Fatty acid synthase activity regulates HER2 extracellular domain shedding into the circulation of HER2-positive metastatic breast cancer patients

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Abstract. Clinicopathological assessment of the functional relationship between the HER2 oncogene and tumor-associated fatty acid synthase (FASN) is largely precluded because immunohistochemical and/or mRNA studies should be performed in biopsies from breast cancer patients. We here sought to determine whether serum FASN (sFASN) could associate with circulating HER2 extracellular domain (HER2 ECD) in the blood of metastatic breast cancer (MBC) patients. Concentrations of serum FASN and HER2 ECD were measured with ELISA in sera retrospectively obtained from 201 patients with metastatic breast cancer (MBC) and 31 healthy subjects. Mechanistical in vitro studies were performed using pharmacological inhibitors of HER2 and FASN as well as cultured cancer cells engineered to overexpress HER2 and FASN human genes. When the upper limit of normal sFASN was defined as the mean + 2SD of the control group, sFASN was elevated above this cut-off (12 ng/ml) in 70 MBC patients (35%). Eighty-nine MBC patients (44%) had elevated levels of HER2 ECD (HER2 ECD cut-off = 15 ng/ml). HER2 ECD-positive MBC patients slightly increased their sFASN levels compared with HER2 ECD-negative MBC patients. sFASN-positive MBC patients had significantly increased levels of HER2 ECD when compared with sFASN-negative MBC patients (mean HER2 ECD=34 ng/ml, 95% CI 26-41 ng/ml and 18 ng/ml -95% CI 15-21 ng/ml, respectively; p=0.002). Sixty percent of sFASN-positive patients concurrently exhibited high levels of HER2 ECD whereas 64% of sFASN-negative patients were negative for circulating HER2 ECD. In vitro studies revealed that BC cells bearing HER2 gene-amplification released higher levels of extracellular FASN than HER2-negative BC cells. Trastuzumab-induced blockade of HER2 ECD shedding failed to prevent FASN release and retrovirally-induced HER2 overexpression in MCF-7 cells did not increase extracellular FASN. Of note, pharmacological inhibition of FASN activity significantly decreased HER2 ECD levels in the supernatant of HER2-overexpressing BC cells while transient overexpression of FASN gene in HBL100 cells promoted FASN protein release and concomitantly increased HER2 ECD shedding into the extracellular milieu. Subsequent studies should explore if quantitative determination of FASN molecules in blood could become a rapid and accurate non-invasive test to monitor disease progression and survival in HER2-overexpressing MBC undergoing HER2-targeted therapies.

Introduction

In breast cancer disease, amplification of HER2 (ERBB2; Her-2/neu) oncogene leading to overexpression of the HER2 receptor tyrosine kinase (p185HER2) occurs in 15-30% of breast carcinomas and associates with unfavourable prognosis, shorter relapse time and low survival rate (1-5). The association of HER2 overexpression with enhanced malignant phenotypes of breast cancer cells, including those with metastatic potential and resistance to chemo- and endocrine-therapies (6,7), provides a plausible interpretation for the poor clinical outcome of breast cancer patients with HER2-overexpressing tumors. It is obvious that identification of genes/proteins that are differentially expressed in HER2 oncogene-related breast carcinomas is essential in elucidating the mechanistic bases of their increased metastatic potential and resistance to several anti-breast cancer therapies.
It is well known that aberrant expression of HER2 can trigger the activation of multiple downstream signaling pathways (e.g. PI3-K/PTEN/AKT pathway and Ras/Raf/MAPK pathways), which appear to be essential in inducing increased cell proliferation and differentiation, decreasing apoptosis, and/or enhancing tumor cell motility and angiogenesis (8-10). However, much less is known about the specific ‘effectors’ regulated by HER2 that ultimately contribute to its tumorigenic effects. Moreover, few studies on HER2-induced changes in protein expression have been reported using tumor-derived human breast cancer cell lines or breast cancer specimens (11-13). Thus, the proteins that regulate the ‘output’ of the HER2 oncogene are not well characterized. Remarkably, protemic studies have revealed that proteins involved in glycolysis, de novo lipid synthesis and detoxification pathways are highly expressed in HER2-positive breast carcinomas (14). Although the cross-talk between HER2 and the bioenergetic phenotype produced from commonly altered metabolic pathways in breast cancer cells (i.e. aerobic glycolysis -Warburg effect-, de novo fatty acid -FA- biosynthesis, etc), remains to be better defined, a body of evidence strongly supports the notion that the oncogenic effects of HER2 largely depend on the preservation of the ‘lipogenic phenotype’ (i.e. activation of the genetic program involved in de novo FA synthesis including lipogenic enzymes and key metabolic regulators) (15,16).

