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ROCK1 and ROCK2 Are Down-regulated in Aggressive and Advanced Skin Melanomas – A Clinicopathological Perspective

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Abstract. *Background: RhoA and its downstream effectors Rho-associated coiled-coil kinases (ROCK) 1 and 2 are central controllers of cytoskeleton dynamics, and therefore influence cell shape, adhesion and migration. Since modulation of these processes holds promise for an effective anticancer strategy, effects of ROCK inhibition have been evaluated in a number of malignancies. Materials and Methods: Using immunohistochemistry, ROCK1 and ROCK2 expression was semi-quantitatively assessed in 129 patient-derived primary melanomas. Results: There was a striking predilection for low melanocytic expression of both kinases in thick, ulcerated and mitogenic tumors, as well as in nodular histological type. ROCK1 and -2 expression in tumor-infiltrating lymphocytes (TILs) was preferentially down-regulated in advanced and aggressive tumors. Moreover, diminished ROCK2 reactivity in melanoma cells and TILs was associated with shorter melanoma-specific and recurrence-free survival. Conclusion: This is the first analysis of ROCK1 and -2 protein expression in clinical melanoma samples and the results indicated the suppression of ROCK signaling in melanocytes of aggressive and late-stage tumors. Functional models that more accurately represent the clinical setting are necessary to dissect the role of ROCK1 and -2 in melanoma. Additionally, our study indicates that ROCK activity in TILs may be involved in the pathogenesis of cancer, and thus merits further investigations.*

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Cellular motility and invasiveness are key determinants of cancer progression and metastatic spread. Since chances for a durable remission are usually significantly diminished by the development of tumor metastases, mechanisms that regulate this process have been vigorously investigated. Cognition of biochemical agents accountable for cancer dissemination is even more alluring in the current era of novel, molecular-based therapies that may specifically target dysregulated pathways.

A number of studies implicated the family of Rho GTPases and their immediate downstream effectors Rho-associated coiled-coil kinases (ROCKs) as central controllers of important cellular processes, such as cell shape, adhesion or migration (1). Mammalian ROCK proteins consist of two highly homologous isoforms, ROCK1 and ROCK2 (1). Their canonical activity involves promoting actomyosin contractility through phosphorylation of myosin light chain and stabilizing actin filaments through activation of LIM kinase (1). This influence on cytoskeleton dynamics endows ROCKs with a regulatory function over several hallmark features of cancer, *e.g.* invasion, cell division and remodeling of the extracellular matrix (2). Consequently, the effects of ROCK inhibition have been extensively studied in various tumor types. Employment of gene knockdown techniques and protein inhibitors, such as fasudil or Y27632, effectively reduced proliferation, invasion and formation of metastases in a majority of investigations on melanoma, breast, pancreatic, hepatic, ovarian and other types of cancer (3-8). Moreover, depletion of ROCKs reduced cell viability and induced apoptosis in bladder and hematological malignancies, as well as in melanoma (9-11). Conversely, several other groups demonstrated deleterious effects of ROCK inhibition. Treatment with ROCK inhibitor Y27632 led to increased migration and mitotic activity in breast, colon and pancreatic cancer cell cultures (12-15). Others showed that

inactivation of ROCK signaling promoted cell survival and attenuated drug-induced apoptosis in leukemia, neuroblastoma and ovarian carcinoma (16-18), as well as increased cancer cell stemness (19, 20). These seemingly contradictory findings may be at least partly attributed to the choice of different cell lines in various studies. In melanoma, substitution of B16F1 culture with UACC257 or UACC62 cells resulted in opposite observations in proliferation and migration assays – ROCK inhibition impaired melanoma cell growth and motility in the first cell line and promoted these functions in the latter two (11, 21, 22). This illustrates an important caveat to past functional studies – they do not account for the considerable molecular heterogeneity of naturally occurring melanomas. Therefore, their conclusions regarding pro- or antitumor activities of ROCKs may not be easily generalizable to a broad clinical spectrum of skin melanomas.

To date, expression profiles of ROCK1 and ROCK2 proteins have not been characterized in clinical melanoma samples. With this aim, we investigated immunoreactivity of ROCK1 and ROCK2 in the neoplastic compartment of primary melanomas and explored associations between the observed protein expression and other pathological and clinical characteristics. Since the RhoA–ROCK axis plays a regulatory role in immune responses (23, 24), we additionally evaluated expression of ROCK1 and ROCK2 in tumor-infiltrating lymphocytes (TILs).

Materials and Methods

Patient samples. Tissue samples were obtained from 129 patients diagnosed with and treated for skin melanoma between 2005 and 2010 in the Regional Oncology Centre in Opole, Poland. Inclusion criteria were: Availability of paraffin blocks and corresponding histopathology slides, and availability of archival medical documentation, including disease staging and original pathology reports. Data regarding applied medical procedures and patient survival were obtained from medical records of the Regional Oncology Centre in Opole and the Civil Register Office. The study was approved by the Bioethics Committee of Wroclaw Medical University (approval no.: KB-574/2017) which waived the necessity for informed consent and was carried out in accordance with the Declaration of Helsinki.

The patients' treatment was concordant with the prevailing guidelines. Following the histopathological diagnosis of cutaneous melanoma, the primary lesions were excised with a margin of 5, 10 or 20 mm depending on tumor thickness and localization. Sentinel lymph node biopsies were performed in cases with no clinical evidence of metastases (cN0) and Breslow thickness exceeding 1 mm (>pT1a). Lymphadenectomies were applied in cases with metastases in the regional lymph nodes (found clinically or by sentinel lymph node biopsy).

Clinicopathological parameters included information about patient age, sex, location of primary tumors, their TNM classification and staging according to the guidelines of American Joint Committee on Cancer (AJCC) (seventh edition) (25), information about disease recurrence and sentinel lymph node biopsy procedures.

Formalin-fixed and paraffin-embedded tissues of primary tumors were used to prepare 4 μm-thick sections that were subsequently stained by hematoxylin and eosin. All slides were blindly reviewed by two pathologists (MK and PD). Recorded histopathological characteristics included: Breslow thickness, Clark level, histological type, mitotic index (per 1 mm^2), and the presence of ulceration, microsatellites, lymphovascular invasion and TILs. The last parameter was assessed semi-quantitatively with three possible grades: Absent, non-brisk and brisk.

