Elevated Expression of the Testis-specific Gene *WBP2NL* in Breast Cancer

Seyedmehdi Nourashrafeddin[1,2], Mehdi Dianatpour[3,4], Mahmoud Aarabi[5], Maryam Beigom Mobasheri[6,7], Golnesa Kazemi-oula[6] and Mohammad Hossein Modarressi[6]

[1]School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. [2]Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. [3]Department of Medical Genetics, Faculty of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. [4]Transgenic Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. [5]Department of Human Genetics, School of Medicine, McGill University, Montreal, Canada. [6]Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [7]Cancer Research Center, Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran.

ABSTRACT: Breast cancer is one of the most common causes of cancer death in women; therefore, the study of molecular aspects of breast cancer for finding new biomarkers is important. Recent studies have shown that WW domain-binding protein 2 (WBP2) is important for the oncogenic property of breast cancer. WWP2 N-terminal-like (*WBP2NL*) is a testis-specific signaling protein that induces meiotic resumption and oocyte activation events. Our previous study revealed that *WBP2NL* gene expression is elevated in actively dividing cells and it might be associated with cellular proliferation and tumorigenic process. However, the clinical relevance and importance of *WBP2NL* gene in cancer has not been understood yet. Therefore, we were interested in analyzing the expression of *WBP2NL* gene in human breast cancer tissues and breast cancer cell lines, for the first time. We used reverse transcription-polymerase chain reaction (RT-PCR) and semi-nested RT-PCR to evaluate the expression of *WBP2NL* in malignant breast cancer and adjacent noncancerous tissue (ANCT) samples, as well as MCF-7 and MDA-MB-231 cell lines. The *WBP2NL* gene was expressed in 45 out of 50 (90%) breast cancer tissues and overexpressed in the MDA-MB-231 cell line. We suggest that *WBP2NL* may play roles in breast cancer activation maybe through binding to a group I WW domain protein. The elevated expression of *WBP2NL* gene in breast cancer and MDA-MB-231 cell line leads us to suggest that *WBP2NL* might be considered as a novel prognostic factor for early diagnosis of breast cancer.

KEYWORDS: breast cancer, *WBP2NL*, gene expression, cancer/testis genes

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CORRESPONDENCE: modaresi@tums.ac.ir

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Introduction

Breast cancer is the most frequent cause of cancer death in women worldwide. Approximately, 1 out of 10 women develops breast cancer during her lifetime.[1] Genetic tests that evaluate the biology of cancer are usually used to understand more about breast cancer, particularly to help predict the cancer recurrence risk, and thereby to help choose appropriate treatment. Therefore, study on the molecular aspects of breast cancer to find the genes associated with breast cancer risk and new biomarkers for early diagnosis of breast cancer is very important.

Sertoli cells in the testis make a blood–testis barrier that provides an immune-privileged site for germ cell's antigens. Cancer/testis (CT) genes are a family of genes that are normally expressed during spermatogenesis, usually in spermatocytes, and also in various human cancers, but absent in normal somatic cells.[2,3] Most CT genes induce cell proliferation and differentiation in normal testis, and have roles in meiosis.[3] The aberrant expression of CT genes in cancerous cells might cause abnormality in chromosome segregation and cell divisions.[3] Because of the blood–testis barrier and the immune-privileged status of testicular tissue, and also because of the probable roles of CT genes in oncogenesis, these immunogenic proteins are being strongly considered as potential targets for cancer vaccines and immunotherapy.[4,5]

Recent studies have demonstrated that WW-binding protein 2 (WBP2) activates the transcription of both estrogen and progesterone receptors (ERα/PR), and thereby is involved in the regulation of ERα/PR target genes and breast cancer.[6–8] It has been revealed that WW domain-mediated interaction with WBP2 is important for the oncogenic properties of the PDZ-binding motif (TAZ) in breast cancer.[9–11]

WWP2 N-terminal like (*WBP2NL*), also known as post-acrosomal sheath WW domain-binding protein (PAWP), is a testis-specific protein and has no expression in other tissues. *WBP2NL* is a 32-kDa signaling molecule localized in the post-acrosomal sheath (PAS) of spermatozoa.[12,13] In humans, the *WBP2NL* gene is located on chromosome 22q13.2 and consists of six exons extending over 29/69 kb.

