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The Onco-genomic Landscape of Malignant Melanoma: The Tumor Microenvironment comes of Age

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Abstract

Malignant melanoma is an aggressive cancer of the melanocytic cellular components of the skin which is responsible for 55,000 annual fatalities worldwide with more than 3 million people affected. Although genetics, age, and cumulative UV radiation exposure are all known risk factors, the knowledge of melanoma genomic profile at the individual patient level is becoming a more stringent factor towards its clinical management. Especially, since a number of molecular therapies have revolutionized the life expectancy of these patients over the last few decades. The present review, summarizes our current knowledge of the melanoma oncogenomic landscape through its commonly altered gene products, in which clearly emerges the critical role of tumor microenvironment as a distinctive factor for the recurrence and the underlying drug resistance linked to molecular target therapies. BRAF pathway-dependent and independent defects, the T-Cell Receptor (TCR) response-modulating systems involved in tumoral immune-evasion, the alterations in Cancer-associated fibroblasts (CAFs)-generated signals and the underlying cellular paracrine and autocrine network are discussed. Such scenario further strengthens the importance of genotranscriptomic profiling towards designing effective and durable therapeutic strategies.

Article Highlights

- Malignant melanoma is an aggressive cancer of the melanocytic cellular components of the skin which is responsible for 55,000 annual fatalities worldwide with more than 3 million people affected.
- Our current knowledge of the melanoma oncogenomic landscape through its commonly altered gene products, clearly points at the critical role of tumor microenvironment as a distinctive factor for the recurrence and the underlying drug resistance linked to molecular target therapies.
- The underlying BRAF pathway-dependent and independent defects, the T-Cell Receptor response modulating systems-mediated tumoral immune-evasion, the alterations in Cancer-associated fibroblasts (CAFs)-generated signals and the quali-quantitative defects in paracrine and autocrine cellular networks strengthen the importance of geno-transcriptomic profiling towards designing effective and durable therapeutic strategies

Introduction

A consistent and growing number of advances in the biological knowledge of Malignant Melanoma (MM) have been observed in the scientific literature following the initial discovery of its key driver mutation $BRAFV^{600E}$ and the studies uncovering the alternative and/or compensative circuitry leading to MAPK reactivation in response to its kinase activation inhibitor, vemurafenib [1-3]. Furthermore, The research focusing on the role of T-Cell Receptor associated factors on the regulation of cell-mediated immune response involved in tumor surveillance led in the late nineties to the discovery of the role of TCR coreceptors molecules such as CTLA-4 (and its counterpart ligand B7) in tumor surveillance [4]. This line of research was further strengthened by the discovery of the parallel role of the PD-1 and PD-L1 ligand receptor system linking the T-Cell mediated antigen recognition to down-modulation of T-Cell activity and to tumor immune evasion

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in those cancer expressing PD-L1 [5-7] and has provided the rational for the development of the second generation immune therapies that have revolutionized the treatment options of melanoma and other tumor types by extending life expectations from a few months to (for some) several years [8,9]. The above discoveries in the biological and pharmacological realm, though, have been speedup by the parallel advances in geno-transcriptomic analysis with widespread use of NGS technologies. In fact, the cumulative results obtained by qualitative and quantitative sequencing analysis on the several genes involved in the above biological processes in each individual cancer patient have confirmed the molecular heterogeneity of cancer involved in the individual sensitivity and responsivity to such treatment and have clearly pointed at the value of pathway-driven transcriptomic profiling as an effective decisional tool towards selecting the most effective molecular therapies tailored to the biologic asset of a patient cancer [10]. A further game-shifting paradigm has been provided by a growing amount of investigations in the last decade shedding light on the mechanistic role of tumor microenvironment to melanoma progression and responsivity to smart drugs. In particular, due to the paracrine signals network between the melanoma cell, stromal active elements (known as cancer-activated fibroblasts, CAFs) and tumor infiltrating lymphocytes (TILs). Such complexity and the additional inter-cellular circuitry underlying the up-regulation and downregulation of the molecular and genetic discriminants of melanoma's drug responsivity, support the growth in single-cell base profiling technologies and dedicated computational tools. These are likely to become the gold standard to guide the most therapeutic strategies in the next few years. The present review summarizes the functional knowledge gained so far in the role of both intrinsic (melanoma) and extrinsic (CAFs and TILs) molecular pathways which should be taken in consideration in the melanoma patient genotranscriptomic profiling.

