HER3 is a Determinant for Poor Prognosis in Melanoma

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Abstract

Purpose: The epidermal growth factor receptor (EGFR) family member HER3 is overexpressed in diverse human cancers and has been associated with poor prognosis in breast-, lung- and ovarian cancer. However, the relevance of HER3 with regard to its prognostic significance and function in primary melanoma and metastases remains largely elusive.

Experimental Design: HER3 protein expression was analyzed immunohistochemically using tissue micro arrays of 130 primary melanoma and 87 metastases relative to established clinical parameters. The possibility of an influence of HER3 on melanoma cell proliferation, migration, invasion and chemotherapy-induced apoptosis was studied in human melanoma cell lines.

Results: We demonstrate that HER3 is frequently expressed in malignant melanoma and metastases at elevated levels. High HER3 expression may serve as a prognostic marker since it correlates with cell proliferation, tumor progression and reduced patient survival. Suppression of *HER3* expression by RNAi reduces melanoma cell proliferation, migration and invasion *in vitro*. In addition, down-regulation of *HER3* synergistically enhances dacarbazine-induced apoptosis. Moreover, monoclonal antibodies specific for the extracellular portion of HER3 efficiently block heregulin-induced proliferation, migration and invasion of melanoma cell lines.

Conclusion: Our results provide novel insights into the role of HER3 in melanoma and point out new possibilities for therapeutic intervention.

Introduction

Melanoma is a common type of skin cancer, which develops from the malignant transformation of melanocytes, and accounts for 80% of deaths arising from skin cancer (1). The underlying molecular mechanisms of melanocyte transformation have been studied extensively in the past (2, 3). However, to date no drugs are available that significantly prolong patient survival once melanoma progresses to the metastatic state (4). Thus, there is an urgent need for novel therapeutic agents and prognostic markers in the treatment of melanoma patients.

The human EGF receptor (HER) family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, -migration, -invasion and -survival (5). The family consists of four members: EGFR (HER1), HER2 (neu or ErbB2), HER3 (ErbB3) and HER4 (ErbB4). To date eleven ligands have been reported including epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGFα), amphiregulin (AR), epiregulin, betacellulin and the heregulins. These ligands bind directly to their cognate receptors, which leads to the formation of receptor homo- or heterodimers that trigger the activation of multiple signaling pathways (6). Dysregulation of members of the HER-family either by activating mutations, receptor overexpression or aberrant ligand release leads to the development of a variety of human tumors (7). HER3 is over expressed in breast-, ovarian- and lung cancer and this genetic feature has been correlated with poor prognosis (8 - 10). Upon activation by heregulins, HER3 dimerizes with HER2 and EGFR to form potent oncogenic receptor heterodimers (11 - 13). Within this complex, HER3 preferentially recruits PI3-kinase to its cytoplasmic docking sites thereby regulating cell proliferation and -survival (14, 15). So far it was assumed that HER3 is kinase-inactive due to apparently aberrant sequence characteristics in its kinase domain and that it requires heterodimerization with a

kinase-intact member of the HER-family in order to initiate signaling events (16). Consistent with this, it was shown that HER2 requires HER3 to drive breast tumor cell proliferation (17). However, recent findings of Htuhn van der Horst and coworkers showed that HER3 is able to phosphorylate Pyk2 which results in the activation of the MAPK pathway in human glioma cells (18). Furthermore, monoclonal antibodies specific for HER3 can inhibit the proliferation and migration of cancer cell lines (19). Interestingly, it was shown recently that cancer cells escape HER-family inhibitor therapy by up-regulation of HER3 signaling (20) and that HER3 inhibition abrogates HER2-driven tamoxifen resistance in breast cancer cells (21). Moreover, resistance to Gefitinib (Iressa) therapy, an EGFR small molecule inhibitor, was shown to be connected to HER3 signal activation (22).

In this study we analyzed the expression and prognostic significance of HER3 in malignant melanoma. We show that HER3 is up-regulated in melanoma and that high HER3 levels correlate with proliferation, tumor progression and reduced overall patient survival. In addition, we demonstrate that HER3 regulates proliferation, migration, invasion and cell survival of human melanoma cell lines. Moreover, blocking HER3 activation with specific monoclonal antibodies substantially reduced the proliferation, migration and invasion of these cell lines *in vitro*. In summary, our findings support a prognostic relevance of HER3 in melanoma and validate this HER-family member as a potential target for the development of novel cancer therapies.

Material and Methods

Melanoma Patients

Formalin-fixed, paraffin embedded tissue of 130 primary cutaneous melanoma and 87 metastases was immunohistochemically analyzed for HER3. The patient age

ranged from 19 to 90 years. Clinical follow-up was available in all of the patients (mean clinical follow up was 56 ± 25 months). There were 60 nodular (NMM), 42 superficial spreading (SSM), 3 lentiginous (LMM), 9 acral lentiginous (ALM) and 16 not otherwise specified (NOS) melanoma. All melanoma had a Breslow tumor thickness between 0.4 and 17 mm. 53 of 130 patients (41%) had metastases during follow up and 24 of 130 patients (18%) died. Matched tumor samples of primary melanoma and metastases were available for 20 patients. 54 of the 130 patients with primary cutaneous melanomas were previously reported in a sentinel lymph node study (23). Approval was obtained from a local Institutional Ethical Committee and written informed consent signed by all study participants.

