RESEARCH ARTICLE

Elevated Serum Haptoglobin is Associated with Clinical Outcome in Triple-Negative Breast Cancer Patients

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Abstract

Background: Breast cancer is the most common malignancy with the highest incidence rates among women worldwide. Triple-negative breast cancer (TNBC) disease is diagnosed more frequently in younger women, and is associated with a poor prognosis. Elevated levels of serum haptoglobin protein (Hp) are observed in many malignant diseases including breast cancer. We evaluated the expression and prognostic value of Hp among patients with TNBC. Materials and Methods: Serum Hp levels were determined by Elisa in 41 patients with TNBC and 10 normal individuals. Hp status was correlated with other clinico-pathological parameters including patient survival. Results: Of the 41 patients with TNBC, Hp over expression was detected in 24 (59%) by Elisa. Hp up-regulation was confirmed by Elisa based quantification in the serum of 41 TNBC patients against lower grades and 10 normal individuals. Survival analysis revealed that Hp ($p=2.016x10^{-5}$), stage ($p=2.166x10^{-5}$), distant metastasis ($p=2.217x10^{-5}$), tumor size ($p=1.053x10^{-5}$), and tumor grade ($p=0.001$), correlated with patient survival on univariate analysis. Multivariate analysis revealed that Hp ($p=0.001$), and grade of the disease ($p=0.008$) were independent predictors of survival. Conclusion: Our results indicate that serum levels of Hp may play a role as a potential serum biomarker and prognostic indicator among TNBC patients. Thus, Hp may present a new promising prognostic biomarker in TNBC patients, but independent validations are now necessary for confirmation.

Keywords: Triple negative breast cancer - Hp - prognosis - TNBC

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Introduction

Triple-negative breast cancer (TNBC) is defined as hormone receptor-negative and HER2/*neu*-negative breast cancer. Triple negative breast cancer (TNBC) is associated with poor prognosis because of aggressive clinical features, and lack of targeted agents such as trastuzumab or tamoxifen (Gluz et al., 2009; Keam et al., 2011). TNBC accounts for about 11%-20% of all breast cancers. It is diagnosed more frequently in younger and premenopausal women and patients with TNBC who have significantly increased risk of relapse and death (Curigliano and Goldhirsch, 2011; Lee et al., 2011; Fornier and Fumoleau, 2012). However, predictive and prognostic values of TNBC phenotype are relatively undetermined. Therefore, there is a clinical need to identify new prognostic biomarkers that can be used to predict a therapeutic response and clinical outcomes in TNBC patients to rationalize treatment decisions. Up-regulation of biomarkers in the serum of cancer patients by Elisa has helped to identify new markers that have diagnostic, therapeutic and prognostic value (Kawai, 1995). Haptoglobin (Hp) is a plasma glycoprotein, the main biological function of which is to bind free hemoglobin (Hb) and prevent the loss of iron and subsequent kidney damage following intravascular hemolysis. Haptoglobin is also a positive acute-phase protein with immunomodulatory properties. In humans, the HP locus is polymorphic, with two codominant alleles (HP1 and HP2) that yield three distinct genotypes/phenotypes (Hp1-1, Hp2-1 and Hp2-2) (Sadrzadeh and Bozorgmehr, 2004). Elevated levels of haptoglobin have been demonstrated in lung cancer (Beckman et al., 1986), bladder cancer (Benkmann et al., 1987), leukemia (Mitchell et al., 1988), breast cancer (Awadallah and Atoum, 2004), malignant lymphoma (Epelbaum et al., 1998), urogenital tumors (Dunzendorfer et al., 1980), esophageal squamous cell carcinoma (An et al., 2004), ovarian cancer (Zhao et al., 2007) and gliomas (Kumar et al., 2010).

Several studies have shown Hp polymorphism in breast cancer patients, others have reported an increase in the rate at which the disease occurred among patients with Hp1-1 type (Tsamantanis et al., 1980; Kaur et al., 1984; Bartel et al., 1985). However, no information is available

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in the literature about the serum specific expression of Hp in TNBC, differential expression between normal and breast cancer patients, involving different grades and also its significance in prognosis.

The aim of this study was to quantify expression levels Hp in the clinical samples of TNBC, and to evaluate their association with clinical-pathological features to identify new prognostic and/or predictive biomarkers for TNBC patients.

Materials and Methods

Patients and samples

A total of 41 triple negative breast carcinoma patients and 10 healthy female individuals were recruited from the tertiary health care centre. The period of study is from 2008 till 2012. Mean age of the patient cohort was 46 years. Serum samples were frozen and maintained at -80˚C until the assay was conducted. All 41 breast cancer patients and volunteers signed informed consent forms. Patients who met the following eligibility criteria were included: (1) all patients who underwent surgery (modified radical mastectomy, lumpectomy), all patients who were triple negatives, (2) availability of follow-up data; (3) no history of familial malignancy. The main characteristics of the patients with respect to age, menopausal status, stage, tumor size, distant metastasis, nodal status and different grades are shown in Table I. The study was approved by the ethics committee of the hospital. Clinical data were reviewed retrospectively from medical records.

