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HER2/HER3 Signaling Regulates NK Cell-Mediated Cytotoxicity via MHC Class I Chain-Related Molecule A and B Expression in Human Breast Cancer Cell Lines

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Overexpression of the receptor tyrosine kinases HER2 and HER3 is associated with a poor prognosis in several types of cancer. Presently, HER2-as well as HER3-targeted therapies are in clinical practice or evaluated within clinical trials, including treatment with mAbs mediating growth inhibition and/or activation of Ab-induced innate or adaptive cellular immunity. A better understanding of how HER2/HER3 signaling in tumors influences cellular immune mechanisms is therefore warranted. In this study, we demonstrate that HER2/HER3 signaling regulates the expression of MHC class I-related chain A and B (MICA and MICB) in breast cancer cell lines. The MICA and MICB (MICA/B) molecules act as key ligands for the activating receptor NK group 2, member D (NKG2D) and promote NK cell-mediated recognition and cytolysis. Genetic silencing of HER3 but not HER2 downregulated the expression of MICA/B, and HER3 overexpression significantly enhanced MICA expression. Among the major pathways activated by HER2/HER3 signaling, the PI3K/AKT pathway was shown to predominantly regulate MICA/B expression. Treatment with the HER3-specific ligand neuregulin 1β promoted the expression in a process that was antagonized by pharmacological and genetic interference with HER3 but not by the ataxia-telangiectasia–mutated (ATM) and ATM and Rad3-related protein kinases inhibitor caffeine. These observations further emphasize that HER2/HER3 signaling directly, and not via genotoxic stress, regulates MICA/B expression. As anticipated, stimulating HER2/HER3 enhanced the NKG2D-MICA/B–dependent NK cell-mediated cytolysis. Taken together, we conclude that signaling via the HER2/HER3 pathway in breast carcinoma cell lines may lead to enhanced NKG2D-MICA/B recognition by NK cells and T cells. The Journal of Immunology, 2012, 188: 2136–2145.

Natural killer cell activity is promoted via NK group 2, member D (NKGD2) on NK cells, CD8+ T cells, and γδ T cells (1, 2). The engagement between NKGD2 and its ligands enhances cell-mediated cytoxicity and cytokine production against transformed cells (3). An important NKGD2 ligand is the MHC class I chain A and B (MICA and MICB). Both MICA and MICB (MICA/B) are expressed by intestinal epithelial cells and at low levels also by other nonmalignant cell types. Primary tumor cells and tumor cell lines frequently express MICA/B molecules, but the mechanisms responsible for the induction of MICA/B proteins and other NGK2D ligands (NGK2DLs) during oncogenesis are poorly understood. They are described as stress-related proteins that can be induced by heat shock and that are regulated by heat shock transcription elements activated during cell transformation (4). Also, there is evidence that the DNA damage pathway, induced by ionizing radiation and inhibitors of DNA replication, can induce NGK2DLs (5). A direct control of MICA by the BCR/ABL oncogene in chronic myelogenous leukemia has also been described (6), but the regulation of MICA/B expression by oncogenes is incompletely understood. A better knowledge of this would seem important, because it is well established that NGK2DLs play an important role in the recognition of tumor cells by NK cells. Additionally, tumor cells expressing NGK2DLs can become susceptible to NK cell killing even if they have normal MHC class I expression (1, 7). Thus, the balance between NGK2DLs and MHC class I expression on transformed cells is important for their survival under the immune surveillance of their host (8).

The epidermal growth factor (EGF) family consists of four closely related transmembrane tyrosine kinase receptors (EGFR, also known as HER1, 2, 3, and 4). On binding small peptide ligand molecules, HER receptors undergo homo- or heterodimerization and autophosphorylate, which activates downstream signaling pathways resulting in enhanced cell proliferation, migration, invasion, and survival (9–11). The HER2/HER3 dimer is considered to be the most active HER signaling dimer and is crucial for signaling in tumors containing amplification of HER2 (12–14). HER3
has several ligands, including the neuregulins (NRG) 1–4, but it lacks intrinsic tyrosine kinase activity. In contrast, HER2 has no defined ligand but possesses an active tyrosine kinase domain (15, 16). Ligand-induced heterodimerization of HER3 with HER2 activates cell growth and cell proliferation via PI3K/AKT and RAS/MAPK signaling pathways (17, 18). HER2 overexpression is one of the poor prognostic factors in breast cancer (19, 20), and HER3 is often expressed together with HER2 in this disease (21–23). Both molecules are considered promising targets for cancer therapy, with HER2 targeting therapies already in clinical practice (24, 25), and HER3 targeting therapies currently in clinical trials (26, 27).

