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Ran signaling in melanoma: implications for the development of alternative therapeutic strategies

Emilia Caputo^{a,b,*}, Ena Wang^{c,f}, Anna Valentino^a, Stefania Crispi^{a,d}, Valeria De Giorgi^c, Annalisa Fico^a, Bartolomea Ficili^b, Mariaelena Capone^e, AnnaMaria Anniciello^e, Ernesta Cavalcanti^e, Gerardo Botti^e, Nicola Mozzillo^e, Paolo A. Ascierto^e, Francesco M. Marincola^{c,f}, and Salvatore Travali^b

^aInstitute of Genetics and Biophysics –I.G.B., A. Buzzati-Traverso–, CNR, Naples, I-80131, Italy

^bUniversità degli Studi di Catania, Dipartimento di Scienze Biomediche, Catania, I-95124, Italy

^cInfectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine (DTM), Clinical Center (CC), and Center for Human Immunology (CHI), National Institutes of Health, Bethesda, MD (NIH), Bethesda, MD

^dInstitute of Biosciences and BioResources-IBB, CNR, Naples, I-8013, Italy

^eIstituto Nazionale Tumori Fondazione G. Pascale, Naples, I-80131 Italy

^fSidra Medical and Research Center, Doha, Qatar

Abstract

We performed a comparative study between two human metastatic melanoma cell lines (A375 and 526), and melanocytes (FOM78) by gene expression profiling and pathway analysis, using Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) software. Genes involved in Ran signaling were significantly over-represented ($p < 0.001$) and up-regulated in melanoma cells. A melanoma-associated molecular pathway was identified, where Ran, Aurora Kinase A (AurKA) and TERT were up-regulated, while c-myc and PTEN were down-regulated. A consistent high Ran and AurKA gene expression was detected in about 48% and 53%, respectively, of 113 tissue samples from metastatic melanoma patients. AurKA down-regulation was observed in melanoma cells, by Ran knockdown, suggesting AurKA protein is a Ran downstream target. Furthermore, AurKA inhibition, by exposure of melanoma cells to MLN8054,

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To whom correspondence should be addressed: Dr. E. Caputo, I.G.B., A. Buzzati-Traverso, CNR, Naples, I-80131, Italy; Tel. +39 081 6132307; Fax: +39 081 6132706; emilia.caputo@igb.cnr.it.

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Competing interests

All authors have disclosed any financial competing interests. PAA has/had a consultant/advisory role for Bristol Myers-Squibb, Roche-Genentech, Merck Sharp & Dohme, GlaxoSmithKline, Ventana, and Novartis. He received research funds from Bristol Myers-Squibb, Roche-Genentech, and Ventana. He also received honoraria from Bristol Myers-Squibb, Roche-Genentech, and GlaxoSmithKline.

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a specific AurKA inhibitor, induced apoptosis in both melanoma cell lines and molecular alterations in the IPA-identified molecular pathway. These alterations differed between cell lines, with an up-regulation of c-myc protein level observed in 526 cells and a slight reduction seen in A375 cells. Moreover, Ran silencing did not affect the A375 invasive capability, while it was enhanced in 526 cells, suggesting that Ran knockdown, by AurKA down-regulation, resulted in a Ran-independent enhanced melanoma cell invasion. Finally, AurK A inhibition induced a PTEN up-regulation and its action was independent of B-RAF mutational status. These findings provide insights relevant for the development of novel therapeutic strategies as well as for a better understanding of mechanisms underlying therapy resistance in melanoma.

Keywords

Targeted therapy; aurora kinase A in melanoma; gene expression; pathway analysis; ran signaling

1. Introduction

The most aggressive form of skin cancer is melanoma, incidence of which has dramatically increased worldwide (Jemal et al. 2009). Metastatic malignant melanoma has a very poor prognosis, with a median survival time of approximately 6 months (Korn et al. 2008). Several of the molecular mechanisms associated with melanoma origin and progression have now been identified. For instance, the mitogen-activated protein kinase (MAPK) pathway is constitutively activated in melanoma cells, while it is only weakly activated by growth factors released from the local microenvironment in melanocytes, leading to an insufficient stimulus to induce proliferation under physiological conditions (Cohen et al. 2002). Activating mutations in N-Ras have been identified in 15–20% of all melanomas (Brose et al. 2002; Davies et al. 2002), while mutations in B-RAF have been observed in about 60% of melanomas, with V600E accounting for over 80% of all mutations. The BRAF (V600E) protein has markedly increased catalytic activity for its substrates and constitutively activates the RAS-RAFMEK-ERK pathway (Davies et al. 2002; Wan et al. 2004). However, MAPK pathway activation alone is insufficient for melanoma progression and it has been shown that full transformation only occurs with concurrent activation of the PI3K-AKT pathway following PTEN suppression (Dankort et al. 2009).

