RESEARCH ARTICLE

# Legumain Protein as a Potential Predictive Biomarker for Asian Patients with Breast Carcinoma

**Mei Wu, Guang-Rui Shao*, Fei-Xue Zhang, Wen-Xiu Wu, Ping Xu, Zheng-Min Ruan**

## Abstract

**Background: Treatment for breast cancer is mainly performed by surgical resection of primary tumors and chemotherapy. However, after tumor invasion and metastases, breast cancer is hard to control. Clarification of the pathogenic mechanisms would be helpful to the prognosis or therapy for the breast cancer. The aim of this study is to investigate the clinical and prognostic implications of legumain protein Materials and Methods: In this study, we examined mastectomy specimens from 114 breast cancer and matching, 26 adjacent non-cancerous tissues using immunohistochemistry. Results: The results indicated that positive expression of legumain protein in breast cancer was 51.8 % (59/114) and the positive expression of legumain protein in adjacent non-cancerous tissue was 11.5% (3/26). It appeared to be related with lymph node metastasis of breast cancer ($p$=0.02) and correlation analysis indicated that legumain expression was correlated positively with the estrogen receptor (ER) and mutant-type p53 expression (both $p$<0.05). Positive legumain expression was significantly associated with shorter overall survival time in breast cancer patients (log-rank $p$<0.01). Multivariate survival analysis suggested that the positive legumain expression was an independent predictor of poorer overall survival in patients with breast cancer (HR=0.24; 95%CI 0.11-0.65, $p$=0.03). Conclusions: Legumain might be a new potential biomarker for breast cancer, which may reflect the prognosis and overall survival.**

**Keywords:** Breast cancer - legumain - prognostic biomarker - positive expression

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## Introduction

Breast cancer is the development of cancer from breast tissue, which is also the leading causes of cancer deaths in females allover the whole world (Jemal et al., 2011). In the past years, the incidence of breast cancer in Asia is increasing year by year, and the survival rate even achieves a half of the patients. In recent years, the scientists in the whole-world explored the systemic therapies against breast cancer, and brought new hope and excitement, but is limited to the varied clinical course of breast cancer (Elzawawy 2008; Taib et al., 2011). Clinically, the therapy of breast cancer is mainly based on surgical resection of primary tumors and chemotherapy. However, when the tumor invasion and metastases, breast cancer is hard to be therapied (Rustogi et al., 2005). Therefore, the clarify and understanding of the pathogenic mechanism of breast cancer would be become a promising tool for exploring the novel biomarkers and therapeutic targets for breast cancer.

Legumain is an enzyme that in humans is encoded by the LGMN gene (previous symbol PRSC1), and is a cysteine endopeptidase of asparaginyl endopeptidase family, displaying high specificity for hydrolysis of asparaginyl bonds. The previous reports have indicated that legumain positively expressed in many types of human solid tumors, such as colon caner, prostate cancer and breast cancers (Lewen et al., 2008; Wang et al., 2012). However, the legumain always expresses with low level or even no expression in normal tissue. Legumain has been proposed to activate the zymogene progelatinase A, which plays an important role in the degradation of extracellular matrix (Chen et al., 2011). Thus, cells that highly express legumain exhibit enhanced migratory and invasive properties (Liu et al., 2003). The previous studies found that legumain expression in tissues could act as the prognostic factor in colorectal cancer, breast cancer, and ovarian cancer (Murthy et al., 2005; Luo et al., 2006; Wu et al., 2006; Guo et al., 2013). These above backgrounds showed that the expression of legumain may play an important role in tumor progression and development. Therefore, we speculate that the highly expression of legumain may indicate the poor prognosis of breast cancer.

In this study, we examined 114 mastectomy specimens obtained from patients with breast cancers to discuss the correlation between legumain expression and clinicopathological features, and by analyzing the immunohistochemical results to investigate the potential function of legumain as the prognostic markers in breast cancer.

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## Materials and Methods

*Patients and tissue specimens*

In this study, 114 patients who had histologically confirmed invasive breast cancer between June 2001 and July 2003 were included. The inclusion criteria were performed according to the following details, including curative operations were carried out, resected specimens were pathologically examined, a complete medical record was available.

