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TRIB2 as a biomarker for diagnosis and progression of melanoma

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Abstract

Malignant melanoma is the most deadly form of skin cancer. There is a critical need to identify the patients that could be successfully treated by surgery alone and those that require adjuvant treatment. In this study, we demonstrate that the expression of *tribbles2* (*TRIB2*) strongly correlates with both the presence and progression of melanocyte-derived malignancies. We examined the expression of *TRIB2* in addition to 12 previously described melanoma biomarkers across three independent full genome microarray studies. *TRIB2* expression was consistently and significantly increased in benign nevi and melanoma, and was highest in samples from patients with metastatic melanoma. The expression profiles for the 12 biomarkers were poorly conserved throughout these studies with only *TYR*, *S100B* and *SPP1* showing consistently elevated expression in metastatic melanoma versus normal skin. Strikingly we confirmed these findings in 20 freshly obtained primary melanoma tissue samples from metastatic lesions where the expression of these biomarkers were evaluated revealing that *TRIB2* expression correlated with disease stage and clinical prognosis. Our results suggest that *TRIB2* is a meaningful biomarker reflecting diagnosis and progression of melanoma, as well as predicting clinical response to chemotherapy.

Introduction

Malignant melanoma is a highly aggressive cancer that arises from the transformation of melanocytes, the pigment-producing cells within the basal epidermal layer in human skin (1). Malignant melanoma accounts for only 5% of all skin cancers, yet is responsible for 80% of skin cancer deaths. Furthermore, the incidence of metastatic melanoma has increased over the past three decades with a mortality rate that continues to rise faster than almost all other cancers (2). This combination of aggressive clinical behaviour, propensity for metastasis and therapeutic resistance has motivated efforts to translate our growing knowledge into more effective treatments. Where possible, disease management incorporates the wide local excision of the tumour offering a 95% 5-year survival for patients with localized melanoma (3). However, this option is restricted due to late stage diagnosis and tumour metastasis. In addition, disease relapse is frequent resulting in an extremely poor clinical prognosis. Currently, the established treatment of metastatic melanoma includes high-dose interleukin-2 or dacarbazine administration associated with response rates of between 10 and 20% with severe patient side effects during treatment (4–6). Recently, two promising groups of therapeutics, the kinase inhibitors (vemurafenib, dabrafenib and trametinib), and the immune checkpoint blockers (ipilimumab) were approved by the FDA and the EMA (7,8). These therapies might prove to be a breakthrough for late stage melanoma treatment but are associated with an extremely high cost. Consequently an

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Abbreviations

AUC	area under the curve
ROC	receiver operating characteristic
S100B	S100 calcium binding protein B
TCGA	The Cancer Genome Atlas
TRIB2	tribbles2

accurate predictive classification of melanoma would allow for the identification of the patient population that would benefit from systemic therapy, minimizing the unnecessary suffering of patients from therapeutic side effects as well as excessive treatment costs.

Currently, melanoma patients are divided into four stages. These are based on Breslow tumour thickness, the presence of ulceration, the extent of nodal involvement for primary and locally metastatic cutaneous melanoma, serum lactate dehydrogenase (LDH) level and sites of metastasis for systemic disease (9). To improve diagnosis and staging of melanoma, the use of several molecular tissue and serum biomarkers have been investigated (10). At present, serum LDH is the only molecular marker that has been incorporated into routine disease staging with many others at various stages of validation. Of these biomarkers, the most intensively examined is the beta subunit of the acidic calcium binding protein S100 calcium binding protein B (*S100B*). *S100B* expression has been shown to correlate with survival, recurrence and clinical melanoma stage (low protein expression in stages I and II, elevated protein expression in stage III and highest protein levels in stage IV) (11,12). While promising, the absence of a standardized protocol for the analysis of serum samples and S100B measurement impede the evaluation of this marker across different studies. As a result, the application of S100B as a routine clinical serum marker of melanoma is still not established. Similarly, tyrosinase oculocutaneous albinism IA (*TYR*), a tissue specific enzyme involved in melanin synthesis (13) has also emerged as a means to detect malignant melanoma cells either within peripheral blood or regional lymph nodes (14). A number of groups have demonstrated that *TYR* is a reliable factor associated with response to treatment, development of metastases, disease progression and overall survival (15) although this is controversial (16). Consequently, the evaluation of standardized protocols for PCR-based techniques in comparison to those available for standardized measurement of enzyme activities or (specific) protein concentration is required. In addition to these two biomarkers there are a number of less extensively investigated melanoma molecular biomarkers (summarized in Supplementary Table 1, available at *Carcinogenesis* Online (12,13,17–26)) that have been associated with disease progression, disease stage and/or clinical outcome. While these represent promising biomarkers, the clinical value of these molecular targets is limited by the inherent heterogeneity of molecular melanoma signatures as well as by an insufficient selectivity and sensitivity. Consequently, there is an urgent need for better, more robust molecular biomarkers to precisely diagnose disease stage, refine the risk of disease progression and accurately predict disease response to therapy.

In this study, we evaluated the potential of *tribbles2* (*TRIB2*) as a novel molecular melanoma biomarker. The kinase-like protein TRIB2 was found to be highly expressed specifically in melanoma and can facilitate the growth and survival of melanomas by down regulating FOXO activity (17). Here, we show that *TRIB2* can serve as a biomarker of high accuracy to detect the presence and progression of human melanoma. In comparison with 12 previously described melanoma markers, we show that *TRIB2* expression provides the second most accurate marker to identify melanoma formation and progression. Crucially, we confirmed these results in *ex vivo* clinical samples, implicating *TRIB2* status affecting clinical prognosis.

