Translation of a Circulating microRNA Signature of Melanoma to a Novel Solid-Tissue Biomarker to Improve Diagnostic Accuracy and Reproducibility.

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Abstract

Background Successful treatment of cutaneous melanoma depends on early and accurate diagnosis of clinically suspicious melanocytic skin lesions. Currently, histopathology examination of excised skin lesions is considered the 'gold standard' for diagnosis of melanoma. Multiple studies have shown the low accuracy and reproducibility of this method, underscoring the urgent need for new diagnostic tools, including disease-specific biomarkers. Previously, a 38-microRNA signature of melanoma ('Mel38') was previously identified in plasma and validated as novel circulating biomarker. In this study, Mel38 expression in solid biopsy tissue is examined to determine its ability to contribute to accurate and reproducible melanoma diagnoses.

Methods Nanostring digital gene expression profiling was used to apply the Mel38 signature in a cohort of 308 formalin fixed paraffin embedded skin biopsies ('Mel38'). Genomic data were interrogated using hierarchical clustering, univariate and multivariate statistical approaches. Mel38 classification scores (range 0 to 10) were compared to consensus histopathology results, including MPATH-DX class, AJCC disease stage, histological subtype as well as technical assay factors.

Results The Mel38 score can identify high-risk melanomas (MPATH-Dx Class IV) from less-malignant forms of the disease with an area-under-the curve of 0.96 ($P < 0.001$). The genomic score ranges from 0 to 10 and is positively correlated with the melanoma progression, from benign naevi to metastatic disease (intraclass correlation coefficient: 0.85). Using a score threshold of > 2.3 identifies higher-risk melanomas, associated with poorer outcomes and more intensive suggested clinical actions. Multivariate analysis showed the score to be a significant predictor of malignancy, independent of technical and clinical covariates. Analysis of the Mel38 signature in spitz naevi reveal an intra-subtype profile, in common to both benign and malignant conditions.

Conclusion Melanoma-specific circulating microRNAs maintain their association with malignancy when measured in the hypothesized tissue of origin. The Mel38 signature is an accurate and reproducible metric of melanoma status, based on changes in microRNA expression that occur as the disease develops and spreads. Inclusion of the Mel38 score into routine practice would give physicians a genomic assessment of a patient's disease status. Combining molecular biomarker data with conventional histopathology data may improve diagnostic accuracy, reproducibility, and patient outcomes.

Background

Over the past two decades, the annual incidence of melanoma has increased by 4–6% in fair-skinned populations of Northern Europe, North America, New Zealand and Australia[1]. Australia leads the world in melanoma incidence, with two out of three people diagnosed with some form of skin cancer by the age of 70[2]. Unfortunately, public awareness campaigns nor advances in diagnosis and therapeutics have

not translated to prolonged improvements in the age-standardised mortality rates for this cancer type in many countries[3-6].

Currently, melanocytic lesions suspected of being malignant are diagnosed almost exclusively using visual methods, including dermoscopy, followed by biopsy and histopathologic examination. Advances in detection and diagnosis have increased the incidence of melanoma diagnoses worldwide but have not reduced the mortality rate[3]. There is consensus among experts that standardisation of pathology terminology and new molecular tests are needed to improve diagnostic accuracy, patient outcomes and resource use efficiency[4, 7-11].

The MPATH-Dx structure for describing melanocytic lesions is increasingly adopted by pathologists as method to improve consistency and communication between healthcare providers [12, 13]. However, Despite this and other improvements in related areas such as dermoscopy, multiple studies into the accuracy and reproducibility of melanoma diagnosis by histopathology alone is not accurate, nor reproducible[9, 10, 14-19]. Concerningly, results from the Elmore et al study showed that 28% of MPATH-Dx Class V melanomas, i.e. those requiring the widest excision margins and consideration of lymph node screening and/or adjuvant therapy, were under-diagnosed by pathologists in the study. Reproducibility of Class V diagnoses was also shown to be challenging, with 17% of Class V lesions examined receiving a lower classification when the same diagnostic slides were re-analysed by the same pathologist.

Genomic profiling of microRNAs is a precise molecular technique that can be performed on many tissue types, including blood, urine, tears, and solid tissue (Figure 1). MicroRNAs are post-transcriptional regulators of gene expression which have tissue- and disease-specific patterns of expression[20]. Their role in melanoma oncogenesis and progression is well documented, including their crucial role in formation of the dermal tumour niche and activation of cancer associated fibroblasts through exosomal secretion[21].

