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Transcriptome Profiling Identifies *HMGA2* as a Biomarker of Melanoma Progression and Prognosis

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Abstract

The genetic alterations contributing to melanoma pathogenesis are incompletely defined, and few independent prognostic features have been identified beyond the clinicopathological characteristics of the primary tumor. We used transcriptome profiling of 46 primary melanomas, 12 melanoma metastases, and 16 normal skin (N) samples to find genes associated with melanoma development and progression. Results were confirmed using immunohistochemistry and real-time PCR and replicated in an independent set of 330 melanomas using AQUA analysis of tissue microarray (TMA). Transcriptome profiling revealed that transcription factor *HMGA2*, previously

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

unrecognized in melanoma pathogenesis, is significantly upregulated in primary melanoma and metastases (*P*-values=1.2×10^{-7} and 9×10^{-5}) compared with N. *HMGA2* overexpression is associated with *BRAF/NRAS* mutations (*P*=0.0002). Cox proportional hazard regression model and log-rank test showed that *HMGA2* is independently associated with disease-free survival (hazard ratio (HR)=6.3, 95% confidence interval (CI)= 1.8–22.3, *P*=0.004), overall survival (OS) (stratified log-rank *P*=0.008), and distant metastases–free survival (HR=6.4, 95% CI=1.4–29.7, *P*=0.018) after adjusting for American Joint Committee on Cancer (AJCC) stage and age at diagnosis. Survival analysis in an independent replication TMA of 330 melanomas confirmed the association of *HMGA2* expression with OS (*P*=0.0211). Our study implicates *HMGA2* in melanoma progression and demonstrates that *HMGA2* overexpression can serve as an independent predictor of survival in melanoma.

INTRODUCTION

The incidence of melanoma in the United States continues to increase; melanoma causes three out of four deaths due to skin cancer (Hayat *et al.*, 2007; Siegel *et al.*, 2012). Early-stage melanoma is frequently curable, in contrast to the poorer prognosis of melanoma with regional lymph node involvement and the dismal outcome of widely metastatic disease. The molecular alterations contributing to the pathogenesis of melanoma are incompletely defined, and few independent prognostic features have been identified beyond the clinical and pathological characteristics of the primary tumor. Melanoma is generally resistant to chemotherapeutic and immunological treatments, and the efficacy of adjuvant therapy of melanoma is modest at best. The contribution of these therapies to overall survival (OS) of melanoma patients is limited. Although the management of melanoma is rapidly changing because of a better understanding of molecular heterogeneity and introduction of novel targeted therapies, new biomarkers can offer opportunities to develop prognostic models and new therapeutic targets.

Clinical trials for melanoma currently concentrate on rational drug development with several classes of drugs, including kinase inhibitors that target BRAF*V600, VEGF/ PDGF receptors, and mutated KIT, as well as drugs that target cell survival signaling pathways involving MEK, PI(3)K, or PTEN/AKT (Gray-Schopfer *et al.*, 2007; Nikolaou *et al.*, 2012; Woodman *et al.*, 2012). Identification of new signaling pathways in melanoma initiation and progression opens new opportunities for targeted melanoma therapy. In 2011, vemurafenib and ipilimumab became the first drugs approved by the Food and Drug Administration for the treatment of metastatic melanoma patients with BRAF V600 mutations and inoperable late-stage melanoma, respectively (Curti and Urba, 2012; Woodman *et al.*, 2012).

Microarray analysis of gene expression has been used to reveal sets of coregulated genes and unpredicted biological relationships in many cancer types, including melanoma (reviewed by Hoek (2007); Schramm *et al.* (2012)). Studies with different tumor source (cell lines or fresh tumors), microarray platform, and hypotheses considered reported gene expression profiles in melanoma (Bittner *et al.*, 2000; Hoek *et al.*, 2004; Pavey *et al.*, 2004; Bloethner *et al.*, 2005; de Wit *et al.*, 2005; Haqq *et al.*, 2005; Mandruzzato *et al.*, 2006; Winnepenninckx *et al.*, 2006; Jaeger *et al.*, 2007; Kauffmann *et al.*, 2008; Riker *et al.*, 2008;

Conway *et al.*, 2009). As demonstrated in a meta-analysis of melanoma gene expression studies, the source of studied material (cell lines or tissue) may affect the list of differentially expressed genes (Hoek, 2007). Partial reporting of gene lists with different significance thresholds also contributes to discordant lists. Although cell lines provide a homogenous and reproducible source of melanoma cells, tissue samples may generate results more relevant to clinical use.

