Predicting the outcome of melanoma: can we tell the future of a patient's melanoma?

Oriol Yélamos¹ & Pedram Gerami^{*,1}

Practice points

Breslow depth, ulceration & mitotic rate

- The depth of the primary tumor measured in millimeters (Breslow depth) remains the most important parameter to determine the management and initial prognosis of patients with cutaneous melanoma.
- The presence of ulceration or mitotic activity is associated with a worse prognosis, especially when evaluating thin melanomas (<1 mm).

Lymph node evaluation & sentinel lymph node biopsy

- Sentinel lymph node biopsy (SLNB) is the best independent prognostic tool currently available. However, up to 70% of patients that develop distant metastases were SLNB negative.
- SLNB will benefit from additional tools that increase capacity to predict the development of metastases.

FISH & comparative genomic hybridization

- FISH and comparative genomic hybridization can show some chromosomal copy number aberrations which are associated with a worse prognosis, such as gains in *CCND1* or *MYC*.
- FISH-negative melanomas targeting *RREB*, *MYB*, *Cep6* and *CCND1* are associated with a better prognosis.
- However, these techniques are not optimal as a staging tool and are currently most optimal for distinguishing benign from malignant melanocytic lesions or for predicting prognosis of intermediate-grade melanocytic tumors.

Mutation analysis & next-generation sequencing

- Certain genes seem to be associated with a worse outcome, such as *BRAF* or *NRAS*. However, the results are controversial and the prognosis of melanoma cannot be assessed with the analysis of isolated mutations.
- The analysis of specific mutations such as *BRAF* mutations allows the use of targeted drugs and predicts which patients are more likely to respond to these new therapies.
- The study of whole arrays of mutations will evaluate the potential therapeutic targets in patients with melanoma.

Gene expression profiling

- Different gene-expression profiling assays classify patients in low risk (class 1) and high risk of developing metastases (class 2). These tests are validated and will probably become very valuable tools to be used in conjunction with conventional American Joint Committee on Cancer assessment.

Conclusion & future perspective

- Currently, the American Joint Committee on Cancer staging is focused on the histological evaluation of the primary melanoma, the lymph node status and the presence of distant metastases.
- New tools such as next-generation sequencing and gene-expression profiling will provide better theragnostic and prognostic information for patients with melanoma.
- In the future, all tumors will be likely to go through very extensive molecular profiling with reports of the key therapeutic targets and the prognostic markers.

¹Department of Dermatology, Feinberg School of Medicine, The Robert H Lurie Cancer Center, Northwestern University, 676 N. St Clair Street, Suite 1765, Chicago, IL 60611, USA

*Author for correspondence: Tel.: +1 312 695 1413; Fax: +1 312 695 0007; pgerami1@nm.org

Cutaneous melanoma is responsible for the greatest number of skin cancer related deaths. For many years there were few therapeutic options. However, in the last years a number of new therapeutic options have emerged showing improved survival rates for advanced melanoma patients. A significant question based on these findings is whether identification and treatment of patients with biologically aggressive melanomas at an earlier clinical stage offer an opportunity for even greater improvement in overall survival. In this review, we will discuss the recent advancements in molecular strategies beyond traditional staging to identify biologically aggressive melanomas, and which are their implications in terms of predicting the prognosis of patients with melanoma.

KEYWORDS

- comparative genomic hybridization • FISH
- gene-expression profiling • melanoma
- next-generation sequencing • prognosis
- sentinel lymph node biopsy

Cutaneous melanoma is responsible for the greatest number of skin cancer related deaths and it is second only to leukemia among all cancers in lost years of life [1,2]. For many years there were few therapeutic options. However, in the last years a number of new therapeutic options showing improved survival for stage IV melanoma patients have become available. This includes novel targeted therapy strategies which specifically inhibit driver mutations in the cancer pathways as well as immunologic based therapies.

More specifically clinical trials have shown that disease-free survival and overall survival can be prolonged with these new therapies [3]. Ipilimumab, a CTLA4 inhibitor, has been shown to increase the disease-free survival of patients with advanced melanomas [4–6], and has an overall survival rate of nearly 50% at 12 months when used in combination with dacarbazine [6]. Nivolumab and pembrolizumab are two newly developed immune modulators which inhibit the programmed cell death 1 pathway (*PD-1*). Recent clinical trials have shown that the *PD-1* blockade in patients with advanced melanomas may result in an overall survival at 12 months of 69% with pembrolizumab [7] and of 73% with nivolumab [8].

