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"Paradoxical" p16 overexpression in cutaneous melanoma: Molecular and immunohistochemical analysis of a rare phenomenon with a focus on cell cycle regulatory molecules☆

Costantino Ricci [a,b,1,2], Emi Dika [c,d,1,3], Barbara Corti [e,4], Martina Lambertini [c,d,5], Francesca Ambrosi [a,b,6], Simone Cappilli [f,g,7], Marco Grillini [e,8], Giorgia Di Filippo [a], Eugenia Franchini [a], Thais Maloberti [b,h,9], Michelangelo Fiorentino [a,b,*,10], Giovanni Tallini [b,h,11], Dario de Biase [h,i,12]

[a] Pathology Unit, Maggiore Hospital, AUSL Bologna, Bologna, Italy
[b] Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy
[c] IRCCS Azienda Ospedaliero-Universitaria Policlinico di Sant'Orsola, University of Bologna, Bologna, Italy
[d] Dermatology Unit, Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy
[e] Pathology Unit, IRCCS Azienda Ospedaliero-Universitaria Policlinico di Sant'Orsola, University of Bologna, Bologna, Italy
[f] UOC di Dermatologia, Dipartimento di Scienze Mediche e Chirurgiche, Fondazione Policlinico Universitario A. Gemelli-IRCCS, Rome, Italy
[g] Dermatologia, Dipartimento di Medicina e Chirurgia Traslazionale, Università Cattolica del Sacro Cuore, Rome, Italy
[h] Solid Tumor Molecular Pathology Laboratory, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy
[i] Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy

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ABSTRACT

Background: One of the most relevant genetic alterations in cutaneous melanoma (CM) is the biallelic inactivation/loss-of-heterozygosis (LOH) of *cyclin-dependent kinase inhibitor 2 A* (*CDKN2A*), which results in the immunohistochemical loss of p16 frequently found in CM. However, we recently described a rare case of dermal/deep-seated melanoma arising in giant congenital nevus (DDM-GCN) with p16 overexpression combined with p53 loss and *tumor protein 53* (*TP53*) mutation. Herein, we reported a case series of CM with p16 overexpression and analyzed their clinicopathologic features, immunohistochemical expression of the cell cycle regulatory molecules (CCRM: p53, p21, Cyclin D1, Rb), and mutational landscape.

Abbreviations: CM, cutaneous melanoma/melanomas; CN, cutaneous nevus/nevi; LOH, loss-of-heterozygosis; CDKN2A gene, *cyclin-dependent kinase inhibitor 2A*; TP53 gene, tumor protein 53; NGS, next-generation sequencing; CCRM, cell cycle regulatory molecules; [p16 + c], component with p16 overexpression; [p16-c], component without p16 overexpression; DDM-GCN, dermal/deep-seated melanoma arising in giant congenital nevus; GCN, giant congenital nevus; PN-GCN, proliferative nodules arising in GCN; SSM, superficial spreading melanoma; NdM, nodular melanoma; SM, Spitz melanoma; DM, desmoplastic melanoma; SLNB, sentinel lymph node biopsy; ITCs, isolated tumor cells; FISH, fluorescence in situ hybridization; FFPE, formalin-fixed paraffin-embedded; VAF, variant allele frequency.

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* Correspondence to: Pathology Unit, IRCCS Policlinico Sant'Orsola-Malpighi, University of Bologna, Via Massarenti 9, Bologna 40138, Italy.
E-mail address: michelangelo.fiorentino@unibo.it (M. Fiorentino).
[1] ORCID: 0000-0001-7254-4195
[2] These two authors equally contributed to the article.
[3] ORCID: 0000-0003-3186-2861
[4] ORCID: 0000-0001-6555-6227
[5] ORCID: 0000-0003-4282-342X
[6] ORCID: 0000-0001-9046-1115
[7] ORCID: 0000-0001-9813-2391
[8] ORCID: 0000-0003-2506-7239
[9] ORCID: 0000-0002-8306-4653
[10] ORCID: 0000-0002-1749-150X
[11] ORCID: 0000-0003-0113-6682
[12] ORCID: 0000-0002-0609-8817