Pathway-driven Genetic Discriminants of Drug-Resistance in Melanoma: BRAF- and Beyond-BRAF Defects

The implication of BRAF mutations in Malignant Melanoma (of which $BRAF^{V600E}$ is the most commonly observed) and the experience gained with the clinical use of the first BRAF kinase-targeting inhibitor in subjects carrying the BRAF mutant have revolutionized our views on the tumor-regression effects achievable with a molecular targeting drug on an otherwise rapidly lethal neoplastic condition such as Melanoma. In fact, it has been established that approximately 40–60% of Malignant Melanoma and 70-80% of Acquired Melanocytic Nevi contain a BRAF mutation, the vast majority of which (86%) result in a single amino acid change at codon 600 ($BRAF^{V600E}$) [11,12]. The development of the ATP-competitive specific BRAF inhibitor PLX4032 (Vemurafenib) [13] has been instrumental in modifying the progression history of this disease. This, due to the sensitivity to inhibition by vemurafenib conferred by the presence of the V600E mutation on the full length BRAF protein which is translated in the clinical setting with a dramatic reduction in tumor size, and a statistically significant increase in disease free survival. This finding, has provided the rational for BRAF mutant pharmacological targeting in malignant melanomas and other cancer types carrying such mutation. However, an almost immediate finding in the treatment of $BRAF^{V600E}$ mutation-carrying patients using BRAF kinase inhibitor as a monotherapy regimen, was the universal recurrence of the disease (measured by PFS) with a median time to disease recurrence of 6.8 months [14,15]. The mechanistic studies performed in cases undergoing disease progression following BRAF inhibition has shown MAPK pathway reactivation mechanisms in 70% of cases, with PI3K-PTENAKT- upregulating geno-transcriptomic alterations present in 22% of the recurrences [16]. As molecular discriminants of the observed MAPK-pathway reactivation in treated patients, two major studies ([16,17], have found BRAF splicing alterations (13%), gene amplification (19%), kinase duplication (10%, [18]) BRAF gene fusion products secondary to gene structural rearrangements [17], RAS and RAS homologous mutations (NRAS 18%, KRAS 7%), MEK1/2 (3%), CDKN2A(7%) and other quali-quantitative defects at the level of NF1, RB1, P53, ATK1, PTEN, RAC1, ARID2, IDH1 and novel candidate genes (DDX3X, MRPS31 and RPS27) [16,17]. Importantly, among the reported signature mutations in melanoma, BRAFV600E and NRAS(Q61) have been shown to be mutually exclusive in their expression pattern [17,19]. A particular consideration, towards understanding Melanoma and BRAF molecular biology, deserves BRAF dimerization. This is required for normal Ras-dependent RAF activation and is impaired in RAF mutants with moderate, low, or impaired kinase activity [20]. In particular, it has been demonstrated that BRAF homologous dimerization as well as CRAF heterologous dimerization in response to RAS activation are mediated by the BRAF N-terminal domain, and that this is required towards downstream MEK trans-activation under physiological circumstances [21,22]. An additional layer of complexity to this basic axiom is provided by the conditional effect of $BRAF^{V600E}$ depending on whether the cells expresses its full length V600E mutant protein or the p61 isoform lacking the RAS-binding region. In fact, the full length BRAF mutant, differently from its wild type counterpart, is not able to dimerize, and his MEK activation ability is strongly inhibited by vemurafenib. On the contrary, p61BRAF-V600E (missing exons 4-8 coding for its RAS binding domain) displays enhanced dimerization and high intrinsic catalytic activity in a RAS independent fashion, but dimerization *per se* does not affect downstream MEK activation and most importantly does not respond to vemurafenib inhibition ([23] and Figure 1). Ultimately, dimerization of the RAF kinases may contribute to several mechanisms that mediate Raf-inhibitor resistance, including mutational activation of N-RAS and K-RAS [2,24], upregulation of receptor tyrosine kinases (RTKs) that drive RAS activation [2], and expression of a V600E-BRAF splice variant with enhanced dimerization/MEK trans-activation potential [23]. Our current knowledge of the effects of BRAF dimerization on Vemurafenib responsivity [25] is graphically summarized below (Figures 1).