Previously, a signature of 38 circulating microRNAs (‘Mel38’) was identified by genomic profiling of plasma from individuals with stage I-IV melanoma and comparison with data from non-melanoma controls. The biological functions of the Mel38 microRNAs include regulators of angiogenesis & inflammation (n=2), invasion & metastasis (n=14), immune response / treatment resistance (n=11) and tumour suppression or oncogene activation (n=8) [22, 23]. To express the information represented by the Mel38 signature in an interpretable and patient-personalised form, a gene weighting algorithm was applied to compute a classification score (i.e., Mel38 score). Mel38 scores ranges from 0 to 10 and are positively associated with increasing levels of melanoma malignancy. In multiple validation cohorts comprised of varied specimen types, the Mel38 score has shown robust and statistically significant associations with melanoma status, treatment response and prognosis[24].

In this study we perform Mel38 microRNA profiling of 308 clinically annotated, formalin fixed paraffin embedded melanocytic skin lesions, representing the most common specimen and histological subtypes analysed in skin pathology practices. The clinical and technical accuracy of the Mel38 signature is

evaluated and contrasted with equivalent metrics for conventional diagnostic approaches to demonstrate the potential benefit of including genomic profiling in the diagnostic workup of melanocytic lesions.

Patients And Methods

Sample population

This retrospective study was performed using melanocytic lesions that were submitted for diagnostic assessment (Australian Clinical Laboratories) as part of normal health care over the prior five years. Specimens were selected to represent the progression from benign naevi through to advanced invasive adult melanoma (18 years and older), including multiple common histological subtypes and disease stages (AJCC 8th Ed[25]). An additional 14 samples of spitz naevi from patients younger than 18 at the time of diagnosis were also included.

Each specimen was reviewed by two or more experienced dermatopathologists, with the majority diagnosis recorded as the result. The study was approved by Australian Clinical Labs internal medical advisory board and satisfies criteria for use of human tissue by diagnostic pathology companies as outlined by the Australian Government's National Health and Medical Research Council [26]. A study sample size of ≥300 specimens was set based on the methods described by Hajian-Tilak, assuming an AUC of 0.78 or higher with 80% power and 95% confidence[27].

RNA extraction and genomic analysis

A minimum of two unstained slides containing a total of 20µm of tissue and >=10% naevi or 20% melanoma cell content was obtained from each specimen. The reviewing pathologist indicated the area of interest for each specimen using a Hematoxylin and eosin stained slide which then guided the macro-dissected process on the unstained slides. Total RNA was extracted from deparaffinated tissue using the miRNeasy FFPE kit (QIAGEN Cat No./ID: 217504) according to manufacturer guidelines and quantified using a Nanodrop 2000 (ThermoFisher, USA). MicroRNA profiling was performed using the Nanostring nCounter Human v3 miRNA Panel with the nCounter SPRINT or nCounter MAX platform (Nanostring Inc, Seattle USA), according to the manufacturer's guidelines.

Data processing and statistical analysis

Log2 scaled raw data were adjusted for technical variation by positive control scaling and 'top 100' normalisation using the NanoStringNorm R library [28]. Normalised data were then examined for quality by comparing 10 quality metrics with predetermined internal standards, including assessments of positive and negative controls, legation efficiency controls, global mean and standard deviation and the degree of normalisation applied. A classification score ('Mel38 score') was calculated for each specimen that passed the data quality control analysis, using the support vector machine (SVM) gene weighting algorithm, described previously[23]. The SVM microRNA expression weights used to compute a Mel38

score were updated for differences between plasma and FFPE derived data by performing a partial retraining using representative subset of nevi (n=12) and invasive melanoma samples (n=12).

All data normalisation, quality control and Mel38-FFPE score calculations were performed using customised R scripts and the results stored in a relational database. Statistical analysis and visualisation were performed using Microsoft Excel and MedCalc [29, 30]. All P-values calculated are two-sided and when <0.05 were deemed to be statistically significant. Receiver operator curve (ROC) analysis was used to assess the sensitivity and specificity of the Mel38-FFPE score and to determine the optimal score threshold to classify specimens as either high or low risk of disease progression and a potentially poorer outcome[31]. Binary logistic regression was used visualise the score as a continuous predictor of malignancy.

Results

Genomic profiling of melanocytic lesions representing the spectrum of benign melanocytic lesion to metastatic melanoma.

The Mel38 signature was examined using total RNA from 308 FFPE tissue specimens with a melanocytic cellular content of 10% or greater. Patient and specimen details are summarised in Table 1. To visualise the relationship between the 38 microRNAs and specimen details, two-dimensional hierarchical clustering of the complete cohort was performed (Figure 2). This resulted in an ordering of samples corresponding to an increasing degree of cancer progression (left to right). The gradual transition in levels of relative up and down microRNA expression reflects the continuum of naevi to melanoma progression which, as commonly seen in clinical practice.

Notably, two microRNAs (hsa-mir-205 and hsa-mir-497) appear to have similarly low expression in the benign naevi and invasive/metastatic melanomas, but high expression in the early-stage invasive melanomas. These microRNAs may therefore have a specific role in aiding a melanoma cell to develop early invasive characteristics and may warrant further investigation.

The Mel38 score is positively correlated with increasing melanoma stage and is statistically significant independent to other variables.

