

Neuromedin U: A Candidate Biomarker and Therapeutic Target to Predict and Overcome Resistance to HER-Tyrosine Kinase Inhibitors

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Abstract

Intrinsic and acquired resistance to HER-targeting drugs occurs in a significant proportion of HER2-overexpressing breast cancers. Thus, there remains a need to identify predictive biomarkers that could improve patient selection and circumvent these types of drug resistance. Here, we report the identification of neuromedin U (NmU) as an extracellular biomarker in cells resistant to HER-targeted drugs. NmU overexpression occurred in cells with acquired or innate resistance to lapatinib, trastuzumab, neratinib, and afatinib, all of which displayed a similar trend upon short-term exposure, suggesting NmU induction may be an early response. An analysis of 3,489 cases of breast cancer showed NmU to be associated with poor patient outcome, particularly those with HER2-overexpressing tumors independent of established prognostic indicators. Ectopic overexpression of NmU in drug-sensitive cells conferred resistance to all HER-targeting drugs, whereas RNAi-mediated attenuation sensitized cells exhibiting acquired or innate drug resistance. Mechanistic investigations suggested that NmU acted through HSP27 as partner protein to stabilize HER2 protein levels. We also obtained evidence of functional NmU receptors on HER2-overexpressing cells, with the addition of exogenous NmU eliciting an elevation in HER2 and EGFR expression along with drug resistance. Finally, we found that NmU seemed to function in cell motility, invasion, and anoikis resistance. *In vivo* studies revealed that NmU attenuation impaired tumor growth and metastasis. Taken together, our results defined NmU as a candidate drug response biomarker for HER2-overexpressing cancers and as a candidate therapeutic target to limit metastatic progression and improve the efficacy of HER-targeted drugs. *Cancer Res*; 74(14): 3821–33. ©2014 AACR.

Introduction

HER2 overexpression occurs in approximately 25% of breast cancer and is associated with poor prognosis (1, 2). Targeting HER2 with trastuzumab (Herceptin; Genentech), a monoclonal antibody directed against the extracellular domain of the growth factor receptor, has shown activity as a single agent and in combination with classical chemotherapy in HER2-overexpressing breast cancer (3–5). HER2 has also been

successfully targeted with lapatinib (Tykerb; GlaxoSmith-Kline), a reversible dual kinase inhibitor directed at the intracellular ATP-binding domain of the receptor and its dimerisation partner, EGFR/HER1, rendering it kinase inactive (6, 7). Unfortunately, not all HER2-positive patients respond to trastuzumab (8, 9) or lapatinib (10–12) and those that initially respond may acquire resistance. More recently developed neratinib (Pfizer/Puma Biotechnology) is an irreversible small-molecule tyrosine kinase inhibitor (TKI) of EGFR/HER1, HER2, and HER4 (13). In advanced HER2-overexpressing breast cancers, including trastuzumab-refractory and trastuzumab-naïve patients, single-agent neratinib demonstrated some clinical benefits in both cohorts (14) and phase III clinical trials are underway. Our preclinical studies show that HER2-overexpressing cell lines acquire resistance to neratinib, as they did to trastuzumab and lapatinib; although it is too early to know whether this will occur in the clinic. Afatinib (BIBW 2992, Boehringer Ingelheim), another irreversible oral small-molecule TKI of EGFR/HER1, HER2, and HER4 has shown single-agent activity in a phase II trial including trastuzumab-refractory HER2-positive metastatic breast cancers (15).

Targeting HER2 and other HER family members is, undoubtedly, providing significant clinical benefit in patients with HER2-overexpressing breast cancers. However, innate/primary

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and acquired resistance represents substantial clinical challenges, in addition to the lack of means to predict patients' response. Predictive biomarkers for HER-targeting drugs (ideally extracellular, minimally invasive) are needed for improved patient selection and thus enhanced patient outcome, as are novel therapeutic strategies to circumvent resistance. Our previous studies indicate that analyzing extracellular RNAs in medium conditioned by cells can be a useful starting point to identifying potential biomarkers, some proving to have functional relevance within the cell (16–20).

Neuromedin U (NmU) is a secreted neuropeptide and a member of neuromedin peptide family originally isolated from porcine spinal cord (21). NmU, originally named because of its potent uterine contraction-inducing activity, is synthesized as a 174 amino acid precursor and cleaved to a 25 amino acid biologically active peptide. Two G-coupled receptors for NmU have been identified, NmU-R1 (predominantly in the periphery, especially gastrointestinal tract) and NmU-R2 (predominantly in central nervous system). Evidence suggests a role for NmU in pain, stress, immune-mediated inflammatory diseases, and feeding regulation (see reviews: refs. 22, 23). A limited number of studies to date have associated NmU with cancer; with conflicting observations depending on the cancer type. Although NmU has never previously been associated with breast cancer or, indeed, with any HER2-overexpressing cancers, our studies point to a role for NmU as a biomarker for HER-targeted drugs. In addition, NmU may serve as a new therapeutic target to help block tumor growth and metastasis, circumvent primary or acquired resistance, and to add value to HER-targeted therapies.

Materials and Methods

Refer to Supplementary Materials and Methods for detail.

Cell culture and treatments

SKBR3, HCC1954, MDA-MB-361, and T47D cells, obtained from ATCC, were cultured in RPMI-1640 (Sigma-Aldrich) with 10% FCS (PAA) and 1% L-glutamine. Trastuzumab-conditioned (resistant) SKBR3 (SKBR3-TR) cells, and both SKBR3 and HCC1954, lapatinib-resistant cells (SKBR3-LR and HCC1954-LR), and neratinib-resistant cells (SKBR3-NR and HCC1954-NR) resulted from continuously exposing cells to the respective drug for over 6 months. To assess whether functional NmU receptors are expressed on cells, SKBR3 and HCC1954 (1×10^6 cells) were seeded in a 25-cm² flask, allowed to attach overnight and subsequently treated with 1 μ M of NmU-25 (Bachem) for 24 to 48 hours. Cell lines were authenticated by short tandem repeat profiling, tested for *Mycoplasma* quarterly, and were impact tested before introducing into murine models. These were most recently tested in December 2013/January 2014. Trastuzumab was obtained from St. Vincent's Hospital (Dublin, Ireland). Lapatinib, neratinib, and afatinib were purchased from Sequoia Chemicals Ltd.

Short-term drug exposure assays

SKBR3 and HCC1954 cells were seeded (5×10^5 cells, 25-cm² flasks) and allowed to grow to 80% confluency before being

exposed, for 48 hours, to lapatinib (1 μ M/L), trastuzumab (12.5 μ g/mL), neratinib (0.5 μ M/L), or afatinib (0.5 μ M/L).

NmU knockdown and overexpression

Two NmU-specific siRNAs (designated NmU-1 and NmU-2; s225456 and s21351, Ambion), and scrambled siRNA (Scr; negative control; AM4611, Ambion; 30 nmol/L) was used. Transient transfections were harvested 72 hours posttransfection for RNA and protein extraction. NmU full-length cDNA and mock controls (plasmid lacking NmU cDNA) used were of pcDNA3.1(+). Lipofectamine 2000 (Invitrogen) was used for transfection following the manufacturer's instructions. Stable transfectants were established by selecting with zeocin (50 μ g/mL and 300 μ g/mL for HCC1954 and SKBR3-transfected cells, respectively; Invitrogen). For *in vivo* studies, HCC1954-LR-LUC⁺ (i.e. acquired resistance cells) cells stably expressing the luciferase gene (HCC1954-LR-LUC⁺) were transfected with shRNAs for NmU cloned in the pLKO.1 vector (shRNA #1–#5; Open Biosystems, Cat. no.: RHS4080_NM-006681). After verifying successful NmU protein knockdown and conferred drug sensitivity *in vitro*, one (shRNA #1) variant was selected for *in vivo* analysis. HCC1954-LR-LUC⁺ cells with no NmU knockdown were used as a control.

Proliferation assays

Cells with acquired resistance compared with aged-parent cells and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 2×10^3 cells/well; SKBR3 variants, 5×10^3 cells/well) were seeded in 96-well plates for 24 hours before drug additions. Subsequently, a range of concentrations for lapatinib, neratinib, trastuzumab, or afatinib was added to the cells. Five days later, proliferation was assessed using the acid phosphatase method.

