LEP as a potential biomarker in prognosis of breast cancer

Systemic review and meta analyses (PRISMA)

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Abstract

Purpose Obesity strongly affects the prognosis of various malignancies, including breast cancer. Leptin (LEP) may be associated with obesity and breast cancer prognosis. The purpose of our study was to determine the prognostic value of LEP in breast cancer.

Method We conducted a multi-omic analysis to determine the prognostic role of LEP. Different public bioinformatics platforms (Oncomine, Gene Expression Profiling Interactive Analysis, University of California Santa Cruz Xena, bc-GenExMiner, PrognoScan database, R2-Kaplan-Meier Scanner, UALCAN, Search Tool for the Retrieval of Interacting Genes/Proteins database, and The Database for Annotation, Visualization and Integrated Discovery) were used to evaluate the roles of LEP. Clinicopathological variables were evaluated.

Results LEP was downregulated in breast cancer tissues compared to levels in normal tissues. By co-expressed gene analysis, a positive correlation between LEP and SLC19A3 was observed. Based on the clinicopathological analysis, low LEP expression was associated with older age, higher stage, lymph node status, human epidermal growth factor receptor 2 (HER2) status, estrogen receptor (ER+) positivity, and progesterone receptor (PR+) positivity. Kaplan–Meier survival analysis showed that low LEP expression indicated a poorer prognosis. LEP is hypermethylated in breast cancer tissues in PrognoScan and R2-Kaplan Meier Scanner, and low LEP expression was correlated with poor prognosis. LEP protein–protein interactions were analyzed using Search Tool for the Retrieval of Interacting Genes/Proteins database. Gene ontology analysis results showed that cellular component is mainly associated with the endosome lumen, cytosol, and secretory granules and is upregulated. For the biological process energy reserve, metabolic processes exhibited the greatest regulation compared to the others. In molecular function, it was mainly enriched in a variety of combinations, but hormone activity showed the highest regulation.

Conclusion Our study provides evidence for the prognostic role of LEP in breast cancer and as a novel potential therapeutic target in such malignancies. Nevertheless, further validation is required.

Abbreviations: BP = biological process, CC = cellular component, DAVID = The Database for Annotation, Visualization and Integrated Discovery, ER = estrogen receptor, GEPIA = Gene Expression Profiling Interactive Analysis, GO = gene ontology, GTEx = genotype-tissue expression, HER-2 = human epidermal growth factor receptor 2, LEP = leptin, MF = molecular function, PR = progesterone receptor, STRINGdb = Search Tool for the Retrieval of Interacting Genes/Proteins database, TCGA = The Cancer Genome Atlas, UCSC XENA = University of California Santa Cruz Xena.

Keywords: bioinformatics, breast cancer, leptin, obesity
1. Introduction
Breast cancer is one of the most common malignancies and the main cause of cancer-related deaths in women. Breast cancer is believed to involve genetic predisposition, but modifiable variables, such as overweight (body mass index 25–30 kg/m²) or obesity (body mass index >30 kg/m²), also play significant roles. Obesity is a growing public health problem that affects almost all countries. Obesity is regarded as a major risk factor for many severe illnesses, including diabetes mellitus, metabolic syndrome, cardiovascular diseases, and certain cancers. During the past decade, an explosion of evidence has linked obesity with an increase in the incidence of cancer and associated mortality. Obesity is also a major risk factor for breast cancer in postmenopausal women. Adipokines, which are bioactive molecules generated and secreted by adipocytes, may also contribute to postmenopausal breast cancer. Adipocytes produce nearly 50 different adipokines, which have various functions, such as regulation of inflammation and acute phase responses, insulin sensitivity, lipid metabolism, appetite, and energy balance. Because of its association with an increased risk of postmenopausal breast cancer, leptin (LEP) is the most studied adipokine and its role in promoting the growth and development of breast cancer in vitro has been recognized.