Tissue micro array construction and Immunohistochemistry

A morphologically representative region of a paraffin “donor” blocks was chosen to prepare the melanoma tissue micro arrays. The representative region was taken with a core tissue biopsy (diameter: 0.6 mm; height: 3-4 mm) and precisely arrayed into a new “recipient” paraffin block using a customer built instrument (24). After the block construction was completed, 4.0 µm sections of the resulting tumor tissue micro array block were cut with a microtome and used for further analysis.

HER3 and Ki-67 immunohistochemistry was performed using a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona). For antigen retrieval, slides were heated with cell conditioner 1 (standard procedure). Endogenous biotin was blocked with the appropriate kit. Primary antibodies against HER3 (Santa Cruz, clone C-17, dilution 1:50) and Ki-67 proliferation antigen (clone MIB-1, dilution 1:20 were applied and revealed with the iVIEW DAB detection kit, yielding a brown reaction product. The signal was enhanced with the Ventana amplification kit. Slides were counterstained with hematoxylin prior to glass cover-

slipping. The specificity of the staining was controlled by using iso-type antibody controls, secondary antibody controls or blocking peptides. The analysis of the tissue micro arrays was done using a Zeiss Axiovert 300 microscope.

Evaluation of HER3 expression

To determine the expression frequencies of HER3, a semi-quantitative scoring system was applied following the German Immunohistochemical scoring (GIS) system in which the final immuno-reactive score equaled the product of the percentage of positive cells times the average staining intensity. Percentage of positive cells was graded as follows: 0 = negative, 1 = up to 10% positive cells, 2 = 11 to 50%, 3 = 51 to 80%, 4 = >80%. Staining intensity of 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive (25 - 27). The extent of KI-67 staining was recorded as the Ki-67 Labeling Index (number of marked nuclei per 100 melanoma cells). All stainings were evaluated by two different investigators (M.R. and D.M.-P.).

Statistical analysis

The protein expression frequency for HER3 was analyzed by dividing the GIS score in three groups with GIS 1-4 = no/low, GIS 5-8 = moderate and GIS 9-12 = high expression. For statistical analysis, two groups were considered which were divided through GIS-dichotomization at the median (low: GIS ≤6 and high: GIS >6). Correlations between HER3 and Ki-67 were analyzed using Spearman's rank correlation. The overall survival of melanoma patients was estimated by the Kaplan Meier method and differences between groups were assessed by the log-rank test. The overall survival was defined as the time of primary tumor diagnosis to the last follow-up visit or patient death. All p-values were calculated using the two-sided

Fisher's exact Test or the paired Student's T-Test and p-values < 0.05 were considered statistically significant. A multivariable Cox regression model was adjusted, testing the independent prognostic relevance of HER3 in melanoma patients. The following clinical variables were considered: age (≤ 60 years vs. > 60 years), sex (male vs female), tumor thickness (≤ 2mm vs > 2mm) and metastases during follow up. The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions. For the analysis of matched tumor-metastases pairs, the GIS values of HER3 in primary melanoma and melanoma metastases were directly compared and analyzed. The statistical analysis was performed with the SPSS 12.0 software (SPSS Inc., Chicago IL).

Cell culture and compounds

Human Colo 829 melanoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, 1% sodium bicarbonate, 10mM hepes and 4,5g/l glucose. Human MM-358, Mel Gerlach and Mel Juso melanoma cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. Dacarbazine and propidium-iodide were purchased from SIGMA. Heregulin ß1 was purchased from R&D Systems and diluted in PBS prior to use.

RNA interference

HER3 siRNAs were obtained from AMBION. Two independent siRNAs were used in all experiments. Sequences for *HER3* siRNAs were sense1 5' GGCUAUGUCCUCGUGGCCAtt 3', antisense1 5' UGGCCACGAGGACAUAGCCtg 3' and sense2 5' GGCAGUGUGUCCUGGGACUtt 3', antisense2 5'

AGUCCCAGGACACACUGCCtg 3'. The *GL-2* siRNA (Dharmacon) was used as a negative control in all experiments. *GL-2*_sense 5' CGUACGCGGAAUACUUCGAtt 3', *GL-2*_antisense 5' UCGAAGUAUUCCGCGUACGtt 3'. Transfection of siRNAs was done using Oligofectamine (Invitrogen, CA) according to manufacturer's recommendation.

Antibodies, RT-PCR and Western Blot analysis

Antibodies against p-HER3 (Tyr 1289), p85, p-AKT (Ser473), CyclinB1, p-ERK1/2, p-mTOR (Ser2448) and p-Rb (Ser780) were all purchased from Cell Signaling (Beverly, MA). HRP-conjugated rabbit secondary antibodies were from BioRad. Anti-Tubulin, anti-ß-actin and HRP-conjugated mouse secondary antibodies were from SIGMA. The anti-HER3 (clone 2F12) antibody was from Upstate and anti-HER3 (C-17) for Immunohistochemistry as well as Akt 1/2 (H-136) were from Santa Cruz. Anti-p27 was purchased from Abcam. Western blot analysis and Immunoprecipitaions were done as described previously (28). Total RNA was isolated using the RNeasy Mini Kit (Quiagen) and c-DNA was synthesized using the AMV Reverse Transcriptase (Roche) according to manufacturer's recommendations. RT-PCR primers for HER3: HER3_fwd 5' CTCCGCCCTCAGCCTACCAGTT 3' and HER3_rev 5' TGCTCCGGCTTCTACACATTGACA 3' (Tm=64°C) and for Tubulin: Tubulin_fwd; 5' AAGTGACAAGACCATTGGGGGAGG 3' and Tubulin_rev 5' GGGCATAGTTATTGGCAGCATC 3' (Tm = 55°C). All PCR reactions were done in an Eppendorf thermocycler (Eppendorf).

