Towards the prognostic significance and therapeutic potential of HER3 receptor tyrosine kinase in human colon cancer

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Running title: HER3 in colon cancer
Translational Relevance

As a member of the EGFR receptor tyrosine kinases family, HER3 pseudo-kinase was shown to play a crucial role in regulation of oncogenic processes. Dysregulation of this receptor is associated with the progression of different human cancers including those of the breast and colon cancer. Our studies show that HER3 over-expression represents a putative prognostic marker since it correlates with reduced patient survival. We also show that HER3 is over-expressed in human colon cancer cell lines and that HER3 is functionally associated with cancer-related cell processes. Our findings identify HER3 as a promising target for colon cancer therapy development.
Abstract

Purpose: Abnormal accumulation and dysregulation of the epidermal growth factor receptor family member HER3 is associated with the development of various human cancers including those of the breast, lung, and ovary. We have previously demonstrated that in melanoma HER3 is frequently overexpressed, and is associated with poor prognosis. However, the importance of HER3 in colon cancer and its putative prognostic significance is still unknown.

Experimental Design: HER3 expression was analysed in primary colon tumors from 110 patients by immunohistochemistry and correlated with time of progression. Parallel to this, the influence of HER3 over-expression on cell proliferation, migration, invasion, and apoptosis was investigated in four different colon cancer cell lines including DLD-1, LoVo, CaCO2, and T-84.

Results: HER3 was detected at high frequency and exclusively at the membrane of the primary tumors. Elevated HER3 expression levels may serve as a putative prognostic marker since it associates with cell proliferation and decreased time to disease progression. High HER3 protein expression as well as phosphorylation levels were detected in tested cells. HER3 down-regulation by RNA interference abrogated cell proliferation, migration, and invasion. In addition, suppression of HER3 resulted in a G2/M cell cycle arrest, induced apoptosis and led to morphological changes in colon cancer cell lines. Furthermore, application of a monoclonal antibody specific to the extracellular portion of the receptor reduced heregulin-ß1-induced migration and invasion, and also induced apoptosis in colon cancer cell lines.

Conclusion: We postulate that HER3 is critically involved in colon cancer progression and may serve as a novel target for therapeutic intervention.
Introduction

Colon cancer is a common type of bowel cancer that originates from the neoplastic transformation of epithelial cells of the colon through an adenoma precursor stage. It is considered as the fourth most common malignancy in the western world and the third most frequent cause of cancer-related mortality (1, 2). Underlying molecular mechanisms of colon cancer formation have been studied extensively during the last years. Multiple genetic and epigenetic aberrations were shown to initiate the development of colon cancer and to drive its progression (3-9). Understanding the genetic basis of colon cancer however, has not yet led to highly effective therapies other than complete surgical removal of the tumor. In this regard, molecular characterization of predictor genes and signalling pathways controlling colon cancer growth is instrumental to new drug design and the refinement of cancer chemotherapy for individual patients.

HER3 (ErbB3) is a member of the human epidermal growth factor receptor (EGFR/HER) tyrosine kinases family. Its gene maps to human chromosome 12q13.2, and results in a protein of ~180 Kda (10-12). HER3 over-expression was illustrated in a wide range of cancers including those of breast, -ovary, -lung, and prostate, as well as melanoma (13-16). This genetic feature has been correlated with poor prognosis in most of the cases (15-18). Subsequent to its activation, HER3 effectively couples to the PI3K/Akt pathway, thereby controlling different biological outcomes including cell proliferation, motility, and cell survival (19). HER3 has in fact six tyrosine phosphorylation sites with YXXXM motifs that serve as binding sites for PI3K (20-23). HER3 is unique in the ErbB family in that it has been shown to have weak or no tyrosine kinase activity (24). Due to this property, HER3 phosphorylation after heregulin activation is dependent on heterodimerization with other ErbB family members (25-27). In human breast cancer HER3 interacts with HER2 and thereby generates a potent oncogenic dimer that promotes tumor development and progression (27). Indeed, the HER3/HER2 heterodimer represents the most potent mitogenic complex among EGFR family members. It contributes synergistically to cell transformation and to malignant properties of cancer cells (28). Recent work of Van der Horst and co-workers showed that heregulin-induced activation of HER3 in glioma-derived cell lines results in the phosphorylation of a cytoplasmic kinase protein, PYK2 (28). Interestingly, a monoclonal antibody targeting of HER3 was
shown to reduce proliferative as well as migratory properties of breast cancer cell lines (33). Consistent with this, it was shown that increased HER3 expression predicts poor prognosis of melanoma patients, and that treatment of melanoma cell lines by a monoclonal antibody targeting HER3 blocks cell proliferation, migration, and invasion (16). Fascinatingly, it was shown recently that treatment of HER2-amplified breast cancers with HER2-targeting TKIs (tyrosine kinase inhibitors) lead to a rapid compensatory increase in HER3 expression, localization, and signalling activity (29). Thus, HER3 is considered as a crucial receptor, which could be a novel target for the treatment of different human cancers.

