

Key regulators of apoptosis execution as biomarker candidates in melanoma

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Abbreviations: APAF-1, apoptotic protease activating factor 1; c-IAP, cellular IAP; CTLA4, cytotoxic T-lymphocyte antigen 4; Cyt-c, cytochrome c; HtrA2, high temperature requirement protein A2; IAP, inhibitor of apoptosis; IHC, immunohistochemistry; ML-IAP, melanoma IAP; MM, metastatic melanoma; PD-1, programmed death 1; PM, primary melanoma; Smac, second mitochondria-derived activator of caspases; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XAF1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis protein.

Resistance to apoptosis is frequently detected in malignant melanoma, a skin cancer with rapidly growing incidence rates. Apoptosis resistance may develop with disease progression and may be associated with the poor responsiveness of metastatic melanoma to apoptosis-inducing treatments, such as genotoxic chemotherapy and radiotherapy. Likewise, the efficacy of novel treatment options (targeted kinase inhibitors and immunotherapeutics) that indirectly lead to cell death may depend on the susceptibility of melanoma to apoptosis. At its core, apoptosis execution is regulated by the interplay between a comparatively small number of pro- and anti-apoptotic proteins, and consequently numerous studies have investigated the potential of these players as biomarker candidates. Here, we provide a comprehensive overview of biomarker discovery studies focusing on key regulators of apoptosis execution, critically review the findings of these studies, and outline strategies that address current limitations and challenges in exploiting regulators of apoptosis execution as prognostic or predictive biomarkers in melanoma.

Introduction

The worldwide incidence of melanoma continues to increase, with approximately 197,000 new cases and 46,000 deaths annually attributable to melanoma of the skin.¹ Worryingly, increases in this incidence are observed despite significant efforts in prevention and risk awareness campaigns, with the highest incidence rates reported for Australia/New

Zealand, Northern America, and Northern and Western Europe.² Fair-skinned individuals constitute a high-risk group for developing melanoma, with high levels of UV radiation due to excessive sun exposure being a main contributor to melanomagenesis. The molecular mechanisms underlying melanomagenesis, disease progression, and metastasis remain poorly understood. With the exception of elevated serum lactate dehydrogenase and mutant *BRAF* status in metastatic disease, molecular markers that improve staging, prognosis, and patient stratification for personalized treatments have not entered the clinic.^{2,3} Instead, the clinicopathologic characterizations of melanoma thickness, mitotic rate, and ulceration remain the major determinants for staging and prognosis.^{3,4} To more reliably prognosticate disease progression, treatment responsiveness, or tumor recurrence, considerable efforts have been made to identify molecular prognostic or predictive markers that could be established as superior tools for improving the clinical decision making and management of melanoma.⁵

In metastatic melanoma (MM), all approved chemotherapies, localized radiotherapy, and B-Raf-targeted therapies are ultimately intended to induce melanoma cell death, either directly or indirectly. Cell death is typically executed by apoptosis, the major programmed cell death modality in multicellular organisms.⁶ Due to the importance of apoptosis execution and the frequent development of apoptosis resistance in melanoma,^{7,8} considerable work has been conducted investigating regulators of apoptosis execution as potential prognostic or predictive biomarkers. Here, we critically review the major studies in this area, present overviews of the analyzed patient cohorts and detection tools (antibodies and probes), and also provide information on correlations between marker candidates, staging criteria, and patient outcome (Tables 1 and 2). We also outline limitations of current biomarker identification approaches, associated knowledge gaps, and present recent advances in biomarker development strategies that may be of particular interest in the context of cell death signaling in highly heterogeneous cancers such as melanoma.

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Table 1. Cohort characteristics and detection approaches for key regulators of apoptosis execution in previous studies

References	Cohorts	Clinicopathologic data and detailed staging information	Analytical methods	Probes/primers/antibodies
Apaf-1				
Soengas et al. ³³	6 PMs 24 MMs	Unknown	gDNA: PCR mRNA: ISH mRNA: NB protein: IB	Microsatellite marker analysis on 12q22–23: D12S393 Riboprobes targeting fragments: 1–461, 581–1363, 2112–2225, 3426–3710 cDNA fragments to nucleotides 272–2908 and 1517–3525 mAb 6–1–19, pAb 8.46
Fujimoto et al. ³⁴	62 PMs 112 MMs	PMs/MMs: Stage, thickness	gDNA: PCR mRNA: PCR	Microsatellite marker analysis on 12q22–23: D12S1657, D12S393, D12S1706, D12S346 Forward 5'-ACATTC TCACGATGCTACC-3'; reverse 5'-CAATTCATGAAGTGGCAA-3'
Fujimoto et al. ³⁵	49 MMs	Stage	gDNA: PCR	Microsatellite marker analysis on 12q22–23: D12S1657, D12S393, D12S1706, D12S346
Niedojadlo et al. ³⁶	75 nevi 53 PMs 24 MMs 30 nevi	PMs: Clark levels, thickness PMs: Thickness	mRNA: FISH protein: IHC	RNA probes prepared from a plasmid provided by Dr. Xang, University of Texas, USA Rat mAb H19, provided by Dr. O'Reilly, WEHI, Melbourne, Australia Rabbit pAb, H-324, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Baldi et al. ³⁷	61 PMs 15 MMs 10 nevi	PMs: Thickness	protein: IHC	mAb, Santa Cruz Biotechnology Inc.
Mustika et al. ³⁸	11 in situ melanomas 26 PMs 15 MMs 13 nevi	PMs: Thickness	protein: IHC	Rabbit pAb, BD Biosciences, Mississauga, Ontario, Canada
Dai et al. ³⁹	70 PMs 8 nevi	Unknown	protein: FC protein: IHC	mAb from Chemicon International, Temecula, CA, USA Rat mAb from Chemicon International
Zanon et al. ⁴⁰	16 PMs 71 MMs 31 nevi 153 PMs	PMs: Thickness	protein: IHC	pAb RB 9236, Neomarkers
Bachmann et al. ⁴¹				
Caspases				
Woenckhaus et al. ⁴⁹	16 nevi 20 PMs 30 MMs 24 nevi	PMs: Thickness	protein: IHC	Cleaved caspase-6, New England Biolabs, Frankfurt, Germany Cleaved caspase-3, New England Biolabs
Chen et al. ⁴²	52 PMs - cutaneous 25 PMs - mucosal	Cutaneous PMs: staging Mucosal PMs: staging	protein: IHC	Caspase-3: rabbit pAb, Santa Cruz Biotechnology, Inc. Caspase-6: goat pAb, Santa Cruz Biotechnology, Inc. Caspase-7: goat pAb, Santa Cruz Biotechnology, Inc.
Liu et al. ⁴⁷	57 PMs	Thickness	protein: IHC	Rabbit anti-active caspase-3, Abcam, Cambridge, UK
XIAP				
Kluger et al. ⁵⁴	540 nevi 232 PMs 299 MMs	Unknown	protein: IHC	Mouse Ab, BD Transduction Laboratories
Emanuel et al. ⁵³	6 nevi	PMs: Thickness	protein: IHC	Mouse mAb, clone 48, BD Biosciences, San Jose, CA, USA
Chen et al. ⁴²	4 in situ melanomas 67 PMs 24 nevi 52 PMs - cutaneous 25 PMs - mucosal	Cutaneous PMs: staging Mucosal PMs: staging	protein: IHC	Rabbit pAb, Santa Cruz Biotechnology Inc.