LEP is directly associated with obesity. The circulatory rate of obese individuals is higher than that of lean individuals. LEP can directly and independently promote the occurrence of breast cancer and indirectly promote the development of breast cancer through signaling pathways involving estrogen and insulin, which provides the basis for hormone-mediated carcinogenic interactions. Many studies have shown that LEP functions far beyond metabolism alone, and is the main mediator of obesity-related cancers, such as colorectal, prostate, and breast cancers.

Herein, using public bioinformatics platforms, we evaluated the expression pattern of LEP in human breast cancer and the prognostic significance of LEP expression in breast cancer. Finally, we analyzed possible correlations involving genes co-expressed with LEP. These results would facilitate a better understanding of the prognostic value of LEP in human breast cancer.

2. Material and methods
2.1. Oncomine database analysis
The Oncomine database (https://www.oncomine.org/resource/login.html) is a web-based data-mining platform that incorporates 264 independent datasets and aims to collect, standardize, analyze, and deliver transcriptomic cancer data for biomedical research. The database was used to determine LEP expression levels in breast cancer. The fold change in LEP expression in clinical cancer specimens compared to normal controls was obtained using the parameters of P value 1e-4, fold-change 2, and gene ranking in the top 10%. The co-expression profile of the LEP gene in breast cancer is here displayed as a heat map.

2.2. GEPIA analysis
Gene expression profiling interactive analysis (GEPIA; http://geopia.cancer-pku.cn/) is a web server for analyzing the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the The Cancer Genome Atlas (TCGA) and GTEx projects, using a standard processing pipeline. The GEPIA box-plot tool was used for tumor/normal differential expression analysis of LEP in different cancers.

2.3. UCSC cancer genomics browser analysis
The heat map and correlation between LEP and SLC19A3 in the same patient cohort were verified and analyzed by data mining in TCGA-BRCA using the University of California Santa Cruz Xena (UCSC Xena) browser (http://xena.ucsc.edu/). Subsequently, the methylation status of LEP was examined using the UCSC Xena browser.

2.4. Breast cancer gene expression miner v.4.2 analysis
Breast Cancer Gene-Expression Miner v.4.2 (bc-GenExMiner v.4.2) (http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php?js=1) was used to analyze the relationship between LEP expression and clinicopathological parameters in breast cancer patients.

2.5. PrognoScan analysis
PrognoScan (http://dna00.bio.kyutech.ac.jp/PrognoScan/) is an online platform used for meta-analysis of the prognostic value of genes. PrognoScan was used to assess the correlation between LEP expression and survival in breast cancer.

2.6. R2 platform analysis
R2 (http://hservr1.amc.nl/cgi-bin/r2/main.cgi) is a genomics analysis and visualization platform with a database, coupled with a web interface that provides a set of analysis tools. R2 supports all types of survival data and can also be used to generate Kaplan–Meier plots for specific datasets. We analyzed correlations between LEP expression and survival in patients with breast cancer.

2.7. UALCAN analysis
UALCAN (http://ualcan.path.uab.edu/) is a website for online analysis and mining of cancer-related data. It is based mainly on the analysis of relevant cancer data from TCGA database. It can help perform biomarker identification, expression profiling, and survival analysis of related genes. It is possible to query related information from other databases through related links.

2.8. cBioPortal analysis
The cBioPortal for cancer genomics (http://www.cbioportal.org) is a widely used online portal that provides visualization and analysis of large-scale cancer genomics databases. In our study, cBioPortal was used to analyze the clinicopathological parameters of LEP in patients with breast cancer.

2.9. Identification of protein components of the LEP axis
We used the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRINGdb) analysis tool (https://string-db.org/cgi/input.pl), a database of known and predicted protein interactions, to identify interacting proteins using LEP as the query.
2.10. GO analysis

GO analysis is a commonly used technique for large-scale functional enrichment research and can be classified into the biological process (BP), cellular component (CC), and molecular function (MF). To characterize the functional roles of the above proteins from STRING, the Database for Annotation, Visualization, and Integrated Discovery tools (https://david.ncifcrf.gov/home.jsp).

