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Identification of gene expression levels in primary melanoma associated with clinically meaningful characteristics

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Summary

Factors influencing melanoma survival include sex, age, clinical stage, lymph node involvement, as well as Breslow thickness, presence of tumor infiltrating lymphocytes based on histological analysis of primary melanoma (TILs), mitotic rate, and ulceration. Identification of genes whose expression in primary tumors are associated with these key tumor/patient characteristics can shed light on molecular mechanisms of melanoma survival. Here we show results from a gene expression analysis of formalin-fixed, paraffin embedded (FFPE) primary melanomas with extensive clinical annotation. The Cancer Genome Atlas (TCGA) data on primary melanomas were used for validation of nominally significant associations. We identified 5 genes that were significantly associated with the presence of TILs in the joint analysis after adjustment for multiple testing: *IL1R2*, *PPL*, *PLA2G3*, *RASAL1*, and *SGK2*. We also identified two genes

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Conflict of interest

There is no conflict of interest for any of the authors.

significantly associated with melanoma metastasis to regional lymph nodes (*PIK3CG* and *IL2RA*), and two genes significantly associated with sex (*KDM5C* and *KDM6A*). We found that *LEF1* was significantly associated with Breslow thickness and *CCNA2* and *UBE2T* with mitosis. *RAD50* was the gene most significantly associated with survival, with a higher level of expression associated with worse survival.

Keywords

cutaneous melanoma; primary tumor; FFPE

Introduction

Gene expression profiling in tumor samples is a powerful tool for understanding how genetic factors influence tumor morphology and progression [1–3]. However, profiling of primary melanomas is challenging because primary melanoma tumors are relatively small, and for lesions that are heavily or moderately pigmented, melanin can inhibit some downstream applications [4]. In addition, nucleic acids obtained from archived formalin-fixed paraffin – embedded (FFPE) samples are usually fragmented and chemically modified by formaldehyde due to fixation and embedding conditions, and DNA or RNA from FFPE samples are often of a lower molecular weight than those obtained from fresh or frozen samples.[5] Because of these challenges, gene expression studies on primary melanomas are limited. Xu, *et. al.* [6] analyzed gene expression in 31 primary melanomas and identified a 150 gene signature associated with survival. Another study reported on a number of genes differentially expressed between 45 primary melanomas and 18 benign skin nevi [7]. Riker, *et. al.* compared gene expression in 16 primary and 40 metastatic melanomas and identified genes associated with metastases [8]. Several other studies focused on developing a prognostic signature based on gene expression in primary tumors with a rather limited number of samples [7–12]. Even though gene expression data generated by those studies are publicly available, the clinicopathologic characteristics of the primary melanomas are not described. Jönsson et al [13] conducted unsupervised hierarchical clustering of global gene expression data from stage IV metastatic melanomas in 57 patients. The authors identified four melanoma subtypes characterized by expression of immune response, pigmentation differentiation, proliferation, or stromal composition genes. Those subtypes were later recapitulated in primary tumors [14,15]. Jewel et al. [16] used 502-gene cancer panel to identify genes associated with ulceration of primary melanomas.

The Cancer Genome Atlas (TCGA) comprises the largest series of melanomas, with 103 primary Skin Cutaneous Melanoma (SKCM) samples; however, gene expression data are available for only 43 of them. Despite incomplete clinical annotation of SKCM, we used TCGA SKCM samples for *in-silico* validation of findings from discovery analyses performed on primary melanoma samples we collected.

The goal of this analysis was to identify genes whose expression level in primary melanomas correlates with clinically relevant characteristics and validate the associations using TCGA data. This research represents a pilot phase of the InterMEL-consortium, which

is seeking to identify biomarker signatures that predict recurrence from primary, early stage cutaneous melanomas. The clinical characteristics examined included sex, presence of tumor infiltrating lymphocytes based on histologic analysis of primary melanoma (TILs), Breslow thickness, tumor mitotic rate (TMR), lymph node status, and ulceration, and were selected because of their association with clinical outcomes. Numerous studies, beginning over 50 years ago, have shown that melanoma mortality is higher in men than women [17–20]. The presence of TILs within a melanoma can predict both survival and response to treatment in melanoma [21]. Breslow's thickness describes how deeply tumor cells have invaded the skin and is an important prognostic factor in melanoma survival [22]. Tumor mitotic rate is defined as the number of mitoses/mm^2 and is a predictor of melanoma survival, with a higher TMR associated with poorer survival [23]. Melanoma involvement in at least one lymph node is an important predictor of survival and is also used in melanoma staging [24]. Finally, the five-year survival rate is lower in ulcerated stage I and II melanomas as compared to non-ulcerated melanomas of the same stage [25].