Assessing effects of NmU siRNAs with HER-targeted drugs

Forty-eight hours following transfection with NmU-1, NmU-2 siRNA, or Scr sequences, cells were exposed to their approximate IC₅₀ concentrations of lapatinib. A fixed concentration of trastuzumab (15 μ g/mL), neratinib (1 μ M/L), and afatinib (0.5 μ M/L) was assessed for all four cell line variants.

qPCR

Total RNA was isolated from cell lines and conditioned medium using Tri Reagent (Sigma-Aldrich), and treated with DNase enzymes. cDNA was prepared from 500 ng cell-derived and 4 μ L conditioned medium-derived total RNA, respectively. *NmU* (Hs00183624_m1, ABI) was quantified using the threshold cycle (C_t) adjusting to the levels of β -actin (4352933E, ABI) as endogenous control.

Immunoprecipitation

Primary antibody was added (2 μ g/100 μ L of cell extract) to total cell protein (1 μ g/ μ L; total volume of 100 μ L), mixed and incubated for 120 minutes at 4°C. Of note, 20 μ L of 50% protein A agarose beads (Sigma-Aldrich) was added to the cell extract mix and incubated for 60 minutes at 4°C. This mixture was centrifuged at 5,000 rpm for 5 minutes. The beads were washed five times with the cold Lysis buffer and centrifuged at 5,000

rpm for 2 minutes. The beads were resuspended in 20 μ L of Laemmli's sample buffer, boiled for 5 minutes, and centrifuged for 5 minutes. Subsequently, 18 μ L of the samples were loaded per lane in a SDS-PAGE.

Immunoblotting and ELISA

Total cellular proteins (30–40 μ g, depending on the specific protein's abundance; but constant for any given protein) were resolved on 6% to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Primary antibodies used included EGFR (Neomarker), HER2 (Calbiochem), HSP27 (Enzo Life Sciences), NmU-R1 (Sigma-Aldrich), NmU-R2 (LifeSpan Biosciences), and β -actin (Sigma-Aldrich). Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and proteins were visualized by chemiluminescence (Millipore). Detection was performed with a Chemidoc exposure system (Bio-Rad Laboratories). An ELISA kit for NmU (Peninsula Laboratories), HER2 (Calbiochem), and EGFR (R&D Systems) was used according to the manufacturer's instructions.

Wound-healing assay

HCC1954-mock and NmU, HCC1954-LR associated Scr- or siRNA-transfected cell variants (5×10^5 cells/well) and SKBR3-mock and NmU variants (1×10^6 cells/well) were seeded on 6-well plates and cultured for 48 hours to confluency. Monolayer was scratched with a pipette tip and the resulting wounded areas were monitored by phase contrast microscopy and determined using NIH ImageJ software.

Migration and invasion assay

Migration assays were performed using 8- μ m pore size 24-well Transwell chambers (BD Biosciences). For invasion assessment, the inserts were precoated with ECM (Sigma-Aldrich). siRNA- or Scr-transfected cells, and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 1×10^5 /insert; SKBR3 variants, 1×10^6 /insert) were seeded in the upper compartment and allowed to migrate for 48 and 72 hours, respectively. Cells in the upper chamber were removed and migrated/invaded cells were stained with crystal violet. Staining was solubilized in 10% acetic acid, and read at 595 nm.

Anoikis assay

siRNA- or Scr-transfected cells, and NmU cDNA- versus mock-transfected cells (HCC1954 variants, 1×10^5 cells/well; SKBR3 variants, 1×10^4 cells/well) were seeded onto a 24-well plates coated with Poly(hydroxyethyl methacrylic) acid (Sigma-Aldrich) or 95% ethanol and cultured for 24 and 48 hours, respectively. Alamar Blue dye (100 μ L; Serotec) was added/well and absorbance read at 570 nm; reference wavelength, 600 nm.

Assessing potential clinical relevance of NmU in breast cancer

NmU expression was evaluated in microarray datasets representing 3,489 breast tumors, including luminal A ($n = 1,521$), luminal B ($n = 676$), HER2 ($n = 476$), and basal ($n = 454$) molecular subtypes. Methods used for obtaining and collating

the data are detailed in Supplementary Materials and Methods and Supplementary Table S1. All calculations were performed in R (<http://cran.r-project.org/>).

In vivo analysis

All *in vivo* work reported was carried out at University College Dublin (UCD, Dublin, Ireland), approved by the UCD Animal research ethics committee and performed according to local guidelines. BALB/c nu/nu mice (Charles River) were implanted in the mammary fat pad with 10×10^6 HCC1954-LR-LUC control ($n = 10$) or HCC1954-LR-LUC-shRNA NmU[−] cells ($n = 20$). Tumor growth was monitored by caliper measurement and bioluminescence imaging (IVIS Spectrum, PerkinElmer). Treatment was due to start once tumor volume reached 200 mm³. When mice reached endpoint, they were sacrificed and *ex vivo* imaging performed to detect any metastatic spread.

Statistical analyses

Statistical analysis on cell line- and conditioned medium-derived data was performed in Excel. *P* values were generated using Student *t* tests, with $P < 0.05$ considered as statistically significant. GraphPad Prism 5.0 was used for graph generation (GraphPad Software Inc).

All statistical analyses performed are detailed in Supplementary Materials and Methods.

Results

Intracellular and extracellular NmU mRNA levels are associated with acquired resistance to lapatinib

To identify extracellular as well as intracellular mRNAs that may be associated with resistance to HER-targeted agents, our initial analysis included HER2-overexpressing cell line models, SKBR3 and HCC1954, and lapatinib-resistant (LR) variant compared with their aged parent populations. Comparing the concentration of lapatinib that inhibits 50% of proliferation (IC₅₀) for SKBR3 that had acquired resistance (SKBR3-LR; IC₅₀ = 3 μ mol/L) in relation to SKBR3 aged (-Ag) cells (IC₅₀ = 0.09 μ mol/L) as controls (and so termed SKBR3-Ag), a 29.8 ± 2.2 -fold resistance to lapatinib was observed. For HCC1954 cells, a similar trend was observed with a 19.1 ± 2.8 -fold resistance to lapatinib in HCC1954-LR (IC₅₀ = 5 μ mol/L) compared with its age-matched population, HCC1954-Ag (IC₅₀ = 0.3 μ mol/L; Fig. 1A).

Evaluating mRNAs in medium conditioned by SKBR3-LR and HCC1954-LR cells, compared with conditioned medium from their age-matched control cell lines, indicated significantly higher levels of extracellular NmU to be associated with lapatinib resistance. This observation was validated by qPCR in conditioned medium from these cells (Fig. 1B, i and ii). The trend of increased NmU mRNA levels observed in conditioned medium from the resistant cells compared with the sensitive cell lines was subsequently found to reflect that in the corresponding cells (Fig. 1B, iii and iv).

Investigating whether the induced expression of NmU may be an early response to drug exposure, we treated cells with lapatinib (1 μ mol/L) for 48 hours and assessed NmU levels by qPCR. Here, we found that the levels of NmU mRNA detectable

