The European Research Journal 2023;9(3):529-535 Original Article

DOI: 10.18621/eurj.1149697 *Molecular Biology*

Evaluation of hematopoietic - and neurologic-expressed sequence 1-like (HN1L) protein levels in tissue and plasma of breast cancer patients

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ABSTRACT

Objectives: Breast cancer is the second leading cause of cancer deaths among women. Therefore, there is a need for new approaches that increase the success of treatment in breast cancer. Cancer stem cells (CSCs) are associated with treatment resistance and metastasis, which are important problems in cancer treatment including breast tumors. In this study, the Hematopoietic- and neurologic-expressed sequence 1-like (HN1L), also known as Jupiter microtubule associated homolog 2 (JPT2) protein levels which is involved in the self-renewal of CSCs were evaluated in common and rare breast tumor types.

Methods: In this context, HN1L protein levels were measured from plasma of 17 patients and from tumor and normal tissues of 9 patients by enzyme linked immunosorbent assay method.

Results: Mean HN1L levels were measured as 1.63 ± 0.88 ng/mL in plasma samples, 2.18 ± 0.75 ng/mL in tumor tissue samples and 2.71 ± 0.88 ng/mL in normal tissue samples. A significant difference was observed between mean HN1L levels in plasma and normal tissue ($p < 0.05$). Correlation of HN1L protein levels with clinicopathological characteristics were analyzed. Accordingly, HN1L levels were positively correlated with tumor size and invasion status ($r = 0.425$; $p < 0.05$ and $r = 0.449$; $p < 0.05$, respectively).

Conclusions: We believe that the importance of HN1L in management of breast cancers will be demonstrated more thoroughly when further studies are conducted with increased number of patients.

Keywords: HN1L, JPT2, breast cancer, cancer stem cell

Cancer is one of the leading causes of mortality and morbidity all over the world and is accepted as one of the most common diseases in the world after cardiovascular diseases [1]. According to the GLOBOCAN database, part of the International Agency for Research on Cancer (IARC), there were 19.3 million new cancer cases and 10.0 million cancer deaths in 2020. In the same year, the most frequently diagnosed cancer in men were lung (25.8%), followed by prostate (14.6%) and colorectal cancer (9%). In women, breast cancer (23.9%) is the leading cause of death, followed by thyroid (10.9%) and colorectal cancer (9.1%). Breast cancer is the most frequently diagnosed cancer type in the world and is estimated to be the leading cause of cancer death in women worldwide. The latest data show that there were 2.26 million

Received: ***July 27, 2022;*** *Accepted:* ***October 19, 2022*** *Published Online:* ***March 10, 2023***

How to cite this article: *Erturk E, Sarimahmut M, Gokgoz MS, Tolunay S. Evaluation of hematopoietic- and neurologic-expressed sequence 1-like (HN1L) protein levels in tissue and plasma of breast cancer patients. Eur Res J 2023;9(3):529-535. DOI: 10.18621/eurj.1149697*

e-ISSN: 2149-3189

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cases of breast cancer in 2020 [2]. With the increase in targeted treatment options in recent years, the need for more effective management of breast cancer treatment has also increased. Research and clinical use of new molecular biomarker candidates will make significant contributions to the treatment of the disease.

Hematopoietic - and neurologic-expressed sequence 1-like (HN1L), also known as Jupiter microtubule associated homolog 2 (JPT2), is a protein with very limited information and is thought to play a role in embryo development [3]. HN1L gene is located on chromosome 16p13.3 that encodes a 20-kDa protein [3]. HN1L protein, which is localized in the nucleus and cytoplasm of the cell, has been found in liver, kidney, uterus, testis and prostate tissues [3]. There are a limited number of studies revealing the role of HN1L protein in various types of cancer. It has been reported that the HN1L gene is altered in a quarter of breast cancer cases and is correlated with low overall survival in triple negative breast cancer (TNBC) cases [4]. On the other hand, it was revealed that the number of breast cancer stem cells has decreased and sensitivity to chemotherapy was improved by silencing HN1L [4]. HN1L plays a role in the self-renewal processes of cancer stem cells (CSCs). Due to the high frequency of mutations in this gene, it has been determined that measuring mutant HN1L gene fragments in the bloodstream can exhibit tumor burden and be effective in tracking progression [5]. In another study, it was shown that HN1L is highly expressed in breast cancer tissues, positively correlated with metastasis in breast cancer patients, and significantly inhibited invasion and metastasis when silenced [6]. In terms of its molecular interactions, the HN1L protein has been associated with the signal transducer and activator of transcription (STAT) pathway and has been reported to upregulate the expression of high-mobility group protein 1 (HMGB1) protein, which plays a key role in breast cancer invasion and metastasis [4, 6].