Methods

Patient population

Tumors from two institutions were used with IRB approval for exempt studies (Exemption 4, no identifiers and patients deceased): Case Western Reserve University (n = 16) and the University of New Mexico (n = 18) provided the samples that were used in this study. Table 5 provides the clinical description of samples. The table includes 32 samples after 2 samples were removed due to QC issues and low RNA concentration.

Clinical characteristics

We abstracted the following characteristics of tumors/patients from medical records, including sex, TILs in primary melanoma, Breslow's thickness, TMR, lymph node status, and ulceration.

RNA extraction

Ten sections, five- or ten microns each, were obtained from archived FFPE tissue blocks, placed on uncharged glass slides, and shipped to the Molecular Epidemiology Laboratory at Memorial Sloan Kettering Cancer Center. Tissue sections were kept at 4ºC while awaiting nucleic acid extraction. Hematoxylin-eosin stained sections were evaluated by a dermatopathologist to confirm the presence of melanoma, determine the histologic features and tumor purity, and guide the macro and micro-dissection. RNA and DNA were extracted with the ALLPrep DNA/RNA FFPE kit (Qiagen) using the Qiagen deparaffinization solution, and manufacturer's recommendations. The RNA quantities and A260/280, A260/230 ratios (to examine presence of proteins or organic solvents) were determined with a Nanodrop 8000 (Thermo Scientific). The RNA quality was assessed with a TapeStation 2200 (Agilent).

Selection of genes and assessment of gene expression

Tumor-derived RNA samples were profiled using NanoString nCounter custom codesets. This involved the use of a digital color-coded barcode technology that enabled us to measure

expression of a number of candidate genes with a high level of precision and sensitivity, and without the interference of melanin present in pigmented tumors. For this, a set of 760 genes were selected for inclusion in the gene expression analysis, based on published evidence of their association with any aspects of melanoma initiation, progression, or response to treatment. Forty housekeeping genes were included as internal controls. The complete list of the gene used in this analysis can be found in Supplementary Material (Table S1).

Expression data normalization and robustness

For each sample, the geometric mean of each of the positive controls was calculated with the nSolver software analysis tool to estimate the overall assay efficiency. Using nSolver software, raw data were first normalized using the average of the geometric means of six expression sequences not present in any known organism that are spiked into the samples and serve as positive controls. Counts were normalized for all target RNAs in all samples based on the positive control RNA to account for differences in hybridization efficiency and post-hybridization processing, including purification and immobilization of complexes (one sample outside the range of 0.3–3 was removed). Housekeeping genes were then removed if the mean intensity was less than two standard deviations above the mean of the negative controls (six random sequences not present in any organism and not spiked into the samples) or if the coefficient of variation of the intensity of each housekeeping gene divided by the geometric mean of all housekeeping genes for each sample was greater than 90% (geNorm algorithm [40]). Subsequently, the mRNA content normalization was performed using the average of the geometric means of the acceptable housekeeping genes as a scaling factor for the endogenous targets. There were no samples with scaling factors outside the range of 0.10–10.

TCGA samples

Clinical and gene expression data were downloaded from the TCGA data portal. Gene expression data in the TCGA were available for 43 primary melanoma samples. A total of 753 out of the original 760 genes assessed in the pilot InterMEL study were also reported in TCGA. None of these genes were nominally significantly associated with any clinical features studied in the discovery analysis.

Survival analysis

Patients were treated by standard therapy including surgery and chemo- or radiation therapy. We used Kaplan-Meier analysis (log-rank test) to compare subjects with high (≥ median) versus low (< median) level of expression by overall survival. Each gene was analyzed separately.

To test relevance of the results of the analysis of primary melanomas to metastatic melanomas, we identified differentially expressed genes between LN-positive versus LN-negative primary tumors. A total of 14 genes that were significant in both InterMEL and TCGA samples have been analyzed: *PIK3CG, PLCG2, TLR4, CASP8, ITGA8, IL2RB, FLT1, RAC2, NTRK1, IL2RA, IL7R, JAK2, RASGRP1*, and *HSP90B1*. All these genes were downregulated in LN-positive samples. The logarithm of the average expression across the genes was used as a score to predict survival. The median score was used to stratify cases

into high or low average expression. Metastatic (total 460) TCGA samples and metastatic (total 214) samples from the GEO GSE65904 dataset [41] were used in the analysis. In GSE65904 gene expression was measured by the Illumina HumanHT-12 microarray platform (48,107 probes). Probe expressions were converted into gene expressions by choosing the probe with the largest average expression across all samples.