Our study used gene expression profiling of primary melanomas, regional and distant melanoma metastases (MM), and normal skin (N) samples to find novel genes associated with melanoma development and progression. We report genes differentially expressed in primary melanoma and MM, including new and previously reported melanoma genes. Furthermore, we demonstrate that transcription factor *HMGA2* is highly overexpressed in melanoma, is strongly associated with regional and distant metastases, and serves as an independent predictor of disease-free survival (DFS) and OS in melanoma.

RESULTS

Sample characteristics for expression microarray and analysis of BRAF/NRAS mutations

Frozen samples were collected from 421 patients out of 7,959 patients treated at the University of Michigan Multidisciplinary Melanoma Clinic. Samples with sufficiently large lesions were selected for the study (*n*=129); this includes frozen tissue from 67 primary melanomas, 20 MM, and 42 N samples collected from 102 patients. There were 27 paired samples from the same excision (primary melanoma-N), and two patients had two distinct primary melanomas. Clinicopathological characteristics of those samples with microarray data that passed quality control (46 primary melanomas, 12 MM, and 16 N samples) were not significantly different from collected samples (Table 1). Median follow-up was 4 years in primary melanomas.

Primary melanomas (*n*=46) had 20 *BRAF*-mutated samples (L597R (*n*=1), L597S (*n*=1), V600R (*n*=1), V600K (*n*=2), and V600E (*n*=15)), 6 *NRAS*-mutated samples (Q61K (*n*=1), Q61R (*n*=3), Q61L (*n*=2)), and 20 samples without mutations in *BRAF* or *NRAS* (Figure 1a). Among MM (*n*=12), eight had mutations in *BRAF* (V600K (*n*=1) and V600E (*n*=7)), two had *NRAS* mutations (Q61K (*n*=2)), and two samples had no mutations in *BRAF*/*NRAS*. *BRAF* and *NRAS* mutations were mutually exclusive in all samples. The observed frequency of *BRAF/NRAS* mutations in primary melanoma (56%) and MM (83%) is consistent with a previous report (Poynter *et al.*, 2006). Mean mutant to wild-type signal ratio was 1.91 for *BRAF* and 1.05 for *NRAS* mutations demonstrating a low level of sample contamination (Supplementary Table S2 online).

Gene expression differences between primary melanoma, MM, and N

Principal component analysis showed that, although the N and metastases could be distinguished by expression profiles, the expression levels of primary melanomas were heterogenous, and principal components were spread across the data matrix (Supplementary Figure S1 online). We effectively separated N and melanoma using gene expression analysis (Supplementary Figure S2 online). Three melanomas *in situ* clustered with N.

Differentially expressed genes were identified in four comparisons: primary cutaneous and MM versus N (PCM+MM vs. N); PCM versus N; MM versus N; and MM versus PCM (Supplementary Table S1 online). Compared with N, upregulated gene lists in both PCM and MM were significantly (*P*-value <1.33E–06) enriched with genes related to nervous system development. The list of genes upregulated in PCM, unlike MM, was significantly (*P*-value <0.0023) enriched with melanocyte development genes. Upregulated genes in MM related to mitosis, cell division, and cell cycle (*P*-value <2.53E–09); cell adhesion (*P*-value <4.36E–07); immune response (*P*-value <4.29E–06); and cell motility and migration (*P*-value <0.0125). In MM versus PCM, upregulated genes were associated with transcription and gene expression (*P*-value <1.71E–04). In all comparisons, downregulated genes were (*P*-value <4.09E–06) enriched with epidermis development, keratinization, and cell adhesion genes (*P*-value <3.03E–04).

In PCM versus N, we found upregulated melanoma stem cell markers (*ABCB5* and *ABCC2*), melanoma-associated tumor antigens (*MAGEA2*, *PRAME*, *ARMC9*, and *SLC45A2*), melanoma progression genes (*MIA*, *GDF15*, *SPP1*, *KMO*, and *S100B*), melanocyte development and melanin biosynthesis genes (*CITED1*, *SILV*, *MLANA*, *TYR*, *MITF*, *PAX3*, and *TFEC*), cell cycle progression genes (*S100A1*), apoptotic genes (*BCL2A1*, *FAIM3*), oncogenic transcription factors (*HMGA2*, *GDF15*, *HOXD13*, and *LZTS1*), and cell adhesion gene (*NRP2*). Obviously, the genes related to keratinocyte differentiation (*KRT25*, *KRT71*, and *KRT85*) appeared downregulated in PCM as compared with N. MM versus N comparison showed upregulated melanoma-associated tumor antigens (*AKT3* and *ARMC9*), melanoma progression genes (*MIA*, *SPP1*, *KMO*, and *IL8*), melanocyte development and melanin biosynthesis genes (*CITED1* and *PAX3*), cell cycle progression genes (*BUB1*), apoptotic genes (*BCL2A1*), oncogenic transcription factors (*HMGA2*, *HOXD13*, *ETV1*, and *ETV5*), and cell adhesion genes (*ADAM10* and *NRP2*). As expected, downregulated genes in MM versus N were involved in keratinocyte differentiation and epidermal development (keratins, sciellin (*SCEL*), and cystatin (*CST6*)). Some cell adhesion genes (*DSC3*, *DSG1*, and *LY6D*), tumor suppressors (kallikreins and *CLCA2*), and cell cycle and apoptosis-regulating genes (*FGFR2*, *BNIPL*, and *TNS4*) were downregulated in MM. Several transcription factors, including *ETV1*, and a melanoma-associated antigen *SPP1* were upregulated in MM as compared with PCM.