2.11. Statistical analysis

Overall survival (OS) was defined as the time from the first diagnosis of primary breast cancer to death from any cause. Survival curves were generated using PrognoScan and the R2-Kaplan–Meier plot. All results are displayed as P values using the log-rank test.

3. Results

3.1. LEP expression in human breast cancer

Oncomine analysis of cancer versus normal tissues showed that LEP was downregulated in breast cancer to a greater extent than in other cancers (Fig. 1A). LEP expression was significantly decreased in invasive breast carcinoma (~16.434), breast phyllodes tumor (~21.654), mucinous breast carcinoma (~15.065), breast carcinoma (~15.496), and medullary breast carcinoma (~15.482) (Table 1). GEPIA analysis showed that LEP expression in tumors was lower than that in normal tissues from the same breast cancer patients (Fig. 1B). Taken together, these results indicated that LEP is significantly downregulated in human breast cancer.

3.2. Co-expression analysis of LEP

Oncomine analysis identified co-expression of LEP with a large cluster of 19,273 genes across 11 breast carcinomas (Fig. 2B). We confirmed this positive correlation for SLC19A3 by analyzing breast cancer patient data in the TCGA database using UCSC Xena (Fig. 2B and C) and by data mining in bc-GenExMiner v.4.2 (Fig. 2D). These data indicated that LEP might be closely related to the SLC13A signaling pathway in breast cancer.

3.3. Clinicopathological parameters of LEP in breast cancer patients

Among breast cancer patients, LEP expression was lower in the ≤51-year-old group than in the >51-year-old group (Table 2). LEP expression was significantly lower at the T1 and T2 stages than in the T3 and T4 groups. There was no significant difference in LEP expression between patients with positive and negative nodal status. According to staging, LEP expression was significantly lower in stages I, II, and III, but not in stage IV. Estrogen receptor (ER) status was positively correlated with LEP expression. Conversely, LEP mRNA levels were lower in patients with human epidermal growth factor receptor 2 (HER2)-positive

Figure 1. Expression of LEP in different types of cancers (Oncomine and TCGA databases). (A) This graphic generated by Oncomine indicates the numbers of datasets with statistically significant (P < .01) mRNA overexpression (red) or down-expression (blue) of LEP (different types of cancer vs corresponding normal tissue). The threshold was designed with the following parameters: P value of 1e-4, fold change of 2, and gene ranking of 10%. (B) Expression of LEP in different types of cancers, as generated by GEPIA using TCGA data. GEPIA = Gene Expression Profiling Interactive Analysis, LEP = leptin, TCGA = The Cancer Genome Atlas.
tumors than in those with HER2-negative tumors. LEP expression was significantly higher in basal-like status (Table 2). We performed Welch test, using bc-GenExMiner v.4.2, to compare LEP mRNA transcription levels among groups of patients according to different clinicopathological characteristics, and the above results were confirmed (Fig. 3). A significant correlation was found between low LEP expression and other clinicopathological variables.

### 3.4. Prognostic value of LEP mRNA expression in breast cancer patients

The prognostic value of LEP expression in breast cancer was explored using the PrognoScan database and R2: Kaplan–Meier Scanner. Higher expression of LEP correlated with better OS, relapse-free survival (RFS), and disease-specific survival (all \( P < .05 \)) (Fig. 4). In our validation with the TCGA dataset, higher expression of LEP was correlated with better OS \( (P < .05) \).