Proliferation Assay

75000 or 250000 cells were seeded in 24 well or 6 cm plates and transfected with *HER3* or *GL-2* siRNAs using oligofectamine (Invitrogen). The cells were either grown

in the presence of medium containing 10% FCS or serum-starved in medium containing 1% FCS and stimulated with 100ng/ml heregulin ß1. The cell number was counted (Coulter counter, Beckton Dickinson) at the indicated time points. The data are shown as mean ± SDM.

Migration and Invasion Assay

200.000 cells were seeded in 6 well plates and transfected with *HER3* or *GL-2* siRNAs using oligofectamine (Invitrogen). The cells were serum-starved in medium containing 0,1% FCS for 24 hours and 25.000 cells were either seeded on to a membrane or on to a growth factor reduced matrigel coated membrane with 8 µM pores of a modified boyden chamber (Schubert and Weiss) containing 500µl serum-free medium. 10% fetal calf serum served as chemo attractant. The cells were allowed to migrate or invade for 20 or 24 hours, respectively. Migrated or invaded cells were stained with crystal violet, washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification cells in at least 10 random fields were counted. The values for control cells were set to 100% and results are shown relative to controls. The data are shown as mean ± SDM.

HER3 blocking antibody – Proliferation, Migration and Invasion experiments

The HER3 blocking antibody (cl. 105.5) was purchased from Upstate, NY. The second HER3 blocking antibody (cl. 2D1D12) was generated in the Department of Molecular Biology at the Max-Planck Institute of Biochemistry. The HER2 blocking antibody (cl. 4D5) used was the non-humanized form of trastuzumab.

The proliferation assay was performed using a MTT-Assay. Briefly, 7500 cells were seeded in 48 well plates. The cells were serum-starved in medium containing 1% FCS for 24 hours, pre-incubated with either 10µg/ml HER3 blocking antibody (cl.

105.5) or an isotype control antibody for 1 hour and stimulated with 100ng/ml heregulin ß1. The cells were allowed to grow for 48 hours and at that time point, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolimbromide; thizolyl blue, Sigma, Taufkirchen) was added to each well at a final concentration of 1mg/ml. The plates were incubated for 2 hours. The yellow MTT dye is reduced by mitochondrial dehydrogenase activity to a purple formazan, which was then solubilized (SDS, 2-Butanol and HCl) and absorbance was measured at 570nm on a micro-plate reader. MDA-MB 468 breast cancer cells were already described to be inhibited by the anti-HER3 antibody (cl. 105.5) (19) and served as a positive control. The data are shown as percent of inhibition relative to control cells.

The migration and invasion assays were performed as described previously (19, 29). Briefly, 300.000 cells were seeded in 6 cm plates and serum-starved in medium containing 0,1% FCS for 24 hours. 200.000 cells per ml were incubated with 10µg/ml HER3 blocking antibody or an isotype control antibody for 1 hour and 50.000 cells were then seeded either on to a membrane or on to a growth factor reduced matrigel coated membrane with 8 µM pores of a modified boyden chamber (Schubert and Weiss) containing 500µl serum free medium. Conditioned NIH 3T3 medium containing 0.01% ascorbic acid and heregulin ß1 (100ng/ml) were used as a chemo attractant. The cells were allowed to migrate or invade for 20 or 24 hours, respectively. Migrated or invaded cells were stained by crystal violet, washed in PBS and analyzed using a Zeiss Axiovert 300 microscope. For quantification at least 10 random fields were counted. The values for control cells were set to 100% and results are shown relative to controls. The data are presented as mean ± SDM.

To asses the HER3 phosphorylation state, cells were serum-starved for 24 hours, incubated with 10µg/ml blocking antibody for 1 hour, stimulated with 50ng/ml

heregulin ß1 for 2 hours, lysed and subjected to Immunoprecipitations using a specific HER3 antibody.

Apoptosis Assay (Propidium Iodide staining)

200.000 cells were seeded into 6 well plates (Nunc) and transfected with *HER3* or *GL-2* siRNAs. Apoptosis was induced by adding either 10 or 20µM dacarbazine in DMSO to the medium. After 48 hours the supernatant of each reaction was collected and the cells were trypsinized. After centrifugation the cells were incubated for two hours in a propidium-iodide buffer (0.1% Na-Citrate, 0.1% Triton X-100, 20µM Propidium-Iodide) and thereafter subjected to flow cytometric analysis (Beckton-Dickinson Biosciences). Apoptotic cells were identified as the sub G0/G1 peak and quantified using the Cell Quest Pro software (Beckton Dickinson).

Results

HER3 is frequently expressed in primary melanoma and metastases

We investigated HER3 protein expression in 130 primary malignant melanoma and 87 metastases using tissue micro arrays. HER3 immuno reactivity was accentuated at the cell membrane. In primary melanoma, no or low HER3 expression was found in 45 out of 130 cases (35%) (Fig. 1A). Strikingly, moderate to high HER3 expression levels were found in 85 of 130 cases (65%) (Fig. 1B and 1C). Furthermore, HER3 was highly expressed in 35 of 87 melanoma metastases (40%) (Fig. 1D). Taken together, HER3 is frequently expressed in primary melanoma and metastases indicating that HER3 may contribute to melanoma development and progression (Fig. 1E). Importantly, HER3 expression was undetectable in primary melanocytes (Fig. 1F). Interestingly, moderate to high HER3 expression significantly correlated with

increased tumor cell proliferation (Ki-67 Labeling Index) in primary malignant melanoma (p=0,008; data not shown).