To identify new melanoma-associated genes, we focused on the top tenth percentile of upregulated genes with the highest fold change (FC>2.68) in PCM+MM versus N (Supplementary Table S2 online). Within this group, we observed previously characterized melanoma-specific markers (*MAGEA3*, *PRAME*, *SLC45A2*, and *SPP1*), anti-apoptotic *BCL2A1*, actin regulator *PHACTR1*, *SPRYD5* involved in cell growth and differentiation, *TUBB4* involved in cell migration, and finally the transcription factor *HMGA2*, which was the only gene associated with melanoma survival as described below.

HMGA2 expression in N, primary melanoma, and MM

Analysis of relative expression of *HMGA2* in the microarray demonstrated overexpression (higher than median=6.29) in 57% of primary melanomas and 83% of MM compared with N (Figure 1a). Expression of *HMGA2* in N was lower than median in 94% of samples. One N

sample (N307) that had higher than median expression of *HMGA2* was also clustered with melanoma in the supervised cluster analysis. It is important to note that the *HMGA2* expression in N sample N307 was similar to the expression in MEL307 melanoma sample from the same patient.

In the discovery set, we validated the microarray *HMGA2* expression using quantitative real-time reverse transcriptase–PCR (RT-PCR) (Figure 1b). Pearson's correlation coefficient was 0.84 between the expression of *HMGA2* microarray probe 208025_s_at and *HMGA2* expression from real-time RT-PCR ($P<0.0001$). To ensure real-time RT-PCR validity, we also ran RT-PCR for *SPP1*, the well-known melanoma-specific marker (Pearson's coefficient for 1568574_x_at and *SPP1* in RT-PCR was 0.84 ($P<0.0001$)).

Continuous *HMGA2* expression in the microarray was significantly associated with *BRAF*/*NRAS* mutations in PCM and MM (odds ratio (OR)=3.4, 95% confidence interval (CI)=1.2–9.3, $P=0.017$). We confirmed this association in RT-PCR (OR=2.38, 95% CI=1.3–4.2, $P=0.003$) and validated it using melanoma cell lines with known *BRAF/NRAS* mutations. Melanoma cell lines A2058 and SK-MEL-31, heterozygous for *BRAF**V600E, and A375 cell lines, homozygous for *BRAF**V600E, showed overexpressed *HMGA2* comparable to melanoma samples. The CHL-1 without *BRAF* and *NRAS* mutations had no detectable expression of *HMGA2* (Supplementary Figure S3 online).

We also found association of continuous *HMGA2* expression with sentinel lymph node status (OR=2.7, 95% CI=1.1–6.5, $P=0.034$).

Immunohistochemical staining of HMGA2 in N, benign nevi, and melanoma

We validated results of HMGA2 expression at the protein level using immunohistochemical staining on 9 N samples, 41 melanoma samples, and 7 MM that were used for the expression microarray (Figures 1c and 2). All N samples demonstrated no nuclear staining. Melanomas with overexpressed HMGA2 had strong nuclear staining. Melanomas with moderate HMGA2 expression were heterogenous, possessing a variable number of cells with moderate nuclear staining. Six out of seven MM had nuclear HMGA2 staining. Twenty benign nevi showed no nuclear staining against HMGA2.