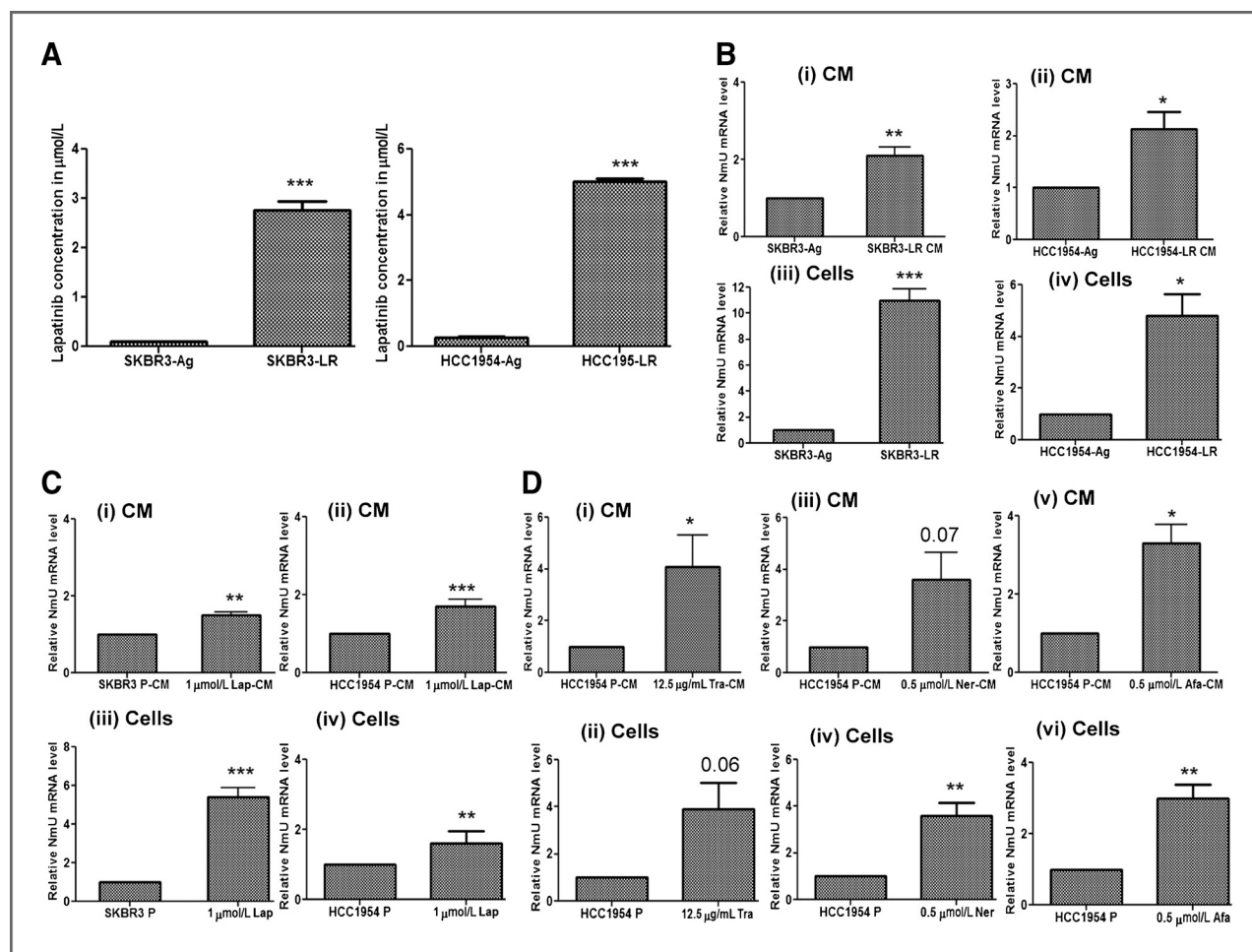


Figure 1. Increased levels of *NmU* mRNA in conditioned medium (CM) and corresponding cells with acquired lapatinib resistance with induced *NmU* also observed after short-term drug exposure. A, HER2-overexpressing cells that were conditioned with lapatinib (SKBR3-LR and HCC1954-LR) compared with their aged-parent control populations (SKBR3-Ag and HCC1954-Ag) demonstrated increased resistance to lapatinib. B, qPCR analysis of *NmU* mRNA in conditioned medium from SKBR3-LR (i) and HCC1954-LR (ii) compared with conditioned medium from their aged-parent cells. Corresponding cellular *NmU* mRNA demonstrated similar trend (iii and iv). C, qPCR analysis of *NmU* mRNA following short-term (48 hours) exposure of the parent cell populations SKBR3 and HCC1954 to lapatinib in their respective conditioned medium (i and ii) and in the corresponding cells (iii and iv). D, qPCR analysis of *NmU* mRNA following short-term exposure of the parent cell populations (HCC1954, as example) to trastuzumab (i), neratinib (iii), and afatinib (v) is shown and *NmU* mRNA levels in the conditioned medium (i, iii, and v) and their corresponding cells (ii, iv, and vi). Data, fold change assigning control an arbitrary 1. All results represent biologic repeats $n = 3 \pm \text{SEM}$, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in conditioned medium from the lapatinib-exposed SKBR3 and HCC1954 cells (Fig. 1C, i and ii) were significantly higher than in the corresponding untreated control conditioned medium, even after this relatively short-term exposure to drug. As expected, a similar trend was found in the corresponding cells (Fig. 1C, iii and iv). Considering a broader range of HER-targeted drugs, treating cells (HCC1954 as example) for 48 hours with trastuzumab (12.5 μg/mL) resulted in induced *NmU* mRNA levels in conditioned medium (3.9 ± 1 -fold; $P = 0.04$) and corresponding cells (3.9 ± 1.15 -fold; $P = 0.06$; Fig. 1D, i and ii). In relation to neratinib (0.5 μmol/L), the resulting induced *NmU* mRNA was 3.6 ± 1 -fold ($P = 0.07$) and 3.6 ± 0.54 -fold ($P = 0.009$) for conditioned medium and cells, respectively (Fig. 1D, iii and iv). The levels of induced *NmU* mRNA for afatinib (0.5 μmol/L) was 3.3 ± 0.49 -fold ($P = 0.01$)

in conditioned medium and 3 ± 0.38 -fold ($P = 0.006$) in cells (Fig. 1D, v and vi).

Induced *NmU* protein expression occurs in cells with acquired resistance to other HER-targeted agents and is not restricted to lapatinib

We questioned whether the changes at the mRNA level in acquired-resistant cells translated to *NmU* protein. In agreement with our mRNA observations, *NmU* protein levels were significantly higher in lapatinib-conditioned cells (SKBR3-LR and HCC1954-LR) compared with their age-matched control cells (SKBR3-Ag and HCC1954-Ag; Fig. 2A, i and ii). Interestingly, this observation was not limited to lapatinib, but was also found in relation to other HER-targeted drugs where acquired resistance populations were available. Specifically, a similar

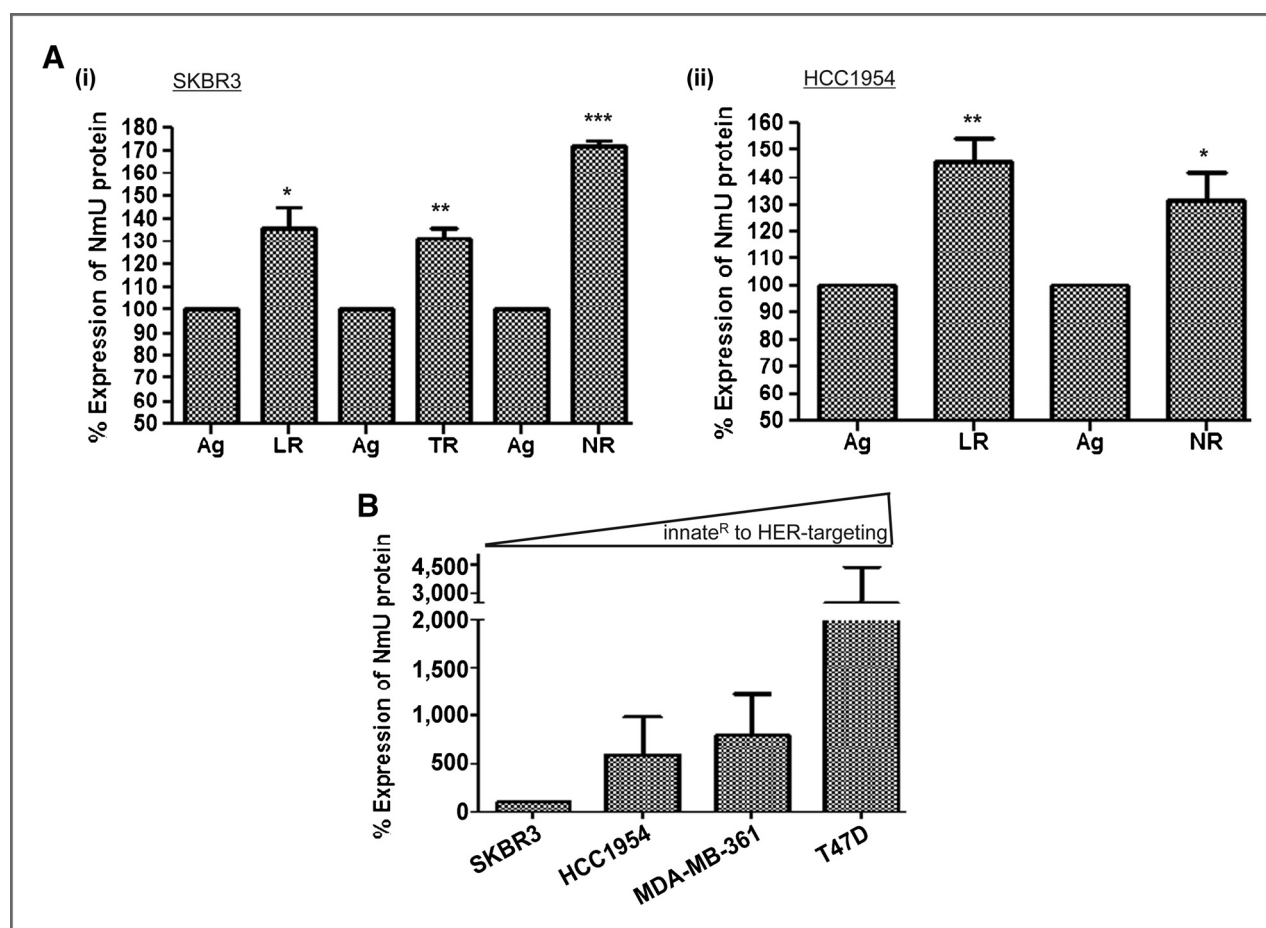


Figure 2. Increased NmU protein expression in cells with acquired-resistance to lapatinib, trastuzumab, and neratinib; also in cells with innate resistance to HER-targeting drugs. A, i, higher levels of NmU protein, analyzed by ELISA, were also found in lapatinib-resistant (SKBR3-LR), trastuzumab-resistant (SKBR3-TR), and neratinib-resistant (SKBR3-NR) cells compared with the corresponding SKBR3-Ag cells. ii, for HCC1954, the same trend was found with lapatinib-resistant (HCC1954-LR) and neratinib-resistant (HCC1954-NR) cells compared with their control HCC1954-Ag cells. Data presented here are NmU protein quantity relative to the amount in the aged control (100%) population. B, increased levels of NmU protein were also associated with innate resistance to HER-targeted drugs. Results represent biologic repeats $n = 3 \pm \text{SEM}$, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

