

ROR α and ROR γ expression inversely correlates with human melanoma progression

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ABSTRACT

The retinoic acid-related orphan receptors (RORs) regulate several physiological and pathological processes, including immune functions, development and cancer. To study the potential role of RORs in melanoma progression, we analysed ROR α and ROR γ expression in nevi and primary melanomas and non-lesional skin and metastases in relation to melanoma clinico-pathomorphological features. The expression of ROR α and ROR γ was lower in melanomas than in nevi and decreased during melanoma progression, with lowest levels found in primary melanomas at stages III and IV and in melanoma metastases. Their expression correlated with pathomorphological pTNM parameters being low in aggressive tumors and being high in tumors showing histological markers of good prognosis. Higher nuclear levels of ROR α and ROR γ and of cytoplasmic ROR γ correlated with significantly longer overall and disease free survival time. Highly pigmented melanomas showed significantly lower level of nuclear RORs. This study shows that human melanoma development and aggressiveness is associated with decreased expression of ROR α and ROR γ , suggesting that RORs could be important in melanoma progression and host responses against the tumor. Furthermore, it suggests that ROR α and ROR γ might constitute a novel druggable target in anti-melanoma management using tumor suppressor gene therapy restoring their normal functions.

INTRODUCTION

Melanoma incidence has been increasing during the last few decades with annual increase of incidence ranging from 3% to 7% in white population, especially in older individuals [1–4]. At the same time the mortality rates have stabilized or declined (2–2.6% annually) in younger population, while in older whites 50 and older mortality

rates have been increased (from 0.2 to 1.1 % per year [5]). The improved survival results mostly from diagnosis of early-stage melanomas [1]. The most efficient treatment mode is surgical excision, but it is limited to the localized disease (stage I and II). The efficacy of conventional therapies as surgery, chemotherapy, or radiotherapy for the treatment of advanced melanomas, is limited and related to adverse effects. In recent years new therapeutic

approaches, based on modulation of immune responses and targeting molecular pathways, have been approved for the treatment of metastatic melanoma [2]. These new anti-melanoma strategies are related to less adverse effects and improved disease free survival. However, they also poses some limitations [1]. Therefore, studies on new regulatory targets and effective approaches in treatment of advanced melanomas are fully justified.

The human nuclear receptor (NR) superfamily is a highly conserved family of transcription factors, which includes receptors for steroid hormones, vitamin D hydroxyderivatives, retinoids, thyroid hormones, and lipids and oxysterols [6–8]. The disturbances in NRs expression are observed in many pathological conditions. Previously we demonstrated that expression of nuclear receptor for vitamin D (VDR) in melanomas decreased with tumor progression. In addition, lack of VDR correlated with shorter overall survival (OS) and disease-free survival (DFS) time [9, 10]. The retinoic acid receptor-related orphan receptor (ROR) family consists of ROR α (NR1F1), ROR β (NR1F2), and ROR γ (NR1F3), members of the NR superfamily [12, 13]. Their activity is regulated by endogenous ligands, including 7-dehydrocholesterol, cholesterol and its derivatives [7, 11–13]. Most recently we demonstrated that ROR α and ROR γ are expressed in normal and pathological human skin and that hydroxyderivatives of vitamin D can act as reverse agonists of these receptors [14]. These novel vitamin D3 hydroxyderivatives are produced ex-vivo by adrenal and placenta fragments as well as epidermal keratinocytes [15], and are detectable in human epidermis and serum [16]. RORs demonstrate a typical NR modular structure, containing a highly conserved DNA binding domain and a less well conserved putative ligand binding domain. They regulate transcription by binding to ROR-responsive elements (ROREs) in the regulatory regions of target genes [6]. RORs are involved in the regulation of diverse, fundamental physiological processes related to cell development, growth, differentiation, apoptosis and immune functions [6, 17–19]. They show a tissue and/or cell type specific pattern of expression [20–30]. ROR α stabilizes p53 and enhances DNA damage-induced apoptosis through p53 [31]. It inhibits angiogenesis [32, 33] and inhibits the NF- κ B pathway [21, 34]. ROR α can also stimulate keratinocytes differentiation [35] and is expressed in all skin cell types [36, 14]. The human ROR γ gene generates two isoforms, which exhibit a distinct tissue-specific expression pattern and play important role in immune regulation (reviewed in [6, 18]. ROR γ t is essential for the polarization of CD4⁺ T helper cells into Th17 cells [37–39] and is involved in (auto)immune responses [40].

Biological functions regulated by RORs are important for the oncogenesis and tumor progression [41, 29, 42, 43]. Lack of ROR γ is related to a high incidence of thymic lymphomas in mice [19, 29]. Although a

tumor suppressive role of RORs was demonstrated in experimental *in vivo* and *in vitro* models [41, 29, 42–44], description of RORs expression in relation to human tumor progression and relation to clinico-pathological features has been superficial, at best. In addition, there is a lack of information on the role of ROR α and ROR γ in skin tumors. Although we detected a strong nuclear expression of both ROR α and ROR γ in human normal skin, ROR expression showed a very heterogeneous pattern in four invasive melanoma samples [14]. Therefore, we decided to examine ROR α/γ expression in benign (nevi) and malignant (melanomas) melanocytic tumors and to correlate their relationship with tumor progression and association with pathomorphological and clinical features including disease free and overall survival, to obtain patient relevant information on their role in human melanomagenesis and melanoma progression.

RESULTS

ROR α and ROR γ expression in human skin and melanocytic lesions

Expression of ROR α and ROR γ protein was detected in all analyzed normal and pathological samples (Figure 1A–1P). ROR α and γ showed both nuclear and cytoplasmic localization; however, the pattern of immunostaining was different for both receptors (Figure 1C–1G, 1K–1O). In keratinocytes of normal skin ROR α showed a predominantly nuclear localization, while in cells of melanocytic lesions (nevi, primary melanomas and melanoma metastases) both cytoplasmic and nuclear expression was equally observed. Detailed analysis revealed significantly higher expression of ROR α in nuclei of normal keratinocytes in comparison to melanocytic cells of pathological samples (Figure 1A), while the cytoplasmic ROR α expression in keratinocytes of normal skin was significantly lower than that in cell of melanocytic nevi and primary melanomas (Figure 1B). In melanocytic tumors, both nuclear and cytoplasmic ROR α gradually decreased with the progression of melanocytic lesions and melanomas (Figure 1B).

ROR γ showed significantly higher expression in both nuclei and cytoplasm of keratinocytes of normal skin in comparison to cells of melanocytic tumors, but exhibited similar cytoplasmic expression vs cells of melanocytic nevi (Figure 1I–1J). There was also a gradual decrease of ROR γ expression during progression of melanocytic tumors from nevus to primary melanoma with lowest expression seen in melanoma metastases. These differences were most pronounced for the nuclear localization.

Comparison of matched primary melanomas and its metastases revealed that in 24 out of 36 cases, nuclear ROR γ was higher in the primary tumors. Wilcoxon

matched-pairs signed rank test for 36 patients showed significant reduction of nuclear ROR γ in metastases when compared to primary lesions ($p = 0.028$).

Similar trends for ROR α and ROR γ expression were also observed for keratinocytes of normal skin vs keratinocytes of skin surrounding nevi and melanomas (Figure 2A–2J). Expression of both nuclear and cytoplasmic ROR γ in keratinocytes of skin surrounding nevi and melanomas gradually decreased being the lowest in skin surrounding melanomas (Figure 2C–2D, 2H–2J). For ROR α , this gradual decrease from keratinocytes of

normal to perilesional skin was found only for nuclear localization (Figure 2A, 2E–2G). Cytoplasmic ROR α was elevated in keratinocytes of skin surrounding nevi in comparison to keratinocytes of normal skin or keratinocytes of skin surrounding melanomas (Figure 2B, 2E–2G).

We also observed significantly higher ROR γ expression in lymphocytes infiltrating primary melanomas localized to the skin in comparison to lymphocytes surrounding metastatic melanomas in the lymph nodes (Figure 3A–3C). There was no relationship between levels

