hVps37A Status Affects Prognosis and Cetuximab Sensitivity in Ovarian Cancer

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Abstract

Purpose: Although prognostic and predictive factors in ovarian cancer have been extensively studied for decades, only few have been identified and introduced to clinical practice. Here, we evaluate hVps37A (HCRP1) as a possible novel predictive marker for ovarian cancer. hVps37A was originally described as a member of the membrane-trafficking ESCRT-I complex mediating the internalization and degradation of ubiquitinated membrane receptors.

Experimental Design: We analyzed an ovarian cancer tissue microarray for HCRP1, EGFR, and HER2 expression. We used a tetracycline inducible ovarian cancer cell culture model to show the effects of hVps37A knockdown in vitro and in vivo. In addition, we studied the effects of epidermal growth factor receptor (EGFR) inhibitors cetuximab and lapatinib on ovarian cancer cells under conditions of hVps37A knockdown.

Results: We find that hVps37A is significantly downregulated in ovarian cancer and modifies the prognostic value of EGFR and HER2 expression. In addition, hVps37A downregulation in ovarian cancer cells leads to cytoplasmic pEGFR retention and hyperactivation of downstream pathways and is associated with enhanced xenograft growth in nude mice and invasion of the collagen matrix. Furthermore, due to subsequent sustained Akt- and MAPK-pathway activation, hVps37A-deficient cells become irresponsive to inhibition by the therapeutic antibody cetuximab.

Conclusion: We propose that hVps37A status could become a novel prognostic and therapeutic marker for EGFR or HER2 driven tumors. Clin Cancer Res; 17(24); 1–12. ©2011 AACR.

Introduction

Reliable prediction of prognosis and therapeutic response based on molecular factors will undoubtedly lead to more personalized cancer medicine. Despite increasing biological knowledge though, only few biological markers for ovarian cancer have entered clinical practice so far. The ErbB family comprises 4 members (ERBB1-4), of which ERBB1 (EGFR) is the most prominent. Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane receptor tyrosine kinase (RTK) that plays an essential role in governing multiple cellular processes, including cell proliferation, survival, and cell migration (1, 2). Targeting of EGFR and other ErbB family members as well as their downstream pathways is a mainstay of treatment in many malignant diseases. Activation by EGF or EGF-like ligands leads to receptor dimerization and autophosphorylation of cytoplasmic residues (3). The phospho-tyrosine residue itself serves as a docking platform for several adaptor molecules, triggering the activation of various pathways including the RAS-RAF-MEK-ERK pathway, the PI3K-Akt pathway, and the PLC-gamma-PKC pathway (4). Hyperactivation of EGFR-dependent signal transduction often accompanies tumor development, and cancer patients with unbalanced EGFR activity generally present with a more aggressive disease leading to unfavorable clinical outcome (5). It is widely believed that hyperactivation of EGFR-mediated signal transduction can be triggered by (a) increased ligand expression, (b) elevated EGFR protein expression, (c) activating EGFR mutations, (d) defective EGFR downregulation, and (e) cross-talk with heterologous receptor systems (6). Introduction of the anti-EGFR...
Translational Relevance

Our study shows that hVps37A is downregulated in a large number of ovarian cancer tissues and influences prognostic relevance of EGFR and HER2 expression. Moreover, we find that loss of hVps37A leads to cetuximab resistance in vitro. We propose that hVps37A expression might be exploitable as a prognostic and predictive marker in ovarian cancer and possibly in other cancer entities as well.

antibody cetuximab (IMC-C225, Erbitux) to clinical practice represented a major breakthrough in cancer therapy. Cetuximab binding leads to inhibition of EGFR-dependent signaling pathways, activation of antibody dependent cellular cytotoxicity (ADCC) and consequently to apoptosis and decreased cellular proliferation, survival, and migration (1, 2). Cetuximab is a chimeric monoclonal antibody binding extracellular domain of EGFR, preventing receptor dimerization and activation as well as triggering its internalization and degradation (3).

EGFR downregulation by the endosomal sorting process depends on receptor internalization via endocytosis, and subsequent vesicular shuttling toward one of 3 distinct cytoplasmic compartments (7). The internalized receptor can (i) be directed back to the cell surface, (ii) enter the trans-Golgi network (TGN), or (iii) be transported into the intraluminal vesicles (ILV) forming multivesicular bodies (MVB), which are subsequently degraded upon fusion with the lysosome (8, 9). The endosomal sorting complex required for transport-1 (ESCRT-1) is one of 3 protein complexes essential for sorting of ubiquitinated transmembrane proteins into internal vesicles of MVBs and subsequent degradation. Vps37A (vacuolar protein sorting 37 homologue A) was initially described in yeast as one of the 3 subunits of ESCRT-1 (10).