ELISA based quantization of serum haptoglobin level

Human Hp Elisa quantitation kit (Cat# 40-288-20080F) from Genway Biotech (SanDiego, CA) was used to measure serum Hp levels as per manufacturer's instructions. In brief, 100/well of primary antibody at concentration 5 mg/ml of 0.05 M $NaHCO_3$, pH 9.6 was coated onto a 96-well Elisa plate, followed by the addition of diluted human serum ($1:10^5$) or Hp standards and then HRP conjugated anti-Hp antibody. The plate was then developed by the addition of 100 micro litres of tetramethylbenzidine; reaction was stopped by 2 M sulfuric acid and read at 450 nm and 570 nm.

Data Analysis and Statistics

We investigated the status of serum Hp by Elisa. Mean age of the patient cohort was 46 years. The period of study is from 2008 till 2012.The mean survival period was (Median: 44 months; Range: 5 months to 3.8 years). DFS survival was defined as the duration between surgery and recurrence, metastasis, death of the patient due to disease or the last follow up. The correlation of expression of Hp gene with survival was assessed by Cox regression method using statistical analysis software SPSS version 15.0. Multivariate analysis was carried out for variables which showed correlation with survival using univariate analysis. Comparison between two groups was performed by Mann-Whitney test using GraphPad Prism 5.01 (www.graphpad.com). Kaplan-Meier method was used to estimate DFS survival. Graph Pad Prism 5.01 software was used for Kaplan-Meier graph plotting and calculation of P-values. P-values less than 0.05 were considered significant.

Results

Elevation of Hp in TNBC serum:

To quantify the Hp amount in case and control serum samples, the Elisa was applied to a population of 41 patients with TNBC, and 10 normal controls. TNBC sera showed elevated levels in comparison to normal controls. TNBC patient's sera had significantly high levels of haptoglobin with a median level of 6.5mg/ml in comparison to normal controls with a p-value of <0.0001. (Figure 2A).

Serum Hp levels in TNBC patients correlate with grade:

Hp serum levels were measured in the available TNBC serum samples belonging to different grades and normal controls. Statistically significant differences were detected among different grades and normal controls (grade 2 v/s grade 3, $p \leq 0.0001$) (Figure 2B).

Correlation between patient survival and Hp expression:

Survival analysis revealed that Hp over expression is found to be poor prognostic indicators. At 44 months time during follow up, 54.0% of Hp negative individuals had DFS. (Figure 1; $\chi^2 = 11.44$, p=0.0007). This suggests an inverse correlation between Hp status and disease free survival as reported earlier.

Univariate Cox regression analysis:

Hp along with different clinical parameters was subjected to univariate Cox proportional hazard regression analysis. Hp, Stage of the disease, distant metastasis, tumor size and grades correlated with TNBC patient survival (Table 2). However, parameters like age,

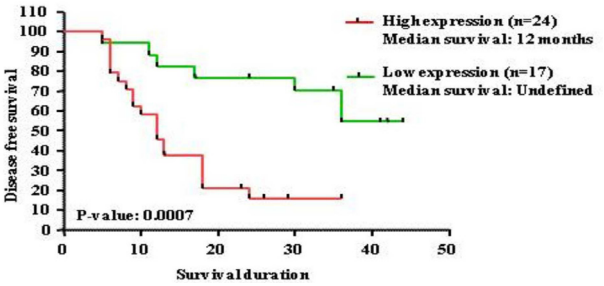

Figure 1. Kaplan-Meier Graph Showing Survival Patterns for Hp Status and Disease Free Survival Among TNBC Patients.

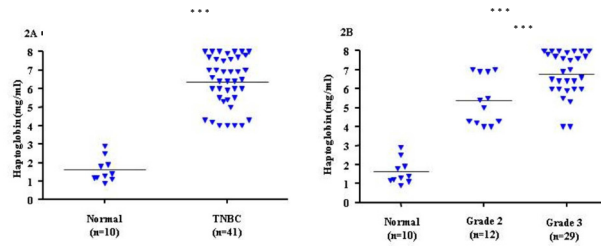

Figure 2. Scatter Plot of Serum Hp Levels. (A) Serum levels of Hp (ng/ml) in normal controls and TNBC are plotted. (B) Serum levels of Hp (ng/ml) in normal controls and different grade levels among TNBC are plotted. Statistical significance Was tested by Mann-Whitney test using Graph pad PRISM software. Asterisks. ***refer to p-value of <0.0001.

