Overexpression of C/EBPβ-LIP, a Naturally Occurring, Dominant-Negative Transcription Factor, in Human Breast Cancer

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Background: When cells fail to maintain a balance between proliferation, terminal differentiation, and programmed cell death, cancer often results. The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors regulates many genes involved in the processes of proliferation and terminal differentiation. The messenger RNA for C/EBPβ is translated into two major isoforms, LAP (liver-enriched activating protein) and LIP (liver-enriched inhibitory protein). LIP levels appear to be elevated in mouse mammary tumors but not in hyperplastic mammary tissues. We tested whether LIP expression is elevated in human breast cancer and whether elevated expression is associated with biologic predictors of the aggressiveness of the disease. Methods: Homogenates of infiltrating ductal carcinoma specimens from 39 women were analyzed for C/EBPβ protein content by western blot analysis, and the ratio of LAP to LIP in specimens containing high levels of LIP (i.e., levels approximately 15 times higher than those in tumor specimens that express little or no LIP) was also determined. Nonparametric statistical analyses were performed. Results: LIP was present at high levels in nine of 39 specimens of infiltrating ductal carcinoma. Eight of the nine specimens of infiltrating ductal carcinoma that contained high levels of LIP were negative for estrogen receptor and progesterone receptor (ER−/PR−); all nine tumors were aneuploid and poorly differentiated, and eight of nine were highly proliferative. Of the tumors that contained LIP at low or nondetectable levels, six of 30 were ER−/PR−, 17 of 29 were aneuploid, eight of 27 were highly proliferative, and 11 of 30 were poorly differentiated. Implication: LIP expression should be evaluated further as a prognostic marker for patients with breast cancer. [J Natl Cancer Inst 1997;89:1887–91]

Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation, and programmed cell death. The CCAAT/enhancer-binding protein (C/EBP) family of basic leucine zipper (bZIP), DNA-binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in proliferation and terminal differentiation (1–3). Currently, the genes for six C/EBPs (C/EBPα, β, δ, γ, ε, and ζ) have been characterized with the use of nomenclature introduced by Cao et al. (3). With the exception of C/EBPε and ζ, all of the genes in this family are intronless and share a conserved carboxyl-terminal DNA-binding domain and bZIP dimerization domain but differ in their amino-terminal trans-activation domains (4).

The gene encoding C/EBPβ is transcribed into a single messenger RNA that is translated in the mammary gland by a leaky ribosome-scanning mechanism, resulting in the synthesis of two principal isoforms designated liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd). Consequently, studies of LAP/LIP gene regulation can be conducted only at the protein level. As with all C/EBP family members, LAP and LIP are capable of forming homodimers or heterodimers with each other as well as with other leucine zipper proteins. LIP displays an increased affinity for DNA, but it lacks a portion of its trans-activating domain, rendering it able to antagonize the transcriptional activation of LAP or other C/EBPs and leucine zipper proteins, in substoichiometric ratios (5).

Recently, we have reported that C/EBPβ-LIP expression is elevated in transplantable and primary mouse mammary tumors of different etiologies but is not expressed in transplantable, preneoplastic mammary hyperplasias (6). These data suggested that LIP expression may play a role in rodent mammary tumorigenesis, but its role in human breast cancer was undetermined. We hypothesized that an increase in the levels or activity of LIP may inhibit terminal differentiation and help facilitate uncontrolled proliferation and tumorigenesis that may result from other genetic alterations known to occur in breast cancer, e.g., amplification of growth factor receptors such as erbB2, increased expression of cyclin D1, and loss or mutation of p53. LIP is of particular interest because it represents a translationally regulated, naturally occurring, dominant-negative C/EBP family member that may play a role in breast cancer. Consequently, the purpose of this study was to determine if LIP expression is elevated in human breast cancer and whether this overexpression is associated with biologic predictors of aggressive behavior in human breast cancer, such as histologic and nuclear grade, cell proliferation, DNA ploidy, and estrogen receptor (ER) and progesterone receptor (PR) status.