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Methods: We retrospectively tested for p16 all cases of CM diagnosed at our institution between January 1st 2019-April 1st 2022. In CM with p16 overexpression, we reported clinicopathologic features, immunohistochemical results for melanocytic markers and CCRM, and mutational landscape investigated with a next-generation sequencing (NGS) panel. In cases with zonal p16 overexpression, the immunohistochemical assessment for melanocytic markers and CCRM, as well as the NGS analysis have been performed in both components {with and without p16 overexpression [p16(+)c and p16(-)]}.
Results: Overexpression of p16 was found in 10/2879 (0.35%) CM [5/10 (50%) diffuse and 5/10 (50%) zonal]. We combined the immunohistochemical results for CCRM and molecular data to classify the cases as follows: a) *Group 1* with altered expression of at least one CCRM but no *TP53* mutations [3/10 (30%), all with Rb altered/lost]; b) *Group 2* with altered expression of at least one CCRM and *TP53* mutations [4/10 (40%), all with p53 altered]; c) *Group 3* with normal expression of CCRM and no *TP53* mutations [3/10 (30%), all with mutations in MAPK pathway genes (*NRAS* and *BRAF*)]. In CM with zonal p16 overexpression, the histologic appearance of p16 (+)c was heterogeneous, whereas combining CCRM profiles and molecular data the cases could be categorized as follows: a) cases with the same CCRM and molecular profiles in both p16(+)c and p16(-)c; b) cases with p16(+)c showing additional genetic mutations and/or modifications of CCRM expression.
Conclusions: p16 overexpression is a rare event, occurring in advanced-stage, clinically- and histologically-heterogeneous CM. These lesions may be classified into three different groups based on CCRM expression and mutational profiles (including *TP53* mutation). The analysis of CM with zonal p16 overexpression suggests that, at least in a subset of cases, this phenomenon could represent a sign of "molecular progression" due to the acquisition of additional genetic mutations and/or modifications of the CCRM profile.

1. Introduction

Our understanding of the mutational landscape of cutaneous melanoma (CM) has made significant progress in the past several years [1–3]. One of the most relevant genetic alterations in CM is the biallelic inactivation/loss-of-heterozygosis (LOH) of *cyclin-dependent kinase inhibitor 2 A* (*CDKN2A*) encoding the tumor suppressor proteins p16 and p14arf [1–8]. Specifically, LOH of *CDKN2A*, through different molecular mechanisms spanning from heterozygous/homozygous deletions and promoter hyper-methylation to missense and truncating mutations has been identified as a distinctive feature of invasive, advanced-stage and/or metastatic CM [1–8]. Consequently, immunohistochemical identification of p16 loss has been adopted in the routine practice for the differential diagnosis between CM and cutaneous nevi (CN) [9–19]. We recently reported an exceptionally rare case of a dermal/deep-seated melanoma arising in giant congenital nevus (DDM-GCN) showing unexpected immunohistochemical overexpression of p16 combined with immunohistochemical loss of p53 and *tumor protein 53* (*TP53*) mutation [20]. We hypothesized the existence of cases of CM with a peculiar combination of genetic mutations and alterations of the cell cycle regulatory molecules (CCRM) that biologically interact with p16, resulting in paradoxical p16 overexpression. Thus, we retrospectively analyzed p16 expression in CM cases to identify those with overexpression of the protein, and here report their prevalence, clinicopathologic features, immunohistochemical melanocytic marker and CCRM (p53, p21, Cyclin D1, Rb) profiles, and genetic alterations identified using a laboratory-developed multi-gene next generation sequencing (NGS) panel [21].

2. Materials and methods

2.1. Case series

We retrospectively reviewed and immunohistochemically tested for p16 all cases of CM diagnosed at our institution (DIAP-Dipartimento InterAziendale di Anatomia Patologica, Bologna, Italy: Pathology Unit, Maggiore Hospital, AUSL Bologna, Bologna, Italy; IRCCS Policlinico Sant'Orsola-Malpighi, University of Bologna Medical Center, Bologna, Italy) between January 1st 2019-April 1st 2022. All cases with p16 overexpression (*see Clinical, histologic, and immunohistochemical analyses* and Supplementary Material 1-Table S1) were included in the present study. All clinicopathologic investigations were conducted according to the principles of the Declaration of Helsinki and all information regarding the human material used in this study has been managed using anonymous numerical codes. The study had been approved by the internal committee of IRCCS Policlinico Sant'Orsola-Malpighi, University of Bologna Medical Center, Bologna, Italy (SkinCancer-18).