First, HER2 overexpression leads to constitutive up-regulation and maintenance of an exacerbated endogenous FA biosynthesis catalyzed by key lipogenic enzymes such as fatty acid synthase (FASN) (17-19). Second, disturbance of the lipogenic phenotype rapidly switches-off the oncogenic activity of the HER2 signaling platform, ultimately resulting in apoptotic cell death of HER2-overexpressing breast carcinoma cells (20-23). Third, an exacerbated endogenous FA biosynthesis is sufficient to trigger a breast cancer-like phenotype functionally dependent on HER2 activity (24). Unfortunately, a functional assessment on the ultimate relationship between the expression and/or activity of HER2 and FASN is largely precluded because immunohistochemical and/or mRNA studies should be performed in tissue biopsies from breast cancer patients. Unexpectedly, recent studies have demonstrated that cultured breast cancer cells can excrete immunoreactive FASN into the extracellular space (25-30). Importantly, significant elevations of 'extra-cellular FASN' have been also detected in the circulation of patients with breast cancer compared to healthy subjects. The current dogma in the field states that the excess intracellular FASN, which increases during progression of tumor cells in the metastatic cascade, is excluded from the cytosol of tumour cells in a stage-related manner compared with healthy subjects. The fact that circulating FASN levels appear to increase in parallel with different clinical stages support the perception that the extracellular form of FASN should be viewed as a tumour marker capable of assessing cancer virulence as its up-regulation in the blood of cancer patients is more pronounced in the late (metastatic) stages of breast carcinomas.

Since cleavage of the HER2 receptor liberates the HER2 extracellular domain (ECD) leaving a fragment with constitutive kinase activity and because this process is clinically relevant since HER2 ECD serum levels in metastatic breast cancer (MBC) patients are associated not only with prognosis but further with responses to chemotherapy, endocrine therapy and trastuzumab (31-37), we herein sought to determine whether serum FASN (sFASN) levels could associate with circulating HER2 ECD in metastatic breast cancer (MBC) patients. Here, we report for the first time that a positive correlation between HER2 ECD levels and circulating sFASN takes place in the blood of HER2 ECD-positive MBC patients. Importantly, higher concentrations of circulating sFASN closely relate with high circulating HER2 ECD levels. By performing in vitro studies with pharmacological blockers of HER2 (i.e. the monoclonal antibody trastuzumab) and FASN (i.e. the small-compound C75) as well as breast epithelial cell lines engineered to overexpress HER2 and FASN human genes, we unambiguously demonstrate that FASN-catalyzed de novo FA biogenesis actively contributes to HER2 ECD shedding in human breast carcinoma cells.

Materials and methods

Reagents. Trastuzumab (Tzb; Herceptin®), kindly provided by Hospital Universitari de Girona Dr. Josep Trueta Pharmacy (Girona, Spain), was solubilized in bacteriostatic water for injection (USP, a sterile, non-pyrogenic preparation of water for injection containing 1.1% -1.1 mg/ml- of benzyl alcohol added as a bacteriostatic preservative), stored at 4 °C (stock solution at 21 mg/ml) and used within one month. C75 was purchased from Alexis Biochemicals (San Diego, CA, USA), dissolved in DMSO, and stored in the dark as stock solution (25 mg/ml) at -20 °C.