Immunohistochemistry. Paraffin blocks with tissues of primary melanomas were cut with a microtome to prepare 4 μm-thick sections which were subsequently mounted on sialinized slides (Agilent DAKO, Santa Clara, CA, USA). The slides then underwent automated dewaxing, rehydration and heat-induced epitope retrieval with EnVision Target Retrieval Solution (Agilent DAKO) in a 30-minute incubation at 97˚C in PT Link Pre-Treatment Module for Tissue Specimens (DAKO). Automated immunohistochemical staining with anti-ROCK1 (rabbit monoclonal, dilution 1:100; Abcam, Cambridge, UK) and anti-ROCK2 (rabbit polyclonal; dilution 1:100; Abcam) was performed in Autostainer Link 48 (DAKO) and Liquid Permanent Red (Agilent DAKO) was utilized as a detection system. Human colorectal adenocarcinoma and human hepatocellular carcinoma tissues were stained as positive controls for ROCK1 and ROCK2 antibodies, respectively. Negative controls were processed using FLEX Rabbit Negative Control, Ready-to-Use (Agilent DAKO) in place of the primary antibody.

Evaluation of immunohistochemistry. Expression of ROCK1 and ROCK2 was evaluated in two compartments, namely melanoma cells and TILs. The semi-quantitative scale of Remmele and Stegner (26) combining two parameters of immunohistochemical reaction, *i.e.* the percentage of reactive tissue and staining intensity, was modified by the authors and employed as described previously for evaluation of immunoexpression in tumor cells (27). In brief, 0-3 points were given for the intensity of reaction and 0-10 points were given (0%: 0 points, 1-10%: 1 point, 11-20%: 2 points, *etc.*) for the percentage of reactive neoplastic cells. Multiplication of these two values gave a product ranging from 0 to 30 points named the immunoreactive score (IRS). Expression of ROCK proteins in TILs was graded as absent/weak, medium or strong based on reaction intensity juxtaposed with the staining intensity of positive controls. An Olympus BX51 light microscope (Olympus America, Inc., Melville, NY, USA) was used for the evaluation of slides.

Statistical analysis. Statistical analyses were performed in R language. Expression of ROCK1 and ROCK2 in cancer cells were transformed into binary variables with the use of maxstat package. In both cases, the cutoff IRS of ≤20 *vs.* IRS>20 was used to differentiate between low and high immunoexpression. Continuous variables, including patient age and Breslow thickness, were characterized with their mean, median, and range values. Kaplan–Meier curves and corresponding log-rank tests were applied with the survminer package, for analysis of melanoma-specific survival (MSS) and recurrence-free survival (RFS). Relationship of ROCK1 and -2 expression in melanocytes and TILs with continuous variables was assessed by the Wilcoxon two-sample test and Kruskal–Wallis test, respectively. Associations of IRS with binary variables were calculated by Fisher's exact test and the relationships with other categorical variables, as well as associations between ROCK 1 and -2 expression in TILs and categorical variables, were analyzed by chi-squared test. Values of $p<0.05$ were accepted as a threshold for statistical significance.

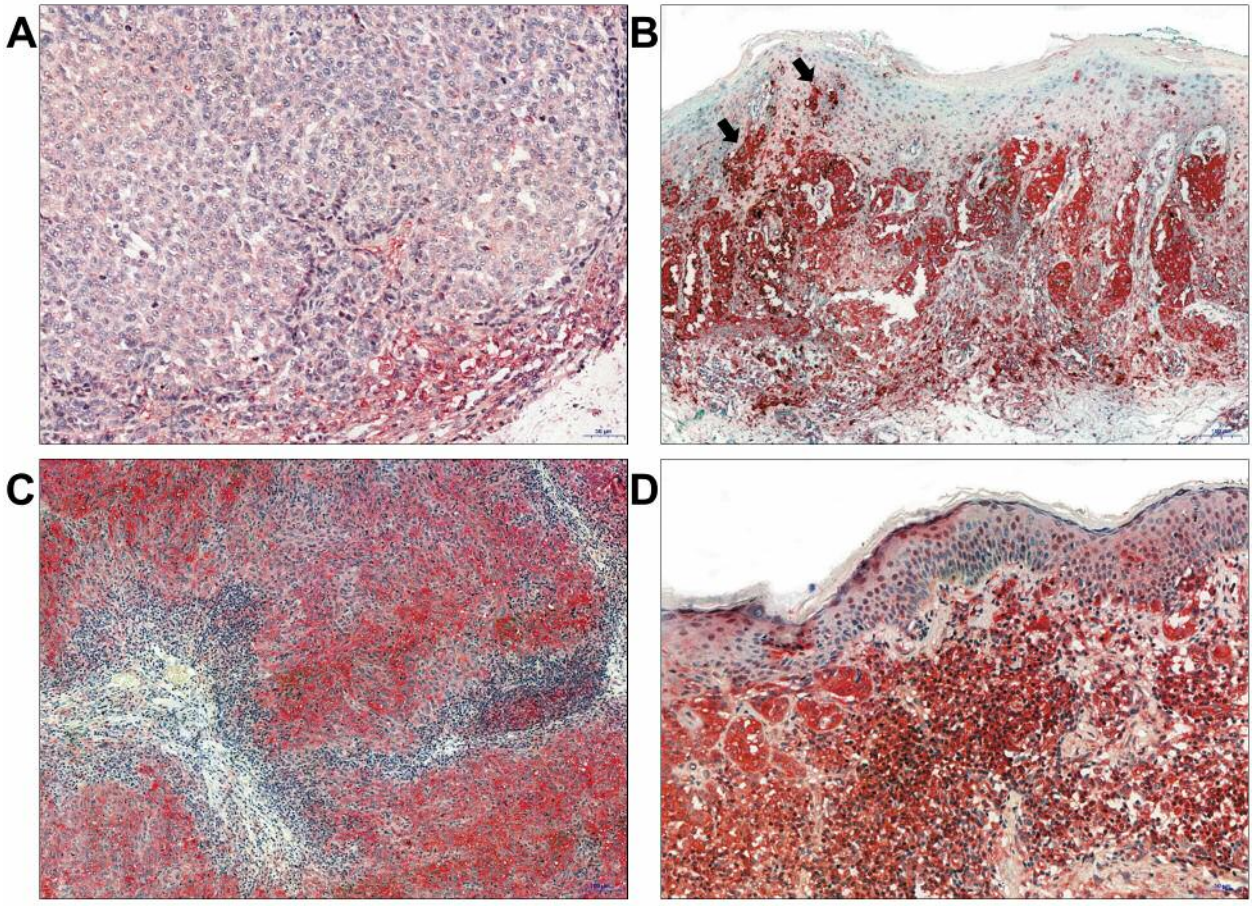

Figure 1. *Immunohistochemical visualization of Rho-associated coiled-coil kinase 1 (ROCK1) expression in cutaneous melanomas. A: Weak cytoplasmic staining of melanoma cells with a rim of stronger-stained immune cells on the lower right (×200). B: Strong staining in melanocytic nests and foci of pagetoid spread (arrows) (×100). C: Neoplastic infiltrate transected by fibrous stroma containing brisk lymphocytic infiltrate with low ROCK1 immunoreactivity (×200). D: Both tumor-infiltrating lymphocytes and melanoma cells highly reactive for ROCK1 (×100).*