Effect of HMGA2 expression on survival end points in melanoma

To evaluate the correlation of *HMGA2* overexpression with survival of melanoma patients, we categorized expression levels of *HMGA2* as high (above the median 6.29) and low (below the median). Multivariate analysis demonstrated a significant association of *HMGA2* overexpression (≥6.29) with regional (OR=10.0, 95% CI=1.5–66.6, $P=0.0176$) and distant metastases (OR=6.8, 95% CI=1.2–39.0, $P=0.0318$) after adjusting for American Joint Committee on Cancer (AJCC) stage and age at diagnosis. Survival analysis showed that expression of *HMGA2* above the median was significantly associated with DFS (hazard ratio (HR)=6.3, 95% CI=1.8–22.3, $P=0.004$), OS (stratified log-rank $P=0.008$), and distant metastases–free survival (DMFS) (HR=6.4, 95% CI=1.4–29.7, $P=0.018$) after adjusting for AJCC stage and age at diagnosis (Figure 3a). Immunohistochemical staining against HMGA2 was also associated with DFS after adjusting for AJCC stage and age at diagnosis

(HR=4.8, 95% CI=1.25–18.1, P=0.0221) (Figure 3b). Leave-five-out cross-validation confirmed these results, with a mean HR of 6.76 for DFS (99.9% of resamples with P<0.05) and a mean HR of 7.12 for DMFS across the 1,000 replicates (90.8% of resamples with P<0.05).

HMGA2 expression and melanoma survival in an independent melanoma replication set

An independent replication tissue microarray (TMA) comprising 580 melanomas (Gould Rothberg *et al.*, 2009a) was used to replicate the association of *HMGA2* expression with survival. Only OS was analyzed because data regarding local recurrence and regional metastases were not available. HMGA2 expression was evaluated using AQUA scores. Although 506 passed quality control, survival data were available for only 330 melanomas.

To estimate OS in the replication TMA, we compared survival between melanomas with 75% quartile of HMGA2 expression measured by AQUA to 25% quartile. We found significant association of HMGA2 overexpression with reduced OS of melanoma patients after adjustment for AJCC stage and age at diagnosis (HR=1.72, 95% CI=1.09–2.73, P=0.0211) (Figure 4).

DISCUSSION

Our study used fresh frozen tissue from patients with newly diagnosed melanoma to compare gene expression and identify new melanoma-associated genes. Some of the previous melanoma expression studies used tissue samples (Haqq *et al.*, 2005; Talantov *et al.*, 2005; Mandruzzato *et al.*, 2006; Winnepenninckx *et al.*, 2006; Conway *et al.*, 2009), but the majority of studies used cell lines. Although fresh tissue analysis is a strength of our study, relatively small sample size and a large number of melanomas with Breslow depth >2mm limit generalization of our findings. We tried to address this issue by replicating our results in an independent melanoma set.

Gene lists generated by our study include both well-known melanoma genes and novel genes that have not previously been reported in relation to melanoma development. One of the new genes is a transcription factor *HMGA2* that was significantly upregulated in both primary melanoma and MM (P-values=1.2×10^{-7} and 9×10^{-5}) compared with N. Moreover, *HMGA2* overexpression in primary melanoma was significantly associated with survival of melanoma patients, probably explained by the increased metastatic potential of *HMGA2*-expressing tumors. The oncogene *HMGA2* is an embryonic architectural transcription factor that is completely silenced or undetectable in normal adult tissues, but has a significant role in the transformation of many cancer types (Miyazawa *et al.*, 2004; Meyer *et al.*, 2007; Malek *et al.*, 2008). HMGA2 binds to the minor groove of DNA in AT-rich regions using AT-hook sequences, changes DNA conformation, and facilitates binding of other transcription factors (Reeves, 2001). The oncogenic role of HMGA2 has not been previously recognized in the pathogenesis of melanoma. However, HMGA2 has been well documented in other types of cancer, where it can be overexpressed, amplified, or fused with other proteins (Fusco and Fedele, 2007).

A large study examining gene expression in 83 PCMs identified a 254-gene prognostic signature, but *HMGA2* was not among these genes (Winnepenninckx *et al.*, 2006). Re-examination of the data shows that *HMGA2* was indeed associated with melanoma thickness, supporting our conclusion about biological relevance of *HMGA2* in melanoma. Another study of nine melanoma cell lines demonstrated 2.96-fold overexpression of *HMGA2* compared with normal human melanocytes (Hoek *et al.*, 2004). Contributing to previously published transcriptome studies of melanoma, our results offer new insights into the pathogenesis of melanoma by highlighting the differential expression and prognostic importance of *HMGA2*.

Several mechanisms may underlie the oncogenicity of HMGA2, including the activation of transcription factor E2F1 through binding of HMGA2 to pRB (Fedele *et al.*, 2006), direct or indirect induction of cyclin A (Pagano *et al.*, 1992), or negative regulation of nucleotide excision repair genes (Borrmann *et al.*, 2003). The chemokine *CXCL1*, which is overexpressed in melanoma and is involved in melanoma progression, has been shown to be regulated by HMGA2 (Nirodi *et al.*, 2001). It is noteworthy that both *CXCL1* and *HMGA2* were significantly overexpressed in melanoma in our study. TGF-beta mediates epithelial–mesenchymal transition by inducing HMGA2 through the SMAD pathway, which may partially explain the association between *HMGA2* overexpression and MM in our study (Thuault *et al.*, 2006). HMGA2 also enhances the NF-kB complex formation (Noro *et al.*, 2003).