HER3 expression in melanoma progression

To investigate the relevance of HER3 expression in primary melanoma we analyzed the relationship between HER3 and clinical parameters. In addition, matched tumor samples of primary melanoma and metastases were studied in 20 patients. Remarkably, 10 of 20 (50%) patients showed an increase of HER3 expression in the metastases compared to the primary tumor. Six of 20 (30%) matched tumor samples showed a similar expression of HER3 and only 4 of 20 (20%) patients showed less HER3 expression in the metastases when compared to the primary tumor (Fig. 2A). These results show that in a majority of cases HER3 expression remains either stable or even increases during disease progression.

Importantly, Kaplan-Meier analysis showed that HER3 expression was significantly associated with tumor specific survival (p=0,014) (Fig. 2B). In a multivariate analysis, an adjusted Cox regression model was developed for the assessment of the overall survival rate. The characteristics of the variables are shown in Table 1. The clinical variables used in the analysis were age, sex, metastases during progression, tumor thickness and HER3 expression (HER3 Immunohistochemistry score). In this model, metastases (p=0,000) and HER3 expression (p=0.041) were correlated with poor prognosis. The hazard ratio for death from melanoma concerning HER3 status was 2.6 (95% Confidence Interval: 1,042-6,671); accordingly, in cases with high HER3 expression, the probability of tumor-related death was almost three times higher than in cases with low HER3 staining. Because of the assumption of proportional hazards, the probability of melanoma-related death was consistently valid during the entire observation period. Notably, HER3 expression did not significantly correlate with any

other clinical parameters (e.g.: tumor thickness, ulceration) tested. Taken together, these results indicate that HER3 is a critical parameter for melanoma prognosis and progression.

HER3 knock-down inhibits melanoma cell proliferation

In order to further characterize the role of HER3 in melanoma, we down-regulated *HER3* by specific siRNAs in Colo 829, MM-358, Mel Gerlach and Mel Juso human melanoma cell lines (Fig. 3A, Suppl.-Fig. 1A and 2A). Strikingly, depletion of *HER3* strongly inhibited proliferation in these cell lines (Fig. 3B, Suppl.-Fig. 1A and 2B). To further characterize the mechanism of the growth inhibition we analyzed proteins implicated in cell cycle control. As shown in Fig. 3B, *HER3* knock-down led to increased p27 levels, reduced Cyclin B1 levels and decreased Rb phosphorylation suggesting that HER3 controls melanoma cell proliferation by interfering with key cell cycle regulators. In addition, *HER3* knock-down strongly inhibited heregulin-induced cell proliferation (Fig. 3B and Suppl.-Fig. 1A). Importantly, HER3 predominantly signals via the PI3K-AKT pathway in the regulation of cell proliferation and -survival. Indeed, AKT activation was impaired in heregulin ß1 stimulated knock-down cell lines indicating that HER3 may signal via the PI3K-AKT pathway in melanoma cells (Fig. 3C and Suppl.-Fig. 1B). In contrast, p-ERK and p-mTOR levels remained unchanged (Fig. 3C and Suppl.-Fig. 1B). Notably, HER2 protein levels were not altered in *HER3* down-regulated cells suggesting that the effects are specific for HER3 (Suppl.-Fig. 3). Our data demonstrate that HER3 appears to promote melanoma cell proliferation which most likely involves the modulation of key cell cycle regulators.

HER3 knock-down inhibits melanoma cell migration and invasion and sensitizes melanoma cells to dacarbazine-induced apoptosis

Melanoma metastases frequently express high levels of HER3 (Fig. 1D and 1E). Given the association between HER3 expression and poor survival, one might hypothesize that HER3 plays a role in melanoma progression. Increased tumor cell migration and invasion are important prerequisites for metastasis. In order to address this question, we analyzed the migration and invasion of Colo 829, MM-358 and Mel Gerlach melanoma cells upon interference with *HER3* expression. To exclude the possibility of measuring inhibition of proliferation we used serum-starved melanoma cells and monitored migration and invasion after 20 and 24 hours, respectively. As shown in Figure 4A, *HER3* knock-down efficiently blocked melanoma cell migration in all three cell lines. In invasion experiments Colo 829 and Mel Gerlach cells were markedly inhibited upon *HER3* suppression (Fig. 4B) while MM-358 cells did not invade the matrix even in untransfected controls (data not shown). These results establish HER3 as a potent mediator of melanoma cell migration and invasion.

We next asked the question whether *HER3* downregulation can induce melanoma cell death. However, in contrast to previously published data in lung cancer cells which undergo apoptosis in the absence of *HER3* (15), suppression of *HER3* did not induce major apoptosis in melanoma cells (Suppl.-Fig. 4). Nevertheless we reasoned that inhibition of *HER3* might synergize with chemotherapy in the induction of apoptosis in melanoma cells. Indeed, we found that dacarbazine-induced apoptosis was significantly increased in *HER3* knock-down melanoma cells (Fig. 4C and Suppl.-Fig. 1C). These results suggest that a combination therapy with HER3 and dacarbazine-like drugs might be useful for the treatment of malignant melanoma.

