Role of ROC1 protein in the control of cyclin D1 protein expression in skin melanomas

Gisele Nai, Mariângela Marques

Department of Pathology, Botucatu Medical School, São Paulo State University (UNESP), Brazil

Abstract

A decrease in the level of the ROC1 protein, which is involved in cyclin D1 degradation, might explain an increase in cyclin D1 protein in the absence of gene overexpression. This study aimed to investigate the relationship between ROC1 and cyclin D1 expression in skin melanomas. A total of 62 cases of primary skin melanomas and 58 cases of compound melanocytic nevi were assessed. Immunohistochemistry was performed using cyclin D1 and ROC1 antibodies, and fluorescent in situ hybridization was used to assess the amplification of the CCND1 gene. ROC1 was expressed in >50% of cells in 87.9% of the melanocytic nevi cases and in 45.2% of the melanoma cases (p = 0.0014). There was a significant negative correlation between ROC1 and cyclin D1 expression in all cases (p = 0.0008985). In comparison with cyclin D1, ROC1 expression was increased in 86.2% of the melanocytic nevi and in 45.2% of the melanomas (p < 0.001). Among the non-amplified melanomas, 50% expressed cyclin D1 in >50% of the cells and expressed ROC1 in <25%. ROC1 expression is negatively correlated with cyclin D1 expression, demonstrating its importance in the degradation of cyclin D1 in melanomas.

Introduction

The transformation of melanocytes to melanoma cells is characterized by an abnormal proliferation that results from alterations in the two major cell cycle regulatory pathways: the retinoblastoma protein (pRb) and p53 tumor suppression pathways [18]. Nearly all the two major cell cycle regulatory pathways: the retinoblastoma protein, characterized by an abnormal proliferation that results from alterations in the two major cell cycle regulatory pathways: the retinoblastoma protein (pRb) and p53 tumor suppression pathways [18].

Cyclin D1 is a nuclear protein encoded by the CCND1 gene, which harbors the cyclin D1 gene. Although cyclin D1 is a well-known growth promoter, it may also function as a survival factor for tumor cells [27,31]. Cyclin D1 amplification or overexpression is a crucial event that leads to melanoma progression [10] and is associated with high proliferation rates in these tumors [17,29]. Failure to downregulate cyclin D1 overexpression in melanocytic cells probably promotes cell proliferation and prevents differentiation [29].

Cyclin D1 is a nuclear protein encoded by the CCND1 gene, which is located at chromosome 11q13. CCND1 amplification has been detected in over 44% of acral lentigious melanomas, but much less frequently in other melanoma subtypes [27,31].

All melanoma cases with an increased number of CCND1 copies overexpress cyclin D1. However, about 25% of melanomas that overexpress cyclin D1 have been found to have a normal number of CCND1 copies, suggesting that cyclin D1 levels are modulated by multiple mechanisms [4,18,27]. It is possible that cyclin D1 overexpression is induced by a defect in its degradation that increases its stability. Cyclin degradation is normally regulated by ubiquitin-dependent proteolysis [19,24].

Different ubiquitin-dependent proteolytic pathways use enzymes conjugated to different structurally similar ubiquitins. These, in turn, are associated with recognition subunits of proteins targeted by a particular degradation sign. The enzyme that, when conjugated, adds ubiquitin to a lysine residue of a target protein and then, subsequently, adds a series of additional ubiquitins, forms a polyubiquitin chain that is recognized by a specific receptor protein in proteasomes [1,14]. Polyubiquitin chains are linked covalently to the target protein through a cascade of three enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). In the last stage of this cascade, the ubiquitin-protein ligase (E3) acts as a central component of the ubiquitination pathway, catalyzing the final transfer of ubiquitin from E2 to the substrate [8,9,11,14,15]. The interaction of the E2 and E3 proteins is through protein fragments called RING finger proteins. The SCF protein (SKP1-CUL1(CDC53)-F-box) and the Anaphase Promoter Complex (APC) are the two major ubiquitin-ligase complexes. They regulate ubiquitin-mediated proteolysis during G1/S phase and anaphase, and they contain the small ROC1 and APC11 RING finger proteins, respectively [6,12,23].
Several studies have demonstrated that the SCF-ROC1 protein is crucial for the ubiquitination of cyclin D1, D2, and D3 in humans, playing a leading role in the regulation of cyclin proteolysis [19,24,32]. However, neither studies of the ROC1 immunohistochemical expression pattern nor studies comparing ROC1 and cyclin D1 expression in melanomas or other tumors are available in the literature.