2.2. Clinicopathologic data and immunohistochemistry

Clinical data (age, sex, and tumor localization) were retrieved from the digital records of the Dermatology Unit, IRCCS Policlinico Sant'Orsola-Malpighi, University of Bologna Medical Center. CM were diagnosed and staged according to the 2023 WHO Classification of Skin Tumours and 8th ed. of the AJCC Cancer Staging Manual [1,22]. From all paraffin-embedded tissue blocks, two consecutive 3-μm thick sections were stained with H&E and p16 (BenchMark ULTRA automated immunostainer; Ventana Medical Systems-Roche Diagnostics, Switzerland), as previously described [14]. In CM with p16 overexpression (Supplementary Material 1-Table S1), nine additional and consecutive 3-μm thick sections were cut and stained with S-100, SOX10, HMB45, MART-1, PRAME, Rb, Cyclin D1, p21, and p53 (BenchMark ULTRA automated immunostainer; Ventana Medical Systems-Roche Diagnostics, Switzerland) [14–16,23–26]. In CM with zonal p16 overexpression (Supplementary Material 1-Table S1), immunohistochemical assessment for melanocytic markers and CCRM was performed in both components {component with and without p16 overexpression [p16(+)c and p16(-)]}. PRAME has been evaluated according to the score proposed by *Lezcano C* et al. [24]. Criteria adopted to evaluate p16 and CCRM (p53, p21, Cyclin D1, and Rb), as well as to dichotomize cases in "normal"/"altered" for the immunohistochemical expression of these markers have been adopted from the previous literature and are summarized in Supplementary Material 1-Table S1 [15,16,25,26]. Expected nuclear and/or cytoplasmatic stain of normal/non-tumoral cells (endothelial cells, fibroblast, keratinocytes, etc.) has been used as internal controls. Clone antibodies, dilutions, and other immunohistochemical technical data are summarized in Supplementary Material 2-Table S2.

2.3. Mutational analysis

One or two 10-μm thick sections were used for DNA extraction using the "QuickExtract™ FFPE DNA Extraction Solution" kit (Lucigen Corporation, Middleton, WI, USA) and quantified using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) (Thermo Fisher Scientific, Waltham, MA, USA). About 30 ng of DNA was used for amplicons preparation, and sequencing was performed using a laboratory-developed multi-gene NGS panel in use at our Solid Tumor

Molecular Pathology Laboratory, IRCCS Azienda Ospedaliero-Universitaria di Bologna, University of Bologna Medical Center (Bologna, Italy) for somatic genetic analysis of several solid tumors including CM (330 amplicons total, human reference sequence hg19/GRCh37) [21]. The genomic regions covered by the NGS panel are reported in Supplementary Material 3-Table S3. To minimize PCR inhibition due to the presence of melanin, 2ul of Betaine 1 N was added to the final reaction mix. Templates were then sequenced using an Ion 530 chip, and results analyzed with IonReporter tools (Thermo Fisher Scientific). According to previously reported NGS panel validation data, only variants identified in at least 5% of the total number of reads analyzed and observed in both sequencing strands were considered for mutational calls [27]. In CM with zonal p16 overexpression, we dissected the two components [p16(+)c and p16(-)] and NGS molecular analysis was performed on the two components separately.