Mel38 scores for each specimen were calculated using support vector machine derived gene weights, as previously described. The scores range from 0 to 10 and are positivity correlated with the degree of malignancy, as shown by box plots of the scores vs AJCC stage (all samples) and vs MPath-Dx classes (primary lesions only), in figure Figure 3A and 3B respectfully. The intraclass correlation of the score vs specimen status (naevi to metastatic melanoma) was 0.85.

To verify that the Mel38 score is a continuous predictor of malignancy, independent to other clinicopathological variables, general linear models (GLM) were computed using was using patient age, gender, histological subtype, and AJCC Stage or MPATH-Dx class[12]. In both models, the genomic score

was significantly different between AJCC stages and MPATH-Dx classes, independent to the other variables in the model (P<0.001).

A separate multivariate analysis of Mel38 scores from invasive melanoma specimens only (n=128) was performed, including, Breslow depth, tumour cell content, patient age, gender, and biopsy site. In this subset, the Mel38 score remained statistically significantly between disease stages (P=0.012), independent of the other variables included in the statistical model, indicating robustness to other factors known to influence microRNA expression.

The Mel38 score is a binary classifier of clinically higher- vs. lower-risk melanocytic lesions.

Whilst the Mel38 score demonstrates a statistically significant continuous association with malignancy, inspection of the MPATH-Dx vs Mel38 box plot (Figure 3B) shows that the largest difference in genomic scores occurs between MPATH-Dx Class IV and V specimens. Notable differences in suggested clinical actions and patient outcome (i.e. 5- and 10-year disease specific survival rates) are also observed between these two classes, as summarised in Table 2. Further assessment of using the Mel38 score as a binary classification tool to assign identify higher-risk (M-PATH Dx Class V) vs lower-risk (Classes I-IV) lesions was then performed.

Receiver operator curve (ROC) analysis on Mel38 scores from 74 MPATH-Dx Class V vs. 181 Class I-IV specimens resulted in area under the curve (AUC) was 0.96 (95% CI 0.92 to 0.98, P<0.001). Inspection of the ROC data showed that a Mel38 score of ≥2.3 was the optimal classification threshold for classifying a specimen as higher-risk. This threshold corresponded to a true positive rate of 95% (i.e.. Sensitivity; 95 CI: 87% to 99%) and true negative rate of 83% (i.e. specificity; CI: 77% to 89%). These performance data suggest that using Mel38 with a threshold of ≥2.3 would result in a underdiagnosis rate of 8.8% (10/113), compared to the observed rate of 27% for conventional pathology alone[14].

Mel38 profiling of Spitz naevi shows similarity to both benign and invasive melanoma.

Spitz naevi are an uncommon type of melanocytic naevus, histologically similar to melanoma and regarded as a challenging subcategory of melanocytic skin lesions to diagnose. Mel38-FFPE analysis was performed on twenty specimens of Spitz naevi submitted for routine histopathologic analysis from individuals ranging from two to thirty years old. As shown in Figure 1, the expression profile of the spitz naevi exhibits similarity to both benign and malignant lesions. Inspection of individual microRNAs in this figure reveals several that appear to have spitz-specific patterns of expression, i.e., hsa-mir-424-5p, hsa-mir-301a-3p and hsa-mir-1537-3p.

One way ANOVA of the Mel38 scores in spitz naevi vs other naevi subtypes revealed statistically significance (P<0.001), with mean scores of 2.7 (95% CI: 2.5 to 3.0) vs 1.5 (95% CI: 1.4 to 1.7), respectively, and significant pairwise differences to all naevi subtypes present. When analysed using a multivariate GLM, incorporating patient age, gender, and naevi subtype, the Mel38 scores of the spitz naevi were only significantly higher than the compound naevi subtype (P=0.015). This suggests that the

younger age of the spitz naevi patients compared to the rest of the cohort may be an influence on the Mel38 expression profile.

The Mel38 score exhibits low technical variability between replicates and in longitudinal control sample analysis.

RNA from 30 naevi and 30 melanoma samples was pooled, aliquoted into 3ul volumes and analysed over a period of 7 (non-sequential) weeks. The Mel38 scores of each pool are shown longitudinally in Figure 5A. The mean score for the naevi control pool is 2.1 (standard deviation: 0.25) and 6.5 (standard deviation: 0.15) for the invasive melanoma pool. Additional control sample data will be generated over time and monitored to assess technical noise using Levey Jennings plots.

To assess the reproducibility of the genomic score throughout its dynamic range (i.e., 0 to 10), RNA from 89 samples was re-analysed and compared to the original Mel38 score. As shown in Figure 5B, there is high consistency between replicate scores, with an intraclass correlation coefficient of 0.96, (95% CI 0.94 to 0.98) and no deviation from linearity (Cusum test, P=0.62). These results demonstrate that the Mel38 signature exhibits a high degree of longitudinal stability and technical reproducibility.

