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Original Articles

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

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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 ($p \leq 0.001$) 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, 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.

Introduction

The most aggressive form of skin cancer is melanoma, the incidence of which has dramatically increased worldwide [1]. Metastatic malignant melanoma has a very poor prognosis, with a median survival time of approximately 6 months [2]. 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 [3]. Activating mutations in N-Ras have been identified in 15–20% of all melanomas [4,5], 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–RAF–MEK–ERK pathway [5,6]. 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 [7].

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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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 [8] and the melanocyte cell line, FOM78 [9] as control. Gene expression was also investigated in 113 tissue samples derived from metastatic melanoma patients.

Materials and methods

Cell lines

Human melanoma cell lines A375 and 526 and melanocyte FOM78 [9] 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 3 mM 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% CO_2 atmosphere.

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 [10,11] and 6 μg of amplified χRNA was 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 αRNA 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/BRB-ArrayTools.html) [12]. Genes with intensity <100 in both channels, spots size <20 um 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.

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.

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).

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

Total RNA (300 ng) from melanocyte and melanoma cell lines was 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′-CACCACCAGCAGCGACTCT-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′-CATCACCAAGTGGTCGTTTCC-3′; TERT Forward 5′-GGCGACATGGAGAACAAGCT-3′, Reverse 5′-CCAACAAGAAATCATCCACCAAA-3′) were designed using Primer Express 2.0 software (Applied Biosystems). Actinβ was used as internal control (Forward 5′-TTCTACAATGAGCTGCGTGTG-3′ and Reverse 5′-GGGGTGTTGAAGGTCTCAAA-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 [13].

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 × *g* at 4 °C) for 15 minutes and the total protein concentration was determined in the supernatant by Bradford assay.

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 (1:10,000; 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 (±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: ***.

Gene expression analysis on melanoma tissues

Total RNA from 113 melanoma metastases from patients treated at the Surgery Branch, NCI, was extracted using miRNeasy minikit (Qiagen). RNA quality and quantity were 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).

Small interfering RNA transfection

Ran gene silencing by small interfering RNA (siRNA) was carried out with control no-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 (75 mM siRNAs and 10 μl of Lipofectamine RNAiMAX, Invitrogen). Cells were analyzed 48 hours post-transfection.

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% CO_2 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, 10×) 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 $P < 0.05$.