Anti-HER3 monoclonal antibodies block heregulin-induced HER3 activation and melanoma cell proliferation, migration and invasion

We have shown above that HER3 is frequently overexpressed in primary melanoma and melanoma metastases and that high HER3 levels confer poor prognosis for melanoma patients. In addition, the RNA interference experiments suggest that HER3 may be a potential target for melanoma therapy. To test this hypothesis *in vitro* we treated Colo 829, MM-358, Mel Gerlach and Mel Juso melanoma cells with anti-HER3 monoclonal antibodies. Remarkably, heregulin-induced activation of HER3 and its association with the PI3-K subunit p85 was completely abrogated in antibody-treated cells when compared to controls (Fig. 5A). In addition, anti-HER3 monoclonal antibodies seem to cause receptor degradation or internalization similar to previously obtained results in breast cancer cells (19). Importantly, anti-HER3 monoclonal antibodies are able to block heregulin-induced proliferation, migration and invasion of human melanoma cell lines (Fig. 5B, 5C, 5D and Suppl.-Fig. 2C) indicating that such antibodies may be effective anti-melanoma therapeutics. Importantly, in Mel Juso melanoma cells, HER2 inhibition by a specific monoclonal antibody had no effect on tumor cell invasion demonstrating that inhibition of HER3 is sufficient to block melanoma invasivity (Suppl.-Fig. 2C).

Taken together, these results suggest that targeting HER3 may be a promising new opportunity for melanoma therapy.

Discussion

The discovery of animal oncogenes that are derived from genes encoding receptor tyrosine kinases has led over the past twenty years to the development of several targeted therapeutics with the HER2 monoclonal antibody Trastuzumab being the first in clinical application for the treatment of metastatic breast carcinoma with HER2

gene amplification. However, in spite of further advances in the development of side-effect-poor therapies for major malignancies such as breast cancer, there is still a great unmet need for better, more effective therapies for other cancer types. Melanoma is a highly aggressive skin cancer and current therapies only show limited efficacy in patients with late stage disease (4, 30). So far it is known that the Ras-Raf-MAPK and the PI3K-AKT pathways are frequently activated in malignant melanoma and that this contributes to tumor progression (30, 31). However, the role of receptor tyrosine kinases in melanoma development remains poorly characterized.

HER3 is highly expressed in many human tumor types and has been associated in some cases with poor prognosis (8 - 10). Moreover, recent studies suggest that breast tumors exposed to EGFR or HER2 targeted therapies escape this inhibition by persistent activation of HER3 and the PI3K-AKT pathway (20 - 22) suggesting that agents targeting HER3 could provide a novel and promising approach towards the treatment of some cancers. We have recently shown that *HER3* is frequently expressed in human melanoma cell lines (32) and macro-array analysis revealed that *HER3* is the most prominently expressed HER-family receptor in these cell lines (P. Knyazev, *unpublished data*). Interestingly, HER3 and HER2 have been described to be expressed in human melanoma cell lines and both receptors were implicated in the control of melanoma cell growth (33). In the present study we show HER3 expression in primary melanoma and its significant association with tumor cell proliferation. Furthermore, we demonstrate frequent and high HER3 expression in melanoma metastases, suggesting that HER3 may be involved in disease progression. In addition, high levels of HER3 significantly correlated with decreased life expectancy of patients establishing HER3 as a novel prognostic marker for melanoma. Importantly, HER3 expression is undetectable in primary melanocytes suggesting that HER3 overexpression specifically occurs during melanoma

development. The mechanism of HER3 overexpression appears to be caused by increased gene transcription since Southern blot analysis of 54 human melanoma cell lines did not reveal HER3 gene amplification (data not shown). So far, HER3 gene amplifications could only be detected in non-small cell lung and breast cancer as well as in synovial sarcomas (34, 35, 36) whereas no amplification was reported for pancreatic, stomach, head and neck and brain tumors (37, 38, 39, 40). To specifically address the role of HER3 in melanoma development and progression, we analyzed human melanoma cell lines upon siRNA interference with *HER3* expression. Reduction of *HER3* expression resulted in reduced cell proliferation, migration and invasion. On the molecular level, suppression of *HER3* led to increased p27 protein levels and reduced Rb phosphorylation which seems to be the cause for the observed growth inhibition. Interestingly, HER3 can inhibit heregulin-induced cell proliferation which may be due to an impaired AKT activity. In this context it is important to note that AKT is known to modulate the cellular localization of p27 thereby promoting cell cycle progression (41, 42, 43, 44); however, in melanoma cells p27 seems to be regulated by an AKT-independent pathway since p27 upregulation could also be seen in unstimulated *HER3* knock-down cells. Interestingly, p27 proteolysis can be regulated by oncogenic signaling through the EGFR and HER2 which can be reversed by targeted inhibition of both receptors (45). It will be the topic of further studies to elucidate the mechanism of HER3-mediated p27 regulation in the control of human melanoma cell proliferation which may be similar to EGFR and HER2-mediated p27 regulation. Notably, *HER3* ablation did not affect the ERK1/2 and mTOR kinases suggesting that inhibition of other downstream pathways seems to be sufficient to block melanoma cell proliferation, migration and invasion. Interestingly, mTOR phosphorylation at serine 2448 is known to be mediated by the PI3K-AKT pathway; however, inhibition of AKT in heregulin-

stimulated cells did not result in downregulation of phospho-mTOR. In fact, mTOR seems to be constitutively activated as shown in unstimulated melanoma cells (Fig. 3C). A possible explanation for this observation is that mTOR might be either activated through yet unknown mutations or directly modulated by the Ras-related GTPase Rheb as described recently (46, 47, 48).