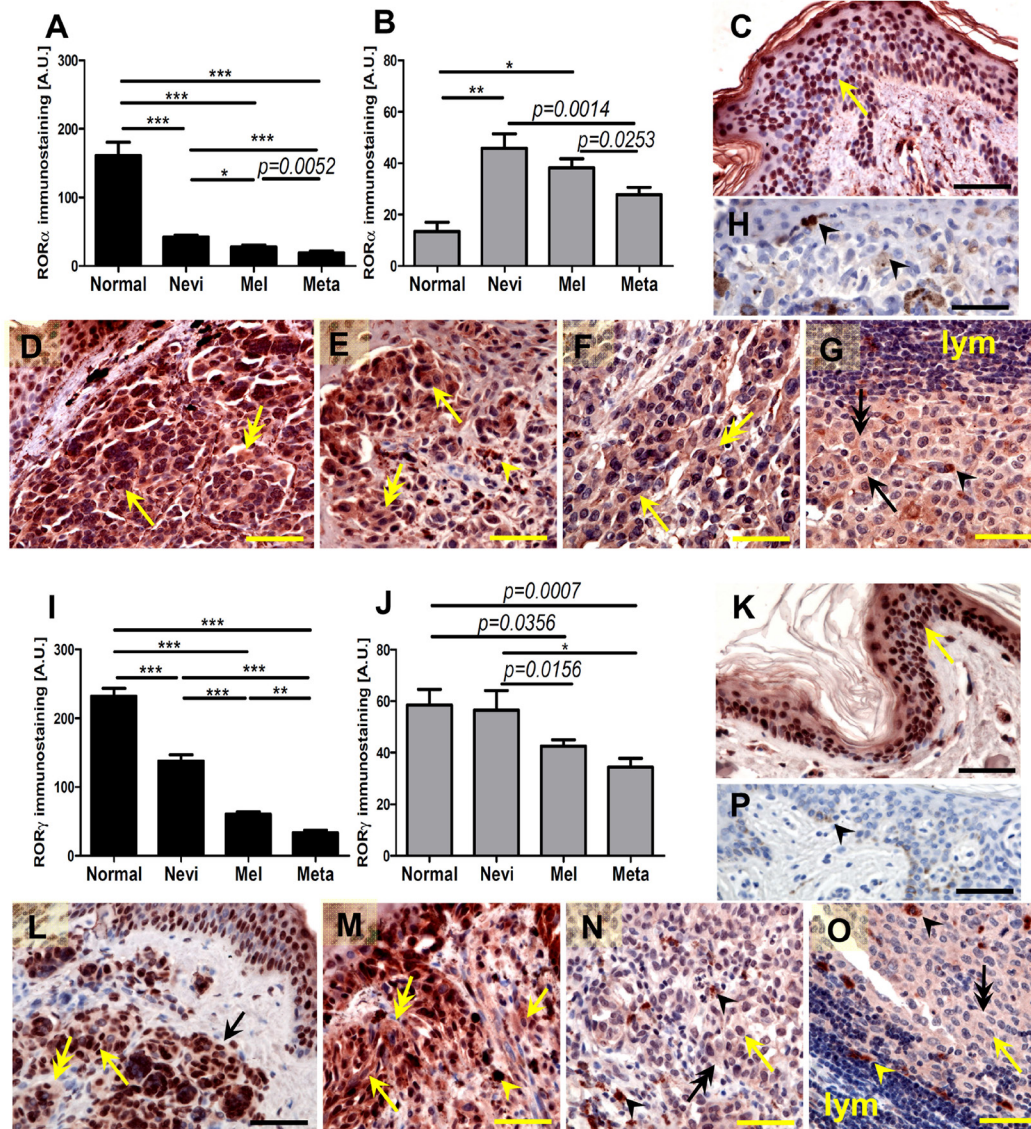


Figure 1: The mean level of nuclear (A, I) and cytoplasmic (B, J) ROR α (A, B) and ROR γ (I, J) in keratinocytes of normal skin, melanocytic cells of nevi, primary melanomas (MM) and metastases (meta). Statistically significant differences are denoted with P values as determined by Student's t -test and with asterisks by ANOVA (* $P < 0.05$, ** $P < 0.01$ and * $P < 0.001$). Representative ROR α (C-H) and ROR γ (K-P) immunostaining of normal skin (C, K), melanocytic nevi (D, L), early-stage melanomas (E, M), advanced melanomas (F, N) and lymph node metastases (G, O). Negative controls are presented at H and P panels. Arrows indicate nuclear staining in melanomas, arrow heads - melanin, double arrows - cytoplasmic staining, lymph - lymphocytes, scale bars - 50 μ m.**

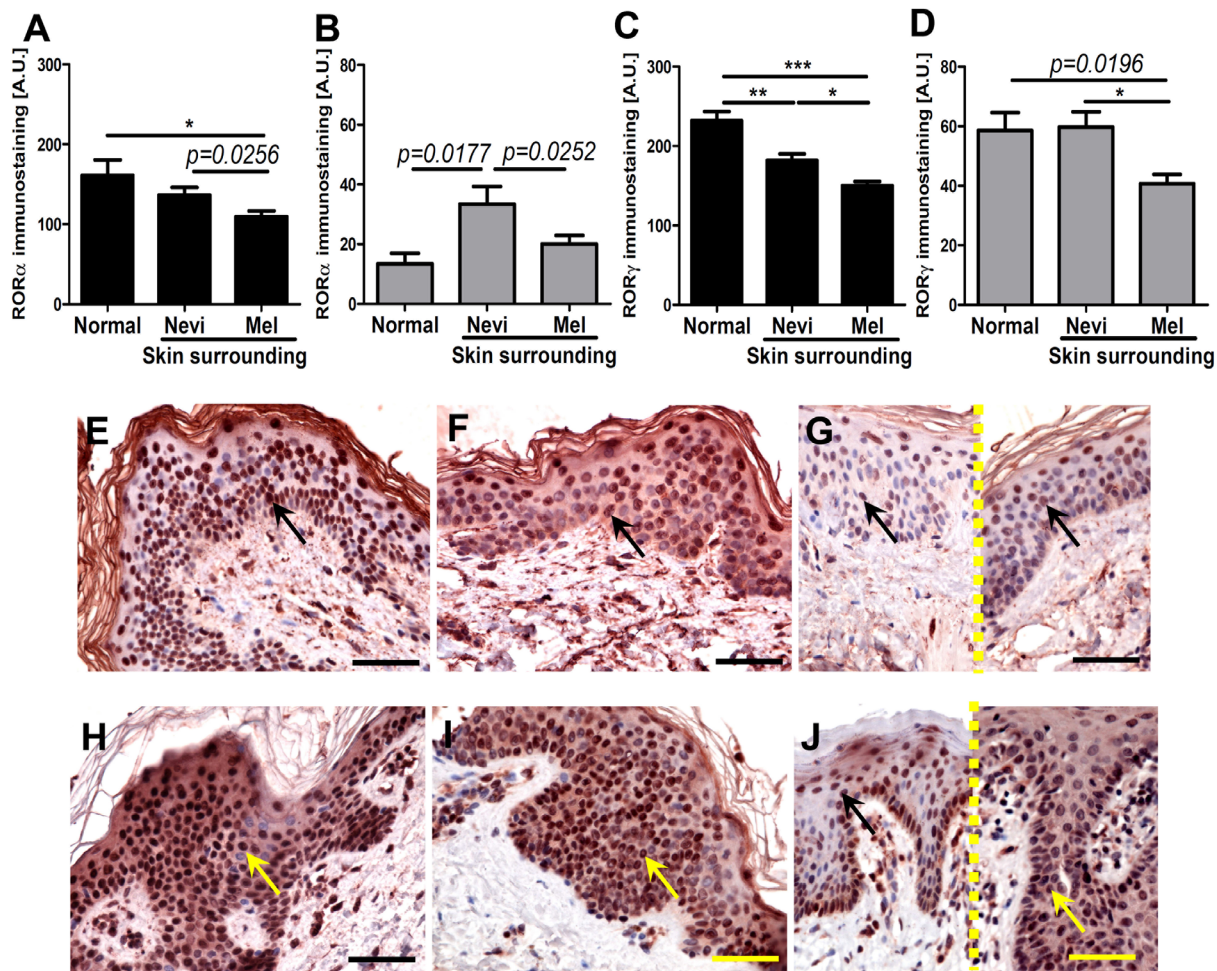


Figure 2: The mean level of nuclear (A, C) and cytoplasmic (B, D) ROR α (A, B) and ROR γ (C, D) in keratinocytes of normal skin and keratinocytes of skin surrounding melanocytic nevi and melanomas. Statistically significant differences are denoted with P values as determined by Student's t -test and with asterisks by ANOVA (* P <0.05, ** P <0.01 and * P <0.001). Representative ROR α (E-G) and ROR γ (H-J) immunostaining of normal skin (E, H), skin surrounding melanocytic nevi (F, I) and skin surrounding melanomas (G, J different cases, separated with dashed line). Arrows indicate nuclear staining in melanomas, scale bars - 50 μ m.**

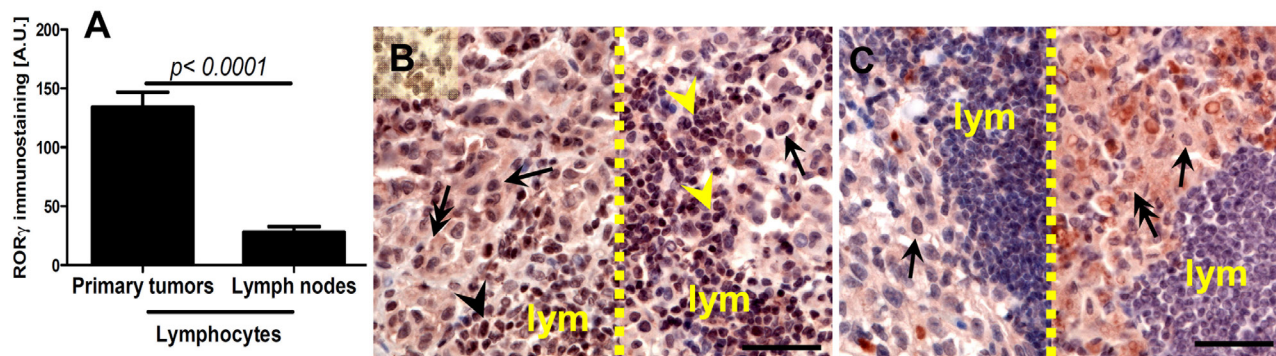


Figure 3: Comparison of ROR γ in lymphocytes in primary melanomas and metastatic lymph nodes (A). Statistically significant differences are denoted with P values as determined by Student's t -test. Representative ROR γ immunostaining of lymphocytes (lym) in primary melanoma (B, two different cases, separated with dashed line) and metastatic lymph node (C, two different cases, separated with dashed line). Arrows indicate nuclear staining in melanomas, arrow heads - staining in lymphocytes, double arrows - cytoplasmic staining in melanomas, scale bars - 50 μ m.

of ROR α and ROR γ expression and anatomic localization of the lesion.

ROR α and ROR γ expression in tissue samples is affected by melanogenesis

We also tested the correlation between melanin pigmentation and ROR α and ROR γ expression in primary melanoma tumors (Figure 4A–4B), and have found that its nuclear immunoreactivity was inversely correlated with high melanin content ($r=-0.2920$, $P=0.0052$ and $r=-0.2109$, $p=0.0399$, respectively). The melanomas with high melanin content showed significantly lower levels of nuclear ROR α and ROR γ than amelanotic and moderately pigmented melanomas (Figure 4A–4B). Moreover, these relationships were more pronounced for ROR α expression and vertical growth phase of melanomas (data not shown). There was no correlation for cytoplasmic ROR α and ROR γ expression and melanin content.

We also tested ROR α and ROR γ expression in cultured melanoma cells using immunofluorescence (IF) and Western Blot (WB) techniques (Figure 5). ROR α and ROR γ antigens were detected in both cytoplasmic and

nuclear compartments of melanoma cells (Figure 5A, 5B, 5E), and in positive controls consisting of HEPA cells overexpressing ROR α or ROR γ , while being absent in negative controls (Figure 5C). The phenotype of amelanotic and melanotic cells is presented in Figure 5D. The IF signals were higher in amelanotic cells vs melanotic melanoma cells (Figure 5A, 5B). The WB confirmed expression of ROR α and ROR γ in both nuclear and cytoplasmic fractions of human SKMEL and hamster AbC1 melanomas, as well as control HEPA cells overexpressing RORs (Figure 5E). WB analysis also showed a decrease (relative to Lamin A as control) in ROR α or ROR γ expression in melanized human melanoma nuclear extracts in comparison to amelanotic cells by 4% and 10%, respectively (Figure 5E).