The human homologue of Vps37A (hVps37A), is located on the short arm of chromosome 8. For this region, 8p22, loss of heterozygosity (LOH) occurs to a high frequency in several human cancers including ovarian cancer. The expression of hVps37A was found to be reduced or undetectable in hepatocellular carcinoma (HCC; ref. 11) by a positional cloning approach and consequently the name HCRP1 (hepatocellular carcinoma–related protein) was suggested. Samples of formaldehyde fixed-paraffin embedded (FFPE) ovarian tumors for establishing the tissue microarray (TMA) were obtained from archival material of 144 patients who underwent radical cytoreductive surgery or between the years 1987 and 2002 at the Medical University of Vienna, Vienna, Austria. Written and oral informed consent was obtained from all patients according to the Ethics Committee of the Medical University of Vienna for further processing and analysis of the clinical data. The clinical characteristics of patients from the cohort used for the tissue microarray are described in Table 1. Microarrays were composed by taking core needle “biopsies” from specific locations in the preexisting paraffin-embedded tissue blocks and reembedding them in an array master block, using techniques and an apparatus developed by Beecher Instruments Inc., Micro-Array Technology (Sun Prairie). To achieve good representation of the tumor, 3 biopsies of tumor material were selected from each patient. None of the patients with borderline tumors died during the follow-up time and were excluded from the survival analysis. No data on chemotherapy treatment was available in this cohort, although most patients received platinum and taxane-based

also involved in ovarian cancer pathogenesis. We set out to characterize the function of hVps37A in an ovarian cancer model in vitro and in vivo as well as define its influence on prognosis of ovarian cancer patients.

Materials and Methods

Patient material

Samples of formaldehyde fixed-paraffin embedded (FFPE) ovarian tumors for establishing the tissue microarray (TMA) were obtained from archival material of 144 patients who underwent radical cytoreductive surgery or between the years 1987 and 2002 at the Medical University of Vienna, Vienna, Austria. Written and oral informed consent was obtained from all patients according to the Ethics Committee of the Medical University of Vienna for further processing and analysis of the clinical data. The clinical characteristics of patients from the cohort used for the tissue microarray are described in Table 1. Microarrays were composed by taking core needle “biopsies” from specific locations in the preexisting paraffin-embedded tissue blocks and reembedding them in an array master block, using techniques and an apparatus developed by Beecher Instruments Inc., Micro-Array Technology (Sun Prairie). To achieve good representation of the tumor, 3 biopsies of tumor material were selected from each patient. None of the patients with borderline tumors died during the follow-up time and were excluded from the survival analysis. No data on chemotherapy treatment was available in this cohort, although most patients received platinum and taxane-based

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<table>
<thead>
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<th>Patients’ characteristics</th>
<th>Tissue microarray N (%)</th>
<th>mRNA N (%)</th>
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<tr>
<td>II</td>
<td>31 (24.8)</td>
<td>19 (16.8)</td>
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<td>IV</td>
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<td><strong>Histology</strong></td>
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<td>70 (60.9)</td>
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<tr>
<td>Nonserous</td>
<td>55 (44.0)</td>
<td>45 (39.1)</td>
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adjuvant regimen according to the institutional standard operating procedures.

Samples of ovarian tumors for mRNA expression analysis were obtained from a second cohort of 115 patients who underwent radical surgery at the Charité University Hospital, Berlin, Germany between years 2000 and 2004. Epithelial-enriched normal ovarian and benign cyst samples came from patients diagnosed without malignant disease at the Medical University of Vienna. In total, 20 benign ovarian samples and 115 primary tumor samples were assessed. Informed consent to sample and data collection by all patients was given according to the Institutional Review Board of the Charité University Hospital Berlin and the Medical University of Vienna, as stated above. The clinical characteristics of these patients are described in Table 1. Eighty-five (78.0%) of the patients with ovarian cancer in the second cohort underwent chemotherapy with a platinum and taxane-based regimen.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

Total RNA from cell lines was prepared with the RNeasy Mini Kit (Qiagen) and quality and quantity assessed on RNA Nano Chips (Lab-on-a-Chip, Agilent Technologies). All steps were accomplished according to the manufacturers’ protocols.

cDNA was synthesized from 1 μg total RNA using the DuraScript RT-PCR Kit (Sigma-Aldrich) in a volume of 20 μL and subsequently diluted to a total volume of 100 μL. The following Assay-on-Demand probes were selected for TaqMan real-time PCR: FLJ32642, Hs00329751_m1 and beta 2-microglobulin (B2M), Hs99999907_m1, HPRT1 and GAPDH (Applied Biosystems). The real-time PCR reaction mix was composed of 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems) supplemented with 2 μL of the obtained cDNA, 1 μL of the probe and 8 μL of ddH2O to a total volume of 20 μL. The reaction was done on the 5700 Sequence Detection System (Applied Biosystems) with default cycle conditions. Expression was relatively quantified as described elsewhere (13) and its log-transformed values were used in the analysis. Briefly, PCR efficiencies were calculated from calibration curves for individual probes and expression rates were compared with control cells (calibrator). All PCR reactions were done from 3 independent experiments, and reverse transcriptase-negative and template-negative controls were included.