The present study was approved by the Ethics Committee of The second Hospital of Shandong University. All of the patients involved in this study have been gave their consent and approved this study.

*Immunohistochemistry experimental procedures*

The thin slices of tumor tissues were treated in 4% formaldehyde solution over one night, and then incubated with paraffin embedding. The paraffin tissues were sliced into 4 μm-thick sections on the glass slides coated with 3-aminopropyl triethoxysilane for immunohistochemistry (staining with hematoxylin and eosin to determine histological type and grade of tumors).

The above sections were treated for immunohistochemistry according t the previous report (Lewen et al., 2008; Wang et al., 2012) . Then the sections were incubated with polyclonal rabbit anti-human legumain antibody (1:500) (Abcam, UK), Monoclonal mouse anti-human ER antibody (1:500) (Santa Cruz, CA, USA), polyclonal rat anti-human mutant-type tumor protein 53 (p53) antibody (1:100) (Santa Cruz, CA, USA) overnight at 4°C. Following washings with PBS, sections were incubated for 20min at 37°C. The secondary antibodies, poly peroxidase-anti-mouse/rabbit immunoglobulin (1:1000) (Zhongshan, Beijing, China) were then applied to the sections for 30min at 37°C. The immunoreactive products were visualized by DAB kits, following extensive washings. Sections were then counterstained in Gill's Hematoxylin and dehydrated in ascending grades of methanol before clearing in xylene, and mounting under a coverslip.

Nuclear staining for ER and mutant-type p53 was graded 1 if<10% of the cells were stained; 2+ if 10%-50% of the cells were stained; 3+ if>50% of the cells were stained. The grades of 2+ and 3+ were considered as positive one.

*Legumain expression judgment criteria*

The legumain expression was analyzed by calculating with semi-quantitatively method. The judging criteria was listed as followings: 0 represents<25% legumain expression in neoplastic cells; 1 represents≥25 and<50% legumain expression in neoplastic cells; 2 represents≥50% legumain expression in neoplastic cells. Among the above judgment, the samples that obtained the score of 1 or 2 were considered as the positive samples.

*Statistical analysis*

The data in this study were analyzed with SPSS statistics software 19.0 (Microfost, IL, USA). Relationships between tumor markers and other parameters were studied using independent t-tests. Spearman correlation analysis was used to study the correlation between ER or wild-type p53 and legumain protein expression. Disease-specific survival was analyzed using the Kaplan-Meier method. The log-rank test was used to analyze survival differences. Multivariate analysis was carried out using the Cox proportional hazards model selected in forward stepwise. The P-value of less than 0.05 was considered as the statistical significance.

## Results

*Legumain expression in breast cancer tissues and adjacent non-cancerous tissues*

The immunohistochemical examination results indicated that the positive expression of legumain protein in breast cancer was 51.75 % (59/114) and the positive expression of legumain protein in adjacent non-cancerous tissue was 11.54% (3/26) (Figure 1).

*Patient characteristic*

The mean age of the 114 patients studied was 50.2 years (range 31-78years). Eighteen cases had ductal carcinoma in situ (DCIS), and 96 cases had invasive ductal carcinomas (IDC). Within the total sample, 61 patients had no lymph node metastasis and 24 with pN1, 19 with pN2, and 10 with pN3 metastasis (Table 1). From the Table 1 we could find that legumain protein expression was related with lymph node metastasis of breast cancer ($p$=0.02). However, the legumain expression was not related with age, tumor type and sex ($p$=0.36, 0.58, and 0.43, respectively).

