Original Article
Low RhoA expression is associated with adverse outcome in melanoma patients: a clinicopathological analysis

Maciej Kaczorowski1, Przemyslaw Biecek2, Piotr Donizy1, Malgorzata Pieniazek3, Rafal Matkowski4,5, Agnieszka Halon1,5

1Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Borowska 213, Wroclaw, Poland; 2Faculty of Mathematics and Information Science, Warsaw University of Technology, Koszykowa 75, Warsaw, Poland; 3Department of Clinical Oncology, Tadeusz Koszarowski Regional Oncology Centre, Katowicka 66a, Opole, Poland; 4Department of Oncology and Division of Surgical Oncology, Wroclaw Medical University, Hirszfelda 12, Wroclaw, Poland; 5Lower Silesian Oncology Centre, Hirszfelda 12, Wroclaw, Poland

Received March 2, 2019; Accepted June 11, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: RhoA GTPase is physiologically involved in the formation of stress fibers, cellular contractility and polarity, maintenance of cell cycle and transcriptional control. During tumorigenesis, it plays roles in cancer cell proliferation, apoptosis, adhesion, invasion and metastasis. While RhoA seems to act as a tumor promotor in most malignancies, data regarding its function in skin melanoma are fragmentary and conflicting. We aimed to clarify the clinical significance of RhoA expression in melanoma by immunohistochemical evaluation of 134 primary tumors and subsequent statistical analysis with clinicopathological profiles of patients. Increased RhoA expression was associated with thinner tumors, higher grade of tumor-infiltrating lymphocytes and lack of disease recurrence. Moreover, we observed a trend towards higher RhoA expression in cases without concurrent metastases. Recurrence-free survival and melanoma-specific survival of patients with high RhoA-expressing tumors were significantly prolonged. Multivariable regression model adjusting for melanoma thickness and status of regional lymph nodes confirmed independent prognostic value of RhoA immunoreactivity. In summary, we found associations between RhoA expression and histopathological phenotype of primary tumors as well as patient survival which suggest a suppressive role of RhoA in skin melanoma.

Keywords: RhoA, malignant melanoma, prognosis, survival

Introduction
Rho family proteins are small GTPases that influence numerous processes including cellular adhesion, polarity, migration as well as cell cycle progression [1]. They operate as molecular switches that become active when the coupled nucleotide GDP is exchanged for GTP [1]. Rho proteins’ activity is dependent on a number of regulators classified as guanine nucleotide exchange factors, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors [1]. Imbalance between these controllers, enhancing Rho signaling, is a frequent finding in cancer [2]. This, as well as overexpression and activating mutations of some Rho GTPases themselves demonstrated in many tumors, is suggestive of their prouncogenic properties [2, 3]. Contrarily, other Rho proteins seem to play significant roles in tumor suppression [3].

RhoA is one of the canonical and most studied members of the family. Its activity may be induced by heterogeneous stimuli such as cytokines, hormones and interactions with extracellular matrix proteins [4, 5]. Besides its physiological functions, it plays roles in hallmarks of cancer development and progression including proliferation, apoptosis, invasion and metastasis [6-9]. The majority of authors have highlighted cancer-promoting activities of RhoA and associated its overexpression with aggressive tumor phenotype and adverse prognosis [2, 3]. However, recent functional studies, notably extensive investigations of colorectal and squa-
mous cell lung cancers, demonstrate engagement of RhoA in important pathways impeding tumorigenesis [10, 11]. Thus, the effect of RhoA signaling in cancer is not universal and seems to be context dependent.

In melanoma, in vitro studies documenting the activity of RhoA in the context of selected features of malignancy gave conflicting results. Some authors reported tumor-promoting functions of RhoA related to increased migration, cell survival and regulation of melanoma cell apoptosis [12-15]. Other experiments endorsed mechanisms of opposite significance such as RhoA-dependent immune modulation and inhibition of invasiveness [16-18].

To date there has been no definitive evidence for the clinical relevance of RhoA expression in skin melanoma. We aimed to address this issue by immunohistochemical analysis of RhoA expression in 134 variably advanced primary cutaneous melanomas. Then, we checked for statistical relationships between RhoA reactivity and other histopathological and clinical parameters, including patient survival.