Materials and methods

Datasets used for the analysis

For analysis, microarray data for human normal skin, benign nevi, primary and metastatic melanoma samples from three independent studies were utilized [Talantov *et al.* (27) (GSE3189), Smith *et al.* (28) (GSE4587) and Riker *et al.* (29) (GSE7553)]. Talantov *et al.* (GSE3189) used Affymetrix Hu133A GeneChips to identify molecular markers for lymph node staging in melanoma tumourigenesis. The dataset consists of 7 normal skin, 18 nevi and 45 melanoma samples. Smith *et al.* dataset (GSE4587) comprise 2 normal skin, 4 nevi, 2 melanoma *in situ* and 7 melanoma samples. These different sample classes are summarized in Supplementary Table 2, available at *Carcinogenesis* Online. Gene expression study was carried out using Affymetrix Human Genome U133 Plus 2.0 Chip to identify molecular changes at the transition of melanoma *in situ* into metastatic melanoma. The third dataset derived from the Riker *et al.* (GSE7553) study were conducted using Affymetrix Human Genome U133 Plus 2.0 Chips. This dataset comprises 15 basal cell carcinoma, 11 squamous cell carcinoma, 14 primary melanoma and 40 metastatic melanoma samples. Importantly, all of the genes present in Affymetrix Human Genome U133A array [also used in the Talantov *et al.* (GSE3189) study] are present in the Affymetrix Human Genome U133 Plus 2.0 array [utilised in both the Riker *et al.* (GSE7553) and Smith *et al.* (GSE4587) reports]. All of the bio-markers investigated in our study are present in both array platforms.

Datasets pre-processing and analysis

Expression data were obtained from Gene Expression Omnibus database (accession numbers: GSE3189, GSE4587, GSE7553). Data analysis was carried out in the R/Bioconductor environment. In particular, data pre-processing, normalization and summary index calculation was performed using the RMA (Robust Multi-array Average) method as implemented in the Bioconductor affy package with a default parameter setting. The Bioconductor limma package was utilised to detect differential expression in metastatic melanoma compared with their corresponding controls. To correct for multiple testing, Benjamini and Hochberg procedure was applied (30). For identification of differentially regulated genes, adjusted *P*-value ≤ 0.05 and absolute log2 fold change ≥ 1.0 was set as criteria. To assess the statistical significance of common up- or down-regulated genes in the three microarray datasets, the hypergeometric test (which is equivalent to Fisher's exact test) was applied.

Receiver operating characteristic curve analysis

To stringently assess the capacity of compared biomarkers for melanoma diagnosis and staging, receiver operating characteristic (ROC) curves were constructed. ROC curves display the sensitivity and specificity of biomarkers across a maximal range of threshold settings and are standard means for the comparison of classifiers. In our case, samples were classified based on the expression levels of marker genes with respect to the chosen threshold. Comparison of the achieved classification with the known samples classes (e.g. normal tissue, nevi or melanoma) provided the number of true positives (TP), false positives (FP), false negatives (FN) and true negatives (TN). The sensitivity or true positive rate (TPR) is defined as TPR = TP/(TP + FN), whereas false positive rate (FPR), that is 1-specificity is given by FPR = FP/(FP + TN). For each microarray studies, TPR and FPR were recorded across a maximal range of expression threshold values for individual biomarker and displayed as ROC curves. As overall measure for classification performance, the total area under the ROC curve (AUC) was calculated. The AUC is equivalent to the average sensitivity across the full range of specificity values and can have a maximum value of 1. AUC was derived for each of the constructed ROC curves, that is for each biomarker and each of the sample classifications.

For comparison, the biomarkers were ranked according to their average AUC in the three datasets.

The Cancer Genome Atlas database analysis

We performed our analysis on skin cutaneous melanoma (SKCM) data sets available in The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/). We downloaded the all the 385 files from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) and extracted normalized counts computed using RSEM software (PMID: 21816040) from these into a data matrix. Further, we mapped sample annotations for the individual datasets to perform several comparisons such as patient survival (alive or dead), Clark's level of invasion and Breslow's depth.

Quantitative real-time PCR

Total RNA was extracted from our clinical samples using Tri-reagent (Sigma). Real-time PCR was performed on a CFX-96 PCR machine (BioRad) using the SSoFastSybr® green master mix (BioRad) and following the manufactures guidelines. The primer sequences for measuring all of our genes of interest were purchased from NZYTech (Portugal) and are shown in Supplementary Table 3, available at *Carcinogenesis* Online. All expression analysis for each clinical sample was conducted in quadruplicate. Normalization was carried out against *GAPDH* and data analysis utilised the $2^{-\Delta\Delta C_t}$ methodology, described by Livak *et al.* (31).

Statistical analysis

All statistical analysis conducted following qRT-PCR used unpaired two-tailed student *t*-test (Graph pad PRISM) generating a *P* value for each comparison. Values ≤0.05 were considered significant in our analysis although for each calculation, the *P* value is clearly indicated. Where N/S is indicated, the *P* value is not significant.

Clinical sample analysis

Surgically excised tissue samples from metastatic lesions of stage IV melanoma patients prior to first-line therapy were freshly frozen and cryo-preserved until processing. The collection and the documentation of clinical samples were performed after obtaining informed consent with Institutional Review Board approval. Specifically, study #123/2008 was under taken with Ethics Committee approval from the Ethics Committee Faculty of Medicine, University of Wuerzburg, Germany. The frozen tissues were split into smaller sections for RNA extraction using TRI-Reagent (Sigma).

Results

To address if *TRIB2* could be a melanoma biomarker we examined three independent whole genome microarray studies that contained a large number of samples in each disease grade (normal skin, benign nevi, basal cell carcinoma, squamous cell carcinoma, primary melanoma and metastatic melanoma).