ROR α and ROR γ expression correlates to melanoma advancement and melanoma prognostic markers (proliferation, ulceration, TILs, histological type)

ROR α and ROR γ expression inversely correlated with melanoma progression, aggressiveness and prognostic

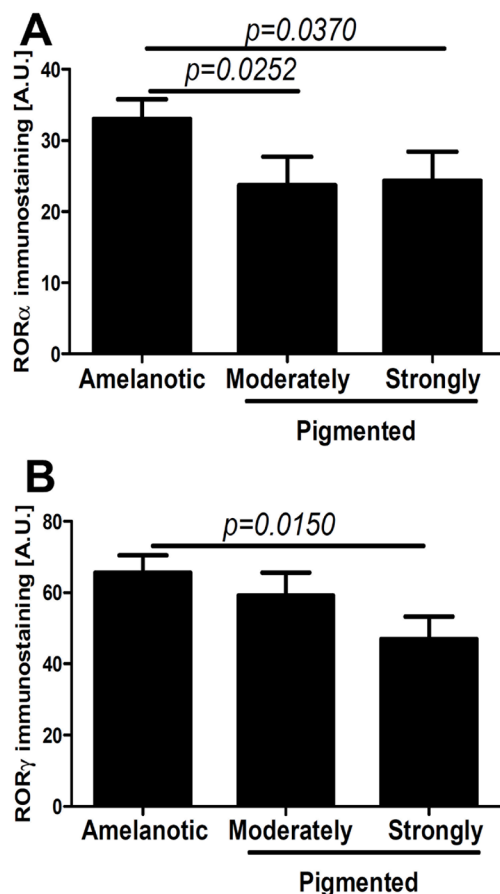


Figure 4: Comparison of nuclear ROR α (A) and ROR γ (B) in amelanotic, moderately and strongly pigmented primary melanomas. Statistically significant differences are denoted with P values as determined by Student's t -test.

markers with less advanced melanomas exhibiting higher expression of ROR α and ROR γ (Figure 6–Figure 11; Table 1). The detailed analysis of ROR α and ROR γ in primary melanomas stratified according to Clark and Breslow levels revealed significant negative correlation between expression of ROR α and ROR γ and progression of melanoma (Table 1). Melanomas at less advanced stages (Clark levels I and II and Breslow thickness ≤ 2 mm) (Figure 1E–1F, 1M–1N, Figure 6, Figure 7) showed highest level of both nuclear and cytoplasmic expression of ROR α and ROR γ . Moreover, a significant decrease of cytoplasmic and nuclear expression of ROR γ was seen in melanoma cells growing in the reticular dermis in comparison to the cells localized in the papillary dermis (Figure 12A). Analysis of mean staining intensity of

ROR γ showed similar trend (Figure 12B), although in some melanoma cases focally nuclear ROR γ was stronger in reticular than papillary dermis.

We found a similar correlation when primary melanomas were classified according to AJCC pTNM stage. The negative correlation was observed between pT stage and pN and nuclear and cytoplasmic level of ROR α and ROR γ (Table 1). The comprehensive analysis of mean level of ROR α and ROR γ revealed that less advanced melanomas (pTis, pT1, and pT2) showed significantly higher ROR α and ROR γ expression than more advanced melanomas (pT3 and pT4) (Figure 8A–8D).

We also analyzed in detail relative expression of RORs in relation to the ability to develop metastases and found that primary melanomas that metastasized showed

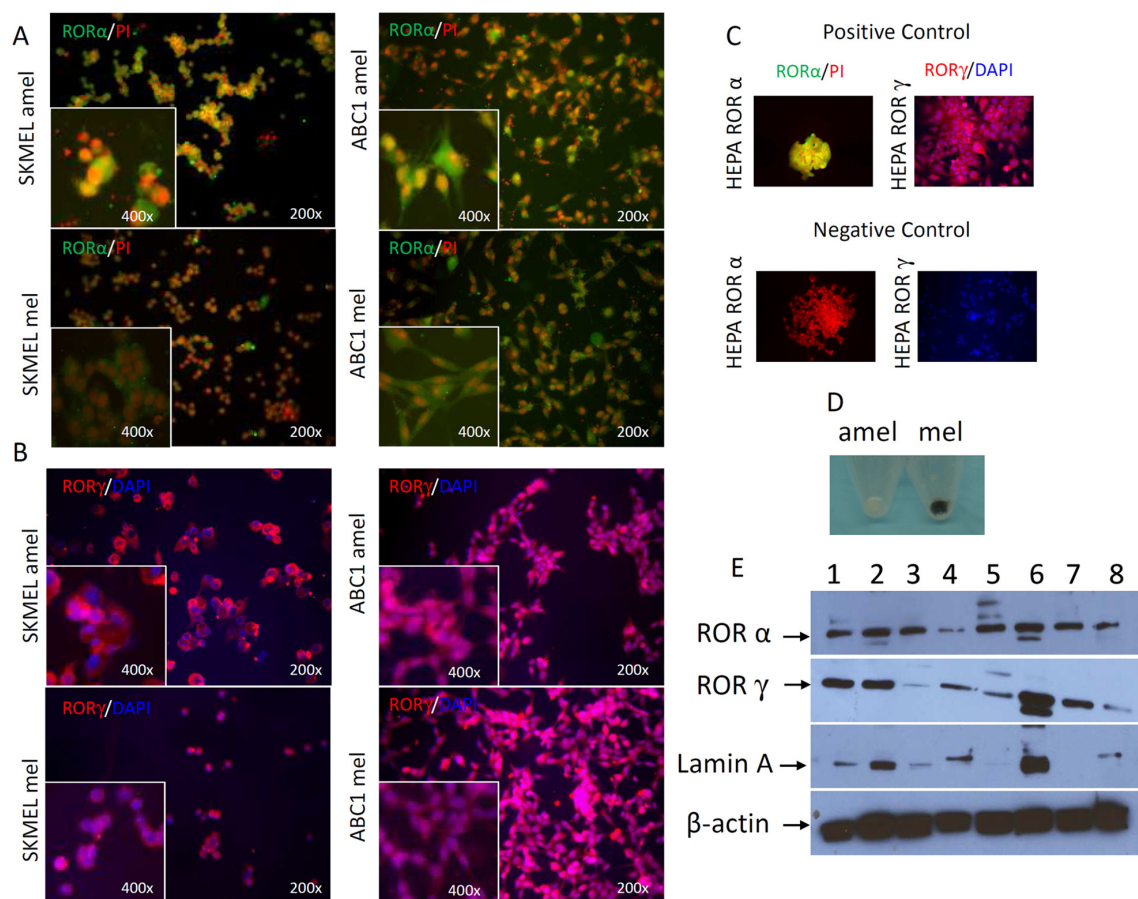


Figure 5: Changes in ROR α and ROR γ expression pattern in cultured melanoma cells after induction of melanogenesis. F-ICC, ROR α expression (green) in SKMEL amelanotic (amel), SKMEL melanotic (mel), ABC1 amelanotic (amel) and ABC1 melanotic (mel); PI was used to counterstain nuclei (A). F-ICC, ROR γ expression (red) in SKMEL amel, SKMEL mel, ABC1 amel, ABC1 mel; DAPI used to counterstain nuclei (B). Examples of positive and negative controls (C). The differences in pigmentation between melanotic (mel) and amelanotic (amel) SKMEL cells (D). WB analysis of ROR α and ROR γ expression (E). Proteins were extracted separately from cytoplasm (1, 3, 5, 7) and nucleus (2, 4, 6, 8) of human SKMEL (amelanotic 1-2, melanotic 3-4), hamster AbC1 (melanotic 5-6) melanomas and HEPA cells as a positive control (7-8) and stained with antibodies against ROR α , ROR γ . Arrows in two upper panels show proteins with an expected molecular mass of ~ 67 kDa for ROR α and of ~ 63 kDa for ROR γ . Two lower panels show, respectively, stains for Lamin A (nuclear marker) and β -actin (expressed in both compartments), which were used as loading controls (E).

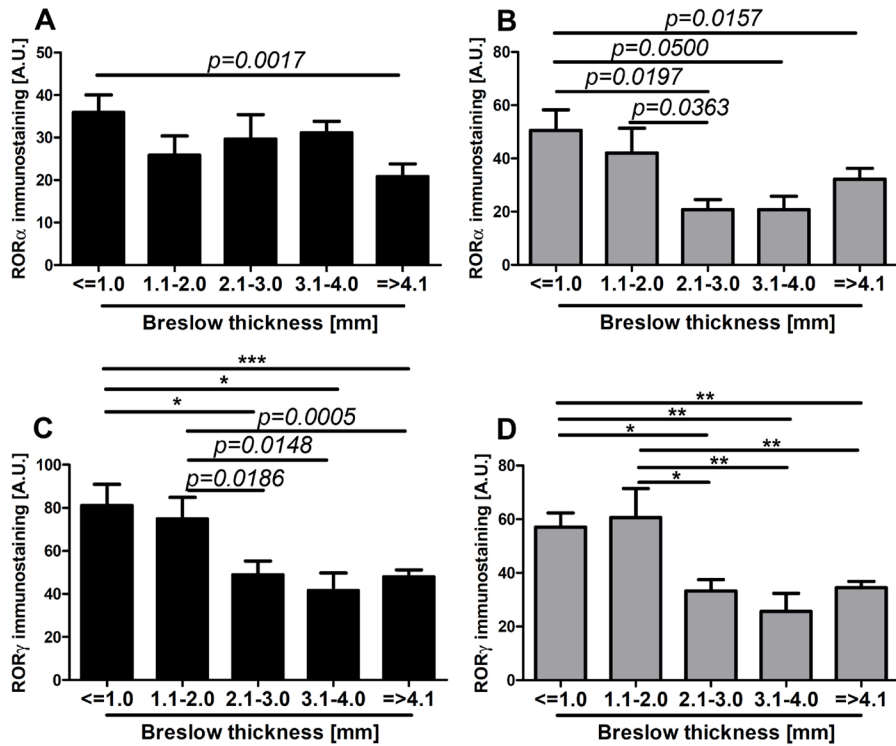


Figure 6: The mean level of nuclear (A, C) and cytoplasmic (B, D) ROR α (A, B) and ROR γ (C, D) in primary melanomas stratified according to the Breslow thickness. Statistically significant differences are denoted with *P* values as determined by Student's *t*-test and with asterisks by ANOVA (P*<0.05, ***P*<0.01 and ****P*<0.001).**

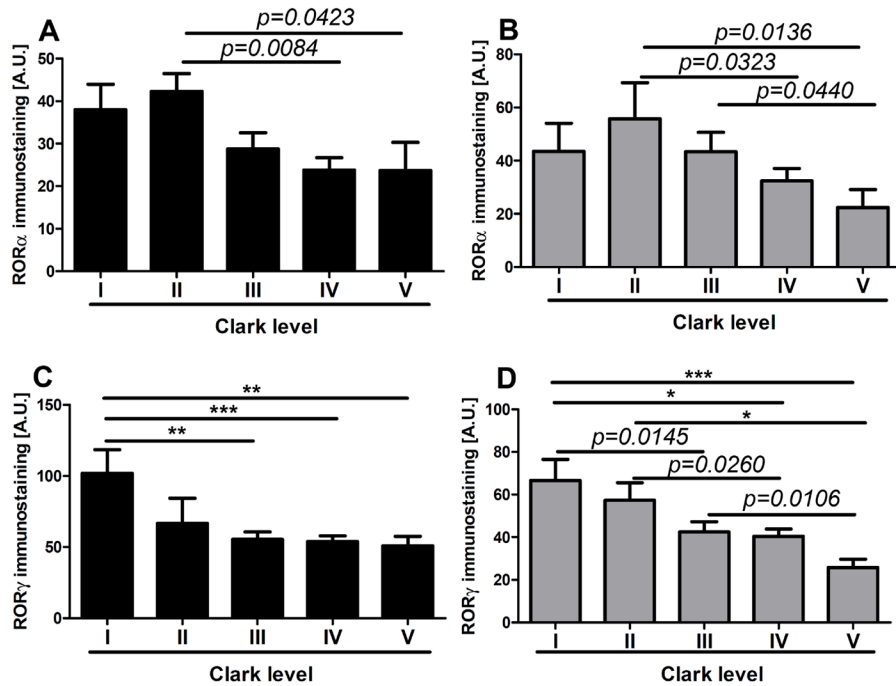


Figure 7: The mean level of nuclear (A, C) and cytoplasmic (B, D) ROR α (A, B) and ROR γ (C, D) in primary melanomas stratified according to the Clark level. Statistically significant differences are denoted with *P* values as determined by Student's *t*-test and with asterisks by ANOVA (P*<0.05, ***P*<0.01 and ****P*<0.001).**