(continued on next page)

Table 1. Cohort characteristics and detection approaches for key regulators of apoptosis execution in previous studies (Continued)

References	Cohorts	Clinicopathologic data and detailed staging information	Analytical methods	Probes/primers/antibodies
Hiscutt et al. ⁵⁵	6 nevi 7 in situ melanomas 42 PMs	Staging and thickness	protein: IHC	mAb, BD Biosciences, Oxford, UK
ML-IAP Nachmias et al. ⁵⁹ Gong et al. ⁵⁷	27 PMs 14 nevi 19 PMs (cut/muc) 15 MMs	Unknown Unknown	protein: IB mRNA: ISH mRNA: RT-PCR	Mouse mAb, clone 88C570, Imgenex antisense 5'-CAAAGACGATGGACACGGC-3'; sense 5'-GCCGTGCCATCGTCTTTG-3' upstream 5'-ATGGGCTCTGAGGAGTTGCGTC-3'; downstream 5'- CATAGCAGAAGAAGCACCTCACCTTG-3'
Takeuchi et al. ⁵⁸	63 MMs	Staging	protein: IHC mRNA: qRT-PCR	Rabbit pAb, R&D Systems, Minneapolis, MN, USA FRET probe: 5'-FAM- TGAGCTGCCACACCCAGGAGAG-BHQ-1-3'
Chen et al. ⁴²	24 nevi 52 PMs - cutaneous 25 PMs - mucosal	Cutaneous PMs: staging Mucosal PMs: staging	protein: IHC	Goat pAb, Santa Cruz Biotechnology Inc.
Apollon Tassi et al. ⁶¹	8 PMs (cell lines) 26 MMs (cell lines)	Unknown	protein: FC, IB	Mouse Ab, BD Biosciences
c-IAPs Chen et al. ⁴²	24 nevi 52 PMs - cutaneous 25 PMs - mucosal	Cutaneous PMs: staging Mucosal PMs: staging	protein: IHC	c-IAP1: rabbit pAb, Santa Cruz Biotechnology Inc. c-IAP2: rabbit pAb, Santa Cruz Biotechnology Inc.
XAF1 Ng et al. ⁶⁵	40 nevi 70 PMs	PMs: Thickness	protein: IHC	Rabbit Ab provided by Dr. Korneluk, University of Ottawa, Canada

Abbreviations: Cut, cutaneous; FC, flow cytometry; FISH, fluorescence in situ hybridization; IB, immunoblot; IHC, immunohistochemistry; ISH, in situ hybridization; MM, metastatic melanoma; muc, mucosal; NB, Northern blot; PCR, polymerase chain reaction; PM, primary melanoma; qRT-PCR, quantitative real time polymerase chain reaction.

The Relevance of Apoptosis Pathways in Melanoma and their Convergence into a Common Execution Phase

Impaired apoptosis execution may allow cancer cells to evade therapeutic triggers of programmed cell death and could result in treatment-induced selection for cell populations with increased stress tolerance, thereby contributing to the recurrence of tumors with acquired apoptosis resistance. Indeed, an increased resistance to apoptosis is a hallmark feature of cancer.^{7,9} Apoptosis can be triggered through 3 major signaling pathways (Fig. 1A). The intrinsic pathway responds to intracellular stress and damage. For example, DNA damage, as induced by ionizing radiation and genotoxic chemotherapy, is a prototype inducer of intrinsic apoptosis. Dacarbazine and temozolomide, the primary chemotherapeutics for the treatment of metastatic melanoma, are pro-drugs that in their activated form result in DNA alkylation and intrinsic apoptosis.^{10,11} Also, other stress situations, including inhibition of protein synthesis and protein degradation as well as the inhibition of kinases crucial for the control of cell survival and proliferation, including B-Raf, can induce intrinsic apoptosis.¹²⁻¹⁴ The extrinsic pathway is initiated by oligomerized death ligands (e.g., TNF-related apoptosis-inducing ligand [TRAIL] or CD95L), which bind to their cognate cell surface receptors. Physiologically, these