The expression of p-cyclins correlates with melanoma malignancy potential and prognosis. Thus, understanding the mechanism underlying p-cyclin overexpression can contribute to the development of therapeutic approaches for melanomas overexpressing these proteins. The purpose of this work was to assess the relationship between ROC1 and cyclin D1 expression in skin melanomas and melanocytic nevi.

**Material and methods**

**Study groups**

This cross-sectional, analytic study included 62 cases of primary skin melanoma that were allocated into four groups, according to melanoma thickness: Group 1: 15 cases of melanoma <1 mm; Group 2: 15 cases of 1.01–2 mm melanoma; Group 3: 15 cases of 2.01–4 mm melanoma; and Group 4: 17 cases of melanoma >4 mm. A total of 58 cases of compound melanocytic nevus were used as controls (Group 5). The melanoma cases did not originate from melanocytic nevi nor did they show histological regression. The sample calculation was based on the prevalence of skin melanomas in the general population.

**Immunohistochemistry**

Tissue sections 4 μm thick were cut, mounted on slides previously treated with poly-D-lysine, and immunostained according to the ABC technique. Incubation with primary antibodies ROC1 (clone RB-069-P, LABVISION, Westinghouse, USA; 1/800 dilution) and cyclin D1 (clone RBT14, BioSB, Santa Barbara, USA; 1/100 dilution) was carried out. The reaction was developed with DAB (Sigma Chemical Co., St. Louis, USA) for five minutes and counterstained with Giemsa [25]. Squamous epithelium of tonsil was used as a positive control for ROC1 immunolabeling, and normal breast tissue was used as the control for cyclin D1.

A semiquantitative scoring system was used for the assessment of immunohistochemical staining. Cell nuclei are either positive or negative for ROC1 and cyclin D1. The percentage of tumor cells with positive staining was determined and classified into four classes: (1) 0–25% of cells stained; (2) 26–50% of cells stained; (3) 51–75% of cells stained; and (4) 76–100% of cells stained [27].

For comparative purposes, the following categories were used to classify the relationship between ROC1 and cyclin D1 expression (ROC1/cyclin D1): (1) increased ROC1 in relation to cyclin D1 expression, or when the percentage of ROC1-positive cells was higher than that of cyclin D1-positive cells; (2) proportional ROC1 and cyclin D1 expression, or when the percentage of ROC1-positive cells was the same as that of cyclin D1-positive cells; and (3) increased cyclin D1 in relation to ROC1 expression, or when the percentage of cyclin D1-positive cells was higher than that of ROC1-positive cells.

**Fluorescent in situ hybridization (FISH)**

For FISH was used the Vysis® LSI® Cyclin D1 (11q13) SpectrumOrange/CEP 11 SpectrumGreen™ Probe (Downers Groove, USA) that is a dual-color probe consisting of a red-labeled locus-specific (CCND1 gene) and a green-labeled specific chromosome 11 centromeric region.

A total of 60 nuclei from each sample were assessed using FISHView/SPOTView (Applied Spectral Imaging, Israel) for the quantification of nuclear gene amplification and analysis of differences in nuclear gene amplification within the same tumor.

Gene amplification was considered negative when the CCND1/CEP11 ratio was <1.8; equivocal when the CCND1/CEP11 ratio was 1.8–2.2; and positive when the CCND1/CEP11 ratio was >2.2 [27].

**Statistical analysis**

In order to detect differences in protein expression associated with age, gender, lesion site, study group, melanoma type, and Breslow thickness, the Chi-square test or the Fisher's exact test was used. A Spearman's coefficient was used to assess correlations between expression levels. Significance level was set at α = 0.05 in all tests.