In the current study, it is aimed to investigate the potential of HN1L as a promising biomarker candidate in breast cancer.

METHODS

Patients and Clinical Characteristics

Nine fresh tissue (tumor and normal) samples and 17 plasma samples were collected from breast cancer patients who were admitted to Breast Surgery Department. The study was approved by the local Clinical Research Ethics Committee (2021-10/44) and conducted in accordance with Helsinki Declaration. Informed consent was obtained from all patients. Clinicopathological characteristics of the patients were obtained from Breast Surgery and Medical Pathology Departments. Histological and molecular subtypes of breast cancer were defined by respective clinical departments.

HN1L Enzyme Linked Immunosorbent Assay

HN1L protein levels were measured from plasma of 17 patients and from tumor and normal tissues of 9 patients by Enzyme linked immunosorbent assay (ELISA) method. Whole blood samples collected into EDTA-coated tubes from patients were centrifuged at 2000 × g for 10 min (Nüve NF800R, Turkey). Then, 1 mL aliquots of the supernatant were transferred into cryovials for long term storage. Tissue samples were cut into thin pieces with a scalpel and subsequently incubated with RIPA lysis buffer supplemented with protease inhibitors and sodium vanadate in a microcentrifuge tube for 20 min. Tumor and normal tissues were homogenized on ice for 1 min at 900 rpm (Schuett Homgenplus, Germany). The homogenized samples were centrifuged at 10,000 × g for 10 min at 4 °C (Nüve NF800R, Turkey). Clean supernatants were transferred to a new 1.5 ml microcentrifuge tube and stored at 4 °C to determine protein concentration and perform ELISA. Bicinchoninic acid assay was utilized to evaluate total protein concentration as described by Smith *et al*. [7]. All accumulated plasma and tissue samples were stored at −80°C until further use.

For ELISA, HN1L protein levels were evaluated according to the manufacturer's instructions (Abbexa, Cambridge, United Kingdom). Briefly, standard solutions, reagents and samples were prepared. Equal amounts of protein at 100 µL volume were loaded into each well of a 96-well plate. Also, 100 µL of standard solutions were pipetted in order to generate a calibration curve. After different incubation times and washing steps, 100 µL of biotin conjugated detection antibody, 100 µL of HRP (horseradish peroxidase) conjugated detection antibody, 90 µl of TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution and

Table 1. Clinicopathological characteristics of patients from plasma and tumor tissue group

Characteristics	Plasma (n = 17)	Tumor tissue (n = 9)	*p* value
Gender, n (%)			
Female	17 (100.0)	8 (88.9)	0.346[a]
Male	-	1 (11.1)	
Age (years), mean ± SD	57.47 ± 12.28	55.44 ± 15.74	0.742[b]
Histological subtype, n (%)			
Invasive ductal	16 (94.1)	1 (11.1)	**0.001[c]**
Invasive lobular	-	2 (22.2)	
Mucinous	-	1 (11.1)	
Tubular	-	1 (11.1)	
Malignant phyllodes tumor	-	1 (11.1)	
Mixed type	1 (5.9)	3 (33.3)	
Molecular subtype, n (%)			
Luminal A	7 (41.2)	8 (88.9)	**0.024[a]**
Luminal B	10 (58.8)	1 (11.1)	
ER positive, n (%)	17 (100.0)	8 (88.9)	0.346[a]
PR positive, n (%)	13 (76.5)	7 (77.8)	0.668[a]
HER2 positive, n (%)	2 (11.8)	-	0.453[a]
Tumor localization, n (%)			
Right	9 (52.9)	5 (55.6)	0.613[a]
Left	8 (47.1)	4 (44.4)	
Tumor stage, n (%)			
I	1 (5.9)	5 (55.6)	
II	13 (76.5)	3 (33.3)	**0.017[c]**
III	3 (17.6)	1 (11.1)	
BRCA negative, n (%)	1 (7.1)	-	0.609[a]
P53 positive, n (%)	3 (17.6)	-	0.262[a]
P63 positive, n (%)	8 (47.1)	3 (33.3)	0.402[a]
Calponin positive, n (%)	8 (47.1)	3 (33.3)	0.402[a]
E-Cadherin positive, n (%)	17 (100.0)	3 (33.3)	0.000[a]
CK5/6 positive, n (%)	10 (66.7)	3 (33.3)	0.122[a]
In situ component, n (%)			
No	6 (35.3)	4 (44.4)	
Under 25%	11 (64.7)	4 (44.4)	0.261[c]
Over 25%	-	1 (11.1)	
Necrosis, n (%)	2 (11.8)	1 (11.1)	0.732[a]
Lymphatic invasion, n (%)	3 (17.6)	-	0.262[a]
Perineural invasion, n (%)	6 (35.3)	-	0.054[a]
Vascular invasion, n (%)	1 (5.9)	-	0.654[a]
Microcalcification, n (%)	7 (41.2)	3 (33.3)	0.517[a]
HN1L (ng/mL), mean ± SD	1.63 ± 0.88	2.18 ± 0.75	0.124[b]
Tumor size, mean ± SD	2.05 ± 1.26	2.66 ± 1.57	0.302[b]
Metastasis status	0.65 ± 0.79	0.67 ± 1.41	0.450[b]
BRH, mean ± SD	1.35 ± 1.46	1.11 ± 0.78	0.587[b]
Ki-67, mean ± SD	155.06 ± 140.31	105.56 ± 124.13	0.383[b]

ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, BRCA = breast cancer susceptibility protein, CK = cytokeratin, BRH = benign reactive hyperplasia, SD = Standard Deviation

[a]Fischer's Exact Test, [b]Independent Samples T-Test, [c]Likelihood Ratio

50 µL of stop solution were added, respectively. Finally, absorbance was measured in a spectrophotometer (EL × 800, BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm. HN1L protein concentration was expressed as ng/mL.

Statistical Analysis

Nominal and ordinal parameters were described as frequencies; whereas, scale parameters were described as mean and standard deviations. Fischer's Exact Test, Chi-Square and Likelihood ratio tests were used to indicate differences between categorical variables. Kolmogorov Smirnov test was used to analyze normality of scale parameters. Independent Samples t-test and One Way ANOVA were used to detect differences of normally distributed scale parameters. Spearman's rho correlation and ROC analysis were implemented for relational analysis. SPSS 17.0 for Windows was used, and 95% Confidence Interval was mentioned at 0.05 significance level.

RESULTS

Management of breast cancer should be improved in order to accomplish better outcomes in clinical practice. CSC hypothesis posits that cells with stem cell like properties take part in processes such as therapy resistance and recurrence; which in turn, lowers the effectiveness of treatment and hinders therapy success [8, 9]. A better understanding of proteins associated with CSCs could shed light on how to overcome the important obstacles in breast cancer treatment. Thus, we have investigated a CSC associated protein, HN1L, to evaluate its levels in plasma and tumor tissue of breast cancer patients and its correlation with clinical parameters. To our knowledge, this is the first study that aims to determine the levels of HN1L protein from the circulation with any type of cancer including breast carcinomas.

The present study included 35 samples from 26 breast cancer patients. The samples were divided into two groups; namely plasma and tumor tissue, to describe the origin of sample. Clinicopathological characteristics of patients is shown in Table 1. Histological subtype distribution in plasma samples was invasive ductal (n = 16) and mixed carcinoma (n = 1); whereas the histological subtype distribution of tissue samples was invasive ductal (n = 1), invasive lobular (n = 2), mucinous (n = 1), tubular (n = 1), mixed (n = 3) carcinoma, and malignant phyllodes tumor (n = 1). Mucinous, tubular and mixed carcinomas and malignant phyllodes tumors are among rare type of tumors that are represented with a frequency of less than 5% within breast carcinomas [10]. There is also a tumor sample collected from a male breast cancer patient which is a rare case as well, with less than 1% frequency of all breast cancer cases [11]. Considering the molecular subtype distribution, plasma and tissue samples of luminal subtypes were selected and included in the study, which in turn contributed to the homogeneity of the study groups.

Circulating HN1L protein levels detected from plasma and levels of HN1L protein measured from homogenized tumor and normal tissue of breast cancer patients are shown in Table 2. Mean HN1L concentration values were ranked from lowest to highest as plasma, tumor tissue and normal tissue. Comparison of HN1L protein levels between plasma, tumor and normal tissue groups demonstrated that HN1L protein levels differ significantly between normal tissue and plasma groups ($p < 0.05$). However, difference in mean HN1L protein levels was statistically insignificant both between tumor and normal tissue groups as well as between tumor tissue and plasma groups ($p > 0.05$). Therefore, the absence of significant difference

Table 2. Comparison of HN1L protein levels between plasma, normal and tumor tissue groups