Understanding the complex regulation of these pathways and the role of novel altered pathways may lead to improvements in the rational development of targeted therapies. In this study, we applied a network identification analysis, based on gene expression profiles and protein-protein interaction networks, to characterize and compare two different melanoma cell lines, A375 and 526 (Cormier et al. 1999) and the melanocyte cell line, FOM78 (Herlyn et al. 1985) as control. Gene expression was also investigated in 113 tissue samples derived from metastatic melanoma patients.

Materials and methods

2.1 Cell lines

Human melanoma cell lines A375 and 526 and melanocytes FOM78 (Herlyn et al. 1985) were kindly provided by Dr M Bettinotti (NIH, Bethesda, MD, USA). Melanoma cell lines were cultured in RPMI 1640 medium (Invitrogen-Gibco, Monza MB, Italy), supplemented with 3mM L-glutamine (Invitrogen-Gibco), 2% penicillin/streptomycin, and 10% FBS. Melanocytes were cultured in MGM-4 cell medium (Euroclone, Milan, Italy). All cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

2.2 Gene expression analysis

Total RNA was isolated from cultured cell lines using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA quality and integrity was evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Two rounds of RNA amplification were performed as previously described (Wang et al. 2000; Wang et al. 2005) and 6 µg of amplified χ RNA were labeled with Cy5 while reference samples derived from peripheral blood mononuclear cells (PBMC) pooled from six normal donors were amplified in the same fashion and labeled with Cy3 using a ULS α RNA Fluorescent Labeling kit (Kreatech, Amsterdam, The Netherlands). A 36k whole transcriptome expression array was fabricated using human Array-Ready Oligo Set (AROS™ V4.0, Operon, Cologne, Germany) at the Infectious Disease and Immunogenetics Section, (NIH, Bethesda, MD, USA). Hybridization was carried at 42°C for 18-24 hours and the arrays were then washed, scanned and analyzed using BRB array software (<http://linus.nci.nih.gov/BRBArrayTools.html>) (Simon et al. 2007). Genes with intensity <100 in both channels, spots size <20µm were excluded. Genes with intensity <100 in one channel only were adjusted to 100. After median normalization over the whole array, data were stringently filtered to remove genes with missing values in more than 40% of the experiments and genes with less than 1.5-fold change in either direction from the median value in less than 20% of the entire array experiments.

2.3 Pathway analysis

Ingenuity Pathway Analysis (IPA7.0, Ingenuity System®, <http://www.ingenuity.com/>) was used to functionally annotate differentially expressed genes from the cell lines analyzed and to identify potential networks each with a specific score, corresponding to the probabilistic fit between the networks and a list of biological functions stored in the Ingenuity Pathways Knowledge Base, a proprietary manually curated database.

2.4 GSEA analysis

Gene Set Enrichment Analysis (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) was used to assess whether an *a priori* defined set of genes showed statistically significant concordant differences between two phenotypes (melanoma cells vs. melanocytes). The primary result of the GSEA is the enrichment score (ES), which reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. Normalized log2

intensities were used in the analysis against a computational gene set defined by mining large collections of cancer-oriented microarray data (C4 computational gene sets, Molecular Signatures Database v4.0).

2.5 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA (300 ng) from melanocyte and melanoma cell lines were converted to cDNA using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, Life Technologies, Grand Island, CA, USA). Primers for the selected genes (AURKA: Forward 5'-CACCTTCGGCATCCTAATATTCTT-3', Reverse 5'-GGGCATTTGCCAATTCTGTT-3'; MYC Forward 5'-CACCAACCAGCAGCGACTCT-3', Reverse 5'-TTCCACAGAAACAACATCGATTTC-3'; PTEN Forward 5'-GGAGATATCAAGAGGATGGATTCG-3', Reverse 5'-CAGGAAATCCCATAGCAATAATGTT-3'; RAN Forward 5'-TTGGTGATGGTGGTACTGGA-3', Reverse 5'-GGAGAGCAGTTGTCTGAGCA-3'; RCC1 Forward 5'-TGCAGGTGCAGCTGGATGT-3', Reverse 5'-CATCACCAAGTGGTTCGTTTCC-3'; TERT Forward 5'-GGCGACATGGAGAACAAGCT-3', Reverse 5'-CCAACAAGAAATCATCCACCAAA-3'), were designed using Primer Express 2.0 software (Applied Biosystems). Actin β was used as internal control (Forward 5'-TTCTACAATGAGCTGCGTGTG-3' and Reverse 5'-GGGGTGTGAAGGTCTCAAA-3'). Experiments were performed in triplicate. Quantitative real-time polymerase chain reaction (qRT-PCR) was done on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The entire procedure for qRT-PCR analysis (primer design, reactions, amplicon specificity and determination of gene target expression levels) was performed as previously described (Crispi et al. 2009).