Statistical analysis

Our hypothesis was that gene expression in primary melanomas is associated with clinically relevant tumor and/or patient characteristics. In univariate analyses, we used nonparametric correlation to detect associations of clinically relevant characteristics with TILs, Breslow's thickness, and TMR. Associations of the gene expression level with binary characteristics (sex, lymph node status, and ulceration status) were assessed using t-statistics. The Benjamini–Hochberg false discovery rate (FDR) method was used to adjust for multiple testing [42]. We separated our analysis into discovery using primary data from the pilot InterMEL samples, and a validation sample, using an in-silico analysis of SKCM TCGA sample expression and clinical data. Associations nominally significant in the discovery analysis were used in the validation analysis. Statistical significance in the validation analysis was decided based on the number of tests in the validation phase. We then performed a combined analysis of both samples by using inverse variance meta-analysis of pilot InterMEL and TCGA results.

For multivariate analysis we have used least absolute shrinkage and selection operator (LASSO) implemented in STATISTICA (StatSoft). Logistic (for binary outcome) or linear (for continuous outcome) regressions were used. Tuning parameter lambda was selected to make number of non-zero beta coefficients not exceeding the number of predictors in univariate analysis with liberal FDR<0.2.

We used gene set enrichment analysis (GSEA) [43] to identity biological functions enriched by the genes up or downregulated in the given phenotype – differentially expressed genes. We used curated hallmark GSEA gene sets. Gene sets with FDR <0.05 were considered to be significant.

Results

Univariate analysis

Of the 34 tested InterMEL-pilot RNA samples, one failed QC and one had insufficient RNA, resulting in 32 evaluable samples used in this study.

Genes differentially expressed by sex

We identified 30 genes differentially expressed between male and female patients. The list of the genes with the corresponding P-values for sex difference can be found in supplementary materials (Table S2). For two genes, *KDM5C* and *KDM6A*, the expression was much higher in female compared to male patients. The differences for these two genes remained significant after the adjustment for multiple testing: p=0.01 and p=0.02, correspondingly.

Those are the only genes that were reproduced by the analysis of TCGA data. Figure 1a shows the heat map of the genes nominally associated with sex.

Genes associated with tumor-infiltrating lymphocytes

TILs were coded as ‘0”, “1” and “2” for absent, nonbrisk, and brisk TILs, correspondingly. For each gene we calculated the Spearman’s correlation coefficient between the gene expression and TILs. We identified 110 genes nominally associated with TILs (Supplementary Table S3). None of these associations remained statistically significant after adjustment for multiple testing. Twenty-four of the nominally significant genes identified in the InterMEL-pilot were also significantly associated with TILs in TCGA: *AQP3, CBLC, CEBPA, CRABP2, EGFR, ERBB2, FLT3, IL1B, IL1R2, IL7R, ITGB4, JAG2, LAMA3, LAMB3, LAMC2, PBX1, PLA2G3, PLA2G4F, PPL, PRDM1, RASAL1, SFN, SGK2, and WNT11*. For the associations that were common to both datasets, the direction of the effect (positive or negative) was the same. In the joint analysis five genes remained significant after the adjusting for multiple testing: *IL1R2, PPL*, *PLA2G3, RASAL1, and SGK2*. Figure 1b shows the heat map of the genes nominally associated with TILs status.

Lymph node status

We identified 131 genes associated with lymph node status coded as LN-positive versus LN-negative. The list of the genes can be found in Supplementary Table S4. We found that 15 genes, *PIK3CG, PLCG2, TLR4, CASP8, ITGA8, IL2RB, FLT1, RAC2, NTRK1, IL2RA, IL7R, ALKBH3, JAK2, RASGRP1*, and *HSP90B1*, were also nominally significant in TCGA. *PIK3CG* expression remained statistically significant in the validation set after the adjustment for multiple testing and *IL2RA*remained statistically significant in the joint analysis of pilot and validation test after the adjustment for multiple testing. Figure 1c shows the heat map of the genes nominally associated with LN status.

Ulceration

Thirty-one genes were detected as nominally statistically significant for an association of their expression with ulceration status, coded as present versus absent (Table S5). Only one gene, *IL23A*, was also nominally significant in TCGA (t=2.4, P=0.02). The association was not significant after the adjustment for multiple testing. Figure 1d shows the heat map of the genes nominally associated with ulceration status.

Breslow thickness

We identified 69 nominally significant genes (Supplementary Table S6), none of which remained significant after the adjustment for multiple testing. Five of them, *HSP90B1, ACVR1C, LEF1, FGF17*, and *VEGFA*, were also significant in TCGA. The sign of the association was the same in all genes except one, *FGF17,* which was negative in the discovery (Correlation Coefficient (CC)=−0.39, P=0.03) and positive in the validation (TCGA) set (CC=0.34, P=0.03). *LEF1* remained significant in the validation set after adjustment for multiple testing. No finding remained significant in the joint analysis.