trend (i.e., significantly increased NmU protein levels) was observed when we compared our trastuzumab-resistant (SKBR3-TR or -NR) and neratinib-resistant (SKBR3-NR or -NR) cells with their age-matched control cells (SKBR3-Ag; Fig. 2A, i) and our neratinib-resistant HCC1954 (HCC1954-NR or -NR) cells with their age-matched cells (HCC1954-Ag; Fig. 2A, ii).

Endogenous NmU protein levels may be associated with innate sensitivity/resistance to HER-targeting drugs

SKBR3 cells are sensitive to both lapatinib and trastuzumab; HCC1954 cells are sensitive to lapatinib but resistant to trastuzumab; conversely, MDA-MB-361 is resistant to lapatinib, but sensitive to trastuzumab (24). Although T47D is described as having normal HER2 expression (25), it is also unresponsive to both drugs (26, 27), and so we elected to include these in our analysis. As shown in Fig. 2B, endogenous levels of NmU protein appeared to correlate with the innate sensitivity versus resistance profile of these four cell lines.

NmU expression is prognostic for poor outcome in breast cancer, particularly for HER2-positive and luminal A subtypes

To determine whether NmU has relevance in human breast cancer rather than being solely a cell line/conditioned medium-related observation, we collated and mined microarray data relating to 3,489 breast tumors. Kaplan-Meier estimates of survival (Fig. 3A) indicated high levels of NmU expression to be associated with poor outcome for patients with breast cancer ($P < 1e-14$). Considering each of the breast cancer molecular subtypes within this general population of breast tumors, we found NmU expression to be particularly associated with poor outcome for those patients who had HER2-positive tumors (Fig. 3B; $P < 5e-6$) and luminal A tumors (Fig. 3C; $P < 8e-6$). These associations were not significant with luminal B (Fig. 3D; $P = 0.081$) or basal-like (Fig. 3E; $P = 0.456$) tumors.

Although complete clinicopathological information was unavailable for all 3,489 patients, multivariate analysis

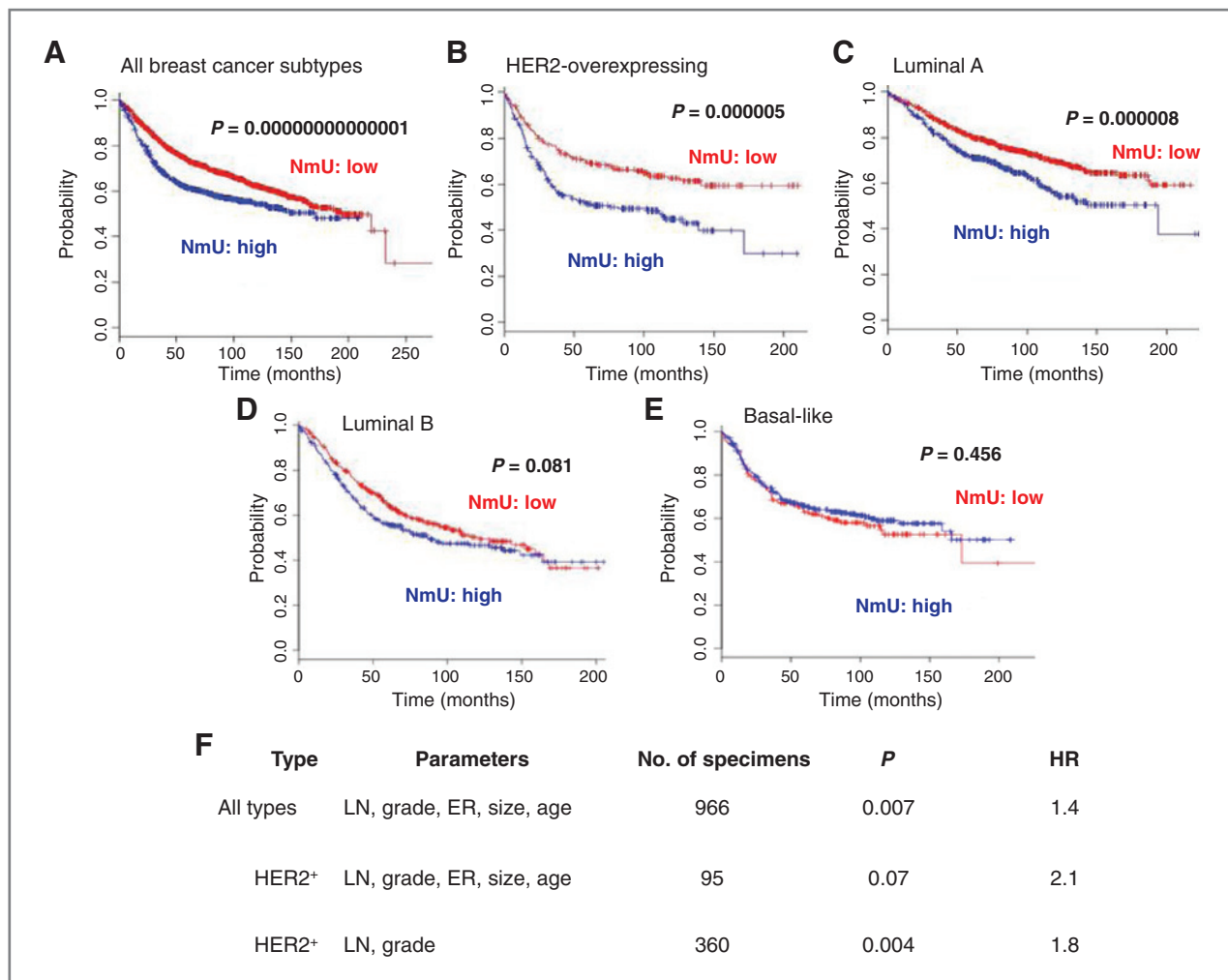


Figure 3. *NmU* expression is prognostic for poor outcome for patients with breast cancer, particularly within the HER2-positive and luminal A molecular subtypes. A, Kaplan-Meier estimates indicate that high levels of *NmU* are associated with poor prognosis in breast cancer ($n = 3,489$). The association of *NmU* expression with patients outcome in relation to each breast cancer molecular subtype was subsequently investigated and shown to be significant for HER2-positive ($n = 476$; B) and luminal A ($n = 1,521$; C) tumors but not luminal B ($n = 676$; D) and basal like ($n = 454$; E). F, multivariate analysis reporting on *NmU* following adjustment for a range of established clinicopathological parameters indicated its independence as a poor prognostic biomarker.

correcting for tumor size, grade, estrogen receptor status, lymph node status, and age of patient, where this information was available, confirmed *NmU* as an independent prognostic biomarker rather than it being simply a surrogate for an already established parameter (Fig. 3F). Specifically, considering all tumor types where information on these five parameters was available ($n = 966$), high levels of *NmU* expression was associated with poor outcome ($P = 0.007$; HR = 1.4). Considering the HER2-overexpressing subtype, which is particularly relevant to this study, detailed clinical information was available for only 95 patients. Following correction, high levels of *NmU* in HER2-overexpressing tumors tended toward statistical significance ($P = 0.07$; HR = 2.1). As information on tumor grade and lymph node status was available for a substantial number of the HER2-overexpressing tumors ($n = 360$ specimens), we evaluated *NmU* in this cohort and found it to be independently

associated with poor outcome for HER2-overexpressing patients ($P = 0.004$; HR = 1.8).