Although HER2 and HER3 are considered potential targets for cancer therapy, they have also been associated with an “immune escape” phenotype. Thus, several groups reported that overexpression of the HER2 oncogene can lead to tumor escape from the host immune system via downregulation of MHC class I (28–30), and overexpression of HER2 severely impaired MHC class I-restricted recognition by CTLs (31, 32). These findings suggest that activation of the HER2 signaling pathways leads to tumor escape from the adaptive immune system. However, because MHC class I molecules activate the inhibitory signaling in NK cells (33), it is of particular interest to understand whether HER2/HER3 signaling also would affect the expression of activating NK cell receptors. In this study, we demonstrate that HER2/HER3 signaling regulates the expression of MICA/B molecules in human breast carcinoma cell lines, resulting in enhanced sensitivity to NK cell-mediated recognition.

Materials and Methods

Cell culture and reagents

Human breast cancer cell lines MDA-MB-231, MDA-MB-453, and T47D were obtained from the American Type Culture Collection. The genotypes of all cell lines were identified by short tandem repeat method using STR Identifier (Applied Biosystems, Foster City, CA) and authenticated. All cell lines were maintained in culture in RPMI 1640 medium with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA) or 50 U/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were maintained in culture in RPMI 1640 medium with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and 50 U/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere with 5% CO2. For cell culture work, LY294002 (Calbiochem, San Diego, CA), PD98059 (Calbiochem), and lapatinib (GlaxoSmithKline, Rockford, IL) according to the manufacturer’s protocol. Equal amounts of protein were separated on 4–12% NuPage Bis-Tris acrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were blocked for 30 min in PBS (−) with 0.5% Tween 20 (Sigma-Aldrich) buffer (PBST) with 2% nonfat dry milk, followed by incubation overnight at 4 °C in PBST with 2% nonfat dry milk and primary Abs against HER2 (Calbiochem), p-H2A (Sigma), p-HER2 (Ty1225), Akt, p-Akt (Ser473), p-p44/42 MAPK (Thr202/Tyr204), and α-tubulin (Sigma-Aldrich). After two washes in PBST, membranes were incubated with HRP-linked goat anti-rabbit or anti-mouse IgG Abs (Cell Signaling Technology) for 1 h at room temperature. After blots were washed three times in PBST, enhanced chemiluminescence (GE Healthcare, Fairfield, CT) was performed, and images were captured using an LAS-1000 camera system (Fujiﬁlm, Tokyo, Japan). Membranes were stripped in strip buffer (PBS (−) with 2% SDS [Invitrogen] and 0.7% 2-ME [Sigma]) for 30 min at 50°C and reprobed up to three times. Densitometric analyses for protein quantification of each blot were calculated using ImageJ 1.42q (http://rsb.info.nih.gov/ij). The experiments were repeated three times.

Cell proliferation assays

Cells were collected and stimulated with 2–250 ng/ml NRG1 β for 15 min, then transfected in triplicate into 96-well flat-bottom plates at 5 × 103 cells per 100 μl per well in RPMI 1640 with 2% FBS and 2 mM L-glutamine and incubated for 3 d. The relative percentage of metabolically active cells was compared with that of untreated cells was determined on the basis of mitochondrial conversion of MTT (Molecular Probes, Eugene, OR) according the manufacturer’s protocol. In brief, after incubation, 10 μl MTT solution (5 mg/ml) was added to each well and incubated for 4 h. Thereafter, 50 μl DMSO was added to 25 μl supernatant and incubated for 10 min and plates were read at 540 nm using Versa Max (Molecular Devices, Sunnyvale, CA).

Apoptotic assays

Cells were seeded into 24-well plates at 104 cells per well and treated with 2–250 ng/ml NRG1 β for 2 d. Both nonadherent and adherent cells were washed in PBS (+), trypsinized, and collected. Cells were pelleted at 450 × g, washed by PBS (−), repelleted, and labeled according to the manufacturer’s protocol. Briefly, each sample was stained with 50 μl mixture regent containing annexin V binding buffer with 2.5 μl FITC-labeled annexin V (eBioscience) in a V-bottom 96-well plate at room temperature for 15 min. The cells were resuspended in 300 μl annexin V binding buffer and transferred to FACS tubes for analysis. Cells were acquired on a FACSCalibur cytometer and analyzed using FlowJo software 6.4.7. The experiments were repeated three times.