Poor melanoma biomarker correlation across each data set

Our first objective was to obtain an overview of the global changes in gene expression within each genome wide study. We identified the differentially expressed genes in each complete data set incorporating a 2-fold increase or decrease in gene expression as the expression change threshold for our analysis, comparing normal skin versus nevus, primary melanoma or metastatic melanoma samples from the Talantov *et al.* (GSE3189) and Smith *et al.* (GSE4587) studies and from the Riker *et al.* (GSE7553) study comparing basal carcinoma versus squamous, melanoma and metastatic melanoma samples. For statistical rigor and robustness we also required an adjusted *P*-value of less than 0.05 for each gene of interest that showed a 2-fold expression change. Across all of the genome wide studies, we found that 86 genes were consistently up-regulated while the expression of 222 genes was significantly reduced in each independent study (summarized in Figure 1A and B, Supplementary

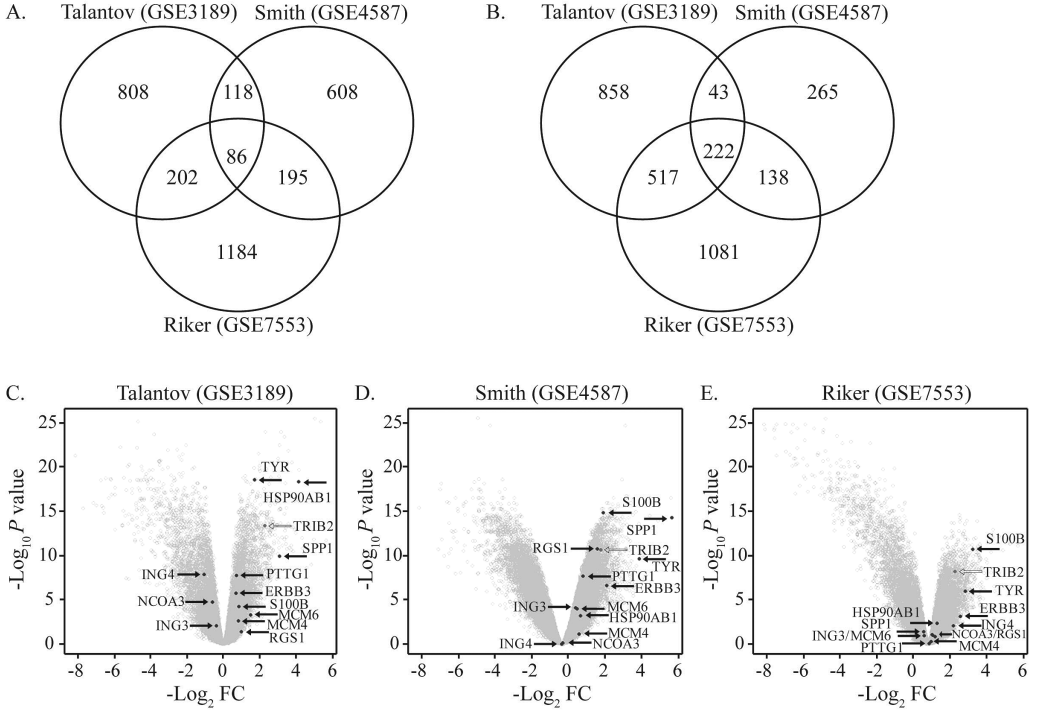

Figure 1. Venn diagram and Volcano plots indicating the differentially regulated genes across each data set. [Talantov *et al.* (GSE3189), Smith *et al.* (GSE4587) and Riker *et al.* (GSE7553)]. (**A**) Conserved up regulated genes. (**B**) Conserved down regulated genes. (**C**) Talantov *et al.* (GSE3189), D. Smith *et al.* (GSE4587) and E. Riker *et al.* (GSE7553). Log2 fold change are plotted along *x*-axis and (–log 10) adjusted *P*-values along *y*-axis. The red dot within each plot indicates *TRIB2* and blue dots show each compared melanoma biomarker.

Tables 4 and 5, available at *Carcinogenesis* Online). It is important to note that while the number of genes with a conserved expression pattern across every study was low, these gene expression changes were highly significant (*P*-value = $1.63E^{-10}$, $1.10E^{-4}$ and $5.00E^{-3}$, respectively). We also note that while their expression changes were not conserved between each study, all 12 biomarkers (as well as *TRIB2*) were present in either the up-regulated or down-regulated gene list (and therefore are present in either Venn diagram).

We next questioned if the transcription of the 12 putative melanoma biomarkers changed from normal to metastatic melanoma disease state. We note that only *S100B*, *SPP1* and *ING4* displayed a conserved statistically significant expression change from normal skin to metastatic melanoma in all of the data sets (Supplementary Table 6, available at *Carcinogenesis* Online). While we observe a poor correlation between the 12 melanoma biomarkers across each genome wide array, we noted that *TRIB2* transcription was both consistently and significantly increased across all three studies. Furthermore, despite the biomarkers *S100B*, *SPP1* and *ING4* showing a statistically significant expression difference across every microarray, only *S100B* displayed a conserved increase in expression during transformation from control (normal skin or basal carcinoma) to metastatic melanoma. While *ING4* demonstrated a statistically significant change between control and metastatic melanoma samples in each array study, the changes observed were however not consistent. *ING4* expression was drastically decreased in metastatic melanoma samples (compared with control groups) within the Riker *et al*. (GSE7553) study, yet it was significantly over-expressed in the Talantov *et al*. (GSE3189) and Smith *et al*. (GSE4587) studies. It should be noted that a potential caveat related to the Riker *et al*. study might be the use of basal cell carcinoma and squamous cell carcinoma samples that are already transformed as controls for nonmelanoma skin cancer. This can account for the discrepancy noted for *ING4* expression. However, while this was a concern for *ING4*, this difference (between Talantov *et al*. (GSE3189) and Smith *et al*. (GSE4587) compared with Riker *et al*. (GSE7553) was only noted for one additional biomarker (*PTTG1*, Supplementary Figure 1F, available at *Carcinogenesis* Online) and was not conserved for any other biomarkers where expression changes were conserved between Talantov *et al*./Riker *et al*. (*ERBB3*) or Smith *et al*./Riker *et al*. (*HSP90AB1*). Overall from this analysis, we can conclude that the expression profiles for each of the 12 melanoma biomarkers were poorly conserved across the three genome-wide microarray studies with only *S100B* and *SPP1* demonstrating a conserved increase in transcription during carcinogenesis (from control to metastatic melanoma). Strikingly, *TRIB2* transcription was significantly increased across all three independent data sets, suggesting that *TRIB2* could be a melanoma biomarker.