ligands are expressed as transmembrane proteins by cytotoxic T lymphocytes and assist in eliminating target cells that present “foreign” antigens. Strategies to exploit the extrinsic pathway for targeted anti-cancer therapies, in particular through the activation of TRAIL receptors, are currently being evaluated in preclinical and clinical phases.^{15,16} A third route toward apoptosis likewise involves the action of cytotoxic T lymphocytes as well as natural killer cells, both of which can deliver proteases of the granzyme family into target cells, with granzyme B being a potent inducer of apoptosis.¹⁷ Data obtained from analyses of the immune infiltration status of melanoma tumor microenvironments indicate that the extrinsic pathway and the granzyme pathway could play a central role in determining the efficacy of targeted immunotherapeutics, such as cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD-1) inhibitors, and probably also B-Raf inhibitors.^{18,19} Apoptosis therefore plays a central role in the prevention of melanoma development as well as in the responsiveness to current treatment options for metastatic disease.

All 3 apoptosis pathways converge in a common terminal phase of apoptosis execution during which members of the cysteine-dependent aspartate-specific protease (caspase) family cleave and inactivate hundreds of cellular substrates, including key structural and pro-survival proteins.^{6,9} Likewise, active processes that further contribute to the degradation of cellular content and

Table 2. Correlations between regulators of apoptosis execution and clinicopathologic or outcome data

Reference	Correlation analyses					Materials		
	Phenotype of interest	Tested for correlation with	Corr.	p value	Test	Analyzed	Tissue/Serum	Sample size
Apaf-1								
Fujimoto et al. ³⁴	<i>APAF-1</i> LOH	Overall survival	no	0.43	Log-rank test	gDNA	tissue	52 PMs
	<i>APAF-1</i> LOH	Overall survival	yes	0.049	Log-rank test	gDNA	tissue	97 MMs
Fujimoto et al. ³⁵	<i>APAF-1</i> LOH	Overall survival	yes	0.046	Log-rank test	gDNA	serum	44 MMs
Niedlojadlo et al. ³⁶	<i>Apaf-1</i> mRNA expression	Clark level	yes	0.03	Pearson's corr coeff	mRNA	tissue	53 PMs
	<i>Apaf-1</i> mRNA expression	Breslow thickness	no	0.38	Pearson's corr coeff	mRNA	tissue	53 PMs
	<i>Apaf-1</i> protein expression	Clark level	yes	0.037	Pearson's corr coeff	protein	tissue	53 PMs
	<i>Apaf-1</i> protein expression	Breslow thickness	no	0.23	Pearson's corr coeff	protein	tissue	53 PMs
Dai et al. ³⁹	<i>Apaf-1</i> protein expression	Breslow thickness	no	> 0.05	Chi-square	protein	tissue	70 PMs
	<i>Apaf-1</i> protein expression	Tumor ulceration	no	> 0.05	Chi-square	protein	tissue	70 PMs
	<i>Apaf-1</i> protein expression	5-year survival	no	> 0.05	Log-rank test	protein	tissue	70 PMs
Zanon et al. ⁴⁰	<i>Apaf-1</i> protein expression	Patient survival	no	Unknown	Unknown	protein	tissue	16 PMs + 66 MMs
Bachmann et al. ⁴¹	<i>Apaf-1</i> protein expression	Tumor thickness	yes	0.05	Unknown	protein	tissue	153 mixed samples
	<i>Apaf-1</i> protein expression	Overall survival	no	0.095	Log-rank test	protein	tissue	153 mixed samples
Caspases								
Woenckhaus et al. ⁴⁹	Active caspase-3 expression	Survival time	no	> 0.5	Cox regression	protein	tissue	66 mixed samples
	Active caspase-6 expression	Survival time	no	> 0.5	Cox regression	protein	tissue	66 mixed samples
XIAP								
Hiscutt et al. ⁵⁵	XIAP protein expression	Stage	yes	< 0.001	Wald chi-square	protein	tissue	55 mixed samples
ML-IAP								
Gong et al. ⁵⁷	ML-IAP protein expression	Patient age	yes	0.0056	Spearman rank corr	not specified	tissue	48 mixed samples
Takeuchi et al. ⁵⁸	ML-IAP mRNA expression	Overall survival	no	> 0.05	Unknown	mRNA	tissue	63 MMs
Nachmias et al. ⁵⁹	ML-IAP protein expression	Response to treatment	yes	0.02	Fisher's exact test	protein	tissue	27 PMs
XAF1								
Ng et al. ⁶⁵	XAF1 protein expression	Tumor thickness	no	0.119	Chi-square	protein	tissue	70 PMs
	XAF1 protein expression	5-year survival	no	0.889	Log-rank test	protein	tissue	69 PMs
	XAF1 nuclear positivity	5-year survival	no	0.896	Log-rank test	protein	tissue	69 PMs

Abbreviations used: Coeff, coefficient; Corr, correlation.

the dismantling of the cell body, including DNA fragmentation, cell contraction, and the formation of apoptotic vesicles, are activated in a caspase-dependent manner. Although all 3 pathways initiate the execution phase by engaging mitochondria in the cell death process, both the extrinsic and the granzyme-dependent pathways also can drive apoptosis execution independent of mitochondria in so-called type-I cells^{6,9,17} (Fig. 1A). Signaling through the mitochondria involves the action of BH3-only proteins, a large subgroup of the Bcl-2 protein family, which initiate the formation of pores in the outer mitochondrial membrane.²⁰ During intrinsic apoptosis, combinations of these BH3-only proteins are transcriptionally upregulated or post-translationally stabilized or modified for activation. In the extrinsic and granzyme pathways, the BH3-only protein Bid is cleaved and activated by the initiator caspase-8 or by granzyme B.^{17,20}