2.6 Protein extracts

Cells were lysed in lysis buffer (1% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA [pH 8], 0.2 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride) for 30 minutes at 4°C with constant agitation. Insoluble material was removed by centrifugation (16000 $\times$ g at 4°C) for 15 minutes and the total protein concentration was determined in the supernatant by Bradford assay.

2.7 Western Blot Analysis

Western blot was performed according to standard procedures. Mouse monoclonal antibodies against p53 (DO-1; diluted 1:1000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), rabbit monoclonal to c-Myc (1:5000, Abcam, Cambridge, UK), rabbit polyclonal to telomerase reverse transcriptase (diluted 1:1000; Abcam), rabbit polyclonal antibodies against PARP (diluted 1:1000, Cell Signaling Technology, Danvers, MA, USA), phospho-MEK1/2 (Ser217/221) (diluted 1:1000, Cell Signaling Technology), MEK1/2 (diluted 1:1000, Cell Signaling Technology), Aurora Kinase A (diluted 1:100; Abcam), Ran (diluted 1:500; Abcam) and β -actin (diluted 1:1,000, Cell Signaling Technology) were used. Detection was achieved by HRP-conjugated anti-mouse (1:10000; Cell Signaling Technology) or HRP-conjugated anti-rabbit (1:1,000,000; Cell Signaling Technology).

antibodies. Immune complexes were visualized by an enhanced chemiluminescence system (ECL Advance™, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Actin was used as a loading control. The image analysis was performed by ImageJ software (<http://rsbweb.nih.gov/ij/>). Results represent the means ($\pm$ SEM) of three independent experiments performed in triplicate. *P*-value was determined by using t-test and value 0.005 was indicated, in the figure, with the symbol: ***.

2.8 Gene expression analysis on melanoma tissues

Total RNA from 113 melanoma metastases from patients treated at the Surgery Branch, NCI, were extracted using miRNeasy minikit (Qiagen). RNA quality and quantity was estimated using Nanodrop (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). First- and second-strand cDNA were synthesized from 300 ng of total RNA according to the manufacturer's instructions (Ambion WT Expression Kit). cDNAs were fragmented, biotinylated, and hybridized to the GeneChip Human Gene 1.0 ST Arrays (Affymetrix WT Terminal Labeling Kit, Affymetrix, Santa Clara, CA, USA). The arrays were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix); scanning was carried out with the GeneChip Scanner 3000 and image analysis with the Affymetrix GeneChip Command Console Scan Control. Expression data were normalized, background-corrected, and summarized using the RNA algorithm at the <http://www.partek.com/> website. Data were log-transformed (base 2) for subsequent statistical analysis. Cluster analysis was performed using Cluster and TreeView software (Stanford University, Stanford, CA, USA).

2.9 Small interfering RNA transfection

Ran gene silencing by small interfering RNA (siRNA) was carried out with control non-targeted and two independent Ran-directed siRNA oligonucleotides (s11769 and s11767, Ambion, Carlsbad, CA) using Lipofectamine RNAiMAX reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Briefly, 1×10^5 cells/well were seeded into six-well plates and incubated with the transfection complexes (75mM siRNAs and 10 μ l of Lipofectamine RNAiMAX, Invitrogen). Cells were analyzed 48 hours post-transfection.

2.10 Invasion assays

An invasion assay was performed in a 24-well transwell chamber, by using a BD BioCoat Matrigel invasion chamber (BD Bioscience, Bedford, MA, USA), according to the manufacturer's instructions. Briefly, equal numbers of cells (2.5×10^4 cells/well) in RPMI 1640 medium complemented with 1% FCS were added to the upper compartment of the chamber. As a chemoattractant, the lower compartment contained RPMI 1640 medium supplemented with 10% FCS. After 18 hours at 37°C in a 5% CO₂ incubator, the Matrigel coating on the upper surface of the filter was wiped off using a cotton swab. Cells that migrated through the filters were fixed, stained, photographed, and counted (4 random fields/insert, 10x) in the light microscope on ten randomly selected fields. The mean number of cells was calculated per field. Three sets of experiments were carried out, each in triplicate. The statistical significance of the results was calculated using the ANOVA procedure. The data were considered to be significant when $P < 0.05$.

