Nischarin expression may have differing roles in male and female melanoma patients

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SUPPLEMENTAL MATERIALS AND METHODS

Public datasets

Two publicly available datasets from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database – GSE3189 (7 normal skin, 18 nevi and 45 primary melanoma samples) [1] and GSE46517 (only samples from Medical University of Vienna, Austria that had available clinical information were selected – 7 normal skin, 9 nevi, 31 primary and 52 metastatic melanoma samples) [2] – were downloaded in pre-processed form. To eliminate multiple probes for the NISCH gene, GEO datasets were uploaded to the GSEA software (Broad Institute) to collapse the dataset from probes to symbols using the max_probe collapsing mode before further statistical analysis between different tissue samples. The Cancer Genome Atlas project (TCGA) skin cutaneous melanoma (SKCM) Broad Institute GDAC Firehose cohort (https://doi.org/10.7908/C11G0KM9) was analyzed using the web-based bioinformatics tool cBioPortal available from https://www.cbioportal.org/ [3, 4] and normalized transcriptomic dataset with 472 samples was downloaded from the site. Considering that TCGA SKCM and GSE46517 contained information about patients and samples, these two datasets were investigated for possible correlation of clinical or pathological data with *NISCH* expression.

For the patient overall survival analysis, TCGA SKCM primary tumor data available on HPA website (https://v21.proteinatlas.org/ENSG00000010322-NISCH/pathology/melanoma) was analyzed using best cut-off value for NISCH expression to obtain maximally separated Kaplan-Meier plots for all samples and individual sex subgroups. Additionally, 31 primary melanoma samples from the GSE46517 [2] were analyzed. Samples were divided into NISCH high and NISCH low expression groups where the median value of *NISCH* expression was used for determining the cut-off because of the small sample sizes of the groups.

Preprocessed DNA methylation data for TCGA SKCM dataset was downloaded from Mexpress (https://mexpress.be - accessed on 13 May 2021). Additionally, GSE86355 dataset [5] with information about methylation status in nevi, primary and metastatic melanoma was downloaded from GEO database. Mean beta values of the CpG probes located up to 1500bp upstream of *NISCH* transcription start site (TSS1500) were plotted. The mean beta value of normal skin and/or nevus samples was taken as the cut-off; and mean±SD was considered as the zone of uncertainty.

TCGA SKCM (https://doi.org/10.7908/C11G0KM9 - accessed on 12 May 2021) and Dana-Farber Cancer Institute (DFCI) [6] datasets with somatic copy-number alteration (CNA) information together with complete genetic and mutation status for NISCH gene were analyzed using cBioPortal platform. *NISCH* mRNA expression data, copy number variations and mutation details were retrieved and plotted using Graph Pad Prism.

Interactive web resource The Human Protein Atlas was used to investigate *NISCH* mRNA and protein expression levels in skin and melanoma samples as well as its localization in normal (https://v21.proteinatlas.org/ENSG00000010322-NISCH/tissue/skin) [7] and tumor cells (https://v21.proteinatlas.org/ENSG00000010322-NISCH/pathology/melanoma) [8].

Tissue samples

Thirty three formalin-fixed and paraffin-embedded (FFPE) tumor samples of 30 melanoma patients of Caucasian descent (3 nevi, 10 primary tumors, 12 lymph node metastases, 8 other distant metastases, non-matched) were retrieved from the tissue repository of the Institute for Oncology and Radiology of Serbia (IORS). The patients were diagnosed with melanoma according to clinical and histological criteria. Average melanoma patient age was 61 years (range 35-81), 18 males and 12 females. Supplementary table S1 lists clinical and histopathological characteristics of patient and tumor samples. The study was approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia (Approval No5549-01, from 11.12.2017) and was performed in accordance with the Helsinki Declaration of 1975, as revised in 2013. All patients signed an informed consent.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from FFPE samples using RNeasy FFPE Kit (Qiagen). The concentration and quality of the RNA were determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech), and cDNA was synthesized with RT² First Strand Kit (Qiagen) according to the manufacturer's instructions. The reactions were performed with Power Up SYBR Green Master Mix (Qiagen) using 50ng of cDNA in a 20µL reaction volume with 300nM primers (primer sequences listed in Supplementary table S2) using the 7500 Fast Real Time PCR system (Applied Biosystems). The

expression level data were normalized to beta actin (ACTB) expression and analyzed by the ΔΔ-Ct method. Expression of *NISCH* mRNA could not be measured in three tumor samples (1, 8 and 20) due to the high melanin content in the samples, and in four tumor samples (16, 21, 23 and 30) and one nevi (N1) due to low quality of the FFPE samples. After quality control, 2 nevi, 8 primary melanoma, 10 lymph node metastases, and 5 other distant metastases were used for further analysis.

