LAPTM4B GENE EXPRESSION AND POLYMORPHISM AS DIAGNOSTIC MARKERS OF BREAST CANCER IN EGYPTIAN PATIENTS

EKSPRESIJA I POLIMORFIZAM GENA LAPTM4B KAO DIJAGNOSTIČKI MARKERI KANCERA DOJKE KOD EGIPATSKIH PACIJENTKINJA

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Summary

Background: The aim of this study was to investigate the association between LAPTM4B gene polymorphism and the risk of breast cancer among Egyptian female patients. Also, measurement was done of its serum level to evaluate its significance as a diagnostic marker for breast cancer.

Methods: This case control study was done on 88 breast cancer patients, 40 with fibroadenoma and 80 healthy subjects. Genotyping of the LAPTM4B polymorphism was determined by PCR. Serum LAPTM4B level was measured using ELISA.

Results: There was a significant difference in the (*1/2+*2/2) genotypes in breast cancer patients (59.1%) compared to the control subjects (43.8%) (P=0.047; OR=1.86; 95% CI=1.01–3.43). The frequency of the allele 2* of the LAPTM4B gene was significantly higher in breast cancer patients (36.4%) than in the control (25.6%) (p=0.034; OR=1.66; 95% CI=1.04–2.65). Genotypes (*1/2+*2/2) were significantly associated with the differential classification of TNM. Serum level of LAPTM4B was significantly higher in breast cancer patients than in control and fibroadenoma and in fibroadenoma patients than in control. In breast cancer patients, serum LAPTM4B was significantly higher in breast cancer patients than in control and fibroadenoma and in fibroadenoma patients than in control. In breast cancer patients, serum LAPTM4B was significantly higher in stage III and in large tumor size. Serum LAPTM4B was significantly higher in the cancer patients’ genotypes (*1/2+*2/2).

Conclusions: Genetic polymorphism of LAPTM4B is a potential risk factor for the development of breast cancer. Serum LAPTM4B may be used as a diagnostic and prognostic marker for breast cancer.

Keywords: breast cancer, LAPTM4B, fibroadenoma

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Kratak sadržaj

Uvod: Cilj studije bio je da se ispitati povezanost između polimorfizma gena LAPTM4B i rizika od kancer dojke kod egipatskih pacijentkinja. Izmereni su i nivoi ovog gena u serumu kako bi se utvrdio uticaj koji on ima kao dijagnostički marker za kancer dojke.

Metode: Ova anamnestička studija obuhvatila je 88 pacijen-kinja sa kancerom dojke, 40 obolelih od fibroadenoma i 80 zdravih ispitanica. Genotipizacija polimorfizma LAPTM4B obavljena je tehnikom PCR. Nivo LAPTM4B u serumu je izmeren pomoću ELISA.

Rezultati: Pronađena je značajna razlika u genotipovima (*1/2+*2/2) kod obolelih od kancer dojke (59,1) u poredenju s kontrolnim ispitanicama (43,8%) (P=0,047; OR=1,86; 95% CI=1,01–3,43). Učestalost alela 2* gena LAPTM4B bila je značajno veća kod obolelih od kancer dojke (36,4%) nego u kontrolnoj grupi (25,6%) (p=0,034; OR=1,66; 95% CI=1,04–2,65). Genotipovi (*1/2+*2/2) bili su značajno povezani sa diferencijalnom klasifikacijom TNM. Nivo LAPTM4B u serumu bio je značajno viši kod obolelih od kancer dojke nego u kontrolnoj i grupi sa fibroadenomnom, kao i kod obolelih od fibroadenoma u poredenju s kontrolom. Kod obolelih od kancer dojke, LAPTM4B u serumu je bio značajno viši u slučajevima tumo- ra III klase i tumora velikih dimenzija. LAPTM4B u serumu je bio značajno viši u (*1/2+*2/2) genotipovima obolelih od kancer.

Zaključak: Polimorfizam gena LAPTM4B potencijalni je faktor rizika za razvoj raka dojke. LAPTM4B u serumu može se koristiti kao dijagnostički i prognostički marker za kancer dojke.