Discussion

Melanoma is a heterogeneous, progressive disease. It originates from melanocytic skin lesions and progresses through a series of well-documented stages of malignancy. In this study we describe the performance of a novel genomic biomarker for melanoma in a different specimen type (FFPE biopsy tissue) from that in which it was discovered (plasma). By comparing the 38-microRNA signature to relevant clinicopathological and specimen variables, its potential to contribute accurate and reproducible information to a patient's diagnostic picture is demonstrated.

The cohort presented in this study was designed to reflect the clinical continuum of melanoma and allowed the microRNA expression data generated to be analysed in relation to a spectrum of disease, from benign naevi to metastatic melanoma, as well as a binary condition, by classifying specimens into MPATH-Dx classes. In the former context, the Mel38 score exhibits a strong positive correlation with the progressive stages of melanoma, as represented by the ICC coefficient (0.85). When analysed as binary condition, with groups formed based on preestablished diagnostic classes with substantially different recommended follow-up actions and associated patient outcomes, a Mel38 threshold of $\geq$2.3 was able to identify higher risk disease with high sensitivity and specificity.

A selection of spitz naevi specimens were included in this study due to their histological similarity with melanoma. Despite the fact they are usually diagnosed in younger patients, misdiagnosis of melanoma as a spitz nevus is an ongoing challenge[15]. Hierarchical clustering showed the expression profile of this subtype to have similarities to both benign and malignant disease, which was also reflected by their Mel38 scores. To date very few genomic studies of spitz naevi have been performed; only 56 out of 47,333 (0.1%) of melanocytic lesion profiles in the NCBI Gene Expression Omnibus being of spitzoid

type[32]. This observation, albeit based on a limited sample size, may indicate there are a subset of spitz naevi which may benefit from additional treatment or monitoring.

Technical reproducibility of Mel38 signature was demonstrated by the low level of variation observed for both a melanoma and naevi control RNA pool, tested repeatedly over several months. Low variation and strong linearity were observed between a series of samples covering the full dynamic range of the score which were analysed in duplicate. These technical data are in line with those of other well validated genomic signatures used for individual patient management. Finally, the demonstrated reproducibility of the Mel38 score analyses exceeds that of conventional pathology based diagnoses, where almost one third of specimens may receive a different MPATH-Dx class when re-reviewed by the same pathologist[14].

Limitations of this study is the small numbers of less-common melanoma and naevi subtypes, such as amelanotic melanoma. Future work will focus on specific subtypes of melanoma and non-cancerous skin lesions which present challenges to accurately diagnose using conventional methods. A further limitation is not directly associating the genomic score to patient outcome, due to the lack access to this information and the recency of the specimens.

It is important to highlight the proposed use of Mel38 is as a complementary molecular biomarker to conventional histopathology, and is not intended to replace any current practices. Genomic testing, microscopic and immunohistochemical assessments of cellular/tissue structure all add unique and sometimes overlapping data points that contribute to a complete diagnostic picture. Incorporating genomic profiling into diagnostic workups may be useful for understanding future clinical events or treatment reactions that are inconsistent with the original diagnosis and stage of disease. Combining diagnostic modalities is becoming standard of care for many cancer types including breast (eg. Oncotype DX, MammaPrint, EndoPredict) and prostate (Oncotype DX, Prolaris, ProstaVysion), both of which have seen promising reductions in mortality over recent years, in part due to increased precision in diagnosis and molecular disease subtyping. Clinical adaptation of Mel38-FFPE has the potential to add novel, robust and personalised genomic information to the diagnostic picture of patients with clinically suspicious melanocytic lesions.

Conclusion

Successful treatment of melanoma begins with accurate evaluation of melanocytic skin lesions. The consequences of over-or under-diagnosis could be avoided by using complementary diagnostic techniques. The Mel38 genomic signature, originally discovered in plasma and now validated in solid tissue, demonstrates a high degree of clinical accuracy and technical reproducibility. These factors make it suitable for use as an adjunct diagnostic biomarker in the clinical setting. By providing physicians with the opportunity to offer patients a genomic ‘second opinion’ of an excised lesion, it is hoped that diagnostic precision and accuracy can be improved and a greater understanding of genotype/phenotype differences in melanoma can be gained.

Abbreviations

Mel38: 38 microRNA signature.

GLM: General linear model.

FFPE: Formalin fixed paraffin embedded.

CI: Confidence Interval

ROC: Receiver Operator Curve

AUC: Area Under the Curve

Declarations

Source of funding:

Institutional funding.

Disclosures:

Author abbreviations

Ryan Van Laar: RVL, Samuel King: SK, Amicel Baynosa: AB, Richard McCoy: RM, Mirette Saad: MS, Sian Fereday: SF, Ingrid Winship: IW, Catherine Uzzell: CU, Anthony Landgren: AL.

Ethics approval and consent to participate.