#### Table 1

| Order | Type of breast cancer vs normal breast tissue | Fold change | \( P \) value | \( t \) test | Source |
|-------|---------------------------------------------|-------------|--------------|-------------|--------|
| 1     | Invasive breast carcinoma                    | −16.434     | 7.67E-44     | −20.133     | Curtis breast |
| 2     | Breast phyllodes tumor                       | −21.654     | 1.94E-44     | 23.768      | Curtis breast |
| 3     | Mucinous breast carcinoma                    | −15.065     | 6.48E-45     | 18.77       | Curtis breast |
| 4     | Breast carcinoma                             | −15.496     | 4.58E-20     | 15.711      | Curtis breast |
| 5     | Medullary breast carcinoma                   | −15.482     | 5.93E-38     | 17.9        | Curtis breast |
| 6     | Ductal breast carcinoma in situ              | −11.683     | 7.58E-09     | 10.338      | Curtis breast |
| 7     | Tubular breast carcinoma                     | −11.709     | 1.22E-40     | 16.702      | Curtis breast |
| 8     | Invasive ductal and invasive lobular breast carcinoma | −12.448     | 4.41E-44     | 17.467      | Curtis breast |
| 9     | Invasive lobular breast carcinoma            | −3.047      | 2.28E-45     | 17.109      | Curtis breast |
| 10    | Invasive ductal breast carcinoma             | −3.201      | 8.87E-51     | 21.993      | Curtis breast |
| 11    | Invasive breast carcinoma                    | −3.184      | 1.09E-18     | 12.46       | Gluck breast  |
| 12    | Mixed lobular and ductal breast carcinoma    | −2.247      | 3.05E-09     | 6.902       | TCGA breast  |
| 13    | Invasive ductal breast carcinoma             | −10.754     | 4.06E-37     | 18.577      | TCGA breast  |
| 14    | Male breast carcinoma                        | −2.024      | 6.27E-07     | 6.055       | TCGA breast  |
| 15    | Invasive breast carcinoma                    | −4.619      | 2.08E-14     | 8.481       | TCGA breast  |

TCGA = The Cancer Genome Atlas.

Figure 2. Co-expression analysis of LEP gene expression. (A) LEP co-expression of genes analyzed using Oncomine. (B) Heat map of LEP expression and SLC19A3 mRNA expression across breast cancer sample types in TCGA database, determined using UCSC Xena. (C) Correlation between LEP and SLC19A3 mRNA expression in TCGA database determined using UCSC Xena. (D) Relationship between LEP and SLC19A3 in breast cancer analyzed using bc-GenExMiner v.4.2. LEP = leptin, TCGA = The Cancer Genome Atlas, UCSC XENA = University of California Santa Cruz Xena.
3.5. Co-expressed genes, correlation, and DNA methylation status of LEP

A correlation heat map and DNA methylation status retrieved from UCSC Xena confirmed that LEP expression gradually decreased with increasing DNA methylation (Fig. 5A). We also found that the methylation level of the LEP promoter was higher in breast cancer tissue than in normal tissue (Fig. 5B).

3.6. Protein–protein interactions

Figure 6 illustrates the direct interactions and predicted functional associations involving LEP and other proteins retrieved from STRINGdb. The following proteins interacted with LEP: signal transducer and activator of transcription 3 (STAT3), peroxisome proliferator-activated receptor gamma (PPARG), glucagon (GCG), C-reactive protein (CRP), insulin (INS), insulin receptor substrate 1 (IRS1), tyrosine-protein phosphatase non-receptor type 1 (PTPN1), leptin receptor (LEPR), tyrosine-protein kinase JAK2 (JAK2), and suppressor of cytokine signaling 3 (SOCS3).

3.7. GO analysis

The above proteins were classified into the 3 functional groups of GO analysis: CC, BP, and MF (Fig. 7). Numerous BPs were identified (Fig. 7). The results of the GO analysis showed that CC

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\begin{array}{|c|c|c|c|}
\hline
\text{Variables} & \text{No.} & \text{LEP expression} & \text{P value} \\
\hline
\text{Age} & \leq 51 & 1312 & \text{Low} \\
& > 51 & 2026 & \text{High} \\
\text{T stage} & T1 & 678 & \text{Low} \\
& T2 & 435 & \text{Low} \\
& T3 & 28 & \text{–} \\
& T4 & 13 & \text{–} \\
\text{N stage} & N0 & 2353 & \text{–} \\
& N1 & 1441 & \text{–} \\
\text{Stage} & I & 630 & \text{Low} \\
& II & 979 & \text{Low} \\
& III & 144 & \text{Low} \\
& IV & 11 & \text{–} \\
\text{ER status} & – & 1382 & \text{Low} \\
& + & 3568 & \text{High} \\
\text{PR status} & – & 776 & \text{–} \\
& + & 1117 & \text{–} \\
\text{HER2 status} & – & 1405 & \text{High} \\
& + & 184 & \text{Low} \\
\text{Basal-like status} & – & 3805 & \text{High} \\
& + & 1037 & \text{Low} \\
\hline
\end{array}
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Table 2

Clinicopathological relationship of LEP mRNA expression in breast cancer.