In this study, our aim was to determine whether HER3 could be considered as a determinant for poor prognosis in colon cancer patients, and whether it has a relevant role in transmitting proliferative, survival, and motility signals in colon cancer. We therefore analysed HER3 expression levels in a panel of 110 colon cancer patients. We performed clinico-pathological correlations using multivariate analysis. Furthermore, we analysed the inhibitory effect of HER3 siRNA as well as of HER3 monoclonal antibody treatment on different oncogenic processes including cell proliferation, survival, migration, and invasion. Our findings support a putative prognostic relevance of HER3 in colon cancer and implicate this HER family member as promising novel target for colon cancer treatment.

**Experimental procedures**

**Clinical samples and statistical analyses**

HER3 expression was evaluated using formalin-fixed, paraffin-embedded (FFPE) colon cancer samples of patients who underwent surgical tumour resection at the Ludwig-Maximilians-Universität München between 1994 and 2005. Follow-up data were recorded by the Munich cancer registry. Paraffin-embedded tissue samples of primary tumours were available in 110 cases. The median age of the patients at the time of diagnosis was 67 years. Clinico-pathological characteristics of the study population are summarized in Supplementary Table 1. For inclusion models, age, gender, tumor grade, and T-category were considered as co-variables. Frequency data were analysed using the $\chi^2$ test. Cancer specific survival was calculated from the date of primary surgery to the date of cancer associated death. Survival data of
patients whose death was not attributable to colorectal cancer or whose follow-up ended before death were censored. Survival analysis was done by the Kaplan-Meier method and the groups were compared with the log-rank test. Univariate and multivariate analyses were based upon the Cox regression model. Cases with missing data were excluded from multivariate analyses. Statistical procedures were done using SPSS version 15.0 (SPSS Inc.). \( P < 0.05 \) was considered as statistically significant.

The study was approved by the ethics committee of the Medical Faculty of the Ludwig-Maximilians-Universität München.

**Immunohistochemistry, scoring of HER3 expression, and statistical analyses**

All tumors were arrayed in quadruplicate on tissue microarrays using 1.0 mm diameter punch sets (Beecher instruments). 5\( \mu \)m sections were prepared for immunohistochemical staining. As the primary antibody we used anti-HER3 (clone C-17, 1:50; Santa Cruz). Staining was performed on a Ventana Benchmark autostainer with the XT ultraView DAB Kit (Ventana Medical Systems). All slides were counterstained with Hematoxylin. To exclude unspecific staining, isotype and system controls were included. HER3 expression was scored semi-quantitatively, considering membranous immunostaining of the tumor cells only. Depending on the intensity of staining, HER3 expression was classified as weak, intermediate, or strong.

**Cell lines, compounds, and culture conditions**

All cell lines were purchased from American Type Culture Collection (ATCC), and propagated in a humified atmosphere containing 7% \( \text{CO}_2 \) at 37\(^\circ\)C. The human DLD-1 colon cancer cell line was maintained in RPMI 1640 supplemented with 10% FCS. Human LoVo and CaCO2 colon cancer cells were cultured in Ham's F-12 and DMEM medium, respectively, both supplemented with 20% FCS and 1% L-glutamine. T-84 cells were maintained in Ham’s F-12/DMEM high glucose (1:1) medium supplemented with 5% FCS and 1% pyruvate. All media were supplemented with 1x penicillin/streptomycin (Gibco). 5-Fluorouracil and propidium iodide were purchased.
from Sigma. Heregulin-ß1 was purchased from R&D Systems and diluted in phosphate-buffered saline (PBS) before use.

**RNA interference**

HER3 siRNA duplexes were synthesized by Ambion. Two independent siRNAs were used in all experiments. The targeted sense and antisense sequences were as follows respectively: sense-1 5´-GGCUAUGUCCUCUAGCCAtt-3´, antisense-1 5´UGGCCACGAGACGAGCCgtg-3´, sense-2 5´GGCAGUGUCCUGGGACUtt-3´, and antisense-2 5´AGUCCAGGACACAGCCgtg-3´. The GL-2 siRNA from Dharmacon was used as a negative control with the following sequence: 5´-CGUACGCGGAAUACUUCGAtt-3´ (sense), 5´-UCGAAGUAUCCGCGUACGtt-3´ (antisense). siRNA transfection was performed using Lipofectamine RNAiMax (Invitrogen, CA) according to the manufacturer’s recommendation.