The study was approved by Australian Clinical Labs internal medical advisory board and satisfies criteria for use of human tissue by diagnostic pathology companies as outlined by the Australian Government's National Health and Medical Research Council.

Consent for publication

N/A

Availability of data and materials

Data available upon request

Declaration of interest

RVL: Employee and shareholder in Geneseq Biosciences.

IW: Board Member and Shareholder in Geneseq Biosciences

SF: Consultant to Geneseq Biosciences

SK, RM, MS, CU, AL: Employee of Australian Clinical Labs

Funding

NA

Authors' contributions

Study design and management: RVL, MS, IW, CU, AL

Laboratory work: SK, AM, RM

Pathology review: CU, AL

Data analysis: RVL, SF

Manuscript drafting and review: All.

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Tables

Table 1: Patient and specimen demographics

Descriptor	Number	Percent
Age:		
Mean	60	
<30	19	7%
31-40	37	13%
41-50	34	12%
51-60	41	14%
>60	157	54%
Gender		
Male:	154	53%
Female:	134	47%
Naevi		
Superficial spreading	1	1%
Intradermal	16	17%
Compound	37	40%
Junctional	16	17%
Spitz	20	22%
Other	3	3%
Melanoma in-situ		
Lentigo maligna	33	69%
Superficial spreading	6	12%
Other	9	19%
Invasive melanoma		
Superficial spreading	84	50%
Nodular	29	17%
Lentigo maligna	8	5%
Other	46	28%
AJCC Clinical Stage:		
IA	58	35%
IB	19	11%
IIA	17	10%
IIB	21	13%
IIC	13	8%
III/IV	39	23%
MPATH-Dx Class		
I	33	11%
II	44	15%
III	50	17%
IV	54	18%
V	113	38%

*Table 2: MPATH-Dx classes, mean Mel38 scores and associated clinicopathological variables, including suggested clinical actions. *Melanoma specific survival figures based on AJCC melanoma staging and outcome data corresponding to the T-stage associated with each MPATH-Dx class. [#]Suggested clinical actions assume representative sampling and are reproduced from Piepkorn et al [12]*

MPATH-Dx Class:	I	II	III	IV	V
Mel38 score (mean)	1.5	1.7	1.7	2.1	5.2
Mel38 risk group	Low risk				High Risk
T stage	N/A	N/A	0	T1a	T1b or more
Perceived risk for progression	Very low risk	Low risk	Higher risk	Substantial risk for local/regional progression	Greatest risk for regional and/or distant metastases
5 /10-year melanoma-specific survival*	100% / 100%	100% / 100%	100% / 100%	98% / 96%	≤93% / ≤89%
Suggested clinical action#:	No further treatment / follow up as required.	Narrow but complete excision (<5mm).	Complete excision with at least 5mm but <10mm margins.	Wide local excision with ≥10mm margins.	Wide local excision with ≥10mm margins. Consideration of sentinel lymph node biopsy, adjuvant therapy.
Clinical action likely to require referral:	No				Yes