*ER and p53 expressing enhanced in legumain positive samples*

In the legumain positive samples, we analyzed the

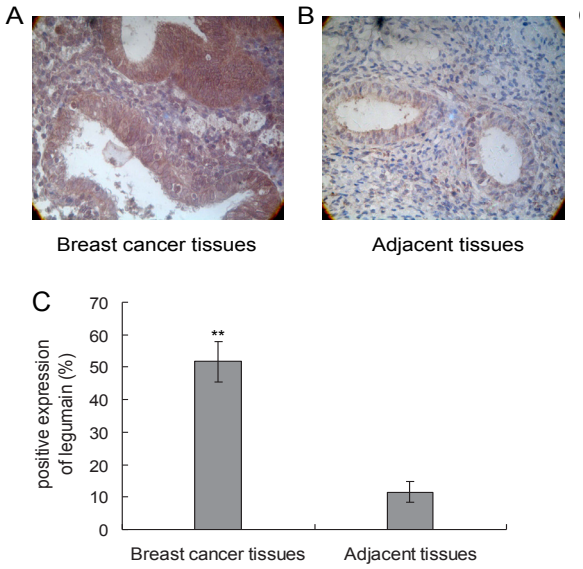


**Figure 1. Expression of Legumain Protein in Breast Cancer Tissues and Adjacent Tissues (SP, 400×). A).** Legumain protien expression in cancer tissues. **B).** Legumain protein expression in adjacent tissues. **C).** Statistical analysis of legumain protein in both tissues. **$p$<0.01 represents the legumain protein expression in cancer tissues compared to in adjacent tissues

**Table 1. Correlations of Legumain Expression with Clinicopathological Parameters**

| Parameters | Total | Positive Legumain Expression (n=59) | Negative Legumain Expression (n=55) | p values* |
|---|---|---|---|---|
| Age | 50.2±2.1 | 50.97±2.3 | 49.84±2.1 | 0.36 |
| Sex | | | | |
| Male:Female | 1.3:1 | 1.2:1 | 1.4:1 | 0.43 |
| Metastasis | | | | |
| No | 61 | 14 | 47 | 0.02 |
| Yes | 53 | 45 | 8 | |
| Tumor types | | | | |
| DCIS | 18 | 27 | 24 | 0.58 |
| IDC | 96 | 32 | 31 | |

*p value less than 0.05 was considered statistically significant.

**Table 2. Multivariate Survival Analysis in the Retrospective Cohort Study of 114 Breast Cancer**

| Parameters | Hazard ratio | 95%CI | p values* |
|---|---|---|---|
| Age | 1.27 | 0.57-2.83 | 0.31 |
| Gender | 0.88 | 0.59-1.46 | 0.68 |
| Tumor types (DCIS) | 0.76 | 0.44-1.35 | 0.65 |
| Metastatis (Yes) | 5.68 | 2.43-9.27 | 0.62 |
| Legumain (Positive) | 0.24 | 0.11-0.65 | 0.03 |

*p values were calculated through Cox proportional hazard model

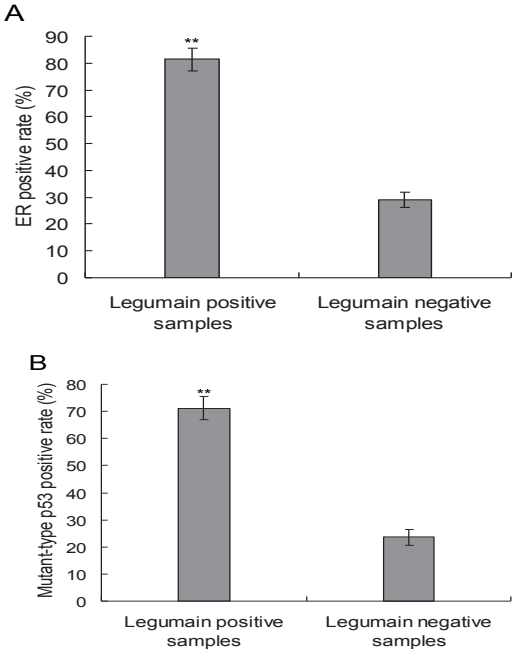


**Figure 2. ER and Mutant-type p53 Expression in Legumain Positive and Negative Samples. A).** ER positive rate in legumain positive and negative samples. **B).** Mutant-type p53 positive rate in legumain positive and negative samples. **$P$<0.01 represents the ER or mutant-type p53 positive rate in legumain positive samples compared to legumain negative samples

ER and p53 expression. The resunt indicated that the ER level was significantly increased in legumain positive samples compared to legumain negative samples (Figure 2A, $p$<0.05). Also, the mutant-type p53 was significantly increased in legumain positive samples compared to legumain negative samples (Figure 2B, $p$<0.05). Thus, the ER and mutant-type p53 expression was found to be related with legumain expression.

