Assessment of Plasma Soluble Human Leukocyte Antigen-G as a Prognostic Marker in Post-Chemotherapy Breast Cancer Patients in Gaza Strip

Gaza’da Plazmada Çözünür İnsan Lökosit Antijen-G’nin Kemoterapi Sonrası Meme Kanseri Hastalarda Prognostik Belirteç Olarak Değerlendirilmesi

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Objective: This study aims to investigate whether plasma soluble human leukocyte antigen-G (sHLA-G) can be used as a potential biomarker for the diagnosis and follow-up of breast cancer in post-chemotherapy patients in Gaza strip.

Patients and methods: The study population was divided into two groups: 60 women with breast cancer and receiving chemotherapy (post- group) and 60 women with breast cancer who did not receive chemotherapy (pre- group). The control group consisted of 60 healthy women. Cancer antigen 15-3 (CA15-3) and carcinoembryonic antigen (CEA) levels were analyzed using the Axsym Immunoassay system.

Results: Plasma sHLA-G levels were significantly higher in breast cancer patients compared to healthy controls (p<0.001). Area under receiver operating characteristic (ROC) curve of sHLA-G for discriminating patients with breast cancer (n=120) from the control group (n=60) was 0.919 (95% CI=0.882 - 0.956) and was smaller than those of CA15-3 (0.998, 95% CI=0.996-1.00) and CEA (0.985, 95% CI=0.973-0.997). The ROC curves of sHLA-G, CEA, and CA15-3 for differentiating the group of patient who did not receive chemotherapy yet (n=60) and the group of patient who received anti-cancer treatment (n=60) were 0.998, 0.781 and 0.698, respectively (p<0.001).

Conclusion: Our study findings provide evidence in further support for the application of sHLA-G as a biomarker for diagnosis, prognosis, and follow-up of breast cancer therapy.

Key words: Breast cancer; chemotherapy; soluble human leukocyte antigen-G.

Amaç: Bu çalışmada plazmada çözünür insan lökosit antijen-G’nin (sHLA-G) Gaza’da kemoterapi sonrası hastalarda meme kanserinin tanı ve takibi için potansiyel bir biyobelirteç olarak kullanılmamayaçağı araştırıldı.

Hastalar ve yöntemler: Hasta popülasyonu iki gruba ayrıldı: meme kanseri olan ve kemoterapi verilen 60 kadın (pre- grup) ve meme kanseri olan ve kemoterapi verilenmeyen 60 kadın (post- grup). Kontrol grubu ise 60 sağlıklı kadından oluşuyor idi. Axsym immünolojik tahlil sistemi kullanılarak, karsinoembriyonik antijen (CEA) ve kanser antijen 15-3 (CA15-3) düzeyleri analiz edildi.

Bulgular: Plasma sHLA-G düzeyleri, sağlıklı kontrollere kıyasla, meme kanseri olan hastalarda anlamlı düzeyde daha yüksek idi (p<0.001). Meme kanserli hastaların (n=120) kontrol grubundan (n=60) aytır edilmesinde sHLA-G’nin alıcı işletim karakteristik eğrisi (ROC) altında kalan alan, 0.919 (%95 CI: 0.882-0.956) oldu, CA15-3 (0.998, %95 CI=0.996-1.00) ve CEA’ninkinden (0.985, %95 CI=0.973-0.997) daha küçük idi. Daha önce kemoterapi verilmemiş hasta grubunun (n=60), daha önce antikanser tedavi verilen hasta grubundan (n=60) aytır edilmesinde sHLA-G, CEA ve CA15-3’in ROC eğrisi sırasıyla 0.998, 0.781 ve 0.698 idi (p<0.001).

Sonuç: Çalışma bulgularımız, sHAL-G uygulamasının meme kanserinin tanıında, prognozunda ve tedavinin takibi için bir biyobelirteç olarak kullanılabilirliğini desteklemektedir.

