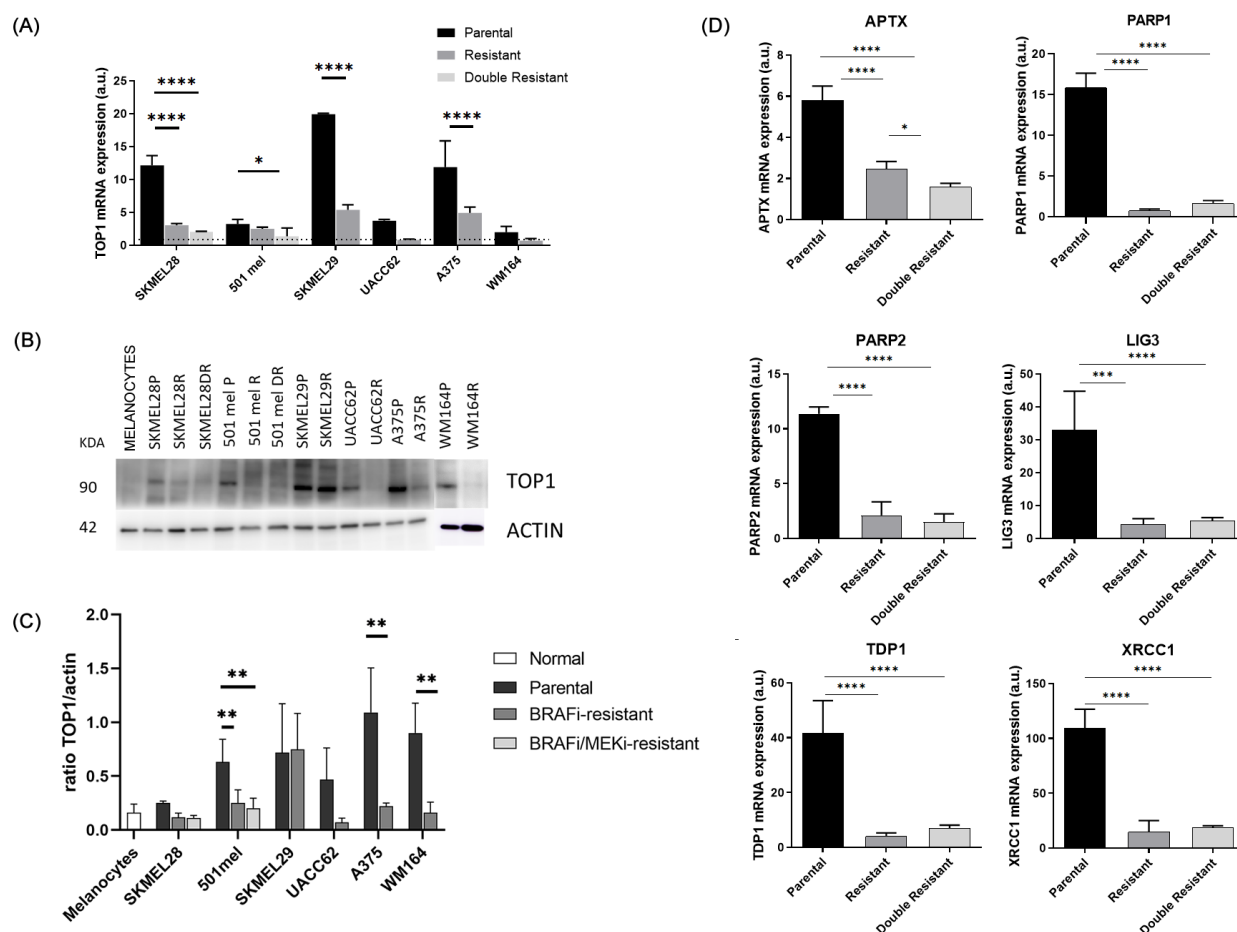


Figure 3 – TOP1 heterogeneous expression in resistance. (A) Relative mRNA expression of TOP1 parental, BRAFi-resistant and BRAFi/MEKi double-resistant cells. Beta-actin was used as the housekeeping gene and all samples were normalized by parental expression. All samples were normalized by melanocyte expression (dotted line). (B) Protein expression of TOP1 in parental, BRAFi-resistant and BRAFi/MEKi double-resistant cells and melanocytes. Actin was used as loading control; (C) Relative protein quantification using a ratio of TOP1 and actin expressions; (D) Relative mRNA expression of Single-Strand Break marker genes in parental, BRAFi- and BRAFi/MEKi-resistant SKMEL28 cells. Error bars correspond to technical and experimental triplicates. Values are expressed as Mean \pm SD. Significance is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.4 TOP1 pharmacological inhibition modulates different pathways in parental and BRAFi/MEKi-resistant melanoma