Results

Expression of ROCK1 and ROCK2 proteins in skin melanomas. Cytoplasmic immunoreactivity for ROCK1 and ROCK2, ranging from weak and focal to strong and diffuse, was observed in neoplastic compartments of all 129 primary tumors (Figure 1A and B, and Figure 2A and B). Median and mean IRS for ROCK1 were 20 and 19.8, respectively, whereas for IRS ROCK2 these values were 21 and 20.9, respectively. Slightly over 40% of melanomas were classified as high ROCK1 expressors and 46% as high ROCK2 expressors. IRS for ROCK1 and ROCK2 were moderately correlated (Spearman's r=0.6, $p<0.0001$; data not shown). In our previous study on skin melanoma, we analyzed expression of RhoA GTPase, a direct controller of ROCK1 and ROCK2 function (28). Interestingly, ROCK1 and ROCK2 immunoreactivity did not correlate with RhoA expression (Figure 3). As regards TILs, at least focal positive staining for ROCK1 was present in 124 cases, being weak in 51, medium in 45 and strong in 28 tumors (Figure 1C and D). Reaction for ROCK2 was also positive in all evaluated cases: Weak in 28, medium in 72 and strong in 24 melanomas (Figure 2C and D). Five tumors with no apparent lymphocytic infiltrate (TIL grade: Absent) were excluded from this analysis.

Expression of ROCK1 and ROCK2 in melanoma cells and clinicopathological characteristics. ROCK1 expression in our cohort was mostly unrelated to the clinical background but ROCK2 was expressed more strongly in older patients. Melanoma recurred more often in those with tumors with low ROCK2 staining, but there was only a trend for a relation with ROCK1, without clear statistical significance. Melanocytic expression of both ROCK proteins dropped with advancing pT stage. This relation was particularly evident with regard to ROCK2 staining, with 93% of high expressors among pT1 tumors and only 20% among pT4 cases.

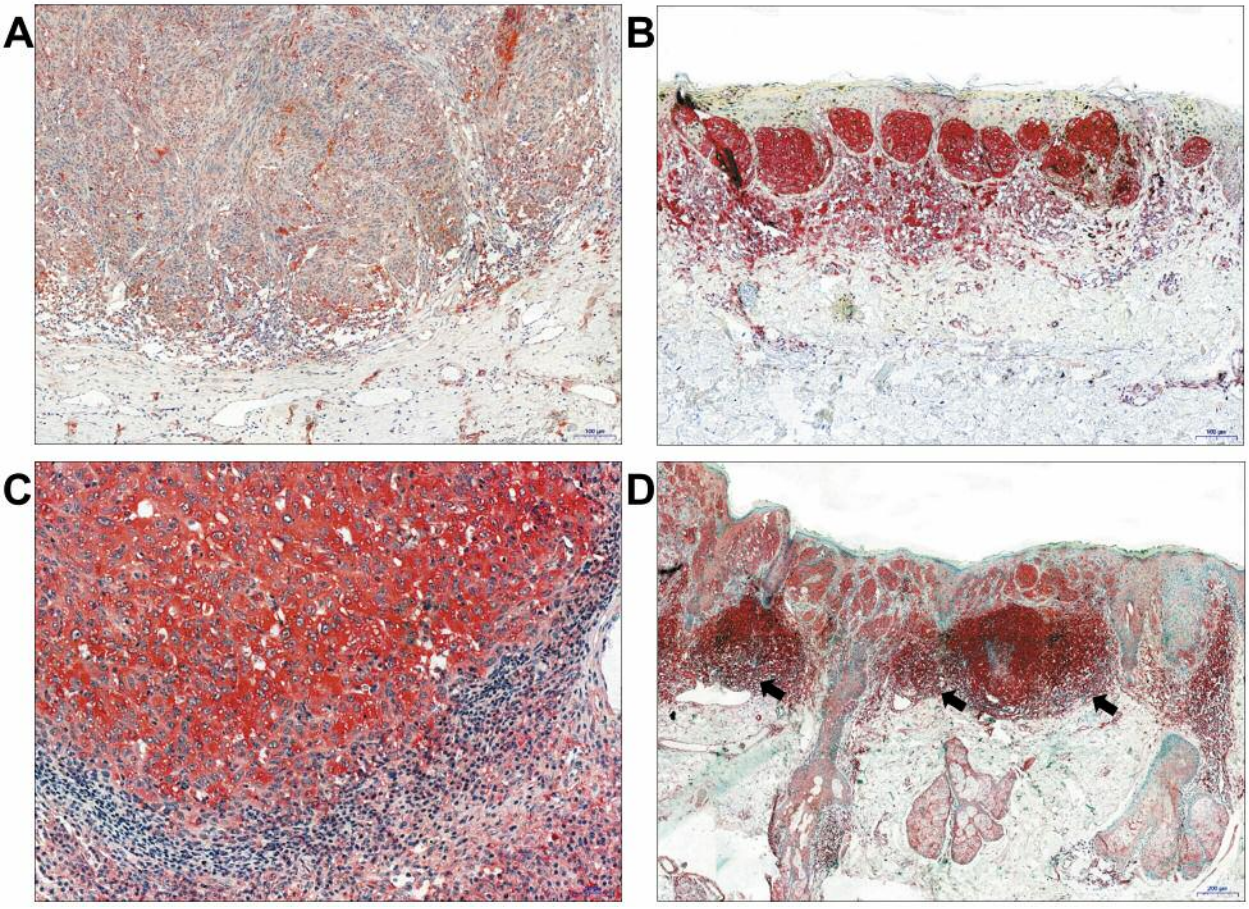

Figure 2. *Immunohistochemical visualization of Rho-associated coiled-coil kinase 2 (ROCK2) expression in cutaneous melanomas. A: Base of a nodular melanoma with weak staining of tumor cells (×100). B: Melanoma nests strongly positive for ROCK2 (×100). C: Nodule of malignant melanocytes surrounded by a rim of lymphocytes that are weakly positive for ROCK2 (×200). D: Brisk accumulation of tumor-infiltrating lymphocytes (arrows) with intense ROCK2 staining (×50).*