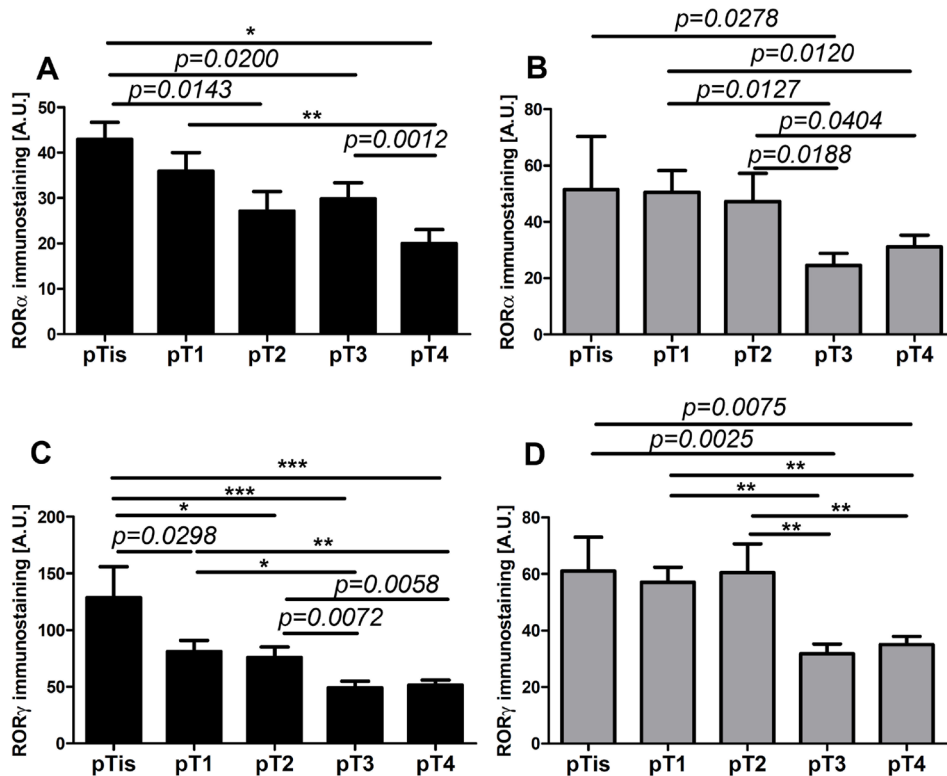


Figure 8: The mean level of nuclear (A, C) and cytoplasmic (B, D) RORα (A, B) and RORγ (C, D) in primary melanomas stratified according to the pT. Statistically significant differences are denoted with *p* values as determined by Student's *t*-test and with asterisks by ANOVA (P*<0.05, ***P*<0.01 and ****P*<0.001).**

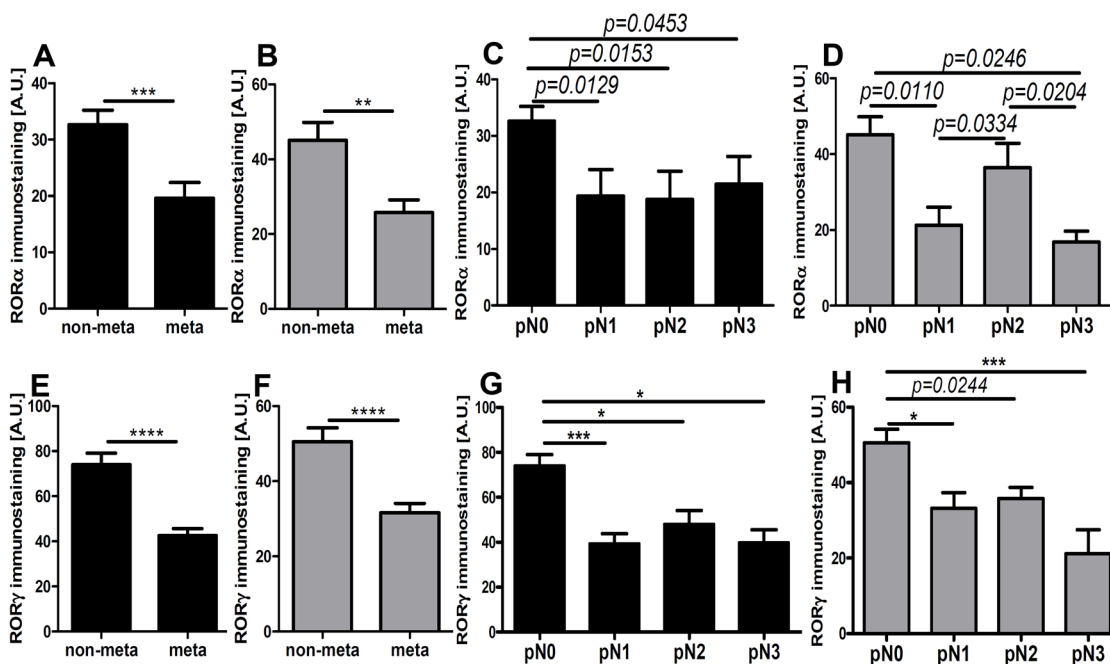


Figure 9: The mean level of nuclear (A, C, E, G) and cytoplasmic (B, D, F, H) RORα (A-D) and RORγ (E-H) in primary melanomas stratified according to the presence of metastases presence and pN status. Statistically significant differences are denoted with *P* values as determined by Student's *t*-test and with asterisks by ANOVA (P*<0.05, and ****P*<0.001).**

reduced nuclear and cytoplasmic expression of ROR α and ROR γ in comparison to non-metastasizing melanomas (Figure 9A–9B, 9E–9F). When primary melanomas were subgrouped according to pN stage, significant reduction of both nuclear and cytoplasmic of analyzed markers was observed in stages pN1, pN2, and pN3 (advanced stages) vs pN0 (Figure 9C–9D, 9G–9H).

Lastly, we analyzed ROR α and ROR γ expression in relation to overall stage of the diseases and found significant negative correlation between overall stage and nuclear and cytoplasmic levels of ROR α and ROR γ (Table 1). Furthermore, analysis of primary melanomas stratified according overall stage demonstrated that melanomas at very early stages of development (stage 0 and 1) had significantly higher both nuclear and cytoplasmic ROR α and ROR γ levels than primary melanomas at stage II and primary melanomas with regional and distant metastases (stages III and IV). This trend was observed for melanomas at stages assessed at the time of diagnosis

(data not shown) and during progression of the disease, as assessed during follow-up (Figure 10A–10D).

Next, we analyzed the correlation between ROR α and ROR γ levels in primary melanomas and the presence of tumor-infiltrating lymphocytes (TILs), proliferation activity and histological type. Both nuclear and cytoplasmic ROR α correlated positively with TILs, but for ROR γ such correlation was found only for the nuclear localization (Table 1). Specifically, significantly higher nuclear and cytoplasmic ROR α and nuclear ROR γ levels were found in melanomas with brisk TILs (Figure 11A–11D). The presence of ulceration was correlated with significant reduction of both nuclear and cytoplasmic stains for ROR α and ROR γ (Figure 11E–11H; Table 1). Similarly, more aggressive nodular type melanomas (NM) showed substantial decrease of ROR α and ROR γ expression (both nuclear and cytoplasmic) in comparison to superficial spreading (SSM) melanomas (Figure 11I–11L). Proliferation activity, as assessed by Ki-67

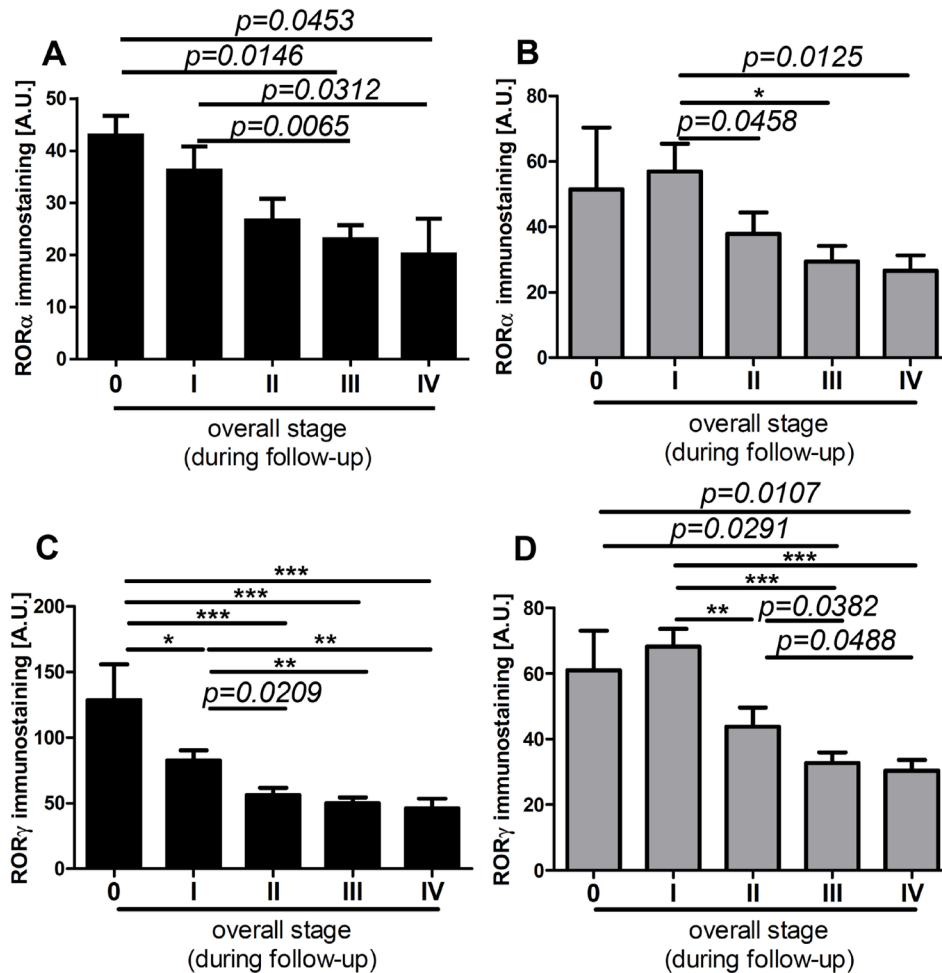


Figure 10: The mean level of nuclear (A, C) and cytoplasmic (B, D) ROR α (A, B) and ROR γ (C, D) in primary melanomas stratified according to the disease stage assessed during follow-up of patients. Statistically significant differences are denoted with *P* values as determined by Student's *t*-test and with asterisks by ANOVA (**P*<0.05, ***P*<0.01 and ****P*<0.001).