BRAF inhibitors vemurafenib and dabrafenib have shown dramatic responses in patients harboring *BRAF* mutations [9,10]. However, although the initial responses with these targeted therapies are very high, disease progression appears in most of the patients since melanoma acquires additional mutations which allow it to escape from the drug effect [9]. This is not surprising since melanomas have the highest mutational rate of all cancers due to a continuous exposure to environmental mutagens such as ultraviolet radiation [11]. Therefore, current clinical trials target both *BRAF* and *MEK*, in order to block the entire Raf/MEK/ERK pathway. In a Phase III clinical trial using the *BRAF* inhibitor dabrafenib plus the *MEK* inhibitor trametinib, a

12-month overall survival was achieved in 72% of patients [12]. In another Phase III trial, the combination of vemurafenib and the new *MEK* inhibitor cobimetinib showed better results than the use of vemurafenib plus placebo, achieving a progression-free survival of 9.9 months and an overall response of 68% in the combined therapy group versus a progression-free survival of 6.2 months and an overall response of 45% in the control group [13].

An obvious and significant question based on these recent findings is whether identification and treatment of patients with biologically aggressive melanomas at an earlier clinical stage, perhaps in the adjuvant setting, offers an opportunity for even greater improvement in overall survival. In this review, we will discuss the recent advancements in molecular strategies beyond traditional staging to identify biologically aggressive melanomas. This includes techniques such as FISH, comparative genomic hybridization, mRNA expression profiling and next-generation sequencing.

Current perspective

The American Joint Committee on Cancer (AJCC) TNM staging system for melanoma is currently the standard method to classify patients with melanoma in different stages according to the primary tumor (T), the involvement of regional nodes (N) and the presence of distant metastases (M). According to these parameters, patients are classified in four stages (stages I–IV) which are used to guide management of the disease, as well to determine the patients' prognosis.

Breslow depth, ulceration & mitotic activity

The depth of the tumor measured in millimeters has been the single most significant prognostic feature of the tumor itself since Breslow described it in 1970 [14]. In general, patients with a T1 stage (Breslow depth of 1 mm or less) typically have

an excellent prognosis with a 20-year survival of 96% [15]. However, a small percentage of thin melanomas, including those less than 1 mm, will still result in aggressive disease [16–18].

From a histological standpoint, ulceration in the setting of a melanoma is associated with a worse prognosis [16], and upstages the T stage from a to b [19,20]. Similarly, the presence of dermal mitoses upstages T1 melanomas from a T1a to T1b. This has important implications in terms of prognosis as the 10-year disease-free survival for T1b patients is lower compared with T1a patients (87 vs 95%) [18]. Hence, although the majority of patients with thin melanomas will do well, up to 11% will have recurrences of their disease [21], indicating that further parameters besides the primary tumor histology need to be evaluated in order to accurately determine the prognosis.

Lymph node evaluation & sentinel lymph node biopsy

The presence of positive lymph nodes is associated with a poor prognosis due to an increased risk of distant metastases, with an overall 10-year survival for stage III patients ranging from 30 to 70% compared with 40–90% for combined stage I and II patients [18,19]. The status of the sentinel lymph node has been shown to be the single most powerful prognosticator of long term survival [20,22–23]. However there are considerable limitations to the sentinel lymph node biopsy (SLNB).

A significant proportion of patients with SLNB are found to have only microscopic levels of disease involving one or two sentinel lymph nodes (N1a or N2a). The 10-year disease-free survival for N1a and N2a patients is 60 and 30%, respectively [19], highlighting the great variability in the outcomes of patients in this cohort. This can also be explained by differences in the SLNB technique as well as in the SLNB pathologic analysis. For example, several studies have shown that the presence of metastatic material in the lymph node is not always related to a higher risk of distant metastases, as certain micromorphometric parameters may be also considered [24–27]. The presence of minimal tumor burden, defined as micrometastases less than 0.1 mm in diameter as included in the Rotterdam criteria, the subcapsular location of the micrometastases and a depth of invasion of <0.3 mm have been associated with a low risk of developing distant metastases and subsequently a better prognosis [24].

In summary, the prognosis for stage Ia patients is generally excellent with a 10-year survival of 95% [18]. Conversely, stage IV patients have very poor prognosis with a 10-year survival less than 30% [19]. For stage Ib, II and III patients there is great variability in prognosis with the 10-year survival of stage II patients varying from 85 to 40% [19]. Therefore, patients in these intermediate groups will be the ones that will obtain more benefit from new additional prognostic techniques combined with the current tools.

