Prognostic molecular testing in melanoma: ready for prime time?

Jennifer Keller¹, Laurence P Diggs¹ & Eddy C Hsueh^{*1}

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Prognostication of patients with cutaneous melanoma has long been dependent on clinical variables. Molecular testing has an increasingly important role in the management of malignancies. Early studies on molecular profiling of melanoma were replete with technical challenges. The early molecular profiling platforms required robust RNA and could only be obtained from frozen tissue. Thus, early studies tend to include mostly patients with advanced stages of melanoma where fresh tissues can be obtained with relative ease. With the advancement in molecular techniques such as cDNA-mediated annealing, selection, extension and ligation technique, genetic information can be reliably obtained from formalin-fixed, paraffin-embedded (FFPE) archival tissues [1]. Recent reports using whole-genome RNA profiling, miRNA profiling, gene-expression profiling (GEP) with 31-gene panel and NanoString technology have further advanced the development of molecular testing toward improving prognostication and integration in the routine management of melanoma patients.

Using global genetic profiling approach evaluating the expression of 3000 genes, Jonsson *et al.* [2] identified four molecular subtypes of melanoma: high-immune, pigmentation, proliferative and normal-like. Each molecular subtype was characterized by gene signatures representing diverse molecular mechanisms. High expression of genes involved in immunological process such as *IFNGR1*, *CXCL12* and *IL1R1* was observed in high-immune subtype. In contrast, high expression of cell-cycle associated genes such as *E2F1*, *BUB1* and *CCNA2* was noted in proliferative subtype. The pigmentation subtype was characterized by higher expression of genes involved in melanin synthesis and melanocyte differentiation, for example, *MITF*, *TYR*, *SILV*, *DCT* and *EDNRB*. High expression of genes involved in epidermis and ectoderm development was observed in normal-like subtype. There was significant difference in overall survival (OS) rates between the four subtypes, with proliferative subtype having with the shortest survival. This molecular-profiling approach

KEYWORDS

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- melanoma prognostication
- molecular testing

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¹Department of Surgery, Saint Louis University, St Louis, MO, USA

*Author for correspondence: Tel.: +1 314 577 8566; Fax: +1 314 771 1945; eddy.hsueh@health.slu.edu

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was subsequently validated in three independent cohorts [3–5]. Harbst *et al.* analyzed gene expression in archival tissues from 210 cutaneous melanoma patients and 13 mucosal melanoma patients [3]. Further refinement of the four molecular subtypes was performed to construct a binary classification into high-grade and low-grade subgroups. Significant difference in OS and relapse-free survival (RFS) among the subgroups was observed using the 4-group and 2-group classification schema. Cirenajwis *et al.* validated the prognostic utility of the 4-group molecular profiling approach in 214 cutaneous melanoma patients using archival tissues [4]. Nsengimana *et al.* validated the prognostic utility of 2-group molecular profiling approach in 300 archival tumors (224 primary melanoma and 76 metastatic melanoma) [5].

Further gene ontology analysis and mutation spectrum analysis within the context of the gene-expression phenotypes revealed wound response, immune response, DNA repair and cell cycle as important determinant biologic processes in melanoma [3–5]. *CDKN2A* alterations were more prevalent in the proliferative subtypes, and pigmentation subtype melanomas were enriched for genetic events in *CTNNB1*, *MITF* or *CCND1* [4]. Hereditary breast cancer signaling and *BRCA1* DNA damage response were two of the most significant networks associated with high-grade tumors [3]. High-grade tumors have increased level of genomic imbalance, and increased expression of *MITF*, its upstream regulator *SOX10* and its downstream targets *TYR* and *MLANA* [3]. High expression of genes such as *CCL21* and *CXCL12* is characteristic of the low-grade tumors [5].

In contrast to global gene-expression profiling approach, Gerami *et al.* evaluated a 31-gene GEP panel in cutaneous melanoma based on a selective approach identifying genes of interest from the analysis of expression data from public databases and from a clinically validated 15-gene prognostic expression profile assay for uveal melanoma [6]. Majority of the genes evaluated were located on chromosomes 1 (*CRABP2*, *TACSTD2*, *CLCA2*, *S100A9*, *SPRR1B* and *S100A8*), 6 (*GJA1* and *ARG1*), 9 (*TYRP1* and *AQP3*) and 12 (*MGP*, *KRT6B* and *BTG1*). Gene ontology analysis revealed a representation of genes involved in epithelial differentiation and development as well as genes representing cellular components such as cell–cell junction and nonmembrane bound organelle classes.