Anahtar sözcükler: Meme kanseri; kemoterapi; çözünür insan lökosit antijeni.
Breast cancer is the most common cancer in women worldwide and second most common cancer overall with nearly 1.7 million new cases diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women.[1]

In Palestine, breast cancer was the most prevalent type of cancer among Palestinians, accounting for 18.8% of all reported cases at the end of 2010.[2] In Gaza Strip, breast cancer is the leading cause of deaths related to cancer for women with a five-year survival rates as low as 30% to 40%.[3]

Human leukocyte antigen G (HLA-G) is a novel tumor marker of which soluble isoforms produce secretory proteins.[4] Human leukocyte antigen, G which belongs to the family of non-classical HLA class I genes, is located within the major histocompatibility complex (MHC) on the p 21.31 region of chromosome 6, presenting a restricted tissue expression pattern and encoding molecules with immune modulatory properties. [4]

Alternative splicing of the primary transcript generates seven different isoforms of the molecule, four of which are membrane-bound (HLA-G1, -G2, -G3 and -G4), while the other three are soluble (HLA-G5, -G6 and -G7).[5] One additional soluble form of the molecule may be generated by shedding of the proteolytically cleaved surface such as HLA-G1. Plasma sHLA-G derives from both the secretion of the soluble isoforms and the shedding form of HLA-G1.[6,7]

In normal tissue, HLA-G protein expression is restricted to fetal trophoblasts, endothelial precursors,[8] as well as to the thymus,[9] cornea,[10] nail matrix,[11] beta cells of the islets of Langerhans[12] and mesenchymal stem cells.[13] Human leukocyte antigen G originally described to be selectively expressed at the maternal-fetal interface on cytotrophoblast cells, thereby contributing to maternal-fetal tolerance.[14]

In this study, we aimed to investigate whether plasma sHLA-G can be used as a potential biomarker for the diagnosis and follow-up of breast cancer in post-chemotherapy patients in Gaza strip.

**PATIENTS AND METHODS**

**Study population**

The study population consisted of women diagnosed with breast cancer who were treatment-naïve or treatment-experienced. The control group consisted of healthy women. All women were aged 25 to 75 years.

A total of 180 women with and without breast cancer aged between 25 to 75 years who agreed to participate in our study were recruited from Al-Shifa hospital between July 2013 and July 2014 and were divided into two groups:

- **Study group (n=120)** consisted of 60 women with breast cancer and receiving anti-cancer treatment (post- group) and 60 women with breast cancer who did not receive chemotherapy (pre- group).
- **Control group (n=60)** consisted of 60 healthy women selected randomly from the women who visited Al-Shifa hospital for physical check-ups and had no clinical evidence of breast cancer or any other overt diseases. Age matching was considered in the three groups.

The participants in the study group registered in the oncology department at Al-Shifa hospital were confirmed as having breast cancer based on pathological features of tissue specimens. Tumor staging was stratified according to the sixth edition of the tumor-node metastasis (TNM) classification by the International Union Against Cancer. Pregnant women and patients with other types of cancers were excluded.

**Ethical consideration**

The study protocol was approved by the Department of Medical Laboratory sciences at Al Aqsa University. A written informed consent was obtained from each subject by the treating physician. Moreover, ethical approval from the local Helsinki Committee at the Ministry of Health in Gaza strip was obtained.

**Blood sampling and processing**

Venous blood samples were collected using plain tubes and ethylenediaminetetraacetic (EDTA) tubes for serum and plasma separation.
- After centrifugation at room temperature for 15 min at 4,000 rpm to obtain serum and plasma, the samples were stored at -80 °C.
Determination of plasma sHLA-G