Immunohistochemistry

Tissues were stained for NISCH expression, as previously described [9], with primary anti-nischarin antibody (BD Biosciences). Vimentin (clone V9, Thermo Lab Vision) and Hematoxylin and eosin (H&E) staining were performed by IORS Pathology service, as part of the diagnostic procedure [10].

Genomic microdeletion analysis

The extraction of genomic DNA from FFPE samples was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer protocol. Control DNA was extracted from lymphocytes of a healthy donor. Concentration and quality of the DNA were determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech). Quantitative RT-PCR was performed with Power Up SYBR Green Master Mix (Applied Biosystems) using 20ng of cDNA and 300nM primers [9] in a 20uL reaction volume. The reactions were performed using the 7500 Fast Real Time PCR system (Applied Biosystems). Standard curves for selected primers were created using $\log_{10}$ dilution series of genomic DNA at concentrations ranging from 0.1 to 100 ng. Slope values were calculated using GraphPad Software. Data normalization and copy number calculation were performed as previously described [11]. Sample was found positive for microdeletion if at least one primer pair indicated microdeletion.

Bisulfite conversion and methylation specific PCR

Previously extracted DNA was bisulfite converted using EZ DNA Methylation-Direct Kit (Zymo Research) according to the manufacturer protocol. Fully methylated DNA was used as a positive control (Human Methylated DNA, Zymo Research) and PCR-amplified un-methylated DNA was used

as negative control. Bisulfite converted DNA was used for PCR reaction using 25ng DNK and 300nM primers [12] in 20uL final reaction with AmpliTaq Gold 360 Master Mix (Applied Biosystems). Analysis was performed on a ProFlex PCR System (NISCH 56°C, ACTB 60°C, 40 cycles). PCR products were visualized on Agilent 2100 Bioanalyzer using DNA 1000 LabChip kit (Agilent Technologies). The relative level of *NISCH* promoter methylation was determined as a ratio of quantified methylated *NISCH* to *ACTB* (reference gene) multiplied by 1000. The mean value of nevi methylation level was set as the cut-off, and the mean±SD area was considered to be the zone of uncertainty. The samples with relative methylation level above the mean+SD were categorized as hyper-methylated compared to the normal tissue.

Gene Set Enrichment Analysis

Primary melanoma samples from GSE4651 and TCGA SKCM datasets were divided by patients' sex and analyzed with GSEA software using the Hallmark, KEGG and Reactome gene sets to find gene expression signatures associated with *NISCH. NISCH* mRNA expression was used as a continuous phenotype. Genes were ranked by Pearson correlation, and 1000 permutations with permutation type set to phenotype were used. A significant gene set was defined as the one with a nominal p value <0.05 and false discovery rate (FDR) <0.25 [13, 14].

Immune Infiltration Analysis

Infiltration Estimation file for all TCGA tumors was downloaded from TIMER2.0 website (http://timer.cistrome.org/) [15]. This file contained results of seven different cell type quantification methods (TIMER, CIBERSORT, CIBERSORT-ABS, EPIC, quanTIseq, xCell, and MCP-counter) that can estimate immune and non-immune cells content in the tumor samples based on the gene expression. All these methods can be used for comparison between samples, except CIBERSORT [15]. TCGA SKCM primary melanoma samples were selected to investigate the differences in high and low NISCH groups according to the best expression cut off survival analysis for both sexes.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 7 (GraphPad Software). For survival analysis the log-rank (Mantel-Cox) test was used. Differences between two groups of samples were determined using unpaired two-sided t-tests, and for three and more groups one-way ANOVA was used with Tukey's or Dunnett's multiple comparisons test. Results were considered significant when p was <0.05.

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