Although these studies have provided specific actionable

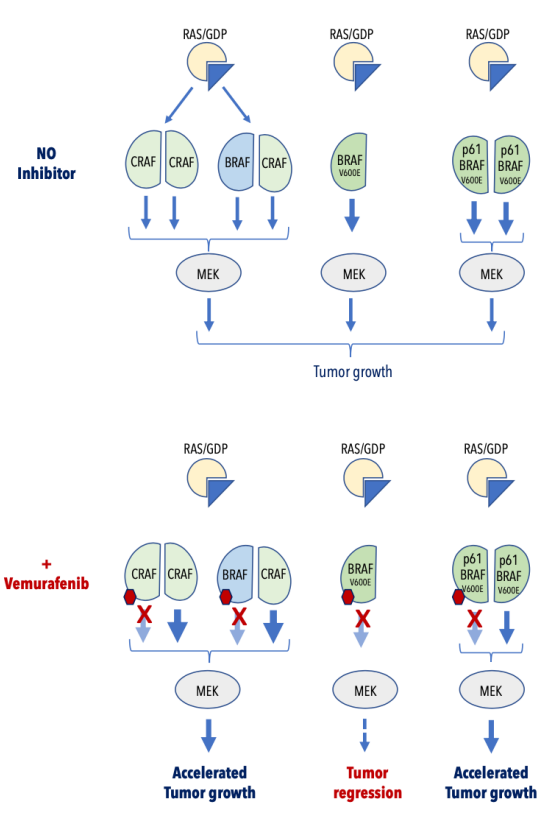

Figure 1: Effects of BRAF dimerization of Vemurafenib block (modified from Molina-Arcas M et al. [25])