3. Results

3.1. Clinicopathologic data and immunohistochemical melanocytic marker profile

Overexpression of p16 was found in 10/2879 (0.35%) CM [5/10 (50%) diffuse and 5/10 (50%) zonal]. The mean age at diagnosis was 64.2 years (range: 37–81 years), with 7/10 (70%) males and 3/10 (30%) females. CM were located in the extremities (6/10, 60%), trunk and dorsum (3/10, 30%), and head & neck region (1/10, 10%). According to the 2023 WHO Classification of Skin Tumours, the histologic subtypes included 1 (10%) DDM-GCN, 5 (50%) superficial spreading (SSM), 2 (20%) nodular (NdM), 1 (10%) Spitz (SM), and 1 (10%) desmoplastic (DM) melanoma [1]. The mean Breslow thickness was 8.5 mm (range: 1.5–19 mm). According to the 8th edition of the AJCC Cancer Staging Manual, the pT stages included 1 (10%) pT2b, 2 (20%) pT3a, 2 (20%) pT3b, and 5 (50%) pT4b [22]. All but one patients (9/10, 90%) underwent sentinel lymph node biopsy (SLNB), and lymph node metastases were identified in 4 (44.4%) cases; pN stages included 1 (10%) pNx, 5 (50%) N0, 2 (20%) pN1a both with isolated tumor cells (ITCs), 1 (10%) N2a, and 1 (10%) N2c with microsatellites [22]. Notably, *case #5* with DDM-GCN did not show lymph node metastases but multiple proliferative nodules histologically comparable to those of the primary cutaneous lesion [20]. Distant metastases to skin and soft tissue (M1a) were found in 1 (10%) patient (*case #1*). Clinicopathological data of the case series are listed in Table 1. Five cases (*cases #2*, *3*, *4, 5*, and *7*) were positive (with focal, moderate, and/or diffuse positivity) for all tested melanocytic markers (S-100, SOX10, HMB45, and MART-1), whereas the other five cases were positive for at least one but not all these markers. All but one case (case *#10*, DM with score 1 *sec. Lezcano* et al.) showed diffuse PRAME stain (score 4 *sec. Lezcano* et al.) [24]. In CM with zonal overexpression of p16, we did not find marked immunohistochemical melanocytic markers expression differences between p16(+)c and p16(-)c, except that for *case #5* [DDM-GCN, giant congenital nevus (GCN), and proliferative nodules arising in GCN (PN-GCN)] [20]. Immunohistochemical results for melanocytic markers of the case series are listed in Supplementary Material 4-Table S4.

3.2. CCRM and mutation profiles

We combined immunohistochemical results of CCRM and sequencing data to categorize CM with diffuse p16 overexpression and p16(+)c of CM with zonal p16 overexpression as follows:

-*Group 1* {altered expression of at least one CCRM but no *TP53* mutations [3/10 (30%), all with Rb altered/lost}.

- *Case #7*: Rb altered/lost /// *BRAF* (p.Val600Glu) and *TERT* (c.−146 C>T) mutations;
- *Case #8*: Rb altered/lost /// *BRAF* (p.Val600Glu) mutation;
- *Case #9*: Rb altered/lost, p21 and Cyclin D1 altered /// *HRAS* (p.Gly12Arg) mutation;

-*Group 2* {altered expression of at least one CCRM and *TP53* mutations [4/10 (40%), all with p53 altered}

- *Case #1*: p53 altered/lost /// *NRAS* (p.Gln61LysC), *TERT* (c.−146 C>T), and *TP53* (c.560-2 A>T) mutations;
- *Case #4*: Rb altered/lost, p53 altered/overexpressed /// *BRAF* (p.Val600Lys), *TERT* (c.−124/−125 delinsTT), and *TP53* (p.Tyr234His) mutations;
- *Case #5*: Rb altered/lost, p53 altered/lost /// *NRAS* (p.Gln61LysC) and *TP53* (p.Arg213Ter) mutations;
- *Case #10*: *p53* altered/overexpressed and p21 altered /// *TP53* (p.Gln165SerfsTer5) mutation;

-*Group 3* {normal expression of CCRM and no *TP53* mutations [3/10 (30%), all with MAPK pathway genes (*NRAS* and *BRAF*) mutations}

- *Case #2*: *BRAF* (p.Val600Glu) and *KIT* (p.Arg804Gly) mutations;
- *Case #3*: *NRAS* (p.Gly12Asp), *TERT* (c.−146 C>T), and *DPYD* (c.1905 +1 G>A) mutations;
- *Case #6*: *NRAS* (p.Gly12Asp) and *TERT* (c.−146 C>T) mutations;

3.3. CM with zonal p16 overexpression

Histologically, CM with zonal p16 overexpression showed heterogeneous appearance:

-p16(+)c with a tumoral/nodular silhouette largely overwhelming p16(-)c, which persists as a residual component at the shoulder of the lesion (*cases #4, #7 and #8*);.

-DDM-GCN [p16(+)c] with tumoral/nodular silhouette intermixed and pushing PN-GCN and the residual component of GCN [p16(-)c] (*case #5*);.

Table 1
Clinical-pathological data of the case series.