Materials and Methods

Western Blot Analysis

Infiltrating ductal carcinoma specimens from 37 patients and normal breast tissue from 10 patients were provided by the Baylor College of Medicine Tissue Bank, Department of Pathology. Specimens of infiltrating ductal carcinoma from two patients were provided by the Providence Health Center, Waco, TX. Seven of the 10 normal tissues were paired controls from patients with infiltrating ductal carcinomas examined in this study, and the remaining three were age-matched controls. Normal tissue is defined as tissue resected greater than 2 cm from the margins of the tumor, containing minimal adipose tissue and a higher proportion of breast parenchyma. The use of discarded human tissue was approved by the Baylor Institutional Review Board. Tissues were disrupted in RIPA buffer—50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid], and 0.2% sodium dodecyl sulfate (SDS)—containing the following kinase, phosphatase, and protease inhibitors: 1 mM NaVO₃, 1 mM NaF, 1 mM Na₂MoO₄, 10 mM okadac acid, and 1 μg/mL benzamidine, aprotinin, soybean trypsin...
hbitor, and antipain. Aliquots of these lysates containing 100 μg of protein were subjected to electro-
phoresis on denaturing SDS–12% polyacrylamide mini gels and then transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) overnight at 75 mA. Blots were blocked 90 minutes in TBST (20 mM Tris [pH 7.5], 150 mM NaCl, and 0.5% Tween 20) containing 3% non-fat dry milk (Carnation, Glendale, CA). They were then incubated for 90 minutes in this solution containing ant-
ibodies (0.5 ng/mL) (Santa Cruz Biochemicals, Inc., Santa Cruz, CA) prepared against C/EBPβ. Blot washes consisted of TBST (without milk); the washing was done three times for 5–10 minutes each, with agitation. Blots were then incubated for 60 minutes in blocking solution containing 200 ng/mL biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, U.K.) and then washed. Lastly, blots were incubated for 30 minutes in block-
ing solution containing 40 ng/mL streptavidin–horseradish peroxidase (Oncogene Science, Union-
dale, NY) and washed as before. Enhanced chemiluminescence (Hyperfilm; Amersham) and chemifluorescence reagents (Storm Fluoroom-
ger; Molecular Dynamics, Sunnyvale, CA) were used for visualization, as per the manufacturer’s in-
structions.

To determine the sensitivity of western blot analy-
sis for detection of C/EBPβ expression, we used both the Amersham enhanced-chemiluminescence (ECL) and the enhanced-chemifluorescence (ECF) detection systems to analyze increasing amounts of a C/EBPβ protein standard isolated from previously characterized mouse mammary tumors generated by overexpression of transforming growth factor-α (TGF-α) (6). The ECL data were analyzed by use of multiple exposures of Hyperfilm and quantitated with Adobe Photoshop (Adobe Systems, Mountain View, CA), and the ECF data were analyzed by use of a STORM imaging system (Molecular Dynamics) and Image Quant software (Molecular Dynamics). Levels of LAP and LIP were linear over a larger range with the use of ECF detection (12.5–400 μg/lane) as compared with ECL detection (6.25–100 μg/lane) (data not shown). Consequently, for pur-
poses of quantitation, each western blot was ana-
alyzed with the use of the ECF detection system, and levels of LAP and LIP were analyzed as "fold" and/or percent change from the C/EBPβ protein standard (100 μg). Because a source of purified C/EBPβ protein was not available, an extract of TGF-
α-induced mammary tumors (in RIPA buffer) that contained large amounts of the C/EBPβ isoforms was used as the standard. The values were plotted on a scatter diagram, and the data for LIP expression were separated into two distinguishable groups (data not shown). Standard values were assigned the value of 1 or 100%. High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, lower LIP (low or nondetectable) values ranged from 0.3-fold below standard values to 0. Notice none of the data points were positioned between the values of 0.53 (53%) and 0.25 (25%). Consequently, values that were greater than 50% below the value for the C/EBPβ protein standard were classified as high, and those that were less than 25% below the standard were classified as low or nondetectable. As a visual check for uniform gel loading, all blots were stained with the Reversible Protein Detection Kit (Sigma Chemical Co., St. Louis, MO).