DNA transfection

The vector pRK5/HER3 was a gift from Prof. Axel Ullrich (Max Planck Institute, Martinsried, Germany). Tumor cells were grown in 12-well plates and transfected upon reaching 50–70% confluence with pcDNA3/empty (Invitrogen) or pRK5/HER3. Cells were transfected in Opti-MEM I cell culture medium (Invitrogen) with 4.0 μg plasmid using 10 μl Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 or 48 h, transfected cells were collected for further experiments.

Small interfering RNA assay

Cell lines were grown in 12-well plates and transfected upon reaching 50% confluence with small interfering RNA (siRNA) targeting HER2 or HER3 (SMARTpools; Dharmacon, Lafayette, CO) along with the control siRNA (Dharmacon). Cells were transfected in Opti-MEM I cell culture medium

mean fluorescence intensity (ΔMFI) was calculated as (MFI with specific mAb − MFI with isotype control)/MFI with isotype control. Relative MFI (rMFI) values were calculated to compare the differences between ΔMFI of a specific treatment and control as: 100 × (ΔMFI of specific treatment/ΔMFI of control treatment).
using Lipofectamine 2000 according the manufacturer’s protocol. After 48 h, transfected cells were used for further experiments.

**NK cell-mediated cytotoxicity assay**

This work was approved by the Institutional Review Board of Karolinska University Hospital. PBMCs were collected from healthy donor buffy coats through density centrifugation. PBMCs cultured with 100 IU/ml IL-2 (Proleukin; Chiron, Emeryville, CA) for 3 d or negatively purified NK cells (NK cell isolation kit II; Miltenyi Biotec, Auburn, CA) rested overnight served as the source of effector NK cells. The phenotype of the cells (CD3 CD56) was confirmed by flow cytometric analysis using PE-labeled anti-CD3 (HIT3a) and allophycocyanin-labeled anti-CD56 (HCD56) Abs (both from BioLegend). Treated or untreated target cells were tested for sensitivity to NK cell-mediated lysis in a standard chromium release assay (35). [51Cr] release in the supernatants was measured by a gamma counter (Wallac Sverige, Upplands Väsby, Sweden). The percentage of specific lysis was calculated as follows: 100 (experimental release – spontaneous release)/(maximum release – spontaneous release). To analyze the involvement of NKG2D in the cytolytic activity of NK cells, effector cells were coincubated with 20 μg/ml anti-NKG2D blocking Ab (1D11; BioLegend) or an isotype-matched control Ab (11711; R&D Systems) at 4˚C for 30 min and washed before the cytolytic assay. To analyze the involvement of MICA/B, anti-MICA/B (6D4; BioLegend) or isotype-matched control Ab (MOPC-173; BioLegend) was added to the target cells at 4˚C for 30 min before the coculture with NK cells to a final concentration of 10 μg/ml.

**Statistical analyses**

Differences in means were evaluated with a Student t test or Fisher exact test. All analyses were performed at a significance level of 5% (p < 0.05) using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**Results**

**HER3 influences expression of MICA/B in breast carcinoma cell lines**

The ability of HER2/HER3 signaling to influence MICA/B expression levels was analyzed in breast cancer cell lines by flow cytometry. MDA-MB-231 (low HER2/HER3 levels; Supplemental Fig. 1) and T47D and MDA-MB-453 (high HER2/HER3 levels; Supplemental Fig. 1) cell lines were chosen for these experiments.

First, tumor cells were treated for 48 h with lapatinib, an EGFR and HER2 tyrosine kinase inhibitor (36) that also can inhibit HER2/HER3 signaling (37). Expression of MICA/B was significantly downregulated by lapatinib in the HER2/HER3 high-expressing T47D and MDA-MB-453 cell lines but was not affected in the HER2/HER3 low-expressing MDA-MB-231 cell line (Fig. 1A). These findings suggest that HER2/HER3 signaling is involved in