Case number	Sex	Age (years)	Localization	Breslow thickness (mm)	Histological subtype	pT	pN
1	F	70	Extremities	6	NdM	pT4b	pN2a^
2	F	65	Extremities	5	SSM	pT4b	pN0
3	F	81	Extremities	9	NdM	pT4b	pNx
4	M	68	Trunk and dorsum	3.4	SSM	pT3b	pN0
5	M	37	Extremities	19	DDM-GCN	pT4b	pN0§
6	M	77	Extremities	3.6	SSM	pT3b	pN0
7	M	56	Trunk and dorsum	9	SSM	pT4b	pN2c¶
8	M	43	Trunk and dorsum	1.5	SSM	pT2b	pN1aØ
9	M	65	Extremities	3.9	SM	pT3a	pN1aØ
10	M	80	Head & neck region	3	DM	pT3a	pN0

ØBoth cases showed isolated tumor cells (ITCs);
^This patient developed distant metastases to skin and soft tissue (M1a);
§This case showed multiple proliferative nodules in the lymph nodes, which were histologically comparable to those of the primary cutaneous lesion;
¶This patient had multiple microsatellites; female (F); male (M); dermal/deep-seated melanoma arising in giant congenital nevus (DDM-GCN); superficial spreading melanoma (SSM); nodular melanoma (NdM); Spitz melanoma (SM); desmoplastic melanoma (DM); isolated tumor cells (ITCs);

-p16(+)c with tumoral/nodular silhouette, zonation aspects and clearly demarcated from p16(-)c in NdM (*case #1*).

We combined the immunohistochemical expression of CCRM and molecular results to categorize cases as follows:

(a) cases with the same CCRM and molecular profiles in both p16(+)c and p16(-)c:

- *Cases #1* and *#7*: in case *#7* the variant allele frequencies (VAF) of the involved genes (*BRAF* and *TERT*) increased passing from p16(-)c to p16(+)c, while in case *#1* the VAF (*NRAS*, *TERT*, and *TP53*) decreased;

(a) cases with p16(+)c showing additional genetic mutations and/or modifications of CCRM expression:

- *Case #4*: p16(+)c [Rb altered/lost, p53 altered/overexpressed /// *BRAF* (p.Val600Lys), *TERT* (c.−124/−125 delinsTT), and *TP53* (p.Tyr234His) mutations], p16(-)c [Cyclin D1 altered /// *BRAF* (p.Val600Lys) mutation];

Table 2
The CCRM and NGS results. The criteria adopted to evaluate p16 and CCRM (p53, p21, Cyclin D1, and Rb) and to dichotomize the cases in "normal"/"altered" for the immunohistochemical expression of these markers have been adopted from previous literature and summarized in Supplementary Material 1-Table S1 [15,16,25,26]. For NGS analysis, we reported the variants and the corresponding VAF.