Cell lines and culture conditions. HBL100, MDA-MB-231, MDA-MB-468, MCF-7 and SKBR3 breast cancer cells were obtained from the American Type Culture Collection (ATCC). JIMT-1 cells were established at Tampere University, Finland, and are available from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de). HBL100, MDA-MB-231, MDA-MB-468, MCF-7 and SKBR3 cell lines were routinely grown in Improved MEM (IMEM; Biosource International) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine. JIMT-1 cells were grown in F-12/DEMEM (1:1) supplemented with 10% FBS and 2 mM L-Glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells were screened periodically for Mycoplasma contamination. Drugs were prepared freshly from stock solutions and diluted with growth medium. Control cells were cultured in medium containing the same concentration (v/v) as the experimental cultures with treatments. The vehicle solutions had no noticeable influence on the proliferation of experimental cells.

Generation of HER2-overexpressing MCF-7 cells. Construction of pBabe/HER2 retroviruses, retroviral infection of MCF-7 cells, and stable selection of MCF-7/HER2 cells were performed as described elsewhere (38,39). Transient overexpression of human FASN gene in HBL100 breast epithelial cells was performed as previously described (24).

Measurements of HER2 ECD and sFASN in serum samples. All laboratory analyses were performed centrally to preclude
variation in analysis and interpretation. The samples were analyzed without knowledge of their provenance. Serum testing was entirely performed at the Molecular Oncology Laboratory at the Catalan Institute of Oncology in Girona (Catalonia, Spain). The levels of circulating HER2 ECD in serum samples were measured by using a sandwich enzyme immunoassay according to the manufacturer's instructions [human neu quantitative immuno-1 enzyme-linked immuno-sorbent assay (ELISA); Bayer Diagnostics; Cambridge]. The HER2 ECD values were expressed in ng/ml. The laboratory’s upper limit of the reference range was set at 15 ng/ml. Intra- and interassays coefficients of variation (CVs) were lower than 8%. sFASN concentrations were measured by a sandwich enzyme immunoassay (FAS-detect ELISA, FASgen Inc., Baltimore, MA, USA) according to manufacturer's instructions. The within- and between-run CVs were less than 10%, and the detection limit was 3.22 ng/ml.

**Measurements of HER2 ECD and sFASN in cultured cells.**

To evaluate the effects of trastuzumab and C75 on FASN and HER2 ECD release respectively, cells were plated in 100-mm tissue culture dishes and cultured in regular medium with 10% FBS until they reached 75-80% confluence. The cells were washed twice with serum-free medium, and incubated overnight upon serum-free conditions. Cells were then cultured in 5 ml of 5% FBS-containing or serum-free DMEM in the presence or absence of 100 μg/ml trastuzumab or 10 μg/ml C75, as specified. After treatment, conditioned media were centrifuged to remove the cell debris and then used immediately or frozen at -80°C until utilization. Cells were washed twice with cold-PBS and then lysed in buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride) for 30 min on ice. The lysates were cleared by centrifugation in an Eppendorf tube (15 min at 14,000 x g, 4°C). Protein content was determined against a standardized control using the Pierce Protein Assay kit (Pierce, Rockford, IL, USA). Concentration of HER2 ECD and sFASN in conditioned media was performed as described above. Although the cell masses of the different experimental conditions did not vary greatly about 30-50 mg from a 100-mm confluent plate, HER2 ECD and FASN release levels were normalized by mg of protein in the whole cell lysate to ensure that the negative or positive detection of both HER2 ECD and extracellular FASN should reflect the capacity of the cells to shed HER2 ECD and/or to release FASN rather than the difference in cell number or cell mass. Data are presented as means (columns) and 95% confidence intervals (bars) of three independent experiments carried out in duplicate.