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**FIGURE 1.** The expressions of MICA/B, MICA, and MICB are regulated by HER2/HER3 signaling in HER2/HER3 high-expressing breast carcinoma lines. (A) Breast carcinoma cell lines were treated with 1 μM lapatinib (LAP) or as a control with 0.01% DMSO for 48 h. (i) The effects on the MICA/B, MICA, and MICB expression were assessed by flow cytometry as shown in the representative histograms. (ii) The rMFI of MICA/B, MICA, and MICB were calculated based on at least three independent experiments and evaluated with a Student t test. (B) T47D cells were transfected with siRNA targeting HER2 (siHER2), HER3 (siHER3), or control siRNA (siCtr). (i) Forty-eight hours after the transfection, the HER2 and HER3 levels were assessed by Western blot analyses. (ii) The blots were semiquantified, and the columns represent the mean values of three independent experiments. The silencing effect was evaluated with a Fisher exact test. (iii) Representative histograms demonstrating the MICA/B, MICA, and MICB expression in cells upon genetic silencing (as indicated), and mean values of (iv) MICA/B and (v) MICB rMFI of three independent experiments were evaluated with a Fisher exact test. Bars indicate SEM. *p < 0.05, ***p < 0.001. A.U., Arbitrary units; ISO, isotype control.
the regulation of MICA/B expression in breast cancer cells, when HER2/HER3 are expressed at high levels.

Next, we silenced either HER2 or HER3 in the high-expressing T47D breast carcinoma line using siRNA. Forty-eight hours after siRNA transfection, the expression of HER2 or HER3 in T47D cells was found to be attenuated equally, as measured in Western blot (Fig. 1Bi, 1Bii). Interestingly, the expression of MICA/B was significantly downregulated by siRNA of HER3 but not by siRNA of HER2 in T47D cells (Fig. 1Bi, 1Biv). MDA-MB-453 cells also showed similar results (Supplemental Fig. 2Ai).

Next, MICA and MICB were assessed separately after lapatinib or siRNA treatment. Whereas lapatinib had a strong effect on MICB and no effect on MICA in T47D cells, the opposite was true in MDA-MB-453 cells (Fig. 1A).

Both HER2 and HER3 siRNA downregulated MCB expression in T47D cells (Fig. 1Bii, 1Bv). The effect, however, was significantly stronger for siRNA of HER3 as compared with siRNA of HER2. Only siRNA of HER3, but not siRNA of HER2, was able to downregulate MICA in MDA-MB-453 cells (Supplemental Fig. 2A). These findings suggested that the expression level of HER3, but not of HER2, is important for the expression of MICA/B molecules, which is why for the subsequent experiments we focused on the role of HER3 in regulating MICA/B expression.

To investigate this further, the expressions of MICA and MICB were analyzed after transient transfection with HER3 in the HER2/HER3 low-expressing MDA-MB-231 cells. In MDA-MB-231 cells transfected with HER3, but not in empty vector transfected cells, HER3 was strongly expressed and phosphorylated (Fig. 2A). In line with the aforementioned observations, marked increases in MICA/B and MICA were observed in the HER3 transfected cells compared with empty vector transfected ones (Fig. 2B), whereas no increase in MICB expression was noticed (Fig. 2Bi).

MICA/B are regulated mainly by the PI3K/AKT pathway in breast carcinomas

The downstream signaling of HER2/HER3 is mainly mediated via the PI3K/AKT and RAS/MAPK pathways (17, 18). To further evaluate whether these pathways regulate the MICA expression, three cell lines (T47D, MDA-MB-453, and MDA-MB-231) were treated with the PI3K/AKT signal inhibitor LY294002 (38) and the RAS/MAPK signal inhibitor PD98059 (39). LY294002 strongly downregulated the expression of MICA/B in T47D and MDA-MB-231 cells at concentrations between 10 and 30 μM and moderately in the MDA-MB-453 cells. In contrast, treatment with PD98059 only resulted in minor changes (Fig. 3). These findings suggest that HER2/HER3 regulates the expression of MICA/B molecules predominantly by the PI3K/AKT pathway.