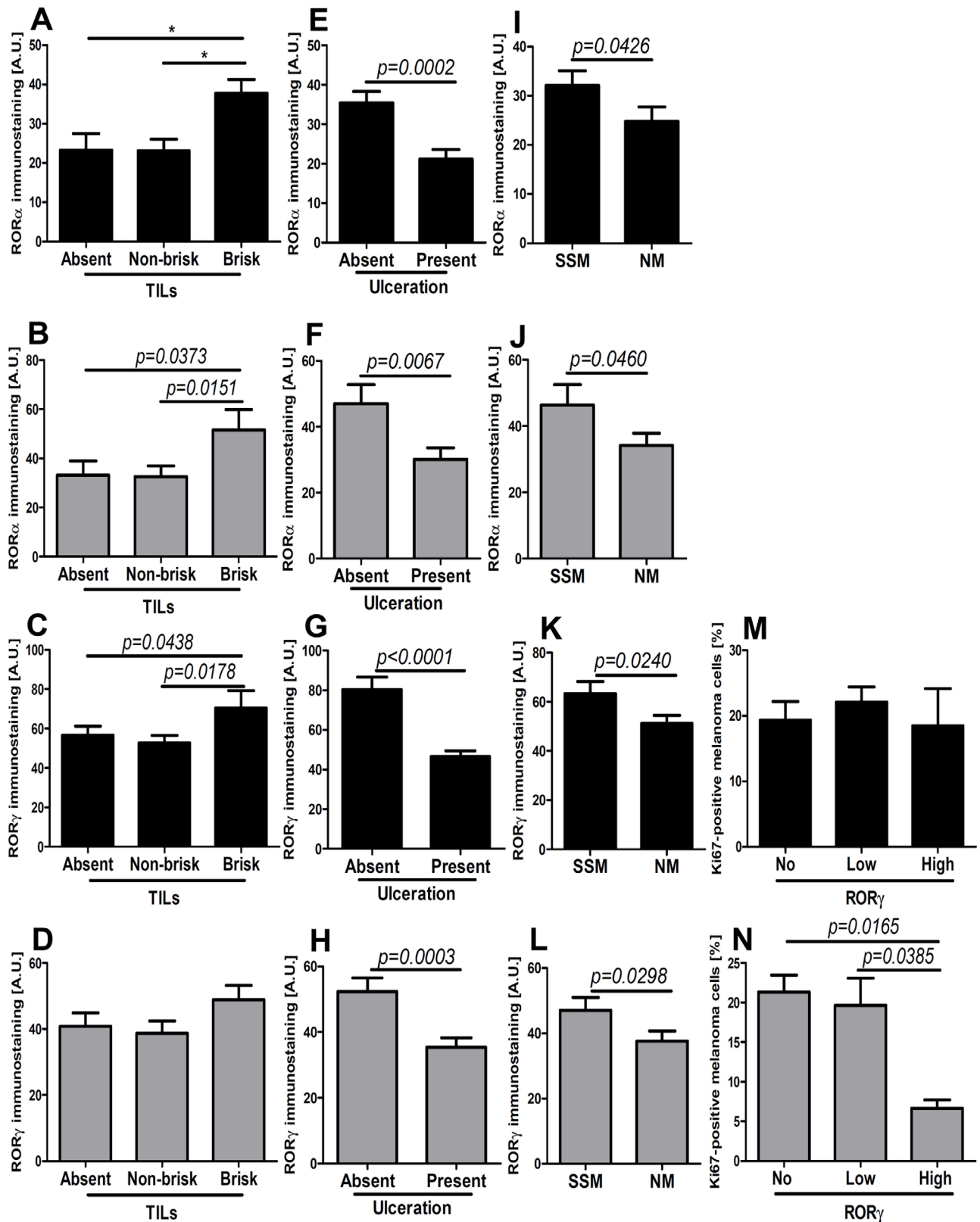


Figure 11: Comparison of mean level of nuclear (black bars) and cytoplasmic (gray bars) ROR α (A, B, E, F, I, J) and ROR γ (C, D, G, H, K-N) in primary melanomas without and with tumor-infiltrating lymphocytes (TILs) (A-D), without and with ulceration (E-H), stratified according histological type (I-L, SSM - superficial spreading melanomas; NM - nodular melanomas) and Ki-67 immunostaining (M, N). Statistically significant differences are denoted with *P* values as determined by Student's *t*-test and with asterisks by ANOVA (P*<0.05, ***P*<0.01 and ****P*<0.001).**

Table 1: Correlation of ROR α and ROR γ levels with pathomorphological features

| | Nuclear ROR α | Cytoplasmic ROR α | Nuclear ROR γ | Cytoplasmic ROR γ |
|--|-----------------------|--------------------------|-----------------------|--------------------------|
| Clark level (n=79) | r=-0.1952 p=0.0424 | r=-0.2521 p=0.0125 | r=-0.3238 p=0.0020 | r=-0.4065 p=0.0001 |
| Breslows thickness (n=79) | NS | r=-0.1918 p=0.0485 | r=-0.2777 p=0.0076 | r=-0.3152 p=0.0028 |
| pT (n=79) | r=-0.3364 p=0.0012 | r=-0.2940 p=0.0043 | r=-0.3696 p=0.0005 | r=-0.3884 p=0.0002 |
| pN (n=79) | r=-0.2810 p=0.0066 | r=-0.2470 p=0.0152 | r=-0.3428 p=0.0011 | r=-0.3442 p=0.0011 |
| Overall stage* (n=79) | r=-0.3134 p=0.0035 | r=-0.3094 p=0.0039 | r=-0.4977 p<0.0001 | r=-0.3857 p=0.0003 |
| Overall stage** (n=79) | r=-0.3260 p=0.0017 | r=-0.2868 p=0.0052 | r=-0.4897 p<0.0001 | r=-0.4410 p<0.0001 |
| TILs (n=76)# | r=0.3589 p=0.0008 | r=0.2213 p=0.0282 | r=0.2623 p=0.0106 | NS |
| Ulceration (n=78)## | r=-0.3725 p=0.0004 | r=-0.1635 p=0.0776 | r=-0.4302 p<0.0001 | r=-0.3318 p=0.0016 |
| Proliferative index (Ki-67 immunostaining) (n=74)### | NS | NS | NS | r=-0.2159 p=0.0333 |

* assessed at the time of diagnosis

** assessed during follow-up

NS- not statistically significant

- missing data for 3 cases

- missing data for 1 case

- missing data for 5 cases

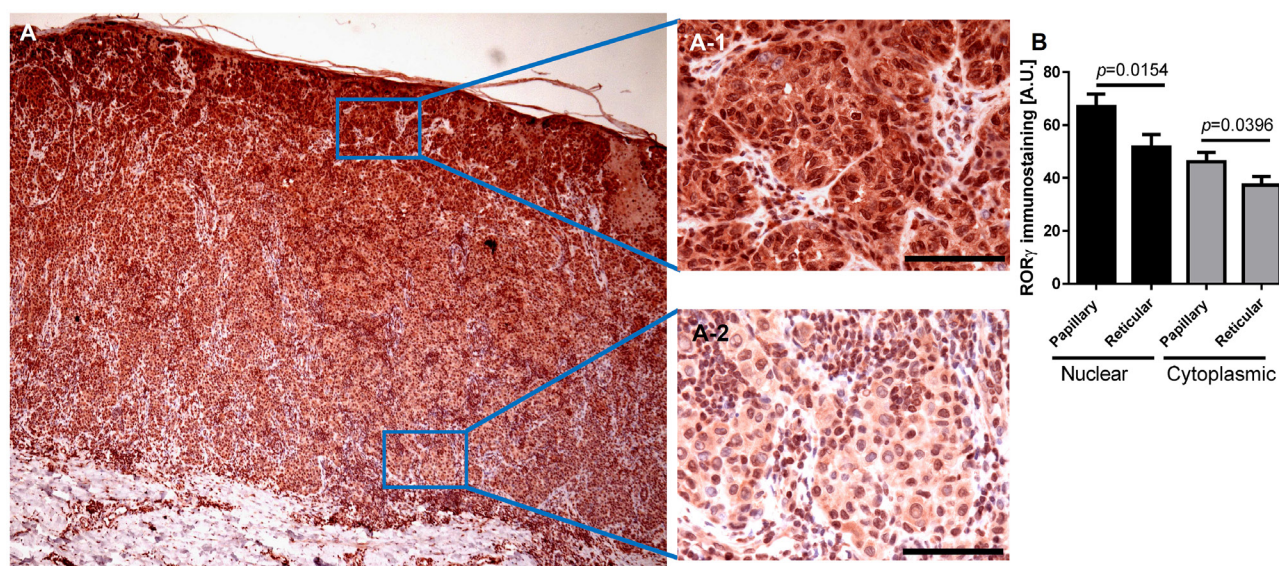


Figure 12: Representative immunostaining of ROR γ in melanoma. ROR γ expression gradually decreased with increasing melanoma depth (A) A-1 and A-2 represent fragments indicated by squares in Figure (A) Scale bars - 100 μ m. (B) Mean nuclear (black bars) and cytoplasmic (gray bars) ROR γ immunostaining in melanoma cells within the papillary and reticular dermis. Statistically significant differences are denoted with *P* values calculated with t-test.

immunocytochemistry, had no correlation with cytoplasmic and nuclear ROR α levels (Table 1) or nuclear ROR γ expression (Figure 11M), except that higher cytoplasmic ROR γ staining was observed in melanomas with lower proliferation levels (Figure 11N). There was no relationship between ROR α and ROR γ levels and mitotic rate calculated as the mean number of mitoses per mm² (data not shown).

ROR α and ROR γ expression affects OS and DFS of melanoma patients

Importantly, there was a correlation between the expression of ROR α and ROR γ in primary melanomas and OS and DFS in cohort of melanoma patients included into this study. Nuclear ROR α and ROR γ affected both OS and DFS (Figure 13A–13F, Table 2). High nuclear level of ROR α and ROR γ correlated with significantly longer OS ($\chi^2 = 9.179$, $P = 0.0102$ and $\chi^2 = 8.335$, $P = 0.0156$, respectively) and DFS ($\chi^2 = 9.947$, $P = 0.0069$ and $\chi^2 = 6.104$, $P = 0.0473$, respectively).