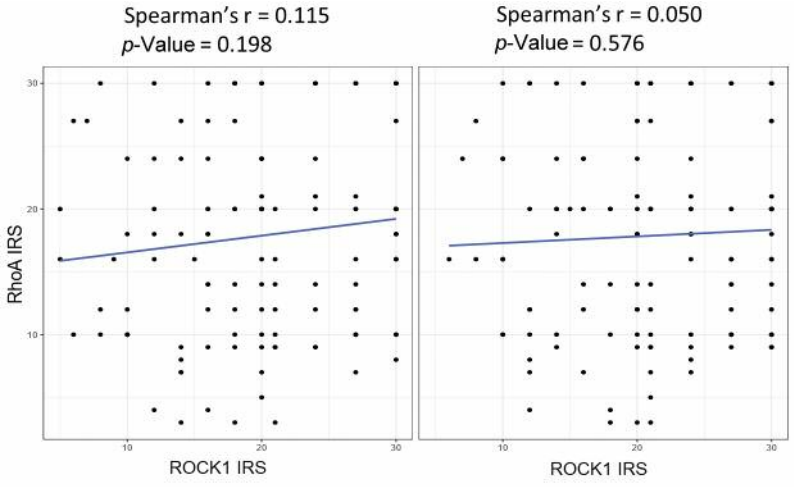

Figure 3. *Scatter plots demonstrating characteristics of immunoreactivity for Rho-associated coiled-coil kinase (ROCK) 1 and 2 throughout the study population together with corresponding values of RhoA immunoreactive score (IRS). Dots represent individual tumors and lines represent trend.*

Table I. *Clinical characteristics of melanoma patients according to Rho-associated coiled-coil kinase 1 (ROCK1) expression.*

Parameter		ROCK1 immunoreactivity						
		Melanoma cells			Tumor-infiltrating lymphocytes			
	Total (n=129)	Low (n=76)	High (n=532)	*p*-Value	Weak (n=518)	Medium (n=45)	Strong (n=28)	*p*-Value
Age, years								
Mean±SD	62±15	63±15	60±14	0.29[a]	64±15	61±14	62±16	0.56[b]
Median	65 (18-87)	67 (18-87)	64 (25-82)		69 (18-86)	62 (24-87)	65 (25-81)	
Gender, n (%)								
Female	67 (52%)	38 (50%)	29 (55%)		26 (51%)	27 (60%)	12 (43%)	
Male	62 (48%)	38 (50%)	24 (45%)	0.72[c]	25 (49%)	18 (40%)	16 (57%)	0.35[d]
Primary tumor location, n (%)								
Head/neck	12 (9%)	10 (13%)	2 (4%)		5 (10%)	3 (7%)	4 (14%)	
Extremities	55 (43%)	29 (38%)	26 (49%)	0.24[d]	24 (47%)	22 (49%)	7 (25%)	0.28[d]
Hand/foot	4 (3%)	3 (4%)	1 (2%)		1 (2%)	2 (4%)	0 (0%)	
Trunk	58 (45%)	34 (45%)	24 (45%)		21 (41%)	18 (40%)	17 (61%)	
AJCC stage, n (%)*								
I	46 (36%)	16 (21%)	30 (57%)		12 (24%)	16 (36%)	17 (61%)	
II	47 (36%)	37 (49%)	10 (19%)	**0.0002**[d]	23 (45%)	15 (33%)	8 (29%)	**0.049**[d]
III	24 (19%)	16 (21%)	8 (15%)		12 (24%)	8 (18%)	2 (7%)	
IV	12 (9%)	7 (9%)	5 (9%)		4 (8%)	6 (13%)	1 (4%)	
Primary tumor (pT), n (%)								
pT1	30 (23%)	8 (11%)	22 (42%)		7 (14%)	9 (20%)	13 (46%)	
pT2	22 (17%)	10 (13%)	12 (23%)	**<0.0001**[a]	6 (12%)	11 (24%)	5 (18%)	**0.0014**[b]
pT3	32 (25%)	22 (29%)	10 (19%)		10 (20%)	14 (31%)	6 (21%)	
pT4	45 (35%)	36 (47%)	9 (17%)		28 (55%)	11 (24%)	4 (14%)	
Regional lymph node status (pN), n (%)								
pN−	98 (76%)	55 (72%)	43 (81%)		35 (69%)	34 (76%)	26 (93%)	
pN+	31 (24%)	21 (28%)	10 (19%)	0.30[c]	16 (31%)	11 (24%)	2 (7%)	**0.045**[d]
Distant metastases (pM), n (%)								
pM−	117 (91%)	69 (91%)	48 (91%)		47 (92%)	39 (87%)	27 (96%)	
pM+	12 (9%)	7 (9%)	5 (9%)	>0.99[c]	4 (8%)	6 (13%)	1 (4%)	0.37[d]
SLNB result (n=56), n (%)								
No metastases	35 (62%)	22 (63%)	13 (62%)		18 (35%)	10 (22%)	6 (21%)	
Metastases	21 (38%)	13 (37%)	8 (38%)	>0.99[c]	9 (18%)	8 (18%)	2 (7%)	0.63[d]
Recurrence, n (%)								
No	85 (66%)	45 (59%)	40 (75%)		30 (59%)	30 (67%)	22 (79%)	
Yes	44 (34%)	31 (41%)	13 (25%)	0.062[c]	21 (41%)	15 (33%)	6 (21%)	0.20[d]

AJCC: American Joint Committee on Cancer Seventh edition (25); SLNB: sentinel lymph node biopsy. [a]Wilcoxon two-sample test. [b]Kruskal–Wallis test. [c]Fisher's exact test. [d]Chi-squared test. Statistically significant results ($p<0.05$) are shown in bold.

Consistently, the highest ROCK1 and ROCK2 reactivity characterized AJCC stage I tumors (Tables I and II). Comparison of ROCK IRS with microscopic features of skin melanoma revealed more correlations suggestive of down-regulation of ROCK1 and ROCK2 expression in advanced and aggressive tumors. Immunoreactivity of both proteins was diminished in thicker, more invasive and mitogenic melanomas. We also noted a predilection for low ROCK1 and ROCK2 levels in a nodular subtype of melanoma, as well as an association with the presence of ulceration. Moreover, ROCK2, but not ROCK1 expression in cancer cells was statistically positively related to the intensity of tumor-associated lymphocytic infiltrate (Tables III and IV).