Considering our findings so far that TOP1 positively correlates with invasion and that both melanocytes and BRAFi resistant cells express less TOP1, we asked whether

pharmacological inhibition of TOP1 in patients would promote phenotypic changes toward melanocytes or resistant subtypes. Topotecan, a water-soluble analog of camptothecin, has been widely used as an anticancer drug for the past 20 years because its selectivity as a topoisomerase I (TOP1) inhibitor that traps TOP1 cleavage complexes. As such, camptothecins are also widely used to interrogate DNA repair pathways associated with DNA-protein cross-links and replication stress (40). Here, we used Topotecan to inhibit TOP1 pharmacologically. Initial cytotoxicity tests performed with the Trypan Blue exclusion assay revealed very similar IC₅₀ values around 0.3 μ M after 24h (**Supplementary Figure 4**).

To analyze multiple protein markers across parental and BRAFi/MEKi-double resistant cells upon treatment with Topotecan, cells were assayed for >290 proteins or phosphoproteins spanning critical signaling programs commonly perturbed in cancer cells using reverse-phase protein arrays (RPPA). The full panel is shown in **Figure 4A**. The proteins that were differentially expressed between parental cells treated with Topotecan and non-treated are presented in **Figure 4B**. Here, the significant proteins upregulated were Cyclin B1, Rb, Notch3, c-Jun, and Src. Enrichment analysis of biological processes was performed for the comparison groups and shown in **Supplementary Table 1**. The enrichment of the biological processes involved for these upregulated proteins included a response to mechanical stimulus, regulation of gene expression, regulation of cell population proliferation, positive regulation of RNA metabolic process, and positive regulation of chromosome segregation.

We also investigated the differentially expressed proteins between BRAFi/MEKi-resistant treated with Topotecan and non-treated (**Figure 4C**). Only the protein Phospho-S6 Ribosomal Protein (Ser240/244), which lies downstream from mTORC1, was upregulated after TOP1 inhibition. Studies showed that overactivation of the PI3K/AKT/mTOR pathway

is linked to the survival of melanoma cells upon BRAF V600E inhibition (52-54). PLK1, Caveolin-1, and PEA-15 were downregulated. The biological processes enriched involved regulation of protein phosphorylation, response to stress, and apoptotic processes.

Lastly, the analysis between BRAFi/MEKi-resistant and parental SKMEL28 cells, treated with Topotecan, revealed a more extensive range of differentially expressed proteins. The most significantly decreased in double-resistant cells were CDK1, RB, FOXO3, 14-3-3 beta, PCNA, YAP, MEK1, p90RSK, and Annexin-1, while those increased were PAR, Cyclin-B1, HSP27, HES1, TAZ, PKC, Histone-H3, Glutaminase, ARID1A, and RBM14 (**Figure 4D**).

For enriched biological processes, the downregulated process included response to stress, cell population proliferation, DNA damage response, epithelial cell differentiation, regeneration, positive regulation of the cellular biosynthetic process, cellular response to oxidative stress, and regulation of the apoptotic process, whereas for the upregulated we found enrichment for regulation of mRNA metabolic process, positive regulation of ATP metabolic process, positive regulation of purine nucleotide metabolic process, tube development, epithelial tube morphogenesis, and tissue development.

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3.5 The invasiveness signature of TOP1 differs from SSB genes, and TOP1 pharmacological inhibition decreases invasion markers in BRAFi/MEKi-resistant melanoma cells

Due to the ability of TOP1 to introduce transient DNA single-strand breaks (51), we considered whether the correlation between TOP1 and invasion reflects a more general difference in DNA-damage repair capacity between different melanoma phenotypes. **Figure 5A** shows that the invasion signature also positively correlates with genes involved in the regulation of DNA repair and response to DNA damage. To be more precise, we undertook the same analysis for a list of genes involved in single-strand break repair (SSB) (APTX, PARP1, PARP2, LIG3, TDP1 and XRCC1), where APTX, PARP1 and LIG3 negatively correlate with invasion (**Figure 5B**), and contrast with the positive correlation between TOP1 and the invasive gene expression signature.

Knowing the importance of the tumor microenvironment in the process of tumor growth and resistance, we also investigated a set of those markers after TOP1 inhibition (55). Inflammation contributes to tumor progression and can be induced by excessive production of pro-inflammatory cytokines such as interleukin-1 alpha (IL-1a), interleukin-6 (IL-6), and interleukin-8 (IL-8)(56, 57). We then analyzed the analytes released in the media after Topotecan treatment for 24h in parental, BRAFi- and BRAFi-/MEKi-resistant SKMEL28 cells. BRAFi-/MEKi-resistant cells treated were significantly increased for IL-1a and generally higher for IL-6, while BRAFi-resistant cells presented higher IL-8 levels (**Figure 5C**).