**Approval by the Research Ethics Committee**

This study was approved by the Research Ethics Committee of Botucatu Medical School – UNESP (OF. 79/2007-CEP).

**Results**

The patient median age was 60.5 years (23–89 years) in the melanoma group and 30.5 years (4–71 years) in the melanocytic nevus group.

The melanoma group was composed of superficial, spreading melanomas (SSM) (41.9%, n = 26), followed by nodular melanomas (20.9%, n = 13), lentigo maligna melanoma (LMM) (19%, n = 12), acral lentiginous melanoma (16.6%, n = 10), and one unclassified melanoma.

ROC1 and cyclin D1 expression did not vary with age, gender, or lesion site in either the melanoma or the melanocytic nevus group (p > 0.05).

The expression of ROC1 correlated with neoplasia type (benign or malignant) (p = 0.0014). Cyclin D1 protein expression also correlated with neoplasia type (p = 0.000). In the melanocytic nevus group, ROC1 was expressed by >75% of the cells in 62.1% of the cases (n = 36), and by >50% of the cells in 87.9% (n = 51) (p < 0.05). Cyclin D1, in turn, was expressed in <25% of the cells in most cases (91.4% n = 53). In only one case was cyclin D1 expressed in 51–75% of the cells, and no cases showed it in >75% of the cells (p < 0.05) (Fig. 1).

In the melanoma cases, ROC1 expression was observed in >50% of the cells in 45.2% of cases (n = 28) and in <25% of the cells in 27.4% of cases, whereas cyclin D1 was expressed in <25% of the cells in 45.2% of cases (n = 28), and in >50% of the cells in 35.5% of cases (n = 22) (p < 0.05) (Fig. 2).

There was no statistical difference between ROC1 and cyclin D1 expression in relation to melanoma histological type (p > 0.05). Similarly, no statistical difference between ROC1 and cyclin D1 expression levels was associated with Breslow thickness (p > 0.05). However, cases with <25% of the cells expressing ROC1 protein (33.3–35.3% of cases) predominated in Groups 1, 3, and 4, while cases with ROC1 expression in >75% of cells predominated in Group 2 (66.8%) (Fig. 3). On the other hand, cyclin D1 expression was <25% in Groups 1, 2, and 3, but >50% in Group 4 (70.6% of the samples). Group 2 showed no cases with >75% of the cells expressing cyclin D1.

A significant negative correlation was observed between ROC1 and cyclin D1 expression levels regardless of neoplasia type (benign or malignant) (p = 0.0008985). Comparisons between ROC1 and cyclin D1 expression in melanomas and melanocytic nevi are shown in Tables 1 and 2, respectively.
In some cases of melanoma, areas with >75% of the cells expressing ROC1 and <25% of cells expressing cyclin D1 were observed adjacent to areas wherein ROC1 was positive in <25% of the cells, and cyclin D1 was expressed in >75% of the cells. This was found to be independent of increased gene expression (Fig. 4).

The ROC1/cyclin D1 relationship did not vary with age, gender, or lesion site in either melanomas or melanocytic nevi (p > 0.05).

Table 1
Comparison of ROC1 and cyclin D1 expression in melanomas (n = 62) (p = 0.008).