Detailed analysis of melanoma patients stratified according to the level of nuclear ROR α expression revealed longer OS in patients with high (median survival 896.1 days) expression of the receptor in comparison to those that lacked nuclear stain (median survival 618.3

days) ($\chi^2 = 7.420$, $P = 0.0064$, Figure 13A, Table 2). Similar differences were observed also in DFS between cases without ROR α and with high nuclear ROR α (median disease free survival 288.3 days vs 689.7 days; $\chi^2 = 4.697$, $P = 0.0302$, Figure 13B, Table 2) and cases with low vs high ROR α expression (median disease free survival 275.4 days vs 689.7 days; $\chi^2 = 8.835$, $P = 0.0030$, Figure 13B, Table 2).

Similar differences were seen in OS curves of patients with melanomas lacking nuclear ROR γ vs those with high nuclear ROR γ ($\chi^2 = 7.768$, $P = 0.0053$) or melanomas with low vs high nuclear ROR γ stains ($\chi^2 = 4.336$, $P = 0.0373$) (Figure 13C, Table 2). Also DFS curves showed significant differences for patients with melanomas expressing high ROR γ vs melanomas negative for ROR γ ($\chi^2 = 6.797$, $P = 0.0091$) and melanomas with low nuclear ROR γ ($\chi^2 = 4.510$, $P = 0.0337$; Figure 13D, Table 2).

Because of low number of cases with cytoplasmic stain for ROR γ ($n = 3$), the patients were stratified into two groups: with and without ROR γ and statistically significant differences were found for OS and DFS curves in these groups ($\chi^2 = 9.093$, $P = 0.0026$ for OS and $\chi^2 = 12.560$, $P = 0.0004$ for DFS) (Figure 13E, 13F, Table 2). Interestingly, no correlation was found for OS and DFS in

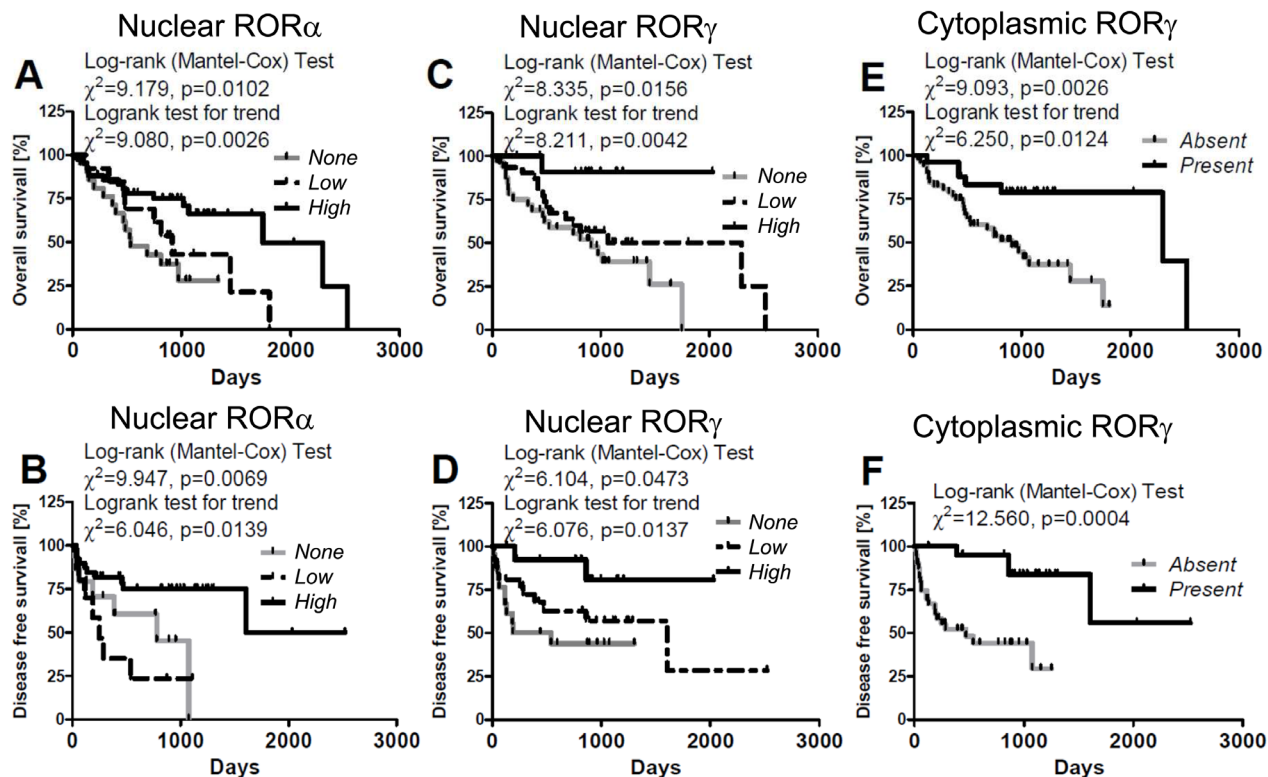


Figure 13: Correlation of overall survival (A, C, E) and disease-free (B, D, F) survival time with relative expression of nuclear ROR α (A, B) and ROR γ (C, D) and cytoplasmic ROR γ in primary melanomas (E, F). Melanomas were classified as “high”, “low” and “none” according to RORs SQ-score; for details see Material and method section: 4.1.3. Evaluation of ROR α and ROR γ immunostained sections.

Table 2: Comparison of overall survival and disease free survival time in patients with ROR α and ROR γ expression in primary melanomas and metastases (Log-rank Mantel-Cox Test)

| Expression of: | Total cases (n) | Deaths (n/%) | Median/mean overall survival (days) | Statistically significant p values | Metastases (n/%) | Median/mean disease free survival (days) | Statistically significant p values |
|--------------------------|-----------------|--------------|-------------------------------------|------------------------------------|------------------|--|------------------------------------|
| nuclear ROR α | | | | | | | |
| None | 22 | 14/63.6 | 618.3/530.0 | | 13/59.1 | 288.3/69.00 | |
| Low | 14 | 9/64.3 | 843.1/876.0 | P>0.05* | 11/78.6 | 275.4/127.0 | P>0.05* |
| High | 43 | 16/37.2 | 896.1/899.0 | P=0.0064* P>0.05^ | 15/34.9 | 689.7/597.0 | P=0.0302* P=0.0030^ |
| cytoplasmic ROR α | | | | | | | |
| None | 14 | 8/57.1 | 720.7/876.0 | | 7/50.0 | 470.3/263.0 | |
| Low | 18 | 11/61.1 | 813.4/819.0 | P>0.05* | 12/66.7 | 338.7/152.0 | P>0.05* |
| High | 47 | 20/42.6 | 841.4/825.0 | P>0.05* P>0.05^ | 21/44.7 | 580.9/429.0 | P>0.05* P>0.05^ |
| nuclear ROR γ | | | | | | | |
| None | 32 | 20/62.5 | 733.9/779.0 | | 19/59.4 | 298.3/114.0 | |
| Low | 33 | 18/55.5 | 872.4/829.0 | P>0.05* | 18/55.5 | 539.2/284.0 | P>0.05* |
| High | 14 | 1/7.1 | 860.7/877.0 | P=0.0053* P=0.0373^ | 2/14.3 | 842.7/877.0 | P=0.0091* P=0.0337^ |
| cytoplasmic ROR γ | | | | | | | |
| None | 53 | 30/56.6 | 713.9/714.0 | | 32/60.4 | 340.5/127.0 | |
| Low | 23 | 8/34.8 | 1007.0/940.0 | P=0.0080* | 7/30.4 | 818.7/916.0 | P=0.0006* |
| High | 3 | 0/0.0 | 1101.0/1204.0 | P=0.0231* P>0.05^ | 1/33.3 | 1101.0/1204.0 | P=0.0405* P>0.05^ |

* versus No ROR

^ versus Low ROR

patients stratified according to cytoplasmic expression of ROR α (Table 2). The t-test analysis confirmed Mantel-Cox test both for OS and DFS (data not shown).

Similarly, a significant better prognosis was also observed for melanoma patients with nuclear ROR α immunolocalization and with nuclear and cytoplasmic ROR γ immunolocalization after adjustment for Breslow thickness (Table 3). After adjustment for overall stage these relationship were significant for DFS both for nuclear ROR α immunolocalization and nuclear and cytoplasmic ROR γ immunolocalization (Table 3) and for OS only for nuclear ROR γ ($\chi^2=22,396$, $p<0.0001$, $b=-0,391$, $SE=0,343$, $p=0,0357$, $Exp(b)= 0,676$, 95% CI of $Exp(b)=1,4475$ to $3,0928$).

DISCUSSION

This study represents the first comprehensive analysis of ROR α and ROR γ in human melanocytic tumors in relation to several clinico-pathomorphological features associated with various stages of melanoma progression. Specifically, nuclear expression of ROR α and ROR γ was lower in cells of melanomas in comparison to melanocytic nevi and keratinocytes of normal skin and decreased during progression of melanoma, with lowest levels found in primary melanomas at stages III and IV and in melanoma metastases. Concerning cytoplasmic expression, ROR α levels were lower in keratinocytes of normal skin vs cells of melanocytic nevus and primary

Table 3: Survival analysis using Cox proportional-hazards regression analysis of risk factors in relation to RORs immunostaining in human melanomas adjusted for Breslow thickness (A) and overall stage (B)

| A | | | | | | | |
|---|----------|---------|--------|-------|---------|--------|----------------|
| | χ^2 | P value | b | SE | P value | Exp(b) | 95% CI |
| Overall survival | | | | | | | |
| Nuclear RORα | 12.557 | 0.002 | -0.804 | 0.357 | 0.024 | 0.448 | 0.222 to 0.902 |
| Nuclear RORγ | 13.859 | 0.001 | -0.864 | 0.349 | 0.013 | 0.421 | 0.212 to 0.835 |
| Cytoplasmic RORγ | 19.810 | <0.0001 | -1.481 | 0.492 | 0.003 | 0.227 | 0.087 to 0.596 |
| Disease-free survival | | | | | | | |
| Nuclear RORα | 7.349 | 0.0254 | -0.660 | 0.439 | 0.132 | 0.517 | 0.219 to 1.221 |
| Nuclear RORγ | 8.586 | 0.0137 | -1.132 | 0.438 | 0.010 | 0.322 | 0.136 to 0.762 |
| Cytoplasmic RORγ | 17.831 | 0.0001 | -2.111 | 0.653 | 0.001 | 0.121 | 0.034 to 0.436 |
| B | | | | | | | |
| Disease-free survival | | | | | | | |
| Nuclear RORα | 44.427 | <0.0001 | -0.954 | 0.453 | 0.035 | 0.385 | 0.159 to 0.937 |
| Nuclear RORγ | 44.757 | <0.0001 | -0.917 | 0.439 | 0.037 | 0.400 | 0.169 to 0.945 |
| Cytoplasmic RORγ | 46.838 | <0.0001 | -1.543 | 0.683 | 0.024 | 0.214 | 0.056 to 0.816 |

b – regression coefficient; SE - standard error; Exp(b) – relative risk; 95% CI - 95% confidence interval for Exp(b); Lack of RORs (= 0) versus RORs present (= 1).

melanomas; however, a decrease of its expression was seen in primary and metastatic melanomas vs melanocytic nevus. Cytoplasmic ROR γ was higher in keratinocytes of normal skin and cells of melanocytic nevi vs cells of localized and metastatic melanomas. Moreover, ROR α and ROR γ expression correlated with pathomorphological pTNM parameters. Higher RORs levels positively correlated with markers of good prognosis such as SSM vs NMM histological type, presence of brisk TILs, absence of ulceration and low proliferation activity.