Tumor mitotic rate

We identified 76 genes nominally associated with TMR. The genes are shown in Supplementary Table S7. Six genes nominally significant in the pilot InterMEL study, *MSH6, RAD51, CCNA2, SUV39H2, CDC6*, and *MSH2*, were also nominally significant in TCGA. For all genes the sign of association was the same in the both datasets. *CCNA2* and *UBE2T* remained significant in the joint analysis after the adjustment for multiple testing.

Clinical stage

We coded clinical stages starting from IIA, IIB, IIC, IIIA, IIIB, IIIC, and IV as consecutive integers and looked at the correlation with expression of each gene. We have identified 13 genes nominally associated with stage (Table S8). None of them remained significant after adjustment for multiple testing or was detected even as nominally significant in TCGA sample.

Survival analysis

A total of 31 nominally significant genes were identified (Supplementary Table S9). None of the genes were significant after adjustment for multiple testing. We did not use TCGA SKCM dataset for validation because survival data were available for only two out of 53 primary melanoma samples. Supplementary Figure S1 shows survival curves for *RAD50* gene, with low expression having a better survival, and for TNFRSF10A gene, with low expression having worse survival.

We found that the average expression of 14 genes in metastatic melanomas predicts survival (Fig. 2). Those 14 genes were identified based on the analysis of primary melanomas (comparison of LN-positive *vs* LN-negative cases).

Overall correlation of differentially expressed genes between pilot InterMEL and TCGA samples

The sample size in this analysis is relatively small, which leads to insufficient statistical power to detect small associations. On the other hand, the large number of genes (and statistical tests) may lead to false positives. To deal with these issues we used TCGA samples for independent validation. True positives are expected to have similar patterns of differential expression in both samples; that is, true positives are expected to be consistent between the discovery (pilot InterMEL) and the validation (TCGA SKCM) samples, while false positives will be uncorrelated. As a measure of differential expression of the gene, we used t-statistics for categorical variables and correlation coefficient for quantitative traits. We looked at parallelism of differential expression in discovery and validation samples. For example, we used t-statistics for gender differences for each of 753 genes. We estimated correlation of t-statistics between the pilot InterMEL and the TCGA samples to estimate the overall consistency between the discovery and the validation samples (Table 1). The strongest overall consistency between pilot InterMEL and TCGA samples was detected for TILs, followed by Breslow's thickness, lymph node status, ulceration status, and clinical stage. No significant associations were detected for TMR or sex. The TCGA sample size was very small for TMR, only being reported for five patients, which may explain why there was no association identified.

Least absolute shrinkage and selection operator (LASSO) analysis

Table 2 shows genes with β≠0 in LASSO analysis. The number of significant genes varies from two for clinical stage to 20 for TILs.

Gene set enrichment analysis

The results of the gene set enrichment analysis (GSEA) are shown in Table 3. Supplementary figure S2 shows an example of a distribution of the genes that are up- (upper panel) or down-regulated (lower panel) in a given pathway. The G2M_CHECKPOINT was the most frequently associated pathway; it was significantly associated with LN status, ulceration status, Breslow thickness, tumor mitotic rate and clinical stage. Tumor mitotic rate is the phenotype with the largest number (six) of associated functional categories. Table 4 shows genes contributing to the enrichment of gene sets listed in Table 3.

Discussion

Our goal was to demonstrate an approach for investigating associations between gene expression in primary melanomas and patient characteristics that are relevant for melanoma progression and survival and may be used to develop a reliable molecular prognostic signature based on gene expression.

We identified two genes in the InterMEL and TCGA samples that were statistically significantly associated with sex after the adjustment for multiple testing: *KDM5C* and *KDM6A*. Both genes are located on the X chromosome in relative proximity to each other (Xp11.3 and Xp11.22, respectively). The gene expression levels for these two genes are about twice as high in female as compared to male patients, suggesting that incomplete inactivation of the X chromosome might contribute to the observed differences in expression levels. *KDM5C* (Lysine Demethylase 5C) is involved in the regulation of transcription and chromatin remodeling, and *KDM6A* (Lysine Demethylase 6A) catalyzes the demethylation of tri/dimethylated histone H3. Although the relevance of these genes in relation to sex differences and melanoma survival is not clear, *KDM5C* mutations have been reported in association with recurrence and survival in renal cell carcinoma [26]. *KDM6A* plays a role in transcriptional regulation in cancer including bladder and pancreatic cancer [27,28].

We have identified five genes significantly associated with quantity of lymphocytes in primary tumors assessed as TILs. Only two of them, *IL1R2* and *PPL*, have evidence supporting an association with immune response. The expression level of *LEF1* was positively associated with Breslow thickness. *LEF1* plays an important role in regulation of growth and differentiation of melanocytes and melanoma [29]. It may influence melanoma development through Wnt/β-catenin signaling pathway in melanoma epithelial-to-mesenchymal-like transition [30]. Two genes found to be significantly associated with lymph node status, *PIK3CG* and *IL2RA*, have been shown to play important roles in melanoma development through regulation of tumor cell adhesion and invasion and immune response [31].