Cellular invasion and metastasis represent complex processes and occur in several stages, and the role of several Matrix Metalloproteinases (MMPs) is essential in these processes (58). For these markers, MMP-2 and MMP-9 analytes demonstrated significantly

elevated values in the BRAFi/MEKi-resistant samples when compared with both parental and BRAFi-resistant groups that decrease after the Topotecan treatment (**Figure 5C**).

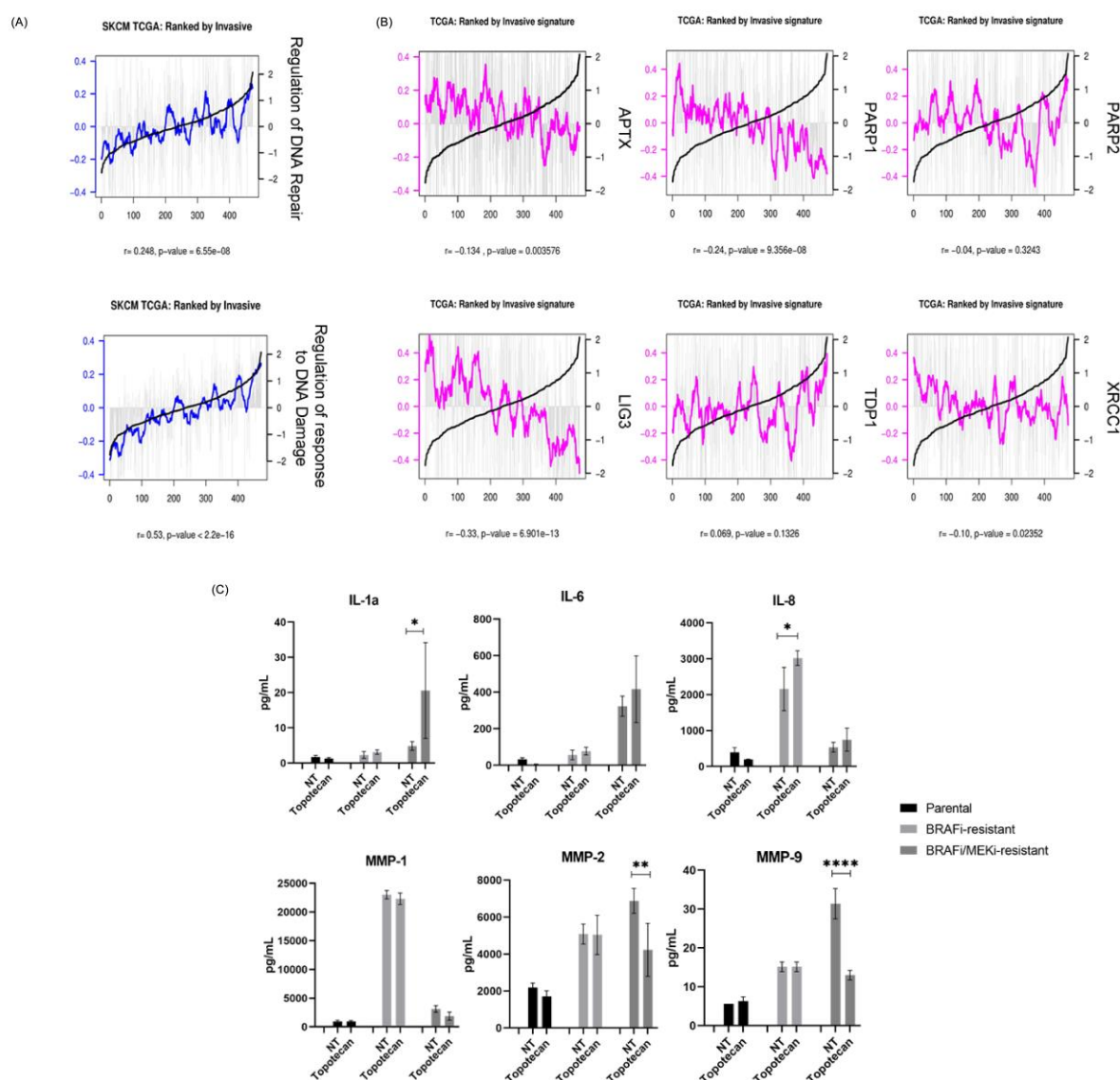


Figure 5 – The invasion signature of SSB genes and the effects of TOP1 inhibition in BRAFi/MEKi-resistant cells (A) Correlation between Regulation of DNA Repair and Regulation of response to DNA Damage markers (blue lines) using TCGA melanoma cohort ranked by the Verfallillie et al. (50) invasive signature (Blackline); (B) Expression of indicated genes (pink line) as in (A) using TCGA melanoma cohort ranked by the invasive signature (Blackline); (C) Multiplex panel for tumor microenvironment markers (IL-1a, IL-6, IL-8, MMP-1, MMP-2, MMP-9). Bar graphs representing cytokine/protease concentrations in the media of SKMEL28 cells (parental, BRAFi-resistant, BRAFi/MEKi-resistant untreated or treated with IC₅₀ Topotecan. MMP-2 and MMP-9 analytes demonstrated significantly elevated values in the BRAFi/MEKi-resistant samples when compared with both parental and BRAFi-resistant groups that decrease after Topotecan treatment. IL-8 was significantly increased

for BRAFi-resistant cells, both treated and untreated. *P < .05; **P < .005; ***P < .0005.
Abbreviations: NT, non-treated.

4. DISCUSSION

The acquisition of an invasive phenotype is crucial in the progression of benign melanocyte hyperplasia to metastatic melanoma. Therefore, understanding what triggers this transition and the mechanisms involved in invasion are essential to plan effective therapy strategies (59). Here, we show that TOP1 mRNA and protein expression increase during melanoma progression from benign melanocytes to metastasis in patient samples. Interestingly, this is seen as a heterogeneous distribution in metastatic samples that we showed that could be associated with the invasive phenotype.

Over the years, the functions of MITF have been strongly connected to the plasticity of melanoma cells which allowed the representation of a schematic model where high MITF activity levels are associated with cell differentiation and reduced proliferation and that progressively decreasing MITF activity levels are associated with proliferation, dedifferentiation/invasion, senescence, and eventually cell death (21, 60). Although TOP1 expression is high in MITF-low melanomas and cell lines, we also