Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis

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Abstract

Lipocalin 2 (LCN2; also known as NGAL) is a secreted glycoprotein and its elevated expression has been observed in breast cancers. However, the importance of LCN2 in breast tumorigenesis is unclear. Here, we employed a spontaneous mammary tumor mouse model showing that MMTV-ErbB2 (V664E) mice lacking mouse LCN2 had significantly delayed mammary tumor formation and metastasis with reduced matrix metalloproteinase-9 activity in the blood. LCN2 expression is upregulated by HER2/phosphoinositide 3-kinase/AKT/Signal transducer and activator of transcription 3kB pathway. Decreasing LCN2 expression significantly reduced the invasion and migration ability of HER2+ breast cancer cells. Furthermore, injecting an anti-mouse LCN2 antibody into mice bearing established murine breast tumors resulted in significant blockage of lung metastasis. Our findings indicate that LCN2 is a critical factor in enhancing breast tumor formation and progression possibly in part by stabilizing matrix metalloproteinase-9. Our results suggest that inhibition of LCN2 function by an inhibitory monoclonal antibody has potential for breast cancer therapy, particularly by interfering with metastasis in aggressive types of breast cancer.

Introduction

Recent studies have implicated lipocalin 2 (LCN2), a member of the lipocalin family, in leukemia (1–3) and other solid tumors (4). The lipocalin family is a large group of small secreted glycoproteins involved in binding and transportation of small lipophilic molecules (5). The physiologic ligand for LCN2 is not known. On bacterial infection, LCN2 tightly binds to bacterial iron-siderophores and deprives the iron source for their growth (6), which is shown by the delayed clearance of bacteria infection in LCN2 knockout mice (7, 8).

It was observed that increased LCN2 levels were present in the plasma of leukemia patients with CML (1–3). We found that mouse LCN2 (mLCN2) is required for BCR-ABL–induced leukemia in a mouse bone marrow transplant model showing that mLCN2 or human LCN2 were generated in our laboratory using recombinant proteins purified from Escherichia coli, a gift from Dr. Roland Strong (6). Antibodies against AKT, pAKT, HER2, pY-HER2, and β-actin were purchased from Cell antisera (Abcam, UK). Dominant-negative and constitutively active AKT constructs are gifts from Dr. Zhou Songyang.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Materials and Methods

Tissue culture and mice. Cell lines were grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin at 37°C in humidified incubator with 5% CO2. LCN2 knockout (LCN2–/–) mice were a gift from Dr. Alan Aderem (8). MMTV-ErbB2(V664E) transgenic mammary tumor mouse model crossed with LCN2–/– mice. Our findings presented here illustrate the importance of LCN2 in breast cancer tumor formation and metastasis and the possible underlying molecular mechanisms promoted by LCN2 expression in breast cancer, indicating that inhibition of LCN2 has potential in breast cancer therapy.

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primers for mLNC2: 5′-AGCCGACATCCGGAGCAGTC-3′ and 5′-ACTTGCCCAAGGCGGTGAACG-3′ and for mouse actin: 5′-GCTGGAAGGTGGA-CAGTGGAC-3′ and 5′-ATGGATAGTGGGATGCTGTC-3′.

Two-chamber migration and invasion assays. Cell migration and invasion assays were done using the two-chamber migration assay (8 μm pore size; BD Biosciences). For SKBr3 cells, 1 × 10^5 cells were seeded in serum-free medium in the upper chamber and migrated toward 10% FCS in the lower chamber for 18 h followed by fixation and staining with 0.2% crystal violet-20% methanol. Quantification was done using unpaired t test.

Western blotting and zymogram of plasma samples. Plasma (1-2 μL) was separated on 20% SDS-PAGE followed by transferring and blotting with purified anti-mLNC2 polyclonal antibody (1:3,000). Plasma (2-5 μL) was analyzed using 10% zymographic gel (Invitrogen).

Imaging, histology, H&E staining, and immunohistochemistry. Freshly collected lung tissues were imaged using Xenogen IVIS 200 Imaging System for green fluorescent protein signals. For histologic analysis, tissues were postfixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E.