2.11 Drug treatment

Cells were treated with medium containing the specific Aurora kinase A (AurkA) inhibitor MLN8054 (Selleck, Munich, Germany) at different concentrations for 72 hours and with B-Raf inhibitor (GSK2118436) (kindly provided by GlaxoSmithKline, London, UK) at a concentration of 30 nM in all experiments. Cells treated with DMSO (0.2%) were used as the vehicle control. Cell number evaluation, following drug treatment was performed using a CyQuant Cell Proliferation Assay Kit (Invitrogen). Results represented the means (percentage values) of three independent experiments performed in triplicate.

2.12 p53 mutational analysis

Genomic DNA was extracted from cells by QIAamp DNA mini kit (Qiagen) and was used as template for amplification of p53 exons 5–8. The primers were: exon 4 forward: 5'-GACCTGGTCCTCTGACTGCT-3' and reverse: 5'-ATACGGCCAGGCATTGAAG-3'; exon 5-6 forward: 5'-AGGAGGTGCTTAGCGATGT-3', reverse: 5'-CACTGACAACCACCCTTAAC-3'; exon 7 forward: 5'-CAGAGCGAGATTCCATCTCA-3', reverse: 5'-ATGTGATGAGAGGTGGATGG-3'; exon 8 forward: 5'-GGAGTAGATGGAGCCTGGTT-3', reverse: 5'-ATCTGAGGCATAACTGCACC-3'. PCR product sequence analysis was performed in both directions using the same primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using an ABI PRISM 310 genetic analyzer (Applied Biosystems). All sequences were confirmed in a second PCR-sequencing reaction.

2.13 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0[®] statistical software (GraphPad Software Inc., La Jolla, CA, USA). Paired t-test was used for comparison of two paired groups. Multiple comparisons were performed by the repeated measures ANOVA test with the Bonferroni correction. Dot plot, reporting gene intensity values obtained from gene expression analysis, was generated across all samples examined, by using Partek Genomics Suite software.

3 Results

3.1 Gene profiling and network analysis of melanoma cell lines and melanocytes

The transcriptional datasets of the human melanoma cell lines A375 and 526 were functionally classified through an Ingenuity Pathways Analysis (IPA) Core Analysis (IPA7.0, Ingenuity System[®]) and compared with that of the melanocytes, FOM78. IPA is a web-delivered application that allows the identification of functional relationships between differentially expressed genes. For each probe set, IPA generates a metadata file, containing information on how to map the given dataset onto molecules associated with specific disease, cellular functions and canonical pathways. Thus, it is possible to obtain indications on the cellular processes, characterizing melanoma cell lines and genes potentially involved in disease development. We examined deregulated genes for molecular, cellular function and canonical pathways for both melanoma cell lines, A375 and 526, and melanocytes, with complete lists reported in the **Supplemental Table 1**.

All the deregulated genes (approximately 1800) obtained from the microarray data were analysed with IPA Core Analysis. IPA associates molecules under analysis with known biological functions and diseases. The link between a category and the associated molecules is defined by a p-value. In general more molecules are involved more likely the association is not due to random chance, and thus more significant is the p-value. We found EEIF2 Signaling, Ran Signaling, Cell Cycle Control of Chromosomal Replication and Pyrimidine Metabolism as the top four categories among the known canonical pathways enriched within the set of differentially expressed genes (Fig. 1A). The canonical pathways are displayed along the x-axis, while the y-axis displays the -log of p-value, calculated by Fisher's exact test right-tailed. The majority of the genes, belonging to these categories, showed an opposite expression between the melanocyte and melanoma samples, as a result of being specifically up-regulated in the two melanoma cell lines compared to control (Fig. 1B). In particular, genes involving Ran signalling were significantly over-represented ($p = 0.001$) and up-regulated in both melanoma cell lines (Fig. 1C).

To further confirm the IPA pathway analysis results, we performed a GSEA based on c4 computational gene sets, defined by mining large collections of cancer-oriented microarray data (**Supplemental Table 2**). Using the gene list reported in **Supplemental Table 1**, we found 80/92 gene sets up-regulated in phenotype MEL; 56 gene sets were significant at a false discovery rate (FDR) $< 25\%$; 22 gene sets were significantly enriched at nominal p-value $< 1\%$; and 45 gene sets were significantly enriched at nominal p-value $< 5\%$. Enrichment of genes involved in Ran signaling was observed in the melanoma cell lines compared with melanocytes, supporting the IPA findings.

