Hepcidin and ferritin blood level as noninvasive tools for predicting breast cancer

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Background: Currently used CA15-3 and CEA have found their clinical application particularly in the follow-up of patients with advanced disease. Novel biomarkers are urgent, especially for improving early diagnosis as well as for discriminating between benign and malignant disease.

Patients and methods: In the present study, we used a proteomic approach based on surface-enhanced laser desorption/ionization–time of flight–mass spectrometry screening with the aim of identifying differentially expressed 2–30 kDa proteins in plasma of patients with malignant (65 cases) and benign (88 cases) breast lesions with respect to 121 healthy controls.

Results: We found that the most promising SELDI peaks were those corresponding to hepcidin-25 and ferritin light chain. We evaluated the capability of these peaks in predicting malignant and benign breast lesions using the area under the receiver operating characteristic curve (AUC). The results showed a good capacity to predict malignant breast lesions for hepcidin-25 [AUC: 0.82; 95% confidence interval (CI) 0.75–0.90] and ferritin light chain (AUC: 0.86; 95% CI 0.79–0.92). Conversely, a weak and satisfactory capability to predict benign breast lesion was observed for hepcidin-25 (AUC: 0.63; 95% CI 0.41–0.85) and ferritin light chain (AUC: 0.73; 95% CI 0.49–0.97). A significant association between HER2 status and hepcidin-25 was observed and the distribution of transferrin and ferritin were found significantly different in patients with breast cancer when compared with that of controls.

Conclusions: This study provides evidence that hepcidin and ferritin light chain level in plasma may be of clinical usefulness to predict malignant and benign disease with respect to healthy controls.

Key words: breast tumors, hepcidin, ferritin, iron metabolism, cancer biomarker, plasma

Introduction

The breast cancer diagnosis is a complex and protracted process. The diagnostic accuracy of mammography and the current screening modality are dependent on age, breast density, and tumor characteristics [1].

The need for novel, specific, and portable biomarkers is still urgent, especially for improving early diagnosis as well as for discriminating between benign and malignant diseases.

Recent advances in genomics and proteomics identified specific cancer biomarker profiles in bio-fluids that however need further development and validation.

Nevertheless, it is evident that early detection of breast cancer in blood is both clinically appealing and technically challenging due to the scarce disease contribution and heterogeneity. Today, even though major breast cancer subtypes have been characterized, i.e. Luminal A, Luminal B, HER2-positive, and Basal-like, little is known about the heterogeneity of breast cancer in blood.

To overcome these limitations and to identify potentially useful biomarkers our pragmatic approach was focused on the identification of changes in plasma peptides/proteins potentially useful to discriminate between patients with cancer and healthy subjects as well as patients with related benign conditions. To do this, plasma from 88 patients with breast benign lesions, 65 with breast cancer, and 121 healthy subjects were analyzed using surface-enhanced laser desorption/ionization–time of flight–mass spectrometry (SELDI-TOF-MS), also known as ProteinChip Array technology.
patients and methods

Cohort 1 included both benign \( (n = 10) \) and malignant \( (n = 65) \) breast tumors patients (cases) together with healthy controls subjects \( (n = 121) \); cohort 2 consisted of only benign breast tumors patients \( (n = 78) \). Among these, 25 cases were hyperplasia, 24 fibroadenoma, 11 mastopathy, 7 papilloma, 5 fibrosis, and 6 other benign tumors (3 benign phyllodes tumors, 1 tubular adenoma, 2 hyperplasia, and papilloma) (see supplementary material File S1, available at *Annals of Oncology* online).

In cohort 1, median age was 45 years (range: 28–79 years) and 59 years (range: 29–82 years) for patients with benign and malignant breast lesions, respectively. In the controls, the median age was 45 years (range: 19–77 years). In the independent series of patients with benign breast lesion (cohort 2), age ranged between 17 and 77 years with a median of 45 years. The clinical–pathological characteristics of the malignant tumors are reported in Table 1. Written informed consent was obtained from all subjects upon approval of the study by the institutional review board and independent ethics committee.

**SELDI-TOF-MS spectrum acquisition**

Samples were processed using IMAC30 surface biochips (IMAC30 Bio-Rad Laboratories, Milan, Italy), as described in supplementary material File S1, available at *Annals of Oncology* online. Peptide mass fingerprints and amino acid sequencing results were entered into the Mascot database (http://www.matrixscience.com) for protein identification (supplementary Files S2 and S3, available at *Annals of Oncology* online).