Identification of *TRIB2* as a biomarker indicating metastatic melanoma

To evaluate more stringently the potential of *TRIB2* expression as a biomarker of metastatic melanoma, we analyzed differential gene expression in each study using Volcano plots (Figure 1C–E). We ranked each biomarker (including *TRIB2*) within each microarray study and as we would have predicted following our global gene analysis, we noted that there was little conservation in expression between the 12 melanoma biomarkers in each data set. For example, the Talantov *et al*. (GSE3189) and Riker *et al*. (GSE7553) studies indicate that *ING3* expression is reduced in metastatic melanoma in contrast to the Smith *et al*. (GSE4587) study that indicated the induction of this gene in metastatic melanoma samples. While there was little conservation between these data sets, for each study, *TRIB2* was significantly over-expressed in a consistent manner and was in the top three of all over-expressed genes. Interestingly, in the Smith *et al*. (GSE4587) and Riker *et al*. (GSE7553) studies *S100B* was the most over-expressed biomarker in the metastatic melanoma samples. In the Talantov *et al*. (GSE3189) data set, *TYR* was the most over-expressed gene between normal skin versus metastatic melanoma. Taking into consideration the inconsistent expression profiles of "bona fide" melanoma markers between these studies we are able to conclude that *TRIB2* and specifically, the over-expression of *TRIB2* is a strong melanoma biomarker candidate.

TRIB2 expression correlates with melanoma progression

Of equal interest and importance for a biomarker is the potential to differentiate various stages of disease. With this question in mind, we examined if *TRIB2* expression could distinguish the various stages of melanoma progression (normal skin, nevus, primary melanoma and metastatic melanoma). To test this, we plotted the expression of *TRIB2* as well as the three melanoma biomarkers (*SPP1*, *S100B* and *ING4*) that demonstrated a statistically significant transcription difference between normal skin versus metastatic melanoma samples (Figure 2A–D). We also plotted the fold expression changes for the remaining nine melanoma biomarkers (Supplementary Figure 1A–I, available at *Carcinogenesis* Online). For each biomarker that demonstrated an overall significant expression change between normal/basal tissues compared with metastatic melanoma tissue, we conducted two-tailed unpaired *t*-tests to address if any of the melanoma biomarkers or *TRIB2* could be used to determine melanoma stage. Of these four genes, *TRIB2*, *S100B* and *SPP1* demonstrated a statistically significant increase in expression from the control groups (normal skin/basal carcinoma) versus metastatic melanoma in all three genome-wide array studies (Figure 2A–C). For the other biomarker [*ING4* (Figure 2D)] we observe a highly significant difference in expression when we compare control samples versus metastatic melanoma; however there is no consistency for these genes between the microarray studies: For example, when we examine *ING4* expression changes, the Talantov *et al*. (GSE3189) and Smith *et al*. (GSE4587) studies reveal a significant expression decrease from normal to metastatic melanoma, whereas the Riker *et al*. (GSE7553) study reveals a highly significant induction of *ING4* expression between basal and metastatic melanoma. We note significantly elevated *TRIB2* expression when normal skin versus nevus samples were analysed in addition to nevus versus melanoma *TRIB2* expression particularly within the large Talantov *et al*. (GSE3189) study. Our analysis also identified that of all the previously described melanoma biomarkers, only *S100B* over-expression was as consistently conserved as *TRIB2*. Based on these results, we were able to statistically rank the 12 melanoma markers evaluating the statistical significance of each gene expression change across all three microarray data sets and to incorporate *TRIB2* into this analysis (Table 1). Equally when we considered only enhanced gene expression associated with increased melanoma grade in our analysis we found (including all 12 melanoma biomarkers) that *TRIB2* over-expression was the second most conserved gene of all evaluated genes Table 2). These results show that *TRIB2* expression is a highly promising biomarker of melanoma presence and progression reflecting the stage of disease by its expression level.