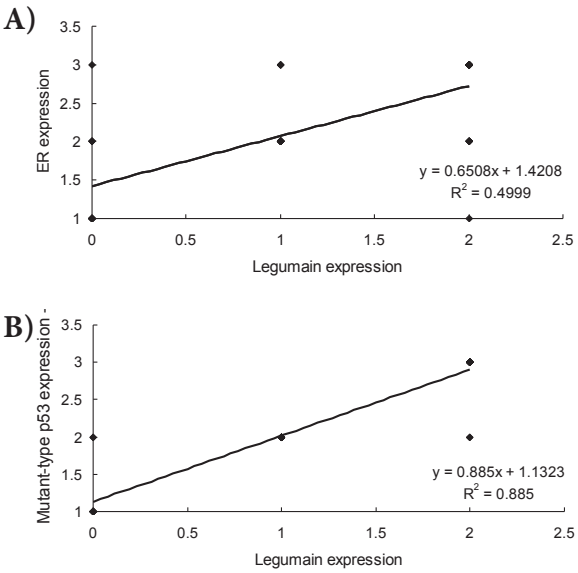


**Figure 3. Correlation Analysis Between the Legumain Expression and Er or Mutant-type p53 Expression. A).** Correlation analysis between legumain expression and ER expression. **B).** Correlation analysis between legumain expression and mutant-type p53 expression

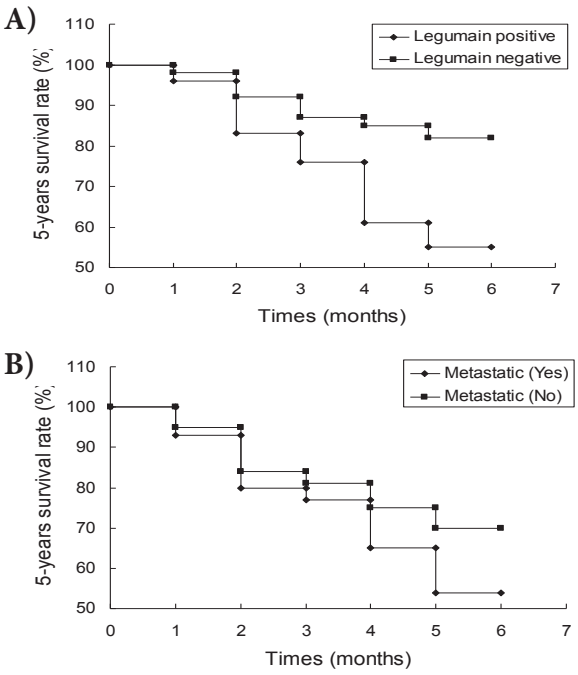


**Figure 4. The 5-year Survival Time of Breast Cancer Patients. A).** The 5-years survival time of breast cancer patient with legumain positive compared to legumain negative expression. **B).** The 5-years survival time of breast cancer patients with lymph metastatisis or without lymph metastatisis

*Correlation between ER or mutant-type p53 and legumain expression*

The correlation analysis results indicated that the legumain expression was correlated positively with the ER expression (Figure 3A, r=0.8763, $p$<0.05). Also, the legumain expression was correlated positively with the mutant-type p53 expression (Figure 3B, r=0.7963, $p$<0.05).

*Legumain levels and overall survival*

The 5-year survival time in patients with high levels of

legumain or positive legumain protein was 55%, while the 5-years survival time in patients with legumain negative levels was 82% months (Figure 4A). Positive legumain expression was significantly associated with shorter overall survival time in breast cancer patients (Figure 4A, Log-Rank $p<0.01$). However, the lymph node-positive group was not associated with the survival time in breast cancer patients (Figure 4B, Log-Rank $p>0.05$).