**biochemical variables**

Determination ferritin, transferrin, and iron was carried out using a COBAS C6000 automatic analyzer (Roche, Milan, Italy). Total transferrin was measured by ELISA assay (R&D Systems, Inc., Italy). In Table 2, some descriptive statistics of the biochemical variables distribution are reported according to the disease status in the cohort 1 and cohort 2.

**statistical analysis**

By considering the cohort 1, the comparison of the distribution of each of the considered continuous variables (peaks, iron, ferritin, transferrin) in patients with malignant breast tumors with that of controls was carried out by using the Wilcoxon–Mann–Whitney test \([2]\). This approach was also used to compare the distribution of each of the considered peak in patients with benign breast lesions with that of controls. The relationship between each peak and the disease status was investigated by resorting to a logistic regression model adjusted by age \([3]\). In order to investigate the form of relationship between the disease status and the considered peaks and detect possible nonlinear effects, we resort to a regression model based on restricted cubic splines \([4]\). We investigated the predictive capability (i.e., diagnostic performance) of each logistic model by means of the area under the receiver operating characteristic (ROC) curve \((AUC) [5]\). Value of AUC is expected to be 0.5 in absence of predictive capability, whereas it tends to be 1.00 in the case of high predictive capacity. To aid the reader to interpret the value of this statistic, we suggest that values between 0.6 and 0.7 be considered as indicating a weak predictive capacity, values between 0.71 and 0.8 a satisfactory predictive capacity, and values >0.8 a good predictive capacity \([6]\). Bonferroni’s correction was used to adjust for multiple comparisons \([7]\).

The relationship between the clinical–pathological variables and each of the considered peaks in patients with malignant breast tumors was investigated by resorting to the Kruskal–Wallis test \([2]\). This approach was also used to assess the relationship between each of the considered continuous variable and the type of lesion in the independent series of patients with benign breast lumps (cohort 2). The strength of association of each of the considered peaks with each of the biochemical variables was assessed with the Spearman correlation coefficient \((r_s)\) and its 95% confidence interval \((CI)\) computed according to the bias-corrected and accelerated (BCa) bootstrap method (95% CI\(_{BCa}\) \([8]\). All statistical analyses were carried out with the SAS software (Version 9.2.; SAS Institute, Inc., Cary, NC) by adopting a significance level of \(\alpha = 0.05\).
results

SELDI-TOF-MS

IMAC 30 ProteinChip arrays were used to analyze plasma samples from subject of both the considered cohorts. Protein profiles were acquired in the m/z range from 2000 to 30 000. After data processing by Biomarker Wizard Software, a total of 22 peaks resulted over-expressed in patients with breast cancer with respect to the controls one. According to the platform algorithm, the most differential peak had an m/z of 2792 (P = 2.1E−22) that was identified as hepcidin-25 by using mass spectrometry-based sequencing of the chip eluate (supplementary File S2, available at Annals of Oncology online). The second most differential peak had an m/z of 19 889 (P = 4.4E−09) and was identified as ferritin light chain (supplementary File S3, available at Annals of Oncology online).

predictive capability of Hepcidin-25 and ferritin light chain

The box-plots reported in Figure 1A (panel I and II) describe the distribution of hepcidin-25 and ferritin light chain in patients with malignant, benign breast lesions and in controls. Using the Wilcoxon–Mann–Whitney test, we found that the difference of the distributions of each peak in patients with malignant breast tumors compared with that of controls (P < 0.001). Conversely the distribution of ferritin was significantly higher in patients with malignant breast tumors compared with that of controls (P < 0.001). By considering the patients of the cohort 2, we did not observe a statistically significant association between the type of lesion and hepcidin-25 as well as between each of the biochemical variables and the type of lesion.