Table 1. Characteristics of TNBC Patients

		Total	Percentage (%)
Haptoglobin	Positive	24	59%
	Negative	17	41%
Age: Range 46 (29-62)	≤46	21	51%
	>46	20	49%
Menopausal status	Pre	21	51%
	Post	20	49%
Stage	I	3	7%
	II	11	27%
	III	12	29%
	IV	15	37%
T-stage	T1	5	12%
	T2	13	32%
	T3	7	17%
	T4	16	39%
Distant metastasis	Positive	15	37%
	Negative	26	63%
Lymph Nodal status	Positive	34	83%
	Negative	7	17%
Grade	2	12	29%
	3	29	71%

Table 2. Univariate Cox Regression Analysis

Variable	Regression coefficient	Hazard ratio (95% CI)	P-Value
Haptoglobin	1.019	2.770 (1.734-4.426)	$2.016x10^{-5}$
Stage	1.316	3.728 (2.031-6.843)	$2.166x10^{-5}$
Distant metastasis	2.155	8.631 (3.535-21.072)	$2.217x10^{-5}$
Lymph node status	0.04	1.041 (0.358-3.025)	0.941
Tumor size	1.182	3.260 (1.927-5.515)	$1.053x10^{-5}$
Grade	2.502	12.203 (2.871-51.870)	0.001
Menopausal status	0.441	1.554 (0.719-3.361)	0.262
Age	0.567	1.763 (0.815-3.813)	0.15

Table 3. Multivariate Cox Regression Analysis

Variable	Regression coefficient	Hazard ratio (95% CI)	P-Value
Haptoglobin	0.828	2.289 (1.421-3.688)	0.001
Grade	1.992	7.334 (1.669-32.224)	0.008

menopausal status and lymph node involvement of the patient showed no significant correlation with patient survival.

Multivariate analysis

The markers which stood significant in univariate analysis were subjected to forward condition multivariate Cox proportional hazard regression analysis. We found that Hp (HR=2.289; B=0.828; p=0.001), and confounding factor grade (HR=7.334; B=1.992; p=0.008) were independent predictors of survival in TNBC patients (Table 3). However, stage of the disease, distant metastasis, and tumor size lost their significance in multivariate analysis.

Discussion

Haptoglobin expression is characteristic of many types of malignant tumors, including breast cancer. In this study, we analysed the serum specific expression of Hp protein in TNBC, differential expression between normal and breast cancer patients, involving different grades and also its significance in prognosis. We showed that levels of Hp protein can be reproducibly measured in serum of TNBC patients. Our data suggest that Serum Hp levels are high in TNBC patients, compare to normal controls. Furthermore, we demonstrate that increasing levels of serum Hp in patients corresponds to different grades. In addition, the patient serum belonging to grade 3 had substantially higher levels of Hp than the lower grade patients with statistical significance. Carlsson, et al has shown Hp over expression in breast cancer patients using small number of samples (Carlsson et al., 2011), no information is available in the literature about TNBC specific expression of Hp, differential expression between different grades of TNBC and also significance of over expression. Survival analysis indicated that TNBC patients expressing higher level of Hp in their serum samples had a shorter survival. At 44 months during follow up 54% of Hp negative individuals had dfs survival (undefined vs 12 months; p=0.0007). In a previous study, over expression of Hp in breast carcinoma, assessed by SELDI-TOF MS, is not associated with prognosis (Gast et al., 2008).

Univariate survival analysis showed that over expression of Hp, advance stage, larger tumor size, distant metastasis, and grade of the tumor were all found to be poor prognostic indicators. Other parameters like age, menopausal status, and nodal involvement were all found to have no effect on survival pattern.

A Cox multivariate regression analysis revealed that Hp was a significant predictor of overall survival (HR=2.289; B=0.828; p=0.001), along with confounding factor grade (HR=7.334; B=1.992; p=0.008). However advance stage, distant metastasis, and larger tumor size lost their significance in multivariate analysis.

Our study is first of its kind to demonstrate Hp over expression by serum Elisa among TNBC patients and also demonstrated that increasing levels of serum Hp in patients corresponds to different grades among TNBC population. Our survival analysis therefore provides evidence that Hp over expression could be a useful independent prognostic indicator in patients with TNBC. Over all our data suggests that lower haptoglobin level is associated with better survival out come. More studies are however warranted to confirm these findings.

However, no information is available in the literature about the serum specific expression of Hp in TNBC, differential expression between normal and breast cancer patients, involving different grades and also its significance in prognosis. In conclusion, Thus the over expression of Hp in TNBC patients may result in poor survival. Indeed the survival analysis in our study suggests a prognostic value for Hp expression. We have identified Hp as a potential grade specific TNBC serum marker of prognostic importance. More importantly, this marker could serve as a potential therapeutic target for TNBC patients.

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