*Stage* tumor stage, *N* stage node stage, *ER* status estrogen receptor, *PR* status progesterone receptor, *HER2* status human epidermal growth factor receptor 2.

Figure 3. Relationship between LEP expression and clinicopathological parameters in breast cancer patients. The box plot was retrieved from bc-GenExMiner v.4.2. Global significant differences between groups were assessed by Welch t test to generate P values, along with Dunnett–Tukey–Kramer testing. LEP = leptin.
Figure 4. Relationship between LEP expression and clinicopathological parameters in breast cancer patients. Relationship between LEP mRNA expression and clinical outcomes in breast cancer patients (Prognostic Scan Database and R2: Kaplan–Meier Scanner). Relationship between LEP mRNA expression and clinical outcomes in breast cancer patients (Prognostic Scan Database and R2: Kaplan–Meier Scanner). (A–D). Survival curve comparing patients with high (red) and low (blue) expression of LEP was plotted from the PrognosticScan database in breast cancer patients. Threshold of Cox $P$ value <.05. (E) Survival curve comparing patients with high (red) and low (blue) expression of LEP was plotted from R2: Kaplan–Meier in TCGA breast cancer patients. The threshold of Cox $P$ value <.05. LEP = leptin, TCGA = The Cancer Genome Atlas.

Figure 5. Correlation between LEP mRNA expression and promoter methylation in breast cancer (TCGA data). (a) Heat map of LEP expression and DNA methylation status retrieved from UCSC Xena. (b) Promoter methylation level of the LEP gene in breast cancer tissues compared to normal tissues. The box plot was retrieved from the UALCAN web server. LEP = leptin, TCGA = The Cancer Genome Atlas, UCSC XENA = University of California Santa Cruz Xena.
is mainly associated with the endosome lumen, cytosol, and secretory granule lumen and is upregulated. For the BP energy reserve, metabolic processes have the highest regulation compared to the others. In MF, it was mainly enriched in a variety of combinations, but hormone activity showed the greatest regulation.

4. Discussion

In the present study, we examined the prognostic significance of LEP in breast cancer. LEP is considered a potential breast cancer susceptibility gene.[15] Most previous studies have shown an association between high LEP expression levels and poor prognosis in several cancers.[8,39] In contrast to these previous studies, low levels of LEP were seen to play an unfavorable role in breast cancer progression in our data analysis. Serum LEP abundance is higher in patients with breast cancer than in controls, and higher levels of serum LEP, intratumoral LEP mRNA, and intratumoral ObR isoform mRNA levels have been observed to be poor prognostic predictors in patients with breast cancer.[8,16,40] In this study, we first analyzed the expression of LEP in breast cancer tissues and other malignancies and found that LEP expression in breast cancer was downregulated compared to that in other tumor types. We validated these results by analyzing the data surrounding LEP expression in different types of cancers generated by GEPIA using TCGA data (Fig. 1). An assessment of the comprehensive median expression of LEP across 15 analyses using the Curtis Breast microarray
dataset showed that LEP expression was downregulated in most breast cancer types (Table 1).