pairwise comparisons between peaks and biochemical variables

By considering the 95% CI_BCA, we observed a relevant positive correlation between hepcidin-25 and ferritin in the cohort 1 for both patients with malignant breast tumors (n = 41, rS = 0.75, 95% CI_BCA 0.55–0.87) and controls (n = 15, rS = 0.92, 95% CI_BCA 0.78–1.00). In the same cohort, we observed, although less relevant, a negative correlation between hepcidin-25 and transferrin in both patients with malignant breast tumors (n = 45, rS = −0.33, 95% CI_BCA −0.60 to −0.03) and controls (n = 29, rS = −0.59, 95% CI_BCA −0.78 to −0.37). Similarly, by considering the patients with benign breast lesions (cohort 2), we observed a positive relationship between hepcidin-25 and ferritin (n = 62, rS = 0.85, 95% CI_BCA 0.75–0.92) and a negative one between hepcidin-25 and transferrin (n = 77, rS = −0.54, 95% CI_BCA −0.68 to −0.36). In addition, we observed a positive correlation between hepcidin-25 and iron in the control subjects of the cohort 1 (n = 29, rS = 0.66, 95% CI_BCA 0.33–0.83) and in the benign ones of the cohort 2 (n = 76, rS = 0.37, 95% CI_BCA 0.13–0.56).

discussion

Using SELDI-TOF-MS, we report the first quantitative measurements of bioactive plasma hepcidin-25 in patients with benign and malignant breast tumors compared with healthy controls women. The results show that breast cancer patients had significantly higher hepcidin-25 and ferritin levels in patients with malignant breast tumors compared with that of controls (P < 0.001). Conversely the distribution of ferritin was significantly higher in patients with malignant breast tumors compared with that of controls (P < 0.001). By considering the patients of the cohort 2, we did not observe a statistically significant association between the type of lesion and hepcidin-25 as well as between each of the biochemical variables and the type of lesion.

Table 2. Descriptive statistics of the investigated biochemical variables

<table>
<thead>
<tr>
<th>Biochemical variables</th>
<th>Modalities</th>
<th>n</th>
<th>Min</th>
<th>25th centile</th>
<th>Median</th>
<th>75th centile</th>
<th>Max</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin (mg/dl)</td>
<td>Malignant BT</td>
<td>45</td>
<td>221.00</td>
<td>262.00</td>
<td>274.00</td>
<td>300.00</td>
<td>395.00</td>
<td>38.00</td>
</tr>
<tr>
<td>Controls</td>
<td>29</td>
<td></td>
<td>226.00</td>
<td>296.00</td>
<td>307.00</td>
<td>346.00</td>
<td>486.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Benign BL*</td>
<td>77</td>
<td></td>
<td>216.00</td>
<td>274.00</td>
<td>301.00</td>
<td>332.00</td>
<td>461.00</td>
<td>58.00</td>
</tr>
<tr>
<td>Iron (µg/dl)</td>
<td>Malignant BT</td>
<td>48</td>
<td>36.00</td>
<td>84.50</td>
<td>96.50</td>
<td>118.00</td>
<td>206.00</td>
<td>33.50</td>
</tr>
<tr>
<td>Controls</td>
<td>29</td>
<td></td>
<td>32.00</td>
<td>78.00</td>
<td>95.00</td>
<td>128.00</td>
<td>180.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Benign BL*</td>
<td>76</td>
<td></td>
<td>18.00</td>
<td>55.50</td>
<td>80.50</td>
<td>111.50</td>
<td>184.00</td>
<td>56.00</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>Malignant BT</td>
<td>41</td>
<td>19.37</td>
<td>44.88</td>
<td>74.22</td>
<td>149.10</td>
<td>410.60</td>
<td>104.22</td>
</tr>
<tr>
<td>Controls</td>
<td>15</td>
<td></td>
<td>7.45</td>
<td>14.62</td>
<td>27.03</td>
<td>50.07</td>
<td>92.99</td>
<td>35.45</td>
</tr>
</tbody>
</table>

*Patients with benign breast lesions patients from cohort 2.
BT, breast tumor; BL, breast lesion; IQR, interquartile range (75th–25th centile).
malignancy. In addition, hepcidin levels result positively correlated with ferritin and inversely correlated with transferrin.