Interestingly, molecules involved in the Ran signalling pathway have been previously reported to be differentially expressed in many human cancers, such as colon adenocarcinoma, prostate adenocarcinoma, renal cell carcinoma, breast adenocarcinoma, lung carcinoma and bladder transitional cell carcinoma (Xia et al. 2008). Moreover, Ran over-expression in stably transfected cells has been reported to trigger an oncogenic transformation indicating that Ran up-regulation is involved in the loss of cellular growth control (Ly et al. 2010). Here, we found RanBP1 and RCC1 molecules up-regulated in both melanoma cell lines (Fig. 1C).

3.2 Functional connections among Ran and genes in microarray dataset

In order to identify functional connections among Ran members and other genes present in our dataset, IPA pathway analysis was performed to correlate molecules that met different criteria (type of molecules, tissues and cells, diseases etc.) starting from molecules of interest. AurkA, c-myc, PTEN (Phosphatase and Tensin homolog on chromosome TEN) and TERT (Telomerase Reverse Transcriptase) genes were connected by a functional relationship (Fig. 2).

To independently validate the transcriptional signatures obtained from microarray analysis, we analyzed the expression of these genes using qRT-PCR and Western blot. The qRT-PCR data confirmed the expression values detected by microarray for all genes (Table 1) present in the pathway (Fig. 2). In Western blot analysis, a high level of expression of AurkA and Ran was observed in both melanoma cell lines compared to melanocytes (Fig. 3 A).

Both Ran and AurkA gene expression was also investigated in 113 tissue samples derived from metastatic melanoma patients. We detected Ran in about 48% and AurkA in about 53% of samples (Fig. 3B). A strong correlation was observed between expression of the two genes, which is expected given that AurkA is a Ran effector molecule (Silverman-Gavrila et al. 2006).

To further confirm AurkA as one of downstream targets of Ran signaling in melanoma, Ran expression was silenced by means of small interfering RNA technology (siRNA), as described in Materials and Methods. Then, the Ran silencing effects on the AurkA expression, on cell survival and on invasion capability of both melanoma cell lines were examined. We observed a reduction in AurkA expression in both melanoma cell lines upon Ran silencing (Fig. 3C). A reduction of cell proliferation was also detected in Ran silenced A375 and 526 cells compared to the non-silenced ones (Fig. 3 D).

This finding may be the result of a cancer cell mitosis reduction, as previously reported (Xia et al., 2008). Ran silencing was confirmed analyzing Ran protein levels by western blot, as showed in Fig. 3C.

We also examined the effect of Ran silencing on invasive capability of these melanoma cell lines. Using an invasion assay, enhanced invasive ability of 526 melanoma cells was observed compared with A375 after Ran blocking (Fig. 3E). This finding is in contrast with data previously reported (Yuen et al. 2013; Deng et al. 2014), and suggests Ran independent mechanisms are involved in the invasive capability of melanoma cells.

Other genes included in the pathway with an opposite expression in melanocyte and melanoma cells were found associated with melanoma aggressiveness. In particular, the loss of PTEN has been related to the E- to N-cadherin profile switching, described during melanoma progression (Hao et al., 2012). TERT expression up-regulation has been previously reported to be related to mutations in the promoter region, causing an increase of its activity. It has also been described in familial and sporadic melanomas (Horn et al. 2013) and its amplification has been observed to be associated with poor outcome in acral lentiginous melanoma (Diaz et al. 2014).

3.3 Exposure of melanoma cells to MLN8054, a specific AurkA inhibitor

AurkA inhibition has been shown to limit tumor growth, impair mitosis and induce senescence in melanoma, suggesting a potential therapeutic role (Liu et al. 2013). Recently, we demonstrated the anti-tumor effects of an Aurk A inhibitor, as single agent and/or in various combinations with B-RAF and MEK inhibitors in a B-RAF (V600E) mutated human melanoma cell line (A375) and a three-dimensional (3D) human skin reconstruction model (Caputo et al. 2014).

Both melanoma cell lines, expressing high level of AurkA, were exposed to the AurkA inhibitor MLN8054 at different concentrations for 72 hours. As shown in Fig. 4A, both cell lines had an approximate 60% survival on exposure to MLN8054 at 1 μ M concentration. To further investigate the cytotoxic effect of MLN8054 on melanoma cells, we investigated PARP cleavage by Western blot. A PARP cleavage in both cell lines exposed to MLN8054

at 1 μ M concentration was seen, although the timing was different, being observed at 24 hours in A375 cells and 48 hours in 526 cells (Fig. 4B). This finding suggested that both melanoma cell types were induced to undergo apoptosis following exposure to MLN8054.