Our results contradict previous research that indicates the involvement of serum LEP in the progression of established breast tumors. However, it also speculates that a high serum LEP level may be associated with breast carcinogenesis since LEP stimulates the proliferation of normal human breast epithelial cells in vitro.[32] The difference in study results could be the following reasons. Firstly, serum LEP levels in breast cancer patients and healthy women are not significantly different. Second, serum LEP levels do not show a significant difference between tumor stages. Third, LEP mRNA levels in tumor tissue are much lower (1/12th) than those in adipose tissue (data not shown). Thus, the reason for the association between serum LEP levels and LEP mRNA levels is different and hence the contradiction.[32]

In this study, through co-expression analysis, we found that LEP expression was positively correlated with SLC19A3 expression. Previous research suggests that SLC19A3 is downregulated in breast cancer by hypermethylation of a CpG-rich region in its promoter.[41,42] The positive correlation between SLC19A3 and LEP expression in our study indicates its role in breast cancer progression and metastasis (Fig. 2).

The main role of LEP is in modulating ER signaling and aromatase activity.[43,44] Increased expression of LEP and LEP receptors in both primary and metastatic breast cancer tissues and correlation of the expression of these proteins with ER status, tumor size, and higher tumor grade have been documented in several studies.[8,45] LEP and estrogen may cooperate in maintaining estrogen-dependent breast cancer growth.[8] LEP can increase aromatase activity, promote the production of estrogen, and thus stimulate the development of ER+ breast cancer.[46] In the current study, ER status was positively correlated with LEP expression. Conversely, basal-like and HER2-positive subtypes were negatively correlated with LEP expression. Nevertheless, more data are required to elucidate the mechanisms underlying LEP expression and HER2 status. The evolution of estrogen-dependent breast cancer is primarily caused by ER signals that can be activated by LEP signaling,[46] which may explain the positive association between ER+ breast cancer patients in the current study and breast cancer recurrence (Table 2, Fig. 3).

One study reported that high expression of Ob-R mRNA in breast tissue indicates a poor prognosis for patients with high serum LEP levels.[47] A previous study reported that a shorter RFS duration was associated with mRNA expression of the short isoform of the LEP receptor (OB-R-S).[47] In contrast, our results showed that low LEP expression correlated with shorter OS, RFS, DMFS, and disease-specific survival, and Kaplan–Meier survival analysis indicated that LEP expression was associated with reduced OS in breast carcinoma patients, indicating that low expression of LEP may be functionally important in tumor progression (Fig. 4).

In addition, DNA methylation is an important regulator of gene transcription, and such epigenetic modifications are common in many tumors and during development. Global hypomethylation is considered a cause of cancer.[48] In our analysis, comparing the LEP expression heatmap and DNA methylation status revealed that LEP expression gradually decreased with increasing DNA methylation, indicating that LEP methylation levels were higher in breast cancer tissue than in normal tissue from the same patients (Fig. 5).

Finally, we analyzed the relationships between LEP and interacting proteins using STRINGdb. We found that STAT3, PPARG, GCG, CRP, INS, IRS1, PTPN1, LEPR, JAK2, and SOCS3 directly interact with LEP (Fig. 6). Using these proteins, we performed GO functional annotation analysis using The Database for Annotation, Visualization and Integrated Discovery. GO analysis, the establishment of the PPI network, and network topological analysis were utilized to identify genes potentially related to the initiation and development of LEP (Fig. 7). GO functional analysis indicated that LEP was enriched in hormone activity for MFs, indicating its role as a hormone.[49]

In summary, LEP may play a role in breast carcinoma development. Nevertheless, there is a lack of in-depth insight into the relevant mechanisms involved, and further research is required. It will also be necessary to determine the normal reference range for LEP levels in the healthy population. Further longitudinal studies are required to explain the relationship between LEP expression levels and the risk of breast cancer. We believe that new medicines could be created to combat breast cancer and other breast diseases with further studies involving LEP. Overall, our study suggested that LEP can be used as a tool to predict breast cancer recurrence and survival.

**Figure 7.** GO analysis using DAVID tools. DAVID = The Database for Annotation, Visualization and Integrated Discovery, GO = gene ontology.
positive prognostic biomarker and a potential therapeutic target in breast cancer.

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None.

Author contributions

TYJ, MS, and KSP conceived the analysis and wrote the manuscript. SEN, YBY, JHY, IJY, and SHK conducted proofreading. All authors have reviewed the manuscript.

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Correction

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