Drug resistance type/ poor outcome marker	Gene/Prot product name Conferring resistance	Type of alteration Observed	Involved mechanism(s)	Reference(s)
BRAF Inhibitors resistance	BRAF V600E	Drug-induced Overexpression	Quantitative response following Targeted treatment	[57]
BRAF Inhibitors resistance	BRAF V600E	Alternative splicing	Enhanced dimerization and increased MEK binding	[20,23,58]
BRAF Inhibitors resistance	ATG7- BRAF/ TAX1BP1-BRAF/ TRAK1-RAF1, RAF1- AGGF1, and CLCN6-RAF1 *(w/intact kinase)* AKAP9-BRAF/ TRIM24-BRAF/ / CDC27-BRAF/ MAD1L1-BRAF/ CDK5RAP2-BRAF/ TMEM178BBRAF *(with dimerization)* -AGK-BRAF/ AGAP3-BRAF fusion/ KIAA1549-BRAF/ ERC1-BRAF/ PAPSS1-BRAF/ ZNF767-BRAF *(w/o dimerization)*	RAF Fusion products by gene rearrangement	MAPK pathway reactivation	[17,32,33,57,59]
BRAF Inhibitors resistance	CRAF over-expression	BRAF-> CRAF switch	*MAPK reactivation*	[60,61]
BRAF Inhibitors resistance	NRAS	Upregulation and NRASQ61H mut	STAT3 activation	[2,62]
BRAF Inhibitors resistance	MEK1	Exon3 mutations	MAPK reactivation	[63-65]
BRAF Inhibitors resistance	PI3KCA	Activating mutation in NRAS-/BRAF-mutated melanomas	Mutationally activated PI3KCA cooperate with MEK to AKT-TORC1/2 Proliferative pathway	[65]
BRAF Inhibitors resistance	CDKN2A/B	Copy number	MAPK reactivation	[66] [57]
BRAF Inhibitors resistance	HGF	CAFs-dependent paracrine loop	Resistance-associated to PI3K and MAPK activation	[3,67]
BRAF Inhibitors resistance	WNT5A	over-expression	Activation of AKT via FZD7 and RYK	[68]
BRAF Inhibitors resistance	IGF1R	over-expression	Resistance-associated to PI3K and AKT activation	[28]
BRAF Inhibitors resistance	VEGFR1	over-expression	Putative VEGF-A autocrine loop	[69]
BRAF Inhibitors resistance	EGFR	over-expression	Activation of EGFR-SFK-STAT3	[70,71]
BRAF Inhibitors resistance	PDGFR	over-expression	MAPK reactivation	[2]
BRAF Inhibitors resistance	EPHA2	over-expression	MAPK reactivation	[72]
BRAF Inhibitors resistance	AKT/PKB	over-expression	?	[73]
BRAF Inhibitors resistance	KIT	Over-expression/ activating mutations	MAPK reactivation	[17,34]
BRAF Inhibitors resistance	SRC	over-expression	Activation of STAT3	[71,73]
BRAF Inhibitors resistance	CDC7, MCM2-7	over-expression	miR-3613-3p downregulation	[74]
BRAF Inhibitors resistance	Nestin	over-expression	PI3K/AKT/mTOR mediated	[75]
BRAF Inhibitors resistance	CEP170-AKT3, AKT3- PLD5, ZEB2-AKT3/ ARHGAP30-AKT3/ MITFFOXP1, CADM2-MITF/ FRMD4BMITF/ PCBP2-HMGA2, TSFMHMGA2, and SENP1-HMGA2/ LBHFLT4/ CPSF4L-ERBB4/ LCLAT1- EPHA3 MOBKL1B-EPHB1	Non-RAF fusion products w/ intact kinase domains (predicted)	MAPK pathway reactivation	[17]
BRAF Inhibitors resistance	DDX3X	X-chromosome-linked ATPase/RNA Helicase LoF	RAS, PI3K and b-Catenin pathways dysregulation	[17,34]
BRAF Inhibitors resistance	STK11	Splite-site mutation 464+1G>C	TBD	[57]
BRAF Inhibitors resistance	VHL	Splite-site mutation E52	TBD	[57]
BRAF Inhibitors resistance	PRKAR1A	PKA catalytic subunit loss of function	PKA signaling activation	[34]
BRAF Inhibitors resistance	ARID2, ARID1A, ARID1B, PBRM1, BRD7	SWI/SNF family members Loss of function	Gene expression dysregulation	[34]
Checkpoint Inhibitors resistance	PTEN	Loss of function mutation	TILs downregulation	[76]
Checkpoint Inhibitors resistance	MIFT	Reduced levels	TILs downregulation	[76]