The association of *CCNA2* with tumor mitotic rate also remained significant after the adjustment for multiple testing; the expression of this gene positively correlates with mitotic

rate. *CCNA2* controls both the G1/S and the G2/M transition in the cell cycle. It was identified as a prognostic marker for melanoma survival [32]. *CCNA2* has been shown to induce autophagy in melanoma cells [33]. Ubiquitin Conjugating Enzyme E2 T (*UBE2T*) gene was also significantly associated with TMR in InterMEL sample after adjustment for multiple testing. The expression level of the gene in tumor was shown positively correlates with cell proliferation and tumor progression in different types of cancer [34,35].

Surprisingly, we did not detect genes whose expression level in primary melanomas was significantly associated with clinical stage. One of the possible explanations may be that selected genes do not show *gradual* changes in expression but rather may have stage specific differences that we cannot detect because of the limited sample size (the number of patients for any specific stage is between 1 and 6 in our samples). In addition, stage at diagnosis does not necessarily relate to tumor aggressiveness which we would not necessarily hypothesize is related to tumor gene expression.

The results of the LASSO regression are consistent with the results of univariate analysis in that if the number of associated genes was high for a given phenotype, the number of significant genes was also high in the LASSO analysis. However, because of a relatively small sample size, we consider the results of the multivariate regression analyses as preliminary.

The most significant gene detected in survival analysis was *RAD50*. We found that low expression of *RAD50* in primary melanoma is associated with a better survival. This finding is consistent with reported studies where low expression level (or mutational alterations) of *RAD50* was associated with a better survival in breast [36], colorectal [37], and gastric [38] cancers [36–38]. Together with our findings, the results suggest that the association between somatic alterations of *RAD50* may be of a pan-cancer nature and likely reflect modifications of basic biological functions, e.g. genomic instability [39].

In GSEA we have identified a number of biological functions that remained significant after correction for multiple testing. In the majority of cases identified functions were logically associated with phenotypes. Examples include “interferon gamma response” associated with TILs, “G2M checkpoint” associated with tumor mitotic rate and “TNFA signaling via NFKB” associated with ulceration status. Surprising findings include “epithelial mesenchymal transition “ associated with sex and “estrogen response” associated with TILs. These findings need to be validated on a larger dataset to be generated by InterMEL project.

Our study had a number of strengths, including analysis of gene expression with respect to many clinically relevant factors in a modestly sized cohort of samples and, importantly, many of our findings were further substantiated using publically available TCGA data. Further, our proof of principle study shows the feasibility of extracting high quality RNA from FFPE samples of SKCM, many of which were over a decade old with 90% of the samples being of sufficient quality for large scale expression analyses. However, our study is not without limitations. We used targeted analysis of preselected genes and this approach may have omitted some important signals from genes that were not included in the analysis. The reason we used a candidate- instead of a whole genome approach is because of the

inherent limitations of archived primary melanomas. Namely, the amount and the quality of the extracted RNA, and the presence of melanin in some cases, precludes us from using other platforms. On the other hand, the amount and quality of the tissue-derived RNA presented no limitations with the Nanostring platform. In addition, we only used samples from patients with stage II and higher melanoma, and thus our findings may not be relevant to stage I melanoma patients.