Immunohistochemistry

Tissues were fixed overnight in 10% neutral buff-
ered formalin, embedded, sectioned, deparaffinized through a graded series of xylenes and alcohols, and then rehydrated in water and phosphate-buffered sa-
line. Antigens were retrieved by boiling the tissue sections for 10 minutes in 2 M urea, and endoper-
oxidases were blocked in a methanol solution con-
taining 3% H2O2 for 15 minutes at room tempera-
ture. After being washed, tissue sections were incubated for 1 hour at room temperature with 10% normal goat serum (Sigma Chemical Co.) and 20% avidin blocking solution (Vector Laboratories, Inc., Burlingame, CA) in 1× PBST (i.e., phosphate-
buffered saline containing 0.1% Tween 20). Excess blocking solution was drained, and polyclonal rabbit primary antibody for C/EBPβ (1:300; Santa Cruz Biochemicals, Inc.) containing 20% biotin blocking solution (Vector Laboratories, Inc.) in 10% normal goat serum was applied overnight at 4°C. Biotinyl-
ated goat anti-rabbit secondary antibody at a 1:200 dilution in 10% normal goat serum was incubated for 30 minutes at room temperature. Tissue sections were then incubated for 30 minutes at room tem-
perature with the preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vecastain Elite ABC; Vector Laboratories, Inc.). Colorimetric detection by use of the chromagen di-
amino benzidine tetrahydrochloride was carried out according to the manufacturer’s instructions (Vector Laboratories, Inc.). Control slides were treated identically except that 6 μg of C/EBPβ peptide (Santa Cruz Biochemicals, Inc.) was incubated with the pri-
mary antibody for 15–30 minutes at 0°C.

Analysis of Prognostic Indicators

Analyses of ER and PR status were performed at OncQuest, Specialty Laboratories, Inc., Santa Monica, CA, by use of a dextran–charcoal-binding assay. For consistency in histologic grading, all slides were examined by one pathologist (R. Laucirica), and the grade for each infiltrating ductal carcinoma was assessed by use of the method of Elston and Ellis (7). DNA flow cytometry analysis of tumors procured at the time of surgery and MIB-1 (Immunotech, Inc., Westbrook, ME) immunohisto-
chemistry, which detects the Ki67 nuclear antigen associated with cellular proliferation (8), were con-
ducted in the Department of Pathology, The Meth-
odist Hospital. The DNA content (ploidy) for 37 of 39 cases was assessed by use of a FACScan flow cytomter (Becton Dickinson Immunocytometry Systems, San Jose, CA) and Modfit software (Verity Software House, Topsham, ME). Nonparametric statistical analysis was performed in consultation with a biostatistician (Dr. Charles Miller, Depart-
ment of Surgery, Baylor College of Medicine). P values were computed by Fisher’s exact test (two-
sided) and the Mann-Whitney t test (two-sided).

Results

LIP is an amino-truncated version of the C/EBPβ LAP isoform. Therefore, C/EBPβ antibodies, which recognize a car-
boxy-terminal epitope in C/EBPβ pro-
tins, detect both LAP and LIP isoforms. Accordingly, the antibodies that recog-
nize the amino-terminal region do not de-
dect LIP. Consequently, no antibodies that can selectively distinguish LIP are cur-
rently available. Size fractionation of the C/EBPβ protein isoforms by SDS–polyacrylamide gel electrophoresis fol-
lowed by western blotting with the use of an antibody to the carboxy-terminus of C/EBPβ, however, permitted the detect-
e of the different C/EBPβ isoforms. At pres-
ent, this is the only technique avail-
ble for the analysis of LIP expression.

To address the question, "Is LIP ex-
pressed in human breast cancer?," we
analyzed infiltrating ductal carcinomas from 39 women, aged 26–83 years old. As evidenced by western blot analyses, levels of LAP and LIP expression with the infiltrating ductal carcinomas were very quite variable among the different pa-
ents (Fig. 1, A, B, and C). Additional protein bands, representing both cross-
active proteins and phosphorylated or other post-translationally processed LAP and LIP isoforms, were also visible in many tumors.

For the determination of the signifi-
ce of elevated levels of LIP expression in some tumors, an expression level was assigned (as described in the “Materials and Methods” section) to the LIP values and was tested for association with various prognostic factors. LIP was expressed at high levels in 23% (nine of 39) of the infiltrating ductal carcinomas. Examples of high levels of LIP expression are shown in Fig. 1, B (lanes 2, 3, and 6). LIP levels designated as “high” (mean value ± 95% confidence interval: 1.59 ± 0.6) were on average 15 times greater than the levels of LIP in the lower expressing (mean value ± 95% confidence interval: 0.107 ± 0.02) or nonexpressing tumors (P < .0001, two-sided Mann-Whitney t test). In "normal" breast tissue, LIP lev-
els were low or nondetectable above background values.