Activation of HER2/HER3 signaling with the HER3 ligand NRG1β enhances the expression of MICA/B

Next, we asked whether a ligand-induced HER2/HER3 signaling will impart MIC expression as tested with Abs both against total MICA/B and as tested separately against MICA and MICB. To study this, NRG1β, the most important HER3 ligand known to activate signaling via heterodimerization of the HER2/HER3 complex (12, 40), was used. A significant upregulation of total MICA/B could be achieved in the HER3 high-expressing MDA-MB-453 and T47D cells following a 24-h exposure to NRG1β. The upregulation was mainly accounted for by an increase in MICB for T47D cells and in MICA in MDA-MB-453 cells (Fig. 4A). In contrast, no effect of NRG1β was observed in the HER3 low-expressing MDA-MB-231 cells. Ligation with NRG1β was confirmed to induce phosphorylation of HER3 and to activate both AKT and MAPK downstream signaling pathways in HER3 high-expressing T47D cells, but not or only marginally in HER3 low-expressing MDA-MB-231 cells, although the MAPK pathway was already constitutively activated in the latter cell line (Fig. 4B). No effect of NRG1β was found on tumor cell proliferation or tumor cell apoptosis in T47D cells (Supplemental Fig. 3A, 3B).

NRG1β-induced MICA/B are regulated by HER2/HER3 signaling and not by genotoxic stress

It has been demonstrated that NKG2D ligand expression can be induced by activation of the DNA stress-sensing ataxia-telangiectasia–mutated (ATM) and ATR. We tested whether the NRG1β as an activator of HER2/HER3 signaling, while topo-isomerase II inhibitor doxorubicin, known to activate ATM, was used as an inducer of MICA/B expression via DNA stress (41–43). Lapatinib was used as an inhibitor of HER2/HER3 signaling, while caffeine was used as inhibitor of ATM and ATR (44, 45). The baseline expression of MICA/B was enhanced by doxorubicin, while being downregulated by caffeine in T47D cells (Fig. 5Aii, 5Aiii). As expected, doxorubicin-induced MICA/B expression was blocked by caffeine in T47D cells (Fig. 5Aiii, Supplemental Fig. 4A). Next, T47D cells were pretreated with 1 mM caffeine or 1 μM lapatinib for 2 h prior stimulation with 50 ng/ml NRG1β for 24 h. Interestingly, NRG1β-induced MICA/B expression was not

FIGURE 2. Overexpression of HER3 results in enhanced MICA/B and MICA expression in MDA-MB-231 cells. (A) Expression and phosphorylation of HER3 in HER3 low-expressing MDA-MB-231 breast carcinoma cells transfected with an empty vector (Ctr) or HER3 vector were determined by Western blot analysis. (B) Histograms demonstrating MICA/B, MICA, and MICB expression in cells transfected with empty vector or HER3 vector as assessed by flow cytometry, and (ii) the according rMFI mean values of MICA/B and MICA were evaluated with a Student t test as a result from three independent experiments. Bars indicate SEM. *p < 0.05, **p < 0.01. ISO, Isotype control.
blocked by caffeine (Fig. 5Bi, Supplemental Fig. 4Aii), but was clearly blocked by lapatinib (Fig. 5Ci, Supplemental Fig. 4Aiii). Immunoblotting showed that lapatinib, but not caffeine, attenuated NRG1β-induced phosphorylation of both HER2 and HER3, resulting in inhibition of the downstream pathways of HER2/HER3 signaling (Fig. 5Bii, 5Cii).

We also assessed whether siRNA of HER2 and HER3 could block the NRG1β-induced upregulation of MICA/B expression. The results showed that silencing of HER3 almost completely blocked NRG1β-induced upregulation of MICA/B, whereas silencing of HER2 had no effect (Fig. 5Di, Supplemental Fig. 4B). Furthermore, silencing HER3 significantly blocked NRG1β-induced phosphorylation of both AKT and MAPK, whereas HER2 silencing had no effect (Fig. 5Di, Supplemental Fig. 4B). These findings suggested that NRG1β-induced upregulation of MICA/B was not enhanced by genotoxic stress but by a direct activation of HER2/HER3 signaling. Additionally, they underscored that HER3 is a more important receptor partner than HER2 for the ligand-induced MICA/B upregulation.