BRAF inhibitors and Checkpoint inhibitors resistance	mTOR (in melanoma); In CD8+: Along with CTLA-4 induces CD8+ tumoral tolerance	mTOR suppression In melanoma;	Suppression pS6 protein by TORC in melanoma reflects effect of anti-BRAF therapy	77] [78]
Checkpoint Inhibitors resistance	β-Catenin	Pathway activation	YAP nuclear translocation in CAFs TILs downregulation WNT5a-MARCKS axis	[79] [76] [80]
Checkpoint Inhibitors resistance	TAZ, YAP, TEAD	Increased PD-L1 gene transcription in in Breast, and Lung ca	T-Cell function inhibition via PD-L1	[81]
Checkpoint Inhibitors resistance	SPHK1	Expression inversely correlated to outcome	FoxP3, TGF-β, IL-10, CCL-17, CCL-22, CCR4, IDO1, IDO2, CTLA4, PDCD1*,CD274**, PDCD1LG2***, TIGIT, LAG3 and HAVCR2****	[82]
Checkpoint Inhibitors resistance	Score based on the 10 stromal genes: Fap, Cpxm1, Serpinf1, Dpt, Lum, Crispd2, Adam33, igfbp6, Dcn, C1s1	10 CAF-s genes profile linked to ICI resistance	CAFs paracrine-mediated effect	[83]
Mediator of melanoma cell growth	SOX10			[84,85]
Mediator of melanoma cell growth	MKRN2	over-expression	P53-dependent pathway	[86]
Mediator of melanoma cell growth	TMEM87a/Elkin1	causal link	PIEZO1-independent pathway	[87]
CAF mediated resistance	Notch1 autocrine stimulation and signaling in CAFs	Autocrine stimulation	Suppression of aggressive and stem-like features	[38-40]
CAF mediated resistance	MMPs expression (by CAFs)	decreased NK mediated melanoma oncolytic effect	downmodulation of NKG2D ligands MICA/B	[37]
CAF mediated resistance	Neregulin1 expression (by CAFs)	paracrine stimulation	Overcome of vemurafenib block on BRAF mutated melanoma by ErbB2/ErbB3 stimulation	[41-43]
CAF mediated resistance/ Immunespecific suppression	TGF-b (by melanoma cells)	Paracrine SerpinF1/ PEDF downregulation - CD8+ suppression	Favors fibroblast to CAF conversion in synergy with PDGF-BB - CXCR3 downregulation in CD8+	[44,46]
CAF mediated resistance	Nodal (by melanoma cells)	Paracrine stimulation	CAF conversion by TGF-b- Snail activation in fibroblasts	[45]
CAF mediated resistance	HAPLN1	Reduced levels in CAF	Its lack reduces immune cells infiltration of MM microenvironment	[47]
Melanoma marker of poor outcome	NME1	reduced levels	Tumor suppressor for genes associated to melanoma malignancy	[88]
Melanoma marker of poor outcome	CYR61	reduced levels	TBD	[89]
Melanoma marker of poor outcome	DUSP1	reduced levels	TBD	[89]
Melanoma marker of poor outcome	RNASE4	reduced levels	TBD	[89]

Table 1: Genes and related gene products involved in MM growth, molecular target- and ICI- drugs resistance and post-treatment survival

knowledge towards identifying those patients responsive to pharmacological targeting of BRAF they have also raised additional challenges. In fact, in spite of the extreme sensitivity of the BRAF mutant to Vemurafenib block, the very same inhibitor displays opposite effects on wild-type BRAF as well as in NRAS-bearing melanomas (not displaying BRAF mutation) in terms of both dimerization and MEK transactivating activity [23,26]. Also, in addition to the short PFS (less than 7 months) achieved with Vemurafenib monotherapy in BRAF mutant-carrying Melanoma patients, the MAPK hyper-activation mechanisms triggered in response to Vemurafenib sustained treatment has been directly linked to the onset of secondary melanomas [27] This had led to the development of dual targeting strategies in the attempt to either prevent and/or circumvent the paradoxical hyper-activation effects on wild type BRAF supported by RTKs up-regulation loops [28] and further overcome or minimize the observed rapid MAPK pathway reactivation and the mid-long term negative effects linked to Vemurafenib monotherapy treatment. In this regard, the BRAF/MEK combined block has been found to bear practical advantages in the clinical setting over monotherapy [27,29] in which it exerts a small but statistically significant survival improvement over anti-BRAF monotherapy in BRAF mutant melanomas ranging 2.4 to 4.1 months measured as Progression Free Survival (PFS) in phase II randomized clinical trials [30,31]. In other cases, where the PI3K or related-downstream pathways activation are involved in vemurafenib-acquired resistance, the use of MEK/PI3K or MEK/CDK4/6 combo therapies have been proposed. Indeed, preclinical studies have experimentally proven their ability to delay MEK inhibitor resistance [32]. Furthermore, this dual block for BRAF-downstream and modulating parallel effectors has been shown to work in 2.6-6.7% of melanomas carrying BRAF fusion products [33]. One of the most impactful attempts to functionally categorize Melanoma has come