Figures

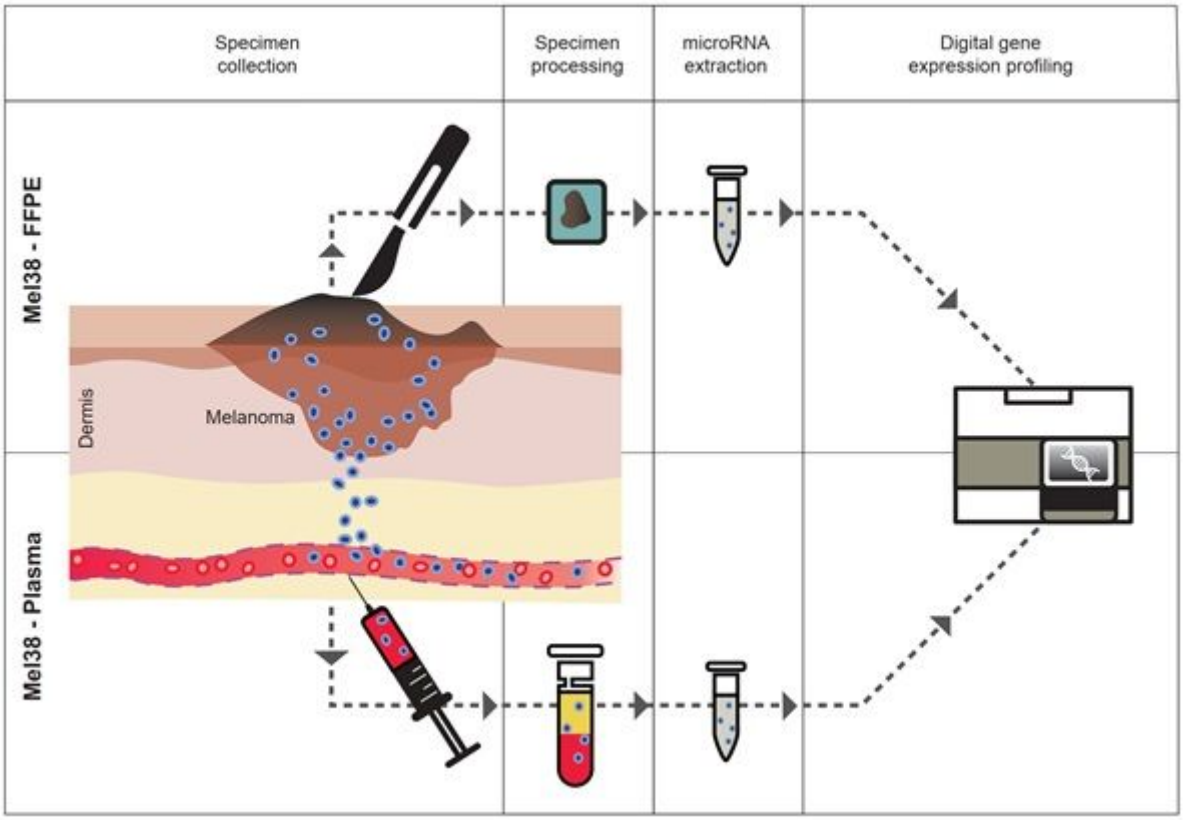

Figure 1

Schematic diagram of the development of Mel38, a microRNA signature of melanoma originally identified in plasma (lower section), and its adaptation to FFPE melanocytic skin lesions (upper section) using digital gene expression profiling methods and classification algorithms previously described.

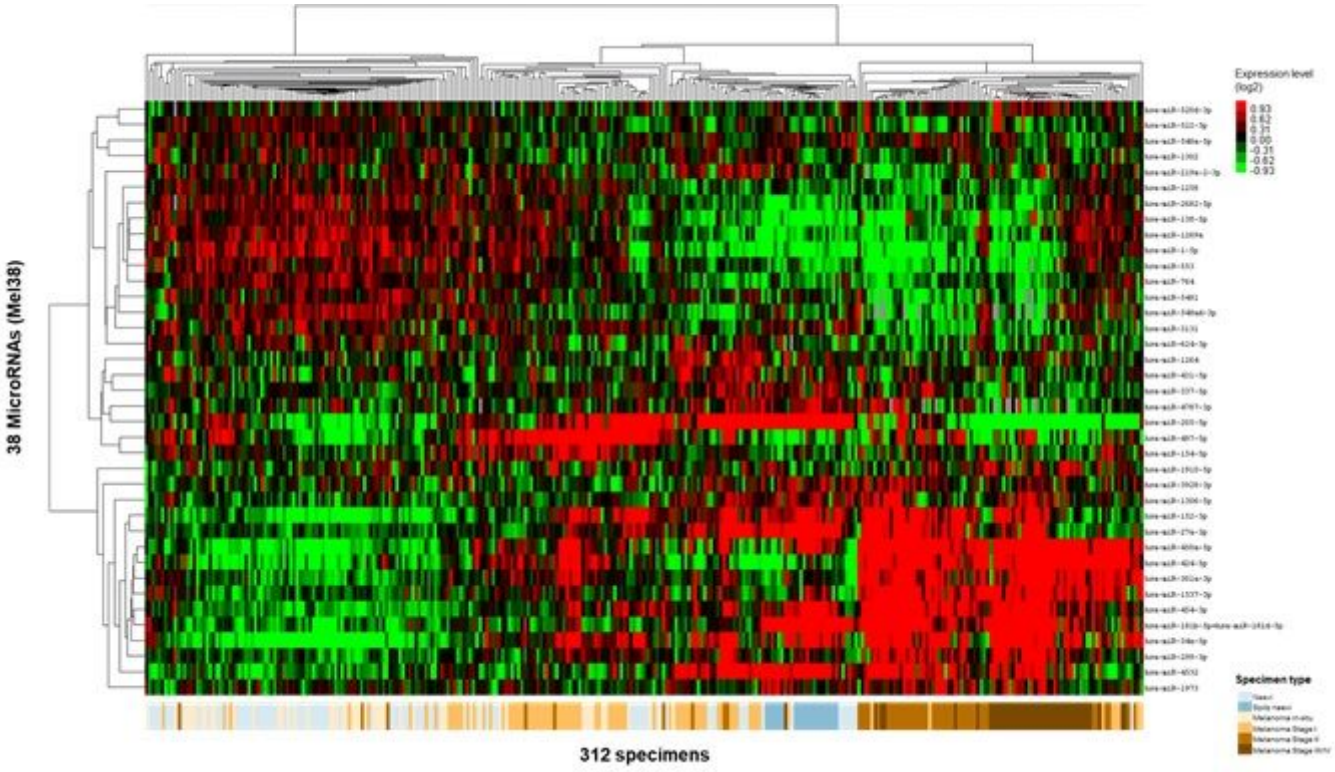

Figure 2

Supervised hierarchical clustering of the Mel38 signature data from 308 FFPE specimens of benign naevi, melanoma in-situ and invasive melanoma. The clustering pattern revealed shows blocks of up and down regulation, reflecting the raw data used by the Mel38-FFPE classifier to compute each patient's individual Mel38-FFPE score.