note some positive correlation in high MITF tumors. We see different MITF responses when we decrease the TOP1 expression; if by transient siRNA silencing, we see an increase in MITF expression, but if pharmacological inhibition is used, MITF remains low. Interestingly, Topotecan treatment decreases the expression of SSB genes in MITF low and medium cells, although in high-MITF cells, there is no change.

Decreased MITF correlates with increased TOP1 levels only in naïve cells. Even though BRAFi-resistant A375 is described as high MITF, we also see a decrease in TOP1 expression. Furthermore, we show that TOP1 decreased expression in BRAFi- and

BRAFi/MEKi-resistant cells are consistent among most of our cell lines (5 out of 6) regardless of the MITF status.

These observations suggest that multiple factors may be controlling TOP1 expression and that MITF may only contribute indirectly. Further work will be necessary to identify which of the many candidate factors such as SOX9, SOX10, SMAD3, NGFR and ERBB3, frequently altered in the undifferentiated and neural crest-like subtypes (61) might be implicated in the high TOP1 expression in MITF-low cells.

Due to the crucial role in DNA supercoil relaxation, TOP1 is often considered an essential chemotherapeutic target. TOP1 transiently generates SSBs as a part of its normal catalytic cycle (34). Under some circumstances, such as the close proximity of other DNA lesions, the ligation activity of TOP1 can be inhibited, resulting in the assembly of an irreversible cleavage complex that is unable to re-ligate the DNA and release TOP1, then requiring SSBR (39, 62).

We, therefore, investigated whether the correlation with invasiveness is a process related to only TOP1 or also to mechanisms of DNA Repair. We see that the genes involved in the regulation of DNA Repair and the regulation of response to DNA Damage are also increased in the invasive phenotype (63). However, in a set of genes related to SSB response, we see PARP1 and LIG3 negatively correlated with invasion, with only TOP1 being positively correlated. Increased levels of single-strand breaks and/or alkali-labile sites (SSB/ALS) in melanoma cells correlates with increased invasive and metastatic capacities (64). To prevent the formation of DSBs, SSB repair must be completed before DNA replication. In that sense, the SSB response helps to prevent the accumulation of cellular lesions that are a factor to be considered in the phenotypic characterization of invasive and metastatic tumor cells and may contribute to the genomic instability characteristic of different tumor cell subpopulations and resistance (65, 66).