- Plasma levels of sHLA-G protein were determined by a specific sandwich enzyme-linked immunosorbent assay (ELISA). sHLA-G levels were quantified using a commercially available ELISA kit (Exbio, Prague, Czech Republic) according to the manufacturer’s instructions. Briefly, calibrators and samples were incubated in micro-titration wells and pre-coated with a monoclonal antibody, mouse anti-HLA-G monoclonal antibody (MEMG/9), which recognize the most abundant soluble isoforms (shed sHLA-G1 and intron 4-containing secreted HLAG5). After 60 min incubation and washing, monoclonal anti-human beta 2 microglobulin antibody labeled with horseradish peroxidase (HRP) was added to the wells and incubated for 60 min, which recognize the immobilized antibody sHLA-G complex. Following another washing step, the substrate (H2O2 with tetra methyl benzidine) solution was added to react with the remaining HRP-conjugated antibody. After the addition of acidic stop solution, the absorbance of resulting yellow product was measured at 450 nm using the ELISA micro plate reader (DIA MED EURO GEN Model DEE READ, USA). According to the absorbance values proportional to sHLA-G concentrations of calibrators, a calibrator curve was constructed and the sHLA-G concentrations of unknown samples were determined by this calibrator curve. Plasma samples from study participants were mixed and performed in batches at the same time.

Determination of serum cancer antigen 15-3 (CA15-3) and carcinoembryonic antigen (CEA)

Serum CA15-3 levels were assayed by using the Axsym Immunoassay system (Ortho-Clinical Diagnostics; Rochester, NY, USA) with Vitros Immunodiagnostic Products CA15-3 (Ortho-Clinical Diagnostics). Tests for CEA were performed using the Axsym Immunoassay System with the CEA test kit (Beckman Coulter Inc.; Brea, CA, USA).

Each assay was performed according to the respective manufacturer’s instructions based on chemiluminescent reactions.

Statistical analysis

Data were analyzed statistically using the PASW version 18.0 software (SPSS Inc., Chicago, IL, USA). Frequencies, cross tabulation, and statistical tests such as chi-square and t-test were performed. The receiver-operating characteristic (ROC) curve was performed to distinguish between two diagnostic groups (diseased/normal).

RESULTS

Carcinoembryonic antigen and CA15-3 levels of the study population

The mean CEA score level was 2.06 ng/mL in the control group which fell within normal range (<2.5 ng/mL in adults), and was 7.6 ng/mL in the breast cancer pre-group, and 5.19 ng/mL in the breast cancer post-group. The median value was 2.05 in the control group, 7.60 in the pre-group, and 5.00 in the post-group. The range of CEA was 0.3-5.0 ng/mL in the control, 4.0-13.0 ng/mL in the pre-group, and 2.1-9.0 ng/mL in the post-group. These results reflected high levels of CEA in breast cancer patients compared to healthy women and that CEA level was higher in breast cancer patients who did not receive chemotherapy, compared to patients who received chemotherapy (Table 1).

Also, Table 1 indicates that the mean score level of CA15-3 was 16.61 u/mL in the control group which is within normal values (<30 u/mL), while higher levels were found in the women with breast cancer, as it was 58.66 u/mL in the pre-group and 49.09 u/mL in the post-group. The median was 15.75 u/mL in the control group, 56.75 u/mL in the pre-group and 47.30 u/mL in the post-group.

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Control group (Normal women (u/mL))</th>
<th>(Pre-group) Breast cancer but did not receive chemotherapy (u/mL)</th>
<th>(Post-group) Breast cancer post or during treatment (u/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEA</td>
<td>CA15-3</td>
<td>CEA</td>
</tr>
<tr>
<td>Mean</td>
<td>2.06</td>
<td>16.61</td>
<td>7.63</td>
</tr>
<tr>
<td>Median</td>
<td>2.05</td>
<td>15.75</td>
<td>7.60</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.86</td>
<td>8.55</td>
<td>2.28</td>
</tr>
<tr>
<td>Range</td>
<td>2.50</td>
<td>27.20</td>
<td>9.00</td>
</tr>
<tr>
<td>Minimum-Maximum</td>
<td>0.3-5</td>
<td>2.8-30</td>
<td>4.13</td>
</tr>
</tbody>
</table>

CEA: Carcinoembryonic antigen; CA15-3: Cancer antigen 15-3.
group. The range of CA15-3 was between 2.8-30.0 u/mL in the control group, 37.5-82.0 u/mL in the pre-group, and 29.4-72.0 u/mL in the post-group. These results reflected high levels of CA15-3 in patients with breast cancer, suggesting higher values in newly diagnosed cases who did not receive treatment, while lower levels were observed in women who received chemotherapy.