Because the amino-truncated LIP iso-
form has a greater DNA affinity than the LAP isoform and can heterodimerize and inhibit the transactivation ability of other C/EBPs at a substoichiometric ratio as low as 1:5 (20%), the ratio of LIP to LAP was determined for the predominant LAP isoform (35 kd) that also has the greatest transactivation potential (5). In the tumors expressing the highest LIP levels, the av-
average ratio of LIP to LAP was determined

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with DNA ploidy. Nine (100%) of the carcinomas, we compared levels of LIP expression in the infiltrating ductal carcinomas. With the exception of lanes 1 and 2 in panel A, each lane represents the infiltrating ductal carcinoma from a different patient. Lanes 1 and 2 in panel A consist of an infiltrating ductal carcinoma (IDC) and its paired normal (N) tissue. One hundred micrograms of protein was analyzed per lane. The estrogen receptor (ER) and progesterone receptor (PR) status from each infiltrating ductal carcinoma is listed above the corresponding lane. The exposure time for panel B is not the same as that for panel A and panel C, and these western blot analyses were not all performed simultaneously. The variations in LIP levels on these ECL blots have been confirmed by use of the quantitative enhanced-chemifluorescence detection methodology described in the “Materials and Methods” section.

![Western blot analysis and enhanced-chemiluminescence (ECL) detection of CCAAT/enhancer-binding protein β (C/EBPβ) isoforms (liver-enriched inhibitory protein [LIP—20 kd] and liver-enriched inhibitory protein [LIP—20 kd]) in human infiltrating ductal carcinomas.](image)

To determine whether an association existed between genetic instability and LIP expression in the infiltrating ductal carcinomas, we compared levels of LIP expression in 38 of 39 tumor specimens with DNA ploidy. Nine (100%) of the nine tumors that expressed LIP at high levels were aneuploid. The tumors that expressed LIP at either low or nondetectable levels were more evenly distributed: 17 (59%) of 29 were aneuploid, whereas 12 (41%) of 29 were diploid (P = .0356, two-sided Fisher’s exact test).

Because loss of ER expression is often associated with a poor clinical outcome (9), we next determined whether steroid receptor status in these tumors was associated with LIP expression. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) were negative for estrogen and progesterone receptor (ER−/PR−), 19 (49%) were positive for estrogen and progesterone receptor (ER+ /PR+), and six (15%) were either estrogen receptor negative and progesterone receptor positive (ER−/PR+) or estrogen receptor positive and progesterone receptor negative (ER+/PR−). High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, low and nondetectable LIP values ranged from 0.3-fold below standard values to 0. Frequency analysis was performed by use of two-sided Fisher’s exact test (P = .0015).

Five tumors (from patients 2, 4, 5, 9, and 10) were classified as polymorphic nuclei (grade III—36.7% [11 of 30]; grade II—30% [nine of 30]; and grade I—33.3% [10 of 30]) were evenly distributed between the tumors expressing LIP at low or nondetectable levels (P = .013, two-sided Fisher’s exact test). The proliferative fraction (determined by either MIB-1 immunochemistry and/or by DNA flow cytometry) also displayed an association with LIP expression. In the infiltrating ductal carcinomas that contained high LIP levels, eight (89%) of nine specimens contained a high fraction of proliferative cells, but only eight (30%) of 27 of the low or nondetectable LIP-expressing tumors contained a high fraction of proliferative cells (P = .0046, two-sided Fisher’s exact test).

Although it was not possible to localize LIP expression to specific cell types, immunocytochemical staining was performed on a limited number of the infiltrating ductal carcinomas and their surrounding tissue to determine which cells expressed the C/EBPβ isoforms. These results were consistent with those obtained from the previous western blot analyses and revealed strong C/EBPβ expression in pleomorphic nuclei of grade III, ER+/PR+ tumors (Fig. 2, A) and weaker C/EBPβ expression in the nuclei of grade I, ER+/PR+ tumors (Fig. 2, A).
Breast cancer is thought to progress through multiple morphologic stages, beginning with typical hyperplasia and progressing through atypical hyperplasia, in situ carcinoma, invasive carcinoma, and eventual metastasis. Invasive carcinomas are also heterogeneous with respect to their proliferative and invasive potential. In this study, C/EBPβ protein isoforms and, in particular, the naturally occurring, dominant-negative LIP isoform have been detected and are more highly expressed in ER+/PR+, aneuploid, Elston–Ellis grade III infiltrating ductal carcinomas than in the less aggressive, ER+/PR+, diploid, Elston–Ellis grade I tumors. This observation is consistent with the hypothesis that the C/EBP isoforms may play a role in regulating terminal differentiation and cell cycle progression, as illustrated in Fig. 3.