**Immunoblotting.**

Fifteen microliters of either conditioned media (analyses of FASN expression in the extracellular milieu) or equal amounts of protein from whole cell lysates (i.e., 50 μg for the analyses of P-Tyr1248 HER2) were resuspended in 5X Laemmli sample buffer (10 min at 70°C), resolved by electrophoresis on 3-8% NuPAGE Tris-Acetate (FASN), and transferred onto nitrocellulose membranes. Non-specific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at RT with TBS-T buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween-20] containing 5% (w/v) non-fat dry milk. The treated filters were washed in TBS-T and then incubated with anti-FASN and anti-Tyr1248 HER2 antibodies for 2 h at RT in TBS-T containing 1% (w/v) non-fat dry milk. The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) in TBS-T were added for 1 h, and immunoreactive bands were visualized with ECL detection reagent. The primary antibodies for FASN immunoblotting were: The clone M3 (1:2000 dilution), mouse anti-FASN monoclonal antibody (kindly provided by Dr Ellen Pizer, Johns Hopkins University, MA, USA), and the mouse anti-phospho-HER2 (Y1248) monoclonal antibody Ab-18 (clone PN2A; NeoMarkers, Fremont, CA, USA).

**Statistical methods.**

Descriptive results of continuous variables were expressed as 95% confidence intervals. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test and then variables were given a log-transformation if necessary. Relation between variables were tested using Pearson's test and stepwise multiple linear regression analysis. We used χ2 test for comparisons of proportions, and ANOVA test with post-hoc Scheffé's test for comparisons of quantitative variables across categories. For a given value of p<0.05, the study had a 99% power to detect...
significant correlations between parameters in the whole sample of subjects in a bilateral test (n=201). The analysis were performed using the program SPSS (version 11.0).

Results

High levels of the extracellular form of FASN occur in the serum of metastatic breast cancer patients. sFASN levels from the control group (n=35 healthy subjects) were 8±2 ng/ml (mean ± SD). sFASN levels in MBC patients (n=201) ranged from 1 to 210 ng/ml (mean sFASN=35 ng/ml; 95% CI 29-41 ng/ml). When the upper limit of normal sFASN was defined as the mean plus two SDs (mean + 2SD) of the control group, this cut-off for sFASN normality (i.e. 12 ng/ml sFASN) revealed that 35% of metastatic breast cancer (MBC) patients (n=70) exhibited elevated levels of sFASN. Circulating HER2 ECD levels ranged from 5 to 209 ng/ml (mean HER2 ECD=33 ng/ml; 95% CI 24-42 ng/ml). Overall, 89 patients (44%) had elevated levels of HER2 ECD (HER2 ECD cut-off = 15 ng/ml).

Positive correlation between HER2 ECD levels and circulating sFASN in HER2 ECD-positive metastatic breast cancer patients. Although circulating HER2 ECD tended to be positively linked to sFASN, HER2 ECD concentrations were not significantly associated with circulating sFASN when analyzing all MBC subjects as a whole (Fig. 1). When we evaluated the association between HER2 ECD and sFASN separately in HER2 ECD-negative (HER2 ECD <15 ng/ml) and in HER2 ECD-positive (HER2 ECD ≥15 ng/ml), circulating HER2 ECD concentrations were not associated with sFASN in HER2 ECD-negative MBC patients (r=0.120, P=0.206). However, the relationship of circulating HER2 ECD with sFASN was statistically significant among HER2 ECD-positive MBC patients (r=0.362, P=0.000).