Materials and methods

Patients

Tissue samples from 134 patients with a diagnosis of skin melanoma made between 2005 and 2010 were analyzed. The patients were diagnosed and treated in the Regional Oncology Centre in Opole, Poland. Inclusion criteria were based on the availability of histopathology slides, paraffin blocks and medical documentation, including archival pathology reports and disease staging. Medical records at the outpatient clinic of the Regional Oncology Centre in Opole and the Civil Register Office were the sources of information about diagnostic and therapeutic procedures applied and patient survival. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Bioethics Committee of Wroclaw Medical University (consent No. 478/2017). The need for informed consent was waived by the Bioethics Committee of Wroclaw Medical University.

The patients’ treatment was up-to-date with the prevailing guidelines. If after removal of the primary lesion the histopathological diagnosis was skin melanoma, the scar was excised with a margin of 5, 10 or 20 mm depending on tumor location and Breslow thickness. Sentinel lymph node biopsy was performed in cases with Breslow thickness above 1 mm (>pT1a), but no evidence of metastatic spread (cN0). If metastases in the regional lymph nodes were found (either by sentinel lymph node biopsy or clinically), lymphadenectomy was performed.

Clinicopathological characteristics of the patients included sex, age, primary tumor location, TNM stratification and staging according to the 7th ed. of American Joint Committee on Cancer guidelines, data on disease recurrence and sentinel lymph node biopsies (Table 1).

Hematoxylin and eosin-stained sections of formalin-fixed and paraffin-embedded tumor tissue were used for histopathological evaluation. All slides were viewed in a blinded manner by two pathologists (MK and PD). Histologic type, Breslow thickness, Clark level, mitotic rate (counted per 1 mm²), presence of ulceration, lymphangioinvasion, microsatellitosis as well as tumor infiltrating lymphocytes (TILs) were recorded (Table 2). To assess TILs, we applied a semi-quantitative system:TILs absent: no lymphocytes present or lymphocytes are present but do not infiltrate the tumor at all; TILs non-brisk: lymphocytes infiltrate melanoma focally or not along the entire front of invasion; TILs brisk: lymphocytes diffusely infiltrate the base of the tumor or the entire invasive component.

Immunohistochemistry

Anti-RhoA antibody (mouse monoclonal, clone 26C4; dilution 1:50; Santa Cruz Biotechnology; Dallas, TX, USA) was used to stain sections from 134 analyzed primary tumors. 4 µm paraffin sections cut with microtome were mounted on sialinized slides (code number S 3003; DAKO, Glostrup, Denmark) and subsequently subjected to automated dewaxing, rehydration and heat-induced epitope retrieval, performed in PT Link Pre-Treatment Module for Tissue Specimens (DAKO), using EnVision Target Retrieval Solution (DAKO) for 30 minute incubation at 97°C. Autostainer Link 48 (DAKO) was used for immunohistochemical staining and EnVision FLEX/HRP (DAKO) was used for detection. Human brain tissue was stained as a positive control. Negative controls were processed using FLEX Mouse Negative Control, Ready-to-Use (DAKO) in place of the primary antibody.
Evaluation of immunohistochemistry

RhoA expression was evaluated in the neoplastic compartment, i.e. in tumor cells, by a semi-quantitative method. Two parameters of immunohistochemical reaction were analyzed: the percentage of positive cells (the percentage of reactive tissue) and staining intensity. Scale of Remmele and Stegner modified by the authors was employed to calculate the final reaction score, as described previously [19, 20]. In short, 0-10 points were given (0%-0 pts, 1-10%-1 pt, 11-20%-2 pts, etc.) for the percentage of positive cells and 0-3 points for the intensity of reaction. These values were multiplied to produce the final result for each case named ImmunoReactiveScore (IRS) ranging from 0 to 30 points. Light microscope Olympus BX51 (Olympus America, Inc., Melville, NY, USA) was used for evaluation of slides.