Proliferative diseases such as cancer often result from failure to withdraw from the cell cycle at the G1 checkpoint. Multiplesignal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late G1 is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). It has been demonstrated that Rb interacts directly with and activates all of the C/EBP isoforms (10); however, how this interaction

Fig. 3. Hypothetical model for the role of CCAAT/enhancer-binding proteins (C/EBPs) in cell cycle progression. The interaction of retinoblastoma protein (Rb) with the C/EBPβ–liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd) isoforms may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland (see text for details). Studies in adipocytes have demonstrated that C/EBPβ and C/EBPδ are involved in early proliferative and differentiative processes, leading to the activation of C/EBPα, which then contributes to terminal differentiation by arresting adipocyte proliferation (16,17). In HepG2 hepatoma cells, C/EBPβ–LAP has been reported to inhibit cell cycle progression before the G1/S boundary, and this effect can be antagonized by expression of the dominant-negative LIP isoform, thereby promoting cell proliferation (11). In the rat mammary gland, C/EBPα expression is highest at lactation, when mammary epithelial cells undergo terminal differentiation, and C/EBPβ expression with an elevated ratio of LIP to LAP is highest during pregnancy, a period of lobuloalveolar proliferation (12). The ratio of LIP to LAP decreases almost 100-fold at the onset of lactation.
affects Rb activity is not known. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. We, therefore, propose that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression (Fig. 3). Support for this hypothesis comes from the observations that the ratio of LIP to LAP is regulated during proliferative phases of both liver and mammary gland development (5,11,12).

One caveat that must be considered in the interpretation of the foregoing results is the cellular heterogeneity that exists between breast tumors of the same type. For example, when infiltrating ductal carcinomas are compared, ER and PR levels may vary considerably in cells within a given tumor (13,14). The degree of desmplasia, lymphovascular invasion, and necrosis can also vary from patient to patient. Homogenization of tumor specimens for western blot or steroid receptor analyses can result in a misleading “dilutional” effect and does not permit direct cellular comparisons. This heterogeneity may be partly responsible for the variations observed in the LAP and LIP levels between infiltrating ductal carcinomas and tempers the associations of LIP expression levels with steroid receptor status and DNA ploidy. Furthermore, these studies do not permit an evaluation of ratios of LIP to LAP within individual cells of differing proliferative potentials within these tumors.

Mammary tumorigenesis is thought to result from multiple molecular changes, such as the activation of oncoprobes by mutation, the overexpression of growth factors and their receptors, and/or the inactivation of tumor suppressor genes. It has been proposed that multiple molecular changes lead to a malignant phenotype, whereas, fewer “hits” are necessary for a benign tumor (15). Our study suggests that overexpression of the naturally occurring, dominant-negative transcription factor C/EBPβ-LIP may be one of the molecular events that predisposes breast cells to a selective growth advantage, resulting in progression of infiltrating ductal carcinomas; however, additional tumors need to be examined to confirm our hypothesis.

These studies do not answer the question of whether overexpression of LIP, in the most advanced, aggressive tumors, facilitates tumor progression or if overexpression is simply a result of increased proliferation. These type of functional studies cannot be performed with patient biopsy specimens but require the use of animal or cell culture models. In this regard, we have recently generated transgenic mice that selectively express high levels of LIP in cells of the mammary gland (Zahnaw CA, Rosen JM: unpublished observations). Studies are also in progress to determine the effects of regulatable LIP expression on cell cycle progression in mammary epithelial cells. These types of experiments will be required to determine if LIP expression is a cause or an effect of mammary tumorigenesis. Notwithstanding this determination, LIP expression may provide a useful additional marker for the identification of breast tumors in patients with a poor clinical outcome.

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Notes

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