Ključne reči: kancer dojke, LAPTM4B, fibroadenom
Introduction

Breast cancer is the most frequent cancer (28% of all cancers) and the most fatal form of malignancy among women, accounting for 15% of cancer deaths (1, 2). In Egypt, breast cancer is estimated to be the most common cancer among females accounting for 37.7% of their total, with 12,621 new cases in 2008. It is also the leading cause of cancer related mortality accounting for 29.1% of their total with 6,546 deaths. The incidence to mortality ratio is poor (1.9:1); these estimates are confirmed in many regional Egyptian cancer registries (3, 4).

Lysosome associated protein transmembrane 4 beta (LAPTM4B), a novel gene upregulated in hepatocellular carcinoma (HCC) and their cell lines, was cloned using fluorescence differential display, RACE, and RT-PCR (5). Studies showed that NIH 3T3 cells transfected with LAPTM4B cDNA displayed profound changes, including increased cell growth and proliferation rates, increased colony formation efficiency in soft agar, reduced serum dependence and morphological alterations such as the increase of microvilli on cell surfaces (6). Increased LAPTM4B expression was also reported in many other solid tumors such as breast cancer, which has increased LAPTM4B expression by 50.9% over normal breast tissues (7). LAPTM4B is composed of 7 exons and 6 introns, and located in chromosome 8q22. It has two alleles, LAPTM4B*1 and LAPTM4B*2 (GenBank accession nos. AY219176 and AY219177, respectively) (8). Allele *1 differs from allele *2 in that it contains only one copy of a 19-bp sequence in the first exon, whereas this sequence is duplicated and tandemly arranged in allele *2. Previous studies showed that the LAPTM4B polymorphism was significantly associated with susceptibility to lung cancer, gastric cancer, colorectal cancers, lymphoma and cervical cancer, but not esophageal carcinoma and rectum carcinoma (9–13). A study that was done by Fan et al. (14) showed that LAPTM4B*2 was associated with an increased risk of breast cancer in the Chinese women population and may be a risk factor of breast cancer.

This study aims to investigate the association between LAPTM4B gene polymorphism and the risk of breast cancer among Egyptian female patients. Also, its serum level was measured to evaluate its significance as a diagnostic marker for breast cancer.

Subjects and Methods

Subjects

The present study included 208 Egyptian women whose age ranged from 20 to 70 years. They were recruited from the General Surgery Department at the Faculty of Medicine, Cairo University. Patients were classified into fibroadenoma or breast carcinoma groups according to history taking, clinical examination and the diagnoses were confirmed by mammography and surgical biopsies. Eighty controls, that were proven to be healthy with no family history of breast cancer, were recruited during routine checkup.

The studied subjects were divided into three groups as follows: Group I: (n=80) healthy females as a control group. Group II: (n=40) patients with fibroadenoma. Group III: (n=88) patients with breast carcinoma; they were classified according to the TNM grading system into 11 cases in stage I, 57 cases in stage III and 20 cases in stage IV. This group included 68 non-metastatic breast cancer patients and 20 metastatic subjects. Inclusion criteria were: adult females, age range 20–70 years, no previous treatment with chemotherapy or radiotherapy. Exclusion criteria: age below 20 and above 70 years, previous treatment with chemotherapy or radiotherapy, other malignancy.

Written consent forms were signed by all participants in this study including controls. Also, this study was approved by the ethical committee of kasr Alainy, Cairo University. All cases were subjected to estimation of the LAPTM4B protein level in serum. The fibroadenoma and carcinoma biopsies were examined histo-pathologically. From each subject, a blood sample was taken and divided into two tubes, one for separation of serum and to be used for the estimation of LAPTM4B level by ELISA. The other tube contained EDTA and was used for DNA extraction and genotyping analysis of LAPTM4B.