Case number	Histological subtype	p16 (+)c					p16 (-)c				
		Rb	**p53**	**p21**	**Cyclin D1**	**NGS**	**Rb**	**p53**	**p21**	**Cyclin D1**	**NGS**
1	NdM	normal: 30%	altered/lost: 0%	normal: 20% (w)	normal: 50% (w)	*NRAS* (p.Gln61Lys): 49%; *TERT* (c.−146 C>T): 25%; *TP53* (c.560–2 A>T): 72%;	normal: 60%	altered/lost: 0%	normal: 20% (w)	normal: 50% (w)	*NRAS* (p.Gln61Lys): 52%; *TERT* (c.−146 C>T): 65%; *TP53* (c.560–2 A>T): 83%;
2	SSM	normal: 35%	normal: 50% (w and m)	normal: 60% (m)	normal: 55% (w and m)	*BRAF* (p.Val600Glu): 17%; *KIT* (p.Arg804Gly): 16%;					
3	NdM	normal: 50%	normal: 60% (w)	normal: 65% (m and s)	normal: 80% (w)	*NRAS* (p.Gly12Asp): 92%; *TERT* (c.−146 C>T): 24%; *DPYD* (c.1905 +1 G>A): 79%;					
4	SSM	altered/lost: 2%	altered/over expressed: 95% (s)	normal: 3% (w and m)	normal: 55% (w and m)	*BRAF* (p.Val600Lys): 32%, *TERT* (c.−124/−125 delinsTT): 40%; *TP53* (p.Tyr234His): 52%;	normal: 70%	normal: 60% (w, m, and s)	normal: 50% (m and s)	altered: 80% (m and s)	*BRAF* (p.Val600Lys): 10%;
5	DDM-GCN	altered/lost: 0%	altered/lost: 0%	normal: 3%	normal: 4%	*NRAS* (p.Gln61LysC): 48%; *TP53* (p.Arg213Ter): 81%; *TERT*: NE;	PN-GCN [altered/lost: 0%]	PN-GCN [normal:60% (w)]	PN-GCN [normal: 70% (w, m, and s)]	PN-GCN [normal: 3%]	*NRAS* (p.Gln61LysC): 46%; *TERT*: NE;
							GCN [altered/lost: 0%]	GCN [normal: 70% (w)]	GCN [normal: 10% (w)]	GCN [normal: 3%]	*NRAS* (p.Gln61LysC): 46%; *TERT*: NE;
6	SSM	normal: 60%	normal: 80% (w)	normal: 35% (m)	normal: 30% (w)	*NRAS* (p.Gly12Asp): 29%; *TERT* (c.−146 C>T): 36%;					
7	SSM	altered/lost: 4%	normal: 5% (w)	normal: 15% (w)	normal: 10% (w)	*BRAF* (p.Val600Glu): 42%; *TERT* (c.−146 C>T): 64%;	altered/lost: 4%	normal: 15% (w and m)	normal: 55% (m and s)	normal: 40% (w and m)	*BRAF* (p.Val600Glu): 14%; *TERT* (c.−146c>t): 7%;
8	SSM	altered/lost: 0%	normal: 40% (w and m)	normal: 25% (w, m, and s)	normal: 10% (w)	*BRAF* (p.Val600Glu): 15%;	altered/lost: 1%	normal: 40% (w and m)	normal: 10% (w)	normal: 5% (w)	wt
9	SM	altered/lost: 2%	normal: 90% (w)	altered: 90% (m and s)	altered: 80% (m and s)	*HRAS* (p.Gly12Arg): 53%;					
10	DM	normal: 30%	altered/over expressed: 70% (s)	altered: 100% (s)	normal: 40% (w and m)	*TP53* (p.Gln165SerfsTer5): 22%					

cell cycle regulatory molecules (CCRM); next-generation sequencing (NGS); p16 protein (p16); p53 protein (p53); p21 protein (p21); protein that in humans is encoded by the CCND1 gene (Cyclin D1); retinoblastoma protein (Rb); dermal/deep-seated melanoma arising in giant congenital nevus (DDM-GCN); giant congenital nevus (GCN); proliferative nodules arising in GCN (PN-GCN); superficial spreading melanoma (SSM); nodular melanoma (NdM); Spitz melanoma (SM); desmoplastic melanoma (DM); component with p16 overexpression [p16(+)c]; component without p16 overexpression [p16(-)c]; wild-type (wt); not evaluable (NE); weak (w); moderate (m); strong (s); variant allele frequency (VAF);

- *Case #5*: DDM-GCN p16(+)c [Rb altered/lost and p53 altered/lost /// *NRAS* (p.Gln61LysC) and *TP53* (p.Arg213Ter) mutations], GCN and PN-GCN p16(-)c [Rb altered/lost /// *NRAS* (p.Gln61LysC) mutation];
- *Case #8*: p16(+)c [Rb altered/lost /// *BRAF* (p.Val600Glu) mutation], p16(-)c [Rb altered/lost /// wild-type (wt)];

The CCRM and molecular results are summarized in Table 2. An illustrative example of CM with zonal p16 overexpression is shown in Fig. 1.