**Western blotting, immunoprecipitation, and immunofluorescence**

Cells were either left non-stimulated or were stimulated with 100ng/ml heregulin-ß1 for 24h and lysed on ice in lysis buffer. Antibodies to phospho-HER3 (Tyr1289), p85, phospho-AKT (Ser473), cyclin B1, phospho-extracellular signal-regulated kinase 1/2, phospho-mammalian target of rapamycin (mTOR; Ser2448), and phospho-Rb (Ser780) were all purchased from Cell Signalling. Horseradish peroxidase-conjugated rabbit secondary antibodies were from Bio-rad. Anti-tubulin, anti-ß-actin, and Horseradish peroxidase-conjugated mouse secondary antibodies were from Sigma. The anti-HER3 (clone 2F12) antibody was from Upstate, and anti-HER3 (C-17) for immunohistochemistry was from Santa Cruz. Anti-p27 was purchased from Abcam. For immunoprecipitation, a specific anti-HER3 (homemade; cl.1B4.C3) antibody and protein A-Sepharose (Pharmacia, Piscataway, NJ) were added to the cleared lysate and incubated overnight at 4°C. Immunoprecipitates were subsequently washed with HNTG buffer [20 mM HEPES (pH7.5), containing 150 mM NaCl, 1 mM EDTA, 1 mM sodium fluoride, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100]. Sample buffer containing SDS and 2-mercaptoethanol was added, and samples were denatured by heating at 95°C for 8 min. Proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, nitrocellulose filters were
first incubated with mouse monoclonal Antibody or rabbit polyclonal primary antibodies overnight at 4°C. Next, HRP-coupled goat anti-mouse or goat anti-rabbit secondary antibody was added, followed by an ECL substrate reaction (Perkin Elmer). The substrate reaction was detected on Kodak (Rochester, NY) X-Omat film. Filters used more than once with different antibodies were stripped according to the manufacturer’s protocol, blocked and re-probed.

For immunodetection, HER3 and GL-2 transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 10 min and blocked in 4% BSA. The sections were incubated with a primary antibody against vimentin (1:100; Santa Cruz) and then with the appropriate secondary antibody (Molecular probes and Jackson Immunoresearch, USA). Nuclear DNA was finally counterstained with DAPI (0, 02 μg/ml) and micrographs were taken using a Zeiss Axioplan 2 with MetaVue software (Molecular Devices).

**Proliferation assay**

Cells were transfected with HER3 or GL-2 siRNA using Lipofectamine RNAiMax (Invitrogen). The cells were either grown in their appropriate media or serum starved in medium containing 1% FCS for 24 h and subsequently stimulated with 100 ng/ml heregulin-ß1. The cell number was counted (Coulter Counter, Becton Dickenson) at the indicated time points. The data are shown as mean ± SD.

**Cell cycle analysis**

To analyze the cell cycle, HER3 knockdown cells as well as control cells were harvested and fixed with 70% ethanol in PBS. Cells were then resuspended in 0.5 ml PBS containing propidium iodide and DNase-free RNase for 2 h and subsequently subjected to flow cytometric analysis (Becton Dickinson Biosciences) to determine cell cycle profiles. At least 10 000 cells were analyzed from each sample.

**Migration and invasion assays**

Cells transfected with HER3 or GL-2 siRNA using Lipofectamine RNAiMax (Invitrogen) were serum starved in medium containing 0,1% FCS for 24h and seeded
either onto a membrane or onto a growth factor-reduced Matrigel-coated membrane with 8 μM pores of a modified Boyden chamber (Schubert and Weiss) containing 500 μl serum-free medium; 10% FCS medium served as a chemo-attractant. After 20 h or 24 h incubation at 37°C, migrating and invading cells respectively were stained with crystal violet and washed in PBS. The cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were examined and photomicrographs were taken (Zeiss Axiovert 300 Microscope). At least 10 random fields per filter were counted for quantification. The data are shown as mean ± SD.

**Apoptosis assay**

HER3 or GL-2 transfected cells were treated with either 10 or 20 μmol/L 5-fluorouracil or DMSO as a negative control. After 72h, floating cells were collected and adherent cells were trypsinized. After centrifugation, cells were incubated for 2 h in a propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 20 μmol/L propidium iodide) and subsequently subjected to flow cytometric analysis (Becton Dickinson Biosciences). Apoptotic cells were identified as the sub-G0-G1 peak and quantified using the CellQuest Pro software (Becton Dickinson).

**HER3 blocking antibody: Migration, invasion and apoptosis experiments**

The HER3 blocking antibody (clone 105.5) was purchased from Upstate.

To assess the HER3 phosphorylation state, cells were serum starved for 24 h, incubated with 10 μg/ml blocking antibody for 1 h, stimulated with 100 ng/ml heregulin-β1 for 2h, lysed, and subjected to immunoprecipitation using a specific HER3 antibody (homemade; cl.1B4.C3).

MDA-MB-468 breast cancer cells were already described to be inhibited by the anti-HER3 antibody (clone 105.5) (33) and served as a positive control. The data are shown as percent of inhibition relative to control cells.