***NmU* affects sensitivity to lapatinib, trastuzumab, neratinib, and afatinib**

To assess whether *NmU* might be functionally involved in resistance to HER-targeted drugs, we stably transfected *NmU* cDNA into SKBR3 and HCC1954 parent cells and established successful overexpression of *NmU* compared with levels in mock-transfected cells, using both qPCR and ELISA (Fig. 4A, i and ii). For all drugs tested, that is, lapatinib (Fig. 4B, i), trastuzumab (Fig. 4B, ii), neratinib (Fig. 4B, iii), and afatinib (Fig. 4B, iv), the resulting anti-proliferative effects were significantly compromised in the *NmU*-transfected cells compared with the mock-transfected cells. The exception to this being the response of HCC1954-*NmU* to trastuzumab (Fig. 4B, ii).

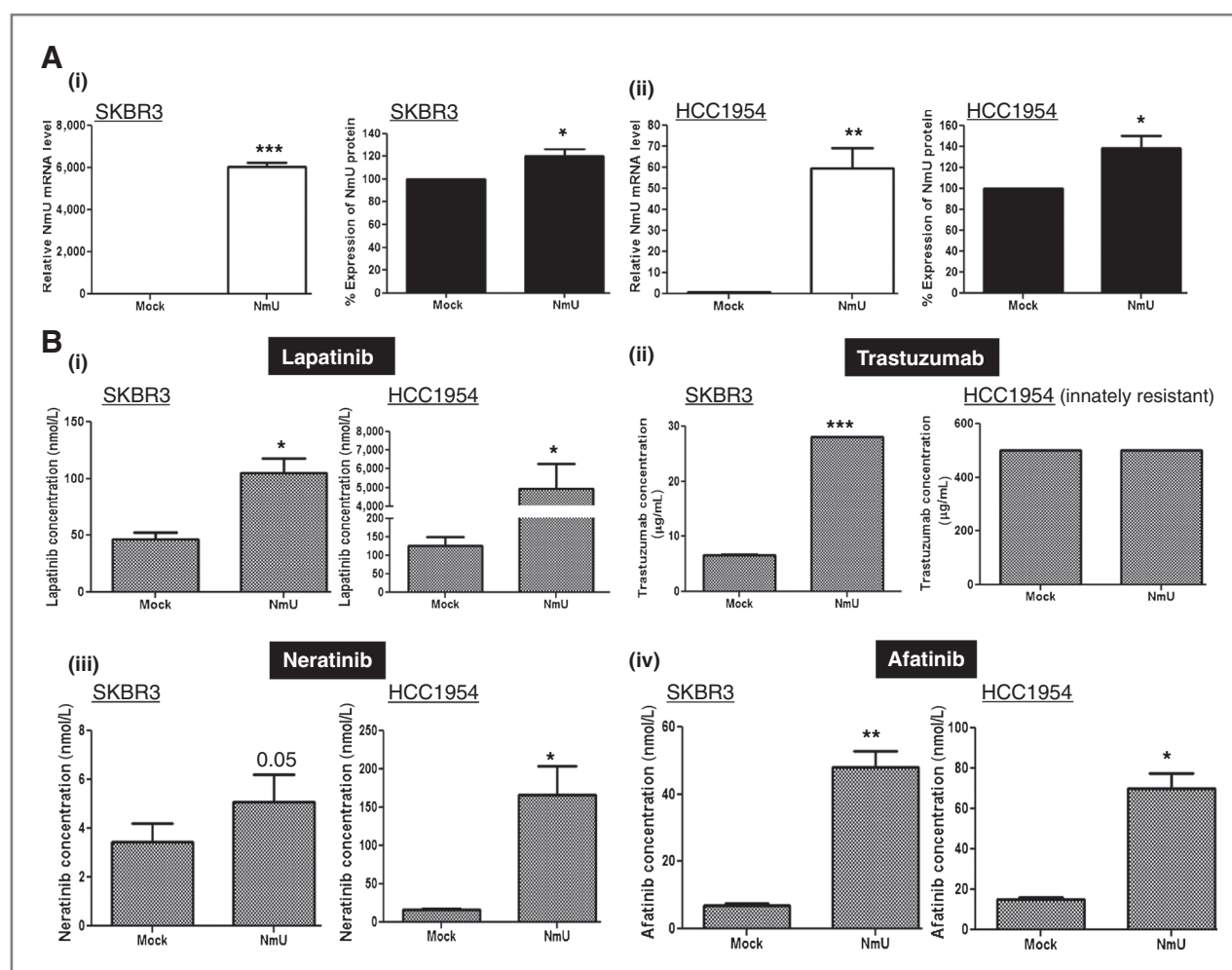


Figure 4. NmU overexpression reduces sensitivity to HER-targeted agents. Following NmU cDNA overexpression (A), levels of *NmU* mRNA (qPCR) and protein (ELISA) were confirmed in SKBR3 (i) and HCC1954 (ii) cells compared with mock-transfected populations. B, comparative influences on proliferation of SKBR3-NmU and HCC1954-NmU cells compared with the mock-transfected control cells in the presence of lapatinib (i), trastuzumab (ii), neratinib (iii), and afatinib (iv). Results represent $n = 3 \pm \text{SEM}$, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To further explore a functional role for NmU in resistance to HER-targeted drugs, NmU was subsequently knocked down in both our acquired resistant cell lines (SKBR3-LR and HCC1954-LR) and innately resistant/unresponsive cells (MDA-MB-361 and T47D). Again, qPCR and ELISAs established significant knockdown of *NmU* mRNA as shown in Fig. 5, i and protein Fig. 5, ii, respectively, compared with levels in scrambled (Scr) control cells. In relation to effects on response to drug, while some variation was observed between cell lines and siRNAs, NmU knockdown (compared with scrambled control, Scr) was found to increase the antiproliferative effects achieved in response to all of the four HER2 drugs tested (Table 1 and Supplementary Table S2A and S2B).

Proposed mechanism-of-action

We next investigated how NmU knockdown may be enhancing the effects of this range of HER-targeted drugs. As HER2 is a target for all four drugs and EGFR is also a target of lapatinib, neratinib, and afatinib, we assessed the levels of

these specific targets—their total amounts and their phosphorylated forms—using ELISAs and immunoblotting. Of great interest, with both SKBR3-LR and HCC1954-LR cells, NmU knockdown was associated with significantly reduced levels of total HER2 protein (Fig. 6A, i and ii).

By mining the literature, we found that HSP27 has previously been reported as highly expressed in HER2-positive breast cancer (28) and, importantly, has been recognized as reducing cellular trastuzumab susceptibility by directly binding to HER2 and thus increasing its stability (29). We hypothesized, therefore, that HSP27 may be the linking molecule in this chain of events facilitating the mechanism by which NmU contributes resistance to HER-targeted drugs. To investigate this, NmU was immunoprecipitated and assessed by immunoblotting for HSP27 binding. NmU immunoprecipitation did, indeed, pull HSP27 out and further increased levels of the NmU:HSP27 complex were observed in NmU over-expressing (HER drug resistant) cell lines variants compared with their sensitive mock-transfected parent lines (Fig. 6B, i and ii). Subsequently,

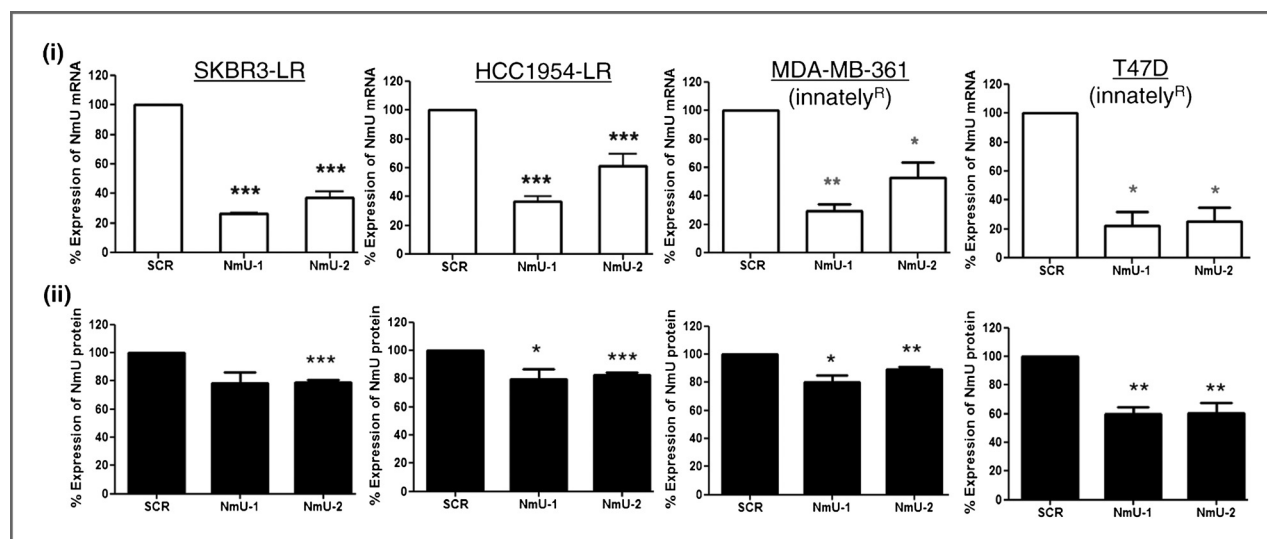


Figure 5. Confirmation of NmU knockdown by siRNAs. Following transfection with two NmU-siRNA (NmU-1 or NmU-2) or scrambled sequence (Scr), levels of NmU mRNA (qPCR; i) and protein (ELISA; ii) showed partial knockdown of NmU expression in both the acquired lapatinib-resistant SKBR3-LR, HCC1954-LR, innately unresponsive T47D, and MDA-MB-361 cells. Results represent $n = 3 \pm \text{SEM}$, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