Until recently, there was a shortage of information on RORs expression in resident cells of human skin, except of few papers analyzing expression of ROR α [35, 45, 36]. In human keratinocytes ROR α promotes epidermal differentiation and expression of structural proteins involved in lipid barrier formation [35, 45]. Importantly, the same authors [35] found reduced ROR α expression in skin squamous cell carcinoma tumors and squamous cell lines, indicating reverse correlation between epidermal cancer and ROR α expression. Previously, we reported ROR α and ROR γ expression in cells of human normal skin, including epithelial cells of the epidermis, hair follicle, sebaceous and sweat glands, dermal fibroblasts, immune cells and cultured melanocytes and melanoma lines [14]. Since initial studies have shown that the expression of ROR α and ROR γ in melanomas was

aberrant and heterogeneous, further detailed examination is presented in this study.

The decreased expression of ROR α and ROR γ during progression of melanocytic lesions and the significant reduction of their expression in most advanced primary melanomas (Breslow thickness >2mm, Clark level III-V, pT3-4, pN1-3, overall stage II-IV) and melanoma metastases suggest that reduced or lack of ROR α and ROR γ expression or defects in the ROR pathways could promote tumor progression. Lack or significant reduction of ROR α and ROR γ expression in primary melanomas and melanoma metastases would be consistent with this concept. As mentioned above, ROR α and ROR γ exhibit, inter alia, anti-tumor effects and disturbances in their functions and expression can contribute to a development and progression of malignant lesions. A reduced level of ROR α has been associated with several other cancers, including hepatocellular, breast, lung, colon, cervical, ovary and prostate cancers [46, 47, 44]. In mouse models activation of ROR α inhibited growth of androgen-independent prostate cancer cells [41]. Increased ROR α expression suppressed also the aggressive phenotype in breast cancer models, including reduced colony size, inhibited cell proliferation, invasion and migration [48]. Moreover, reduced ROR α levels correlated with shorter OST in patients with breast and hepatocellular

cancers [46, 48]. The crucial role of RORs as anti-tumor factors is further supported by studies showing a negative correlation of ROR α and ROR γ expression with pathological grade, increased incidence of tumor recurrence, vascular invasion, clinical outcome, including distance metastasis free survival and OST [46, 49, 50]. Correspondingly, in the present study the significant reduced ROR α and ROR γ expression in melanomas correlated to poorer clinical outcome, as determined by overall and disease-free survival time. Furthermore, in melanomas lower level of ROR α and ROR γ was associated with markers of poor prognosis such as absent or non-brisk TILs, nodular histological type, presence of ulceration and higher proliferation index, indicating that a reduced level of ROR α and ROR γ promotes a more aggressive melanoma phenotype. Together, these results suggest that RORs are very important in oncogenesis and tumor progression. Positive correlation between decreased ROR α and ROR γ expression with progression of melanomas suggests the potential usefulness of ROR α and ROR γ as new, immunohistochemical marker in melanoma diagnosis, predicting future behavior of the lesion. Additionally, the shorter overall and disease-free survival time when ROR α and ROR γ were absent suggests that their presence or absence could serve as a prediction marker of clinical outcome.

The reduced ROR α and ROR γ levels were also observed in melanized melanomas. This was further substantiated by testing of melanoma lines in which induction of melanogenesis by L-tyrosine [51, 52] was accompanied by reduced levels of nuclear ROR α and ROR γ . This is consistent with our previous observation showing the reduction of OS and DFS of patients with a pigmented melanomas at stage 3 and 4 [53], and on expression of several vitamin D related pathways such as VDR, CYP24A1 and CYP27B1 [9, 54, 10] and HIF-1 α [55]. These results are also in accordance with a hypothesis that active melanogenesis can promotes melanoma progression [56, 57, 52, 58], and with previous research showing that melanogenesis can increase immune-, chemo- and radioresistance of melanoma cells [59–64], and affects survival of melanoma patients after radiotherapy [65], which suggests that inhibition of melanogenesis can be considered as an adjuvant treatment in melanoma therapy [66, 67].

The reduced RORs expression in melanoma progression might promote tumor growth via multiple pathways, which are linked to the role of RORs in the regulation of circadian rhythm, glucose and lipid metabolism, or immune system (reviewed in [68]). For example, disturbance of circadian rhythm can promote cancer development [69], and a reduction of RORs expression promotes aerobic glycolysis and tumor cell growth (reviewed in [70, 68]). Finally, RORs, especially ROR γ , are important regulators of inflammation and Th17 immune responses [68, 71]. Therefore, a reduced expression of RORs may affect a delicate balance between anti- and pro-tumorigenic actions of immune response.

Ligands for RORs, include several intermediates of the cholesterol biosynthetic pathway, cholesterol, and hydroxy derivatives and esters of cholesterol, which can act either as agonists or inverse agonists [7, 11–13]. Furthermore, previously we found that VDR expression decreased during progression of melanocytic lesions [9, 10] and that ROR α and ROR γ can act as receptors for novel vitamin D hydroxyderivatives [14]. Therefore, we suggest that disturbances in expression of ROR α and ROR γ in melanomas in combination with perturbations in skin endogenous metabolic pathways related to production of active forms of vitamin D or other secosteroids or sterols with their respective phenotypic actions could contribute to melanomagenesis and melanoma progression. This concept could be evaluated using appropriate animal models including patient-derived orthotopic xenografts, which mimic clinical tumor growth and metastasis [72, 73]. We also plan to perform an in depth mechanism oriented studies to define modes of action of RORs in melanomagenesis using patient-derived orthotopic xenografts and transgenic mice.

In conclusion, our studies indicate that development and progression of melanoma is associated with decreased expression of ROR α and ROR γ , and that downregulation of RORs level is associated with unfavorable clinical prognosis. These data indicate that expression and localization of RORs may serve as diagnostic tools and prognostic markers of the diseases outcome. We also suggest that targeting ROR α and ROR γ signaling may represent a promising strategy for melanoma treatment.

MATERIALS AND METHODS

Clinico-pathological studies

Tissue samples of human cutaneous melanocytic tumors

The patients were qualified into this retrospective study based on preliminary clinic-pathological data obtained from database of the Oncology Center in Bydgoszcz, Poland. Preliminary selection of patients was random and the criterion for the searching the electronic database of patients included the diagnosis of primary melanoma in the Oncology Center, followed by verification of paraffin formalin-fixed paraffin-embedded tissue blocks quality and the availability of tissue tumor in the section after routine diagnostic procedures. Normal skin samples [n=11] were obtained from patients who underwent surgery not related to skin diseases, while primary melanoma samples [n=79], melanoma metastases [n=39] and nevi [n=27] were excised from patients treated in the Oncology Centre in Bydgoszcz during the period 2003-2010 [54, 9]. Clinico-pathomorphological characterization of the patients included into this study is presented in Table 4. The mean follow-up time was 26.8 months (ranging from 1.4 to 84.0 months).

Table 4: Patient and melanoma characteristics

| Clinico-pathologic features | n |
|------------------------------------|-----------------------------------|
| Type of lesions | |
| All samples | 156 |
| Melanocytic nevi | 27 |
| Primary melanomas | 79 |
| Nodular | 39 |
| Superficial spreading | 38 |
| Acral | 2 |
| Melanoma metastases | 39 |
| Normal skin | 11 |
| Age (y) | |
| Melanocytic nevi | Mean 40, median 35 (range 20-85) |
| Melanomas | Mean 61, median 59 (range 25-100) |
| Male/female ratio | |
| Melanocytic nevi | |
| M | 7 |
| F | 20 |
| Melanomas | |
| M | 44 |
| F | 35 |
| Anatomical Site | |
| Melanocytic nevi | |
| Extremities | 6 |
| Head and neck | 5 |
| Trunk | 16 |
| Melanomas | |
| Acral | 2 |
| Anogenital | 3 |
| Extremities | 28 |
| Head and neck | 16 |
| Trunk | 30 |
| Breslow thickness (mm) | |
| in situ | 5 |
| 0-1 | 18 |
| 1.1-2 | 11 |
| 2.1-3 | 8 |
| 3.1-4 | 5 |
| >4.0 | 32 |
| Clark level | |
| I | 10 |
| II | 7 |
| III | 23 |
| IV | 27 |
| V | 12 |

(Continued)

| Clinico-pathologic features | n |
|--|-------|
| pT | |
| pT0 | 5 |
| pT1 | 18 |
| pT2 | 11 |
| pT3 | 13 |
| pT4 | 32 |
| pN | |
| pN0 | 51 |
| pN1 | 12 |
| pN2 | 10 |
| pN3 | 6 |
| pM (at the time of diagnosis/during follow-up) | |
| pM0 | 79/70 |
| pM1 | 0/9 |
| Overall stage (at the time of diagnosis/during follow-up) | |
| 0 | 5/5 |
| 1 | 21/20 |
| 2 | 24/17 |
| 3 | 29/28 |
| 4 | 0/9 |

M – males, F - females.

Melanomas were classified AJCC Melanoma Staging and Classification [74, 75], briefly: pTis - melanoma in situ; pT1 – melanoma ≤ 1.00 mm in thickness, pT2 - 1.01-2.00 mm in thickness, pT3- 2.01-4.00 mm in thickness, pT4 - > 4.00 mm in thickness and N0 – no regional lymph node metastases detected, N1 - 1 metastatic lymph node presents, N2 – 2-3 metastatic lymph nodes present, N4 - 4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes. The study using human skin samples was approved by the Committee of Ethics of Scientific Research of Collegium Medicum of Nicolaus Copernicus University, Poland (approval number KB 448/2009).

