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CHAPTER 3

Autotaxin expression profiling in human breast cancer
Autotaxin expression profiling in human breast cancer

Anna J.S. Houben¹, Hugo M. Horlings²³, Jelle Wesseling², and Wouter H. Moolenaar¹

¹Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
²Division of Medical Oncology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
³Present address: Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

ABSTRACT

Autotaxin (ATX) is a secreted phosphodiesterase that produces the lipid mediator LPA, a mitogen and motility factor for many cell types. ATX is essential for embryonic development, while excessive ATX-LPA signaling promotes tumor formation, angiogenesis and experimental metastasis. In particular, overexpression of ATX in mouse mammary glands promotes late-onset, invasive mammary cancer. However, little is known about ATX expression in human breast cancer. Here, ATX immunohistochemistry was performed in 193 breast cancer samples, and associations between ATX expression, clinico-pathological characteristics and survival were evaluated. Virtually all carcinoma samples were ATX-positive, regardless of tumor size, grade or lymph node status, while the surrounding stroma showed little or no ATX expression. Despite the high amount of ATX-immunoreactivity, there was no significant correlation between ATX protein and mRNA expression and clinical parameters. We conclude that, although breast cancer cells express ATX and are known to be LPA-responsive, the ATX expression profile by itself is not significantly correlated with clinical outcome.
INTRODUCTION

Lysophosphatidic acid (LPA) signaling is implicated in both physiological as well as pathological processes by inducing proliferation, migration and many other cellular functions (reviewed in (1)). LPA acts on specific G protein-coupled receptors, which are expressed in virtually every cell type (2). Autotaxin (ATX) is a secreted phosphodiesterase that hydrolyzes lysophosphatidylcholine into the lipid mediator lysophosphatidic acid (LPA) (3). ATX was originally identified as an autocrine motility factor for human melanoma cells (4) and later found to be identical to plasma lysophospholipase D, the main enzyme accounting for LPA production in the circulation (5;6).

ATX is essential for embryonic development (7;8) and, when overexpressed in mice, it is an effector of tumor formation, angiogenesis and metastasis (reviewed in (9)). Of particular interest, transgenic expression of ATX or individual LPA receptors (LPA1-3) in mouse mammary glands induces late-onset, invasive mammary cancer (10). Furthermore, injection of ATX-expressing mammary carcinoma cells into nude mice enhances the formation of osteolytic bone metastasis (11).

In human malignancies, high ATX expression has been implicated in development and progression of prostate cancer (12), early-stage colorectal cancer (13) and Hodgkin lymphoma.
Thus far, ATX expression patterns in breast cancer patients have not been adequately analyzed. In the present study, we examined ATX expression by immunohistochemical (IHC) analysis on tissue microarrays derived of 193 mammary carcinomas, and explored possible correlations with clinico-pathological and prognostic characteristics.

RESULTS

We analyzed ATX protein expression in a series of primary tumors by IHC on tissue microarray sections using anti-ATX monoclonal antibody 4F1 (16). Specificity and reactivity of the 4F1 antibody in tissue sections has been assessed previously (12;17). ATX IHC staining was observed specifically in carcinoma cells, with little or no ATX expression detectable in the surrounding stroma (Fig. 1A). Positive staining was found in the majority of tumors, with moderate to strong staining (scores 2 and 3) in 86% of all cases, while only very few tumors were ATX negative (Fig. 1B and Fig. 1C). Thus, ATX expression is a common feature of primary breast cancer, suggesting that ATX-LPA receptor signaling might play a role in mammary tumorigenesis and progression.

Using the known gene expression data of this patient cohort (15), we analyzed the correlation between ATX mRNA and protein expression. Unexpectedly, the IHC scores did not correlate with the mRNA expression pattern (p=0.28). Whereas the ATX IHC profile showed clear differences among patients, the mRNA expression pattern did not show much variability (Fig. 2). It should be noted that ATX mRNA expression levels represent the sum of intracellular and secreted ATX, while IHC analysis only detects the intracellular pool representing newly synthesized ATX. It is currently not clear how intracellular ATX levels relate to the secreted, LPA-generating, ATX pool. It seems likely that intracellular and extracellular levels are closely correlated. However, future analysis is needed to test this hypothesis.

![Figure 2. Correlation between ATX mRNA and protein expression. Boxplot showing the ATX mRNA expression per group of ATX IHC score (n=193). There is no clear correlation between ATX mRNA and protein expression (p=0.28, Kruskall Wallis test, n=193).](image-url)
Next, we examined the association of ATX immunoreactivity with patient and tumor characteristics, including age, tumor size, differentiation grade as well as lymph node, Her2 and progesterone receptor status. Breast cancer is a heterogeneous disease with different histological and molecular subtypes (18). Due to the known distinct behavior of ER positive breast cancer versus ER negative breast cancer, we analyzed these subgroups separately. As summarized in Table 1, no association between ATX expression and clinico-pathological parameters could be demonstrated. In addition, Kaplan-Meier analyses showed no significant correlation between ATX IHC score and patient survival (Fig. 3A). The ER-negative patient group showed a trend towards better survival, although this difference was not statistically