Statistical data analysis. The Kaplan-Meier method (log-rank test) was used to determine the survival curves of tumor formation in MMTV-ErbB2 (V664E) mice with variations in mLNC2 expression in human breast cancer, consistent with a western analysis of LCN2. Statistical data were analyzed with Stata 9.2 (Stata Corp). The log-rank test was used in intergroup comparisons. The Wilcoxon rank-sum test was used to analyze the fluorescence imaging results of the in vivo antibody studies.

Results

Upregulation of LCN2 (NGAL) through HER2/neu signaling pathway in human breast cancer. We observed a significant clinical correlation of higher LCN2 expression with HER2 positivity among 318 newly diagnosed breast cancer patients, implying that LCN2 might be a downstream target of HER2 signaling pathway. When blocking HER2 function with Herceptin in HER2 SKBr3 cells, we found that LCN2 expression was inhibited (Fig. 1A). LCN2 expression in SKBr3 cells was reduced in a dose-dependent manner by the phosphoinositide 3-kinase inhibitor LY294002 and by Bay 11-7082, which specifically blocks IκB kinase (Supplementary Fig. S1). Secretion of LCN2 into conditioned medium was also effectively inhibited under these conditions (Supplementary Fig. S1). In addition, overexpression of dominant-negative Akt led to reduced expression of LCN2, whereas overexpression of constitutively active Akt increased LCN2 levels (Fig. 1D). These results indicate a role of the HER2/phosphoinositide 3-kinase/AKT/NF-κB signal cascade in inducing LCN2 expression in human breast cancer, consistent with a recent study showing that LCN2 expression was largely dependent on the NF-κB pathway in thyroid neoplastic cells (18).

Mice lacking mLNC2 have impaired ErbB2(V664E)-induced mammary tumor formation. We tested mLNC2 effects in a spontaneous mouse mammary tumor model. Transgenic mice carrying the mutant form of ErbB2(V664E) driven by the mammary-specific promoter MMTV develop multiple primary breast tumors and lung metastases (17). By breeding MMTV-ErbB2(V664E) mice (FVB) and mLNC2−/− mice (C57BL/6; ref. 8), we generated three groups of mice expressing ErbB2(V664E) with variations in mLNC2 alleles (mLNC2+/−, mLNC2−/−, and mLNC2−/−; Supplementary Fig. S2A-C).

In this genetic study for the effects of mLNC2 on ErbB2(V664E)-induced breast tumors, we observed striking differences in the timing of tumor formation as well as the number and the size of primary tumors among the three groups. Groups carrying one or two alleles of mLNC2 started to develop multiple large (>1 cm) mammary tumors ~170 days after birth. In contrast, mLNC2−/− mice did not form similar size of tumors until ~260 days, a time when >60% mice in the groups expressing mLNC2 were already terminated due to excessive tumor burden (Fig. 2A). The overall tumor occurrence was significantly delayed in the mLNC2−/− group (260-500 days with T50 = 303 days) compared with the mLNC2−/− mice (170-340 days with T50 = 210 days; Fig. 2A). Although the mLNC2−/− group showed a slightly delayed course of tumor occurrence compared with the mLNC2−/− group, no significance was observed regarding the tumor occurrence and the tumor volume between these two groups (Fig. 2A and B), indicating that one allele mLNC2 deficiency was not sufficient to interfere with the formation of ErbB2(V664E)-induced breast tumors. We observed greater numbers and larger volumes of tumors per mouse in the groups expressing mLNC2 compared with the mLNC2−/− group (Fig. 2B; Supplementary Fig. S2D). Notably, the lung metastases in mLNC2−/− mice were significantly delayed compared with the mLNC2−/− mice (P < 0.05; Fig. 2C). By Kaplan-Meier analysis, the T50 for lung metastasis in the mLNC2−/− group is ~260 days. In contrast, the T50 value was not reached in the mLNC2−/− and the mLNC2−/− groups, suggesting that deficient mLNC2 expression also impairs lung metastasis in this model.