Once mitochondria are permeabilized, the execution phase of apoptosis is initiated by the release of mitochondrial intermembrane space proteins.²¹ Cytochrome c (cyt-c) activates apoptotic protease activating factor 1 (Apaf-1) in the cytosol. Activated Apaf-1 oligomerizes into the heptameric apoptosome, which then can recruit the inactive pro-form of caspase-9 (procaspase-9)^{22,23} (Fig. 1B). Once procaspase-9 binds to the apoptosome, it is activated, autocatalytically processed, and begins to proteolytically activate its 2 major targets, the downstream effector procaspases-3 and -7.^{6,9} Activated caspases-3 and

-7 drive the terminal phase of apoptosis execution and also activate another effector caspase, caspase-6, that may play a crucial part in the demolition of the nuclear envelope²⁴ (Fig. 1B). X-linked inhibitor of apoptosis (XIAP) is the major cellular inhibitor of caspases involved in apoptosis execution, being able to inhibit caspases-9, -3, and -7^{25,26} (Fig. 1B). In addition, XIAP can ubiquitinate binding partners and thereby may permanently inactivate caspases or enforce their degradation. Importantly, IAP proteins are highly multi-functional and were shown to play important roles outside of the apoptosis execution phase.²⁶⁻²⁸ The main antagonist of XIAP is second mitochondria-derived activator of caspases (Smac), a mitochondrial protein that is co-released with cyt-c (Fig. 1B). Smac dimers bind to XIAP and thereby alleviate caspase inhibition and promote apoptosis execution. The XIAP binding motif of Smac has also been found in other mitochondrial proteins, including the protease Omi/HtrA2 (high temperature requirement protein A2).²⁹ An additional XIAP antagonist is XIAP-associated factor 1 (XAF1), a protein that reduces cytosolic amounts of XIAP by promoting its nuclear translocation.³⁰ The relative amounts of caspases, XIAP, and IAP antagonists therefore determine whether apoptosis execution can proceed in a rapid, switch-like manner or is suppressed.^{31,32} Overall, the execution phase of apoptosis is therefore controlled by the interplay of a small number of core regulatory proteins.

Apoptotic Protease Activating Factor 1 (Apaf-1)

Since loss of Apaf-1 expression confers resistance to apoptosis execution, various studies have investigated the *APAF1* gene and transcript or protein levels as potential biomarkers in primary and metastatic melanoma.

Studies on APAF1 at the Gene Level

In a study of 24 metastatic melanomas (MMs), 10 patients (42%) presented with a loss of heterozygosity (LOH) on the 12q22–23 region that encompasses the *APAF1* gene.³³ Correspondingly, a subsequent study reported *APAF1* LOH in 36 out of 98 MMs analyzed (37%).³⁴ The frequency of *APAF1* LOH in primary melanomas (PMs) was significantly lower (10 out of 54 cases; 19%, $p = 0.020$),³⁴ suggesting that LOH may be associated with disease progression. This was indeed substantiated in an analysis of 10 paired PM and MM samples, which showed that LOH in *APAF1* increased from 20% to 70%.³⁴ Although *APAF1* LOH in patients with PM was not associated with reduced overall survival (OS) ($p = 0.43$), a statistically significant association was identified in a cohort of 97 patients with stage III/IV MM ($p = 0.049$; 27 months follow-up).³⁴ These findings indicate that *APAF1* LOH status in MM may carry prognostic value. Evaluation of 12q22–23 LOH status may also be possible through analysis of circulating DNA in patient blood sera. In a study of pre-treatment sera obtained from 49 patients with stage IV MM who underwent chemotherapy (combination treatment including dacarbazine, cisplatin, vinblastin, interferon α -2b, interleukin-2, and tamoxifen) the frequency of 12q22–23 LOH was significantly lower in the responder group (21%) than in the non-responder group (55%) ($p = 0.029$).³⁵ Furthermore, patients without *APAF1* LOH in pre-chemotherapy serum survived significantly longer than patients with *APAF1* LOH ($p = 0.046$).³⁵ These findings therefore correspond to results from tissue-based analyses and further indicate that the *APAF1* LOH status may carry predictive capacity for treatment responsiveness.

Interestingly, *APAF1* LOH tends to associate with an overall lack of *APAF1* mRNA in patients with MM,³³ indicating that the remaining *APAF1* gene is not effectively transcribed. Concordant with these findings in MMs, Soengas et al. found that human metastatic melanoma cell lines likewise frequently present with a loss of *APAF1* mRNA and protein expression.³³ Many of these cell lines also harbored only one copy of the *APAF1* gene, corresponding to the LOH phenotype found in tissue samples. Mutations were not found within the *APAF1* gene in 8 cell lines

tested, suggesting other means of transcriptional repression.³³ The DNA methyltransferase inhibitor 5aza2dC increased *APAF1* mRNA and Apaf-1 protein amounts in otherwise Apaf-1-negative cell lines, but enhanced methylation of the *APAF1* promoter region could not be detected in melanoma cell lines.³³ Likewise, Fujimoto et al. failed to detect elevated methylation in the *APAF1* promoter region in 49 tumor samples analyzed.³⁴ This indicates an *APAF1* haplo-insufficiency and a non-linear potentiation of trans-mediated suppression of *APAF1* transcription.