Table 1. Association of ATX protein expression with clinico-pathological variables in primary breast tumors. The number of patients is indicated, with the column percentage between brackets. The p-values were calculated with the use of Chi square test, unless indicated otherwise. ATX low is defined as ATX IHC score 0-2; ATX high is defined as ATX IHC score 3; Her2: Her2 receptor (expression determined by IHC); PR: progesterone receptor (expression determined by microarray analysis); chemo- and hormonal therapy: adjuvant therapy. $^5$ Fisher exact test; $^6$ Chi square test for trend; n=193.
significant (univariate Cox regression for ER-positive group: hazard ratio (HR) 0.79, p=0.39, and for ER-negative group: HR 1.6, p=0.25; multivariate Cox regression for ER-positive group: HR 0.78, p=0.38, and for ER-negative group: HR 1.6, p=0.29). Similar as for the IHC scores, ATX mRNA expression profiles also failed to show a correlation with patient outcome (Fig. 3B). In line with our results, a recent study reported absence of a correlation between ATX mRNA levels and classical prognostic parameters in breast cancer patients (11). Thus, we conclude that ATX expression profiles are not significantly correlated with clinical outcome in human primary breast cancer.
FUTURE PROSPECTS

Mammary carcinoma cells are highly responsive to LPA, particularly with respect to cell migration (19), and a causal link between ATX-LPA receptor signaling and mammary cancer has been reported in a transgenic mouse model (10). This suggests that excessive ATX-LPA signaling is sufficient for tumor initiation and progression in mice. We thus hypothesized that ATX would play a role in human breast cancer, and might serve as a prognostic factor. However, the results of our tissue microarray analysis show that ATX expression in breast cancer does not correlate with clinical and prognostic characteristics. Of note, high ATX expression does not necessarily imply enhanced LPA signaling, as the interplay between substrate availability, product degradation and the LPA receptor expression profile together will determine the outcome of ATX action. A previous study showed that LPA$_2$ expression is linked to invasive ductal breast carcinoma (20). It will be interesting to examine how LPA receptor expression profiles, either by itself or in combination with ATX expression, correlate with clinical outcome in human breast cancer.

EXPERIMENTAL PROCEDURES

Patients
This study was carried out using tumor samples from a breast cancer patient cohort of the fresh-frozen tissue bank of the Netherlands Cancer Institute (15). All patients were younger than 53 years and were diagnosed with stage I or II breast cancer, lymph node negative or positive, containing both estrogen receptor (ER) negative and ER positive tumors. The median duration of follow-up (defined as time to any metastatic event) among all patients was 9.9 years, with 12.1 years for the patients without a metastasis event and 2.9 years for the patients with a metastasis event. All patients had been treated by breast conserving or ablative therapy followed by radiotherapy and/or adjuvant chemotherapy and/or hormonal therapy. The study was approved by the medical ethics committee of the Netherlands Cancer Institute.

Immunohistochemistry
Blocks of formalin-fixed, paraffin-embedded primary breast cancer tissue were prepared using a tissue arrayer. For immunohistochemistry, sections were heated at 75°C for 30 min., deparaffinized in xylene and rehydrated via a series of graded alcohols. After washing with TBS (J.T. Baker BV) for 5 min., sections were boiled for 15 min. in citrate buffer pH 6 (trinatrium-citrate-dihydrate 8.2 mM, citric acid 0.18 mM) for antigen retrieval and cooled down for 1 hour. Sections were then incubated with peroxidase blocking solution (Immunovision Technologies) for 10 min., washed with TBS for 5 min. and incubated overnight with rat monoclonal antibody 4F1 raised against a human ATX polypeptide (amino acid 58-182) (16), 1:50 dilution at 4°C. Next, sections were washed for 5 min. with TBS, incubated with post-antibody blocking/polymer penetration enhancer solution (Immunovision Technologies) for 15 min., washed, incubated with polymerized HRP-anti-mouse/rabbit IgG (Immunovision Technologies), washed, and incubated with DAB and hematoxyline. ATX staining in the tumor sections was scored by a consultant breast pathologist (JW) as 0, 1, 2, 3 corresponding respectively to negative, faint, moderate, strong staining intensity. The highest score out of three cores from the same tumor was used for analysis. If one or two cores out of three failed, the highest value of the remaining core(s) was included. Only the tumor component
was considered when judging staining and sections containing stromal tissue without carcinoma cells were discarded from the analysis. This resulted in a total number of 193 patient samples that were scored for ATX expression.

**Statistical analysis**

Time to metastasis for two ATX groups (IHC score 0-2 versus 3) was estimated according to the Kaplan-Meier method. In addition, we tested for trend, using the significance of the coefficient for the continuous ATX IHC score 0-3. The association between ATX IHC score (4 groups: score 0, 1, 2 and 3) and ATX mRNA expression was tested using the Kruskall Wallis test. Associations between ATX IHC score and clinico-pathological variables were evaluated by a Chi square test, unless otherwise indicated.

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