Furthermore, *HER3* suppression led to a marked decrease in melanoma cell migration and invasion. It will be of importance to elucidate the exact role of HER3 in these processes and to identify factors which mediate HER3 responses during melanoma metastasis (e.g.: matrix-metallo proteinases).

Interestingly, *HER3* knock-down in lung cancer cells did lead to a significant increase in apoptosis (15). However, interference with HER3 function was not sufficient to induce major apoptosis of melanoma cells. Based on these results we tested whether a combination of *HER3* downregulation with chemotherapeutic drug treatment would increase cell death. To date, dacarbazine is the only FDA-approved drug for the treatment of advanced melanoma with a tumor response rate of about 15-20% (4). Indeed, *HER3* knock-down cells were highly sensitive to dacarbazine-induced apoptosis suggesting that a combination with agents interfering with HER3 might prove effective in the treatment of malignant melanoma.

To further analyze whether HER3 may qualify as a novel target in melanoma therapy, we treated human melanoma cells lines with anti-HER3 monoclonal antibodies. We found that such antibodies can inhibit heregulin-induced HER3 phosphorylation leading to receptor internalization or degradation. Furthermore, we show that binding of p85, the regulatory subunit of PI3K, to HER3 is abrogated upon antibody-incubation demonstrating that signaling via the PI3K/AKT signaling pathway is inhibited in these cells. Importantly, melanoma cell proliferation, migration and invasion are significantly reduced in antibody-treated cells when compared to

controls. These data demonstrate that anti-HER3 antibodies can inhibit HER3 signaling most likely through the PI3K-AKT pathway in melanoma cell lines and thereby seem to block melanoma cell functions. Importantly, we could show in Mel Juso melanoma cells that a monoclonal antibody specific for HER2 did not affect melanoma cell invasion suggesting that inhibition of HER3 alone is sufficient to block melanoma invasiveness (Suppl. Fig. 2C). It will be essential in the future to test the efficacy of anti-HER3 blocking antibodies on melanoma development and metastasis in preclinical animal models in order to further validate HER3 as a possible target for melanoma therapy.

Taken together, our results establish HER3 as a potential target for melanoma therapy development and interference with its function may offer a new and promising approach to improve clinical patient outcome.

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Figure legends

Fig. 1 HER3 expression in primary melanoma and metastases. Immunohistochemical staining of HER3 in primary melanoma and metastases. **A** Low HER3 expression (20x), **B** Moderate HER3 expression (40x) and **C** High HER3 expression (40x) in primary melanoma. HER3 immunoreactivity is accentuated at the cell membrane (black arrows). **D** High HER3 expression in melanoma metastases (40x). **E** HER3 expression frequencies in primary melanoma and

metastases. **F** Absence of HER3 expression in primary melanocytes. HER3 expressing and non-expressing melanoma cell lines served as positive and negative controls, respectively. Tubulin served as a loading control.

Fig. 2 HER3 protein expression confers poor prognosis in melanoma patients. **A** HER3 expression increases during melanoma progression. 20 primary tumors with matching melanoma metastases where evaluated based on their GIS score for HER3. **B** Kaplan-Meier analysis of tumor-specific survival according to HER3 expression levels (p = 0,014).

Fig. 3 *HER3* knock-down inhibits melanoma cell proliferation

A *HER3* knock-down in Colo 829 and MM-358 melanoma cells. Western blots are shown for HER3 and Tubulin. **B** *HER3* knock-down inhibits the proliferation of Colo 829 and MM-358 melanoma cells. The growth curves were done as described in Material and Methods. The data are shown as mean ± SDM. Western blots for p27, p-Rb and Cyclin B1 are shown. Tubulin served as a loading control. **C** *HER3* knock-down impairs AKT activity upon heregulin ß1 stimulation in Colo 829 and MM-358 melanoma cells. Western blots are shown for HER3, p-AKT, p27, p-ERK1/2 and p-mTOR. ß-actin served as a loading control.

Fig. 4 *HER3* knock-down inhibits melanoma cell migration and invasion and induces apoptosis in response to chemotherapeutic drugs. **A** *HER3* knock-down inhibits the migration of Colo 829, MM-358 and Mel Gerlach melanoma cells. For quantification, pictures of migrated cells were taken on a Zeiss Axiovert 300 microscope and cells were counted in at least 10 random fields. The values for control cells were set to 100% and results are shown relative to controls. The data

are shown as mean ± SDM. **B** *HER3* knock-down inhibits the invasion of Colo 829 and Mel Gerlach melanoma cells. Quantification of invaded cells was done as described in A. **C** Induction of apoptosis in Colo 829 and MM-358 *HER3* knock-down cells upon treatment with increasing amounts (10µM and 20µM) of dacarbazine. Data are shown as mean ± SDM.