Table 1. RhoA immunoreactivity in melanoma cells and clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>RhoA IRS</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low</strong></td>
<td><strong>High</strong></td>
<td>( n = 46 )</td>
</tr>
<tr>
<td>Age in years (18-87)(^a)</td>
<td>(18-86)</td>
<td>(24-87)</td>
</tr>
<tr>
<td>mean: 61.6±14.8; median: 64.5</td>
<td>64.6±14.0; 68</td>
<td>60.0±15.0; 60.5</td>
</tr>
<tr>
<td>Gender(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>45</td>
</tr>
<tr>
<td>Primary tumor location(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head/neck</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Extremities</td>
<td>17</td>
<td>39</td>
</tr>
<tr>
<td>Hand/foot</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Trunk</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td><strong>7th ed. AJCC stage(^c)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Primary tumor (pT)(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pT1)</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>(pT2)</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>(pT3)</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>(pT4)</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Regional lymph nodes status (pN)(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases absent (pN-)</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>Metastases present (pN+)</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Distant metastases (pM)(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases absent (pM-)</td>
<td>38</td>
<td>83</td>
</tr>
<tr>
<td>Metastases present (pM+)</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Sentinel lymph node biopsy status(^d) (60 patients)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastases</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>Metastases present</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Recurrence(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>22</td>
<td>66</td>
</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^a\)\(p\) value of Wilcoxon two sample test; \(^b\)\(p\) value of Fisher's exact test; \(^c\)\(p\) value of \(\chi^2\) test.
RhoA expression in skin melanoma

At least weak and focal expression of RhoA was detected in all 134 primary melanomas. The staining pattern was predominantly cytoplasmic. IRS ranged from 3 to 30 with the mean value of 17.7 and median value of 18. High RhoA expression (IRS≥14) characterized 88 melanomas while low RhoA reactivity (IRS<14) was found in the remaining 46 tumors (Figure 1).

RhoA expression and clinico-pathological characteristics

Clinical characteristics like gender, age and tumor location were not associated with RhoA expression. We observed a shift towards low RhoA reactivity in advancing AJCC stages; this finding was on the borderline of statistical significance. Analogously, there was a relationship between RhoA immunoreactivity and pT variable-while the majority of early tumors expressed high levels of RhoA, slightly over half of pT4 melanomas were low RhoA expressors (P = 0.028). Down-regulation of RhoA was more prevalent in cases with concurrent nodal and/or distant metastases compared with non-metastatic primary tumors, but this was another observation without definitive statistical significance. Finally, the disease recurred more frequently among low RhoA-expressing cases (P = 0.002) (Table 1). Considering pathological parameters of the pri-
RhoA expression in skin melanoma

Over, we found a distinct trend towards low RhoA expression in metastatic cases. These findings advocate for tumor-suppressive activity of RhoA in skin melanoma that is diminished in advanced disease.

Data supporting a link between melanoma invasiveness and downregulation of RhoA function come from a study of Díaz-Núñez et al. on histone deacetylase inhibitors [17]. Treatment of melanoma cell lines with these agents showed a pro-invasive effect accompanied by upregulation of N-cadherin and decrease of RhoA function [17]. Moreover, application of Rho inhibitor C3T and transfection with dominant-negative RhoA led to similar results, which confirms that RhoA is functionally involved in modulation of melanoma invasion [17]. On the other hand, Klein and Higgins showed that RhoA signaling was significantly upregulated and determined melanoma invasiveness following treatment with BRAF inhibitor [12]. In the absence of BRAF inhibition, however, depletion of RhoA had no effect on melanoma cell movement [12].

A recent study described a novel, non-immunological role of CD70 molecule in melanoma pa-

**Figure 1.** Immunohistochemical staining of skin melanomas with anti-RhoA antibody. Nests of melanoma cells with strong RhoA expression in a case of superficial-spreading melanoma; band-like, brisk infiltrate of lymphocytes is surrounding the tumor base (A: 40×; hematoxylin). High cytoplasmic expression of RhoA in malignant melanocytes (B: 400×; hematoxylin). Weak RhoA immunoreactivity in a case of nodular melanoma; stronger-stained cells are intratumoral lymphocytes (C: 100×; hematoxylin). Highly mitogenic (arrows) melanoma expressing minimal RhoA (D: 400×; hematoxylin).
Figure 2. Kaplan-Meier plots of melanoma patient survival in groups stratified according to RhoA expression. Low RhoA reactivity is associated with shorter recurrence-free survival (A) and melanoma-specific survival (B) (p levels of log-rank tests).