Following development phase of the assay utilizing archival tumor tissues from 107 stage I and II primary melanoma patients from three separate institutions, the assay was further refined in a training set composed of 164 stage 0–IV melanoma patients and validated in an independent 104 stage 0–IV melanoma patients. Patients were classified as either class 1 (low risk) or class 2 (high risk). The accuracy of the predictive modeling as determined by area under the receiver operating characteristic curve was 0.91 with an overall risk prediction accuracy of 83%. The gene-expression profiling classification correlated significantly with disease-free survival (DFS) and OS. Its prognostic utility was further confirmed on multivariate analysis with 5-year DFS of 97% in class 1, and 31% in class 2 validation set patients. The negative predictive value was 93% with a positive predictive value of 72%. This assay was further validated in another independent cohort of 217 melanoma patients who had undergone sentinel node biopsy [7]. In this separate validation study, archival primary melanoma tissues from 217 melanoma patients who had undergone sentinel node biopsy were evaluated with the 31-gene GEP assay. GEP classification and sentinel node status were independent prognostic variables for DFS, distant metastasis-free survival and OS. In addition, it was observed that combining GEP classification and sentinel node status further improved metastatic risk prognostication.

Over the last decade, miRNA has attracted significant interests from cancer researchers. The miRNAs are small noncoding RNAs that modulate gene expression of several key cellular processes by inhibiting protein translation and inducing mRNA degradation [8]. Because of their imperfect sequence complementarity with their mRNA targets, each miRNA has the potential to modify the expression of multiple genes. Because they are quantifiable, genome-wide and highly stable in FFPE tissues, miRNAs may serve as ideal biomarkers for cancer [9,10].

Several recent studies have demonstrated the utility of altered miRNA expression as prognostic biomarker for melanoma. Segura *et al.* reported a panel of six miRNAs (miR-150, miR-342, miR-455-3p, miR-145, miR155 and miR-497) that could predict postrecurrence survival with 80.2% accuracy rate [11]. Caramuta *et al.* proposed another panel of six miRNAs (miR-191, miR-193b, miR-365, miR-338, miR-193a and let-7i) in association with shorter survival [12].

In a study of 128 primary melanoma tissues, Satzger *et al.* reported a significant association of high miR-15b expression with RFS and OS on multivariate analyses [13]. Tembe *et al.* performed global miRNA expression profiling of 45 American Joint Commission on Cancer (AJCC) stage III metastatic melanoma patients [14]. The miRNAs: miR-142-3p, miR-142-5p, miR-146b-5p and miR-150-5p were observed to have differential expression of >1.5-fold in good prognosis compared with poor prognosis patients. Further evaluation in an independent cohort of 86 stage III and IV patients confirmed significant correlation of miR-142-5p and miR-142-3p expression with survival. Hanniford *et al.* reported that a 4-miRNA signature panel comprising of miR-150, miR-15b, miR-16-5p and miR-374b-3p, in combination with AJCC stage, was predictive of subsequent development of brain metastasis [15].

The miRNAs are thought to be shed from tumor in exosomes which would protect them from degradation by endogenous RNases [16]. Thus, they are very stable in the bloodstream. Tembe *et al.* observed an association of serum miR-142-3p expression with stage of disease [14]. Serum miR-150-5p expression correlated with survival in stage IV patients [14]. Fleming *et al.* analyzed serum levels of 12 miRNAs in 283 melanoma patients and observed miR-15b levels significantly increased over time in patients who recurred [17]. Friedman *et al.* screened 355 miRNAs using a quantitative reverse transcription-polymerase chain reaction panel in sera from 80 melanoma patients at primary diagnosis (discovery cohort) [18]. Expression of five miRNAs (miR-150, miR-15b, miR-199a-5p, miR-33a and miR-424) were significantly correlated with RFS on multivariate analysis. Further analysis separating the patients into high- and low-risk groups by using expression level cut-offs confirmed significant correlation with RFS with sensitivity of 0.84 and specificity of 0.76. This association was further confirmed in an independent validation cohort.

Recently, a unique nucleic acid bar-coding technique (NanoString®, WA, USA) has been

introduced for molecular testing. This novel technology uses unique digital color-coded barcodes that hybridize to specific nucleic acid targets and allow for direct detection and quantitation of gene transcripts in a single reaction in a highly reproducible and sensitive manner [19]. Utilizing NanoString technology, Sivendran *et al.* analyzed RNA from 40 stage II–III FFPE primary melanomas with a 53-immune gene panel [20]. The gene panel signature was significantly associated with DFS and RFS. The proposed 53-gene panel was further confirmed in an independent test population of 48 patients. The gene signature was an independent predictor of nonprogression ($p < 0.001$), RFS ($p < 0.001$) and disease-specific survival ($p = 0.024$) in the validation cohort. Multivariate logistic regression showed that the 53-gene signature score was the best predictor of progression.

The future of molecular testing utilizing techniques such as whole-genome RNA profiling, miRNA profiling, 31-gene GEP assay and NanoString technology is bright. Although the reports were based on assays performed on archival tumor samples, they have shown promising utility in risk stratification of cutaneous melanoma. Significant work remains to be done in order for molecular testing to be available on a widespread basis and for these techniques to reach their prime. Further research is needed to improve the availability and ease with which molecular testing is implemented.

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