knockdown of NmU in lapatinib-resistant SKBR3-LR and HCC1954-LR (Fig. 6C, i and ii) demonstrated relative decreased levels of HSP27. A proposed model of this mechanism of action is outlined (Fig. 6D, i-ii).

Upon NmU silencing, the total amounts of EGFR present was not significantly affected in both cell lines (Supplementary Fig. S1A, i and ii), but phosphorylation of the EGFR that was present was significantly reduced (Supplementary Fig. S1B).

To further explore the functional role of NmU, after establishing that both NmU receptors (NmU-R1 and NmU-R2) are expressed by SKBR3 and HCC1954 cells, we observed that treating these cells with exogenous NmU (NmU-25) induced expression of both HER2 and EGFR proteins (Supplementary Fig. S2A). This suggests that either or both of NmU-R1 and NmU-R2 are functionally active on these cells. Interestingly, exposure to exogenous NmU also induced a low, but significant, level of resistance to lapatinib (SKBR3, $P = 0.004$; HCC1954, $P = 0.00004$), trastuzumab (SKBR3, $P = 0.003$; HCC1954, $P = 0.000002$), neratinib (SKBR3, $P = 0.005$; HCC1954, $P = 0.01$), and afatinib (SKBR3, $P = 0.002$; HCC1954, $P = 0.0017$) in both SKBR3 and HCC1954 cell lines (Supplementary Fig. S2B).

NmU expression is also associated with other phenotypic characteristics, including cell motility, invasion, and resistance to anoikis

To assess what other functional role(s) NmU may have, NmU-overexpressing and NmU knockdown cells were further evaluated. Events associated with more "aggressive" cancers include the ability of the cells to move (migration assays), digest, and migrate through extracellular matrix during intravasation and extravasation (invasion assays) and to survive in suspension, as necessary to survive in the peripheral circulation en route to metastasize (anoikis assays). NmU cDNA

overexpression in HCC1954 compared with HCC1954-mock was associated with increased motility as evaluated by wound healing (Supplementary Fig. S3A, i), increased migration through Transwell (Supplementary Fig. S3B, i), increased invasion through extracellular matrix-coated Transwells (Supplementary Fig. S3C, i), and resistance to anoikis (Supplementary Fig. S3D, i). Conversely, NmU knockdown in HCC1954-LR was associated with opposite effects, that is, decreased cellular motility (Supplementary Fig. S3A, ii), decreased migration (Supplementary Fig. S3B, ii), decreased invasion (Supplementary Fig. S3C, ii), and increased sensitivity to cell death by anoikis (Supplementary Fig. S3D, i; representative NmU cDNA transfected in SKBR3 results are summarized in Supplementary Fig. S4).

In vivo, NmU knockdown reduced tumor growth and metastasis

To establish the relevance of NmU in the orthotopic *in vivo* setting, HER drug-resistant HCC1954-LR cells that expressed luciferase underwent NmU knockdown *in vitro* and were subsequently injected into the mammary fat pad of immunocompromised animals. As indicated in Fig. 7A, orthotopic growth of HCC1954-LR shRNA NmU knockdown (NmU⁻) tumors was significantly impaired relative to control ($P = 0.0056$). This is indicated by representative bioluminescence images taken at day 56 (Fig. 7B). Within the timeframe of the current study (65 days), tumors from 7 of 10 of the control animals had reached endpoint (15 mm diameter), and animals sacrificed. Metastatic lesions were found in the lymph nodes, liver, and lungs (Fig. 7C and D). At this time (day 65), only 2 of 20 animals bearing HCC1954-LR NmU⁻ reached endpoint. In these animals at time of sacrifice, metastatic lesions were found in the liver and lungs, but not in the lymph nodes. Tumors in the remaining 18 of 20 animals were significantly below endpoint diameter with no

Table 1. NmU knockdown partly restores sensitivity in cells with either acquired or innate resistance to HER targeting

Drug	Drug concentration	Cell line	Drug + SRC (% proliferating cells)	(+) Antiproliferative benefit with NmU-1	P	(+) Antiproliferative benefit with NmU-2	P
Calculations based on when nontransfected is set to 100%							
Lapatinib	3 μ mol/L	SKBR3-LR	50	10.2	0.01	10.4	0.008
	5 μ mol/L	HCC1954-LR	50	24.4	0.006	17.0	0.03
	1 μ mol/L	MDA-MB-361	50	21.7	0.00005	7.0	0.001
	5 μ mol/L	T47D	50	7.7	0.03	5.9	0.05
Tras'mab	15 μ g/mL	SKBR3-LR	60	25.2	0.0009	12.0	0.09
	15 μ g/mL	HCC1954-LR	85	42.1	0.0001	27.8	0.001
	15 μ g/mL	MDA-MB-361	39	14.7	0.0004	6.9	0.03
	15 μ g/mL	T47D	67	18.0	0.11	11.1	0.30
Neratinib	1 μ mol/L	SKBR3-LR	26	14.9	0.014	5.4	0.19
	1 μ mol/L	HCC1954-LR	25	14.5	0.002	11.1	0.004
	1 μ mol/L	MDA-MB-361	25	12.8	0.10	6.2	0.45
	1 μ mol/L	T47D	39	15.1	0.20	6.8	0.62
Afatinib	0.5 μ mol/L	SKBR3-LR	49	20.2	0.08	9.1	0.40
	0.5 μ mol/L	HCC1954-LR	68	26.7	0.002	18.8	0.002
	0.5 μ mol/L	MDA-MB-361	87	21.6	0.001	20.8	0.001
	0.5 μ mol/L	T47D	85	18.1	0.02	15.5	0.04
Calculations based on when Scr + drug is set to 100%							
Lapatinib	3 μ mol/L	SKBR3-LR	50	20.2	0.006	20.7	0.01
	5 μ mol/L	HCC1954-LR	50	49.2	0.006	34.3	0.03
	1 μ mol/L	MDA-MB-361	50	43.4	0.00007	13.9	0.002
	5 μ mol/L	T47D	50	15.5	0.002	11.8	0.02
Tras'mab	15 μ g/mL	SKBR3-LR	60	41.8	0.0002	20.5	0.009
	15 μ g/mL	HCC1954-LR	85	49.6	0.0000004	32.8	0.000002
	15 μ g/mL	MDA-MB-361	39	37.3	0.0005	17.7	0.02
	15 μ g/mL	T47D	67	26.2	0.0009	15.7	0.04
Neratinib	1 μ mol/L	SKBR3-LR	26	58.0	0.0004	20.2	0.03
	1 μ mol/L	HCC1954-LR	25	57.3	0.00002	43.9	0.000004
	1 μ mol/L	MDA-MB-361	25	48.1	0.001	23.7	0.004
	1 μ mol/L	T47D	39	36.7	0.002	18.9	0.004
Afatinib	0.5 μ mol/L	SKBR3-LR	49	42.8	0.002	19.8	0.04
	0.5 μ mol/L	HCC1954-LR	68	39.4	0.0001	27.6	0.000002
	0.5 μ mol/L	MDA-MB-361	87	24.9	0.0002	24.0	0.00004
	0.5 μ mol/L	T47D	85	21.5	0.002	18.3	0.004

NOTE: NmU knockdown in these cells enhanced the effectiveness of lapatinib, trastuzumab, neratinib, and afatinib at decreasing proliferation compared with that observed in corresponding Scr-transfected cells.