Fig. 5 An anti-HER3 monoclonal antibody (cl. 105.5) inhibits HER3 activation and blocks melanoma cell proliferation, migration and invasion. A Anti-HER3 antibody treatment blocks heregulin-induced HER3 activation, its association with p85 and leads to receptor internalization or degradation. Colo 829, MM-358 and Mel Gerlach melanoma cells were serum-starved, incubated with 10µg/ml HER3 blocking antibody (cl. 105.5) or an isotype control antibody, stimulated with heregulin ß1, lysed and equal amounts of protein was subjected to Immunoprecipitations using a specific HER3 antibody. Western blots for p-HER3 (Y1289), HER3 and p85 are shown. **B** An anti-HER3 antibody (cl.105.5) inhibits heregulin-induced melanoma cell proliferation *in vitro*. Cell proliferation was measured by a MTT assay as described in Material and Methods. MDA-MB 468 breast cancer cells served as a positive control as described previously (19). **C** An anti-HER3 antibody (cl.105.5) blocks melanoma cell migration *in vitro.* The cells were either incubated with 10µg/ml anti-HER3 antibody or an isotype control antibody. The migration assay was done in a modified boyden chamber. Conditioned NIH3T3 medium containing 100ng/ml heregulin ß1 was used as a chemo attractant. The quantification was done as described in Figure 4. The data are shown as mean ± SDM. **D** An anti-HER3 antibody (cl. 105.5) blocks melanoma cell invasion *in vitro*. The assay was done as in B using growth factor-reduced matrigel in a modified boyden chamber.

Table 1. Multivariate analysis of factors possibly influencing overall survival (forward LR method)

Variable	Characteristics	Hazard ratio (95%CI)	*p*
Age	0 ≤ 60 years 1 > 60 years	—	NS
Sex	0 = male 1 = female	—	NS
Tumor thickness	0 ≤ 2mm 1 > 2mm	—	NS
HER-3	0 low 1 high	2.6 (1.04-6.6)	**0.041**[a]
Metastases during follow up	0 no metastases 1 metastases	22.2 (5.2-95.5)	**0.000**

[a] Bold type representing data with p < 0,05.
Abbreviations: NS, not significant; HR, hazard ratio; 95% CI, 95% confidence interval

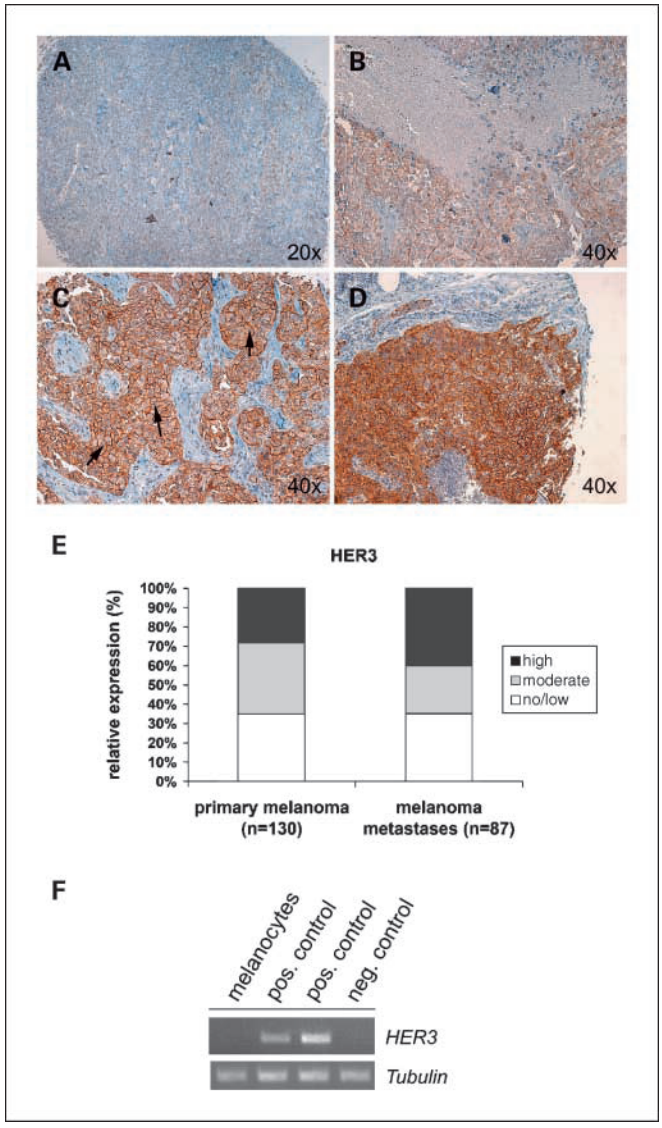
A
20x
B
40x
C
40x
D
40x
E
HER3
relative expression (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
high
moderate
no/low
primary melanoma (n=130)
melanoma metastases (n=87)
F
melanocytes
pos. control
pos. control
neg. control
HER3
Tubulin

Fig. 1

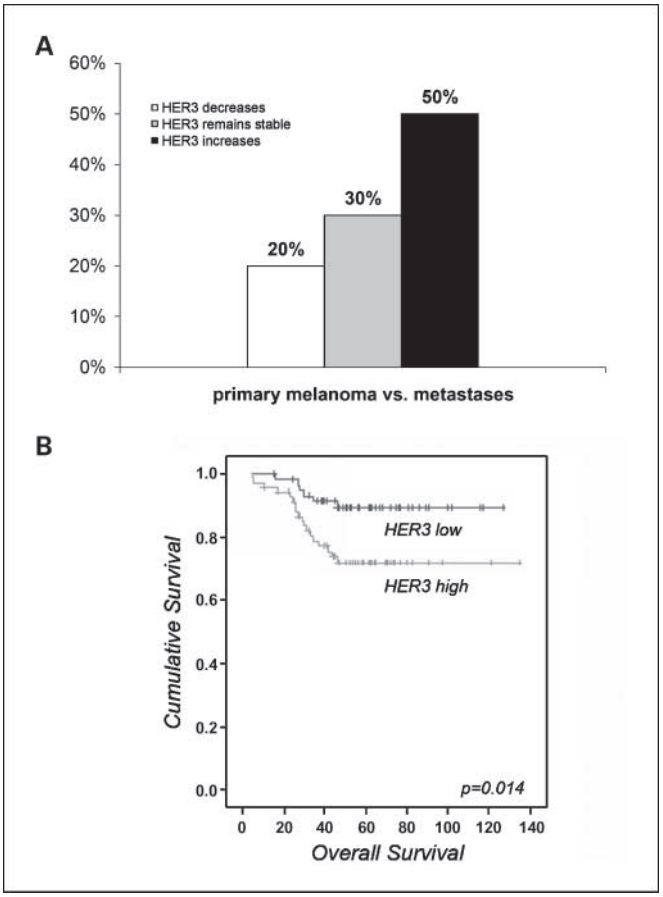
A
60%
50%
40%
30%
20%
10%
0%
HER3 decreases
HER3 remains stable
HER3 increases
20%
30%
50%
primary melanoma vs. metastases
B
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
HER3 low
HER3 high
p=0.014
0
20
40
60
80
100
120
140
Overall Survival