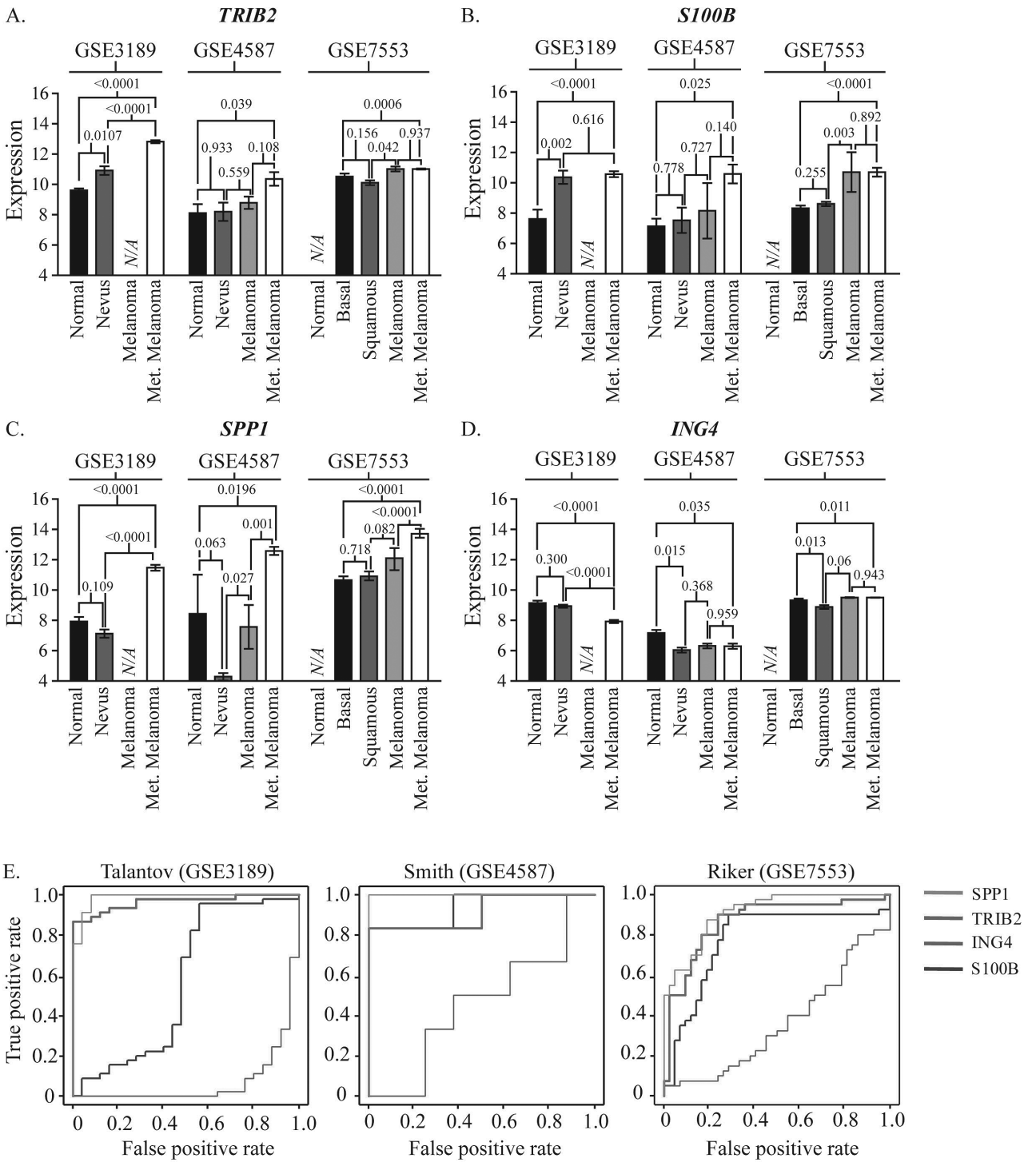

Figure 2. Expression and ROC curve analysis for *TRIB2* and each melanoma biomarker within each data set [Talantov *et al.* (GSE3189), Smith *et al.* (GSE4587) and Riker *et al.* (GSE7553)]. **A–D** indicates the expression changes within each melanoma stage (normal/basal, nevus/squamous, melanoma or metastatic melanoma) for *TRIB2*, *S100B*, *SPP1* and *ING4*. For each plot the *x*-axis shows the average log2 normalized gene expression (measured by quantification of the fluorescent signal from the microarray hybridization) from each set of samples on *y*-axis. **E–G** shows the classification performance of the molecular melanoma biomarker *TRIB2*, *S100B*, *SPP1* and *ING4* from each dataset [E. Talantov *et al.* (GSE3189), F. Smith *et al.* (GSE4587) and G. Riker *et al.* (GSE7553)]. On each plot, the *x*-axis indicates the TPR and sensitivity and the *y*-axis indicates the FPR 1-specificity.

TRIB2 is confirmed as a biomarker of melanoma progression via ROC analysis

A crucial caveat we next addressed was to critically evaluate the performance of *TRIB2* as a biomarker for metastatic melanoma. To determine the strength of *TRIB2* as a predictive marker we calculated the TPR and FPR specificity for *TRIB2*, *S100B*, *SPP1* and *ING4* from the three genome wide array studies. ROC curves were generated for each biomarker following our TPR and FPR analysis (Figure 2E). For the remaining biomarkers we also plotted ROC curves (Supplementary Figure 2, available at

Table 1. Increased biomarker expression within melanoma

	Biomarker	Average *P* value
1	*SPP1*	0.007
2	*S110B*	**0.008**
3	*TRIB2*	**0.013**
4	*ING4*	0.015
5	*MCM6*	0.048
6	*NCOA3*	0.106
7	*PTTG1*	0.128
8	*TYR*	0.146
9	*ERBB3*	0.187
10	*HSP90AB1*	0.213
11	*MCM4*	0.348
12	*RGS1*	0.35
13	*ING3*	0.545

When we analysed increased gene expression for each of the 12 metastatic biomarkers (including *TRIB2*) we were able to rank the previously described melanoma biomarkers. Besides *S100B*, *TRIB2* displayed the most conserved, increased gene expression across all three data sets across each grade of melanoma. The significance of the two values is ** and **, respectively.