The cell migration and invasion assays were performed as described previously (16, 30, 33). Briefly, cells were seeded in 10-cm plates and serum starved in medium containing 0.1% FCS for 24 h. Cells (400,000/ml) were then incubated with 10 μg/ml HER3 blocking antibody or an isotype control antibody for 1 h. In both assays, cells were plated in triplicate at a density of 100,000 cells per well either onto a membrane.
or onto a growth factor-reduced Matrigel-coated membrane with 8 μM pores of a modified Boyden chamber (Schubert and Weiss) containing 500 μl serum-free medium. Conditioned NIH3T3 medium containing 100 ng/ml heregulin-β1, and 0.01 % ascorbic acid served as a chemo-attractant. The cells were allowed to migrate or invade for 20 or 24 h, respectively. Migrated and invaded cells were stained by crystal violet, washed in PBS, and analyzed using a Zeiss Axiovert 300 microscope. For quantification, 10 random fields at least were counted per filter. The data are shown as mean ± SD. The apoptosis assay was done using a propidium iodide staining. Briefly, cells were serum starved in medium containing 1 % FCS and afterwards pre-incubated with either 10μg/μl blocking antibody (clone 105.5) or an isotype control. After 72 h floating cells were collected and adherent cells were trypsinized. After centrifugation, cells were incubated for 2 h in a propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 20 μmol/L propidium iodide) and subsequently subjected to flow cytometric analysis (Becton Dickinson Biosciences). Apoptotic cells were identified as the sub-G0-G1 peak and quantified using the CellQuest Pro software (Becton Dickinson).

Results

Increased HER3 expression is associated with decreased survival of colon cancer patients

We analysed HER3 protein expression in 110 primary colon carcinoma using tissue microarrays. Immuno-staining revealed differential expression of HER3 among these cases with 25.5%, 53.6%, and 20.9% showing low, intermediate, and high expression, respectively (Fig. 1A-E). Using Kaplan-Meier statistics, we observed that patients with intermediate or high HER3 expression levels had significantly shorter survival times than cases with low HER3 expression (Fig. 1F). Because intermediate and high HER3 expression did not show a significant difference in this survival analysis, we next categorized all cases into two groups only. Low expressing cases were defined as HER3-low while intermediate and high expressing cases were defined as HER3-high (Fig 1F). In order to learn about the dependence of these two survival groups on other clinical factors, we next analysed their association with variables such as age, gender, T-stage, tumor grade, and tumor location and
observed no significant correlation (Suppl. Table 1). To determine whether these survival groups thus are independent of these clinical variables, we then used multivariate Cox-regression analysis. In this analysis, we found HER3-high to be an independent prognostic marker for low survival in our study collection, associated with a relative risk of 3.29 (Table 1). Taken together our data suggests that HER3 expression is of clinical relevance when assessed in colon cancer samples and indicates a higher risk of tumor associated death when highly expressed.

**HER family member expression in colon cancer cell lines**

In order to select a model system which is suitable for our investigation, we screened a panel of 5 colon cancer cell lines (DLD1, LoVo, CaCO2, RKO, and T-84) for the protein expression levels of three members of the HER family, namely HER3, HER2, and EGFR. HT-29 and HCT-116 colon cancer cell lines were tested previously (31) and considered as positive and negative control for HER3 expression, respectively (Fig. 2). A considerable difference by the HER3 expression was observed among the five tested cell lines. Whereas DLD-1 and T-84 cell lines are characterized by a very high to a high HER3 levels respectively, CaCO2 and LoVo express HER3 moderately. No detectable HER3 was observed in the RKO cell line. In order to further validate our results, basal phosphorylation levels of HER3 of the same cell lines was assessed (Supplementary Fig. 1). While HER2 expression levels vary from one cell line to the other, all tested cell lines showed moderate EGFR expression levels (Fig. 2).

**HER3 knockdown affects proliferation of colon cancer cell lines and interferes with activation of downstream targets**

To validate our clinical results, we focused on the function of the HER3 receptor tyrosine kinase *in vitro*, by testing its influence on diverse biological processes including proliferation, -motility, -invasion, and survival. We therefore reasoned to down-regulate HER3 in DLD-1, LoVo, CaCO2, and T-84 colon cancer cell lines by small interfering RNA (siRNA) (Fig. 3A; Supplementary Fig. S4A and S5A).

First, we addressed the question whether HER3 could be involved by the regulation of colon cancer cell proliferation. As expected, depletion of HER3 strongly inhibited
proliferation in all four tested cell lines (Fig. 3B; Supplementary Figs. S4B and S5B). In addition, HER3 knockdown significantly inhibited heregulin-β1-induced cell proliferation (Fig. 3B and supplementary Figs. S4B and S5B). To further characterize the molecular mechanism behind this growth inhibition, we checked expression levels of main proteins implicated in the regulation of the cell cycle before and after HER3 knockdown. As shown in Fig. 3C and supplementary Figs. S4C and S5C, HER3 suppression lead to a significant decrease in cyclin B1 expression as well as to decreased Rb phosphorylation, suggesting that HER3 controls cell proliferation by interfering with key cell cycle regulators.