	Plasma (n = 17)	Tumor tissue (n = 9)	Normal tissue (n = 9)	Total	*p* value
HN1L (ng/mL) mean ± SD	1.63 ± 0.88[a]	2.18 ± 0.75	2.71 ± 0.88[a]	2.05 ± 0.94	**0.014**[b]

[a]Tukey's Post hoc, plasma and normal tissue (mean difference: -1.08; $p = 0.011$)
[b]One Way ANOVA

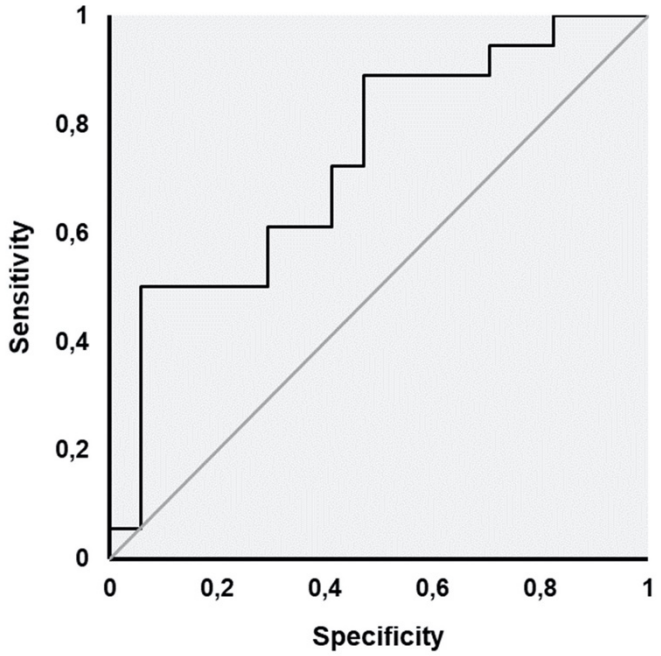

Fig. 1. ROC analysis results for predictive value of HN1L for plasma. According to the results of the ROC analysis AUC was 73.2% and was found to be statistically significant ($p < 0.05$).

between plasma and tumor tissue HN1L protein levels suggests that evaluation of plasma HN1L protein levels may prove to be useful as a surrogate of tumor HN1L protein levels. We have further examined the difference between plasma and normal tissue HN1L levels in detail by analyzing receiver operating characteristic (ROC) curve to determine the discriminatory accuracy (Fig. 1). Predictive value of HN1L for plasma was 73.2% with statistically significant area under curve ($p < 0.05$). Sensitivity for 1.55 cut off level was 52.9% and specificity was 88.9%. For 2.08 cut off level of HN1L, sensitivity was 70.6% and specificity was 61.1%. Overall, this model indicates a good discriminatory ability for the prediction of HN1L concentration from plasma and normal tissue. Then, the correlation between HN1L protein levels and clinical parameters were explored. Spearman's correlation analysis revealed that tumor size (r = 0.449; $p < 0.05$) and lymphatic invasion (r = 0.425; $p < 0.05$) had significant positive correlation with HN1L protein levels (Table 3). However, there was no significant correlation with age, presence of metastasis or any of the assessed pathological parameters. This finding suggests that HN1L protein levels might accurately reflect the tumor size and lymphatic invasion in breast cancer.

Table 3. Spearman's rho correlation analysis results between HN1L protein levels and clinicopathological characteristics

Characteristics	r	*p* value
Sample type	0.286	0.157
Age	0.162	0.430
Histological subtype	0.134	0.514
Molecular subtype	0.057	0.782
ER status	-0.280	0.166
PR status	-0.073	0.723
HER2 status	0.184	0.379
Tumor localization	0.010	0.960
Tumor stage	-0.027	0.897
BRCA status	-0.354	0.098
P53 status	0.120	0.558
P63 status	-0.151	0.463
Calponin status	-0.151	0.463
E-Cadherin status	-0.280	0.166
CK5/6 status	-0.151	0.481
In situ component	-0.106	0.606
Necrosis	-0.088	0.668
Lymphatic invasion	0.425*	**0.030**
Perineural invasion	0.110	0.594
Vascular invasion	0.200	0.327
Microcalcification	-0.179	0.381
Tumor size	0.449*	**0.024**
Metastasis status	-0.093	0.652
BRH	-0.119	0.563
Ki-67	0.200	0.326

ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, BRCA = breast cancer susceptibility protein, CK = cytokeratin, BRH = benign reactive hyperplasia