Higher concentrations of circulating sFASN predict high circulating HER2 ECD levels in metastatic breast cancer patients. HER2 ECD-positive MBC patients tended to significantly increase their sFASN levels compared with HER2 ECD-negative MBC patients [mean sFASN=39 ng/ml -95% CI 30-48 ng/ml, and 27 ng/ml, 95% CI 20-34 ng/ml, respectively; p=0.044] (Fig. 2, left). Though 75% of HER2 ECD-negative patients were sFASN-negative, a similar number of sFASN-positive (47%) and sFASN-negative (53%) patients was observed among HER2 ECD-positive subjects (Fig. 2, right). Interestingly, sFASN-positive MBC patients had significantly increased levels of HER2 ECD when compared with sFASN-negative MBC patients [mean HER2 ECD=34 ng/ml, 95% CI 26-41 ng/ml and 18 ng/ml, 95% CI 15-21 ng/ml, respectively; p=0.002] (Fig. 3, left). Thus, 64% of sFASN-negative patients were negative for circulating HER2 ECD while 60% of sFASN-positive patients concurrently exhibited high levels of HER2 ECD (Fig. 3, right).

Trastuzumab-induced blockade of HER2 does not prevent FASN release in HER2-overexpressing breast cancer cells. BC cells naturally-bearing HER2 gene-amplification (i.e. JIMT-1, SKBR3 and BT474) released higher levels of extracellular FASN than BC cells expressing physiological levels (i.e. one gene copy) of HER2 (i.e. MDA-MB-231, MDA-MB-468). To evaluate whether prevention of HER ECD shedding could lead to changes in the release of FASN to the extracellular medium, HER2-overexpressing breast cancer cells were cultured in the absence or presence of the
anti-HER2 monoclonal antibody trastuzumab (100 \( \mu \)g/ml) for 72 h. Although Tzb exposure slightly decreased FASN release in BT-4T4, Tzb-induced down-regulation of HER2 ECD shedding failed to prevent FASN release in JIMT-1 and SKBR3 cells (Fig. 4, left panel). This lack of correlation between HER2 ECD status and FASN release was further confirmed when evaluated the extracellular levels of FASN prior and after retroviral-induced ectopical overexpression of HER2. Thus, we failed to detect a significant increase in the expression of extracellular FASN when HER2-negative MCF-7 cells were engineered to stably overexpress HER2 (Fig. 4, right panel).

FASN activity regulates HER2 ECD shedding in cultured cancer cells. Since we previously demonstrated that FASN blockade strongly suppressed HER2 oncogene upon long-
transiently transfected with plasmid pCMV6-XL4 carrying full-length human FASN cDNA (gi: NM_004104) (24). HBL100 cells transiently transfected with pCMV6-XL4/ 
FASN exhibited an enhanced endogenous lipid synthesis as well as an increased phosphorylation status of the 1248 Tyr residue of HER2 (24). Here, immunoblotting analyses revealed that FASN protein accumulated significantly in the extracellular milieu obtained from HBL100 cells engineered to overexpress human FASN gene (Fig. 5B). A concomitant increase of HER2 ECD was measurable in the conditioned media of FASN-overexpressing HBL100 cells whereas HER2 ECD remained undetectable in mock-transfected HBL100 cells (Fig. 5B).

Discussion

Several cellular receptors, including HER2, require their correct localization within ordered lipid microdomains (lipid rafts) for efficient signaling (40-43). Lipid rafts are rich in cholesterol and sphingolipids, products largely generated in tumor cells by FASN (15,44). Studies from our laboratory have shown that pharmacological blockade of FASN lipogenic activity rapidly inactivates HER2 tyrosine kinase activity (15,20). When using fluorophores conjugated to cholera-toxin B-subunit, which binds to the lipid raft constituent ganglioside GM1, we concluded that FASN blockade-related disruption of HER2 activity paralleled FASN blockade-induced down-regulation of GM1 signals at the tumor cell membrane (15). We recently revealed that exacerbated endogenous FA biogenesis triggered upon forced overexpression of the lipogenic enzyme FASN functions as an intrinsic stimulus that is sufficient to hyper-activate an oncogenic and chemoresistance signaling through the HER network (24). Our in vitro approach revealed that low levels of basal HER1/HER2 tyrosine kinase activity in non-cancerous breast epithelial cells were dramatically enhanced following FASN up-regulation. These findings, altogether, strongly suggested that FASN-catalyzed de novo FA biogenesis appears to significantly affect the formation of HER1/HER2 signal transduction complexes at the membrane lipid rafts, changing their state from ‘off’ to ‘on’.