from a multi-dimensional genomic study published in 2015 by the Cancer Genome Atlas Network [17]. The study was performed on more than three hundred patient-derived matched specimen from both primary and metastatic melanomas. Besides confirming and refining previously identified geno-transcriptomic alterations and identifying the novel cancer-driver DDX3X (Table I), the study stands out for the original functional framework provided which allows to classify all melanomas according to: (a) four main genomic sub-types based upon their main (cancer) driver defect, and (b) three transcriptomic sub-classes. Specifically, at the genomic level melanomas were classified in (1) the BRAF mutant sub-type (52%), (2) the RAS sub-type (28%), (3) the NF1 sub-type (14%), and (4) a Triple negative wild-type (6%) with the latter group being characterized by lack of specific hotspot mutations for BRAF, N/H/K-RAS or NF1. In this regard, under the proposed genomic clustering most hotspot mutations attributable to UV signature (affecting both cancer drivers coding regions and TERT promoter) were mostly found in the first three sub-types (BRAF 90.7%, RAS 93.5% and NF1 92.9%, respectively, against a 30% in the Triple negative sub-type) while somatic copy number alterations and structural rearrangements were exclusively found in the triple negative sub-type (the fourth group). Additionally, based upon transcriptomic clustering of 1,500 genes corresponding to the most represented mRNA transcript variants from 329 sample cases, the study proposes three novel transcriptomically-defined new functional classes named after each cluster as: (a) “Immune” type, (b) “Keratin” type, and (c) “MITF low” type, accounting respectively for: (a) 51%, (b) 31% and (c) 18%, of the screened sample cases. The clinical significance of this last classifications bears high practical value, since the immune transcriptomic sub-class was associated with improved post-accession survival in patients with regional metastatic melanoma. This was assessed via (1) a novel Lymphocyte Score, LS, and (2) the associated high levels of the non-receptor tyrosine kinase LCK, which was also strongly associated to this sub-class with favorable outcome. Another more recent study analyzed 1000 exomes out of 470 patients from the cancer genome ATLAS [34]. The study conclusions support the 2015 study and further deepen the previous findings [17]. Among these, the authors confirm the role of DDX3X, an X-Chromosome linked tumor suppressor gene which codifies for an ATPase,-RNA Helicase protein, as a significant mutated gene (SMG) in melanoma. Using both (a) transcriptomic signature between the DDX3X mutated and wild type population and (b) DDX3X binding analysis on putative target transcripts, they strengthen the link between DDX3X mutations and its Loss of Function (LoF) in melanoma with ~75% of such mutations attributable to UVR (by NMF analysis). They also find its LoF to be linked to dysregulation of RAS, PI3K and b-Catenin pathways in the analyzed melanoma samples and ultimately suggest that DDX3X loss my play a role in de-differentiation, invasiveness and reduced proliferation in agreement with a published functional study [35]. Additional novelties of the study, relate to the finding that specific members of the SWI/SNF transcriptional modulators family (ARID2, ARID1A, ARID1B, PBRM1, BRD7) and PRKAR1A, a catalytic subunit of PKA with tumor suppressor ability, are significantly enriched in the LoF melanoma population. Finally, the study confirms the significance of the tumor mutational burden, TMB (higher in males) with the patient post-accession survival (survival relative to time of tumor sample procurement) along with UVR signature, Immune signature, age (at time of sample procurement), tumor site (with Immune signature, UVR signature and TMB carrying the best predictors of overall survival).