FISH & comparative genomic hybridization

Like most malignant neoplasms, the vast majority of melanomas have been shown to carry clonal chromosomal copy number aberrations [28]. Alternatively these changes are uncommon in nevi with some small exceptions. Hence, techniques such as comparative genomic hybridization and FISH have been utilized to evaluate melanocytic tumors for such aberrations to improve classification and diagnosis of histologically borderline lesions [28,29]. These techniques have contributed greatly to the classification of borderline melanocytic neoplasms. These same techniques have been evaluated in a prognostic setting as well.

An example can be found in certain subsets of FISH-negative melanomas. Although FISH can discriminate melanomas from nevi with a high sensitivity and specificity [29,30], some melanomas show negative FISH results when using a 4 probe diagnostic FISH assay targeting *RREB1*, *MYB*, *Cep6* and *CCND1* [31]. This subset of FISH-negative melanomas has shown to have a better prognosis than the ones with chromosomal copy number aberrations, with only 2% of patients presenting with distant metastases in the FISH-negative group compared with the 27% of patients in the FISH-positive group [31].

Additionally, certain chromosomal aberrations have been specifically associated with aggressive disease. In one study using FISH targeting a wide array of chromosomal loci frequently altered in melanoma, it was found that melanoma with high level copy number gains involving *CCND1* or *MYC* had particularly aggressive disease, with these findings being associated with the development of distant metastatic disease [32]. Furthermore, in the case of *CCND1*, higher level gains particularly in the form of an homogeneous staining regions, which are intrachromosomal continued repeats of a gene with sometimes more than ten or 15

copies, have been linked to uniformly aggressive disease [33,34]. The limitation with FISH as a prognostic tool is that chromosomal copy number aberrations exist in the vast majority of melanomas including those of an earlier stage. Hence, many melanomas which have not yet acquired the potential for metastatic spread may have a positive FISH. The strength of the current FISH assay used for melanoma is that it could potentially eliminate some low grade melanocytic tumors classified as melanoma based on histology but without significant malignant potential at the biologic level. Alternatively, one could use highly stringent criteria to ensure a positive result is indicative of highly aggressive disease. However, since there is a vast array of loci that may be aberrant as a melanoma acquires metastatic capability, a targeted FISH assay with highly stringent criteria would likely have low specificity in identifying aggressive tumors.

Mutation analysis & next-generation sequencing

The platforms for sequencing of tumors have rapidly advanced. With next-generation sequencing it is possible to obtain an extensive mutational profile of a melanoma with a single experiment. As targeted oncologic therapies have now shown proven benefit in extending survival in stage IV melanoma patients, the mutational profile is a highly valuable information. For example, patients with mutations in *BRAF* show a significantly better response to targeted inhibitors of *BRAF* such as vemurafenib or dabrafenib than patients without this mutation [9,10]. Furthermore, patients escaping from the *BRAF* inhibition often have additional mutations such as mutations in *PD-1* or in its ligands [35], *PTEN* mutations [36] or *NFI* mutations which might activate the MAPK, PIK3/AKT and/or mTOR pathways [37]. Identifying the mutational pattern can help predict which therapies a given melanoma will respond to. For example, patients with *PD-1* mutations are more likely to respond to pembrolizumab or nivolumab. Hence, the data obtained from sequencing at this time are critical in guiding targeted therapy.

Some studies suggest that melanomas harboring *BRAF* or *NRAS* mutations have a poor prognosis in patients with advanced stage disease [38–40]. However these findings have been inconsistent [41–43]. This is not surprising since these mutations and many of the other driver mutations identified in melanoma are early

events and a considerable number of additional mutagenic changes are acquired before the tumor acquires metastatic capabilities. Furthermore, many of the changes that occur in the tumor that allow it to obtain metastatic capability may involve epigenetic changes in addition to genetic changes [44].

In summary, the mutational profile obtained from most standard cancer panels used in next-generation sequencing can provide valuable information regarding which driver mutations are present in a tumor, and can guide targeted therapy. However, these panels are less focused on the genetic and/or epigenetic changes that a given tumor acquires to obtain metastatic capability. Therefore, they may not provide an entirely accurate presentation of which pathways are activated and at current are limited as a prognostic tool.