Methods

Analysis of LAPTM4B polymorphisms

DNA Extraction

DNA was extracted from whole blood of both patient and control group with QIAamp DNA mini kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. DNA concentration and purity of each sample were measured by the NanoDrop®-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

Polymerase chain reaction (PCR)

Genotyping of the LAPTM4B polymorphism was determined by PCR. Specific primers were used: forward primer 5’-GCCGACTAGGGGACTGGC GGA-3’ and reverse primer 5’-CGAGAGCTCGAGGCTTCTG GCC-3’. In each 25 µL reaction, 100 ng/µL DNA was amplified by the 2X Taq master mix Taq DNA polymerase (0.05 U/µL), 2X Vibuffer A, 0.4 mmol/L dNTPs and 3 mmol/L MgCl2 (Vivantis, Malaysia) with 1 ul of each primer. The PCR conditions were set as follows: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s and a final extension step of 72 °C for 7 min. After electrophoresis on
agarose gel 3% stained with ethidium bromide, photographs were taken under an ultraviolet light transilluminator. The homozygous LAPTM4B*1 and LAPTM4B*2 genotypes were identified by a 204-bp band and a 223-bp band, respectively. The heterozygous LAPTM4B*1/2 genotype exhibited the two bands of 204-bp and 223-bp, respectively (Figure 1).

Measurement of LAPTM4B in serum

LAPTM4B was determined in serum by using human lysosomal associated protein trans-membrane 4 beta ELISA kit (Cat No: E0109h) provided by Wuhan Eiaab Science co., LTD (Wuhan, China). The microtiter plate provided in this kit has been pre-coated with an antibody specific to LAPTM4B protein. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for it and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5,5'-tetramethylbenzidine) substrate solution is added to each well. Only those wells that contain biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of the samples is then determined by comparing the O.D. of the samples to the standard curve (15).

Statistical analysis

Data were coded and entered using SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA) version 17. Data were statistically described in terms of mean ± standard deviation, frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables was done using Mann-Whitney test when comparing two subgroups and Kruscal-Wallis when comparing more than 2 subgroups. For comparing categorical data, Chi square (χ²) test was performed. Exact test was used instead when the expected frequency was less than 5. Genotype and allele frequencies were compared between the disease and the control groups using Chi-square tests. Odds ratio (OR) with 95% confidence intervals was calculated. Receiver operator characteristic (ROC) curves were derived and area-under-the-curve (AUC) analysis was performed to get the best cutoff value of LAPTM4B for detecting cancer and fibroadenoma cases. A probability value (P value) less than 0.05 was considered statistically significant.

Results

The present study was conducted on 208 female subjects. They were classified into 3 groups: Group 1: control; 80 (38.5%) healthy control females with no family history of breast cancer. Group 2: fibroadenoma patients; 40 (19.2%) females. Group 3: breast cancer patients; 88 (42.3%) females.

Table I shows demographic data of the studied groups. As regards the age of the studied groups, it was 50.81±9.27 years in the control group, 32.55±9.73 in the fibroadenoma patients group and 51.9±8.59 in the breast cancer patients group. There was a statistically significant difference between the control group versus fibroadenoma (P₁=0.001) and breast cancer versus fibroadenoma (P₂=0.001). On the other hand, there was no statistically significant difference between the control group versus breast cancer group. There was a statistically significant difference in family history between the fibroadenoma group and breast cancer group (P=0.002).
The number of fibroadenoma patients with positive family history was 11 (27.5%), while among the breast cancer patients there were 51 (58%).

Clinico-pathological data of the breast cancer patients show that the number of invasive ductal breast cancer patients was 79 (89.8%) and that of invasive lobular type subjects was 9 (10.2%). There were 62 cases (70.5%) with tumor size < 5 cm while 26 subjects had tumor size > 5 cm (29.5%). Sixty-eight cases (77.3%) did not show metastasis, whereas 20 subjects (22.7%) had metastases. Regarding TNM staging, 11 cases (12.5%) were of stage II, 57 cases (64.8%) were of stage III and 20 cases (22.7%) were of stage IV. As regard tumor grade, 5 subjects (5.7%) were grade one, 73 subjects (83%) were grade 2 and 10 subjects (11.4%) were grade 3. Fifty-eight cases (65.9%) show -ve ER/PR status, 15 cases (17%) show +ve ER/PR status and the ER/PR status of 15 cases (17%) was unknown.