Cell culture and shRNA silencing of hVps37A

The human ovarian carcinoma SK-OV-3 cell line was cultured in McCoy’s medium enriched with 10% FBS and cultured in humidified incubator (37°C) at 5% CO2. For applying the Tet-Off inducible system, founder cell lines were generated by transfecting SK-OV-3 cells with the pTet-Off vector (neo+) encoding a tetracyclin repressible transactivator (tTA). Resistant colonies were selected with 200 μg/mL G418 and characterized by transient transfection with the luciferase reporter plasmid (pTRE-Luc) containing luciferase under the control of tTA. Cell lines with highest response were used to establish cells inducible for hVps37A-specific shRNA (Open Biosystems; #V2HS_21202, #V2HS_21203), which were subcloned into the SIN-TREmiR30-PIG vector (pur’) downstream of the tetracycline inducible promoter. Presence of tetracycline (doxycycline, Dox) in the culture medium then suppresses shRNA expression, while its withdrawal would induce the knockdown of hVps37A. Puromycin-resistant colonies were isolated in the presence of Dox, and screened for silencing efficiency of hVps37A by qRT-PCR and Western blotting.

The human ovarian carcinoma cell line MDAH-2774 was cultured in RPMI medium with 10% FCS (fetal calf serum), 50 units/mL penicillin G, and 50 μg/mL streptomycin sulfate at 37°C in a humidified atmosphere of 95% air with 5% CO2. Three different shRNA constructs complementary exclusively to the hVps37A mRNA in addition to one nonsense construct serving as a control, were cloned into the vector pSilencer 4.1-CMV neo. Transfection was done with Lipofectamine 2000 according to the manufacturers’ protocol (Invitrogen). Stable clones were selected with 700 μg/mL G418, picked, and subcultured with 350 μg/mL G418. Silencing efficiency was quantified by qRT-PCR and Western blot.

Antibody production

Polyclonal and monoclonal peptide antibodies against hVps37A were used for immunohistochemistry experiments and Western blotting. Tissue microarrays were analyzed using (at that time available) polyclonal antibody, whereas immunohistochemistry of mouse tumors and Western blotting were done using the monoclonal antibody. The following peptide sequence was selected: MSPYASQPFPPLPP. Rabbit antiserum raised against this peptide sequence was prepared and affinity purified by Eurogentec. Rabbit monoclonal antibody was also prepared by Eurogentec.

Immunohistochemistry

After deparaffinization and rehydration, the samples were treated with 0.3% H2O2/PBS (pH 7.4) for 10 minutes to quench endogenous peroxidase activity and blocked with serum of the secondary antibody diluted 1:50 in PBS. Primary antibodies against EGFR [EGFR (1005) Santa Cruz Biotechnology, Inc.] and hVps37A were diluted 1:100 in serum/PBS and applied on the samples for 1 hour. HER2 was stained using the Dako HercepTest (Dako). The secondary anti-rabbit antibody was applied for 30 minutes. For Ki-67 stainings, epitope retrieval was done using Deppp-9; endogenous peroxidase activity was quenched using 0.3% H2O2 in Methanol. The dilution for both the primary monoclonal mouse, anti-human Ki-67 (M7240, Dako) and secondary anti-mouse antibody (Vector Laboratories) was 1:200. After visualization with DAB+ (Dako) and counterstaining with hematoxyline & eosin, the slides were mounted in Eukitt (O. Kindler GmbH) and analyzed on an Olympus BX50 upright light microscope (Olympus Europe) equipped with the Soft Imaging system CC12.

The tissue microarrays were treated in an identical manner and the entire cohort was analyzed in one batch.
Binary clinical variables were calculated using Spearman’s rank correlations between expression levels and ordinal or 5-year survival prognosis of EGFR and HER2. Nonparametric ROC curves) was used to calculate specificity and sensitivity for the available staining for EGFR, HER2, and hVps37A were included in the survival analysis. Logistic regression (ROC analysis) was done with a median follow-up of 40.0 months (range 0.4–168.7 months) and event rate of 30.4%. Kaplan–Meier estimates of overall survival were obtained and groups divided into 2 groups with low (0, 1) or high (2, 3) expression (Table 2). Patients were dichotomized into 2 groups at the median of hVps37A expression. Because 40% of the tumor samples stained negatively or very weakly positively for EGFR, we also dichotomized the samples into 2 groups of low and high EGFR expression (Table 2). Overall survival analysis was done with a median follow-up of 40.0 months (range 0.4–168.7 months) and event rate of 30.4%. Kaplan–Meier estimates of overall survival were obtained and groups were compared using the log-rank test. Only patients with available staining for EGFR, HER2, and hVps37A were included in the survival analysis. Logistic regression (ROC curves) was used to calculate specificity and sensitivity for the 5-year survival prognosis of EGFR and HER2. Nonparametric correlations between expression levels and ordinal or binary clinical variables were calculated using Spearman’s p.