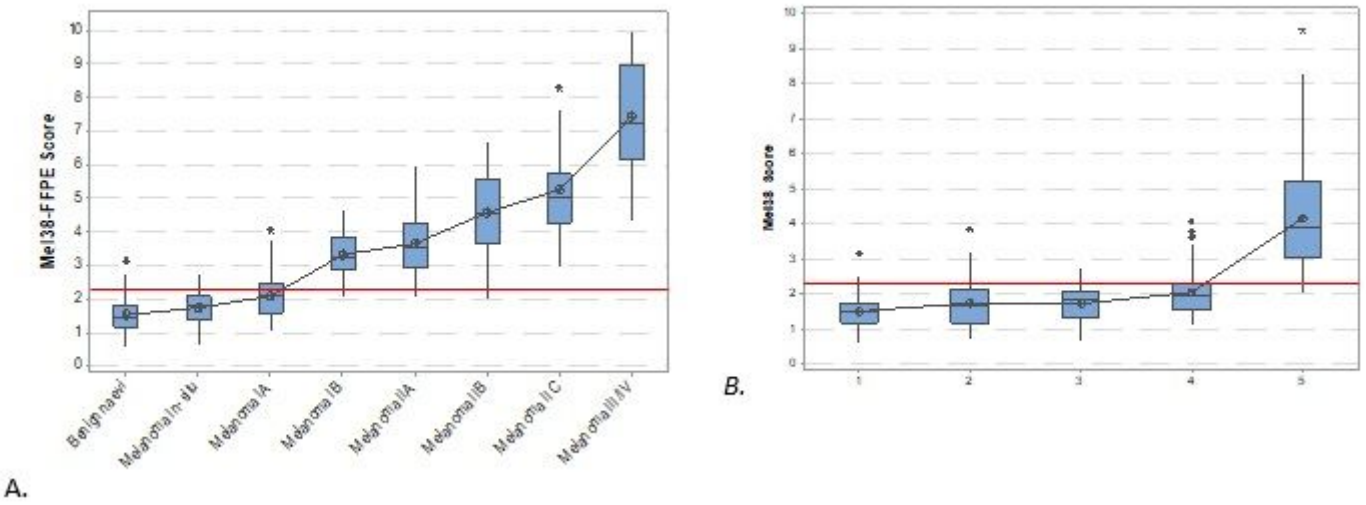

Figure 3

(A) Box plot of Mel38-FFPE scores grouped by AJCC clinical stage (8th ed). Connecting lines between boxes correspond to mean values. (B) Box plot of Mel38 scores of primary melanocytic lesions grouped by MPATH-Dx melanoma class.

