Aim of the study: Breast cancer is the most common cause of death in women. Obesity has been associated with increased risk of breast cancer in post-menopausal women. It induces chronic inflammation, which increases local and systemic levels of cytokines and adipokines such as leptin. Leptin (LEP) and leptin receptor (LEPR) genes have several polymorphisms in humans. This study aims to assess the association between blood levels of leptin and LEPR Q223R gene polymorphism in patients of breast cancer.

Material and methods: The current study was carried on 48 female breast cancer patients and 48 healthy female subjects. Carcinoembryonic antigen (CEA), cancer antibody CA15-3, and leptin hormone were determined. Single nucleotide polymorphism of LEPR Q223R was assessed by PCR/RFLP. Statistical analysis used: The statistical analysis of data was done by using SPSS version 20.

Results: There were significant increases in the concentrations of CEA ($p = 0.004$), CA15-3 ($p < 0.001$), and leptin hormone ($p < 0.001$) in BC patients in relation to the respective concentrations in control subjects. CEA and CA 15-3 showed significant differences between various BC stages. As regard to LEPR Q223R gene polymorphism, AA genotype showed significantly higher frequency in BC patients when compared to their respective controls, with higher risk to develop BC.

Conclusions: Leptin hormone shows significantly higher concentrations in BC patients. As regard to LEPR Q223R gene polymorphism, AA genotype showed significantly higher frequency in BC patients.

Key words: leptin, leptin receptor, LEPR Q223R gene polymorphism, breast cancer.
Numerous studies have confirmed the association of LEPR Q223R gene polymorphism with body mass index, insulin resistance, and postmenopausal breast cancer [15].

Hence, the present study aims to assess the effect of LEPR Q223R polymorphisms on breast cancer risk in Egyptian females, and their relation to different stages.

Material and methods

Subjects

This study was carried out on two groups. The first patient group comprised of 48 breast cancer females (mean age 47.7 ±7.5 years; mean BMI 34.37 ±6.08 kg/m²) who were selected from the Oncology Centre (MUOC) at Mansoura University through 2014–2015. Informed consent was obtained from all individuals included in the study. Approval of the Local Ethics Committee of Mansoura University was obtained, with reference code MS/190.

The second control group consisted of 48 healthy subjects (mean age 43.5 ±9.2 years; mean BMI 27.28 ±3.52 kg/m²) with no family or personal history of cancer breast.

Subjects with non-adenocarcinoma epithelial tumours, non-epithelial tumours, Li Fraumeni syndrome, or a history of ionising irradiation were excluded.

Methods

Five millilitres of peripheral blood was collected from the antecubital vein of overnight fasted patients. Then 1 ml peripheral blood was collected in EDTA vacutainer tube from patients and controls for DNA extraction and restriction fragment length polymorphism (RFLP). 4 ml blood was added to polypropylene tubes with a stopper, left to clot for 20 minutes, and the resulting serum was further divided into three aliquots. The aliquots were kept at –20°C for other laboratory tests.

Liver function tests (SGPT, SGOT, albumin, and bilirubin) and kidney function tests (creatinine) were assessed spectrophotometrically using COBAS Integra 400 plus Roche Diagnostics Ltd. CH-6343 Rotkreuz Switzerland. Cholesterol was estimated by CHOD-POD liquid [16], triglycerides by GPO-POD liquid [17], and HDL by precipitating reagent [18]; all were supplied by SPINREACT, S.A./S.A.U. SPAIN. Indirect measurement of LDL cholesterol was performed using the Friedewald equation:

\[
\text{LDL-c (mg/dl)} = \text{TC (mg/dl)} - \text{HDL-c (mg/dl)} - \text{TG (mg/dl)} / 5 \quad [19].
\]

The carcinoembyronic antigen (CEA) and cancer antigen 15-3 (CA15-3) were estimated by electrochemiluminescence immunoassay (ECLIA) using ELECSYS 2010 Roche Diagnostics, Germany. Leptin hormone was estimated by sandwich ELISA technique using DBC Leptin ELISA, Diagnostics, Germany. Leptin hormone was estimated by electrochemiluminescence immunoassay (ECLIA) using ELECSYS 2010 Roche Gen 15-3 (CA15-3)

For amplification of the region Gln223Arg polymorphism, the following primer was used: forward primer, 5-d ACCC TTG CGT GCT CCAAATGA-3; reverse primer, 5-d CTA GCAAATA TTTTT GTAA GCAA TT -3. PCR amplification was done using DreamTaq PCR Green Master Mix (2X) (cat. No. k1081, Lithuania, EU). DreamTaq PCR Master Mix (2X) was gently vortexed and briefly centrifuged after thawing. The reaction mixture of total volume (25 µl) contained 15 µl PCR Master Mix, 0.5 µl forward primer (100 pmol), 0.5 µl reverse primer (100 pmol), 5.0 µl extracted DNA (100 ng), and 4 µl nuclelease-free water. The samples were gently vortexed and PCR was done using the thermal cycle (Biorad PTC-100, peltier, USA) [21].