Conclusion

In conclusion, we show that the expression of several genes in InterMEL and SKCM is associated with clinically relevant tumor features as well as patient characteristics. Findings from this study might suggest targets for future drug development to treat melanoma. Future studies will be needed to validate these, as well as assess their association with other variables, such as patient survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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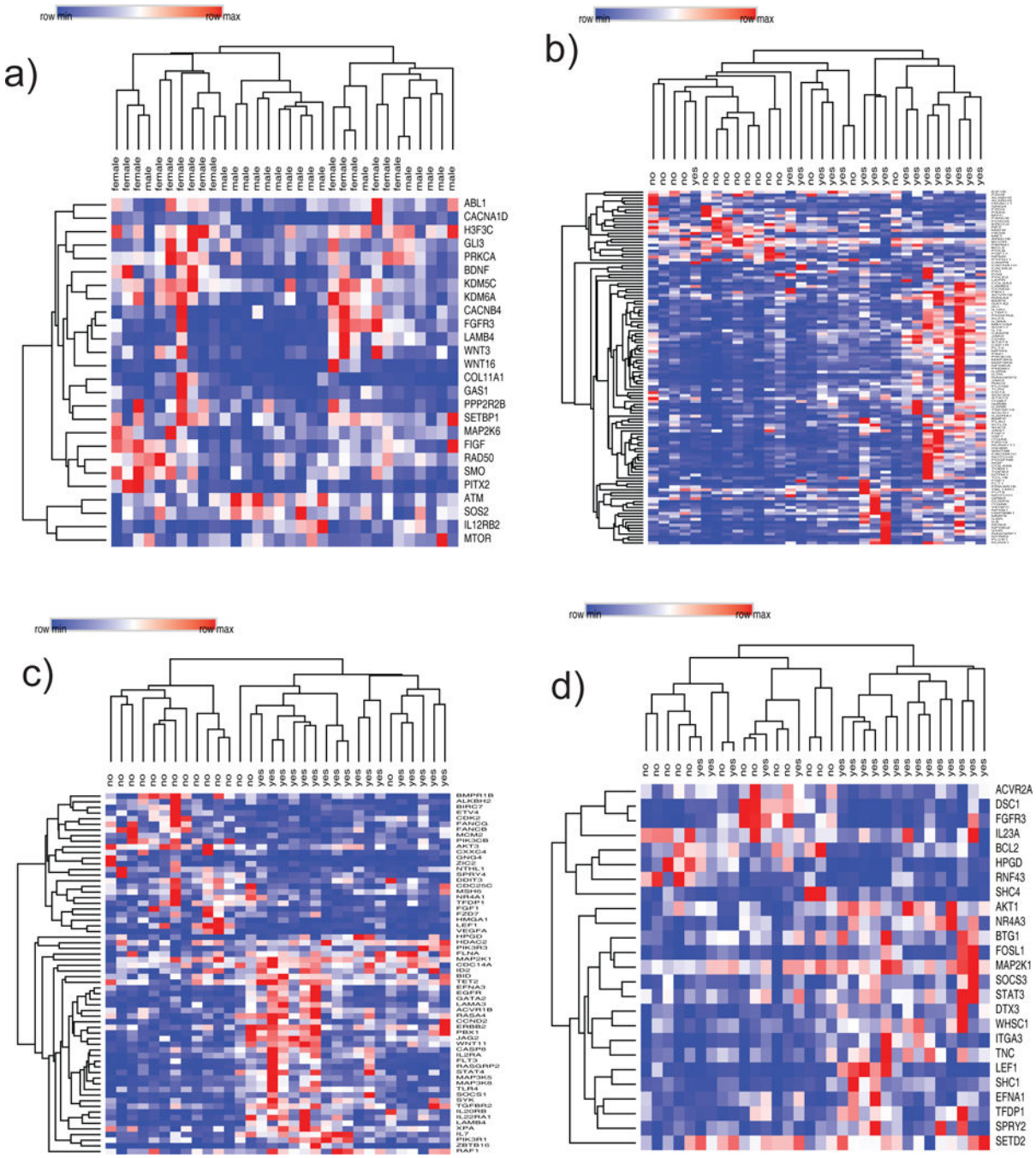

FIGURE 1.

Heat maps of genes nominally significant in analyses of binary traits. (a) sex; (b) TILs; (c) LN status; (d) ulceration status.