Unlike the healthy wild-type mice with minimal levels of mLNC2 in the plasma (Fig. 2D, top, last three lanes), we detected a dramatically increased mLNC2 level in the plasma of tumor-bearing MMTV-ErbB2(V664E) mice (Fig. 2D, top). Interestingly, we observed elevated MMP-9 gelatinase activity and the presence of higher molecular weight gelatinase activity in the plasma of tumor-bearing mice expressing mLNC2 compared with mLNC2−/− group (Fig. 2D, bottom, outlined with red box). We also noticed the relatively weak MMP-9 gelatinase activity in the plasma of tumor-bearing mLNC2−/− mice (Fig. 2D, bottom), and these samples displayed distinct gelatinase bands with molecular weight lower than the MMP-9 band, suggesting the beneficial effects of mLNC2 in maintaining MMP-9 activity potentially through stabilizing MMP-9 as implied by the previous studies (15, 16).

LCN2 expression in HER2 breast tumor cells stimulates cell invasion in vitro and their metastatic potential in mouse xenograft model. Using HER2 SKBr3 cells (high LCN2 expression),
Figure 2. Effects of mLCN2 on the mammary tumor formation and metastasis in the MMTV-ErbB2(V664E) transgenic mouse model. A, time course of primary mammary tumor development in MMTV-ErbB2(V664E) transgenic mice in the three genetic backgrounds: mLCN2+/+, mLCN2+/-, and mLCN2−/−, depicted by the Kaplan-Meier analysis. T50 is a calculated statistical value incorporating both time and incidence of tumor formation when 50% of the mice in the same group developed mammary tumors. B, summary of total breast tumor weights per mouse in the three mice groups. C, Kaplan-Meier analysis of lung metastasis of the three mice groups. D, levels of mLCN2 (top) in the plasma of the three mice groups at euthanization. mLCN2 levels in the plasma from normal healthy mice were shown in the last three lanes. MMP activity in the plasma of the three mice groups was analyzed using 10% zymogram gel (bottom). Conditioned medium from MMP-9–transfected 293T cells was used to mark MMP-9 position. Red box outlined the high molecular weight of MMP activity (HMW MMP).
we observed a significant reduction in cell migration and invasion on knocking down LCN2 expression compared with either parental cells or cells expressing nonspecific shRNA (Fig. 3A and B). We used MDA-MB-468 (high LCN2 expression) to test LCN2 effects in mouse xenograft model. Note, we did not observe any change in cell growth rate in culture on lowering LCN2 expression by shRNA in either SKBr3 or MDA-MB-468 cells (data not shown; Supplementary Fig. S3A). We injected mammary fat pads of nude mice with 1 million of either parental MDA-MB-468 cells or its derivatives expressing either the nontargeted shRNA or the shRNA for LCN2. We analyzed the primary tumor and the surrounding tissues 42 days after implantation. No significant differences in the primary tumor size/weight were found among the three groups (Supplementary Fig. S3B). However, the capacity of tumor cells for invasion and metastasis, as measured by the events of lymphovascular invasion (Fig. 3C, b), intramammary lymph node metastasis (Fig. 3C, c), and chest/abdominal wall invasion (Fig. 3C, d), were significantly reduced in the group injected of MDA-MB-468 cells with the LCN2 shRNA knockdown (Fig. 3D).