**sHLA-G levels of study population**

As shown in Table 2, the mean score for sHLA was 73.5 U/mL in the control group, 367.3 U/mL in the pre-group, and 132.9 U/mL in the post-group, while the median was 75.6, 356.0 and 120 U/mL respectively.

The range was between 20.0-141.6 U/mL in the control group, increased to 242.4-512.0 U/mL in the pre-group, and 65.4-264.0 U/mL in the post-group. The two groups of patients exhibited significantly higher sHLA-G in breast cancer patients compared to healthy controls (p<0.001). However, the patients receiving chemotherapy showed lower levels of sHLA-G compared to the patients who did not receiving chemotherapy.

**Receiver operating characteristic curve**

Receiver operating characteristic curves were used to evaluate the performance of sHLA-G, CEA and CA15-3 in discriminating the breast cancer group (n=120) from the control group (n=60) and to evaluate the performance of sHLA-G, CEA and CA15-3 in discriminating the pre-group (n=60) from the post-group (n=60).

**Area under the ROC curve (AU-ROC) of sHLA-G, CA15-3, and CEA in predicting breast cancer**

The sHLA-G levels were significantly higher in breast cancer patients than in healthy controls (p<0.001). We found that the AU-ROC for sHLA-G was 0.919 for breast cancer patients. 

That sHLA-G can be used as a tumor marker in breast cancer patients.

The AU-ROC for CEA was 0.985 and higher than the AU-ROC for sHLA-G. At 96.7% specificity, the highest sensitivity to detect breast cancer was 93.3% (95% CI =0.973-0.997%) at a cut-off value of 3.09 ng/mL.

The AU-ROC for CA15-3 for breast cancer patients versus healthy controls specimens was higher than those of sHLA and CEA; the AU-ROC for CA15-3 was 0.998 at 100% specificity, the highest sensitivity to detect malignancy was 96.7% (95% CI =0.996-1.000%) at a cut-off value of 32.8 u/mL (Figure 1).

Data showed that plasma sHLA-G expression was significantly higher in the pre-group compared to the post-group (p<0.001). The AU-ROCs of sHLA-G, CEA, and CA15-3 for differentiating the group of women who did not receive chemotherapy yet (n=60) and the group of women who received anti-cancer treatment (n=60) were 0.998, 0.781, and 0.698, respectively. When sHLA-G cut-off value was taken as 216.0 U/mL, a sensitivity of 100% (95% CI=0.994-1.00%) and a specificity of 93.3% were achieved.

At a sensitivity of 96.7% and specificity of 46.7% (95% CI =0.701-0.861%), CEA had a cut-off value of 4.2 ng/mL, whereas at a sensitivity of 96.7% and specificity of 36.7%, CA15-3 had a cut-off value of 42.45 u/mL (95% CI=0.606-791%). The ROC curves indicated that sHLA-G levels were superior to CEA and CA15-3 in differentiating between the pre- and post-groups (p<0.001), as shown in Figure 2.

**DISCUSSION**

**Levels of CEA, CA15-3 and HLA-G of study population**

As indicated in the present results, CEA mean score level was significantly higher than normal range (<2.5 ng/mL in adults) in diseased women who did not receive chemotherapy (7.6 ng/mL) and who received chemotherapy (5.19 ng/mL); however, it was within normal range in controls (2.06 ng/mL). Similar results were

The present results showed that the mean plasma level of sHLA-G was higher in patients who did not receive chemotherapy (367.3 U/mL), and lower values were found in patients who received chemotherapy (132.9 U/mL), while much lower values were observed in healthy women (73.5 U/mL). These results indicated that plasma sHLA-G increased in patients with breast cancer, compared to healthy controls and that receiving chemotherapy may reduce sHLA values. This result is consistent with the findings of Davidson et al., who found reduced expression of HLA-G in effusions obtained after the start of chemotherapy. In addition, the study conducted by Jeong et al., showed that levels of sHLA-G were significantly higher in patients with breast cancer (median 117.2), compared to the control group (median 10.1 U/mL) and the AU-ROC curve values of sHLA-G for differentiating breast cancer from normal controls was 0.89. They concluded that sHLA-G concentrations could be used as a diagnostic marker for detecting breast cancer.