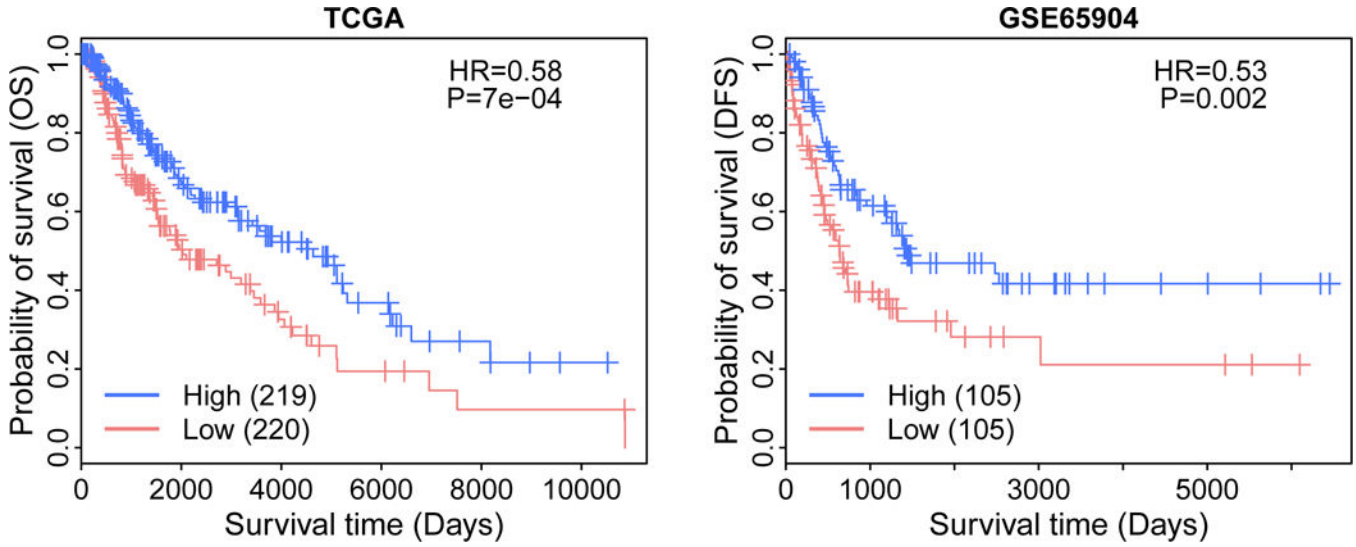

FIGURE 2.

Survival curves for high *vs* low expression of the 14 genes differentially expressed in primary melanomas. A median of the score based on the average, normalized, log-transformed expression of 14 genes was used to stratify cases in high and low expressed.

Table 1.

Overall agreement between InterMEL-pilot and TCGA data in differentially expressed genes.

Variable	Statistics Used	CC	N	p-value
TILs	T statistics	0.35	749	$\mathbf{3.2\times10^{-10}}$
Breslow's thickness	CCs	0.22	748	$\mathbf{1.3\times10^{-7}}$
Lymph node status	T statistics	0.16	748	$\mathbf{1.1\times10^{-5}}$
Ulceration	T statistics	0.12	748	$\mathbf{1.5\times10^{-3}}$
Clinical stage	CCs	0.08	749	$\mathbf{2.2\times10^{-2}}$
Mitotic rate	T statistics	0.06	737	1.3×10^{-1}
Sex	T statistics	0.03	749	5.7×10^{-1}

CC - correlation coefficient; N - sample size (number of genes)

Table 2.

Genes that remained significant in LASSO models.

Outcome	Significant genes
Sex	*KDM5C, KDM6A, BDNF*
TILs	*BID, CDC25C, ETV4, FANCB, FLNA, GNG7, HPGD, ID2, LEF1, MAP2K1, MCM2, MLF1, NRAS, PIK3R1, PIK3R3, PPP2R1A, SFRP4, STMN1, SYK, TGFBR2*
Breslow thickness	*CACNB2, EGF, EPO, H3F3A, ITGB4, LIF, MAD2L2, MAP3K12, MCM6, MMP9, PTEN, SHC1, TLX1, TNFRSF10C, WNT7A*
Tumor mitotic rate	*AKT1, CACNB2, CASP7, CCNA2, COL2A1, H3F3A, HELLS, ITGA7, MCM6, MMP9, PTEN, SHC1, UBE2T*
Lymph node status	*ALKBH2, ARID1B, BCOR, CALML6, CCND2, EPO, FZD9, GZMB, IL2RA, MET, NFKB1, NPM2, PBRM1, POLE2, PTPN11, TNC, VDR*
Ulceration	*AKT1, DSC1, HPGD, IL23A, NR4A3, SETD2, SHC4, SOCS3, WHSC1*
Clinical stage	*ABL1, LTA4H*

Table 3.

Functional categories enriched by differentially expressed genes in GSEA. We used GSEA Hallmark pathways for this analysis.

Gene set	Sex	TILs	LN status	Ulceration	Breslow's	TMR	Stage
E2F_TARGETS			1.1E-03		1.1E-03	1.0E-04	
EPITHELIAL_MESENCHYMAL_TRANSITION	4.1E-03						
ESTROGEN_RESPONSE_LATE		9.9E-03				2.4E-02	
G2M_CHECKPOINT			1.1E-03	3.6E-02	1.1E-03	1.0E-04	4.9E-02
IL6_JAK_STAT3_SIGNALING		5.9E-03				2.3E-03	
INFLAMMATORY_RESPONSE	1.6E-02					4.9E-02	
INTERFERON_GAMMA_RESPONSE	1.1E-02	2.6E-03				2.2E-02	
KRAS_SIGNALING_DN				2.2E-02			
MYC_TARGETS_V1			1.0E-03		1.0E-03	1.0E-05	
TNFA_SIGNALING_VIA_NFKB				8.4E-03			

Table 4.