We subsequently asked, whether HER3 knockdown has an effect on known downstream substrates namely ERK1/2 and Akt, which are, respectively, effector-proteins responsible for MAPK cascade activation and lipid signalling (27, 32). Akt activation as well as phospho-mTOR levels were impaired subsequent to HER3 knockdown in both heregulin-β1 stimulated and non-stimulated cells, indicating that HER3 may signal via the PI3K-Akt pathway in colon cancer cells (Fig. 3D; Supplementary Fig. S4D). Moreover, HER3 knockdown cells showed increased p27 protein expression levels in comparison to control cells (Fig. 3D; Supplementary Fig. S4D). In contrast, phospho-extracellular signal-regulated kinase remained unchanged (Fig. 3D; Supplementary Fig. S4D).

**HER3 knockdown induces a G2/M cell cycle arrest in colon cancer cell lines**

Since HER3 suppression by siRNA leads to inhibition of Rb phosphorylation as well as to decreased cyclin B1 protein expression levels, we asked whether the absence of HER3 could influence progression of the cell cycle. In order to address this question, we performed a cell cycle analysis and checked elements of the cell cycle before and subsequent to HER3 knockdown. Interestingly significant increased amounts of cells by the G2/M phase were observed in all 4 HER3 knockdown cells when compared to the controls (GL-2 siRNA as well as untransfected cells). Parallel to this, a shift in the G1 and S phases was detected (Fig. 4A-B).

Surprisingly, the morphology of HER3 knockdown cells changed towards a flat appearance, whereas control transfected cells retained their compact cell shape (Supplementary Fig. S2). This observation prompted us to examine HER3 impact on migration and invasion.
HER3 knockdown suppresses migration and invasion and induces apoptosis in colon cancer cell lines

HER3 has been shown to be implicated in the regulation of several oncogenic processes, particularly migration and invasion in different cancer types including lung, skin, and breast cancers (14, 16, 33). To address the question, whether HER3 is required for colon cancer motility and invasion, we performed migration and invasion assays in DLD-1 and LoVo cell lines in the presence or absence of small interfering RNA (siRNA) targeting HER3. To exclude the possibility of measuring inhibition of proliferation, we used serum-starved conditions and we monitored migration and invasion after 20 and 24 h, respectively. As shown in Fig. 5A, HER3 siRNA effectively abrogated migration of both cell lines. Furthermore, invasivity of both DLD-1 and LoVo was markedly inhibited upon HER3 suppression (Fig. 5B). These results suggest a prominent role of HER3 in regulating migration as well as invasivity of colon cancer cell lines.

To further characterize the role of HER3 in vitro, we asked whether HER3 down-regulation by small interference RNA induces cell death in colon cancer cell lines. Interestingly, in line with previously published data in lung cancer cell lines which undergo apoptosis in the absence of HER3 (14), suppression of HER3 lead to a moderate induction of apoptosis which varies from one cell line to the other inside our model system (Fig. 5C; Supplementary Fig. S6). We next checked whether 5-fluorouracil, a common chemotherapeutic used for the treatment of colon cancer patients, could increase the already obtained apoptotic effect upon HER3 knockdown. Importantly, HER3 inhibition synergizes with chemotherapy in apoptosis induction. Treatment of HER3 knockdown cells with 5-fluorouracil but not of control cells gave rise to a significant increase in the apoptosis rates of the treated cells, reaching ~45% and ~32% for DLD-1 and LoVo cell lines respectively (Fig. 5C). The same effect was also observed in both other colon cancer cells CaCO2 (~45%) and T-84 (~30%) (Supplementary Figs. S4E and S5D). These results suggest that a combinatorial therapy with HER3-inhibitory and 5-fluorouracil-like drugs might be useful for the treatment of malignant colon cancer.
Anti-HER3 monoclonal antibody blocks heregulin-ß1-induced HER3 activation, inhibits colon cancer cell migration and invasion, and induces apoptosis in colon cancer cell lines

To test the hypothesis whether HER3 could be considered as a potential target for colon carcinoma therapy, we analysed the effects of a HER3 monoclonal blocking antibody (clone 105.5), which specifically antagonizes the heregulin-ß1 binding to HER3 in breast and skin cancer cell lines (16, 33). We therefore immunoprecipitated HER3 in cells treated with either HER3 mAB 105.5 or a control antibody and stimulated with heregulin-ß1. We then assessed the phosphorylation of HER3 and the expression levels of the regulatory subunit of PI3K, the p85 protein. Remarkably, anti-HER3 monoclonal antibody interferes with the activation of heregulin-ß1-induced activation of the HER3 receptor and its association with the PI3K subunit, p85 in DLD-1 and LoVo. However, inhibition was incomplete in the CaCO2 cell line (Fig. 6A). Similar to previous reports (16, 33), HER3 seemed to be degraded or internalized through the anti–HER3 monoclonal antibody in DLD-1 and LoVo colon cancer cell lines (Fig. 6A). Interestingly the HER3 blocking antibody was able to block migration and invasion and to induce apoptosis in the DLD-1 and LoVo colon cancer cell lines (Fig. 6B; 6C; 6D), indicating that such antibodies may be effective for the treatment of colon cancer patients. Taken together, these results have potential therapeutic significance of HER3 as new promising target for colon cancer treatment.