Figure 2 shows LAPTM4B genotypes in the different groups. The frequencies of *1/1, *1/2, *2/2 and (*1/2 + *2/2) genotypes of the LAPTM4B gene were 56.2%, 36.2%, 7.5% and 43.8%, respectively, in the control group. The frequencies of *1/1, *1/2, *2/2 and (*1/2 + *2/2) genotypes of the LAPTM4B gene were 60%, 30%, 10% and 40%, respectively, in the fibroadenoma group. Meanwhile, the frequencies of *1/1, *1/2, *2/2 and (*1/2 + *2/2) genotypes of the LAPTM4B gene were 40.9%, 45.5%, 13.6% and 59.1%, respectively, in the cancer group.

There was a significant difference in the (*1/2 + *2/2) genotypes (59.1%) and *1/1 (40.9%) genotypes in cancer patients compared to the control subjects (43.8 and 56.2%) respectively (P=0.047;
The frequency of the allele 2* of the LAPTM4B gene was significantly higher in breast cancer patients (36.4%) than in the control subjects (25.6%) (p=0.034; OR=1.66; 95% CI=1.04–2.65).

Table II shows the association between LAPTM4B genotypes and the clinico-pathological parameters of breast cancer (binary logistic regression adjusted for age). Patients that carry genotypes (*1/2+*2/2) had tumor size > 5 cm and tumor size < 5 cm 44.2% and 55.8% respectively, while these values for patients that carry genotype *1/1 were 8.3% and 91.7% respectively (P =0.001), but the association was weak (OR=0.11; CI=0.03–0.41).

Figure 3 shows the serum level of LAPTM4B protein in the studied groups. It was 357.56±117.14 pg/mL in control, 586.03±281.35 pg/mL in fibroadenoma and 1358.88±672.09 pg/mL in breast cancer. A statistically significant difference was found between the control group and both fibroadenoma cases (P=0.033) and the breast cancer group (P=0.001). Also, there was a statistically significant difference between the fibroadenoma group and breast cancer cases (P=0.001).

Table III shows the relation between serum LAPTM4B and the clinico-pathological data of the breast cancer group. Serum LAPTM4B was significantly higher in tumor size > 5 cm (1688.07±588.87) compared to tumor size < 5 cm (1220.84±660.72) (P=0.001). Also, it was significantly higher in stage III (1467.69±679.66) compared to stage II (1037.01±594.58) (P=0.022). On the other hand, no statistically significant difference was found regarding the age, family history, tumor type, breast cancer – whether metastatic or not, tumor grade and ER/PR status.

Figure 4 shows the association between serum LAPTM4B and genotypes in the studied groups. In
the control group, serum LAPTM4B in subjects with genotype *1/1, *2/2 and *1/2 was 329.67±101.74, 264.98±44.15 and 419.98±122.38 pg/mL respectively. This resulted in a statistically significant difference between the patients carrying genotype *1/2 versus *1/1 and *2/2 (P=0.001). Also, in the breast cancer group, a statistically significant difference was found between *1/1 versus *2/2 and *1/2 (P=0.001). Serum LAPTM4B in the cases with genotype *1/1, *2/2 and *1/2 was 955.57±435.59, 1628.24±701.1 and 1670.95±568.9 pg/mL respectively. No statistically significant difference was found when comparing serum LAPTM4B with its genotypes in the fibroadenoma group (P=0.67).

When comparing serum LAPTM4B in patients carrying genotype *1/1 among the studied groups, there was a statistically significant difference (P=0.001) between control versus fibroadenoma and cancer groups and also between cancer versus fibroadenoma. When comparing serum LAPTM4B in the patients that carry genotypes *2/2 and *1/2 among the studied groups, there was a statistically significant difference (P=0.001) between cancer versus fibroadenoma.