Geno-transcriptomics and Molecular Profiling of Cancer-Associated Fibroblasts (Cafs) in Melanoma Microenvironment

An intense and specific tumorigenic stage-dependent and sequentially occurring cross-talk among the stromal elements, the melanoma cells, and immune system cellular elements in the tumor microenvironment of melanoma has emerged in the last decade. Among the evidences supporting this view, it has been demonstrated that melanoma cells carrying BRAFV600E mutation activate stromal fibroblasts and enhance tumorigenicity through secretion of MMP-1 [36]. A study has linked the secretion of active matrix metallo-proteinases by CAFs to the decreased NK mediated oncolytic targeting of melanoma cells via downmodulation of the NKG2D ligands MIC-A/B [37]. Among the membrane tyrosine kinase receptors and their autocrine and paracrine networks modulating melanoma tumor microenvironment, Notch1 autocrine stimulation and signaling in CAFs [38] has been shown to suppress aggressive and stem-like features in melanoma cells [39,40]. Furthermore, CAF-expressed Neuregulin-1 has been found to promote vemurafenib-treated/BRAF mutated melanoma cells proliferation via paracrine stimulation of ErbB3/ErbB2 signaling; this effect was reversed by ErbB3 antibody-mediated neutralization [41-43]. Interestingly, PEDF (also known as SerpinF1) which has been shown to be a tumor suppressive factor in normal fibroblasts, is downregulated by melanoma-secreted PDGF-

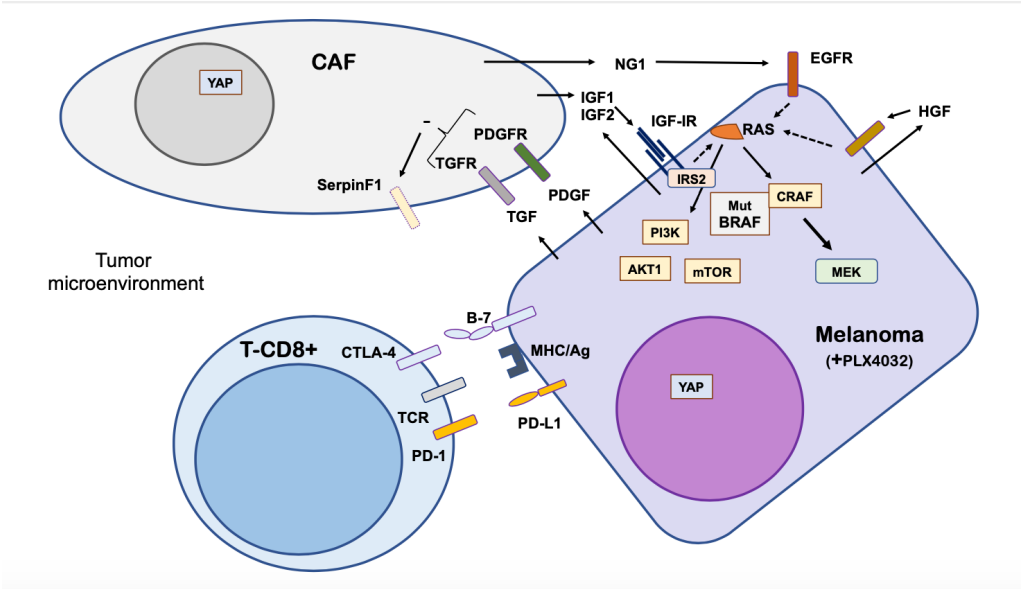

Figure 2: Key cellular elements and gene products emerging in Melanoma Tumor microenvironment involved in molecular drugs target resistance (see also Table I for an extended list of factors).

BB and TGF-β in order to favor CAF conversion [44]. A similar stromal conversion effect to CAF feature is exerted by Nodal, a cancer-expressed TGF superfamily member, along with activation of the TGF-β - Snail signaling axis in fibroblasts [45]. The immunosuppressive role and targetable value of TGF-β in the tumor microenvironment of Malignant Melanoma has been further strengthened by the observation that TGF-β inhibits tumor infiltration of CD8+ T cells and their oncolytic effect *in vivo*. This, through inhibition of their CXCR3 expression which impairs their migration towards melanoma cells expressing CXCL10 [46]. More recently, a study has shown that loss of HAPLN1, a secreted proteoglycan component of the ECM which is lost in aged fibroblasts, is a permissive event in melanoma metastasis. Notably, its experimental reconstitution selectively stimulates the motility of mononuclear immune cells, ultimately restoring the immune microenvironment [47]. A schematics of the relationship between the key cellular components affecting malignant melanoma response to current therapies (including CAFs) is provided in Figure 2 below. The findings linking such mechanisms to drug-resistance in melanoma are summarized in Table I.