HMGA2 expression is negatively regulated by the miRNA let-7 family (Peng *et al.*, 2008). Loss of expression of *let-7* increases the expression of *c-Myc*, *RAS*, *CDK4*, *integrin-β(3)*, and *HMGA2* (Johnson *et al.*, 2005; Park *et al.*, 2007; Muller and Bosserhoff, 2008; Schultz *et al.*, 2008). Clusters of *let-7a-1_let-7f-1_let-7d* are located in 9p22.3, which is deleted in 81% of cases of cutaneous melanoma (Bastian *et al.*, 1998). The MAPK pathway, activated by *BRAF/NRAS* mutations, negatively regulates *let-7* by inducing *LIN28* expression through *Myc* transcription (Dangi-Garimella *et al.*, 2009). This mechanism may explain the association of *HMGA2* overexpression with *BRAF/NRAS* mutations in our study. A recent study showed that *let-7* is downregulated in highly invasive melanoma cell lines (Mueller *et al.*, 2009).

Activated oncogenes in normal cells trigger senescence as a key protective mechanism against cancer (Mooi and Peeper, 2006). For example, *INK4/ARF* products are essential activators of p53-dependent and p53-independent melanocyte senescence (Bennett and Medrano, 2002). In addition, oncogenic BRAF and NRAS can induce *p16* expression and senescence in melanocytes *in vitro* (Gray-Schopfer *et al.*, 2006). During melanoma progression, *c-Myc* overexpression continuously suppresses BRAF*V600E- and NRAS*Q61R-dependent senescence programs, independently of p16 and p53 senescence mechanisms (Zhuang *et al.*, 2008).

HMGA2 is also required for normal proliferation and self-renewal of fetal and young adult neural stem cells through repression of the *INK4/ARF* locus; however, during aging, *let7b* blocks *HMGA2* and contributes to declining neural stem cell function (Tzatsos and Bardeesy, 2008). We speculate that BRAF- and NRAS-activated senescence is overridden

by both p16- and p53-independent *c-Myc* overexpression and by HMGA2 repression of p16- and p53-induced apoptosis and cell senescence. It is noteworthy that *HMGA2* is directly regulated by *c-Myc* (Wood *et al.*, 2000).

Overexpressed *HMGA2* correlates with amplification of 12q harboring *HMGA2* in gastric cancer, liposarcoma, and carcinoma ex pleomorphic adenoma (Yang *et al.*, 2007; Persson *et al.*, 2009). This correlation was associated with copy number variability in other tumor suppressor genes and oncogenes (*MDM2* and *CDK4)*, showing a specific pattern in tumor subtypes.

HMGA2 analysis may have some important clinical applications. Observational noninvasive clinical trials are needed to further estimate the prognostic value of *HMGA2* expression in melanoma. Although immunohistochemistry and quantitative PCR are still the methods of choice, recent improvements in next-generation sequencing technology may introduce targeted RNAseq for routine testing of *HMGA2* expression in melanoma. The melanoma patients with overexpressed *HMGA2* might benefit from more aggressive treatment and closer follow-up. In addition, HMGA2 may be a potential therapeutic target, as *HMGA2* expression seems to be non-essential for normal cell survival, and therapeutic gene silencing would be relatively safe. This approach has been successful in ovarian carcinoma, where short-hairpin RNA silencing of *HMGA2* inhibited cell proliferation, with G1 cell cycle arrest and increased apoptosis *in vitro*, as well as tumor growth inhibition *in vivo* (Malek *et al.*, 2008).

Our study highlights a new aspect of melanoma biology by implicating the well-known oncogene HMGA2 in melanoma progression. We propose that HMGA2 has an important role in melanoma development and serves as an independent prognostic factor. Further studies of HMGA2 in melanoma are warranted to better understand its role in melanoma development and progression.

MATERIALS AND METHODS

Tissue samples

Our report was written to comply with REMARK criteria (McShane *et al.*, 2005; Gould Rothberg *et al.*, 2009b); therefore, we provide relevant information about study design, hypotheses, patient and specimen characteristics, assay methods, and statistical analysis methods. In addition, we conducted a replication study.