DISCUSSION

The importance of guiding therapeutic strategy and the management of patients has increased dramatically in recent years with the expansion of available treatment options such as targeted therapies and immunotherapy [12]. In this respect, especially the utilization of circulating tumor DNA analyses into the routine clinical practice has the potential to be an extremely effective

tool in the management of cancer therapy [13-15]. Furthermore, the analysis circulating proteins as a minimally invasive and an inexpensive tool in determination of disease risk, treatment adjustment, prognostication and disease progression monitoring has been a trending research area [16]. In fact, new breakthroughs in early detection of cancer has been achieved with the adoption of both circulating nucleic acids and proteins as new instruments in this field [16-18]. In this regard, investigation of circulating levels of CSC associated proteins might contribute to the improvement of clinical outcome in breast cancer.

There is a limited number of studies regarding the analysis of circulating levels of CSC associated cellular proteins in many types of malignancies including breast cancer. Mirzaei *et al*. [19] has assessed the levels of double cortin like kinase 1 (DCLK1) extracted from peripheral blood mononuclear cells of patients diagnosed with colorectal cancer and healthy individuals who displayed significantly different levels of the protein by ELISA method. Later, Christman *et al*. [20] utilized the same protein and showed that DCLK1 may have potential as a circulating biomarker for therapy response in esophageal adenocarcinoma. Another example is Glutaredoxin3 (GLRX3), a secretory protein that is upregulated in pancreatic ductal adenocarcinoma CSC enriched spheres. GLRX3 protein levels were measured by ELISA from the plasma of both healthy individuals and pancreatic cancer patients which revealed that there was a significant difference between the two groups, a finding that may have implications in diagnosis of pancreatic cancer. Majority of the previous studies on the role of HN1L in different types of cancer demonstrate that overexpression of this protein promotes cell migration, invasion and metastatis in in vitro and in vivo models and is an indication of worse disease free survival in non-small cell lung cancer patients and poor prognosis in hepatocellular carcinoma patients [21, 22]. Furthermore, silencing HN1L expression lowered the capability of breast CSCs to initiate tumor and inhibited tumor formation in vivo in mice injected with prostate cancer cells [4, 21]. According to immunohistochemistry analysis with tumor and non-tumor tissue samples from breast cancer and esophagogastric junction adenocarcinoma patients, an overexpression of HN1L was reported in tumor tissues compared to adjacent normal tissue of the patients [6, 23]. Our findings however did not confirm this data, most probably due to low number of tumor samples and partly because of diverse histopathological background of the collected tumor samples.

To the best of our knowledge, there is only a couple of studies investigating the plasma levels of HN1L in cancer patients which were conducted by our research group. In these studies, the mean HN1L plasma levels were measured as 1.05, 1.70, 0.95 and 1.03 ng/mL from 29 luminal A, luminal B, triple negative and HER2+ subtypes, respectively [24, 25]. These mean values are comparable with our current findings. Moreover, in the previous study, HN1L levels in luminal subtype samples were in positive correlation with lymphatic invasion and tumor size which is in agreement with our findings considering that sample group was selected from luminal subtype of breast cancer cases as well [25]. The fact that the results obtained are mutually supportive strenghtens our study. The significance of correlation between lymphatic invasion and tumor size with HN1L levels may be associated with the aggressiveness of the tumor. Therefore, this result might gain importance in the future for the follow-up of the disease.

CONCLUSION

To conclude, we have determined the HN1L protein levels in plasma, tumor and normal tissues in breast cancer patients and shown that these levels did not differ significantly between plasma and tumor tissue. We have found out that HN1L protein levels were positively correlated with tumor size and lymphatic invasion. Our analyses also included cases of rare breast cancers with different histological background. Future studies with a higher number of cases would ensure a more comprehensive investigation of the relationship between the change in HN1L protein levels and clinical parameters.

Authors' Contribution

Study Conception: EE, MS, MSG; Study Design: EE, MS, ST; Supervision: EE, MS, MSG, ST; Funding: MS; Materials: MSG, EE, MS; Data Collection and/or Processing: ST, EE, MS; Statistical Analysis

and/or Data Interpretation: EE, MS; Literature Review: EE, MS; Manuscript Preparation: EE, MS and Critical Review: EE, MS.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

This work was financially supported by Bursa Uludag University Scientific Research Coordination Unit (Project No: FHIZ-2021-602).

Acknowledgments

The authors would like to thank Prof. Ferda Ari for her support and Ahmet Sari Mahmout for English editing and proofreading of the manuscript.

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