Expression of ROCK1 and ROCK2 in TILs and clinicopathological characteristics. Strong reactivity for either ROCK1 and ROCK2 in TILs was more frequently found in thinner tumors and cases without concurrent nodal disease. Moreover, weak ROCK2 staining in lymphocytes was associated with melanoma recurrence (Tables I and II). Similarly to the melanocytic expression, the intensity of ROCK staining in TILs was negatively associated with a wide range of microscopic characteristics of primary tumors, *e.g.* Breslow thickness, Clark level of invasion, mitotic rate and the presence of ulceration. Low ROCK1 and ROCK2 expression was more typical of nodular melanomas. In the case of ROCK1, 70% of cases with weak staining were

Table II. *Clinical characteristics of melanoma patients according to Rho-associated coiled-coil kinase 2 (ROCK2) expression.*

Parameter		ROCK2 immunoreactivity						
		Melanoma cells			Tumor-infiltrating lymphocytes			
	Total (n=1299)	Low (n=60)	High (n=69)	*p*-Value	Weak (n=28)	Medium (n=72)	Strong (n=24)	*p*-Value
Age, years								
Mean±SD	62±15	60±14	65±16	**0.024**[a]	65±17	61±15	63±14	0.57[b]
Median	65 (18-87)	62 (18-87)	70 (25-85)		70 (18-86)	64.5 (24-87)	65.5 (33-82)	
Gender, n (%)								
Female	67 (52%)	27 (45%)	35 (51%)		16 (57%)	43 (60%)	6 (25%)	
Male	62 (48%)	33 (55%)	34 (49%)	0.60[c]	12 (43%)	29 (40%)	18 (75%)	**0.011**[d]
Primary tumor location, n (%)								
Head/neck	12 (9%)	9 (15%)	3 (4%)		6 (21%)	3 (4%)	3 (12%)	
Extremities	55 (43%)	25 (42%)	30 (43%)	0.19[d]	14 (50%)	35 (49%)	4 (17%)	**0.0027**[d]
Hand/foot	4 (3%)	1 (2%)	3 (4%)		0 (0%)	3 (4%)	0 (0%)	
Trunk	58 (45%)	25 (42%)	33 (48%)		8 (29%)	31 (43%)	17 (71%)	
AJCC stage, n (%)								
I	46 (36%)	8 (13%)	38 (55%)		4 (14%)	25 (35%)	16 (67%)	
II	47 (36%)	33 (55%)	14 (20%)	**<0.0001**[d]	12 (43%)	28 (39%)	6 (25%)	**0.0072**[d]
III	24 (19%)	13 (22%)	11 (16%)		8 (29%)	12 (17%)	2 (8%)	
IV	12 (9%)	6 (10%)	6 (9%)		4 (14%)	7 (10%)	0 (0%)	
Primary tumor (pT), n (%)								
pT1	30 (23%)	2 (3%)	28 (41%)		3 (11%)	14 (19%)	12 (50%)	
pT2	22 (17%)	7 (12%)	15 (22%)	**<0.0001**[a]	2 (7%)	14 (19%)	6 (25%)	**0.0005**[b]
pT3	32 (25%)	15 (25%)	17 (25%)		7 (25%)	18 (25%)	5 (21%)	
pT4	45 (35%)	36 (60%)	9 (13%)		16 (57%)	26 (36%)	1 (4%)	
Regional lymph node status (pN), n (%)								
pN−	98 (76%)	43 (72%)	55 (80%)		17 (61%)	56 (78%)	22 (92%)	
pN+	31 (24%)	17 (28%)	14 (20%)	0.31[c]	11 (39%)	16 (22%)	2 (8%)	**0.034**[d]
Distant metastases (pM), n (%)								
pM−	117 (91%)	54 (90%)	63 (91%)		24 (86%)	65 (90%)	24 (100%)	
pM+	12 (9%)	6 (10%)	6 (9%)	>0.99[c]	4 (14%)	7 (10%)	0 (0%)	0.17[d]
SLNB result (n=56), n (%)								
No metastases	35 (62%)	18 (58%)	17 (68%)		7 (47%)	22 (69%)	5 (83%)	
Metastases	21 (38%)	13 (42%)	8 (32%)	0.58[c]	8 (53%)	10 (31%)	1 (17%)	0.21[d]
Recurrence, n (%)								
No	85 (66%)	33 (55%)	52 (75%)		13 (46%)	48 (67%)	21 (88%)	
Yes	44 (34%)	27 (45%)	17 (25%)	**0.017**[c]	15 (54%)	24 (33%)	3 (12%)	**0.0074**[d]

AJCC: American Joint Committee on Cancer Seventh edition (25); SLNB: sentinel lymph node biopsy. [a]Wilcoxon two-sample test. [b]Kruskal–Wallis test. [c]Fisher's exact test. [d]Chi-squared test. Statistically significant results ($p<0.05$) are shown in bold.

nodular type, while over 70% of tumors with high expression were superficial spreading melanomas. TIL grade was only weakly associated with ROCK2 immunoreactivity, but no such association was found for ROCK1 (Tables III and IV).

Expression of ROCK1 and ROCK2 and survival of patients with melanoma. To check whether expression of ROCK proteins is associated with clinical outcomes Kaplan–Meier analysis was performed. Although melanocytic ROCK1 immunoreactivity did not stratify the study population with regard to MSS, we noted a trend towards longer RFS in high ROCK1 expressors (Figure 4A). On the other hand, elevated ROCK2 expression in cancer cells characterized patients with favorable MSS and RFS (Figure 4B). Differences in ROCK1 expression in TILs were not associated with patient survival (data not shown) but stronger ROCK2 staining was significantly related to longer MSS and RFS (Figure 4C). However, neither tumoral nor lymphocytic expression of ROCK2 were independent predictors of survival when adjusted for Breslow thickness and pN status in multivariable regression models (data not shown).