Our current findings confirm and extend further the above mentioned in vitro findings in a clinical setting. FASN, an intracellular protein, appears to exit MBC cells and this release into the blood stream is increased in HER2 ECD-positive MBC patients. It could be argued that enhanced levels of sFASN might arise, at least in part, from stimulatory effects of HER2-driven signaling on FASN gene expression which, in turn, will result in enhanced release of FASN protein. At the bench, however, trastuzumab-induced blockade of HER2 ECD shedding failed to completely prevent FASN release to the extracellular medium in HER2-overexpressing BC cells. These findings, together with the fact that we also failed to detect a significant increase in the expression of extracellular FASN when HER2-negative MCF-7 cells were engineered to overexpress HER2, strongly suggest that HER2 activation at the cell membrane may not be sufficient to explain high levels of sFASN in the blood of HER2 ECD-positive MBC patients. Indeed, a similar number of sFASN-positive and sFASN-negative patients could be
observed among HER2 ECD-positive individuals. Interestingly, our study revealed that the higher the concentration of circulating FASN, the higher of circulating HER2 ECD. Thus, sFASN-positive MBC patients had about two times higher blood serum levels of HER2 ECD than sFASN-negative MBC patients. Moreover, these unexpected clinical findings were validated when we monitored a functional relationship between FASN and HER2 ECD levels in cultured breast cancer cells. On the one hand, pharmacological blockade of FASN activity suppressed HER2 tyrosine kinase activity and HER2 ECD shedding in MCF-7/HER2 cells. On the other hand, nearly-normal HBL100 breast epithelial cells engineered to overexpress FASN significantly increased HER2 ECD levels in the extracellular milieu. Therefore, it is tempting to suggest that high levels of FASN-catalyzed endogenous FA biogenesis should significantly promote kinetically favorable interactions for HER2 and signal-transduction-correlated proteins in lipid raft microdomains which, in turn, will enhance HER2 ECD shedding into the extracellular milieu.

In summary, we described above for the first time that a significant association occurs between the circulating levels of the extracellular form of FASN (sFASN) and the HER2 ECD in MBC patients. Although further studies are required in order to define the biological significance and the ultimate role of circulating FASN in MBC patients, the quantitative determination of FASN molecules in blood might represent a previously unrecognized tool to monitoring aggressiveness during progression of HER2-positive MBC. Nevertheless, the quantitative determination of sFASN may be relevant in monitoring responses of breast cancer patients undergoing anti-HER2 therapies such as the anti-HER2 monoclonal antibody trastuzumab. Proteolytic cleavage of the HER2 ECD has been shown to initiate receptor phosphorylation representing HER2 activation in vitro and, as such, it represents a key molecular event on the mode of action of trastuzumab (32,46,47). Indeed, serum HER2 ECD can be found significantly elevated in patients with tumor exhibiting activated HER2, a predictor of the efficacy of trastuzumab-based therapy in patients with MBC (32). We recently provided experimental evidence that FASN blockade efficiently reverses acquired autoresistance to trastuzumab in high-dose trastuzumab-conditioned SKBR3 cells (48). Subsequent studies should explore how circulating levels of sFASN relate to clinical prognostic variables and/or clinical responses to trastuzumab-based therapies. Experiments are currently underway in our laboratory to definitely establish whether measurement of sFASN could become a rapid and non-invasive test of adequate prognostic/diagnostic accuracy to monitor the effects of anti-HER2 therapies on disease progression and survival in HER2-overexpressing breast carcinomas.

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