NRG-induced NK cytotoxicity is NKG2D-dependent

Because NRG1β enhanced the expression of MICA/B in HER3 high-expressing cells, but not in HER2 low-expressing ones, we next evaluated whether NRG1β treatment would affect their sensitivity to NK cell-mediated cytotoxicity. Tumor cells were treated with 50 ng/ml NRG1β for 24 h, and thereafter their sensitivity to NK cell cytotoxicity was evaluated using IL-2–activated PBMCs or purified NK cells as effector cells. As a consequence of NRG1β treatment, a strong increase in sensitivity for both IL-2–activated PBMCs or purified NK cells was noted in HER3 high-expressing T47D cells (Fig. 6Aii, 6Aiii), but not in HER3 low-expressing MDA-MB-231 cells (Fig. 6Aiv, 6Aiv). We next analyzed whether lapatinib-induced suppression of MICA/B expression would result in decreased sensitivity to NK cell-mediated killing in HER2/HER3 overexpressing T47D and MDA-MB-453 cells. This was shown to be the case, as a significant decrease in sensitivity to purified NK cells was found as a consequence of lapatinib treatment of both the T47D and the MDA-MB-453 tumor cell lines (Fig. 6B). Because neither treatment with NRG1β nor with lapatinib affected MHC class I expression on any of the tested target cells (data not shown), this argues for a direct causal relationship between the upregulation of MICA/B expression by NRG1β and the increase in tumor NK sensitivity.

MICA/B are recognized by the NKG2D receptor expressed on NK cells and CD8+ T cells (1, 45). To verify that this receptor is involved in the NRG1β-induced sensitivity to NK cell killing, purified NK cells were pretreated with anti-NKG2D blocking Ab or target cells were pretreated with anti-MICA/B blocking Ab before the NK assay. The anti-NKG2D blocking Ab inhibited NRG1β-induced NK cytotoxicity against the T47D tumor target, whereas treatment with an isotype control Ab had no effect (Fig. 6C). Additionally, anti-MICA/B blocking Ab also inhibited NRG1β-induced NK cytotoxicity in T47D cells (Fig. 6D), indicating that the NRG1β-induced NK cell-mediated cytotoxicity was dependent on NKG2D–MICA/B interaction.

Discussion

In the present study, we report several findings supporting an important role for HER2/HER3 in regulating MICA/B expression in breast carcinoma cell lines. Our observations support the notion that oncogenes directly can induce the expression of NKG2DL. This is in line with the report by Boissel et al. (6), who reported that the BCR/ABL oncogene can regulate the expression of MICA in chronic myelogenous leukemia, and extends these findings to solid tumors expressing the HER2/HER3 oncogenes. MICA/B proteins induced by oncogenes such as HER2/HER3 and BCR/ABL could be one mechanism whereby the innate immune system is alerted to the presence of a developing tumor. The regulation of MICA/B expression by oncogenes has not previously been investigated in solid tumors, which is why we evaluated in breast carcinoma cell lines the HER2/HER3 signaling pathways involved in MICA/B regulation as well as their impact on NK cell-mediated killing.

We demonstrated that lapatinib downregulates the expression of MICA/B in HER2/HER3 high-expressing breast cancer cell lines. Although lapatinib is mainly recognized as a dual inhibitor of EGFR and HER2 (36), lapatinib was also shown to block the ligand-induced phosphorylation of HER3, induced by the heterodimerization of HER3 and HER2, and as a consequence to inhibit p-AKT and p-MAPK (37). These initial findings suggested that HER2/HER3 signaling may be involved in the regulation of the expression of MICA/B.

Several of our observations suggested a dominant role for HER3 as compared with HER2 in enhancing the expression of MICA/B.
Thus, we found a marked increase of MICA in HER3 transfected HER2/HER3 low-expressing MDA-MB-231 cells. We also demonstrated that silencing HER3 in cells expressing high levels of HER2/HER3 significantly attenuated the expression of MICA in MDA-MB-231 cells and that of MICB in T47D cells, whereas silencing of HER2 had less impact. Taken together, this suggested that HER3 but not HER2 levels represent an important rate-limiting factor for the regulation of both MICA and MICB expression in breast carcinoma cell lines. In support of this interpretation of our data, the expression level of HER2 is much higher than that of HER3 in both T47D and MDA-MB-453 cells. As an alternative explanation to the seemingly dominant role of HER3 as compared with HER2, other receptors may take over as heterodimerization partners to HER3 when HER2 is silenced. Thus, also EGFR can...
undergo heterodimerization with HER3 (46), although arguing against this the expression of EGFR is relatively low in T47D cells. Finally, we cannot entirely exclude that the greater effect on signaling as a result of silencing HER3 as compared with HER2 may depend on a more efficient silencing of HER3, although this was not apparent from our Western blot analysis. There is evidence for a differential regulation between MICA and MICB and that they could respond differently depending on the type of