### Data analysis and statistics

In the tissue microarray analysis, staining intensities were evaluated by 2 independent investigators and classified as 0 (missing expression), 1 (low expression), 2 (moderate expression), and 3 (high expression). The tissues were graded positive (high or moderate expression) only when more than 10% cells were positive for the respective staining. For EGFR and HER2, results of triplicates and both interpretations were averaged and rescaled (0–3). Staining for HER2, according to standard procedures for breast cancer, was divided into 2 groups with low (0, 1) or high (2, 3) expression (Table 2). Patients were dichotomized into 2 groups at the median of hVps37A expression. Because 40% of the tumor samples stained negatively or very weakly positively for EGFR, we also dichotomized the samples into 2 groups of low and high EGFR expression (Table 2). Overall survival analysis was done with a median follow-up of 40.0 months (range 0.4–168.7 months) and event rate of 30.4%. Kaplan–Meier estimates of overall survival were obtained and groups were compared using the log-rank test. Only patients with available staining for EGFR, HER2, and hVps37A were included in the survival analysis. Logistic regression (ROC curves) was used to calculate specificity and sensitivity for the 5-year survival prognosis of EGFR and HER2. Nonparametric correlations between expression levels and ordinal or binary clinical variables were calculated using Spearman’s p.

### Table 2. hVps37A expression and clinicopathologic variables

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<tr>
<th>hVps37A</th>
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In vivo xenograft experiments were analyzed using unpaired Student t test. The mRNA expression analysis used relative normalized expression values for hVps37A expression of the normal (N) and ovarian patient (PT) cohort as measured by qRT-PCR. Group means were compared using Student t test. All statistical analyses were done using the SPSS statistical software.

### Protein isolation and Western blotting

The following antibodies were used in the dilution indicated: hVps37A 1:200 (established as described above); primary antibodies (EGFR 1:300, pEGFR 1:100, HER2 1:200, pHER2 1:200, ERK1 1:5,000; ERK2 1:5,000; and beta-actin 1:300) and HRP-conjugated secondary antibodies (anti-goat 1:10,000 and anti-rabbit 1:10,000) were obtained from Santa Cruz Biotechnology, Inc. Akt and pAkt were purchased from Cell Signaling Technology. Upon treatment, cells were lysed and protein was prepared with RIPA+ buffer; protein concentration was determined by a standard Bradford absorbance assay (Sigma Aldrich). Equal amounts of proteins (30 μg) were separated by SDS-PAGE, blotted on polyvinylidene difluoride membranes (GE Healthcare), incubated with the appropriate primary antibody, and visualized via HRP-conjugated secondary antibodies and treatment with the ECL chemiluminescent detection system (GE Healthcare).

### Fluorescence microscopy and TUNEL assay

Cells were seeded on chamber slides, grown for 48 hours, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.1% Triton X-100. The subsequent staining procedure was done as described for immunohistochemistry. The cells were incubated with the primary antibodies directed against pEGFR or EEA1 (1:100) for 1 hour. Alexa 610/350 secondary antibodies (1:200, pHER2 1:200, HER2 1:300) and HRP-conjugated secondary antibodies were applied for 45 minutes. Afterwards the cells were analyzed on a confocal microscope.

The “In Situ Cell Death Detection Kit, Fluorescin” (TUNNEL Assay) was obtained from Roche and conducted according to the manufacturer’s protocol. DNaseI-treated samples served as positive controls.

### Proliferation assays

A total of 2 × 10⁵ SK-OV-3 (with and without Dox) and MDAH (hVps37A silenced, nonsense control, and wild type) cells were seeded in 6-well plates in media complemented with 10% FCS and treated with 20 μg/mL cetuximab (Merck), 4 μmol/L lapatinib (GlaxoSmithKline plc.), or were mock treated. To maintain the logarithmic phase, cells were split at intervals of 48 hours, along with the determination of the cell number by a CASY cell counter (Innovatis AG). This procedure was done in triplicate over a time-span of 6 days and confirmed by a replication of the whole experiment. From the obtained data, doubling times (dt) were calculated and depicted as dt⁻¹.

### 3D cell culture experiments

SK-OV-3 ovarian cancer cells bearing a Dox inducible shRNA construct specific against hVps37A (described...
above) were induced to form multicellular spheroids in nonadhesive 96-well plates in the presence (control) or absence (knockdown) of Dox and grown for 72 hours. The spheroids were transferred to collagen gels (rat Collagen 1, Becton Dickinson, 1.75 mg/mL Collagen) and incubated in FGM supplemented with 2.5% serum (± Dox) and grown for further 72 hours. Spheroids were photographed and the areas of invasive structures were determined with the Axiosvision software (Zeiss).

Mice xenograft experiments
Each treatment group consisted of 10 8-week old athymic Foxn1null mice, which were maintained under specific pathogen-free conditions at the Institute for Cancer Research of the Medical University of Vienna. All mice were subcutaneously inoculated with SK-OV-3 ovarian cancer cells bearing a Dox inducible shRNA construct specific against hVps37A (described above). Dox (125 μg/d) was injected intraperitoneally in 50% of the mice to suppress hVps37A knockdown. The remaining mice were treated with PBS under the same conditions. Tumor size was measured every third day in 2 axes using a calliper. Tumor volume was calculated using the formula: volume = \( \frac{1}{2} \) (length \( \times \) width\(^2\)). Animal experiments were done according to protocols approved by the Austrian Federal Ministry for Education, Science, and Art.