Together, these findings indicate that the *APAF1* gene is frequently inactivated during melanoma progression, not only by LOH but also by transcriptional repression, although the exact mechanism of the latter is not understood. A loss of Apaf-1 expression or a reduction of Apaf-1 is associated with reduced survival, indicating that *APAF1* LOH status could have potential as a prognostic marker, especially in the context of chemotherapy responsiveness. Given the role of Apaf-1 in facilitating the execution of apoptosis upon chemotherapy, a direct functional link between *APAF1* expression, melanoma cell death, and response to therapy can be hypothesized. Further studies on larger cohorts are required to independently validate the above findings.

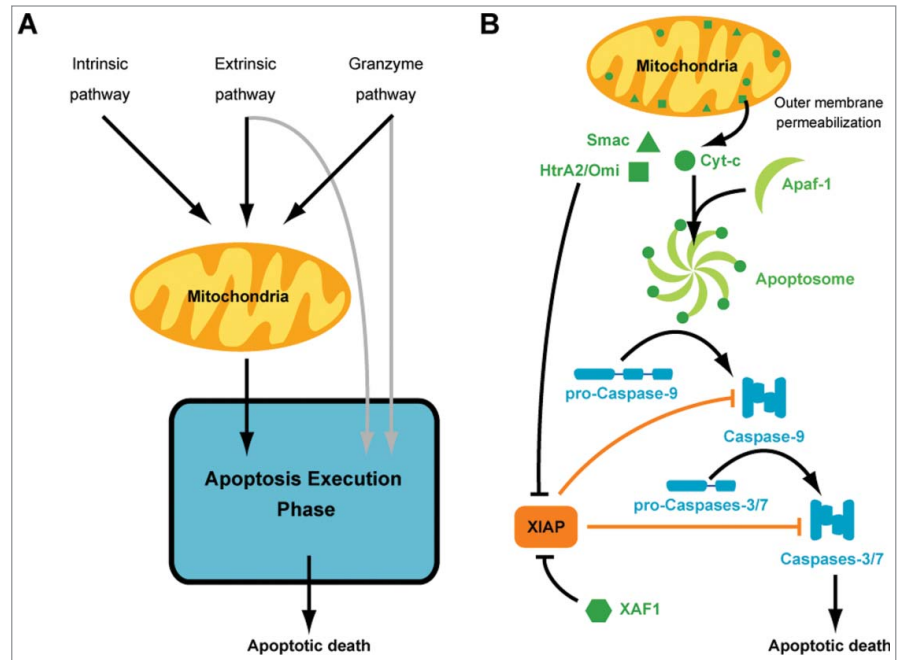


Figure 1. Simplified overview of the apoptosis execution phase. (A) Apoptosis execution is triggered by 3 major pathways that converge in the apoptosis execution phase. All pathways engage the mitochondria, with the extrinsic and granzyme pathways additionally promoting apoptosis execution by the activation of downstream effector caspases. (B) Core regulators and their interplay during apoptosis execution. Cytochrome c (cyt-c), Smac, and Omi/HtrA2 are released from mitochondria upon permeabilization of the outer mitochondrial membrane. An activation cascade of caspases drives the execution of apoptotic cell death. XIAP and other inhibitors of apoptosis (IAPs) serve as caspase inhibitors and are themselves antagonized by Smac, Omi/HtrA2, and XAF1.

Studies on *APAF1* at the Transcript Level

As alluded to above, *APAF1* mRNA levels are frequently affected in melanoma patients. Measurements of *APAF1* transcripts by in situ hybridization (ISH) demonstrated reduced expression in MM tumors with 12q22–23 LOH (9 out of 10 tumors analyzed).³³ In contrast, only 1 out of 6 PMs studied exhibited decreased *APAF1* expression.³³ The association of the heterozygosity status with reduced *APAF1* mRNA levels was further substantiated by comparison of tumors with or without 12q22–23 LOH ($n = 22$). Reduced expression of *APAF1* mRNA was found in 7 out of 10 of 12q22–23 LOH tumors (70%) compared to 5 out of 12 in tumors with both *APAF1* alleles present (42%; $p = 0.03$).³⁴ When comparing benign nevi to primary cutaneous melanomas, *APAF1* mRNA was undetectable in 8 out of 75 benign nevi (11%) and in 27 out of 53 PMs (51%).³⁶ Similar trends for an association of *APAF1* mRNA with disease progression could be found within the group of PMs; *APAF1* mRNA expression decreased from Clark II to Clark III stages ($p = 0.012$), and *APAF1* mRNA was frequently absent in tumors with a thickness greater than 4 mm (11 out of 14 [79%], compared with 5 out of 17 [29%] tumors with a thickness < 1 mm; $p = 0.008$).³⁶ None of the 24 MMs analyzed expressed *APAF1* mRNA.³⁶ Taken together, these results further support the hypothesis that melanoma progression is associated with *APAF-1* downregulation.