Discussion

Pseudo-kinases such as HER3 are emerging as crucial regulators of diverse cellular functions, despite lacking the ability to directly phosphorylate substrates (34). Recent studies highlighted HER3 as a fundamental signalling receptor involving by the modulation of different biological processes in a wide range of cancer types including pancreatic, -breast, -prostate, -gastric, -ovarian and skin cancer (15, 16, 27, 35, 36, 37). In some of these tumors, HER3 expression was even shown to be correlated with poor prognosis (15-18). Additionally, recent works demonstrated that tumors escape from EGFR and HER2 targeted therapy by a rapid compensatory increase in HER3 expression, and in a PI3K-Akt signaling pathway (29), suggesting that agents
targeting HER3 could provide a novel and promising approach toward the treatment of such cancers.

HER3 represents a trans-membrane receptor that is activated by ligand binding. Therefore, one might assume that the predominant localization of HER3 should be the cell membrane. Nevertheless, several publications using different anti-HER3 antibodies for staining of tumor samples demonstrated a cytoplasmic rather than a membranous staining pattern for this receptor (1, 15). In our study we detected a clear membranous localization of HER3 in all 110 analysed paraffin embedded specimens of primary colorectal tumors as well as a strong correlation of HER3 expression with overall survival of patients, establishing HER3 as a putative novel independent prognostic marker for colon cancer. This is of great importance as several therapeutic antibodies aimed at targeting the membrane localized receptor are currently being tested in clinical trials and will hopefully be available as a treatment modality soon. Indeed, these results are in accordance with previous studies, where HER3 expression tightly correlated with poor survival of colorectal cancer patients (38,39). Nevertheless further investigations concerning the HER3 status versus the genetic classification of the CRC tumors, namely LOH, MSI, HNPCC etc. would be of interest to get further insights into the relevance of HER3 expression in different colon cancer subtypes.

To specifically address the function of HER3 in colon cancer development and progression, human colon cancer cell lines were analysed by siRNA interference with HER3 expression. As described above, down-regulation of HER3 induced a reduction in proliferation as well as a G2/M cell cycle arrest in all 4 tested colon cancer cell lines. On the molecular signalling level, HER3 knockdown resulted in increased p27 expression levels. Additionally, phosphorylation of Rb, and cyclin B1 expression were reduced upon HER3 knockdown in comparison to the control cells. Indeed, these molecules are well established and known as essential cell cycle regulators. Their modulation seemed to be the direct cause of the observed growth inhibition and the cell cycle arrest in colon cancer cells.

HER3 down-regulation in both heregulin-ß1 stimulated and non-stimulated cells results in a reduction of the Akt phosphorylation (Fig. 3D; Supplementary Fig. S4D). In fact, up-regulation of p27 may be a direct consequence of the observed impairment of the Akt activity since Akt is known to trigger p27 degradation and cell cycle progression (40-43). Furthermore, it is important to note that several already
published reports have demonstrated the well-established potential of HER3 to activate downstream PI3K-Akt pathway signalling by virtue of six consensus phosphotyrosine sites, which are absent in EGFR or HER2 (16,28,44,45,46). Interestingly, the impaired Akt activity shown in cells upon HER3 knockdown was accompanied by a reduction in the phosphorylation of mTOR at Ser\textsuperscript{2448}, which is known to be mediated by the PI3K-AKT signalling pathway (47). Notably, HER3 ablation did not affect the extracellular signal-regulated kinase 1/2, suggesting that inhibition of other downstream pathways seemed to be sufficient to block colon cancer cell proliferation. Taken together, our results suggest that HER3 expression and phosphorylation controls colon cancer cell proliferation majorly through the PI3K-AKT signalling pathway.

We subsequently checked the role of HER3 as a regulator of cell survival in colon cancer. Interestingly, HER3 depletion in all four tested colon cancer cell lines resulted in an induction of apoptosis, which varied form one cell line to another. Our observations are in fact in accordance with previous studies demonstrating the involvement of the HER3 receptor in regulating cell survival. Indeed, Lee and co-workers showed that deletion of HER3 induced a tumor-specific apoptosis in mouse intestinal epithelium (48). Additionally, HER3 down-regulation in lung cancer cells led to a significant induction of apoptosis (14). Moreover, HER3 knockdown synergistically enhanced dacarbazine-induced apoptosis in melanoma cell lines (16). We next asked, whether a combination of HER3 down-regulation with chemotherapeutic drug treatment would increase cell death in our model system. To date, 5-Fluorouracil is the frequently used chemotherapeutic for the treatment of colon cancer patients. 5-Fluorouracil was also approved to be effective towards different colon cancer cell lines (49, 50). Interestingly the treatment of colon cancer cells using increasing 5-Fluorouracil concentrations upon HER3 down-regulation resulted in increased rates of apoptosis, suggesting that blocking HER3 might prove effective in the treatment of malignant colon cancer.