Here, we see a decrease in TOP1 expression in BRAFi and MEKi resistance. The reduction of TOP1 leads to the accumulation of DNA strand breaks (SSBs and indirectly DSBs) that is expected to enhance cytotoxicity (67). Instead, we see the resistance phenotype that progresses to worse survival in patients. We show that BRAFi- and BRAFi/MEKi-resistant melanoma cells have decreased DNA SSB expression levels. Unrepaired SSBs usually do not accumulate in mitotic cells but rather progress to DNA DSBs that induce catastrophic cell death during mitosis (68).

TOP1 inhibitors are already used in the clinic to treat ovarian and colorectal cancers (38); however, we showed that the resistance phenotype also had decreased TOP1, making it unclear if TOP1 inhibition would benefit melanoma patients. Here we see that TOP1 inhibition modulates different genes in parental and BRAFi/MEKi-resistant cells through our RPPA analysis. In parental cells, Topotecan modulates pathways related to proteins included in the response of DNA damage and stress, that are the known mechanisms of action for Topotecan (69-71), whereas, in BRAFi/MEKi-resistant cells, Topotecan upregulates different metabolic processes and downregulates the metalloproteinases 2 and 9 that are particularly important in the invasion process.

5. CONCLUSIONS

Our data shows that it is evident that high expression of TOP1 can work as a strong indication of melanoma progression. Conversely, we have a different scenario when TOP1 is poorly expressed. First, non-cancer cells, like melanocytes, that do not invade and therefore, follow the premise of the positive correlation of TOP1 with invasion; and secondly, BRAFi- and BRAFi/MEKi-resistant cells, that have decreased TOP1 expression but also present strong invasion capacity based in the higher levels of MMP-2 and -9 and as

previously described (30) for BRAFi-resistant cells. Together, these results highlight a potential contribution of TOP1 to melanoma.

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ETHICAL APPROVAL

This study was approved by the Ethics in Research Committee of the School of Pharmaceutical Sciences of the University of São Paulo (849.540/2014/CAAE 76737917.5.1001.0067) and by the Ethics in Research Committee of the University of Maringa (Maringa, Brazil) (CAAE 76737917.5.1001.0067).

REFERENCES

1. Zhang T, Dutton-Regester K, Brown KM, Hayward NK. The genomic landscape of cutaneous melanoma. *Pigment Cell Melanoma Res.* 2016;29(3):266-83.
2. Menzies AM, Haydu LE, Visintin L, Carlino MS, Howle JR, Thompson JF, et al. Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. *Clin Cancer Res.* 2012;18(12):3242-9.
3. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature.* 2002;417(6892):949-54.
4. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med.* 2012;367(18):1694-703.
5. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med.* 2010;363(9):809-19.
6. Paraiso KH, Smalley KS. Fibroblast-mediated drug resistance in cancer. *Biochem Pharmacol.* 2013;85(8):1033-41.

- 574 7. Johnson DB, Flaherty KT, Weber JS, Infante JR, Kim KB, Kefford RF, et al.
575 Combined BRAF (Dabrafenib) and MEK inhibition (Trametinib) in patients with
576 BRAFV600-mutant melanoma experiencing progression with single-agent BRAF
577 inhibitor. *J Clin Oncol*. 2014;32(33):3697-704.
- 578 8. Trunzer K, Pavlick AC, Schuchter L, Gonzalez R, McArthur GA, Hutson TE, et
579 al. Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients
580 with metastatic melanoma. *J Clin Oncol*. 2013;31(14):1767-74.
- 581 9. Broman KK, Dossett LA, Sun J, Eroglu Z, Zager JS. Update on BRAF and MEK
582 inhibition for treatment of melanoma in metastatic, unresectable, and adjuvant settings.
583 *Expert Opin Drug Saf*. 2019;18(5):381-92.
- 584 10. Yu Q, Xie J, Li J, Lu Y, Liao L. Clinical outcomes of BRAF plus MEK inhibition
585 in melanoma: A meta-analysis and systematic review. *Cancer Med*. 2019;8(12):5414-
586 24.
- 587 11. Flaherty KT, Hodi FS, Fisher DE. From genes to drugs: targeted strategies for
588 melanoma. *Nat Rev Cancer*. 2012;12(5):349-61.
- 589 12. Ribas A, Flaherty KT. BRAF targeted therapy changes the treatment paradigm
590 in melanoma. *Nat Rev Clin Oncol*. 2011;8(7):426-33.
- 591 13. Rambow F, Marine JC, Goding CR. Melanoma plasticity and phenotypic
592 diversity: therapeutic barriers and opportunities. *Genes Dev*. 2019;33(19-20):1295-
593 318.
- 594 14. Johannessen CM, Johnson LA, Piccioni F, Townes A, Frederick DT, Donahue
595 MK, et al. A melanocyte lineage program confers resistance to MAP kinase pathway
596 inhibition. *Nature*. 2013;504(7478):138-42.