To confirm that MLN8054 was inhibiting AurkA protein, we analyzed p53 protein level, a downstream target molecule of AurkA. p53 has been reported to be phosphorylated by AurkA, leading to its ubiquitination and proteolysis (Katayama et al. 2004). As expected, p53 protein level was increased at 72 hours in both cell lines exposed to MLN8054 (Fig. 4C). We also analyzed p53 DNA sequence, identifying a single base pair substitution at codon 72 in the exon 4. This substitution is responsible for one amino acidic residue change from Pro (CCC) to Arg (CGC) in heterozygosis in the A375 cell line. No mutations in the p53 gene were observed in the 526 cell line.

Since both melanoma cell lines carried the B-RAF V600E mutation, we also tested if AurkA inhibitor had a cytotoxic effect in SK23Mel, a B-RAF wild-type melanoma cell line. We found that MLN8054 action was independent of B-RAF mutational status (Fig. 4D), suggesting its potential in all melanomas over-expressing AurkA irrespective of B-RAF status.

Since the V600E mutations in B-RAF are known to activate MEK, we also monitored MEK activation upon B-RAF inhibition by treating B-RAF mutated and wild-type melanoma cell lines with a B-RAF inhibitor. A decrease in the activated MEK1/2 protein level in both B-RAF-mutated melanoma cell lines exposed to B-RAF inhibitor was observed, while MEK activation was not altered in B-RAF wild-type melanoma cells (Fig. 4E).

3.4 Molecular pathway analysis of melanoma cells exposed to MLN8054

To investigate the effect of MLN8054 on the expression of genes involved in the above described molecular pathway, we analyzed c-myc, PTEN, AurkA, TERT and RCC1 molecules by Western blot and/or qRT-PCR. An increased c-myc protein level was observed in 526 melanoma cells, while a slight reduction was noted in A375 cells (Fig. 5A). It has previously been demonstrated that c-myc is involved in cancer cell invasion ability (Zhao et al., 2013). Our findings suggests that Ran knockdown was not sufficient to inhibit cell invasion in melanoma cells since the signalling triggered by Ran silencing through the AurkA down-regulation resulted in a stable and/or increased c-myc protein level in A375 and 526 melanoma cell lines, respectively, suggesting a c-myc-dependent and Ran-independent invasion mechanism in melanoma. In addition, an increased PTEN level was observed in both cell lines, with reduced AurkA and TERT mRNA level in 526 melanoma cells only, as shown in Fig. 5B. A decrease in TERT protein level was also observed in 526 melanoma cells by western blot, while no significant change was found in A375 cells (Fig. 5C).

These data suggest that, although MLN8054 showed a similar cytotoxic effect on both melanoma cell lines, the molecular mechanisms, underlining the cellular response to the treatment were different and dependent on the heterogeneity of cancer cells.

4 Discussion

Melanoma is characterized by chemo-resistance, aggressive clinical behavior and high metastatic spreading. Understanding the biological differences between normal and cancer cells is critical for the study of this malignancy since it will help enable the design of therapies that selectively kill cancer cells without toxicity to normal cells.

In this study, we described the molecular characterization of two melanoma cell lines with a melanocyte cell line as control, through the use of a combination of microarray and GSEA / IPA software analysis. Both of the melanoma cell lines, A375 and 526, have been reported to be positive for HLA-A2 phenotype but differ in the expression of the melanoma-associated antigens (MAAs), MART-1/Melan A and PMel 17/gp100, with 526 cells showing high expression of both MAAs compared to the A375 cells (Cormier et al., 1999). These melanoma cells were characterized for their B-RAF mutational status, with a homozygous mutation in A375 cells and a heterozygous mutation in 526 cells detected at the V600E site (data not shown), although this is not concordant with a previously published report (Sumimoto et al. 2004).

Gene expression analysis with IPA software provided interesting data on the potential proteins and molecular pathways associated with melanoma. Genes involved in the Ran signalling pathway were significantly over-represented and over-expressed in both melanoma cell lines compared with melanocytes. The GTPase Ran protein is one of the four key components (CRM1 / Exportin 1, RAN / RAN-GTPase, RANGAP1, and RANBP1) of the nucleocytoplasmic transport machinery (Pathria et al. 2012; Huang et al. 2009; van der Watt et al. 2009). In melanoma, a deregulation of nucleocytoplasmic transport has recently been reported with a consequent Erk cellular mislocalization, leading to an Erk aberrant cytoplasmic / prosurvival activity (Pathria et al. 2012). Interestingly, all the other nucleocytoplasmic transport component genes included in our dataset were found to be up-regulated in both melanoma cell lines. This finding confirms a deregulation of nucleocytoplasmic transport in melanoma, supporting an additional mechanism of enhanced cytoplasmic accumulation of several proteins that is adopted by cancer cells to achieve proliferative potential (Turner et al., 2008).