Notably established here for its first time is also the importance of HER3 in modulating migration, and invasion of colon cancer cell lines. Both processes were markedly inhibited by HER3 siRNA interference in DLD-1 and LoVo cell lines. Indeed, stimulation of tumor migration and invasion appears to be an essential function of HER3 in some other model systems since HER3 down-regulation abolished migration and migration/invasion in melanoma and breast cancer cell lines.
respectively (16, 33). The involvement of HER3 in regulating motility and migration was additionally demonstrated in the A549 lung cancer cell line (14). It will be of importance to elucidate the exact role of HER3 in these processes and to identify factors, which mediate HER3 responses during colon cancer metastasis like (e.g., metalloproteinases).

Interestingly, HER3 knockdown cells display a shift in the cell morphology, which is characterized by the change of cells towards a flat and round appearance. We believe that HER3 knockdown shifted the cells towards an epithelial-like cell morphology. In order to address this hypothesis in more detail, additional experiments were performed. We investigated changes in expression of the mesenchymal marker vimentin after HER3 down-regulation in LoVo colon cancer cells as an example. Decreased expression levels of vimentin were observed by Western Blot analysis and by immunofluorescence (Supplementary Fig. S3). Since these findings indicate HER3 as a potential regulatory factor for mesenchymal marker expression within colon cancer, it is possible that HER3 expression is linked to epithelial to mesenchymal transition (EMT), a common principle during colon cancer progression.

The employ of blocking antibodies for the treatment of cancers over-expressing pseudo-kinases such as HER3 is promising, since the use of small molecules inhibitors is thought to be inefficient in this case due to the absence of phosphotyrosine sites (51). We therefore treated human colon cancer cell lines using anti-HER3 monoclonal antibody, thereby testing the aptitude of HER3 to be considered as a novel target in colon cancer therapy. Our results indicate that an antibody directed against the extracellular domain of HER3 efficiently inhibits its phosphorylation after heregulin-ß1 stimulation. Anti-HER3 monoclonal antibody accelerates the endocytosis of HER3, resulting from its clearance from the cell surface, and inhibits the activation of downstream effectors since the binding of p85, the regulatory subunit of PI3K, to HER3 was abrogated. Remarkably, an induction of apoptosis in DLD-1 and LoVo colon cancer was shown to be induced after the treatment of cells using the HER3 blocking antibody for the first time. Furthermore, migration and invasion were considerably reduced in antibody-treated cells when compared to controls. Indeed, our results are well in line with previous studies, which established the efficiency of the same blocking antibody towards HER3-mediated migration and migration/invasion in breast and skin cancer, respectively (16, 33).
Taken together, previous as well as our present data indicate that anti-HER3 antibodies can inhibit HER3 signalling most likely through the PI3K-Akt pathway in breast, melanoma, and colon cancer thereby blocking several cellular functions. It will be necessary in the future to test the efficacy of anti-HER3 blocking antibodies on colon cancer development and metastasis in preclinical animal models to further validate HER3 as a possible target for colon cancer therapy. Collectively our data confirm that HER3 emerges as an important determinant of colon cancer behaviour as well as a possible novel target for drug design.

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References


Figure legends

Figure 1. HER3 confers poor prognosis in colon cancer patients

(A-E) Immunohistochemical staining of HER3 in primary tumors of colon cancer patients

A: Low membranous HER3 expression (arrows)
B: Moderate membranous HER3 expression (arrows)
C and D: Strong membranous HER3 expression (arrows)
E: Frequencies of HER3 expression in primary colon carcinomas. 110 primary tumors were evaluated for HER3 expression.

F: Kaplan-Meier curve for overall survival of patients with high and low HER3 expression levels ($P = 0.0131$).

Figure 2. Expression levels of EGFR, HER2, and HER3 in colon cancer cells

Equal amounts of protein lysates from T-84, DLD-1, LoVo, CaCO2, RKO, HT-29, and HCT-116 colon cancer cell lines were subjected to immunoprecipitation using a specific HER3 antibody as well as to Western blot analysis. HT-29 and HCT-116 were considered as a positive and a negative control, respectively. Representative
immunoblots for EGFR, HER2, and HER3 are shown. Tubulin served as loading control.