- 597 15. Konieczkowski DJ, Johannessen CM, Abudayyeh O, Kim JW, Cooper ZA, Piris
598 A, et al. A melanoma cell state distinction influences sensitivity to MAPK pathway
599 inhibitors. *Cancer Discov.* 2014;4(7):816-27.
- 600 16. Muller J, Krijgsman O, Tsoi J, Robert L, Hugo W, Song C, et al. Low MITF/AXL
601 ratio predicts early resistance to multiple targeted drugs in melanoma. *Nat Commun.*
602 2014;5:5712.
- 603 17. Rambow F, Rogiers A, Marin-Bejar O, Aibar S, Femel J, Dewaele M, et al.
604 Toward Minimal Residual Disease-Directed Therapy in Melanoma. *Cell.*
605 2018;174(4):843-55 e19.
- 606 18. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, 2nd, Treacy D, Trombetta JJ, et
607 al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-
608 seq. *Science.* 2016;352(6282):189-96.
- 609 19. Shaffer SM, Dunagin MC, Torborg SR, Torre EA, Emert B, Krepler C, et al. Rare
610 cell variability and drug-induced reprogramming as a mode of cancer drug resistance.
611 *Nature.* 2017;546(7658):431-5.
- 612 20. Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, et
613 al. Integrative genomic analyses identify MITF as a lineage survival oncogene
614 amplified in malignant melanoma. *Nature.* 2005;436(7047):117-22.
- 615 21. Goding CR, Arnheiter H. MITF-the first 25 years. *Genes Dev.* 2019;33(15-
616 16):983-1007.
- 617 22. Lister JA, Capper A, Zeng Z, Mathers ME, Richardson J, Paranthaman K, et al.
618 A conditional zebrafish MITF mutation reveals MITF levels are critical for melanoma
619 promotion vs. regression in vivo. *J Invest Dermatol.* 2014;134(1):133-40.

- 620 23. Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, et al. MEK1
621 mutations confer resistance to MEK and B-RAF inhibition. *Proc Natl Acad Sci U S A*.
622 2009;106(48):20411-6.
- 623 24. Puzanov I, Burnett P, Flaherty KT. Biological challenges of BRAF inhibitor
624 therapy. *Mol Oncol*. 2011;5(2):116-23.
- 625 25. Solit DB, Rosen N. Resistance to BRAF inhibition in melanomas. *N Engl J Med*.
626 2011;364(8):772-4.
- 627 26. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al.
628 Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF
629 secretion. *Nature*. 2012;487(7408):500-4.
- 630 27. de Souza N, de Oliveira EA, Faiao-Flores F, Pimenta LA, Quincoces JAP,
631 Sampaio SC, et al. Metalloproteinases Suppression Driven by the Curcumin Analog
632 DM-1 Modulates Invasion in BRAF-Resistant Melanomas. *Anticancer Agents Med*
633 *Chem*. 2020.
- 634 28. Faiao-Flores F, Alves-Fernandes DK, Pennacchi PC, Sandri S, Vicente AL,
635 Scapulatempo-Neto C, et al. Targeting the hedgehog transcription factors GLI1 and
636 GLI2 restores sensitivity to vemurafenib-resistant human melanoma cells. *Oncogene*.
637 2017;36(13):1849-61.
- 638 29. Oliveira EA, Lima DS, Cardozo LE, Souza GF, de Souza N, Alves-Fernandes
639 DK, et al. Toxicogenomic and bioinformatics platforms to identify key molecular
640 mechanisms of a curcumin-analogue DM-1 toxicity in melanoma cells. *Pharmacol Res*.
641 2017;125(Pt B):178-87.
- 642 30. Sandri S, Faiao-Flores F, Tiago M, Pennacchi PC, Massaro RR, Alves-
643 Fernandes DK, et al. Vemurafenib resistance increases melanoma invasiveness and