In addition, Ran plays a direct role in mitosis, by promoting bipolar spindle assembly (Gruss et al. 2001) through a group of effector proteins, including several microtubule-associated proteins (TPX2, NuMA, NuSAP and HURP), kinesin 5 and AurkA (Vader et al. 2008). Interestingly, in addition to over-expression of AurkA, a kinesin family member (KIF 1B) was also observed to be over-expressed in melanoma cell lines. AurkA is one member of a serine/threonine kinases family, which comprises three elements (AurK A, B, and C) that are essential components of the mitotic pathway (Vader et al., 2008); they ensure the proper chromosome assembly, the formation of the mitotic spindle, and cytokinesis. Over-expression of these kinases has been observed in several tumor types, including colon, breast, prostate, pancreas, thyroid, and head and neck, and is associated with advanced clinical stage and poor prognosis (Xu et al. 2006; Reiter et al. 2006; Nishida et al. 2007; Nadler et al. 2008).

We demonstrated that components of the mitotic pathways, Ran and AurkA, were highly expressed in tissue specimens deriving from patients with metastatic melanoma. Ran was detected in about 48% and AurkA in 53% of the 113 tissue samples analysed. As expected, given that AurkA is a Ran effector, the two genes showed a coordinated expression in melanoma tissue samples, assessed through mRNA profiling. The molecular pathway built by the IPA software tool, where Ran and AurkA were involved, also showed functional connections with c-myc, PTEN and TERT; c-myc and PTEN were down-regulated while TERT was up-regulated in both melanoma cell lines compared to melanocytes (Diaz et al, 2014). PTEN allele loss has previously been reported in at least 58% of melanoma metastases (Birck et al. 2000).

AurkA alone is not a potent inducer of cellular transformation and additional oncogenic events, such as Ras activation, are needed to reveal the oncogenic potential of the cells. However, evidence of its deregulated expression in tumorigenesis and its established role in mitosis suggest that inhibiting these kinases may constitute the basis for the development of powerful anti-cancer strategies in many tumors, including melanoma (Gautschi et al. 2006). Several small-molecules have already been developed as AurkA inhibitors (Gautschi et al. 2006; Marumoto et al. 2003; He et al. 2013) and we recently demonstrated the potential of AurkA inhibitors in the treatment of melanoma (Caputo et al. 2014).

We also found that melanoma cells had a PARP cleavage following exposure to the AurkA inhibitor, MLN8054, suggesting apoptosis in both cell lines, although the timings were different. Treatment of melanoma cell lines with AurkA inhibitors has previously been reported to induce an up-regulation of its downstream target p53 (Katayama et al. 2004), indicating that these inhibitors could regulate cancer cell survival in a p53-dependent manner, although the reduced cell viability that we observed in SK23Mel, a homozygous p53-mutant strain, upon exposure to MLN8054 did not exclude a p53-independent cell death regulation.

Changes in mRNA level of genes involved in the above identified pathway were studied upon MLN8054 exposure. A significant PTEN up-regulation was detected in both melanoma cell lines, suggesting that it may inhibit proliferation following AKT inactivation (Manning et al. 2007; Salmena et al. 2008). However, further experiments need to be performed in other melanoma cells to address this issue and the involvement of other molecular pathways, e.g. NF- κ B, cannot be excluded (Akca et al., 2011).

More interestingly, down-regulation of AurkA and TERT mRNAs was observed in 526 melanoma cells but not in A375, suggesting different molecular mechanisms triggered by MLN8054 exposure in the different cell types. Exposure to MLN8054 also resulted in increased c-myc protein level in 526 melanoma cells and a slight reduction in A375 cells, suggesting AurkA inhibition may interfere with MAPK molecular signaling in A375 melanoma cells only. Ran silencing induced AurkA down-regulation as well as reducing proliferation of both melanoma cell lines, supporting the potential role of Ran and AurkA as targets for melanoma treatment as previously reported (Xia et al. 2008; Simula et al. 2009; Caputo et al. 2014). Ran silencing also induced increased invasive capability in the 526 melanoma cell line but not in A375 cells. This finding is in contrast with data previously

reported (Yuen et al. 2013; Deng et al. 2014), and suggests a secondary role for Ran in the invasive properties of melanoma. It has been recently reported that c-myc plays a key role in enhancing the invasive capability of hepatocellular carcinoma cells (Zhao et al. 2013), suggesting that the increase of the 526 cell invasive capability was Ran-independent and may be c-myc-dependent, although further experiments are needed to address this issue.