Frozen cutaneous melanomas were obtained from the University of Michigan Multidisciplinary Melanoma Clinic where 7,959 patients were treated from 2002 through 2008. The study has been approved by the institutional review board. Samples were selected from 421 frozen primary or metastatic samples including pediatric and non-Caucasian cases consecutively collected during this period. All participating patients received standard-of-care treatment with surgical excision of lesions. OS, DFS, and DMFS data were collected from chart reviews, University of Michigan Cancer Tumor Registry, and by communication with the patient or his/her family. OS was confirmed using the National Death Index.

Detailed description of sample preparation, isolation of RNA, DNA and microarray hybridization, as well as mutational analysis, quantitative RT-PCR, and immunohistochemistry, can be found in Supplementary Methods online.

For independent replication of survival analysis, we obtained well-annotated melanoma TMA slides from Dr David L Rimm (Yale University) comprising 580 primary melanomas and MM. The available de-identified data included clinicopathological features, OS, and demographic characteristics.

Statistical analysis

Microarray expression data were analyzed using Bioconductor for R (Gentleman *et al.*, 2004). Detailed description of statistical analysis of the expression microarray can be found in Supplementary Materials online.

Kaplan–Meier survival curves and log-rank tests were used to analyze OS, DFS (without local recurrence, regional, or distant metastases), and DMFS. Multivariate Cox proportional hazard regression model was adjusted for AJCC stage (stages I/ II vs. stage III) and age at diagnosis. Wald tests based on maximum likelihood estimates of the log-HR parameters in the Cox model were deemed significant at $P<0.05$. OS was evaluated by the stratified log-rank test adjusted for AJCC stage and age at diagnosis because of sparsity of events. Leave-five-out cross-validation (1,000 random permutations of 46 melanomas) evaluated the consistency and robustness of the survival analysis. We use multivariate logistic regression to estimate the association between *HMGA2* expression and metastases, and linear regression for the association between *HMGA2* expression and *BRAF/NRAS* mutations. All analyses were performed in SAS 9.1 (SAS Institute, Cary, NC) using FREQ, LIFETEST, PHREG, and LOGISTIC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CI	confidence interval
DFS	disease-free survival
DMFS	distant metastases–free survival

HR	hazard ratio
MM	melanoma metastases
N	normal skin
OR	odds ratio
OS	overall survival
PCM	primary cutaneous melanoma
REMARK	reporting recommendations for tumor marker prognostic studies
RT-PCR	reverse transcriptase–PCR
TMA	tissue microarray

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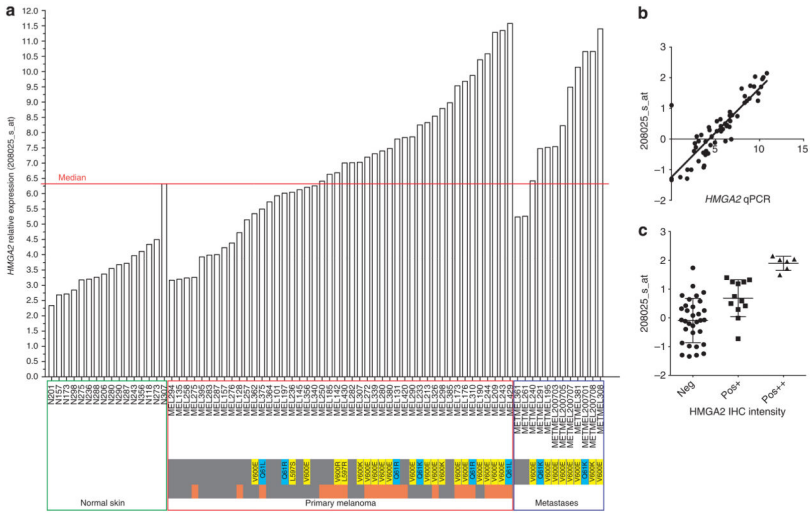

Figure 1. Expression of HMGA2 in normal skin, primary melanoma, and melanoma metastases

(**a**) Overexpression of *HMGA2* probe 208025_s_at was considered above the median (6.29). Orange–gray bar represents primary melanomas that metastasized later (orange). *BRAF* mutations are shown in yellow and *NRAS* mutants are showed in blue bars. (**b**) Pearson correlation between microarray and quantitative PCR *HMGA2* expression data (0.84, $P<0.0001$). (**c**) Correlation between *HMGA2* expression data from microarray and nuclear immunohistochemistry (IHC) intensity staining against HMGA2 (neg, negative staining; pos +, moderate intensity; pos+ +, strong intensity).