Results

Prognostic value of EGFR and HER2 is dependent on hVps37A status
Because hVps37A was suggested to be involved in endosomal RTK-degradation (12), we thought that it might potentially influence the impact of EGFR/HER2 expression on the survival of ovarian cancer patients. Thus, we undertook a tissue microarray (TMA) analysis to test this hypothesis. Median follow-up for patients with malignant ovarian tumors was 40.0 months (range 0.4–168.7 months), and 38 patients (30.4%) had already died. The TMAs were analyzed for protein expression of EGFR and HER2, as these receptors represent well-studied markers for ovarian carcinogenesis and progression and are also targets of hVps37A-mediated receptor degradation. The representative staining intensities for hVps37A, EGFR, and HER2 in the ovarian TMA are shown in Fig. 1. Not surprisingly, high protein expression of the oncogenes EGFR (\( P = 0.005 \)) and HER2 (\( P = 0.002 \)) was associated with unfavorable overall survival rates, thus confirming our previous results and those of others (14; Fig. 2A and D). Furthermore, we looked at hVps37A expression in the same tumor samples. Consistent with the notion that hVps37A is involved in the endosomal sorting process of RTKs, it was primarily detected in the cytoplasm (Fig. 1). hVps37A expression was graded according to staining intensity (Fig. 1) and the patient cohort was divided at the median into 2 subgroups according to hVps37A expression. We observed a relatively high percentage (62%) of tumors with low or missing hVps37A expression in this patient population (Table 2), though hVps37A expression alone did not have an impact on overall survival of ovarian cancer patients (Fig. 2G). No significant differences regarding Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage, grade or histologic subtype were observed between high and low hVps37A expressing patients (Table 2). There was a weak, but significant
positive correlation between EGFR and hVps37A expression (Spearman’s \( r = 0.348, P < 0.001 \)).

To further identify and confirm the loss of hVps37A in ovarian cancer, we analyzed an independent cohort of ovarian cancer patients and measured hVps37A expression at the mRNA level. In this cohort, we also found hVps37A mRNA downregulation in ovarian cancer when compared with normal (epithelialy enriched) ovarian tissue (Fig. 3A), again indicating a deficiency of hVps37A expression in ovarian cancer. Further survival analysis of this cohort was not possible due to the short follow-up period.

To evaluate the impact of hVps37A expression on the prognostic impact EGFR and HER2, we stratified overall survival of our patients based on hVps37A expression. Interestingly, we can observe the strong impact of HER2 (\( P < 0.001 \)) and EGFR (\( P = 0.003 \)) expression on overall survival only in tumors with low or missing hVps37A protein expression (Fig. 2C and F). This well-known prognostic influence of HER2 and EGFR is largely lost in cases with regular (high) hVps37A expression (Fig. 2B and E). Logistic regression (ROC curves) was used to calculate specificity and sensitivity for the 5-year survival prognosis of EGFR and HER2 (Supplementary Fig. S1A–D). In line with corresponding Kaplan–Meier curves, we observe that the prognostic specificity and sensitivity of EGFR and HER2 is dependent on hVps37A expression, as reflected by the area under the curve (AUC). This observation points toward a possible influence of the receptor degradation mechanism on clinical relevance of growth factor receptor expression. Consequently, EGFR or HER2 overexpression may have an
impact on patients' prognosis only in tumors with decreased hVps37A expression.

**Activated EGFR and HER2 accumulate in hVps37A-negative cell lines**

We used ovarian cancer cell lines to gain closer insights into the causal biological mechanisms behind our findings. SK-OV-3 and MDAH-2774 ovarian cancer cell lines, which have detectable expression of EGFR and HER2, were established as model system for functional studies. hVps37A mRNA SK-OV-3 and MDAH-2774 was stably knocked down via a Dox inducible shRNA (SK-OV-3) or constitutive approach (MDAH-2774), respectively, and silencing efficiencies were determined on mRNA and protein levels (Fig. 3B and C, Supplementary Fig. S2A and B). We obtained clones with 60% to 70% reduction of hVps37A mRNA/protein, leading to elevated levels of activated EGFR and HER2 in the SK-OV-3 and MDAH-2774 cell lines. We juxtaposed hVps37A expression with activated EGFR receptor levels and found elevated phosphorylated EGFR while EGFR levels remained constant (Fig. 3B, Supplementary Fig. S2B). This resulted in a significant increase of the pEGFR/EGFR ratio. These observations suggest that activated EGFR accumulates within hVps37A-deficient cells as a result of defects in receptor degradation. We further aimed to define the cellular compartment harboring the aberrantly retained pEGFR protein using immunofluorescence. In control cells, pEGFR was hardly detectable (Fig. 3C). In contrast, pEGFR was detected at much higher levels in the cytoplasm of hVps37A knockdown cells. Frequent colocalization with the early endosomal marker EEA1 (Fig. 3C) indicated that pEGFR may accumulate primarily in sorting endosomes.