<table>
<thead>
<tr>
<th>Cyclin D1 protein expression</th>
<th>0–25% (%)</th>
<th>26–50% (%)</th>
<th>51–75% (%)</th>
<th>&gt;75% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROC1 protein expression</td>
<td>0–25%</td>
<td>26–50%</td>
<td>51–75%</td>
<td>&gt;75%</td>
</tr>
<tr>
<td>0–25%</td>
<td>4/17 (23.5%)</td>
<td>2/17 (11.7%)</td>
<td>7/17 (58.7%)</td>
<td>4/17 (23.5%)</td>
</tr>
<tr>
<td>26–50%</td>
<td>2/5 (40%)</td>
<td>2/5 (40%)</td>
<td>0/5</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>51–75%</td>
<td>5/12 (41.6%)</td>
<td>3/12 (25%)</td>
<td>3/12 (25%)</td>
<td>1/12 (8.4%)</td>
</tr>
<tr>
<td>&gt;75%</td>
<td>17/28 (60.7%)</td>
<td>5/28 (17.9%)</td>
<td>3/28 (10.7%)</td>
<td>3/28 (10.7%)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>28/62 (45.2%)</td>
<td>12/62 (19.3%)</td>
<td>13/62 (21%)</td>
<td>9/62 (14.5%)</td>
</tr>
</tbody>
</table>
Increased ROC1 protein expression, as compared with cyclin D1 expression, predominated in all samples (65% of cases; n = 78).

In the melanocytic nevus group, the ROC1 expression increase was remarkably predominant in relation to cyclin D1 expression (86.2% of the cases). In melanomas, this ROC1 expression predominance was also observed, but in only 45.2% of the cases (p < 0.001) (Table 3).

Although ROC1 and cyclin D1 expression levels were predominantly proportional in melanomas with thickness >2 mm, and although a great number of cases with melanomas >4 mm (35.3%) showed increased cyclin D1 expression in comparison with ROC1 levels, no statistically significant difference was seen among the groups (p = 0.166).

Only in the acral lentiginous melanoma group was cyclin D1 expression greater than that of ROC1 in a large number of cases (40%). On the other hand, this group also showed the largest number of cases with increased ROC1 expression as compared to cyclin D1 expression (50%). No statistically significant difference in the ROC1/cyclin D1 relationship was observed in relation to melanoma histological type (p = 0.605).

Six cases (five melanomas and one melanocytic nevus) exhibited CCND1 gene amplification. In two amplified cases, one was acral lentiginous melanoma and the other was nodular melanoma with Breslow thickness of >4 mm. Cyclin D1 was expressed in 51–75% of the acral lentiginous melanoma cells and in >75% of the nodular melanoma cells. In both the acral lentiginous and nodular melanomas, ROC1 expression was present in <25% of the cells. In the other amplified melanomas (2 SSM and 1 LMM), in one case, the Breslow's thickness was <1 mm, in another it was 1.01–2 mm, and in the other it was 2.01–4 mm. Of these three amplified melanomas, two showed cyclin D1 and ROC1 expression in 51–75% of the cells, while in the other case, cyclin D1 positivity was <25%, and ROC1 was expressed in >75% of the cells.

The sixth amplified case was a genital melanocytic nevus where 51–75% cyclin D1 expression was associated with ROC1 expression in 26–50% of the cells (Fig. 5). This melanocytic nevus was the only...
Fig. 4. Case 1.13: superficial spreading melanoma. (A) Area 1, hematoxylin–eosin, 400×. (B) Area 1, ROC1 expression in >75% of the cells. Immunostaining with anti-ROC1 antibody, clone RB-069-P, LABVISION, 400×. (C) Area 1, cyclin D1 expression in <25% of the cells. Immunostaining with anti-cyclin D1 antibody, clone RBT14, BioSB, 400×. (D) Area 2, hematoxylin–eosin, 400×. (E) Area 2, ROC1 expression in <25% of the cells. Immunostaining with anti-ROC1 antibody, clone RB-069-P, LABVISION, 400×. (F) Area 2, cyclin D1 expression in >75% of the cells. Immunostaining with anti-cyclin D1 antibody, clone RBT14, BioSB, 400×. (G) Normal number of copies of the CCND1 gene, FISH, CCND1 gene labeled in red and CEP11 in green, 1000×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
one to exhibit increased cyclin D1 expression as compared to ROC1 expression. In the majority of melanomas with amplification, protein expressions were proportional (40% of the cases) or cyclin D1 expression was increased when compared with ROC1 expression (40% of the samples). Among non-amplified melanomas, 50% of those with >50% cyclin D1 positivity exhibited ROC1 expression in <25% of cells (Fig. 6), and 43.7% showed ROC1 expression in >50% of cells. No correlation between the amplification of the CCND1 gene and the relationship between protein expression levels was found ($p = 0.500$).