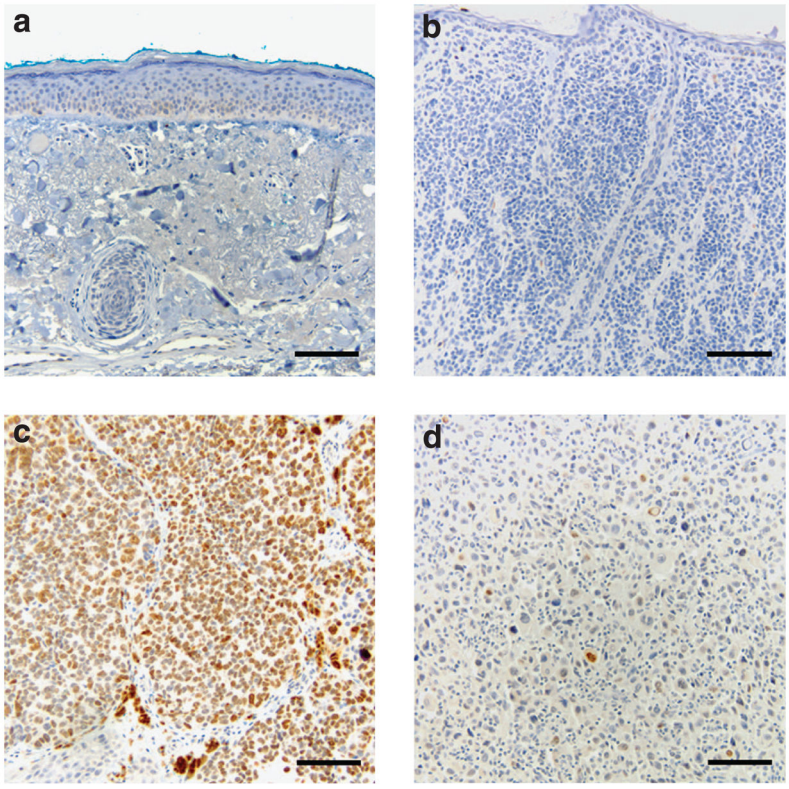

Figure 2. Representative sections with immunohistochemical staining against HMGA2

(**a**, **b**) Normal skin and benign nevus have no nuclear staining, (**c**, **d**) whereas both primary melanoma (diffuse) and melanoma metastases (patchy) have strong nuclear staining against HMGA2. Bar=50 μm.

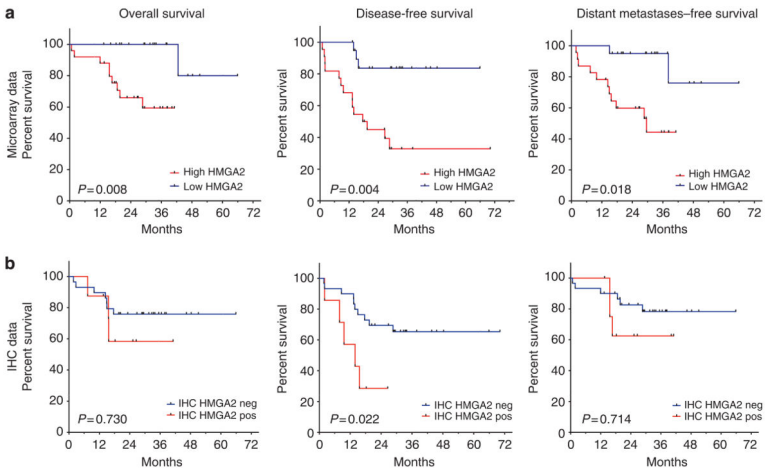

Figure 3. Kaplan–Meier curves for overall, disease-free, and distant metastases–free survival associated with *HMGA2* expression in 46 primary melanomas

Cox proportional hazard regression adjusted for American Joint Committee on Cancer stage and age at diagnosis was used for analysis, except for overall survival from microarray data, where stratified log-rank test was used because of sparsity of events. (a) In microarray expression data, *HMGA2* overexpression was significantly associated with overall survival (*P*=0.008), disease-free survival (hazard ratio (HR)=6.29, *P*=0.004), and distant metastases–free survival (HR=6.41, *P*=0.018). (b) In immunohistochemistry (IHC) expression data, *HMGA2* overexpression was significantly associated with disease-free survival (HR=4.76, *P*=0.022) and showed the same direction of association for overall and distant metastases-free survival as microarray data, although not statistically significant.