Table 2. ROC curve and AUC analysis of each biomarker and *TRIB2*

	GSE3189		GSE4587		GSE7553		Overall	
Gene	AUC	Rank	AUC	Rank	AUC	Rank	Average	Rank
SPP1	0.987	1	1.00	1	0.913	1	0.967	1
TRIB2	0.964	3	0.917	6	0.867	2	0.916	2
PTTG1	0.970	2	1.000	1	0.702	6	0.891	3
MCM6	0.889	5	0.972	3	0.632	8	0.831	4
RGS1	0.737	7	0.896	7	0.803	3	0.812	5
MCM4	0.856	6	0.944	4	0.512	11	0.771	6
TYR	0.958	4	0.667	11	0.671	7	0.765	7
HSP90AB1	0.711	8	0.861	8	0.717	5	0.763	8
S100B	0.565	10	0.944	4	0.775	4	0.762	9
ERBB3	0.668	9	0.833	9	0.612	9	0.704	10
ING3	0.296	11	0.806	10	0.582	10	0.561	11
NCOA3	0.218	12	0.639	12	0.290	13	0.382	12
ING4	0.068	13	0.472	13	0.361	12	0.300	13

Talantov *et al.* (GSE3189), Smith *et al.* (GSE4587) and Riker *et al.* (GSE7553) data and probe sets were analysed for each melanoma biomarker and *TRIB2* and within each data set, based on TP versus FP, we ranked each biomarker within each microarray study. Across each data set, *TRIB2* was consistently one of the highest ranked biomarkers throughout each grade of melanoma disease.

Carcinogenesis Online) and for every biomarker, including *TRIB2* we calculated the AUC. This analysis identified biomarker(s) that can classify each melanoma sample more effectively, where the higher the AUC value, the stronger and more robust the gene of interest. In all three datasets *TRIB2* had a consistently high AUC, [Talantov *et al.* (GSE3189) AUC = 0.963 (ranked third overall), Smith *et al.* (GSE4587) AUC = 0.916 (ranked sixth overall) and Riker *et al.* (GSE7553) AUC = 0.866 (ranked second overall)] and is summarized in Table 2. Importantly in large datasets [such as the Talantov *et al.* (GSE3189) and Riker *et al.* (GSE7553) studies] *TRIB2* has a higher AUC than the majority of all other biomarkers examined suggesting that *TRIB2* performs better in large datasets, an absolute prerequisite for a diagnostic biomarker. Our analysis also highlights that *TRIB2* (with an average AUC = 0.9156) ranked second overall among all 12 biomarkers with only *SPP1* (with an average AUC = 0.966) being ranked higher across three whole genome array studies. These results strongly support our previous findings that indicate *TRIB2* as a very strong candidate for both the diagnosis and progression of melanoma disease.

TRIB2 expression is significantly increased in freshly obtained metastatic melanoma samples compared to normal skin

We next wanted to validate our results in freshly obtained clinical samples from metastatic melanoma patients prior to standard systemic treatment. We analyzed cryo-preserved tumour tissue samples from 20 stage IV metastatic melanoma patients and 12 tissue samples from normal healthy skin (summarized in Supplementary Table 7, available at *Carcinogenesis* Online). The melanoma samples were grouped based on the clinical response of the respective patient to chemotherapy (complete response, stable disease or progressive disease).

We extracted total RNA from the samples and conducted qRT-PCR analysis for each melanoma biomarker including *TRIB2* (Figure 3A white bar). In strong support of our meta-analysis, we note that *TRIB2* expression was significantly higher in melanoma samples compared to normal skin samples. Considering our previous results that suggested *TRIB2* expression could be indicative of disease stage, we questioned if *TRIB2* or the other biomarkers expression correlated with clinical prognosis (Figure 3B). We also questioned if *TRIB2* or any of the three key biomarker expression identified by our ROC/AUC analysis correlated with clinical outcome (Figure 3C). These biomarkers (*TRIB2*, *S100B*, *TYR* and *PTTG1*) were selected as they were the strongest performers in out ROC/AUC analysis and are, in this analysis, the most robust biomarkers from the meta-analysis. Strikingly, we note that *TRIB2* expression correlated strongly with disease response to chemotherapeutic intervention, where the highest average expression was observed in progressive disease samples Figure 3B and C). Conversely, there is no correlation between the various chemotherapeutic regimens, the chemo-sensitivity index (CSI) and the clinical outcome (Supplementary Table 7, available at *Carcinogenesis* Online). The expression level of the other known melanoma markers showed a significant expression increase in metastatic melanoma samples compared to normal tissue although there was not a conserved grouping for any of these based on final disease outcome with the exception that only *TYR* showed a conserved expression change based on treatment outcome. Interestingly *TRIB2*, *S100B*, *TYR* and *PTTG1* showed a statistically significant expression difference between complete response versus either stable disease or progressive disease (Figure 3C). Finally, we questioned if TCGA database supported our *ex vivo* clinical sample studies. We observed that for any of the 13 melanoma biomarkers investigated in our study, only two melanoma biomarkers (*SPP1* and *TYR*) showed a statistically significant expression change between melanoma versus metastatic melanoma samples (Supplementary Figure 3, available at *Carcinogenesis* Online). Unexpectedly, within the TCGA database, *TYR* expression significantly decreased between primary tumour patients versus metastatic melanoma patient samples. While there was no statistically significant expression difference for any of the other tested biomarkers we examined whether there was a significant difference in gene expression in relation to patient survival (alive or dead) (Figure 3D), Breslow score (Figure 3E) or the Clark melanoma index (Figure 3F) for the three biomarkers (*S100B*, *TYR* and *PTTG1*) and *TRIB2* that showed a statistically significant difference regarding clinical outcome (Figure 3C). We detected no statistically significant difference between biomarker expression and patient survival (Figure 3D), but we did note a trend for *TRIB2* and *TYR* that show lower gene expression