Abbreviation: Tras'mab, trastuzumab.

evidence of metastases. A significant survival benefit was, thus, observed ($P \leq 0.0001$; Fig. 7E).

Discussion

NmU has previously been associated with cancer, but an association between NmU and breast cancer or with HER2 overexpression in any cancer type has not previously been established. Specifically, in typically small studies of acute myelogenous leukemia (AML; ref. 30), ovarian (31), pancreatic (32), lung (33), bladder (34), and renal cancer (35), NmU has been associated with oncogenic characteristics. Conversely, preliminary studies of esophageal (36) and oral (37) cancers

suggest tumor suppressor activities. Here, we report that increased *NmU* mRNA expression is significantly associated with poor outcome in breast cancer; particularly for those patients with HER2 or luminal A tumors. Our data also suggest that *NmU* expression has independence as a prognostic factor for poor outcome and is not simply a surrogate for an already established prognostic biomarker.

Our initial observation of NmU being associated with resistance to HER-targeting drugs was made through analysis of medium conditioned by cells that had acquired lapatinib resistance compared with conditioned medium from their respective aged parent populations. This observation reflected

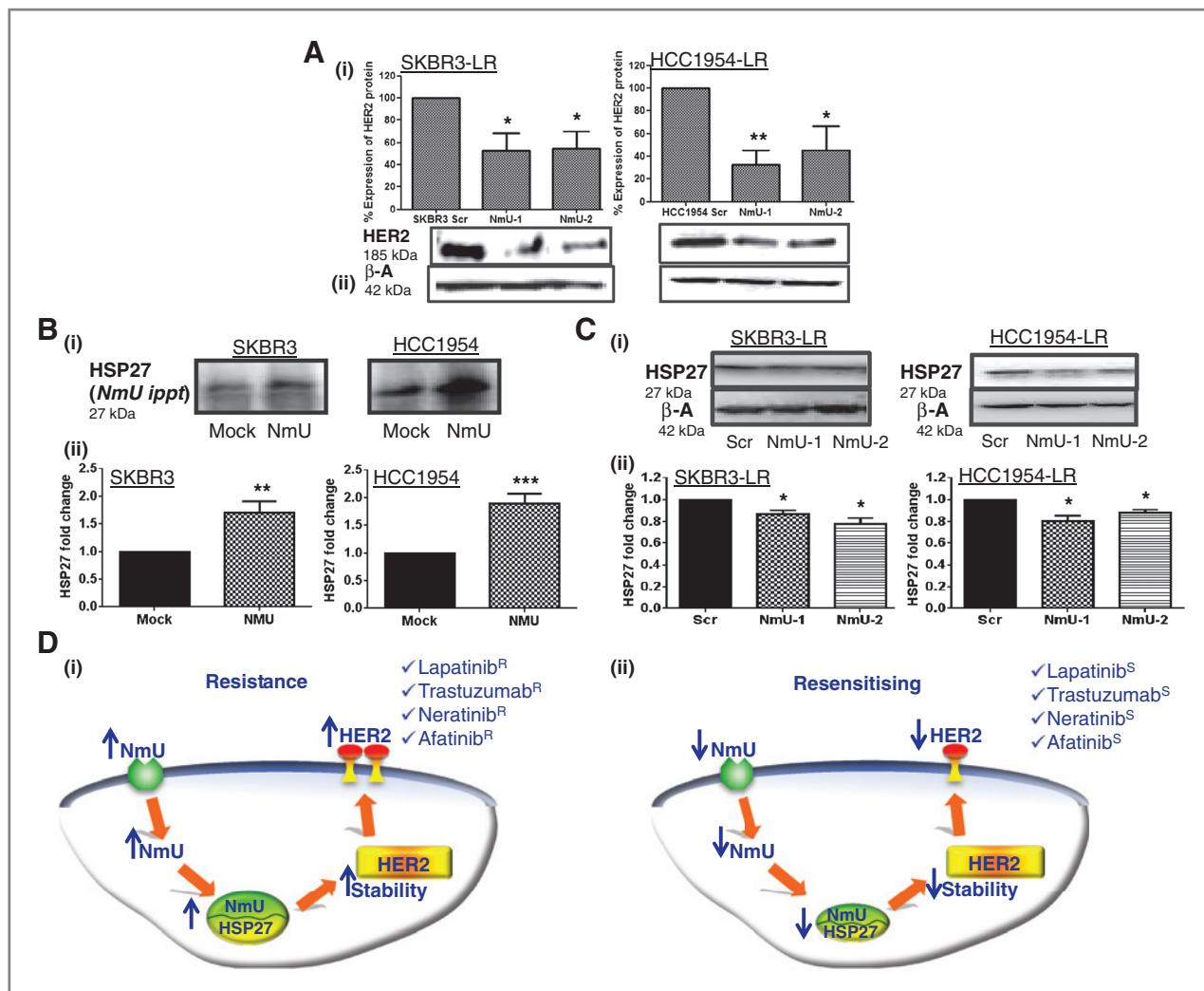


Figure 6. NmU's mechanism-of-action involves complexing with its partner protein HSP27, increasing HER2 stability thus inducing resistance. NmU siRNA knockdown in both SKBR3-LR and HCC1954-LR cells was associated with significantly reduced levels of HER2 protein (A), as shown by ELISA (i) and immunoblotting (ii). Immunoprecipitation of NmU in NmU-overexpressing cells, compared with mock-transfected cells, was associated with significantly increased levels of HSP27 (B) as shown by immunoblotting (i) and corresponding densitometry (ii). NmU siRNA knockdown in both SKBR3-LR and HCC1954-LR cells was associated with significantly reduced levels of HSP27 protein (C), as shown by immunoblotting (i) and corresponding densitometry (ii). D, our proposed mechanism-of-action by which increased NmU results in increased NmU:HSP27 complexity, subsequent increased HER2 stability, and thus resistance (i) and how our NmU knockdown is, therefore, resensitizing the resistant cells to these drugs (ii).

that of the cells themselves. This is in keeping with our previous observations from a range of cell lines (16, 17, 19) and pilot study analysis of serum and corresponding tumor tissue from patients with breast cancer (18). These findings indicate that full-length mRNAs can be selectively expelled from cells. Similar observations, studying different mRNAs, have been reported by others (38). To further explore whether or not the response of increased intracellular and extracellular *NmU* mRNA is an early event, sensitive cells (SKBR3 and HCC1954 parent cells) were exposed to lapatinib short term, showing some increased *NmU* mRNA transcription and expulsion from cells within this time frame. To establish whether the increase in NmU levels (intracellular and extracellular) is specific to lapatinib or whether *NmU* mRNA may have relevance as a

biomarker of response to other HER-targeting drugs, our analysis was expanded to also investigate the response to trastuzumab, neratinib, and afatinib. In all cases, increased transcription and expulsion of *NmU* mRNA resulted. The lower fold changes observed in conditioned medium compared with cells may suggest that this is not a dynamic event; that cells limit the amount of mRNAs they select to send out into the extracellular environment; and/or that some of extracellular mRNA was degraded. However, the *NmU* mRNA levels in conditioned medium from resistant cells were always significantly higher than that in conditioned medium from sensitive cells and evidence suggests that this effect is partly an early event; albeit the levels detected in short-term treated cells were not as enhanced as that in acquired resistance cells. This