The reaction mixture was heated to 94°C for five minutes, followed by 30 cycles each consisting of 60 seconds at 94°C (denaturation), 60 seconds at 55°C (annealing), 60 seconds at 72°C (extension), and a final seven-minute extension at 72°C.

The PCR product was digested with MspI (ThermoScientific Fast Digest, cat. No. FD 0014, Lithuania) [21]. The reaction mixture of total volume (50 µl) contained 23 µl nuclease-free water (#R0581), 15 µl buffer, 10 µl PCR product, and 2 µl MspI restriction enzyme. The components were incubated at 37°C for five minutes. The digested products were separated by electrophoresis in 2% agarose gels with ethidium bromide. The PCR product (440 bp) with G allele was digested by MspI to two fragments (300 and 140 bp), whereas the PCR product with A allele could not be digested and yielded one fragment at 440 bp. So the homozygous GG gave two bands at 300 and 140 bp, homozygous AA gave one band at 440 bp, and the heterozygous GA gave three bands at 440, 300, and 140 bp.

Lanes 1, 3, 5 represent AA genotypes (440 bp), lanes 2, 6, 7, and 9 represent GA genotypes (440, 300, and 140 bp), and lanes 4 and 8 represent GG genotype (300 and 140 bp) (Fig. 1).

Statistical analysis

Excel (Microsoft Office 2013) and SPSS version 20 (statistical package for social science) (SPSS, Inc., Chicago, IL) were used for statistical analysis. The χ² test was used for assessing differences from Hardy-Weinberg equilibrium expectations. The associations between breast cancer and leptin receptor Q223R polymorphism were measured by odds ratio and their 95% confidence interval. N.B: p ≤ 0.05 is significant at confidence interval 95%.

![Fig. 1. PCR-RFLP with MspI restriction enzyme](image-url)
Results

Serum cholesterol, triglycerides, and LDL-cholesterol showed significantly higher concentrations in breast cancer patients when compared to control subjects (0.004, 0.003, 0.006, respectively). There were significant increases in the concentrations of CEA (p = 0.004), CA15-3 (p < 0.001), and leptin hormone (p < 0.001) in BC patients in relation to their concentrations in control subjects (Table 1).

CEA and CA 15-3 showed significant differences between various BC stages; this significance was attributed to significant increases in CEA and CA15-3 in stage IV when compared to stage I (p = 0.002, 0.009, respectively); stage II (p = 0.002, 0.001, respectively), and stage III (p = 0.003, 0.043, respectively) (Table 2).

AA genotype showed significantly higher frequency in BC patients when compared to control subjects, with higher risk of developing BC (p = 0.025, OR = 5.308, 95% CI: 1.082–26.040). As regard to AG, GG genotypes, and A, G alleles, there were no significant differences in frequency between breast cancer patients and controls without risk of breast cancer (Table 3).
Table 4. Distribution of Leptin Receptor Q223R (alleles and genotypes) in BC patients according to stages

| Parameter | BC stage I and II (n = 24) | BC stage III and IV (n = 24) | p    | OR     | 95% CI |
|-----------|---------------------------|-----------------------------|------|--------|--------|
| Genotypes|                           |                             |      |        |        |
|           | Genotypes | n  | %   | n  | %   |       |       |
| AA        |            | 7  | 29.2| 2  | 8.3 | 0.137 | 0.221 |
| AG        |            | 5  | 20.8| 10 | 41.7| 0.119 | 2.714 |
| GG        |            | 12 | 50.0| 10 | 50.0| 1.000 | 1.000 |
| Alleles   |                           |                             |      |        |        |
| A         |            | 19 | 39.6| 14 | 29.2| 0.283 | 1.591 |
| G         |            | 29 | 60.4| 34 | 70.8|       |       |

Table 5. Comparison of BC pathological types between different Leptin Receptor Q223R genotypes in all studied groups

| Parameter | BC patients (n = 48) | p    |
|-----------|---------------------|------|
|           | AA (n = 9)          |      |
|           | AG (n = 15)         |      |
|           | GG (n = 24)         |      |
| IDC       | 6  | 66.7  | 9  | 60.0 | 14 | 58.3 | 0.009* |
| ILC       | 3  | 33.3  | 5  | 33.3 | 1  | 4.2  |       |
| Paget’s disease | 0  | 0     | 1  | 6.7  | 9  | 37.5 |       |

IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma

More advanced BC stages (stage III and IV) showed no significant change in frequency in leptin receptor Q223R genotypes and alleles when compared to stage I and II (Table 4).

There was significant association between BC pathologic types and leptin receptor Q223R genotypes (p = 0.009) because the frequency of GG leptin receptor Q223R genotype was significantly high in Paget disease (Table 5).

Discussion

Breast cancer is the most common cause of death in women. It accounts for 14% of the total cancer deaths [1].