Thogenesis [23]. Expression of CD70 was high in primary lesions and decreased significantly in metastases [23]. Moreover, high CD70 expression impaired migration, invasiveness and formation of metastases [23]. Interestingly, treatment with anti-CD70 antibody promoted trimerization of CD70 which restored aggressive melanoma phenotype by activation of MAPK pathway [23]. In a follow-up study, the same group reported that RhoA enhances promoter activity of CD70 gene and is a key regulator of CD70 protein expression [24]. Although the authors did not investigate the levels of RhoA over time during melanoma progression, in the light of both studies it seems possible that downregulation of CD70 in aggressive and metastatic melanomas results from low RhoA expression in these tumors. This assumption harmonizes with our observations on clinical samples, in which RhoA immunoreactivity was negatively correlated with Breslow thickness—one of the most important prognostic parameters in malignant melanoma. Although the relation between low RhoA and presence of metastases only formed a trend in our cohort, it would most likely be statistically significant in a larger sample.

Tumor-suppressive activity of RhoA may also be associated with regulation of apoptosis and modulation of immune response. In a murine B16F10 model, RhoA inhibition resulted in membranous FasL expression on melanoma cells [16]. Furthermore, it effectively induced Fas-triggered apoptosis in cocultured B lymphoma cells [16]. Thus, our observation that tumors with low RhoA immunoreactivity are less infiltrated by TILs might be related to increased lymphocyte apoptosis induced by FasL-expressing tumor cells. Conversely, Goundiam et al. showed that inhibition of RhoA activity led to inhibition of tumor growth by stimulation of anoikis in malignant melanocytes [14].

Publications indicating tumor-inhibiting activities of RhoA, including this study, are contrasted by several experiments in which pharmacological inhibition of RhoA led to the suppression of melanoma cell motility and tumor growth [15, 25, 26]. Usage of different cell lines may be one of the reasons for these discrepancies, but more studies on animal models and human tissue are necessary to elucidate the contribution of RhoA to melanoma pathogenesis.

Epithelial to mesenchymal transition (EMT) is a phenotype switch that promotes dissemination of many epithelial tumors. Similarly, EMT-like process inducing a migratory phenotype of malignant cells plays a crucial role in melanoma progression [27]. Transforming growth factor β (TGFβ) which displays potent prooncogenic activity in advanced cancers, including melanoma, is one of the best studied activators of EMT [28, 29]. Although exact mechanisms that trigger EMT in response to TGFβ are not fully understood, RhoA activity appears to be one of the important factors. Inhibiting RhoA or its downstream kinase ROCK blocked TGFβ-induced EMT in mouse mammary epithelial cells [30]. Interestingly, the same group reported that proliferative arrest mediated by TGFβ is associated with signaling through RhoA and ROCK [31]. Dependence on RhoA was also observed in TGFβ-stimulated EMT of rat lens epithelial and mesothelial cells as well as during embryonal development of chicken heart. Conversely, EMT in colon cancer seems to be related with a de-
crease in RhoA activation [32]. Requirement of RhoA and Cdc45 GTPases for rearrangements of actin cytoskeleton was demonstrated in TGFβ-treated human prostate carcinoma cells [33]. Notably, EMT-related phenotypic changes were at least partly independent of SMAD signaling [30, 33]. In our previous study on melanoma we found an association between overexpression of SMAD7, an inhibitor of TGFβ/SMAD pathway, and disease progression [19]. However, levels of RhoA and SMAD7 were not correlated in our cohort (data not shown). The extent to which RhoA regulates EMT-like switch in cutaneous melanoma remains to be established.

In summary, our paper indirectly endorses the significance of RhoA in tumorigenesis. Unlike most data on RhoA expression and function in other cancers, our results argue for its engagement in suppression of melanoma. Previous studies in melanoma gave conflicting conclusions, but their direct comparison is often hindered due to methodological differences and focus on selected, different aspects of malignancy such as invasiveness or apoptosis. To the best of our knowledge this is the first work to demonstrate a more generic, clinical relevance of RhoA in skin melanoma. Therapeutic modulation of Rho/ROCK pathway has been proposed in a number of cancers, but deeper understanding of how it influences the natural history of melanoma progression is prerequisite to its clinical use in this setting.

Acknowledgements

A statutory subsidy by the Polish Ministry of Science and Higher Education as part of grants STM.B131.17.008, ST. B130.18.030 and SUB.C280.19.050 (record numbers in the Simple system); PB was financially supported by the National Science Centre grant 2016/21/B/ST6/02176.

Disclosure of conflict of interest

None.
RhoA expression in skin melanoma

Address correspondence to: Dr. Maciej Kaczorowski, Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Boryswa 213, Wroclaw 50-556, Poland. Tel: +48717-343960; Fax: +48717343968; E-mail: octopuso@wp.pl

References

RhoA expression in skin melanoma