FIGURE 5. Effects of caffeine, lapatinib, and HER2 or HER3 silencing on doxorubicin- or NRG1β-induced upregulation of MICA/B in T47D cells. (A) Histograms demonstrating MICA/B expression in cells treated with (i) 0.05 μM doxorubicin or (ii) 1 mM caffeine for 24 h were assessed by flow cytometry. The cells were pretreated with (Aiii) 1 mM caffeine for 2 h followed by doxorubicin (0.05 μM), (Bi) 1 mM caffeine for 2 h followed by NRG1β (50 ng/ml), (Ci) 1 μM lapatinib for 2 h followed by NRG1β (50 ng/ml), (Di) underwent gene silencing (as indicated) for 48 h followed by NRG1β (50 ng/ml) for 24 h. Then the cells were collected and MICA/B surface levels (expressed as rMFI) were assessed by flow cytometric analysis and evaluated with a Fisher exact test. (Bii, Cii, and Dii) Western blot analyses were performed with the cell lysates from the indicated treatment groups with/without NRG1β treatment for an hour using Abs specific for p-HER2, HER2, p-HER3, HER3, p-AKT, p-p44/42 MAPK, and β-actin. Representative data and the according mean values from the semiquantative analysis (expressed in arbitrary units are shown and evaluated with a Student t test. The experiments were repeated three times. Bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001. A.U., Arbitrary unit; CAF, caffeine; CTR, control siRNA; DOX, doxorubicin; ISO, isotype control; LAP, lapatinib; NT, no treatment control.
FIGURE 6. Effects of HER2/HER3 signaling on NK cell-mediated cytotoxicity. (A) T47D (HER3 high-expressing) and MDA-MB-231 (HER3 low-expressing) cells treated with/without NRG1β (50 ng/ml) were subjected to 51Cr-release assay for 4 h using IL-2-activated PBMCs (upper graphs) or purified NK cells (lower graphs) as effector cells. (B) NK cell-mediated cytotoxicity against target cell pretreated with or without 1 μM lapatinib were evaluated in both T47D cells for 4 h (left graph) and MDA-MB-453 cells for 18 h (right graph). (C) Purified NK cells were pretreated with blocking Ab of NKG2D or isotype control before cytotoxicity assay with NRG1β-stimulated T47D cells for 4 h and cytotoxicity was evaluated. Data are presented as mean of triplicate samples and are representative of three independent experiments. (D) T47D cells were treated with/without NRG1β for 24 h and pretreated with blocking Ab of MICA/B or isotype control before the cytotoxicity assay with NK cells for 4 h at an E:T ratio of 5:1. Data are presented as mean of triplicate samples and are representative of two independent experiments. Bars indicate SEM. *p < 0.05. ISO, Isotype control; LAP, lapatinib; NRG, NRG1β; NT, no treatment.

The two downstream pathways of the HER2/HER3 signaling are the PI3K/AKT and RAS/MAPK pathways (26), and thus we next assessed which of these is more important for baseline expression of MICA/B in breast cancer lines. Based on the results from experiments with signal inhibitors, we demonstrate that PI3K/AKT is the dominant pathway in the regulation of MICA/B expression. These findings are in line with the studies on the BCR/ABL signaling (6), where exposure of BCR/ABL+ K562 cells to the same PI3K inhibitor LY294002 resulted in a dose-dependent (in ranges similar to ours) MICA down-modulation. Also in line with our findings, inhibition of MAPK by the MEK1 inhibitor PD98059 did not affect MICA expression in the same study. Therefore, the PI3K/AKT pathway seems to be the dominant one in inducing MICA/B molecules for the HER2/HER3 oncogenes and MICA molecule for the BCR/ABL oncogene.