**Figure 3. HER3 Knockdown inhibits colon cancer cell proliferation**

**A:** Western blot analysis of HER3 and β-actin in DLD-1 and LoVo colon cancer cell lines after HER3 ablation.

**B:** Inhibition of DLD-1, and LoVo colon cancer cell proliferation by HER3 siRNA. The impact of HER3 Knockdown on cell proliferation was shown in both- unstimulated, and with heregulinβ-1 stimulated cells. The growth curves were done as described in Materials and Methods. Mean ± SD.

**C:** HER3 knockdown induces changes in the expression and phosphorylation of key cell cycle regulators, cyclin B-1 and phospho-Rb. Western blots are shown for phospho-Rb, and cyclin B-1. β-actin served as a loading control.

**D:** HER3 Knockdown impairs AKT and m-TOR activities in DLD-1, and LoVo colon cancer cell lines. Western blot analysis for HER3, phospho-AKT, p27, phospho-extracellular signal-regulated kinase 1/2, and phospho-mTOR are shown. Tubulin served as a loading control.

**Figure 4. HER3 knockdown induces a G2/M cell cycle arrest in colon cancer cell lines**

**A:** Representative flow cytometry histograms of DLD-1, T-84, CaCO2, and LoVo cell lines. 48 h post-transfection, cell cycle kinetics of HER3 knockdown cells and control cells were determined by propidium iodide staining.

**B:** Data represent means of triplicate experiments.

**Figure 5. HER3 Knockdown induces apoptosis and blocks migration and invasion of colon cancer cells**

**A:** HER3 Knockdown inhibits migration of DLD-1, and LoVo colon cancer cells. For quantification, migrated cells were fixed, stained, and counted. A total of at least 10 random microscopic fields were counted. The values for control cells were set to 100% and results are shown relative to controls. Mean ± SD.
B: HER3 Knockdown blocks DLD-1 and LoVo colon cancer cell invasion. Quantification of invaded cells was done as described in A.

C: HER3 depletion by siRNA induces apoptosis in DLD-1 and LoVo colon cancer cell lines. The percentage of apoptotic cells was increased upon treatment of HER3 Knockdown cells with increasing 5-fluorouracil concentrations (10 and 20 μmol/L). Mean ± SD.

Figure 6. An anti-HER3 monoclonal antibody (clone 105.5) inhibits HER3-mediated signalling, blocks migration and invasion and induces apoptosis in colon cancer cells

A: Anti-HER3 antibody treatment blocks heregulin-ß1-induced HER3 activation and its association with p85 and leads to receptor internalization or degradation. Serum-starved DLD-1, LoVo, and CaCO2 colon cancer cell lines were pre-treated with 10μg/ml of either an isotyp control antibody or HER3 blocking antibody (clone 105.5) for 60 minutes. Cell stimulation with heregulinß-1 was carried out for 120 minutes. Cell lysates were then subjected to immunoprecipitation using a specific HER3 antibody. Western blots of phospho-HER3 (Tyr 1289), HER3, and p85 are shown.

B: An anti-HER3 antibody (clone 105.5) inhibits colon cancer cell migration. Serum-starved DLD-1 and LoVo colon cancer cell lines were detached using 10 mM EDTA, incubated with 10μg/ml of either an isotyp control antibody or HER3 blocking antibody (clone 105.5) for 60 minutes, and transferred to the upper well of a Boyden chamber. Conditioned NIH3T3 medium containing 100 ng/ml heregulinß-1, and 0, 01% ascorbic acid was used as a chemoattractant. The quantification was done as described in Fig. 5A. Mean ± SD.

C: An anti HER3 antibody (clone 105.5) blocks colon cancer cell invasion. The assay was done as in B using growth factor-reduced Matrigel in a modified Boyden chamber. MDA-MB-468 breast cancer cell line was served in both migration and invasion assays as a positive control as described previously (33).

D: An anti HER3 antibody (clone 105.5) induces the apoptosis in DLD-1 and LoVo colon cancer cell lines. Apoptosis rates were determined by a propidium iodide staining as described in Materials and Methods.

Table 1. Multivariate analysis of cancer specific survival.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6

A

![Graph A]

B

![Bar graph B]

C

![Bar graph C]

D

![Bar graph D]
<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% confidence interval)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (≥ 68 vs. ≤ 67)</td>
<td>1.72 (0.91-3.22)</td>
<td>0.09</td>
</tr>
<tr>
<td>Gender (M vs. F)</td>
<td>0.93 (0.47-1.81)</td>
<td>0.82</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>0.89 (0.37-2.18)</td>
<td>0.80</td>
</tr>
<tr>
<td>Tumor location (rectum vs. colon)</td>
<td>1.75 (0.88-3.46)</td>
<td>0.11</td>
</tr>
<tr>
<td>T stage</td>
<td>3.95 (2.04-7.66)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HER3 (high vs. low)</td>
<td>3.29 (1.28-8.45)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Table 1
Towards the prognostic significance and therapeutic potential of HER3 receptor tyrosine kinase in human colon cancer

Abdelhamid Beji, David Horst, Jutta Engel, et al.

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