Ferritin is currently considered an indicator of iron status and is used to monitor the presence of malignant disease; it is regarded as a predictor of positive lymphonode involvement in patients with breast cancer [9]. Although the source of serum ferritin in physiological or pathological conditions is still unclear, several studies indicate that plasmatic ferritin is produced and secreted by hepatocytes, macrophages, and cancer cells [10]. The elevation in serum ferritin in breast cancer patients, which

Figure 1. (A) Distribution of hepcidin-25 and ferritin light chain in cohort 1. Box plots reflecting the distribution of hepcidin-25 (panel I) and ferritin light chain (panel II) in patients with malignant, benign breast lesions and in control subjects. Each box indicates the 25th and 75th centiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values. (B) Receiver operating characteristic (ROC) curves in cohort 1. ROC curves derived from the univariate logistic analysis corresponding to hepcidin-25 (panel I) and ferritin light chain (panel II).

Table 3. Logistic regression analysis in cohort 1

<table>
<thead>
<tr>
<th>Model</th>
<th>Malignant BT versus controls</th>
<th>Benign BL versus controls</th>
<th>Malignant BT versus controls</th>
<th>Benign BL versus controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin-25</td>
<td>1.423</td>
<td>0.702</td>
<td>1.423</td>
<td>0.702</td>
</tr>
<tr>
<td>Ferritin light chain</td>
<td>7.520</td>
<td>6.986</td>
<td>7.520</td>
<td>6.986</td>
</tr>
</tbody>
</table>

*Bonferoni adjusted.

aAdjusted for age at blood withdrawal.

BT, breast tumor; BL, breast lesion; OR, odds ratio; CI, confidence interval; AUC, area under the ROC curve.
correlates with tumor stage may partly reflect an inflammatory state involving tumor-associated macrophages [11]. Recent studies indicate that ferritin light chain expression in tumor-associated macrophages may be prognostic in node-negative breast cancer [12].

The results of the present study are consistent with previous studies [13] showing that ferritin was closely correlated with plasma hepcidin. Hepcidin is mainly synthesized by the liver and its expression in hepatocytes increases in response to infection/inflammation. If macrophages are an important source of serum ferritin, it may explain the fact that serum ferritin is elevated in inflammation, when increased hepcidin levels inhibit iron recycling from macrophages, causing macrophage iron retention, systemic iron deficiency, and anemia.

Studies in patients with multiple myeloma show that hepcidin is upregulated in these patients by both IL-6-dependent and IL-6-independent mechanisms that may play a role in the anemia often observed in these patients. The IL-6-independent pathway may be attributed to increased bone morphogenetic protein-2 concentrations, which have been suggested to act synergistically with IL-6 to induce hepatocyte hepcidin synthesis [14, 15]. For Hodgkin lymphoma, it has been recently demonstrated that increased IL-6 production is associated with induction of hepcidin, which contributes to the iron-restricted anemia of chronic disease often observed at Hodgkin lymphoma diagnosis [16].

Iron metabolism is frequently altered in breast cancer tissues and upregulation of iron import and downregulation of iron export may enable breast cancer cells to acquire and retain excess iron [17].

Which is the reason why hepcidin is increased in plasma of malignant breast tumor patients? Hepcidin upregulation in the...
liver can be determined by the interplay of pathways controlled by iron status, erythropoietic activity, and inflammation, and the relative strength of each of the individual signals (Figure 2). The induction of hepcidin in response to either inflammatory stimuli or elevated serum iron could be related to IL-6 tumor secretion IL-6 and/or dependent on signals provided by a subset of BMPs (Figure 2). Of note, we found higher hepcidin increase in HER2-positive tumors in agreement with a recent article in which high levels of IL-6 were found to be correlated with HER2 overexpression [11]. Several studies indicate that BMP signaling is hyperactivated during breast cancer initiation and progression and that it enhances tumor stem cell populations and epithelial–mesenchymal transition, indicating a possible involvement.

Of special interest, recent data established in a mouse model supports the concept that also the estrogens are implicated in modulating iron homeostasis by governing hepatic hepcidin expression [18]. These findings regarding the effects of estrogen on iron metabolism might explain the increase in iron accumulation in estrogen-deficient conditions such as menopausal disorders.

In conclusion, we found that hepcidin-25 and ferritin light chain have both a good capacity to predict breast cancer. Additionally, the observed borderline statistical significance for ferritin light chain would suggest its potential capability to satisfactory predict also benign breast lesions. A significant association between HER2 status and increased hepcidin-25 was also observed.

The mechanistic basis of the relationship between breast cancer, hepcidin, and ferritin light chain remains only in part elucidated, suggesting the urgent need for research in this area.

disclosure
The authors have declared no conflicts of interest.

references

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