Studies on Apaf-1 at the Protein Level

Transcript levels frequently do not correlate with protein levels as a result of post-translational regulation of protein stability. Measurements of protein amounts by immunohistochemistry (IHC) may therefore be more informative for prognosis or treatment responsiveness. Apaf-1 protein amounts were significantly reduced in melanomas (pooled from 61 PMs and 15 MMs) compared to 30 benign nevi ($p < 0.00001$), and were further reduced in 15 MMs compared to the group of PMs ($p = 0.01$).³⁷ These results were independently validated in another study which likewise showed reduced Apaf-1 protein expression in 52 melanomas (pooled from in situ melanomas, PMs, and MMs) compared to 10 benign nevi ($p < 0.0001$).³⁸ Complete absence of Apaf-1 protein was detected in 27 out of the 53 PMs analyzed (51%), and all 24 MMs were negative for Apaf-1 protein expression.³⁶ Interestingly, none of the 5 PMs that developed metastases showed detectable Apaf-1 expression.³⁶ Similar associations between reductions in Apaf-1 and the progression stage of PMs were reported by others. Apaf-1 protein expression was significantly decreased in 39 more invasive PMs (thickness > 0.76 mm) compared to 22 less invasive PMs (thickness < 0.76 mm; $p < 0.00001$).³⁷ Other authors likewise reported a significant decrease in Apaf-1 protein expression in more invasive PM tumors, as assessed by melanoma thickness criteria ($p < 0.003$).³⁸ In addition, Apaf-1 protein expression was significantly decreased in 9 out of 12 Clark III stage melanomas (75%) compared to 6 out of 19 Clark II stage tumors (32%),³⁶ consistent with the mRNA measurements reported in the same study. Lack of Apaf-1 protein correlated with disease progression, when defined by Clark stages (I to V, $p = 0.037$),³⁶ but not when determined by

thickness (Breslow thickness, $p = 0.23$).³⁶ Because Clark staging has been replaced by the mitotic rate in the latest staging and classification guidelines,³ Breslow thickness is the only remaining size criterion that is currently routinely assessed. However, the authors argue that Clark stages might be informative in this context, since the transition from horizontal (local invasion) to vertical growth (increased metastatic potential) correlates with transition from Clark stage II to stage III.³⁶

Overall, the above studies on protein levels are in agreement with previously described mRNA expression data, showing decreases in Apaf-1 expression from benign nevi to PMs and from PMs to MMs. The fact that none of the PMs that later developed into MMs expressed Apaf-1 reinforces the hypothesis of a potential role for Apaf-1 downregulation during melanoma progression and increased therapy resistance. However, some other studies failed to identify correlations between lack of Apaf-1 protein and poor clinical outcome. For instance, although significantly decreased Apaf-1 protein expression was found in 70 PMs compared to 13 benign nevi ($p = 0.014$), no correlation was found between Apaf-1 protein expression in PMs and tumor ulceration, tumor thickness, or 5-year patient survival.³⁹ Similar indications for reduced Apaf-1 amounts but a concomitant lack of association with patient clinical outcome were found in another study that measured Apaf-1 expression by IHC.⁴⁰ IHC staining results demonstrated Apaf-1 loss in none of the 8 benign nevi, 2 out of 7 PMs, and 3 out of 6 MMs.⁴⁰ Interestingly, flow cytometry experiments showed that Apaf-1 protein expression was lost in 1 out of 5 melanocytes, 6 out of 16 PMs, but only 9 out of 71 MMs. The authors reported that the Apaf-1 phenotype in the panel of 16 PM and 66 MM tumors was not significantly associated with patient survival. They concluded that reduced Apaf-1 expression, while frequent in melanoma, is not a predominant feature, and that the Apaf-1 phenotype has limited potential as a predictive marker for responsiveness to chemotherapeutics.⁴⁰ A larger study that investigated Apaf-1 expression in PM and MM using tissue microarrays likewise failed to identify an association of Apaf-1 loss with poor outcome;⁴¹ rather, Apaf-1 protein expression tended to increase with disease progression (31 benign nevi, 133 nodular melanomas, 20 superficial spreading melanomas, and 58 paired MMs analyzed). The authors found a significantly higher Apaf-1 protein expression in melanomas compared to benign nevi ($p = 0.002$), and a stronger expression in MMs than in PMs ($p = 0.002$). These authors also showed that increased tumor thickness correlated with a higher Apaf-1 protein expression in PMs ($p = 0.05$), but a statistically significant association between increased Apaf-1 protein expression and survival was not detected ($p = 0.095$).⁴¹ However, no data on staining quality or specificity were presented.

Overall, trends of reductions in the amount of Apaf-1 with disease progression have been observed repeatedly at both transcript and protein level, and data indicating the prognostic or even predictive potential of Apaf-1 levels have been presented.^{33,35-38} However, some studies reported a less frequent loss of Apaf-1 and a statistically insignificant, or even an inverse, relationship between Apaf-1 levels and clinical outcome.³⁹⁻⁴¹

Caspase-9

Since caspase-9 is the only initiator caspase in the mitochondrial apoptosis pathway, its presence is essential for triggering apoptosis execution through the activation of effector caspases-3 and -7. Given its importance, it might be expected that caspase-9 has already been studied in detail in the context of melanoma progression and treatment responsiveness. However, apart from one study that found insignificant increases in caspase-9 positivity between nevi and melanoma in IHC-based analyses ($n = 24$ and 77 , respectively),⁴² if and how caspase-9 transcript or protein amounts are associated with disease prognosis and therapy success has not been reported to date. Intriguingly, the situation is similar for other cancers, with little to no information provided on the potential of caspase-9 as a biomarker. This could be reflective of a general problem in measuring amounts of caspase-9. Reliable detection at the protein level requires not only high-quality antibodies but also sufficient protein abundance to separate specific signals from background staining. Absolute quantification of 15 apoptosis proteins in melanoma cell lines demonstrated that out of all proteins investigated, caspase-9 expression was the lowest.¹⁰ Similar findings were also reported in panels of glioblastoma and colorectal cancer cell lines, most of which express caspase-9 in low nanomolar concentrations.^{43,44} Therefore, despite the well-known requirement for caspase-9 for apoptosis execution, its evaluation as a potential biomarker in melanoma is still uncertain.