Obesity is associated with a high incidence of many serious diseases, including cancer [22]. Obesity due to lifestyles and unhealthy diets raises the risk of cancer, and it is predicted as a bad prognostic factor among survivors of breast cancer. Many studies reported that obesity is accompanied with high death rates from all cancers [23]. However, the mechanism by which obesity can develop breast cancer remains unclear [24, 25].

In the present study, cholesterol, triglycerides, and LDL-cholesterol showed significantly higher concentrations in breast cancer patients when compared to controls (Table 1). These findings are in accordance with Abdel-salam et al. [26] and Florenza et al. [27], who found that lipid profiles were increased significantly in all stages of breast cancer.

Leptin reduces body overweight and decreases the obesity rate as it regulates appetite and size of adipose tissue [28]. Although obese individuals have high levels of plasmatic leptin, they cannot control appetite as a result of development of non-responsive hypothalamic stage for the regulation of appetite and energy expenditure [29].

In the present study leptin hormone showed significantly higher concentrations in BC patients when compared to control subjects (p < 0.001). Conversely, leptin hormone did not show significant differences between various BC stages. These findings are concordant with the findings of Cleary et al. [30]; Tessitore et al. [31]; Han et al. [32]; Chen et al. [33]; Hou et al. [34]; Liu et al. [35] and Taaban et al. [36], who reported that the serum levels of leptin were significantly higher in breast cancer patients than in controls. This can be explained by the fact that the main component of human breast is adipose tissue, which is the chief site of leptin secretion. It has been reported that leptin has a role in mammary glands development [37]. In addition, cancerous cells present in the mammary gland overexpress OB-R and respond to leptin stimulus by increasing vascular endothelial growth factor and its receptor 2, proliferation, and survival [38–40].

However Mantzoros et al. [41], Coskun et al. [42], Sauter et al. [43], Stattnin et al. [44] and Woo et al. [45] reported that there was no relationship between breast cancer and serum leptin levels in postmenopausal women. This conflict can be explained by the failure of control of some potential factors, such as food intake, which affect leptin concentrations.

In the present study CEA showed significantly higher concentrations in breast cancer patients when compared to controls (p = 0.004) (Table 1). This finding is in accordance with the results of Samy et al. [46], who found that preoperative serum levels of CA15-3 and CEA were significantly higher in breast cancer patients compared with the levels of the control group, and these markers decreased after operation.

In the present study CA15-3 showed significantly higher concentrations in breast cancer patients in comparison to control subjects (p < 0.001) (Table 1); also, CEA and CA 15-3
showed significant differences between various BC stages. This significance was attributed to significant increases in CEA and CA15-3 in stage IV when compared to stage I ($p = 0.002, 0.009$, respectively), stage II ($p = 0.002, 0.001$, respectively), and stage III ($p = 0.003, 0.043$, respectively). This is in agreement with Hashim et al. [47], who reported increased levels of CA15-3 in breast cancer patients, when compared to women with benign tumours and healthy controls, and this increase is associated with advanced stages.

Paracchini et al. [48] demonstrated that a specific phenotype characterised by morbid obesity was produced due to genetic mutations in the leptin gene and the LEPR gene. Several polymorphisms related to an obese phenotype have been recognised in humans in the leptin and LEPR gene [49].

In the present study, the frequency of AA genotype of LEPR gene Gln223Arg polymorphisms was significantly high in BC patients when compared to controls ($p = 0.025$). In agreement with our results, Snoussi et al. [50] and Anuradha et al. [51] reported that increased risk and poor prognosis of breast carcinoma are associated with leptin and LEPR polymorphisms. Also, Han et al. [21] described that the GA/AA genotypes of the LEPR gene (Gln223Arg) in combination with elevated lipids and leptin play a major role in the progression of breast cancer.

In contrast to our results, some researchers confirmed no association between Gln223Arg polymorphisms in the LEPR gene and breast cancer [52]. These differences may be attributed to gene-gene interaction, racial differences, and environmental factors. Also, changes in environmental temperature and stress can affect the expression of leptin [53] and change the risk of breast cancer.

Moreover, no significant differences were found between different leptin receptor Q223R genotypes in BC stages ($p > 0.05$ for each).

The frequency of Leptin Receptor Q223R genotypes and pathologic parameters (ER, PR, HER2, cancers stage) in breast cancer patients were not significantly associated [54], although our study shows significant association between breast cancer pathologic types and leptin receptor Q223R genotypes ($p = 0.009$). This is because the frequency of GG leptin receptor Q223R genotype was significantly high in Paget disease. Snoussi et al. [50] found that the LEPR 223G allele is accompanied by short survival and bad prognosis in breast cancer.

One limitation of our study is the higher BMI in patients than in controls, which may influence the leptin level, in addition to the role of cancer.

In conclusion, leptin hormone shows significantly high concentrations in breast cancer patients when compared to controls. It did not show significant differences between various breast cancer stages. As regard to LEPR Q223R gene polymorphism, AA genotype frequency was significantly high in breast cancer patients when compared to controls, with higher risk of developing breast cancer. Otherwise, other genotypes and alleles show no significant differences between breast cancer patients and controls with no risk of breast cancer.

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