Discussion

The RhoA–ROCK pathway is a master regulator of cytoskeletal dynamics, influencing multiple cell functions,

Table III. *Microscopic features of primary melanoma tumors and Rho-associated coiled-coil kinase 1 (ROCK1) expression.*

Parameter		ROCK1 immunoreactivity						
		Melanoma cells			Tumor-infiltrating lymphocytes			
	Total (n=129)	Low (n=76)	High (n=536)	*p*-Value	Weak (n=516)	Medium (n=456)	Strong (n=286)	*p*-Value
Breslow thickness, mm								
Mean±SD	5.1±6.3	6.3±6.2	3.3±6.3	**<0.0001**[a]	7.2±7.0	4.3±6.4	2.5±3.3	**0.0014**[b]
Median	2.7 (0.3-40)	3.7 (0.4-30)	1.5 (0.3-40)		4.7 (0.4-30)	2.3 (0.5-40)	1.2 (0.3-15)	
Clark level, n (%)								
II	37 (29%)	11 (14%)	26 (49%)		8 (16%)	14 (31%)	14 (50%)	
III	41 (32%)	24 (32%)	17 (32%)		19 (37%)	13 (29%)	9 (32%)	
IV	36 (28%)	31 (41%)	5 (9%)		18 (35%)	10 (22%)	5 (18%)	
V	15 (12%)	10 (13%)	5 (9%)	**<0.0001**[a]	6 (12%)	8 (18%)	0 (0%)	**0.014**[b]
Histological type, n (%)								
Superficial spreading	58 (45%)	20 (26%)	38 (72%)		14 (27%)	22 (49%)	20 (71%)	
Nodular	67 (52%)	53 (70%)	14 (26%)		36 (71%)	21 (47%)	8 (29%)	
Acral-lentiginous	4 (3%)	3 (4%)	1 (2%)	**<0.0001**[c]	1 (2%)	2 (4%)	0 (0%)	**0.0012**[c]
Mitotic rate, n (%)								
0	33 (26%)	9 (12%)	24 (45%)		9 (18%)	11 (24%)	12 (43%)	
1-2	20 (16%)	9 (12%)	11 (21%)		3 (6%)	9 (20%)	6 (21%)	
>2	76 (59%)	58 (76%)	18 (34%)	**<0.0001**[c]	39 (76%)	25 (56%)	10 (36%)	**0.0051**[c]
Ulceration, n (%)								
No	76 (59%)	37 (49%)	39 (74%)		21 (41%)	32 (71%)	19 (68%)	
Yes	53 (41%)	39 (51%)	14 (26%)	**0.0062**[d]	30 (59%)	13 (29%)	9 (32%)	**0.0062**[c]
TILs, n (%)								
Absent	5 (4%)	4 (5%)	1 (2%)		n/a	n/a	n/a	
Non-brisk	81 (63%)	51 (67%)	30 (57%)		38 (75%)	29 (64%)	14 (50%)	
Brisk	43 (33%)	21 (28%)	22 (42%)	0.25[c]	13 (25%)	16 (36%)	14 (50%)	0.094[c]
Microsatellites, n (%)								
No	123 (95%)	71 (93%)	52 (98%)		48 (94%)	42 (93%)	28 (100%)	
Yes	6 (5%)	5 (7%)	1 (2%)	0.40[d]	3 (6%)	3 (7%)	0 (0%)	0.50[c]
Lymphovascular invasion, n (%)								
No	121 (94%)	71 (93%)	50 (94%)		46 (90%)	43 (96%)	28 (100%)	
Yes	8 (6%)	5 (7%)	3 (6%)	>0.99[d]	5 (10%)	2 (4%)	0 (0%)	0.18[c]

TILs: Tumor-infiltrating lymphocytes. [a]Wilcoxon two-sample test. [b]Kruskal–Wallis test. [c]Chi-squared test. [d]Fisher's exact test. Statistically significant results ($p<0.05$) are shown in bold.

such as polarity, movement and proliferation (1). Consequently, many studies argued for an oncogenic role of Rho–ROCK signaling in various cancer types, especially during invasion and metastasis (2). In our previous study, we demonstrated on clinical samples that RhoA is down-regulated rather than overexpressed in advanced and aggressive skin melanomas (28). Here, we tested whether expression of effector kinases intimately regulated by RhoA, *i.e.* ROCK1 and ROCK2, is also related to the tumor stage and other clinicopathological features. We found a striking predilection for low melanocytic immunoreactivity of both kinases in thick, deeply invasive, ulcerated and mitogenic tumors. Moreover, ROCK1 and -2 were preferentially down-regulated in nodular melanomas and diminished ROCK2 expression was associated with ominous prognosis. This apparent suppression of the RhoA–ROCK pathway that occurs in aggressive and late-stage tumors suggests its inhibitory role in melanomagenesis, which is contrary to the majority of reports concerning other cancer types.

The current notion of the role that ROCKs play in skin melanoma originate from a number of mechanistic experiments involving cell cultures and murine models. However, the particularities of each study, *i.e.* focus on different specialized contexts, utilization of different ROCK-blocking agents and, notably, different cell lines, resulted in discordant conclusions. Most investigations used B16 mouse melanoma cells and showed that ROCK inhibition impaired tumor growth, invasiveness and formation of metastases (3, 29-32). While observations of human A375 cells and several uveal melanoma lines corroborated these results (3, 30), ROCK inhibition in HT-144 cells was associated with a pro-invasive effect (33). This lability was recently evidenced by

Table IV. *Microscopic features of primary melanoma tumors and Rho-associated coiled-coil kinase 2 (ROCK2) expression.*