Multivariate survival analysis suggested that the positive legumain expression was an independent predictor of poorer overall survival in patients with breast cancer (HR=0.24; 95%CI 0.11-0.65, $p=0.03$; Table 2). Similar to the Log-Rank analysis, the lymph node-positive was not an independent predictor of poorer overall survival in breast cancer patients (HR=5.68; 95%CI 2.43-9.27, $p=0.62$; Table 2).

## Discussion

Breast cancer is the most common cancer among the women all over the world. Breast cancer is usually treated with surgery, which may be followed by chemotherapy or radiation therapy, or both. Hormone receptor-positive cancers are often treated with hormone-blocking therapy over courses of several years (Asiaf et al., 2014). Monoclonal antibodies, or other immune-modulating treatments, may be administered in certain cases of metastatic and other advanced stages of breast cancer (Casas et al., 2013). However, all of the above therapeutic methods are limited to a subset of patients whose tumors express hormone receptor, ER, or targeting the immune modulating treatments. Therefore, the clinical significant biomarker in breast cancer is very important for judging the optimal therapeutic time or with optimal therapeutic methods (Parajuly et al., 2012).

Legumain is recognized as lysosomal protease, which has an extremely restricted specificity desiring an asparagine of substrates (Santamaria et al., 2012). The lysosomal protease always highly expressed in neoplastic cells, which might contribute to neoplastic progression via control signaling molecules and their receptors (Sevenich et al., 2010). The above characteristic of protease may be helpful to diminish apoptosis and enhance neoplasms proliferation. Legumain has been detected in several types of human cancers, including breast carcinomas, colon carcinomas, and central nerve system neoplasms (Gawenda et al., 2007). Legumain has also been utilized as a biomarker of initiation and progression in a few tumors (Reisfeld, 2013), however, the functions or role as a predictor or prognostic biomarker for breast cancer has not been explored.

It's the first time that our discussed the correlation between legumain expression and breast cancer in Asian breast cancer patients. A previous study reported that amplification of legumain was detected in 24% breast cancer on western patients (Gawenda et al., 2007). In our study, the positive legumain protein rate in breast cancer patients was 51.75%, which may be caused by the definition of the positive legumain expression between our and the former study.

In this study, in order to explore the mechanism of the changes of legumain in breast cancer, we examined the expression of ER and p53 protein expression by using the Immunohistochemistry assay. The results indicated that the expression of ER and mutan-type p53 was significantly enhanced in legumain positive samples. The correlation analysis results indicated that the legumain expression was correlated positively with the ER expression (r=0.8763, $p<0.05$). Also, the legumain expression was correlated positively with the mutant-type p53 expression (r=0.7963, $p<0.05$). These results indicated that the relationship between legumain positive expression and breast cancer may be caused by the ER or mutant-type p53 expression in tumor tissues. However, the specific pathway that the regulating the legumain protein also needs to be studied in the further researches.

Our study shown that the positive legumain expression was significantly associated with shorter overall survival time in breast cancer patients (55% overall survival, Log-Rank $p<0.01$), compared to 82% in legumain negative expression patients. This result could guidance the clinical doctors make a prognostic diagnose or pre-therapy for the breast patients. Multivariate survival analysis suggested that the positive legumain expression was an independent predictor of poorer overall survival in patients with breast cancer.

In the recent years, researchers have discovered some efficient predictive marker for the breast cancers. He et al. (2014) found that FOXA1 was a novel promising prognostic biomarker in the breast cancer. Velaiutham et al. (2008) found that the serum CA15-3 correlates with the survival of breast cancer. Through all of the above markers with a relative higher sensitivity and specificity, the clinical confirmation also have not been obtained. Therefore, for the biomarker or predictive marker for breast cancer, the large sample of restrospective study analysis is needed in the future breast cancer study.