Fig. 2

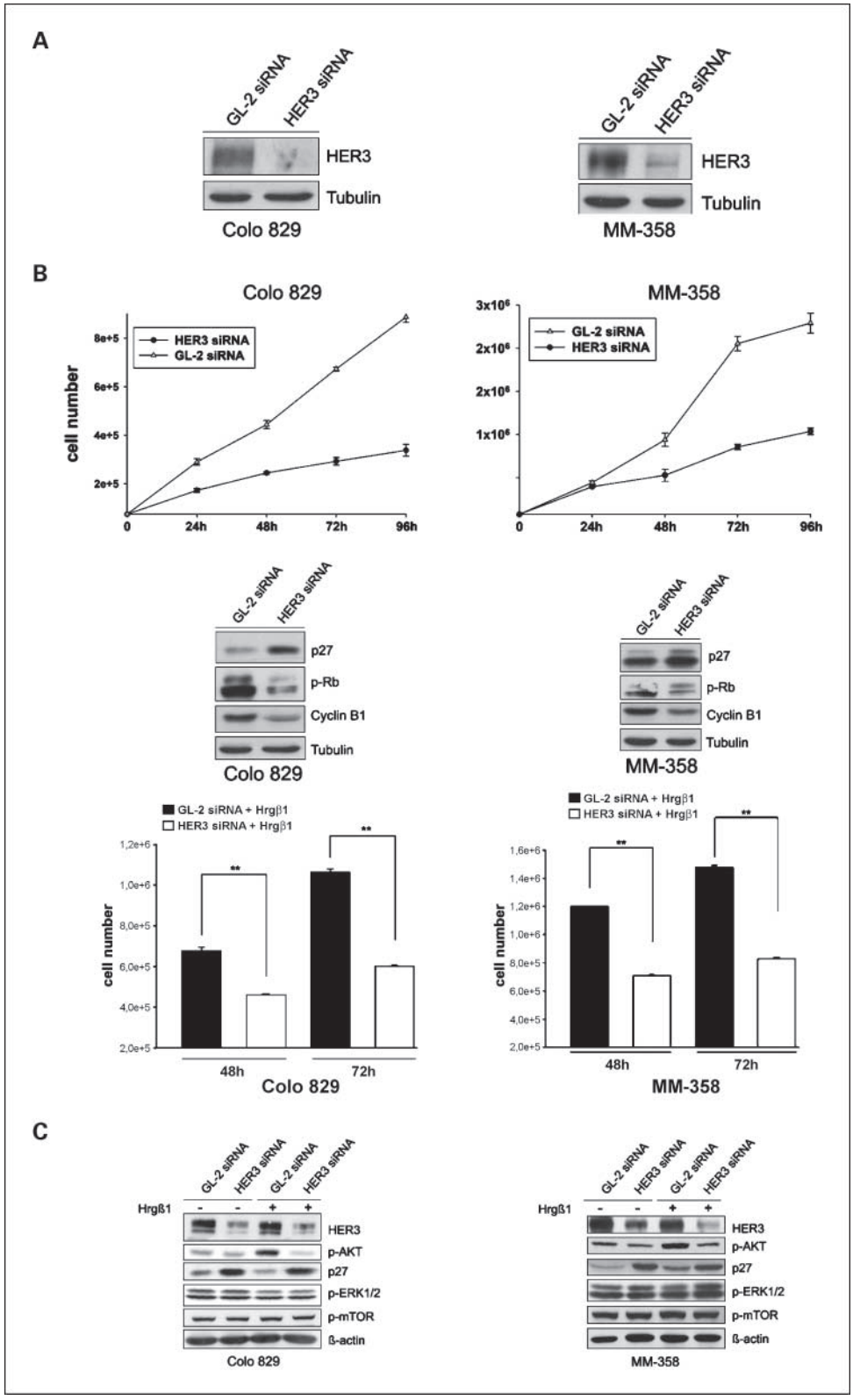
A
GL-2 siRNA
HER3 siRNA
HER3
Tubulin
Colo 829
MM-358
B
Colo 829
MM-358
HER3 siRNA
GL-2 siRNA
cell number
0
24h
48h
72h
96h
p27
p-Rb
Cyclin B1
Tubulin
GL-2 siRNA + Hrgβ1
HER3 siRNA + Hrgβ1
**
C
Hrgß1
HER3
p-AKT
p27
p-ERK1/2
p-mTOR
ß-actin

Fig. 3