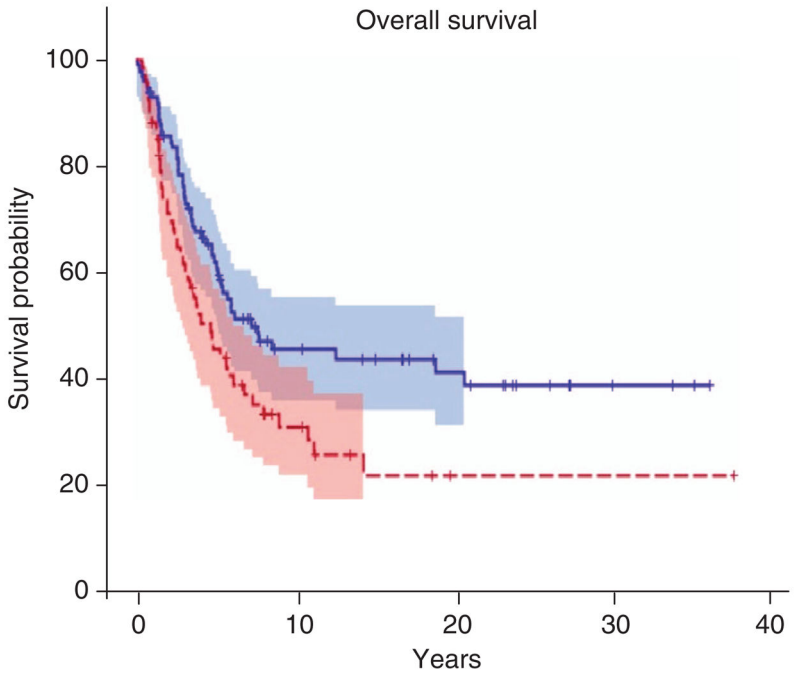

Figure 4. Kaplan–Meier curves for overall survival associated with the expression of HMGA2 protein in an independent set of 330 melanomas

HMGA2 expression was studied in melanoma tissue microarray (TMA) and analyzed by AQUA. HMGA2 expression was compared between 75% quartile (red) and in 25% quartile (blue). Overall survival was analyzed using stratified multivariate log-rank test including age at diagnosis and American Joint Committee on Cancer stage. HMGA2 overexpression was significantly associated with reduced overall survival of melanoma patients (hazard ratio=1.72, P=0.0211).

Table 1

Clinicopathological characteristics of study

Characteristics[1]	Discovery set (*n* =74)	Replication set (*n* =330)	*P*-value
Normal tissue (*n*)	16	—	
Primary melanoma (not metastatic) (*n*)	46	330	
Melanoma metastases (*n*)	12	—	
Mean age, years (range)[2]	59.4 (20–92)	54.5 (48–87)	0.0072
Median Breslow thickness, mm (range)	3 (0.6–19)	2.45 (0.25–16.3)	0.5898
Sex, no. (%)			0.0916
Male	33 (72%)	183 (56%)	
Female	13 (28%)	147 (44%)	
Site, no. (%)			0.2810
Head and neck	10 (22%)	35 (14%)	
Trunk	12 (26%)	81 (32%)	
Legs	19 (41%)	79 (32%)	
Arms	10 (22%)	48 (19.2%)	
Others[3]	0	7 (3%)	
Not available	0	80	
Melanoma type, no. (%)			0.5897
Superficial spreading melanoma	28 (61%)	88 (64%)	
Nodular melanoma	8 (17%)	26 (19%)	
Acrolentiginous melanoma	3 (7%)	7 (5%)	
Lentigo maligna melanoma	1 (2%)	2 (2%)	
Desmoplastic melanoma	1 (2%)	1 (1%)	
Nevoid melanoma	0	1 (1%)	
MIS	2 (4%)	0	
Unclassified	0	0	
Others[4]	3 (7%)	12 (9%)	
Not available	0	193	
AJCC stage at diagnosis			0.2390
0	2 (4%)	0	
I	10 (22%)	191 (69%)	
II	16 (36%)	11 (4%)	
III	18 (40%)	60 (22%)	
IV	0	14 (5%)	
Not available	0	54	
Ulceration, no. (%)			0.2516
Absent	28 (61%)	83 (61%)	
Present	18 (39%)	54 (39%)	

Characteristics[1]	Discovery set (*n* =74)	Replication set (*n* =330)	*P*-value
Not available	0	193	
Mitotic rate, no. per mm²			—
0	13 (28%)	—	
1–6	19 (41%)	—	
>6	13 (31%)	—	
Not available	0	330	
Median follow-up, days	875	1,602	<0.0001

Abbreviation: AJCC, American Joint Committee on Cancer.

[1] The characteristics are presented for primary melanomas only.

[2] Age of the patients calculated at the first biopsy.

[3] Oral, male genital, and female vaginal location.

[4] Polypoid, mucosal melanoma, spindle-cell melanoma, Spitz-type melanoma, and amelanotic melanoma.