Effector caspases

Effector caspases-3 and -7 have largely overlapping specificities and cleave hundreds of substrates during apoptosis execution. Caspase-3 also activates caspase-6, an additional effector caspase that contributes to the cleavage of the nuclear envelope.⁴⁵ The levels of effector procaspase expression in cell-based analyses indicate that procaspase-3 and -7 expression may increase with disease progression.⁴⁶ A study in 77 PMs and 24 benign nevi investigated the expression of caspase-3, -6 and -7 by IHC.⁴² Although it is unclear whether the proforms, active forms, or both were detected, staining intensities for all caspases were high in PM yet statistically significant differences were found only for caspase-3 ($p < 0.01$).⁴² More precise information is, however, available on the amounts of basal effector caspase processing in the context of melanoma disease progression and disease stages. One study investigated the amounts of cleaved caspase-3 in 53 PMs and analyzed a potential link with the rate of progression to metastatic disease.⁴⁷ Progression toward metastatic disease was observed in 16 out of 20 PMs (80%) with high amounts of cleaved caspase-3. In contrast, only 10 out of the 37 PMs (20%) with low amounts of cleaved caspase-3 developed metastatic disease.⁴⁷ These differences were statistically significant ($p < 0.05$) and indicate that basal amounts of caspase-3 activation in PMs may promote disease progression. The authors attribute this to increased migration and invasion, features that may be associated with non-apoptotic roles of caspases at conditions of sublethal

activation.⁴⁸ Active caspase-3 protein expression was not related to patient age, gender, or tumor size, excluding these as confounding factors. In another study on 30 MMs, 20 PMs and 16 nevi, cleaved caspase-3 was found to be significantly increased in MMs ($p < 0.0001$).⁴⁹ Similar results were also found for comparisons of cleaved caspase-6 in the same cohort.⁴⁹ However, elevated basal levels of cleaved caspase-3 or -6 in MM did not correlate with patient survival. Taken together, these findings indicate that caspase levels may increase with disease progression, in particular for caspase-3. Furthermore, basal caspase activity may be present in melanoma, as demonstrated by small amounts of processed caspase-3, and might be a potential factor contributing to disease progression.

X-linked inhibitor of apoptosis protein (XIAP)

XIAP, a prominent inhibitor of caspases-9, -3 and -7 with additional non-apoptotic roles,^{25,50} was found to be overexpressed in various cancers.^{51,52} Several studies investigated the potential of XIAP protein levels as a biomarker in melanoma based on IHC detection. Emanuel et al. found that both benign nevi ($n = 6$) and in situ melanomas ($n = 4$) tested negative for XIAP expression, whereas XIAP was detected in 30 out of the 67 PMs tested (45%).⁵³ Out of these PMs, 37 had a thickness of less than 1 mm and 9 of these cases (24%) tested positive. A much higher positive rate was found in PMs with a thickness greater than 1 mm (21 out of 30 cases; 73%). With follow-up data for up to 6 years available for some of the cases, the authors also analyzed whether XIAP expression was associated with disease progression. In thin PMs, metastasis occurred in 1 out of 9 XIAP-positive melanomas and none of the 28 XIAP-negative melanomas. In contrast, in thick melanomas, metastasis occurred in 17 out of 22 (77%) of XIAP-positive PMs and in 5 out of 9 (56%) XIAP-negative PMs.⁵³ This study showed that XIAP expression tends to be more prevalent in thick melanomas; however, due to a lack of further statistical evaluation and limited sample numbers, it remained unclear whether XIAP expression is significantly associated with disease progression and metastasis. More conclusive data were provided by a larger study on 336 benign nevi, 195 PMs, and 241 MMs, which indeed demonstrated that XIAP expression was significantly elevated in melanomas ($p < 0.0001$).⁵⁴ Also within the melanoma group, XIAP protein expression was found to increase from PMs to MMs ($p < 0.0001$).⁵⁴ A similar trend could also be observed within the group of PMs when separated by thickness criteria (cut off = 2 mm thickness), in which thick PMs were found to express higher amounts of XIAP ($p = 0.0264$). In a more recent study, XIAP protein expression in 6 benign nevi, 7 in situ melanomas, and 42 PMs was likewise found to be significantly elevated in stage II disease compared to benign nevi ($p < 0.05$).⁵⁵ Trends for higher XIAP levels in more progressed disease were also identified when applying the American Joint Committee on Cancer criteria ($p < 0.001$).⁵⁵ Although another study on 77 PMs and 24 nevi showed that XIAP expression was more frequent in PMs than in nevi (70% vs. 58%), this difference was

not statistically significant.⁴² Overall, publications studying XIAP expression at the protein level tend to indicate that XIAP expression increases during melanomagenesis and with disease progression.