Figure 5 shows the receiver operating curve (ROC) for detecting cancer from control. The best serum LAPTM4B cutoff value for detecting cancer was 549.75 pg/mL with an area under the curve of 0.989, 94.3% sensitivity and 96.4% specificity. Figure 6 shows the receiver operating curve (ROC) for detecting cancer
from fibroadenoma patients with the best serum LAPTM4B cutoff value for detecting cancer in fibroadenoma patients of 805.1 pg/mL, area under the curve of 0.886, sensitivity 78.4% and 87.5% specificity.

Discussion

LAPTM4B is a novel cancer-related gene which is upregulated in most solid tumors (7). Previous studies have shown that LAPTM4B plays an important role in the occurrence, development, migration and prognosis of tumors (6, 13). Therefore, the present study aimed to investigate the association between LAPTM4B gene polymorphism and susceptibility to breast cancer among Egyptian female patients, together with measurement of its serum level to evaluate its significance as a diagnostic marker for breast cancer.

A case control study was conducted on eighty-eight breast cancer female patients (forty-eight without metastases and forty patients with metastases). In addition, forty female patients with fibroadenoma and eighty healthy female donors as controls were included in the study. In the present study, the age of the control group was 50.81±9.27, that of fibroadenoma group was 32.55±9.73 and that of breast cancer group was 51.9±8.59. This resulted in a statistically significant difference between fibroadenoma versus both control and breast cancer groups (P=0.001 each). This result coincided with that of Allen et al. (16) who stated that the risk of getting breast cancer increases with age and women are more likely to develop breast cancer in their sixties than in their twenties. At the same time, this finding was consistent with the study done by Dupont et al. (17) which demonstrated a 1.3- to 2.1-fold increased risk of breast cancer in women with fibroadenomas compared with the general population. Moreover, our results agreed with Katz and Dotters (18) who stated that fibroadenoma is the most common breast tumor in women at young age, especially under the age of 30 (19).

Regarding family history, it was positive in 27.5% of fibroadenoma patients and in 58% of breast cancer patients. The result was statistically significant (P=0.002). This was consistent with Dite et al. (20) who stated that positive family history of breast cancer seems to be the risk factor most strongly associated with the disease, as women with any family history of breast cancer represent clinically a higher risk than the general population.

We investigated the LAPTM4B genotypes with PCR assays in 88 breast cancer patients, 40 fibroadenoma patients and 80 healthy controls. We found higher frequency of genotypes *1/2 and *2/2 in breast cancer patients (45.5% and 13.6%, respectively) compared with healthy controls (36.2% and 7.5%, respectively). Meanwhile, the distribution of genotypes (*1/2+*2/2) was statistically significantly higher in breast cancer (59.1%) compared to control (43.8%) group (P=0.047; OR=1.86; CI=1.01–3.43). The frequency of allele *2 was higher in breast cancer cases (36.4%) than in controls (25.6%). Patients carrying the LAPTM4B *2 allele had 1.66-fold higher risk of developing breast cancer than those carrying allele *1 (P=0.034; OR=1.66; CI=1.04–2.65).

These results were consistent with those shown by Fan et al. (14) and Li et al. (21) who found that subjects carrying at least one *2 allele were more likely to develop breast cancer than subjects with LAPTM4B *1/1. This suggested that allele *2 might be a risk factor of breast cancer.

Moreover, we studied the association of LAPTM4B genotypes with the clinico-pathological parameters of breast cancer after adjustment of age. We found that among the subjects with tumor size < 5 cm there were 91.70% of those carrying genotype *1/1 and 55.80% of those carrying genotypes (*1/2+*2/2). In the case of tumor size > 5 cm, there

![Figure 5](image-url) ROC curve for detecting cancer from control.

![Figure 6](image-url) ROC curve for detecting cancer from fibroadenoma.
was a statistically significant difference as the percentage was 8.3% and 44.2% respectively (P=0.001). This finding was not consistent with that shown by Li et al. (19) who stated that LAPTM4B genotypes (*1/2+*2/2) are not significantly associated with tumor size. This may be due to the difference in tumor size in their study (tumor size less than or more than 2 cm).