Gene-expression profiling

Gene-expression profiling (GEP) allows, with a single experiment, to identify which genes are activated and which genes are inactivated by evaluating the amount of expression of a given gene mRNA relative to several control house-keeping genes. This allows the evaluation of an extensive number of cancer pathways simultaneously. Initially, GEP assays were developed using cells lines, mouse models or tissue coming from metastatic tumors, and did not allow the comparison between primary and distant disease [45,46]. However, more recent studies have compared tissue coming from primary melanomas and metastases, and as far as a prognostic indicator for melanoma, they have suggested that GEP may be the optimal platform at this time, with a number of independent investigators having demonstrated the utility of GEP for melanoma prognosis [46–50].

Winnepenninckx *et al.* [47] studied 83 frozen primary melanomas and described a 254-gene assay with a gene signature that was associated with a higher risk of metastatic disease. These genes were involved with the cell cycle, mitotic activity, DNA replication, intracellular signaling, ubiquitination and other cell mechanisms. Alonso *et al.* [46] analyzed 34 vertical growth phase melanomas and reported that genes implicated in the epithelial–mesenchymal transition are closely related with the development of metastases. Another study identified 308 genes to be differently expressed in primary melanomas and in metastases, and suggested that

different subtypes of melanoma also have different genetic signatures [51]. Additional studies have also been published reporting different arrays of gene expression found in localized and advanced melanomas [50,52–53]. However, these studies used fresh-frozen tissue which can be difficult to obtain in routine practice, and therefore did not include large samples of both primary and metastatic melanomas [47,50,54].

With the development of newer technologies capable of analyzing partially degraded DNA, the genetic analysis from formalin fixed paraffin-embedded (FFPE) tissue led to the development of new GEP assays which identified additional prognostic biomarkers and allowed the analysis of larger samples coming from archived FFPE samples [45,55]. Recently, Harbst *et al.* [49] analyzed 223 archived FFPE melanomas and developed a GEP assay which allowed the classification of patients in low-grade and high-grade. This also correlated with the tumor thickness, mitotic activity and degree of ulceration, and was associated with a poorer prognosis. Additionally, Conway *et al.* [55] studied 254 FFPE-archived primary melanomas and identified that osteopontin (encoded in *SPPI* gene) is related to a higher rate of relapse in patients with melanoma.

The first commercial GEP assay for melanoma was developed for uveal melanoma (UM) through collaboration between Castle Biosciences and the laboratory of Dr William Harbour. This GEP assay classifies UM into low risk (class 1) and high risk (class 2) categories with greater accuracy than traditional cytogenetics in predicting the development of distant metastases [56,57]. Traditionally, UM were tested with conventional cytogenetic techniques, as it was recognized that UM with deletions involving the short arm of chromosome 3 were associated with a higher risk of metastases [58]. With the development of new molecular techniques, the mutation was found to be located in the chromosome region 3p21 [59]. This encodes for a protein called Bap1 (*BRCA*-associated protein 1) which is a tumor suppressor protein associated with the cell cycle [60]. In patients with UM, loss of expression of this gene is associated with a more aggressive course. However, a significant proportion of patients with aggressive disease may not have this cytogenetic aberration. This may be the result of loss of function of the *BAP1* gene by means other than chromosomal deletion or via activation of other less frequent pathways resulting in aggressive disease. With the development of a specific 15 gene assay directed at classifying UM

into low and high risk categories, the accuracy of prognostication has improved significantly with almost 26% of patients with a class 2 developing metastatic disease compared with 1.1% of patients classified as class 1 [61]. Since its inception in 2009, GEP profiling for UM has become the standard of care by over 95% of ocular oncology centers [62,63] and it has now become part of the AJCC staging for UM patients [64].

As a follow-up to the development of the UM GEP test, Castle Biosciences has subsequently developed a commercially available GEP test for cutaneous melanoma, known as DecisionDx-Melanoma. To develop this test, the genetic profile of primary cutaneous and uveal melanomas was compared with the genetic profile of metastatic melanomas, identifying 54 genes that were significantly different between primary tumors and metastatic tumors, either downregulated or upregulated [65]. Based on genetic loci, and on previous genes selected in the UM GEP such as *BAP1*, and after adding four control genes (genes present in both primary and metastatic tumors), a 28-gene classifier was developed (*AQP3*, *ARG1*, *BAP1* 5' region, *BAP1* 3' region *BTG1*, *CLCA2*, *CRABP2*, *CST6*, *CXCL14*, *DSC1*, *EIF1B*, *GJA1*, *ID2*, *KRT14*, *KRT6B*, *LTA4H*, *MGP*, *PPL*, *RBM23*, *ROBO1*, *S100A8*, *S100A9*, *SAP130*, *SPPI*, *SPRR1B*, *TACSTD2*, *TRIM29*, *TYRP1*). Analysis of these genes revealed they are involved in the transition from localized melanoma to metastatic melanoma [65].