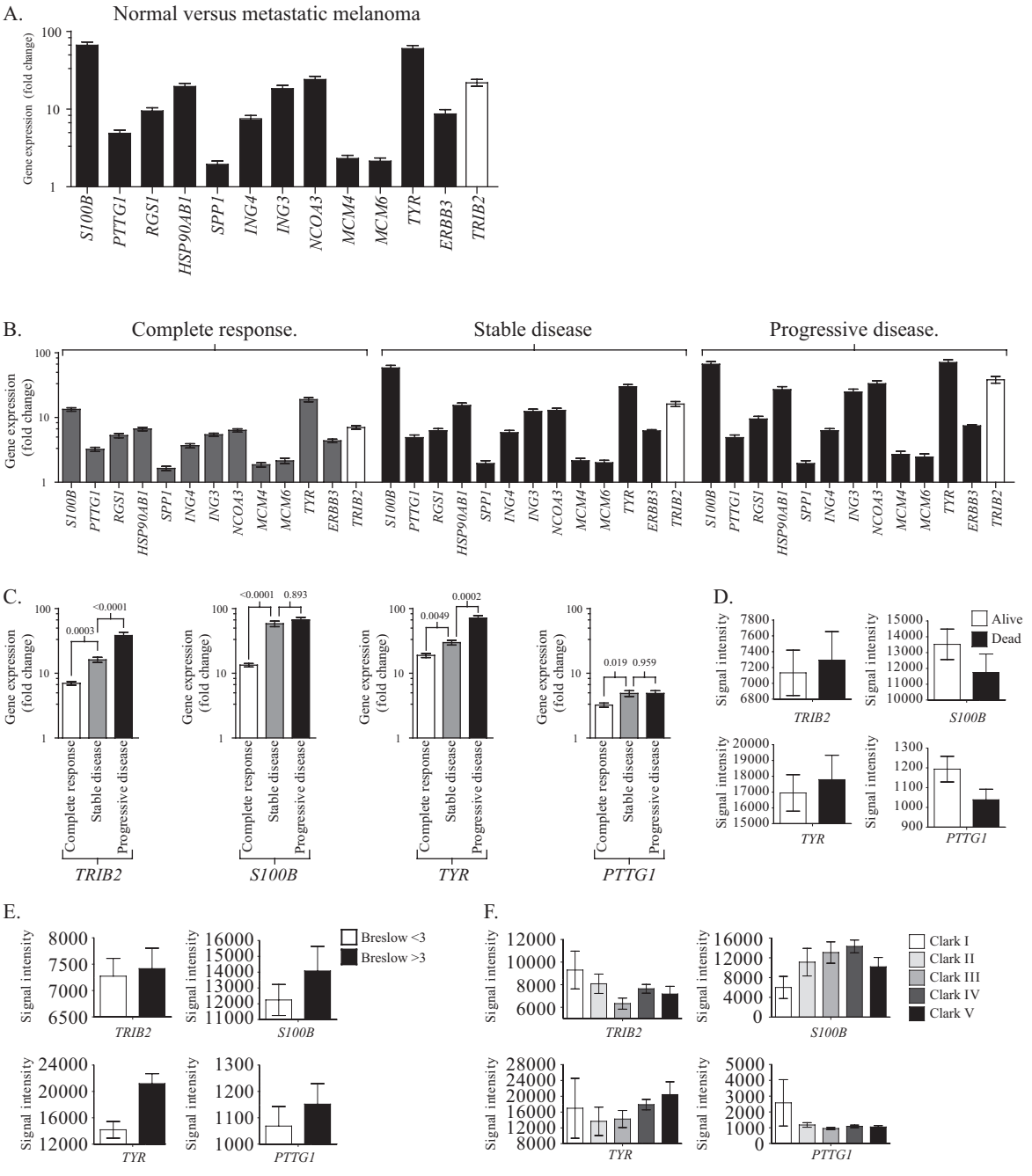

Figure 3. TCGA data analysis of *TRIB2* and each biomarker including transcriptional analysis in *ex vivo* primary stage IV metastatic melanoma samples. (**A**) The average gene expression change from normal tissue compared to metastatic melanoma samples (*N* = 20). **B** shows the analysis of gene expression in melanoma samples (prior to chemotherapeutic administration) separated based on final clinical outcome (complete response *N* = 5, stable disease *N* = 5 and progressive disease *N* = 10). **C** highlights the three strongest biomarkers and *TRIB2* expression based on final patient outcome (including statistical analysis between each group). For all qRT-PCR studies, samples were analyzed in quadruplicate and normalized to *GAPDH* as described in our materials and methods. For each clinical outcome, non-paired two tailed Student *t*-tests were conducted. *P* values are shown. Error bars indicate standard deviation (SD). (**D**). Gene expression from TCGA data set for *TRIB2*, *S100B*, *TYR* and *PTTG1* in alive (white bars *N* = 231) and dead (black bars *N* = 140) patients. Error bars indicated are representative of standard deviation (SD). (**E**) Gene expression analysis of *TRIB2*, *S100B*, *TYR* and *PTTG1* separated based on Breslow classification (<3 mm white bars *N* = 143, >3 mm black bars *N* = 137) groups within the TCGA dataset. Error bars indicated are representative of standard deviation (SD). (**F**) Gene expression analysis of *TRIB2*, *S100B*, *TYR* and *PTTG1* separated based on Clark's index (stage I white bars *N* = 5, stage II light grey bars *N* = 16, stage III grey bars *N* = 59, stage IV dark grey bars *N* = 127 and stage V black bars *N* = 46) within the TCGA dataset. Error bars indicated are representative of standard deviation (SD).

in alive patients. The opposite trend was noted for *S100B* and *PTTG1*. These trends were supported by the Breslow index analysis where high *TRIB2 and TYR*, *S100B* and *PTTG* expression correlated with <3 mm Breslow thickness. However, this trend was not conserved for Clarks index score (Figure 3F).

Discussion

In this study, we evaluated the potential of *TRIB2* to detect the presence and stage of human melanoma compared to 12 previously described melanoma biomarkers. Ideally, melanoma

biomarkers would detect subclinical metastatic disease and accurately reflect tumour progression enabling a personalized disease management strategy and individual risk assessments for patients with melanoma. Although no single biomarker can currently predict patient's clinical outcome several biomarkers have been shown to reflect some aspects of the disease (10).