Signaling via BCR/ABL and HER2/HER3 is different in that HER2/HER3 signaling can be activated by ligands whereas BCR/ABL cannot. This gave us the possibility of studying both the baseline and ligand-induced MICA/B expression in HER2/HER3-expressing breast carcinomas. ATM–ATR, which plays a key role in the DNA damage response pathway, is frequently activated in tumor cells and was found to regulate NKG2DLs via transcriptional regulation (5). Our results in T47D cells treated with caffeine, an inhibitor of the ATM–ATR pathway, are in line with a role also for this pathway in maintaining baseline levels as well as DNA stress-induced MICA/B in HER2/HER3-expressing breast cancer cell lines. In contrast, when analyzing the NRG1β ligand-induced MICA/B in breast carcinoma cell lines, this was found not to be blocked by caffeine but only by lapatinib. These findings indicated that NRG1β-induced HER2/HER3 activation does not regulate MICA/B through genotoxic stress but does so directly mainly via the PI3K/AKT pathway. Because also BCL/ABL regulates MICA expression mainly via PI3K/AKT signaling (6), the role of this signaling pathway for the induction of MICA/B in other types of cancer merits further investigations. We also assessed the effect of silencing HER2 or HER3 in the NRG1β-induced MICA/B. In line with our findings related to the regulation of basal MICA and MICB expression, HER3 and not HER2 was found to be important for NRG1β-induced upregulation of MICA/B. Also, we found that NRG1β-induced phosphorylation of AKT was blocked more by siRNA of HER3 than by that of HER2. These findings suggested that HER3 is an important rate-limiting factor for both basal and NRG1β-induced expression of MICA/B.

A seemingly contradictory finding was that treatment with NRG1β promoted less changes in MICA/B expression in MDA-MB-453 cells than in T47D cells, whereas MDA-MB-453 cells expressed more HER2/HER3 as well as MICA/B than did T47D cells. A possible reason for this could be that the high HER2/HER3 expression in MDA-MB-453 cells has already activated close to a maximum of downstream signaling, whereas the lower HER2/HER3 expression in T47D cells leaves room for additional activation. Alternatively, the intensity of HER2/HER3 signaling depends not only on HER2/HER3 expression levels but also on activating mutations of downstream signaling molecules (48), or on hitherto unknown mechanisms.

Because MICA/B are important ligands for the NK-activating receptor NKG2D, it was of primary importance for us to deter-
mine the functional consequences of HER2/HER3-mediated MICA and MICB regulations on tumor target cells. As anticipated, activation of HER2/HER3 signaling by NRG1γ enhanced sensitivity to NK cell-mediated cytotoxicity in HER3 overexpressing breast carcinoma cells. Conversely, inactivation of HER2/HER3 signaling by lapatinib attenuated the sensitivity to NK cell-mediated cytotoxicity. These findings implicated a role for the NKG2D–MICA/B interaction in the modulation of the NK cell sensitivity in these breast cancer lines. To more directly prove this, we demonstrated that NRG1β-enhanced NK cell-mediated cytotoxicity could be blocked with an anti-NKG2D Ab. This result could not rule out that the interaction between NKG2D ligands other than MICA/B could be of importance for the observed NRG1β-induced enhancement of NK cell activity. Arguing for a predominant role for MICA and MICB as compared with other NKG2D ligands in this regard, we were able to block the enhanced activity by an Ab against MICA/B molecules. It is notable, however, that neither blocking with anti-NKG2D Abs nor with anti-MICA/B Abs significantly decreased NK cell sensitivity of the untreated T47D line (data not shown). The reason for this could be either that NKG2D is not involved in conferring “baseline” NK sensitivity of this cell line, or alternatively and more likely that these Abs are able to only partially block receptor/ligand interaction.

NRGs are regarded as growth factors for cancer cells, as NRGs activate cell growth and cell proliferation via activation of the downstream signaling pathway of HER3 (17, 18). Indeed 1 nM NRG1β was shown to enhance the cell proliferation in 7 of 14 ovarian cancer cell lines (49). We found that up to 250 ng/ml NRG1β could not affect tumor cell proliferation or apoptosis in the T47D cell line. These findings support the conclusion that a high dose of NRG1β enhances breast carcinoma sensitivity for NK cell-mediated cytotoxicity without accelerating tumor growth. Conversely, the implication from our findings is that the HER2/HER3 signaling pathway targeting therapy may suppress tumor sensitivity to NK cells via downregulation of MICA/B, as was shown by the attenuation of NK cell-mediated cytotoxicity by lapatinib. In theory, this indicates that therapies targeting HER2/HER3 signaling may be a double-edged sword as they block tumor cell-activating signaling while suppressing NKG2D-MICA/B–dependent immune surveillance by NK cells, CD8+ T cells, and γδ T cells. However, because lapatinib pretreatment enhanced trastuzumab-induced Ab-dependent cellular cytotoxicity by increasing the cell surface expression of HER2 through inhibition of the degradation of HER2 (50, 51), the clinical net effect of lapatinib treatment when it comes to NK cell-mediated tumor elimination remains to be established.

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Disclosures

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References