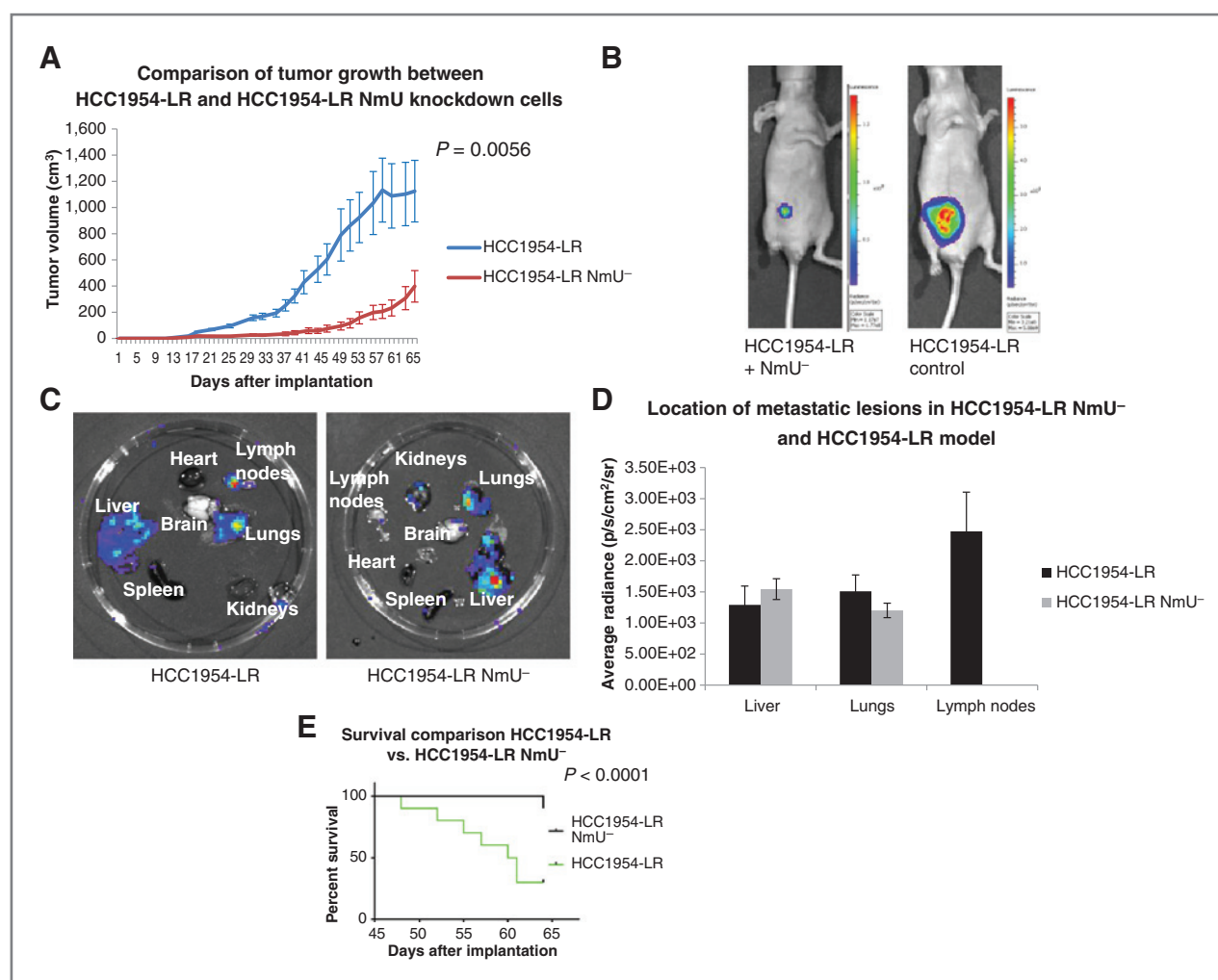


Figure 7. *In vivo*, NmU knockdown dramatically impairs tumor growth and metastatic spread. Ten million cells were implanted orthotopically into the mammary fat pad of BALB/c nude mice (HCC1954-LR, $n = 10$; HCC1954-LR NmU⁻, $n = 20$) and allowed to grow. Animals were sacrificed once they reached endpoint (15-mm tumor diameter). A, tumor growth curve comparison of HCC1954-LR NmU⁻ and HCC1954-LR cell lines. B, representative bioluminescence images of HCC1954-LR NmU⁻ and HCC1954-LR cell lines taken 56 days postimplantation. C, *ex vivo* imaging was performed for assessment of metastatic burden. Metastasis was observed in the liver (7/7 mice), lungs (4/7 mice), and lymph nodes (2/7 mice) in mice bearing the HCC1954-LR cell lines. In the HCC1954-LR NmU⁻ cell line, two mice reached end-point and were sacrificed on day 64. Metastatic deposits were observed in liver and lungs only. D, quantification of metastatic burden in HCC1954-LR and HCC1954-LR NmU⁻ cell lines at time of sacrifice. E, survival comparison of HCC1954-LR and HCC1954-LR NmU⁻ cell lines. By day 64, 70% of mice bearing HCC1954-LR tumor had reached endpoint compared with 10% of those with HCC1954-LR NmU⁻ tumor. This was a significant difference ($P \leq 0.0001$, Mantel-Cox test).

observation of NmU as a potential extracellular, as well as intracellular, predictive biomarker (while not the main focus on this manuscript) warrants further investigation.

For a more comprehensive understanding, we also elected to expand these studies to evaluate NmU protein, in addition to mRNA. Considering innately sensitive and resistant cells, here we found NmU protein levels to correlate with innate resistance versus sensitivity to HER-targeting drugs, that is, with the low levels in SKBR3 (sensitive to trastuzumab and lapatinib) progressive increasing in HCC1954 and MDA-MB-361 (resistant to one or other of these drugs) and at highest levels in T47D (unresponsive to both HER-targeted drugs considered here) cells. Furthermore, in relation to acquired resistance,

NmU proteins level were always found to be significantly higher in cells with acquired resistance to lapatinib, trastuzumab, and neratinib compared with their aged control populations. This suggests that NmU protein, as well as its mRNA, potentially has broad application as a predictive biomarker for response to HER-targeted drugs.

Investigating a functional role of NmU in relation to HER targeting, our overexpression (in sensitive cells) and knockdown (in both acquired-resistant and innately-resistant cells) studies showed NmU levels to inversely correlate with cellular response to all four HER-targeting drugs tested. Specifically, NmU overexpression generally conferred resistance to the antiproliferative effects of all drugs tested. (The exception to

this was the HCC1954 cells, where NmU overexpression did not affect response to trastuzumab. This is likely to be due to the fact that HCC1954 are innately resistant to trastuzumab; ref. 24). Our NmU immunoprecipitation and associated investigations to identify the direct mechanism by which NmU is acting and thus conferring resistance to these drugs indicated NmU to directly partner with HSP27. By directly binding to HER2, HSP27 has previously been shown to increase HER2 stability and thus reduce cellular sensitivity to this drug (29). This is also in keeping with our observation of knock-on effects of NmU expression increasing HER2 protein levels.

Conversely, although there were some differences between cell lines and effectiveness of the two NmU siRNAs used, NmU knockdown added substantially to the antiproliferative effects of lapatinib, trastuzumab, neratinib, and afatinib in both innately- and acquired-resistant cell lines studied. Interestingly, this knockdown in NmU was directly associated with subsequent reduced expression of its identified partner HSP27, HER2, protein and the phosphorylated/activated form of EGFR, the latter possibly being a consequence of a reduced transactivation by HER2. Of note, NmU knockdown also resulted in increased sensitivity of T47D cells to all four drugs despite the fact that T47D cells express normal levels of HER2, that is, T47D is not a HER2-overexpressing cell line (25). As sensitivity to these drugs (targeting HER2 for all four drugs; also EGFR for lapatinib, neratinib, and afatinib) was directly affected by the relatively modest NmU knockdown (up to 40% achieved with siRNA), this suggests that cotargeting NmU, while targeting HER2/EGFR, may help circumvent resistance to these drugs.

In keeping with our hypothesis that NmU is involved in regulating response to HER-targeted drugs, treating sensitive HER2-overexpressing cells (SKBR3 and HCC1954) with exogenous NmU was found to slightly, but significantly, reduce the antiproliferative effects of lapatinib, trastuzumab, neratinib, and afatinib, in association with increased expression of HER2 and EGFR receptors.

The potential benefits of targeting/cotargeting NmU in HER2 breast cancer is further supported by our observations that NmU knockdown significantly decreases migration/motility, invasion, and resistance to anoikis, whereas its overexpression has the opposite effects. Importantly, *in vivo* studies implementing an orthotropic model of HER2-positive tumors resistant to lapatinib (and to other HER-targeted drugs) further supports this, as NmU stable knockdown dramatically

impaired tumor growth and metastatic spread. These observations of NmU being associated with more "aggressive" phenotypic characteristics of cancer cells concur with the reports of NmU being associated with oncogenic characteristics in other cancer types, including AML, pancreatic, ovarian, lung, and bladder cancers (30–32, 35).

In conclusion, here we provide evidence that NmU is associated with poor outcome from breast cancer, particularly being prognostic of unfavorable outcome for patients with HER2-overexpressing tumors; that it has potential as a predictive biomarker of response to a broad range of HER-targeting drugs; and that it is mechanistically involved in conferring this resistance by working in concert with its partner protein HSP27. Our results provide a strong rationale for more extensive analysis of NmU as a companion biomarker for HER-targeted drugs and as a cotarget to help circumvent resistance and thus add value to these drugs.

Disclosure of Potential Conflicts of Interest

N. O'Donovan reports receiving commercial research grants from GSK and Boehringer Ingelheim. J.P. Crown has received speakers' bureau honoraria from GSK. No potential conflicts of interest were disclosed by the other authors.

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Neuromedin U: A Candidate Biomarker and Therapeutic Target to Predict and Overcome Resistance to HER-Tyrosine Kinase Inhibitors

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