**Discussion**

The ROC1 RING finger protein (RING of Cullins), also called Rbx1 and Hrt1, is a highly stable protein that belongs to the C3H2C3 (or RING-2) subclass of RING finger proteins and acts as an essential subunit of ubiquitin-ligase SCF protein [13,19]. It was first isolated in yeast [21] and was biochemically purified as a common component of both the human and yeast SCF complexes [16,28,30], as well as of the von Hippel-Lindau tumor-suppressor complex (CBCVHL or Cul2-Elongin BC-VHL) [7,15] (for review, see Nai and Marques – [20]).

ROC1 protein is encoded by the human gene Rbx1, which contains five exons and is located on chromosome 22q13 [22]. Point mutations in a single amino acid in the ROC1 protein domain can completely disrupt ubiquitin-ligase activity [13,19,21,26]. It mediates the degradation of substrate proteins required for cell cycle progression, signal transduction, and tumor-suppressing activities [7]. It plays an important role in labeling cyclin D1 for proteosomal degradation [19,24,32].

In this study, the expression of ROC1 correlated with neoplasia type (benign or malignant). In the melanocytic nevus group, ROC1 was expressed in >50% of cells in most cases, and in <25% of cells in only one case. However, in the melanoma group, low ROC1 levels (<25%) were seen in a large number of cases, demonstrating a ROC1 deficiency in this group. Nonetheless, no correlations of ROC1 expression with Breslow’s thickness or melanoma histological type were found. Cyclin D1 expression also correlated with neoplasia type. Moreover, in the melanoma group, cyclin D1 expression showed no correlation with Breslow thickness or melanoma histological type.

Although no significant correlations of Breslow thickness with ROC1 and cyclin D1 expressions were detected, increased ROC1 positivity predominated in melanomas of 1.01–2 mm thickness while higher cyclin D1 levels were seen in melanomas thicker than 4 mm. In melanomas with a Breslow thickness between 1.01 and 2 mm, it is possible to observe the beginning of a neoplasia vertical growth phase. The increased ROC1 expression found in tumors of this thickness may reflect an attempt of the host to restrain the progression of the lesion. Similarly, the higher expression of cyclin D1 in melanomas over 4 mm in thickness may be interpreted as indicative of uncontrolled proliferation in thicker tumors at the full tumorigenic phase.

A significant negative correlation was observed between ROC1 and cyclin D1 expression in the study cases. When ROC1 expression increased, cyclin D1 expression decreased, and vice-versa. Melanomas containing areas of high ROC1 protein expression and low cyclin D1 positivity were observed alongside areas of high cyclin D1 expression and low ROC1 expression, making evident the presence of different cell clones in these lesions, as visualized by light microscopy.

The amplification of the CCND1 gene in melanocytic nevi is rare, and so is cyclin D1 expression increase [5,29]. Strikingly, one of the melanocytic nevus cases included in this study showed CCND1
amplification and the highest level of cyclin D1 expression of all melanocytic cases studied (51–75%), associated with a decreased ROC1 expression (26–50%). This case of melanocytic nevus was observed in the genital region of a 20-year-old female. It was characterized by intense junctional activity and cellularity and by areas with morphologically distinct cells contiguous with each other in the likeness of clones. Interpreting an isolated case is difficult, but one explanation for the partial reduction in ROC1 may be the consumption of this protein for the degradation of the increased cyclin D1 that is found in a lesion in the proliferative stage.

In this study, both melanomas with all cells amplified showed cyclin D1 expression in >50% of cells and ROC1 expression in <25% of cells. The lower ROC1 expression observed in the amplified melanomas as compared to the amplified nevus suggests a ROC1 deficiency and not just its consumption for the labeling of the increased cyclin. This assumption is corroborated by the fact that in focally amplified melanomas, no significant ROC1 decrease occurred even when cyclin D1 was increased. It is also confirmed by non-amplified cases that showed increased cyclin D1 expression and a significant ROC1 decrease.