Other IAP Proteins

As described earlier, additional multifunctional IAP proteins, some of which being known interactors of caspases relevant for apoptosis execution, have been described.²⁶ Melanoma IAP (ML-IAP, also named Livin) inhibits caspase-9 and, as the name suggests, was found to be highly expressed in melanoma cell lines.⁵⁶ Similar to XIAP, ML-IAP protein levels were found to be significantly higher in PMs and MMs compared to benign nevi ($p = 0.0033$), as determined by IHC-based comparison of 14 benign nevi (4/14 positive, 21%), 19 PMs (14/19 positive, 74%), and 15 MMs (10/15 positive, 67%).⁵⁷ Data from ISH experiments corresponded with these findings, with *ML-IAP* mRNA expression detected in 1 out of 10 benign nevi (10%), 8 out of 13 PMs (62%), and 10 out of 15 MMs (67%).⁵⁷ However, in contrast to XIAP, no significant differences were detected between PMs and MMs.⁵⁷ In addition, ML-IAP expression may correlate with patient age ($p = 0.0056$).⁵⁷ An independent study in 77 PMs and 24 nevi found similar increases in ML-IAP in PM ($p < 0.01$),⁴² and an additional study successfully detected *ML-IAP* mRNA in 60 out of 63 MMs (95%).⁵⁸ Although a correlation between ML-IAP mRNA amounts and prognosis could not be identified in the latter study, the group of low-expressing MMs was very small ($n = 7$) and limited the statistical power.⁵⁸ A potential value of ML-IAP protein amounts in predicting chemotherapy responsiveness was reported by Nachmias et al.,⁵⁹ who compared 8 responders to 7 patients with disease progression. Although ML-IAP expression was absent or low in responders, intermediate or high expression was found in non-responders ($p = 0.02$).⁵⁹ Bruce/Apollon is an IAP that binds to caspase-9 and accelerates its proteasomal degradation.⁶⁰ Only one study has reported data on Apollon expression in melanoma tissue to date, with expression reported to be higher than in benign melanocytic lesions.⁶¹ Although cellular IAP (c-IAP) 1 and 2 can bind to caspases-9, -3 and -7, they are poor caspase inhibitors and instead primarily seem to function outside of the apoptosis execution phase.^{28,62} In a study of 77 PMs and 24 nevi, PMs more frequently expressed c-IAP1 and c-IAP2 ($p < 0.01$), but expression did not correlate with survival.⁴² Although the IAP survivin can interact with caspases-3 and -7, its main role appears to be the co-regulation of mitosis and cell cycle progression. Numerous previous studies analyzing survivin expression in melanoma suggest that survivin expression increases with disease progression and may indicate poor prognosis (comprehensively reviewed in McKenzie and Grossman 2012).⁶³

XIAP antagonists

Several proteins interfere with the interaction of XIAP and other IAPs with caspases, thereby ensuring that apoptosis execution proceeds swiftly and efficiently. Smac and Omi/HtrA2 are

the best characterized IAP antagonists, and both are co-released with cyt-c from the mitochondria during apoptosis (Fig. 1B). Despite significant interest in novel anti-cancer strategies and synthetic compounds mimicking the IAP antagonizing function of Smac and Omi/HtrA2,⁶⁴ to date no studies have investigated the expression amounts of these proteins in melanoma tissues. XAF1, another XIAP antagonist, reduces cytosolic XIAP levels by translocating it to the nucleus. Using tissue microarrays, XAF1 expression was investigated in 70 PMs and 40 benign nevi by Ng et al.⁶⁵ XAF1 protein amounts appeared to be reduced in PMs compared to benign nevi (33% vs. 13% showing weak staining, $p < 0.05$), and these reductions were observed in both cytosolic and nuclear regions ($p < 0.0001$). Of note, none of the PMs or nevi were negative for XAF1 expression, and XAF1 expression in PMs did not correlate with tumor thickness ($p = 0.119$). Even though this demonstrates a trend toward more anti-apoptotic conditions in PM, neither XAF1 protein amounts nor XAF1 nuclear positivity alone correlated with patient survival (5-year survival; $p = 0.889$ and $p = 0.896$, respectively).

Conclusions

Here, we review studies investigating key regulators of apoptosis execution as biomarker candidates for melanoma disease progression and survival. Changes in expression point toward the development of increased apoptosis resistance with progressing disease stages, as exemplified by reductions in Apaf-1 and XAF-1, and increases in XIAP. Interestingly, decreases in the Apaf-1/XIAP ratio have previously been reported as a prominent feature during the development of apoptosis resistance in postmitotic cells such as cardiomyocytes and sympathetic neurons.^{66,67} It seems surprising that very little information on changes in procaspase levels is available since the amount of caspases is a critical determinant of the capacity to execute apoptosis within the context of altered Apaf-1 and XIAP abundance.^{31,68} This knowledge gap will need to be closed in future studies. Overviews on cohort sizes, analytical tools (including information on antibodies), and staging criteria used in previous studies are presented in **Tables 1 and 2** and may be helpful resources to this end. In this context it is important to note that the information on antibody sources and data on the validation of staining specificities is frequently incomplete or absent in the published literature, and also that staging and sub-staging criteria, in particular thickness criteria, differ considerably between studies. A stricter standardization, following the most recent guidelines on melanoma staging and classification,^{3,4} is therefore desirable for future studies.

A more fundamental question that needs to be asked is whether individual regulators of apoptosis execution can be expected to become reliable biomarkers at all. This question arises from the signaling topology of apoptosis execution itself. In contrast to signaling pathways that are continuously active, such as cellular metabolism, the apoptosis execution network is largely dormant unless triggered by pro-death signals. Once activated, the interplay of Apaf-1, caspases, XIAP, and XIAP antagonists features multiple positive feedback loops that ensure

swift and efficient cell death in apoptosis-competent cells.^{68,69} This inherent non-linearity indicates that it may be impossible to identify a single “master regulator” that could serve as a reliable prognostic or predictive biomarker. Rather, apoptosis competence may need to be investigated in the context of multiple regulatory proteins or the entire signaling module. Although parallel measurements of multiple apoptosis execution proteins in melanoma, based on categorical analyses of IHC staining intensities, has had limited success so far,⁴² initial quantitative studies conducted in other cancer entities, such as colorectal cancer and glioblastoma multiforme, demonstrate the potential of combinatorial biomarker approaches that take network topologies of apoptosis execution into account.^{43,70} Additional apoptosis regulators upstream of the execution phase, such as members of the Bcl-2 family,⁷¹ may add further value and indeed might be

considered equally important for cell death decisions in melanoma. Novel technologies that allow more comprehensive, parallel, and quantitative IHC-based tissue analyses^{72,73} may therefore be of fundamental importance in order to optimally exploit regulators of apoptosis execution as biomarkers in melanoma and other heterogeneous cancers.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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