In a multicenter study involving 268 melanomas, it was shown that this assay could accurately separate melanoma into low risk (class 1) and high risk (class 2) with high accuracy [65]. Specifically in the validation cases, the disease-free survival for class 1 patients was 91% at 5 years, whereas for class 2 patients it was 31% at 5 years. In multivariate analysis comparing AJCC staging to GEP classification in determining prognosis, the hazard ratio for GEP was 9.55 when comparing class 2 to class 1 patients, compared with 5.4 for AJCC status when comparing IIC and greater patients to those with stage IIB or less disease. GEP status was also a stronger predictor of prognosis than ulceration or mitotic rate [65].

In a follow-up study of 217 patients [66], SLNB status was compared with Castle Biosciences GEP classification, showing that GEP was a stronger predictor of prognosis than SLNB status. Among the 217 patients, 58 had a positive SLNB. Thirty-two of these 58 patients had a distant metastasis

and 18 ultimately died. The positive predictive value (PPV) for metastasis was 55% and the negative predictive value (NPV) was 67%. In total, 53 SLNB-negative patients developed distant metastasis and 44 died. A total of 141 of 217 patients were classified as high risk by GEP classification. A total of 71 of these patients developed distant metastasis and 53 died of disease. The PPV for GEP testing for metastasis was 50% and the NPV was 82%. Hence, while SLNB and GEP testing had comparable PPV values, the NPV was considerably stronger for GEP testing. The overall survival at 5 years for all SLNB positive patients was 62% compared with 70% for SLNB-negative cases. The overall survival at 5 years was 55% for all cases with a class 2 GEP classification compared with 89% for all class 1 cases.

Conclusion

Currently the most important parameters to predict the prognosis of a melanoma are provided by the AJCC staging system such as the Breslow depth, the presence of ulceration and mitotic rate, the SLNB result, the extent of lymph node involvement and the presence or absence of distant metastasis. Currently, among the molecular ancillary assays, GEP has been most extensively evaluated for prognosis and offers the only commercially available prognostic assay for melanoma.

The current commercially available GEP assay for cutaneous melanoma evaluates mRNA expression of 28 genes and classifies cutaneous melanoma into a high or low risk signature depending on the expression pattern. This assay has been shown in multivariate analysis to be an independent prognostic marker with stronger predictive ability than SLNB. In combination with SLNB, over 80% of patients who eventually develop distant metastasis can be identified. It is not surprising that among the various molecular platforms, GEP has shown to be the most promising tool as a prognostic marker since it gives an overall picture of which genes are on and which genes are off. However, this test does not clearly reveal which is the specific mechanism of activation, whether it is related to an activating mutation, methylation, copy number changes or other mechanisms. Additionally, it is important to note that most of the melanomas evaluated in the development of Castle's GEP assay were of tumor stage T2 or higher. More data are needed to determine whether the assay can accurately identify the small proportion of thin melanomas which eventually result in distant metastasis

and death. Additionally prospective studies are needed to determine whether incorporating the test into clinical trials involving adjuvant therapy can affect overall survival for patients, and therefore these questions are yet to be answered.

Next-generation sequencing using cancer gene panels is also highly valuable but as a therapeutic tool. With this modality, in a single experiment it is possible to determine all the targetable mutations present in a given tumor. This information can be critical in guiding targeting inhibitor therapy.

Future perspective

In the era of personalized medicine, molecular diagnostics will play a significant role in complementing traditional staging systems. It is likely that in the future all melanomas which are beyond a given stage using conventional staging parameters will undergo further molecular prognostication. The optimal platform for this at current is likely mRNA expression profiling, since it can assess which genes are on or off independently of the mechanism. This gives a global pattern of which pathways are activated. Further advancements may also be able to predict which specific organ sites are likely to be involved by metastasis based on the pathways that are activated, and specific genetic signatures correlating with specific organ metastases may be developed in the future.

In addition to this, based on the combined conventional and molecular staging findings, patients at high risk for metastasis will likely have next-generation sequencing analysis using customized tumor panels. These panels will likely be dynamic including all the potentially targetable mutations. Protocols could then be designed based on the patients' molecular staging, likely organ sites of metastasis and targetable mutations to allow for personalized monitoring and treatment protocols and clinical trial enrollment.

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