Another significant association with the genotypes (*1/2+*2/2) was the differentiation classification of TNM (P=0.01). Among breast cancer patients carrying genotype *1/1 there were 22.20% in stage II and 47.20% in stage III, while among those carrying genotypes (*1/2+*2/2) there were 5.80% in stage II and 76.90% in stage III. No significant difference was found regarding tumor grade and metastasis. These findings disagreed with those of Li et al. (21) who revealed significant association between genotypes (*1/2+*2/2) and tumor grade (P=0.0027) in breast cancer patients. The similarity between this study and ours was the nonsignificant association of genotypes (*1/2+*2/2) in breast cancer with tumor type and ER/PR status. On the other hand, the study done by Fan et al. (14) stated that no association was observed between LAPTM4B genotypes and age, family history, tumor type, tumor size, lymph node metastasis and ER/PR status. To the best of our knowledge, this is the first study using the ELISA technique to measure the serum LAPTM4B protein in breast cancer.

In the present study, we investigated the serum LAPTM4B of healthy subjects, fibroadenoma and breast cancer patients. LAPTM4B was 357.56±117.14 pg/mL in control, 586.03±281.35 pg/mL in fibroadenoma and 1358.88±672.09 pg/mL in breast cancer. This resulted in a statistically significant difference between control versus both fibroadenoma and breast cancer groups (P=0.033 and P=0.001, respectively). Another significant difference was found between fibroadenoma and breast cancer (P=0.001). We observed that the LAPTM4B protein level was progressively increasing from control to breast cancer. This coincided with another study done by Xiao et al. (22) who found that LAPTM4B protein expression in malignant tumor tissues was significantly higher than that in benign tissues and the more advanced malignancy of breast cancer, the higher the expression of LAPTM4B would be. His research group detected LAPTM4B over-expression by using immunohistochemistry (IHC) in both breast cancer and benign breast tumor patients. This finding agreed with several studies (6, 7, 23) which stated that LAPTM4B mRNA and/or protein was over-expressed in a wide variety of cancers such as HCC, gall bladder cancer, uterine and ovarian cancers and extrahepatic cholangiocarcinoma. When we studied the relationship between LAPTM4B protein and the features of fibroadenoma (age, family history and parity), we did not find any statistically significant differences. Meanwhile, in breast cancer such a difference was found regarding tumor size, whether less than or more than 5 cm (P=0.001), and TNM stage II versus III (P=0.022). No statistically significant difference was found regarding metastasis. Xiao et al. (20) revealed that the advanced extent of breast cancer such as later clinical stage is correlated with a high expression of LAPTM4B protein. This was consistent with our results regarding correlations with tumor size and lymph node metastasis.

As far as we know, no other study showed the association between the LAPTM4B genotypes and its protein level in the serum. In the control group, serum LAPTM4B was statistically significantly different in those carrying genotypes *1/1, *1/2 and *2/2 (329.67±101.74, 419.98±122.38 and 264.98±44.15 pg/mL respectively) (P=0.001). In the fibroadenoma group, the LAPTM4B level showed no significant difference in those carrying genotypes *1/1, *1/2 and *2/2 (537.91±225.44, 685.95±385.19 and 575.02±191.68 pg/mL respectively). In the breast cancer group, LAPTM4B protein was statistically significantly different in those carrying genotypes *1/1, *1/2 and *2/2 (955.57±435.59, 1670.95±568.9 and 1628.24±701.1 pg/mL respectively) (P=0.001). Receiver operating characteristic (ROC) curve was done for detecting breast cancer. It revealed that the best cutoff value of serum LAPTM4B for detecting cancer in control was 549.75 (AUC=0.989, sensitivity = 94.3% and specificity = 96.4%). Meanwhile, the best cutoff value of serum LAPTM4B for detecting cancer in fibroadenoma patients was 805.1 pg/mL (AUC=0.886, sensitivity = 78.4% and specificity = 87.5%).

**Conflict of interest statement**

The authors stated that they have no conflicts of interest regarding the publication of this article.
References


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