Area under the ROC curve of sHLA-G, CA15-3, and CEA in predicting breast cancer

The ROC curve analyses were performed to evaluate the feasibility of sHLA-G as a diagnostic marker for breast cancer. Data showed that plasma sHLA-G expression was significantly higher in breast cancer patients, compared to healthy controls (p<0.001) and sHLA-G could predict 91.9% of the patients, which suggests that the test can discriminate between normal individuals and patients. In this study, the AU-ROC for CA15-3 and CEA was 0.998.
and 0.985, respectively for breast cancer patients than in healthy controls specimens. The AUROC for CA15-3 was higher than CEA and sHLA-G.

These results are consistent with the findings of Provatopoulou et al.,[25] who examined sHLA-G plasma expression in 120 patients with breast cancer and 40 healthy controls using ELISA and showed that plasma sHLA-G levels were significantly higher in breast cancer patients, compared to healthy controls with a AU-ROC curve of 0.735 (95% CI=0.630-0.841, p<0.001). Similar results were obtained by He et al.,[26] who found significantly higher sHLA-G levels in breast cancer patients, compared to healthy controls (p<0.001) and the AU-ROC curve was 0.95. Yet, another study found that plasma sHLA-G level was significantly higher in breast cancer patients, compared to healthy controls (median 82.19 vs. 9.65 U/mL, p<0.001).[27] The AU-ROC curve for sHLA-G was 0.953 (95% CI=0.926-0.981, p<0.001). The authors concluded that HLA-G might play a critical role in the prognosis of breast cancer and plasma sHLA-G levels might be a useful preoperative biomarker for the diagnosis. Furthermore, Badr[28] reported that there was an increased serum sHLA-G level in patients, compared to the controls and sHLA-G might be used as a tumor marker in breast cancer patients.

Area under the ROC curve of sHLA-G, CA15-3, and CEA in the comparison of pre- and post- groups

The current study is the first to investigate plasma sHLA-G expression in women with breast cancer who did not receive chemotherapy yet and women who received anti-cancer treatment. Data presented in this study demonstrated that plasma sHLA-G expression was significantly higher in the pre group, compared to the post- group (p<0.001). The ROC curves indicated that sHLA-G was superior to CEA and CA15-3 in differentiating between the pre- and post-groups (p<0.001). The AU-ROCs of sHLA-G, CEA, and CA15-3 for differentiating the group of women who did not receive chemotherapy yet and the group of women who received anti-cancer treatment were 0.998, 0.781 and 0.698, respectively. Our findings indicated that sHLA-G levels decreased in patients receiving anti-cancer treatment. This result was supported by Rulten et al.,[29] who found that sHLA-G levels decreased during the treatment of high-grade ovarian carcinomas (p=0.038).

In conclusion, sHLA-G can be used as a tumor marker in the diagnosis of breast cancer and follow-up of the chemotherapy treatment. Furthermore, as Lumachi et al.,[20] also confirmed serum markers CEA and CA15-3 are exclusively correlated with the size of the tumor in patients with breast cancer, and that both have low sensitivity. There is no significant relationship with other prognostic factors. Thus, preoperative CEA and CA15-3 serum levels measurements are of little value in patients with early-stage breast cancer, in particular, and are not useful in the therapeutic decision-making for treating patients with breast cancer. Based on these results, it is obvious that tumor markers are significant tools for the detection and staging of breast cancer and follow-up of the disease progression. In Gaza hospitals, the main tumor markers used in breast cancer are CEA and CA15-3. The results of this study suggest that sHLA-G can be used in the diagnosis and prognosis of the disease.

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