4. Discussion

One of the most relevant onco-suppressor involved in the biology of CM is *CDKN2A*, which encodes for two proteins acting as tumor suppressors by negatively regulating the cell cycle (specifically the G1 phase): p16 and p14arf proteins [1–8,28–31]. Specifically, the G1 phase of the cell cycle is regulated by two different pathways: p16/Rb and p53 pathways [28–31]. Recent data are showing that, even if these pathways may act independently, they are highly interconnected (especially in the biology of CM) by numerous loop mechanisms [32]. The p16/Rb pathway is frequently impaired in CM and the molecular alterations resulting in LOH of *CDKN2A* and in p16 loss of expression detected by immunohistochemistry occur in a relevant percentage of invasive, advanced-stage, and metastatic CM [1–8,32]. Specifically, somatic genetic alterations involving *CDKN2A* are found in up to 40–45% of CM, spanning from missense mutations, truncating mutations, and in-frame deletions (together occurring in less than 10% of CM) to more common heterozygous and homozygous deletions (25–33%) [1–8]. Subsequent studies focused on epigenetic alterations in CM have shown that promoter hyper-methylation of *CDKN2A* (related to transcription silencing of the gene and loss of p16 expression) can be found in approximately 20–55% of CM [1–7]. This knowledge on the genetic landscape of CM has relevant implications for its histologic diagnosis, since immunohistochemical loss of p16 is highly predictive of malignancy [1–3,9–19]. Immunohistochemical loss of p16 not only correlates with homozygous deletion of *CDKN2A* detected by fluorescence in situ hybridization (FISH), but can also reveal functional inactivation occurring through molecular mechanisms not necessarily affecting gene loss (mutations, promoter hyper-methylation, post-transcriptional changes, etc.), thus making immunohistochemistry more sensible than sequencing to detect p16 alterations [1–5,17–19]. As result, immunohistochemical analysis of p16 has become a very practical approach to identify p16 inactivation and a useful diagnostic tool for the differential diagnosis of melanocytic lesions [9–19]. Driven by our incidental finding of a DDM-GCN with paradoxical overexpression of p16 and intrigued by this phenomenon, we decided to analyze clinicopathologic features, immunohistochemical profile of melanocytic markers and expression of those proteins that cooperate with p16 in directing the activity of the cell cycle (CCRM), and the mutational landscape of CM with p16 overexpression [20]. We also meticulously searched the scientific literature to verify if other authors had previously investigated this rare event. We found that p16 overexpression had been reported only in DM in two previous studies and with a higher prevalence compared to that of our institution [in our institution 1/15 (6.7%) DM (*case #10*) showed p16 overexpression, *data not shown*; *Plaza JA* et al.: 31/40 (77.5%), *Blokhin E* et al.: 6/22: 27.3%) [11,12]. Other authors found different results, with no cases of DM showing p16 overexpression and/or rare cases with only focal/weak stain [1,13,14]. These discrepancies may depend both on the criteria to define p16 overexpression (nuclear, cytoplasmic, nuclear and cytoplasmatic stain; cut-off of positive cells, etc.) and different laboratory methods (clone, dilution, company, etc.) adopted in the studies [1,11–14]. Noteworthy, *Roth A* et al. recently found that truncating mutations in *CDKN2A* (as well as in *NF1*, *TP53*, and *ARID2*) were exclusive and highly diagnostic of DM, not being found in other histotypes of CM and/or desmoplastic CN (common, blue, Spitz, and other histotypes) [33]. However, the authors did not analyze the immunohistochemical expression of p16 in these cases, not attempting to show if truncating mutations of *CDKN2A* could be the molecular justification for such a high frequency of p16 overexpression in DM previously reported [34]. Our results show that p16 overexpression is really rare (0.35% of all tested CM), and occurs in advanced-stage but clinically and histologically heterogenous CM. We found p16 overexpression in different histologic subtypes of CM (classified as distinct clinical-histologic-molecular entities in the 2023 WHO Classification of Skin Tumours), suggesting that this phenomenon crosses different melanocytic lesions rather than be a category-specific signature as potentially suggested by its previous detection only in DM [1,11,12]. In addition, our data show that this phenomenon occurs in CM with different CCRM expression profiles and molecular landscapes (especially regarding *TP53* mutational status). Despite the potential correlation between p16 overexpression and CCRM expression and/or *TP53* mutations suggested by our findings in *Groups 1* and *2* and that mirrors what is observed in other tumors pathologically related to p16 overexpression (squamous cell carcinoma of the cervix, penis, and anus, high-grade serous carcinoma of the ovary, etc.), *Group3* (about 30% of