The ROC1/cyclin D1 relationship correlated with neoplasia type. In melanocytic nevi, there was a predominance of increased ROC1 expression in relation to cyclin D1 (86.2% of the cases), whereas in melanomas, ROC1 expression was higher than cyclin D1 expression in 45.2% of the cases. The only case of a melanocytic nevus in which cyclin D1 was higher than ROC1 expression showed CCND1 amplification, which is in contrast with the melanomas where the majority of cases showed increased cyclin D1 as compared to ROC1 expression and no gene amplification (85.7%). This fact, and the absence of correlations between ROC1/cyclin D1 and gene amplification observed here, supports the idea of ROC1 deficiency in melanomas as part of the phenomenon responsible for the increase in cyclin D1.

The amplification of the CCND1 gene is more common in acral lentiginous melanomas, followed by SSM. In the former, amplification may occur early, even before the neoplasia in situ stage, different from other melanoma types that show amplification later, during progression [4,31]. In this study, however, in the acral lentiginous melanomas showing the highest proportional number of cases of cyclin D1 increase in relation to ROC1 (40%), ROC1/cyclin D1 was not associated with melanoma histological type or Breslow thickness. This shows that ROC1 expression alteration may be an event of melanoma oncogenesis not related to histological type. Even if a correlation of ROC1/cyclin D1 relationship with Breslow thickness does not occur, the large number of cases with ROC1 expression higher than that of cyclin D1 among melanomas <2 mm in thickness may show a stage during which the host response is still effective in restraining tumor progression.

Of the 20 melanoma cases with proportional ROC1 and cyclin D1 expressions (32.3%), amplification of the CCND1 gene was seen in only two. In the melanocytic nevus group, both proteins were proportionally expressed in six cases (10.3%), and none of them showed gene amplification. In the non-amplified melanocytic nevi with proportional ROC1/cyclin D1, cyclin D1 was expressed in <25% of cells and in most cases. On the other hand, in the melanoma group, only five cases showed cyclin D1 in <25% of cells, while six cases exhibited cyclin D1 expression in >50% of cells associated with ROC1 expression also in >50% of the cells. This finding suggests that, despite a ROC1 expression decrease in some cases, cyclin D1 levels in melanocytic nevi remained unchanged possibly due to a predominating cyclin D1 gene expression control mechanism. In melanomas, the mechanism regulating cyclin D1 expression may be something other than gene expression increase and ubiquitination failure. It might include the deficiency of other proteins involved in cyclin ubiquitination, such as cullins proteins.
In most melanocytic nevi, ROC1 protein was expressed by >75% of cells. Deficient ROC1 expression was associated with skin melanomas, where ROC1 expression negatively correlated with cyclin D1 expression, demonstrating the leading role of ROC1 in cyclin D1 degradation within these tumors.

The ROC1/cyclin D1 expression relationship correlated with neoplasia type. In melanocytic nevi, there was a predominance of increased ROC1 in relation to cyclin D1 expression, whereas in the melanoma group, about one fourth of the cases showed increased cyclin D1 as compared to ROC1 expression.

Neither ROC1 levels nor the ROC1/cyclin D1 expression relationship correlated with Breslow thickness or melanoma histological type. However, studies including a larger number of cases with 1.01–2-mm-thick melanomas and acral lentiginous melanomas are necessary to determine whether these parameters actually correlate.

Although this study has few cases with CCND1 gene amplification, the lower ROC1 expression observed in the amplified melanomas compared to amplified nevi, and the great number of non-amplified melanomas that had an increase in cyclin D1 expression and a significant ROC1 decrease, both suggest a ROC1 deficiency in melanomas.

Studies aiming at better understanding the causes of low ROC1 expression which might increase cyclin D1 expression in skin melanomas could highly contribute to the investigation of novel treatments for these tumors.

Acknowledgements

To MedGen Comércio e Importação Ltda, for providing anti-ROC1 antibody aliquots for testing, and to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for their financial support (grant # 07/53269-6).

References