**mLCN2 expression correlates with aggressive tumor formation in murine mammary tumor cell lines.** The association of LCN2 expression with aggressive human breast cancer types was further shown when using a series of mouse breast tumor cell lines (67NR, 168FARN, 4T07, and 4T1), which were derived from the mammary tumors of the same mouse but with distinct metastatic potentials (19). 4T07 and 4T1 cells are the most aggressive and develop lung metastases. We found that only 4T07 and 4T1 cells have mLCN2 transcripts and secrete mLCN2, with the most aggressive 4T1 cells having the highest levels of mLCN2 (Fig. 4A). Knocking down mLCN2 by shRNA in 4T1 cells (Supplementary Fig. S4A, left) reduced MMP-9 activity (Supplementary Fig. S4A, right). Reduction of mLCN2 expression greatly decreased 4T1 invasive ability (Supplementary Fig. S4B) and colony formation in soft agar (Supplementary Fig. S4C). Similar to our findings in the plasma from...
ErbB2-induced breast tumor-bearing mice, we detected high levels of mLCN2 in the plasma of breast tumor-bearing mice implanted with 4T1 cells and increased MMP-9 activity in the plasma compared with normal healthy mice (Supplementary Fig. S4D). I.v. administration of anti-mLCN2 antibody to mice with established mammary tumors reduces lung metastases. To explore the possibility that inhibition of the secreted LCN2 might block distant metastasis, we i.v. injected an affinity-purified rabbit polyclonal antibody against mLCN2 (anti-mLCN2) into nude mice 7 days after implantation with 5,000 green fluorescent protein-labeled 4T1 cells, when visible breast tumors (~2 mm) were formed. Antibody injection (~100 μg) was done once per week for four times, with purified rabbit IgG as control. We observed a dramatic decrease in lung metastases in the anti-mLCN2 antibody-treated group compared with the control IgG-treated group as measured by green fluorescent protein signal intensities in freshly collected lung tissues (P = 0.014; Fig. 4B and C; Supplementary Fig. S5A). Histologic examination confirmed significant differences in lung metastases between the two groups (Fig. 4D). We also found that the levels of circulating 4T1 tumor cells were reduced in the blood of anti-mLCN2 antibody-treated mice (Supplementary Fig. S5B), suggesting that the antibody was able to block the emergence of disseminating tumor cells from the primary tumor sites. Taken together, our results strongly suggest that LCN2 could serve as a new therapeutic target for treating breast cancer metastasis.

**Discussion**

The results presented here provide strong preclinical and experimental evidence that LCN2 is a critical factor that facilitates breast tumorigenesis and metastasis, and it is a potential therapeutic target for aggressive forms of breast cancer. We were the first to use a spontaneous mouse breast cancer model to study the function of LCN2 in breast tumorigenesis. Mice that lack mLCN2
expression had a significant delay in the formation of breast tumors in the MMTV-ErbB2[V664E] background, and when formed, these tumors displayed reduced tumor numbers and size compared with that from mLCN2<sup>−/−</sup> and mLCN2<sup>+/−</sup> mice. The timing of lung metastases was correspondingly delayed in the mLCN2<sup>−/−</sup> mice compared with the mLCN2<sup>+/−</sup> mice. This result indicates that although breast tumor formation and metastasis may not absolutely require LCN2 expression, LCN2 plays an essential role in enhancing the aggressiveness of breast cancer formation and metastasis. Our studies using human breast cancer cell lines and murine mammary tumor cells indicate that LCN2 expression led to more aggressive behavior including lung metastasis, tumor cell migration and invasion, and anchorage-independent growth in vitro.

How LCN2 is involved in solid tumor formation and progression is not fully understood. We observed an overall increased MMP-9 gelatinase activity, formation of higher molecular weight MMP complexes (20), and increased MMP-9 stability in tumor-bearing mice expressing LCN2. In contrast, in tumor-bearing mice with ablated LCN2 expression, an overall decrease in higher molecular weight MMP activities and MMP-9 activity was observed, consistent with the previous findings of the protective role of LCN2 on MMP-9 (16). The importance of these higher MMP activities in tumorigenesis is not known. The effects of loss of LCN2 expression on these MMP-related events are not yet understood and need further investigation.

Most importantly, we provided evidence that i.v. injection of an affinity-purified antibody made against mLNC2 strongly interfered with lung metastasis in an aggressive mouse 4T1-induced mammary tumor model. The primary breast tumors were not prevented from continued growth by i.v. injection of the mLCN2 antibody, which may be due to the insufficient levels of the mLCN2 antibody in reaching an already established tumor microenvironment. However, we did observe that anti-mLNC2 antibody-treated mice had slightly smaller primary tumors (10% reduction on average; data not shown). We found less 4T1 cells in the blood of anti-mLNC2 antibody-treated mice. Possible mechanisms include that the anti-mLNC2 destabilizes LCN2/MMP-9 complex, thereby reducing the exit of 4T1 cells from the primary tumor mass and decreasing distant tumor metastasis. More studies are needed to clarify the mechanism of how anti-LCN2 reduces metastases. Our findings suggest that blocking LCN2 in blood circulation using a neutralizing monoclonal antibody against LCN2 might be an effective therapeutic option to interfere with breast cancer metastasis either alone or in combination with current therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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