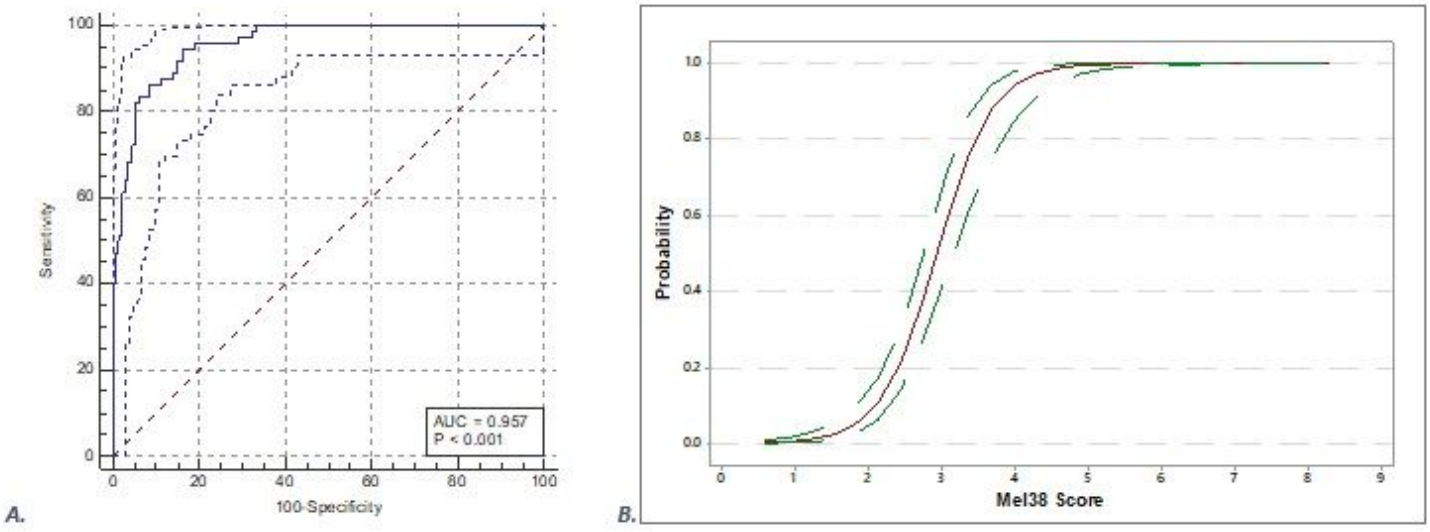

Figure 4

(A) Receiver operator curve analysis of Mel38 scores from 252 primary melanocytic skin lesions (range 0 to 10) vs. histopathology diagnosis (i.e. MPATH-Dx Classes I- IV vs. Class 5). 95%CI: 0.93 to 0.98. (B)

Binary fitted line plot from logistic regression analysis of Mel38 scores indicating the relationship between the genomic score and the corresponding probability of MPATH-Dx Class V melanoma.

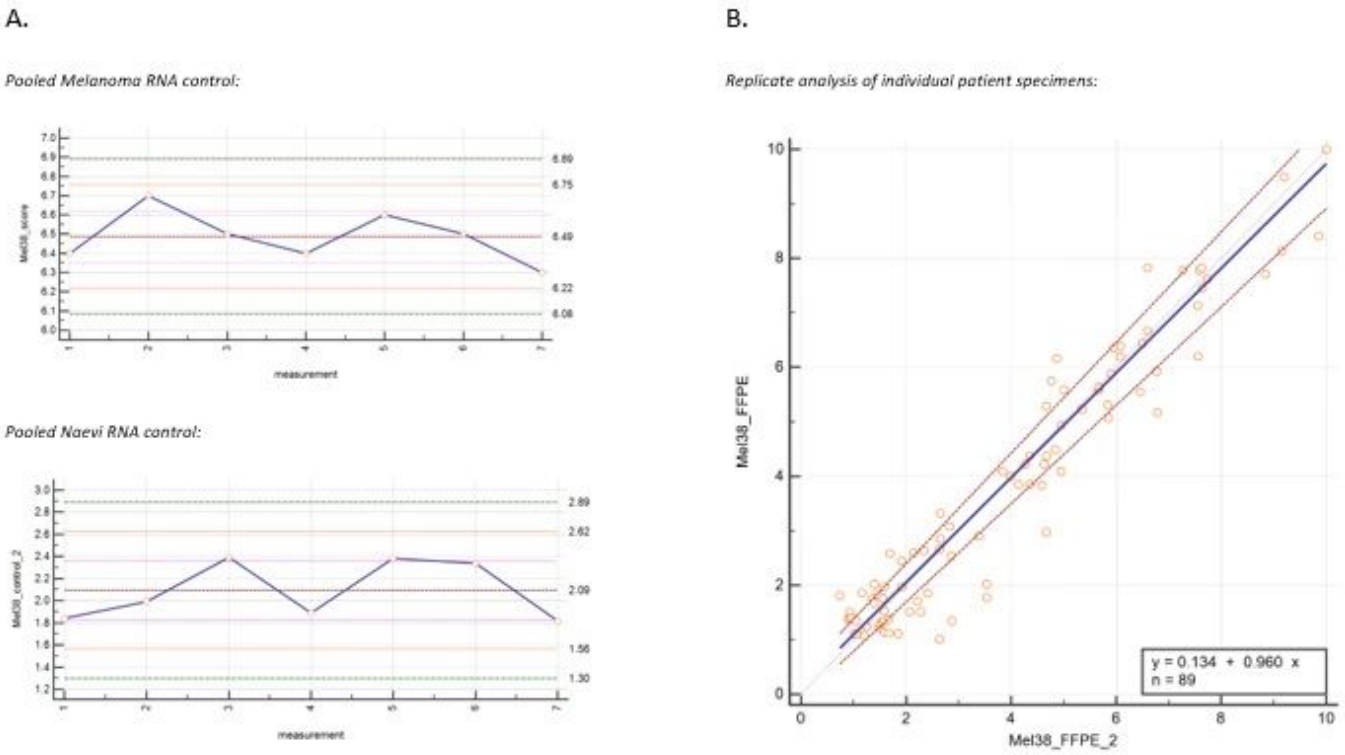

Figure 5

(A) Longitudinal analysis of control sample Mel38 scores. Dashed horizontal lines corresponding to 1s, 2s and 3s Westguard rules indicated[33]. (B) Passing and Bablok regression of replicate Mel38-FFPE specimens. No significant deviation from linearity is detected (Cusom test P=0.62) and intraclass correlation of 0.96 (0.94 to 0.98) reflects the signatures high degree of reproducibility.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.pdf