ROR α and ROR γ immunostaining

ROR α and ROR γ immunostaining was performed on standard formalin-fixed paraffin-embedded 4 μ m sections of human samples, as described previously [14]. Briefly, after antigen retrieval sections were incubated overnight at 4°C with ROR α or ROR γ antibodies (respectively, goat anti-ROR α antibody, clone C-16, Santa Cruz Biotechnology, Dallas, TX, dilution 1:25 and rabbit anti-ROR γ antibody, generated and tested as previously described [14, 76], dilution 1:50. Then, sections were incubated for 30 min with secondary antibody (VECTASTAIN® Elite ABC Goat IgG, Vector

Laboratories Inc., Burlingame, CA), followed by 30 min with ImmPACT™ NovaRED™ HRP substrate (Vector Laboratories Inc., Burlingame, CA) and visualisation with ImmPACT NovaRED (Vector Laboratories Inc., Burlingame, CA) for ROR α immunostaining. For ROR γ detection, sections were further incubated with secondary anti-rabbit antibody EnVision™ FLEX /HRP (Dako, Carpinteria, CA) and Vector NovaRED (Vector Laboratories Inc., Burlingame, CA). After counterstaining with hematoxylin, sections were dehydrated and mounted in permanent medium (Consul Mount; Thermo Fisher Scientific Inc. Waltham, MA, USA). Formalin-fixed paraffin embedded samples of brain and spleen of wild type and knockout mice were used for the assessment of the specificity of the ROR α and ROR γ antibodies, respectively (Figure 14). The ROR γ KO mice (B6;129-Rorc^{tm1Amj}) were described previously [77] and the ROR α -deficient mice (ROR α sg/sg) were obtained from The Jackson Laboratory. Tissue sections were immunostained as described above with either the ROR α antibody (1:500 dilution) or the ROR γ antibody (1:250 dilution). Slides were either analyzed by Nikon Eclipse 80i light microscopy (equipped with Nikon Digital Sight DS Fi1-U2 digital camera and NIS-Elements BR 3.0 software (Nikon Instruments Europe BV, Badhoevedorp, The Netherlands).

Evaluation of ROR α and ROR γ immunostained sections

Immunostained sections of normal and pathological skin were analyzed without knowing the detailed histopathological diagnosis, malignancy grade and other clinical data. ROR α and ROR γ immunostaining intensity of melanomas was scored semiquantitatively, taking into consideration both staining intensity and percentage of immunostained tumor cells or, in normal skin, epidermal cells. The staining intensity was evaluated with reference to immunostaining of control, epidermal cells, scored as strong. The semiquantitative score was calculated as: SQ=mean (IR x SI), where IR is the percentage of immunoreactive cells and SI is the staining intensity from 0 to 3 arbitrary units (A.U.) with 0 as negative (0), weak (1), moderate (2) and strong (3). Then, cases were stratified according to RORs SQ-score as follows: SQ 0.0-10.0=none ROR α , SQ 10.1-50.0=low ROR α , SQ 50.1-300.0=high ROR α , SQ 0.0-50.0=none

ROR γ , SQ 50.1-100.0=low ROR γ , SQ 100.1-300.0=high ROR γ .

Proliferative activity, melanin content evaluation and TILs were performed as previously described [53, 9, 54, 10, 78, 79].

Analysis of OS and DFS time

Clinico-pathological data related to the time between primary treatment (surgical excision) and histopathological diagnosis of melanoma to diagnosis of metastases were obtained from electronic database of the Oncology Center, Bydgoszcz Poland. Metastases were diagnosed by histopathological diagnosis of resected lesions, fine needle aspiration biopsy and/or diagnostic imaging techniques (magnetic resonance imaging, computed tomography). The date of deaths was obtained from the Department of Registry Office in Bydgoszcz, Poland. All data are presented according to REMARK guidelines [80].

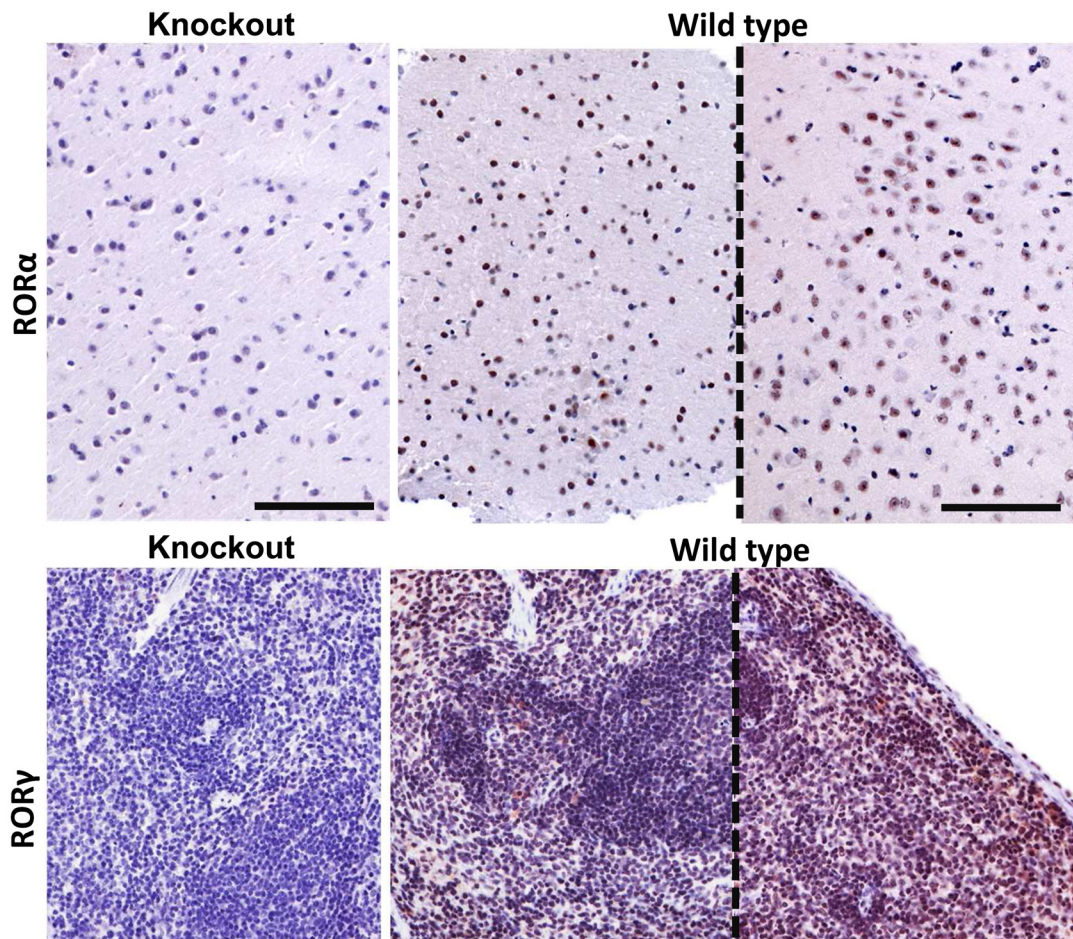


Figure 14: ROR α (upper panel) and ROR γ (lower panel) immunostaining of brain and spleen tissue sections of ROR α or ROR γ knockout mice and wild type mice (sections from two different mice, separated with dashed line). The mouse brain samples were stained with anti-ROR α antibody and mouse spleen with anti-ROR γ antibody. Scale bars - 100 μ m.

Cell culture-based study

Cells

Human SKMEL-188 and AbC1 hamster melanoma cells were cultured in Ham's F10 supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin/amphotericin, Sigma-Aldrich, St. Louis, MO) as described previously [51, 14]. Cells cultured in this medium remained amelanotic [51, 14]. Cells were seeded in chamber slides (for immunofluorescence study) or 60 mm dishes (for western blot study) in triplicate and after achieving 60% confluence, the media were change to the Ham's F10 medium supplemented with L-tyrosine (400 μ M) to induce melanin pigmentation or Ham's F10 without addition (to maintain amelanotic phenotype) and after 4 days, pigmented and non-pigmented cultures were fixed in 4% paraformaldehyde or harvested by trypsinization for western blot analyses (WB). Human hepatoma (HEP) control cells overexpressing either ROR α or ROR γ were cultured as described previously [14] and processed for immunofluorescence or WB as above.

Fluorescent immunocytochemistry (F-ICC)

We have followed protocols described previously in [81, 14]. Briefly, permeabilization and blocking was performed with 0.2% Triton X-100 and 5% donkey serum in PBS for 1h, RT. Primary polyclonal antibody against goat ROR α (#sc-6062, Santa Cruz Biotech, Santa Cruz, CA) and rabbit ROR γ (developed at NIEHS [14, 76]) diluted in the same blocking solution were applied overnight at 4 °C. After extensive rinsing in PBS, the secondary species specific antibody [donkey anti goat Ig γ conjugated Alexa488 (green) or donkey anti rabbit Ig γ conjugated Alexa594 (red); both from Santa Cruz] were applied for 1 h, RT. The positive control consisted of hepatoma cells (HEPA) overexpressing either ROR α or ROR γ , the negative control constituted the same F-ICC procedure except of omitting of the primary antibody. After extensive rinsing the slides were topped with mounting media allowing for nucleus visualization with either propidium iodide (PI, red) or 4',6-diamidino-2-phenylindole (DAPI, blue) and examined under fluorescent microscope (Leica, Digital DM4000B, Buffalo Grove, IL) equipped with appropriate filters capable for visualization of green, red and blue fluorophores and conjugated to a digital camera. Pictures were further analyzed for intensity of immunocomplexes with the use of ImageJ software (National Health Institute, Bethesda, MD).

Western blot (WB)

Detailed description of WB method is provided in [14]. Shortly, harvested cells were subjected to nuclear and cytoplasmic protein isolation with Nuclear Extraction Kit (#400100, Active Motif, Carlsband, CA) according to the manufacturer instructions. Equal amounts (30 μ g) of protein were denatured with Laemli buffer, subjected to SDS/PAGE and transferred to a PVDF membranes and incubated with

antibodies [rabbit ROR α (#GTX100029, GeneTex, Irvine, CA); rabbit ROR γ (see above); rabbit Lamin A (#sc20681, Santa Cruz); and mouse monoclonal β -actin conjugated-HRP (#A3854, Sigma, Saint Louis, MO). After extensive washing in TBS-T the membranes were incubated with secondary species specific Ig γ conjugated-HRP for 2 h, RT and detection of immunocomplexes was performed with chemiluminescence. Calculation of normalized expression levels of RORs antigens was achieved by dividing the mean signal intensity (density) of expressed proteins (evaluated with ImageJ, NIH) with the internal (Lamin A, for nuclear samples) and loading (β -actin) controls. Next, we subtracted the values from amelanotic out of melanized samples and provided them as %.

Statistical analyses

Statistical analysis was performed with the Prism 5.00 (GraphPad Software, San Diego, CA). Results were considered as statistically significant when $P < 0.05$. For comparison two groups the Student's t -test were used, and for comparison three or more groups – ANOVA. To evaluate the association between ROR α and ROR γ staining and categorical variables, the Pearson's correlation was used. Survival analysis was performed using Log-rank test. For analysis of survival after adjustment of Breslow thickness and overall stage the Cox proportional-hazards regression analysis was performed using NCSS10 (NCSS, LLC, Kaysville, Utah, USA). Data are presented as mean \pm SD.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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