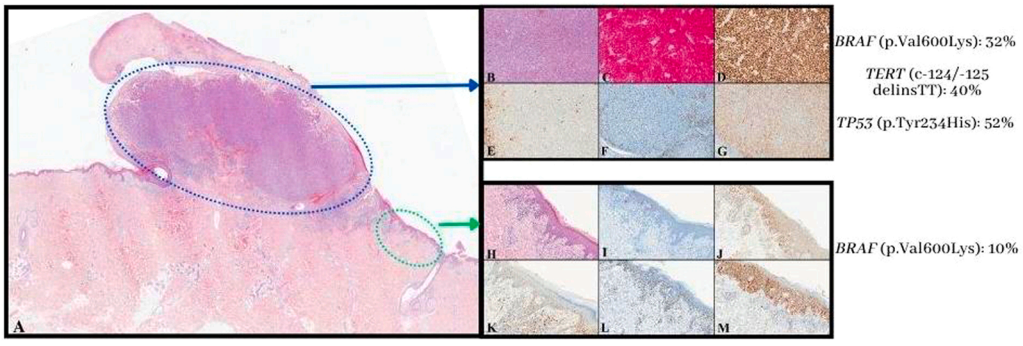

Fig. 1. Cutaneous melanoma (CM); cell cycle regulatory molecules (CCRM); hematoxylin and eosin (H&E); p16 protein (p16); p53 protein (p53); p21 protein (p21); protein that in humans is encoded by the CCND1 gene (Cyclin D1); retinoblastoma protein (Rb); **CM with zonal p16 overexpression (*cases #4, see* Table 2) and immunohistochemical results for CCRM.** At low-power magnification (A: H&E, original magnification x20), the tumoral/nodular component with vertical growth phase [p16(+)c, *upper portion of the Figure*] largely overwhelming the residual component at the shoulder of the lesion and with radial growth phase [p16(-)c, *lower portion of the Figure*] are easily recognizable: -p16(+)c (*upper portion of the Figure*) shows p16 overexpression, Rb altered/lost, and p53 altered/overexpressed (B: H&E, original magnification x200; C: p16, original magnification x200; D: p53, original magnification x200; E: p21, original magnification x200; F: Rb, original magnification x200; G: Cyclin D1, original magnification x200), with *BRAF* (p.Val600Lys), *TERT* (c.–124/−125 delinsTT), and *TP53* (p.Tyr234His) mutations. -p16(-)c (*lower portion of the Figure*) shows Cyclin D1 altered (H: H&E, original magnification x200; I: p16, original magnification x200; J: p53, original magnification x200; K: p21, original magnification x200; L: Rb, original magnification x200; M: Cyclin D1, original magnification x200) with only *BRAF* (p.Val600Lys) mutation.

the cases) showed as p16 overexpression can also be independent of CCRM expression and *TP53* mutation [26,34–36]. All these findings support the hypothesis that p16 overexpression in CM has different biological bases and may not be restricted to a specific mutational status and/or CCRM profile, as observed in other tumors [26,34–36]. Finally, we found a zonal p16 overexpression in 50% of the cases, with two clearly identifiable p16(-)c and p16(+)c. Comparison of CCRM profiles and mutational data between the two components suggests that in a subset of these cases [cases with p16(+)c showing additional genetic mutations and/or modifications of CCRM expression, *see Results: CM with zonal p16 overexpression*], the p16(+)c may represent a "molecular progression" characterized by the occurrence of additional mutations and/or complex biological mechanisms affecting the CCRM expression profile. In the other cases [cases with the same CCRM and molecular profiles in both p16(+)c and p16(-)c, *see Results: CM with zonal p16 overexpression*], the histologic features [p16(+)c overwhelming and/or pushing p16(-)c] again suggests that p16(+)c may represent progression of a neoplastic sub-clone. NGS analysis of the two components again supports the above-mentioned hypothesis that, in a subset of cases, p16 overexpression may be related to mechanisms not involving CCRM and mutational status. Potential limitations of our study include: (a) relatively small sample size, (b) no follow-up data, (c) no comprehensive analysis of *CDKN2A* (sequencing does not identify many of the alterations affecting the gene such as homozygous deletions and promoter hyper-methylation).

To conclude, our results demonstrate that a small group of CM exhibits paradoxical and diagnostically challenging p16 overexpression. This phenomenon characterizes a very small proportion of advanced-stage CM that are clinically and histologically heterogenous, with complex and different CCRM expression profiles and mutational landscapes. Interestingly, in half of these rare p16 overexpressing CM, the overexpression is zonal and probably represents a progression that may or may not be related to the acquisition of additional mutations and/or changes involving the CCRM expression.

Supporting Information

No supporting information for this study.

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CRediT authorship contribution statement

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Declaration of Competing Interest

All the Authors present in this article declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154564.

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