As there has been low agreement between DNA microarray studies sourcing cell line versus tissue samples (32), we conducted our meta-analysis combining datasets based on clinical patient samples from both independent research groups and the TCGA database. Interestingly, apart from *TRIB2* only four out of the 12 melanoma biomarkers displayed significant expression changes in each dataset. The poor overall correlation of the three microarray data sets may be somewhat surprising, but this has generally been observed in such meta-analyses (32). Whereas this characteristic might have initially casted doubts on microarray measurements, it has become more and more obvious that it can be highly beneficial to distinguish between true biological variability and variability merely due technical procedures. In fact, we have shown in a recent study that the divergence observed between microarray studies can be utilized to produce more biologically reliable sets of differentially expressed genes (33).

It is important to note that *S100B* and *TYR* which turned out to be the most consistently over-expressed genes or having the strongest AUC values in the melanoma samples of our meta-analysis are the best-studied factors among the melanoma biomarkers used in this work. *S100B* is a well-established and widely used immunohistochemical marker of pigmented skin lesions (34–36). In fact, German and Swiss guidelines recommend the use of *S100B* as a serum marker for patients with lesions with a Breslow thickness of over 1 mm (10). Importantly, we found *S100B* to be the most over-expressed metastatic melanoma biomarker in two out of three datasets suggesting that the detection of its transcript level might be a sensitive method to detect metastatic disease in peripheral blood of patients. Conversely, in one dataset, *TYR* was the most over-expressed gene between normal compared to melanoma samples. While *TRIB2* was not the most over-expressed gene in melanoma versus normal in any of the datasets, it ranked within the top three genes in every dataset and turned out to be the most consistent melanoma biomarker in our meta-analysis. We also observed through our ROC curve analysis that the performance computed by estimating AUC of *TRIB2* as melanoma biomarker is considerably better in large datasets [Talantov *et al.* (GSE3189), ($n = 70$) (27) and Riker *et al.* (GSE7553), ($n = 80$] (29) when compared to other markers such as *MCM6* and *MCM4* which are ranked higher than *TRIB2* in smaller dataset [Smith *et al.* (GSE4587), ($n = 15$)] (28). Importantly, both *S100B* and *TRIB2* displayed a gradual, highly significant increase in their expression level for each melanoma grade analyzed.

Nevertheless, it is important to stress that finding robust biomarkers for disease progression and staging remains extremely challenging, a caveat highlighted by the analysis of other far more bona-fide biomarkers (such as *S100B* and *TYR*) that show incoherencies in disease staging when comparing the results of the microarray data with the TCGA dataset. This caveat is also indicated by the results for *TRIB2* (as well as for any of these tested biomarkers), as no statistically significant difference based on patient survival (alive/dead), Breslow or Clark index was detected. However, some trends in expression profiles could be noted within this large dataset. Considering the difficulty in detected consistent expression changes of individual genes, it might be necessary to carry out simultaneous assessment of several markers to gain a more robust disease staging definition.

Besides analysing published expression data, we also validated our findings in a number of *ex vivo* melanoma samples. Importantly, a strong correlation of *TRIB2* expression with treatment response por chemotherapeutic intervention was observed. Taken altogether our results suggest that the measurement of *TRIB2* expression might be useful to both diagnose human melanoma and if present, disease stage. Stringent clinical analysis should validate the usefulness of monitoring *TRIB2* expression for melanoma staging in particular the capability to detect the presence of melanoma metastasis in regional lymph nodes. Since the presence of regional lymph node metastasis is the strongest predictor of relapse rates and survival, accurate identification of metastatic disease within the regional lymph nodes is of key importance to identify patients which should be treated with adjuvant therapy. Standard procedure for the examination of regional lymph nodes involves histopathological examination by haematoxylin and eosin and immunohistochemical staining. However, this approach may underestimate the presence of metastatic disease. The effort to develop more accurate and sensitive methods to identify micro-metastatic disease in the regional lymph nodes or the peripheral blood led to the introduction of molecular techniques. The identification of circulating melanoma cells via the PCR-based detection of *TYR* in peripheral blood has been introduced in the early 90s and allowed for a 10 000-fold increased sensitivity when compared to immunohistochemical analysis (14). However, the clinical value of this procedure is limited by the proportion of patients with clinical confirmed distant metastasis being *TYR* negative in almost all the studies. It has been suggested that the combination of *TYR* with additional melanoma specific molecular markers could increase the accuracy of detection (37).

Our present work suggests that *TRIB2* might not only be useful as a marker to monitor the progression of cutaneous skin lesions but also opens the possibility to test its usefulness for combinational use with other biomarkers to detect circulating tumour cells in lymph nodes or peripheral blood. Furthermore, the striking correlation between the expression level of *TRIB2* and the response to different chemotherapeutic regimens suggests its utility as a biomarker of chemotherapy response. Predictive biomarkers may help to guide therapy in a personalized medicine strategy selecting those patients more likely to benefit from chemotherapy while avoiding toxic effects in those patients not deriving benefit from chemotherapy (38). It should be noted that the current work only lays the groundwork for future large-scale studies in independent data sets and verification in a clinical setting preferentially from clinical trials. These studies should also address the correlation of *TRIB2* with the main clinical and histopathological prognosis factors such as tumour depth, diameter, ulceration, anatomic site and sentinel-lymph node status and ultimately with patient survival. A further understanding of *TRIB2* cellular functions and how these could impact cell transformation and disease progression would identify a wide range of novel therapeutic targets that could significantly reduce the mortality associated with metastatic melanoma.

Supplementary material

Supplementary Tables 1–7 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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