Finally, the finding that SK23Me1, a B-RAF wild-type melanoma cell line, was sensitive to treatment with AurKA inhibitor suggested its possible role in the treatment of melanoma without B-RAF mutations. Our data showed the potential of AurKA and of the components of the nucleocytoplasmic transport machinery as targets for personalized therapy in melanoma. This finding, if confirmed, could result in a further possible option for personalized therapy in melanoma, alongside treatments targeting BRAF (Flaherty et al. 2010) and c-KIT (Carvajal et al. 2009). These targeted therapies offer an alternative to conventional chemotherapy, which has been disappointingly ineffective (Ascierto et al. 2010; McDermott et al. 2009). Our findings highlight the need for larger studies involving other melanoma cell lines that could lead to the identification of genes and pathways involved in the clinical progression of disease and the discovery of new markers for early diagnosis, helping to transform care through a personalized approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AurkA	Aurora kinase A
MM	metastatic melanoma
IPA	Ingenuity Pathway Analysis
GSEA	Gene Set Enrichment Analysis
FDR	false discovery rate

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Figure 1.

The most significant canonical pathways across the melanocyte (FOM78) and two melanoma cell lines (A375, 526), detected by Ingenuity. (A). Details of the number of up-regulated or down-regulated molecules in each canonical pathway for all the analyzed cell lines (B). IPA Ran signalling canonical pathway with the relative gene expression (red up-regulated; green down-regulated) in melanocytes and melanoma cell lines. The analysis showed that most of examined genes had a strong association with EIF2 Signaling, Ran Signaling, Cell Cycle Control of Chromosomal Replication and Pyrimidine Metabolism (C). The shape of the node indicated the function of the protein. The line denotes binding between two proteins, while the line with an arrow indicates an 'action on' of a molecule. A dotted line was used to indicate an indirect action.

Figure 2.

IPA functional pathway analysis. AurKA functional relationship with other differentially expressed genes observed in melanocytes (FOM78) and in melanoma cell lines (A375 and 526). For each gene the relationship and the expression (red up-regulated; green down-regulated) are shown. Arrows indicate the direction of the relationship.

Figure 3.

Western blot analysis of AurkA and Ran in melanoma cells and melanocytes (**A**); Dot plot showing differential distribution of AurkA and Ran genes intensities across 113 melanoma tissue samples. High intensities red dots, low intensities blue dots (**B**). Western blot analysis of AurkA and Ran expression in melanoma cells after Ran silencing (**C**). Melanoma cell proliferation analysis after Ran silencing (**D**). Invasive capability analysis of melanoma cell lines after Ran silencing. The percentages of invading (t=16 hours) cells were calculated as reported in Materials and Methods. The pictures highlight the differences in cell number between control no-targeted and Ran-directed siRNA oligonucleotides melanoma transfected cells, able to invade to the lower surface of transwells (**E**).

Figure 4.

Melanoma cell analysis on exposure to MLN8054. Cell viability analysis of A375 and 526 melanoma cells treated with MLN8054 (A). Western blot analysis of PARP (B) and of p53 (C) in melanoma cells upon MLN8054 treatment. *** indicates P -value 0.005. Cell viability analysis of SK23-Mel cells treated with MLN8054 (D). MEK activation analysis of melanoma cells, carrying and not-carrying B-RAF (V600E) mutation, upon treatment with B-RAF inhibitor (E).

Figure 5.

Aurka, PTEN, c-myc, RCC1 and TERT analysis in melanoma cells on exposure to MLN8054 and MLN8054 cytotoxic effect on B-Raf wild-type melanoma cell line. Western blot analysis of c-myc in melanoma cells on exposure to MLN8054 (A). mRNA expression levels of Aurka, PTEN, RCC1 and TERT genes in A375 and 526 cells non-treated (NT) and MLN8054 Treated (MLN8054) (B). TERT protein level analysis in melanoma cells on exposure to MLN8054 (C).

Table 1

Genes validation by RT-PCR primer sequences.

Gene	Description	Fold change		q-PCR	
		A375	526	A375	526
AURKA	aurora kinase A	1,951	1,262	3,320	4,679
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	−4,569	−10,053	−4,832	−6,209
PTEN	phosphatase and tensin homolog	−2,288	−1,080	−2,453	−1,873
RAN	RAN, member RAS oncogene family	7,545	5,045	10,560	7,453
RCC1	regulator of chromosome condensation 1	6,563	6,461	6,870	5,904
TERT	telomerase reverse transcriptase	7,359	5,306	8,402	6,004