Figure 3. hVps37A mRNA is decreased in ovarian cancer as compared with normal ovaries. hVps37A knockdown is associated with pEGF/pHER2 accumulation. A, hVps37A mRNA expression in tumor tissue of an independent cohort of 115 ovarian cancer patients (PT) and 20 age matched normal controls (N). Student t test was used to compare mean mRNA expression levels of hVps37A. B, hVps37A is silenced upon Dox treatment in SK-OV-3tet-off cells, while hVps37A mRNA expression remains unaffected by Dox in SK-OV-3 wild-type cells. C, silencing of hVps37A leads to an increase in HER2 and EGFR phosphorylation. SK-OV-3tet-off cells containing the hVps37A shRNA construct were cultivated in presence or absence of Dox and analyzed by SDS-PAGE and immunoblotting. D, pEGFR (red) accumulates in hVps37A silenced cells and is colocalized with early endosomal marker (EEA1, blue). SK-OV-3tet-off cells were cultivated in presence or absence of Dox on chamber slides, probed against pEGFR and EEA1 and analyzed by confocal microscopy. Colocalization of the pEGFR (red) and EEA1 (blue) signal is indicated by arrows. Green fluorescence of the nuclei arises from the presence of an EGFP cassette in the Tet-Off vector.
This observation further supports the notion of ineffective receptor sorting in hVps37A-depleted cells. To see whether this concept can be extended to other cell lines of ovarian and mammary origin, we quantified hVps37A, (p)EGFR and (p)HER2 by immunoblot analysis in 15 ovarian and breast cancer cell lines and plotted the ratios of activated to total receptor versus hVps37A (Fig. 4B). No significant association between hVps37A and EGFR ($r = 0.111; P = 0.695$) was found, yet we observed a significant ($P < 0.01$) reciprocal correlation between hVps37A and pEGFR protein expression ($r = -0.683; P = 0.007$) as well as between hVps37A and pEGF/EGFR protein ratio ($r = -0.719; P = 0.005$). A statistically weak correlation was also found between hVps37A and pHER2 protein expression, but did not reach the level of significance (data not shown). All these observations lead us to the conclusion that phosphorylated EGFR receptor accumulation in ovarian cancer may be caused by defects in endosomal protein degradation. To further validate this hypothesis, we studied the dynamics of pEGFR degradation in wild type and hVps37A knockdown cells. After incubation in a serum-free medium, cells were stimulated with 100 ng/mL EGF for 15 minutes to achieve receptor activation followed by reincubation in serum-free medium. At defined time points, cells were harvested and pEGFR expression determined by Western blotting. pEGFR degradation was significantly impaired in hVps37A-deficient cells while total receptor levels remained constant in SK-OV-3 and MDAH-2774 cell lines (Fig. 4A, Supplementary Fig. S2C), suggesting sustained EGFR signaling in these cells. This led us to investigate the effects of hVps37A upon treatment with EGFR inhibiting agents.

**hVps37A interferes with EGFR signal transduction and anti-EGFR antibody treatment**

Characterizing changes in EGFR-downstream signalling upon hVps37A knockdown might shed further light on the interaction between hVps37A and EGFR signal transduction. To further determine the effect of hVps37A knockdown on EGFR downstream signalling, we monitored the expression of pAkt, Akt, pErk1/2, Erk1/2, and actin in SK-OV-3tet off cells cultivated in presence or absence of Dox and exposed to 20 μg/mL cetuximab, or 4 μmol/L lapatinib or remained untreated. Protein levels of pAkt, Akt, pErk1/2, Erk1/2, and actin were then determined by Western blotting. D, hVps37A silenced cells are resistant to cetuximab. A total of $2 \times 10^5$ SK-OV-3tet off cells were cultivated in presence or absence of Dox and exposed to 20 μg/mL cetuximab, or 4 μmol/L lapatinib or remained untreated (U). Cell number was determined every 48 hours by a CASY cell counter over 8 days. Doubling time (dt) was calculated, normalized to untreated control and depicted as 1/dt. The columns indicate the mean of 3 independent experiments; error bars represent ± SEM.