Parameter		ROCK2 immunoreactivity						
		Melanoma cells			Tumor-infiltrating lymphocytes			
	Total (n=129)	Low (n=60)	High (n=69)	*p*-Value	Weak (n=28)	Medium (n=72)	Strong (n=24)	*p*-Value
Breslow thickness, mm								
Mean±SD	5.1±6.3	7.8±6.6	2.7±5.0	**<0.0001**[a]	7.8±7.0	5.1±6.6	1.7±2.0	**0.0005**[b]
Median	2.7 (0.3-40)	4.9 (0.6-30)	1.5 (0.3-40)		4.4 (0.6-23)	2.8 (0.3-40)	1.0 (0.3-10)	
Clark level, n (%)								
II	37 (29%)	3 (5%)	34 (49%)		3 (11%)	18 (25%)	15 (63%)	
III	41 (32%)	20 (33%)	21 (30%)		8 (29%)	26 (36%)	7 (29%)	
IV	36 (28%)	25 (42%)	11 (16%)		9 (32%)	22 (31%)	2 (8%)	
V	15 (12%)	12 (20%)	3 (4%)	**<0.0001**[a]	8 (29%)	6 (8%)	0 (0%)	**0.0002**[b]
Histological type, n (%)								
Superficial spreading	58 (45%)	9 (15%)	49 (71%)		5 (18%)	30 (42%)	21 (88%)	
Nodular	67 (52%)	50 (83%)	17 (25%)		23 (82%)	39 (54%)	3 (12%)	
Acral-lentiginous	4 (3%)	1 (2%)	3 (4%)	**<0.0001**[c]	0 (0%)	3 (4%)	0 (0%)	**<0.0001**[c]
Mitotic rate, n (%)								
0	33 (26%)	2 (3%)	31 (45%)		4 (14%)	15 (21%)	13 (54%)	
1-2	20 (16%)	6 (10%)	14 (20%)		1 (4%)	11 (15%)	6 (25%)	
> 2	76 (59%)	52 (87%)	24 (35%)	**<0.0001**[c]	23 (82%)	46 (64%)	5 (21%)	**0.0001**[c]
Ulceration, n (%)								
No	76 (59%)	22 (37%)	54 (78%)		12 (43%)	41 (57%)	19 (79%)	
Yes	53 (41%)	38 (63%)	15 (22%)	**<0.0001**[d]	16 (57%)	31 (43%)	5 (21%)	**0.030**[c]
TILs, n (%)								
Absent	5 (4%)	1 (2%)	4 (6%)		n/a	n/a	n/a	
Non-brisk	81 (63%)	48 (80%)	33 (48%)		22 (79%)	48 (67%)	11 (46%)	
Brisk	43 (33%)	11 (18%)	32 (46%)	**0.0004**[c]	6 (21%)	24 (33%)	13 (54%)	**0.048**[c]
Microsatellitosis, n (%)								
No	123 (95%)	56 (93%)	67 (97%)		25 (89%)	69 (96%)	24 (100%)	
Yes	6 (5%)	4 (7%)	2 (3%)	0.42[d]	3 (11%)	3 (4%)	0 (0%)	0.20[c]
Lymphovascular invasion, n (%)								
No	121 (94%)	55 (92%)	66 (96%)		23 (82%)	70 (97%)	24 (100%)	
Yes	8 (6%)	5 (8%)	3 (4%)	0.47[d]	5 (18%)	2 (3%)	0 (0%)	0.010[c]

TILs: Tumor-infiltrating lymphocytes. [a]Wilcoxon two-sample test. [b]Kruskal–Wallis test. [c]Chi-squared test. [d]Fisher's exact test. Statistically significant results ($p<0.05$) are shown in bold.

Chang *et al*. who compared consequences of ROCK inhibition across several cell lines (22). Treatment with Y27632 reduced proliferation and migration in B16F1 and MeWo cells, but employment of UACC257, UACC62 and M14 lines yielded contrary results, which our findings are consistent with (22). This variation was attributed to B-Raf proto-oncogene, serine/threonine kinase (BRAF) status as only *BRAF*-mutant cells displayed enhanced growth and motility following ROCK inhibition (22). Since *BRAF* mutations are found in around 60% of skin melanomas (34), the peculiarity of ROCK function in these cases may be a major factor influencing the role of ROCK1 or ROCK2 in the clinical conditions. Interestingly, other groups revealed resistance to BRAF inhibitors to be associated with up-regulation of RhoA/ROCK signaling and that coordinated inhibition of BRAF and ROCK may be therapeutically appealing (35, 36). Overall, the significance of ROCK signaling appears to be highly cell- and context-dependent, and individual experimental models likely do not mirror the complexity and heterogeneity of clinical disease. To the best of our knowledge, our results are the first documentation of clinicopathological profiles of ROCK1 and -2 protein expression in patient-derived melanoma samples.

The RhoA–ROCK pathway is known to affect immune responses (24); therefore, we decided to include TILs in the evaluation of ROCK1 and -2 expression in primary melanomas. Since RhoA–ROCK signaling is required for migration of lymphocytes through the endothelial barrier and perivascular collagen (23, 37), we anticipated a relationship between TIL grade and ROCK reactivity. However, only ROCK2 expression in TILs was positively correlated with the intensity of lymphocytic infiltrate, and yet even this association was weak. Nevertheless, we observed significant correlations between lymphocytic staining for either ROCK1