However, there are also a few conflicts of our study compared to the previous study. We found that positive expression of legumain protein was related to lymph node metastasis, however, the lymph node metastasis was not related with the overall survival of breast patients. There may be some reasons causing these differences, such as different sub-groups and ethnic differences. Therefore, the specific role of legumain in breast cancer also needed to be clarified.

In conclusion, legumain might be a new potential biomarker for breast cancer, which may reflect the prognosis of the overall survival of the breast cancer patients.

## References

Asiaf A, Ahmad ST, Aziz SA, et al (2014). Loss of expression and abberrant methylation of the CDH1 (E-cadherin) gene in breast cancer patients from Kashmir. *Asian Pac J Cancer Prev*, **15**, 6397-403.

Casas A, Llombart A, Martin M (2013). Denosumab for the treatment of bone metastases in advanced breast cancer. *Breast*, **22**, 585-92.

Chen JM, Fortunato M, Stevens RA, et al (2001). Activation of progelatinase A by mammalian legumain, a recently discovered cysteine proteinase. *Biol Chem*, **382**, 77-83.

Elzawawy A (2008). Breast cancer systemic therapy: the need for more economically sustainable scientific strategies in the world. *Breast Care*, **3**, 434-8.

Gawenda J, Traub F, Luck HJ, et al (2007). Legumain expression as a prognostic factor in breast cancer patients. *Breast Cancer Res Treat*, **102**, 1-6.

Guo P, Zhu A, Sun Z, et al (2013). Expression of legumain correlates with prognosis and metastasis in gastric carcinoma. *PLoS One*, **8**, 73090.

Hu Q, Luo Z, Xu T, et al (2014). FOXA1: a promising prognostic marker in breast cancer. *Asian Pac J Cancer Prev*, **15**, 11-6.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Lewen A, Zhou H, Hu HD, et al (2008). A legumain-based minigene vaccine targets the tumor stroma and suppresses breast cancer growth and angiogenesis. *Cancer Immunol Immunother*, **57**, 507-15.

Liu C, Sun X, Huang H, et al (2003). Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res*, **63**, 2957-64.

Luo Y, Zhou H, Krueger J, et al (2006). Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest*, **116**, 2132-41.

Murthy RV, Arbman G, Gao J, et al (2005). Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. *Clin Cancer Res*, **11**, 2293-9.

Parajuly SS, Lan PY, Yu MB, et al (2012). Diagnostic potential of strain ratio measurement and a 5 point scoring method for detection of breast cancer: Chinese experience. *Asian Pac J Cancer Prev*, **13**, 1447-52.

Reisfeld RA (2013). The tumor microenvironment: a target for combination therapy of breast cancer. *Crit Rev Oncog*, **18**, 115-33.

Rustogi A, Budrukkar A, Dinshaw K, et al (2005). Management of locally advanced breast cancer: evolution and current practice. *J Cancer Res Ther*, **1**, 21-30.

Santamaria ME, Hernandez-Crespo P, Ortego F, et al (2012). Cysteine peptidases and their inhibitors in *Tetranychus urticae*: a comparative genomic approach. *BMC Genomics*, **13**, 307.

Sevenich L, Schurigt U, Sachse K, et al (2010). Synergistic antitumor effects of combined cathepsin B and cathepsin Z deficiencies on breast cancer progression and metastasis in mice. *Proc Natl Acad Sci USA*, **107**, 2497-502.

Taib NA, Akmal M, Mohamed I, Yip CH. (2011). Improvement in survival of breast cancer patients- trends over time periods in a single institution in an Asian Pacific country, Malaysia. *Asian Pac J Cancer Prev*, **12**, 345-9.

Velaiutham S, Taib NA, Ng KL, Yoong BK, Yip CH (2008). Does the pre-operative value of serum CA15-3 correlate with survival in breast cancer. *Asian Pac J Cancer Prev*, **9**, 445-8.

Wang L, Chen S, Zhang M, et al (2012). Legumain: a biomarker for diagnosis and prognosis of human ovarian cancer. *J Cell Biochem*, **113**, 2679-86.

Wu W, Luo Y, Sun C, et al (2006). Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms. *Cancer Res*, **66**, 970-80.