**Figure 4.** Loss of hVps37A impairs hVps37A degradation and cetuximab sensitivity in ovarian cancer cells. A, time-pulsed activation of pEGFR is sustained in hVps37A silenced cells. SK-OV-3tet off cells cultivated in presence or absence of Dox were starved by serum withdrawal, then pulsed with 100 ng/mL EGF and cultivated for indicated time in serum-free medium. pEGFR and EGFR levels were determined by SDS-PAGE and immunoblotting. B, hVps37A negatively correlates with pEGFR/EGFR ratio. hVps37A, EGFR and pEGFR protein amounts were determined in 15 ovarian and breast cancer cell lines by immunoblotting. Statistical significance was tested by Pearson Correlation ($r = -0.719$) with a $P$ value of 0.005 (C) hVps37A silencing leads to resistance against cetuximab-mediated EGFR inhibition. SK-OV-3tet off cells cultivated in presence or absence of Dox were incubated for 24 hours with either 20 μg/mL cetuximab, or 4 μmol/L lapatinib or remained untreated. Protein levels of pAkt, Akt, pErk1/2, Erk1/2, and actin were then determined by Western blotting. D, hVps37A silenced cells are resistant to cetuximab. A total of $2 \times 10^5$ SK-OV-3tet off cells were cultivated in presence or absence of Dox and exposed to 20 μg/mL cetuximab, or 4 μmol/L lapatinib or remained untreated (U). Cell number was determined every 48 hours by a CASY cell counter over 8 days. Doubling time (dt) was calculated, normalized to untreated control and depicted as 1/dt. The columns indicate the mean of 3 independent experiments; error bars represent ± SEM.
role of hVps37A in ovarian carcinogenesis. We therefore analyzed differences in ErbB-signalling pathways in hVps37A knockdown versus control cells. Consistent with extensive receptor phosphorylation and downstream pathway activation, knockdown of hVps37A was associated with increased phosphorylation of Erk1/2 (Fig. 4C, Supplementary Fig. S2D). Unlike Erk, Akt phosphorylation at serine 473 was not changed in the 2 cell lines studied, arguing for baseline Akt phosphorylation not being affected by sustained growth factor signalling upon hVps37A knockdown in ovarian cancer cells. Next, we wondered whether EGFR inhibition by anti-EGFR antibody cetuximab or EGFR/HER2 tyrosine kinase inhibitor (TKI) lapatinib might have any consequences on EGFR phosphorylation in hVps37A knockdown cells. Lapatinib treatment did inhibit EGFR downstream signalling in both, hVps37A knockdown and wild-type cells, as evidenced by dephosphorylation of Erk1/2 and Akt in the SK-OV-3 and MDAH-2774 cell lines (Fig. 4C, Supplementary Fig. S2D). Interestingly, this was not the case for cetuximab. After cetuximab treatment, Erk1/2 and Akt remained phosphorylated in hVps37A knockdown cells. Cytoplasmic degradation of phosphorylated EGFR seems to be crucial for cetuximab-dependent inhibition of its downstream pathways. On the other hand, EGFR inhibition by lapatinib is rather mediated by its direct interaction with the tyrosine kinase domain, independent of endosomal processing and consequently unaffected by hVps37A status.

Dysfunctional activation of Erk-signal transduction leads to increased cellular proliferation, invasion, and tumorigenesis. We studied the proliferation of control cells and hVps37A knockdown SK-OV-3 cells, which reveals comparable rates (25.5 vs. 24.9 hours doubling time, respectively), thus indicating a limited impact of hVps37A on cellular proliferation under unstressed, untreated in vitro conditions (data not shown). Incubation with cetuximab, however, significantly decreases the proliferation potential of the control cells expressing hVps37A (P < 0.01), whereas the respective hVps37A knockdown cells remain unaffected. This fact indicates that the presence of hVps37A is essential for proper degradation of the receptor-antibody complex, which has been shown to be essential for efficient cetuximab mediated antitumor activity (15). EGFR/HER2 inhibition by lapatinib significantly reduced cell proliferation irrespective of hVps37A expression levels (P < 0.01) in SK-OV-3 and MDAH-2774 cell lines (Fig. 4D, Supplementary Fig. S2E).

Enhanced growth of hVps37A knockdown cells in mouse xenografts

Next, we were curious about the in vivo growth characteristics of the Vps37A-knockdown SK-OV-3 cells in a mouse xenograft model. A total of 5 × 10^6 cells were subcutaneously inoculated into hind flanks of nude mice, half of which were treated with Dox to obtain suppression of the shRNA construct. The mice were sacrificed according to the institutional ethics committee protocols. Tumor growth was increased in mice with hVps37A-knockdown xenografts, resulting in final mean tumor volumes of 1,027 mm^3 compared with 683 mm^3 for the uninduced tumors (P < 0.001, Fig. 5C). The tumors were paraffin embedded for subsequent analysis and successful knockdown of hVps37A was confirmed via IHC staining (Fig. 5D). Interestingly, cellular proliferation was not significantly affected as determined by a Ki67 assay (Fig. 5F). However, we observed significantly decreased apoptosis rates for the Vps37A-knockdown tumors (Fig. 5E). These data indicate that the tumor growth effect can be traced back to sustained survival rather than elevated proliferation.

Discussion

Activation of ErbB RTK family isoforms is often associated with tumor development and progression. One of the mechanisms leading to hyperactivation of ErbB receptor signalling is defective receptor degradation. Recently, hVps37A has been recognized as a member of the ESCRT-I complex. Although we could not detect mutations in the coding region of hVps37A (M. Wittinger and D. Pils, unpublished results), we observed hVps37A mRNA and protein expression to be significantly reduced in primary ovarian cancer, indicative for a negative selection pressure against hVps37A expression.