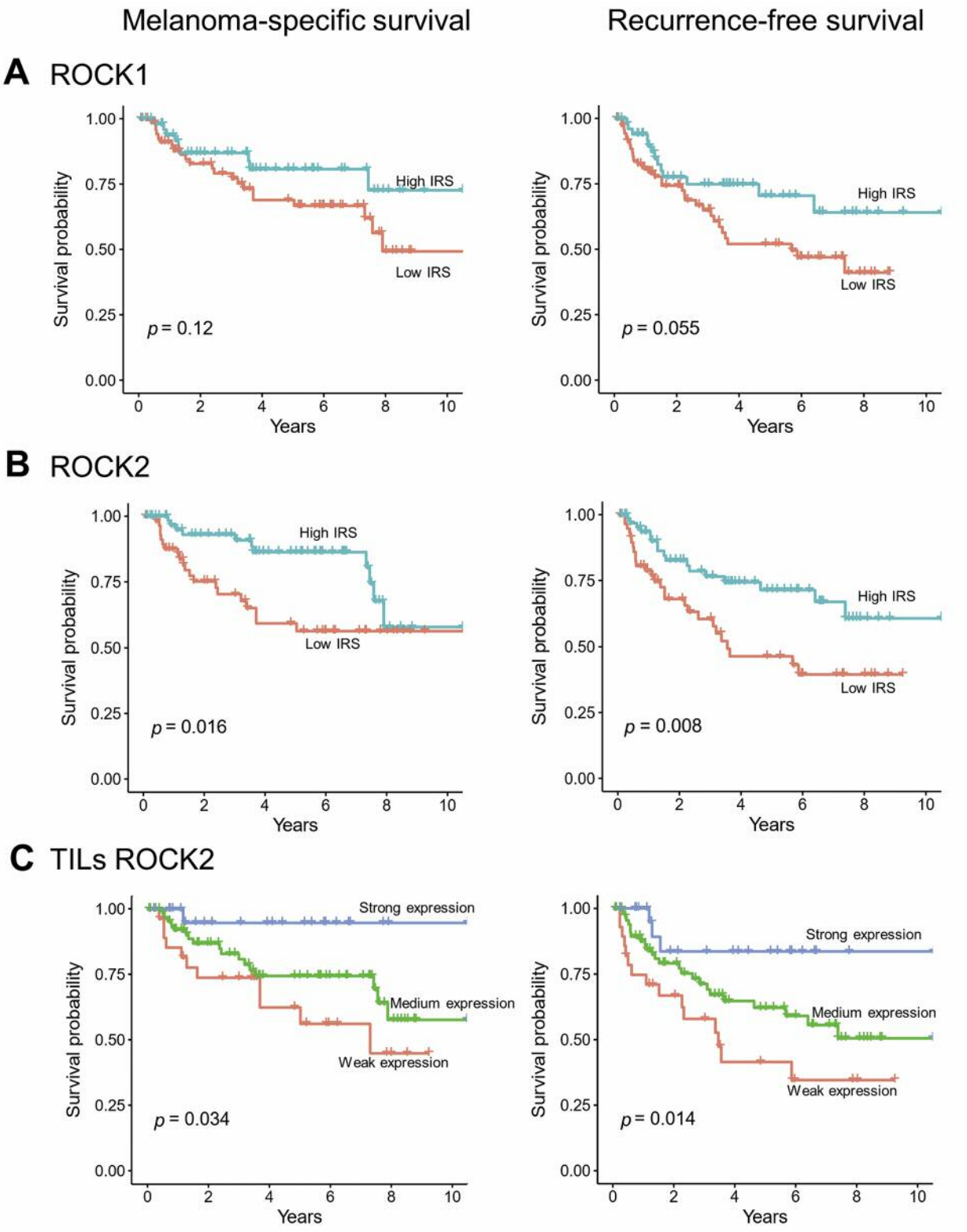

Figure 4. *Kaplan–Meier plots representing patient survival according to characteristics of Rho-associated coiled-coil kinase (ROCK) 1 and 2 expression in primary melanoma tumors. Differences between melanocytic expression of ROCK1 were not related to clinical outcome (A), but lower melanocytic expression of ROCK2 characterized patients with significantly shorter melanoma-specific survival and recurrence-specific survival (B, respectively). Moreover, reactivity for ROCK2 in tumor-infiltrating lymphocytes (TILs) positively correlated with patient prognosis (C).*

and -2 and other characteristics of melanoma, *e.g.* Breslow thickness, histological type, mitotic rate or ulceration. Overall, both proteins were expressed more strongly in tumors with favorable histological features and low clinical advancement. Interestingly, cases with nodal metastases at presentation accounted for 7% and 8% of tumors with strong lymphocytic ROCK1 and ROCK2 expression, respectively, but these rates escalated to 31% and 39% for melanomas with weak staining for ROCK1 and ROCK2 in TILs, respectively. In addition, reduced ROCK2 expression in TILs correlated with poor prognosis. What seems an alluring hypothesis is that TILs with higher activity of ROCKs are more effective at disease containment. This would be in line with previous studies reporting involvement of the Rho–ROCK pathway in T-cell activation and cytotoxicity (38-40). The role of ROCKs expressed by tumor-reactive immune cells remain virtually unexplored but our study indicates that it may be involved in the pathogenesis of cancer, and thus merits further investigation.

ROCK1 and ROCK2 are highly homologous isomers with serine/threonine kinase activity, sharing 65% of their entire amino acid sequence and over 90% of the catalytic domain (1). Both proteins have traditionally been considered to play similar roles in the control of cytoskeletal dynamics, cell-cycle progression and cellular adhesion (1). More recently, development of selective inhibitors and techniques of isoform-specific ROCK depletion allowed for appreciation of nonsynonymous functions of ROCK1 and -2 in various settings, including cancer. In one study, inhibition of ROCK2, but not ROCK1, triggered invasion of clustered colorectal carcinoma cells, and reduced *ROCK2* mRNA correlated with shorter patient survival, whereas no such correlation was observed for *ROCK1* expression (13). Functional investigations of glioblastoma cells revealed that selective knockdown of *ROCK1* or *ROCK2* exerted opposite effects on proliferation (41). Moreover, inhibition of ROCK1 or ROCK2 reduced or enhanced, respectively, glioblastoma cell migration on laminin-coated surface (41). Wang *et al.* demonstrated mRNA and protein expression of ROCK1 and ROCK2 in Y79 retinoblastoma cells but inhibition of only the former isoform affected cell adhesion and invasion (42). Most of the limited studies related to ROCK signaling in melanoma did not aim to differentiate between the roles of ROCK1 and -2 isoforms but Kümper *et al.* compared them in a mouse model of *BRAF*-mutated melanoma. Conditional knockout of *Rock2* resulted in earlier onset of tumorigenesis and reduced survival when compared with depletion of *Rock1* or both genes (43). In our study, expression of either ROCK1 and -2 was negatively correlated with Breslow thickness, ulceration and mitotic index. High analogy between ROCK1 and ROCK2 with regard to these clinicopathological associations advocate for a rather similar role of these proteins in the clinical setting. Moreover, melanocytic immunoreactivity of ROCK1 was moderately correlated to ROCK2 throughout the entire cohort with a coefficient of 0.63 between IRS for ROCK1 and ROCK2. Thus, mechanisms that regulate cellular concentrations of either ROCK isoform may be at least partially mutual. Contrarily, neither ROCK1 nor ROCK2 expression correlated with RhoA reactivity in our cohort. Such associations were reported previously in testicular and bladder tumors (44, 45), as well as in breast cancer, in which coordinated expression of RhoA and ROCKs was induced by hypoxia (46). Our results suggest that in melanoma, transcription or cellular turnover of RhoA and ROCK isoforms are regulated independently.

In summary, this and our previous studies demonstrated that constituents of Rho–ROCK pathway are down-regulated in aggressive and clinically advanced melanomas (28). Although definitive conclusions about the mechanistic involvement of ROCK1 and -2 in melanoma pathogenesis cannot be directly drawn from analysis of protein expression, our results raise the question of whether prior observations based on experiments with cell lines may be easily translatable into clinically valid conclusions. Comparison of ROCK1 and -2 expression in primary and metastatic melanoma lesions, as well as clinical verification of the relationship between BRAF status and RhoA–ROCK signaling, may help clarify the potential for therapeutic modulation of this pathway in cutaneous melanoma.

Conflicts of Interest

The Authors report no conflicts of interest in regard to this study.

Authors' Contributions

MK: Study design, resources, evaluation of immunohistochemistry, analysis of the results, drafting the article; PB: resources, statistical analysis, analysis of the results; MP: clinical data, analysis of the results; PD: resources, evaluation of immunohistochemistry, analysis of the results; RM: resources, analysis of the results; AH: analysis of the results, supervision. All Authors read, revised and approved the final article.

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