- modulates the tumor microenvironment by MMP-2 upregulation. *Pharmacol Res.* 2016;111:523-33.
31. Tiago M, de Oliveira EM, Brohem CA, Pennacchi PC, Paes RD, Haga RB, et al. Fibroblasts protect melanoma cells from the cytotoxic effects of doxorubicin. *Tissue Eng Part A.* 2014;20(17-18):2412-21.
32. Alves-Fernandes DK, Oliveira EA, Faiao-Flores F, Alicea-Rebecca G, Weeraratna AT, Smalley KSM, et al. ER stress promotes antitumor effects in BRAFi/MEKi resistant human melanoma induced by natural compound 4-nerolidylcatechol (4-NC). *Pharmacol Res.* 2019;141:63-72.
33. Champoux JJ. DNA topoisomerase I-mediated nicking of circular duplex DNA. *Methods Mol Biol.* 2001;95:81-7.
34. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol.* 2002;3(6):430-40.
35. Ryan D, Rafferty M, Hegarty S, O'Leary P, Faller W, Gremel G, et al. Topoisomerase I amplification in melanoma is associated with more advanced tumours and poor prognosis. *Pigment Cell Melanoma Res.* 2010;23(4):542-53.
36. Lynch BJ, Komaromy-Hiller G, Bronstein IB, Holden JA. Expression of DNA topoisomerase I, DNA topoisomerase II-alpha, and p53 in metastatic malignant melanoma. *Hum Pathol.* 1998;29(11):1240-5.
37. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol Cell.* 2010;40(2):179-204.
38. Li F, Jiang T, Li Q, Ling X. Camptothecin (CPT) and its derivatives are known to target topoisomerase I (Top1) as their mechanism of action: did we miss something in CPT analogue molecular targets for treating human disease such as cancer? *Am J Cancer Res.* 2017;7(12):2350-94.

- 669 39. Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev*
670 *Cancer*. 2006;6(10):789-802.
- 671 40. Thomas A, Pommier Y. Targeting Topoisomerase I in the Era of Precision
672 *Medicine*. *Clin Cancer Res*. 2019;25(22):6581-9.
- 673 41. Brohem CA, Massaro RR, Tiago M, Marinho CE, Jasiulionis MG, de Almeida
674 RL, et al. Proteasome inhibition and ROS generation by 4-nerolidylcatechol induces
675 melanoma cell death. *Pigment Cell Melanoma Res*. 2012;25(3):354-69.
- 676 42. Pennacchi PC, de Almeida ME, Gomes OL, Faiao-Flores F, de Araujo Crepaldi
677 MC, Dos Santos MF, et al. Glycated Reconstructed Human Skin as a Platform to Study
678 the Pathogenesis of Skin Aging. *Tissue Eng Part A*. 2015;21(17-18):2417-25.
- 679 43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-
680 time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-
681 8.
- 682 44. Rebecca VW, Nicastrì MC, McLaughlin N, Fennelly C, McAfee Q, Ronghe A, et
683 al. A Unified Approach to Targeting the Lysosome's Degradative and Growth Signaling
684 Roles. *Cancer Discov*. 2017;7(11):1266-83.
- 685 45. Zhang G, Frederick DT, Wu L, Wei Z, Krepler C, Srinivasan S, et al. Targeting
686 mitochondrial biogenesis to overcome drug resistance to MAPK inhibitors. *J Clin*
687 *Invest*. 2016;126(5):1834-56.
- 688 46. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J,
689 et al. STRING v10: protein-protein interaction networks, integrated over the tree of life.
690 *Nucleic Acids Res*. 2015;43(Database issue):D447-52.
- 691 47. Anaya J. OncoLnc: linking TCGA survival data to mRNAs, miRNAs, and
692 lncRNAs. *PeerJ Computer Science*. 2016;2.

- 693 48. Riesenberger S, Groetchen A, Siddaway R, Bald T, Reinhardt J, Smorra D, et al.
694 MITF and c-Jun antagonism interconnects melanoma dedifferentiation with pro-
695 inflammatory cytokine responsiveness and myeloid cell recruitment. *Nat Commun.*
696 2015;6:8755.
- 697 49. Thul PJ, Lindskog C. The human protein atlas: A spatial map of the human
698 proteome. *Protein Sci.* 2018;27(1):233-44.
- 699 50. Verfaillie A, Imrichova H, Atak ZK, Dewaele M, Rambow F, Hulselmans G, et al.
700 Decoding the regulatory landscape of melanoma reveals TEADS as regulators of the
701 invasive cell state. *Nat Commun.* 2015;6:6683.
- 702 51. Nitiss JL, Nitiss KC, Rose A, Waltman JL. Overexpression of type I
703 topoisomerases sensitizes yeast cells to DNA damage. *J Biol Chem.*
704 2001;276(28):26708-14.
- 705 52. Penna I, Molla A, Grazia G, Cleris L, Nicolini G, Perrone F, et al. Primary cross-
706 resistance to BRAFV600E-, MEK1/2- and PI3K/mTOR-specific inhibitors in BRAF-
707 mutant melanoma cells counteracted by dual pathway blockade. *Oncotarget.*
708 2016;7(4):3947-65.
- 709 53. Sanchez-Hernandez I, Baquero P, Calleros L, Chiloeches A. Dual inhibition of
710 (V600E)BRAF and the PI3K/AKT/mTOR pathway cooperates to induce apoptosis in
711 melanoma cells through a MEK-independent mechanism. *Cancer Lett.*
712 2012;314(2):244-55.
- 713 54. Shimizu T, Tolcher AW, Papadopoulos KP, Beeram M, Rasco DW, Smith LS,
714 et al. The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and
715 RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res.*
716 2012;18(8):2316-25.