Due to the cytosolic orientation of the phosphorylated tail of RTKs, receptors targeted for endosomal degradation are still signaling competently, an effect which is probably increased by the observed cytoplasmic retention of pEGFR (16). In fact, we describe an increase in Erk1/2 phosphorylation upon knockdown of hVps37A, while basal phosphorylation of Akt remains unchanged. A possible explanation for this effect may be that endosomally located phosphorylated EGFR can not activate PI3K signaling (17), whereas other signaling pathways remain unaffected. MAPK activation leads to different responses dependent on the cellular background or intensity and duration of the signal (18–21).
Our findings resemble closely those established for Tsg101, another component of the ESCRT-I protein complex. It has been reported that the endosomal degradation of EGFR is impaired in Tsg101-deficient cells (22). In addition, nonfunctional Tsg101 causes EGFR accumulation within the cytoplasm (23). However, the function of Tsg101...
as a tumor suppressor gene is controversial, because its
knock-out did not result in cellular transformation and
increased proliferation in mouse embryonic fibroblasts
(24). Our results argue in favor of the occurrence of non-
functional ESCRT-I complexes under conditions of
hVps37A knockdown. We assume that in contrast to
tsg101, hVps37A might indeed constitute a novel tumor
suppressor in ovarian cancer, as this function is assumed for
the functional ESCRT-I complex (24).

EGFR is overexpressed in about 60% of ovarian epithelial
cancers and its activation is correlated with increased tumor
growth and invasion as well as poor patient outcome (25,
26). We found that hVps37A significantly affects the prog-
nostic impact of EGFR and HER2 expression in ovarian
cancer (14, 27). Under conditions of low hVps37A expres-
sion, both EGFR and HER2 expression levels were highly
prognostic, a trait which was completely abrogated under
conditions of high hVps37A expression. Obviously,
hVps37A deficiency tips the fine balance between RTK
activation and subsequent degradation, furthermore influ-
encing patients’ survival. Multiple marker testing would
thus be beneficial to obtain a more reliable and accurate
picture of the disease. If our results can be confirmed in
more comprehensive clinical studies, hVps37A testing
could define a novel path in EGFR and HER2 testing of
ovarian cancer.

The clinical use of several EGFR-targeted therapies in
ovarian cancer has been limited due to their low efficacy
in phase I and II trials (26, 28). EGFR is at the same time
a putative target for hVps37A-mediated receptor degrada-
tion (12, 29). We propose that individual tumor cells can
enhance their selective advantage in the course of disease
progression via downregulation of hVps37A. This subset of
tumors could be more sensitive to a disruption of the EGFR
pathway by EGFR-targeted therapy using monoclonal anti-
body or small molecule TKIs. So far, cetuximab has been
studied and found ineffective in ovarian cancer in several
phase II clinical trials (25, 30–32). Cetuximab is known to
downregulate EGFR signal transduction by various mechan-
isms, including induction of an immune response to the Fc-
region in vivo (33) also known as antibody-dependent
cellular cytotoxicity (ADCC). The role of ADCC in cetux-
imab-mediated antitumor activity has been studied exten-
sively (34), though the proportion to which ADCC and the
immune system contribute to the clinical activity of cetux-
imab is controversial (35). In our study, we focused on
inhibition of EGFR downstream signaling by cetuximab,
which is abrogated by hVps37A loss and could presumably
be a determinant of its clinical activity.

Although there are various mechanisms leading to resis-
tance against anti-EGFR-therapies, including autocrine
EGFR activation, mutation of downstream signaling effec-
tors, and cross-activation of alternative RTKs, recently pub-
lished studies add an additional twist: Cancer cell lines
resistant to cetuximab by long-term exposure developed
increased EGFR levels as a result of defective receptor
degradation (15). The genetic knockdown of hVps37A and
the resulting resistance to cetuximab in our model confirm
this molecular mechanism and propose a model of a pre-
existing cetuximab resistance in advanced ovarian cancer.
Furthermore, in addition to the antagonistic effects, cetux-
imab also possesses the potential to trigger EGFR activation
and dimerization prior to its downregulation (36, 37).
Following incubation with cetuximab, MAPK pathway,
and Akt-pathway remained active in hVps37A-deficient cell
lines, arguing for sustained functional signaling of the
EGFR-cetuximab complex, resulting in resistance to cetux-
imab and unrestrained cell proliferation. In contrast to
cetuximab, lapatinib directly blocks EGFR activation by
targeting the ATP-binding domain. This leads to defective
EGFR-dependent signal transduction independently of
ESCRT-mediated receptor downregulation (38). This is in
line with our observations, that PI3K/Akt and MAPK path-
ways, as well as cell proliferation, were inhibited by lapa-
tinin irrespective of hVps37A expression levels.

Overall, we see hVps37A as a novel tumor suppressor
gene with an essential role in receptor tyrosine kinase
degradation pathway. We propose a clinical relevance for
measuring hVps37A expression, to evaluate it further as a
potential biomarker in ovarian cancer and beyond.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors’ Contributions

M. Wittlinger, P. Vanhara, B. Brenner, A. El-Gazzar, M. Anees, and M. Holmzann performed the experiments. M. Wittlinger, P. Vanhara, P. Horak, and M. Krainer designed the study and wrote the manuscript. M. Sibilia supervised the in-vivo experiments. M. Schemper performed the statistical analysis. M. Sibilia, D. Plh, R. Zeillinger, T. Grunt, and P. Horak provided crucial ideas. R. Horvat, C. Schöfer, and H. Dolznig provided crucial reagents and protocols. All authors discussed and interpreted results.

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