The T-Cell Receptor Co-Receptorial Systems in Malignant Melanoma Unleashing the Potential of Onco-genomic-driven Immuno-oncology

Although the idea of triggering the immune system against tumor cells with the use of traditional vaccination is not new, the failure of those initial strategies has provided biological answers and immediate alternatives with the discovery of the role of T-Cell receptor, co-receptors and associated modulating molecules in the context of processed antigen presentation by macrophages. In fact, the possibility to guide an endogenous effective anti-tumoral immune response strongly relies on the functionality of these T-Cell membrane molecules and their ligands counterparts expressed in the targeted antigen-carrying cancer cell. The T-cell gene products that have been proven crucial to step-up immunotherapies in the last few decades include the CTLA-4/B7 system [48,49] and the PD-1/PD-L1 coreceptor/ ligand system [50] with PD-L1 being co-expressed with the antigen carrying target cells. Indeed, the expression of PD-L1 in cancer has been associated to a worse outcome in agreement with its role in tumor immune-evasion [51]. The success of the animal studies with the first human anti-CTLA-4 monoclonal antibody (Ipilimumab) and the following phase II and III clinical trials displaying greater than 20% responsivity in malignant melanomas (independently on BRAF status) with more than 10 years survival in some patients [52] have shown the potential of the "immune checkpoint" therapies in a deadly cancer such as malignant melanoma, for which the survival achieved using single or combined targeted BRAF therapy is still below one year. The following demonstration of the additive effect between CTLA- 4 and PD-1 blocking combinations in melanoma patients with >50% response and >80% tumor regression, has further established the effectiveness of immune checkpoint strategy in the treatment of Malignant Melanoma [53-55]. Additional TCR response co-modulating factors have been identified such as LAG3, TIM3, TIGIT, VISTA, ICOS, OX40, GITR, 4-IBB, CD40, CD27 and their cellular ligands [56]. The clarification of their exact role in cancer immune-evasion in the micro-environmental cellular context will likely provide valid novel strategies to fine tune future immune-therapy in malignant melanoma and other tumor types. In this context, is not surprising that Immune signature in the two largest cohort studies available to date [17,34] has demonstrated a predictive value which is consensual to the one shown by the overall Tumor Mutation Burden (TMB) towards predicting patient survival, therefore suggesting immune signature profiling to be a parameter to be taken in consideration both in translational research and clinical setting for melanoma. The findings disclosing mechanisms causing checkpoint inhibitors resistance in melanoma are summarized in Table I, and a graphic summary of essential key infiltrating T-Cells interactions in the melanoma tumor microenvironment is also provided in Figure 2.

Conclusions and Perspectives

Unprecedented advances have been possible in malignant melanoma in the last few decades thanks to the specific understanding of the role of the tumor specific pathway-driven signatures, the escape from T-Cell mediated tumor surveillance and the specific role and consequent clinical use of BRAF-pathway and TCR co-receptors targeting strategies. The cumulative scenario emerging in the literature with the growing understanding of the role of Cancer-Associated Fibroblasts and Tumor Infiltrating Lymphocytes in the tumor micro-environmental integrated response to current biologic therapies, supports a higher level of biological complexity than the one currently considered for therapeutic decisions. The geno-transcriptomic profiling of malignant melanoma patients, is becoming an essential approach for evidence-based personalized treatments of patients affected by this disease. The NGS-based panels currently used will further benefit from the extension of the current analysis of the single parenchymal component (the melanoma cells) to the tumor surrounding stromal elements (Cancer-Associated Fibroblasts) as well of the study of the infiltrating T-lymphocytes (CD8+). Bioptic tissue micro-dissection and single-cell-based methods, along with computational tools meant to clarify the contextual functional dynamics in the patient's tumor microenvironment, will play a major role towards the effectiveness of therapeutic strategies and the long-term outcome of this neoplasia.

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