- 717 55. Masjedi A, Hashemi V, Hojjat-Farsangi M, Ghalamfarsa G, Azizi G, Yousefi M,
718 et al. The significant role of interleukin-6 and its signaling pathway in the
719 immunopathogenesis and treatment of breast cancer. *Biomed Pharmacother.*
720 2018;108:1415-24.
- 721 56. Ma Y, Ren Y, Dai ZJ, Wu CJ, Ji YH, Xu J. IL-6, IL-8 and TNF-alpha levels
722 correlate with disease stage in breast cancer patients. *Adv Clin Exp Med.*
723 2017;26(3):421-6.
- 724 57. Gelfo V, Romaniello D, Mazzeschi M, Sgarzi M, Grilli G, Morselli A, et al. Roles
725 of IL-1 in Cancer: From Tumor Progression to Resistance to Targeted Therapies. *Int J*
726 *Mol Sci.* 2020;21(17).
- 727 58. Hofmann UB, Westphal JR, Van Muijen GN, Ruiter DJ. Matrix
728 metalloproteinases in human melanoma. *J Invest Dermatol.* 2000;115(3):337-44.
- 729 59. Gaggioli C, Sahai E. Melanoma invasion - current knowledge and future
730 directions. *Pigment Cell Res.* 2007;20(3):161-72.
- 731 60. Hartman ML, Czyz M. MITF in melanoma: mechanisms behind its expression
732 and activity. *Cell Mol Life Sci.* 2015;72(7):1249-60.
- 733 61. Tsoi J, Robert L, Paraiso K, Galvan C, Sheu KM, Lay J, et al. Multi-stage
734 Differentiation Defines Melanoma Subtypes with Differential Vulnerability to Drug-
735 Induced Iron-Dependent Oxidative Stress. *Cancer Cell.* 2018;33(5):890-904 e5.
- 736 62. Pouliot JJ, Robertson CA, Nash HA. Pathways for repair of topoisomerase I
737 covalent complexes in *Saccharomyces cerevisiae*. *Genes Cells.* 2001;6(8):677-87.
- 738 63. Broustas CG, Lieberman HB. DNA damage response genes and the
739 development of cancer metastasis. *Radiat Res.* 2014;181(2):111-30.

- 740 64. Meade-Tollin LC, Pipes BL, Anderson SJ, Seftor EA, Hendrix MJ. A comparison
741 of levels of intrinsic single strand breaks/alkali labile sites associated with human
742 melanoma cell invasion. *Cancer Lett.* 1990;53(1):45-54.
- 743 65. Khoronenkova SV, Dianov GL. ATM prevents DSB formation by coordinating
744 SSB repair and cell cycle progression. *Proc Natl Acad Sci U S A.* 2015;112(13):3997-
745 4002.
- 746 66. Mei C, Lei L, Tan LM, Xu XJ, He BM, Luo C, et al. The role of single strand
747 break repair pathways in cellular responses to camptothecin induced DNA damage.
748 *Biomed Pharmacother.* 2020;125:109875.
- 749 67. Xu Y, Her C. Inhibition of Topoisomerase (DNA) I (TOP1): DNA Damage Repair
750 and Anticancer Therapy. *Biomolecules.* 2015;5(3):1652-70.
- 751 68. Caldecott KW. Single-strand break repair and genetic disease. *Nat Rev Genet.*
752 2008;9(8):619-31.
- 753 69. Kollmannsberger C, Mross K, Jakob A, Kanz L, Bokemeyer C. Topotecan - A
754 novel topoisomerase I inhibitor: pharmacology and clinical experience. *Oncology.*
755 1999;56(1):1-12.
- 756 70. Jain RK, Hong DS, Naing A, Wheler J, Helgason T, Shi NY, et al. Novel phase
757 I study combining G1 phase, S phase, and G2/M phase cell cycle inhibitors in patients
758 with advanced malignancies. *Cell Cycle.* 2015;14(21):3434-40.
- 759 71. Ohneseit PA, Prager D, Kehlbach R, Rodemann HP. Cell cycle effects of
760 topotecan alone and in combination with irradiation. *Radiother Oncol.* 2005;75(2):237-
761 45.