Genes contributing to the enrichment of functional categories listed in Table 4

Gene set	Differentially expressed genes
E2F_TARGETS	*MYC, MSH2, RAD50, CDK4, BRCA2, RAD21, MCM4, WEE1, PRKDC, HELLS, RFC3, CDC25A, MCM3, MCM7, CDKN2A, SRSF2, STMN1, CHEK2, BRCA1, MCM2, KPNA2*
EPITHELIAL_MESENCHYMAL_TRANSITION	*INHBA, IL15, TNC, FLNA, ITGA2, COMP, JUN, THBS1, PDGFRB, DKK1, VEGFA, FZD8, COL1A1, VEGFC, IGFBP3, COL1A2, ITGB3, COL3A1, ID2, GJA1, SFRP4, COL5A1, FN1, FAS, COL5A2, GAS1, COL11A1, BDNF*
ESTROGEN_RESPONSE_LATE	*BCL2, SFN, FOS, IDH2, JAK1, CACNA2D2, FGFR3, KLF4, CXCL14, TIAM1, LAMC2, JAK2, ID2*
G2M_CHECKPOINT	*MYC, MSH2, RAD50, CDK4, BRCA2, RAD21, MCM4, WEE1, PRKDC, HELLS, RFC3, CDC25A, MCM3, MCM7, CDKN2A, SRSF2, STMN1, CHEK2, BRCA1, MCM2, KPNA2*
IL6_JAK_STAT3_SIGNALING	*STAT1, STAT3, GRB2, CD14, CRLF2, PIM1, CSF2, PIK3R5, JUN, TNF, CSF3R, PLA2G2A, IL3RA, IL1R2, IL1R1, TLR2, MAP3K8, IL2RA, IL7, SOCS1, ACVR1B*
INFLAMMATORY_RESPONSE	*CCR7, PIK3R5, IL7R, IL1B, IL2RB, IL1A, IRAK2, IL8, RASGRP1, OSM, RELA, NFKBIA, CSF3R*
INTERFERON_GAMMA_RESPONSE	*PIM1, NFKB1, PLA2G4A, ITGB7, IL2RB, NFKBIA, CD40, JAK2, TNFSF10, IL15, STAT4, IL7, CASP8, SOCS1*
KRAS_SIGNALING_DN	*WNT16, EGF, FGF22, TLX1, CALML5, TGFB2, CNTFR, FGFR3*
MYC_TARGETS_V1	*MYC, CDK2, CDK4, MCM4, U2AF1, MCM7, SRSF2, MCM2, KPNA2, MCM5, HDAC2, PCNA, RFC4*
TNFA_SIGNALING_VIA_NFKB	*NR4A3, SOCS3, TNC, BTG1, EFNA1, FOSL1, LIF, IL6, VEGFA, DUSP5, BIRC3, IL1A, LAMB3, INHBA, NFKBIA, IL1B, CLCF1, RELA, NR4A1*

Table 5.

Clinical characteristics of primary melanoma samples used in the analysis.

Patient ID	Sex	LN	Clinical stage	Breslow thickness (mm)	Ulceration	Mitosis (#/mm^2)	TILs
IML-0001	male	yes	IIB	3.6	Yes	4	no
IML-0002	male	no	IIB	3	Yes	3	no
IML-0003	female	yes	IIC	10.5	Yes	8	no
IML-0004	male	no	IIC	11	Yes	12	no
IML-0005	male	yes	IIIC	18	Yes	16	no
IML-0006	female	yes	IIB	3.8	Yes	5	yes
IML-0007	male	yes	IIIC	6.9	No	4	no
IML-0008	male	no	IIC	14	Yes	24	no
IML-0009	male	no	IIA	2.5	No	1	no
IML-0010	male	yes	IIIC	1.2	Yes	1	yes
IML-0011	female	yes	IIIA	1.8	Yes	2	yes
IML-0012	female	yes	IIIA	1.5	No	1	no
IML-0013	male	no	IIIB	2.8	Yes	3	yes
IML-0014	female	yes	IIA	3.7	No	2	yes
IML-0015	male	no	IIB	6	no	6	no
IML-0016	female	no	IIB	2.4	Yes	3	yes
IML-0017	male	yes	IIIA	1.5	Yes	1	yes
IML-0019	female	yes	IIIB	12	Yes	23	no
IML-0020	male	yes	IIIC	6	Yes	7	yes
IML-0021	female	no	IV	5	No	2	no
IML-0022	male	no	IIC	3	Yes	21	yes
IML-0024	male	yes	IIIB	6	No	4	yes
IML-0025	male	no	IIIB	3	No	7	yes
IML-0026	female	no	IIB	3	YEs	6	no
IML-0027	female	no	IIC	6	Yes	9	no
IML-0028	male	no	IV	5	Yes	7	yes
IML-0029	female	yes	IIIA	1.4	No	1	yes
IML-0030	female	yes	IV	15	Yes	27	yes
IML-0032	male	yes	IIIB	1.2	No	0	yes
IML-0033	female	no	IIIC	4	No	1	no
IML-0034	female	no	IV	1.4	No	1	yes
IML-0035	female	yes	IIIC	12	No	12	yes