The N-terminal part of *WBP2NL* has homology with WW domain binding protein-2, whereas the C-terminal part contains a PPXY motif that allows it to interact with group I WW domain proteins such as TAZ and the yes-associated protein (YAP). The *WBP2NL* protein enters the oocyte during fertilization, and induces meiotic resumption and oocyte activation events.[14–16]

A previous study by Aarabi et al has shown that *WBP2NL* contributes to sperm-induced calcium release and thereby initiates oocyte activation events.[17] Our previous study revealed that *WBP2NL* is expressed in embryonic stem cells and actively dividing cancerous cell lines, and we suggested that it might be associated with cellular proliferation and tumorigenic processes.[18] However, the mechanisms of *WBP2NL* in cell proliferation and its clinical relevance have not been understood yet. In the present study, we examined the expression of *WBP2NL* in normal and breast cancer tissues as well as in breast cancer cell lines in order to get more insights into its potential role in cellular proliferation and carcinogenesis, for the first time.

Materials and Methods

Patient and normal tissue samples. The study included 50 breast cancer tissues and 30 adjacent noncancerous tissue (ANCT) samples obtained from Imam Khomeini Hospital affiliated to Tehran University of Medical Sciences (Table 1). All patients had been diagnosed on the basis of clinical and laboratory findings (Table 2). Normal testis tissue, as positive control for *WBP2NL* gene expression, was obtained from a prostate cancer patient after orchiectomy. The study complied with the good clinical practice according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects participating in the study.

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from Pasteur Institute of Iran and cultured according to the manufacturer's instruction with slight modification. Cells were cultured in RPMI medium with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. After 2 days, the cells were harvested and about 2×10^6 cells were separated for RNA extraction, cDNA synthesis, and reverse-transcription polymerase chain reaction (RT-PCR) experiments.

RNA extraction and cDNA synthesis. Total RNA was extracted from tumor and normal samples and also breast cancer cell lines by TriPure isolating reagent (Roche) according to the manufacturer's instructions with slight modifications. RNA was dissolved in DEPS-treated water and its concentration was determined by a spectrophotometer (NanoDrop 2000). About 2 µg of total RNA from various samples were subjected for cDNA synthesis using the M-MLV reverse transcription kit (Invitrogen) and random primer (Pharmacia, Sweden).

RT-PCR and semi-nested PCR. A couple of specific primers of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase-1 (*HPRT1*) were used to ascertain the cDNA quality. PCR amplification of *HPRT* was performed in five minutes at 94°C as start denaturation, 30 cycles of denaturation at 94°C for 40 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, and a 7-minute final extension at 72°C. A pair of specific primers was designed to amplify 567 base pair fragments of human *WBP2NL*. The total reaction volumes were 25 µL containing 1 µL cDNA, two specific primers, PCR set, and smart Taq polymerase (Invitrogen). The PCR amplification of *WBP2NL* was performed with 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a 7-minute final extension at 72°C following an initial denaturation of 4 minutes at 94°C. The primers were positioned in different exons of each gene to avoid false positive results because of probable genomic DNA contamination. If no expression of *WBP2NL* was detected in RT-PCR runs on agarose gel, semi-nested PCR was performed using 2 µL of the first PCR reactions. Negative control reaction of the first PCR was used for the semi-nested PCR to identify false positive results caused by genomic DNA contamination. In order to carry out semi-nested PCR, an internal forward primer was used to amplify 168 base pair fragments of *WBP2NL*. The semi-nested amplification of *WBP2NL* was performed with 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 20 seconds, extension at 72°C for 30 cycles, and a 7-minute final extension at 72°C, following an initial denaturation of 4 minutes at 94°C. PCR products were subjected to electrophoresis in 1.5% agarose gel and then visualized under ultraviolet light after DNA staining with ethidium bromide. Amplification reaction carried out using following designed primers:

HPRT Forward: 5-ACCAGTCAACAGGGGACATAA-3′
Reverse: 5′-CTTCGTGGGGTCCTTTTCACC-3′
WBP2NL Forward: 5′-GCCATTGAATTTGCCCAGT TG-3′
Reverse1: 5′-TTAAGAATGGACCTGAGAAGAGG-3′
Reverse2: 5′-GGCTCCATAGACAATAACTGAAC-3′

Table 1. Major pathological features and characteristics of patients.

SUBJECT CHARACTERISTICS	NO. OF SUBJECTS (*n* = 50)
Age (years)	35–65 (mean: 50)
Histology	
Ductal	46 (92%)
Others	4 (8%)
Grade	
1	10 (20%)
2–3	40 (80%)
HER2/neu	
Negative	37 (74%)
Positive	13 (26%)
ER/PR	
Negative	12 (25%)
Positive	38 (75%)

Table 2. Classification of pathological features and *WBP2NL* expression from breast cancer patients.

NO.	PATIENT CODE	AGE	CANCER HISTOLOGY	CANCER GRADE	HER2/neu EXPRESSION	ER EXPRESSION	PR EXPRESSION	*WBP2NL* OVER EXPRESSION (FIRST PCR)	NESTED PCR
1	T201	47	ISDC	II	–	+++	+	–	+
2	T202	57	IDC	II	+++	–	–	+	+
3	T203	62	IDC	III	++	+	+	++	+
4	T204	44	ISDC	I	–	+	+	–	+
5	T205	57	IDC	II	++	–	–	–	+
6	T206	48	ISDC	II	–	+	–	–	–
7	T207	35	IDC	III	++	++	+	+	+
8	T208	39	IDC	II	–	–	+	–	+
9	T209	52	ISDC	II	–	+	++	–	+
10	T210	41	IDC	III	+++	–	–	+++	+
11	T211	46	ISDC	I	–	+	++	–	–
12	T212	63	ISDC	II	–	+	+	–	+
13	T213	51	IDC	II	–	–	–	++	+
14	T214	49	ISDC	I	–	+++	–	–	+
15	T215	55	ISDC	II	–	+	++	–	+
16	T216	44	ISDC	II	–	–	–	–	+
17	T217	61	ISDC	I	–	++	+	–	+
18	T218	42	IDC	III	+++	–	–	+++	+
19	T219	47	ISDC	II	–	+	+++	–	+
20	T220	35	IMC	II	+	+	+	–	+
21	T221	58	IDC	II	+++	–	–	–	+
22	T222	53	IDC	II	++	–	–	++	+
23	T223	50	ISDC	II	–	++	+	–	+
24	T224	48	IMC	II	–	+	++	–	+
25	T225	37	ISDC	II	–	–	–	–	+
26	T226	47	IDC	II	++	+	–	+++	+
27	T227	60	ISDC	II	–	++	+	+	+
28	T228	45	ISDC	I	–	+	++	–	+
29	T229	63	IDC	II	–	+	–	–	+
30	T230	55	IDC	II	–	++	+	+++	+
31	T231	38	ISDC	I	–	++	++	–	–
32	T232	65	ISDC	II	–	++	+	–	+
33	T233	44	IDC	II	–	–	–	++	+
34	T234	51	ISLC	II	–	+	++	+	+
35	T235	58	IDC	III	+++	–	–	+	+
36	T236	43	IDC	II	–	–	++	–	+
37	T237	41	ISDC	I	–	+	+++	–	–
38	T238	57	ISDC	I	–	++	+	–	+
39	T239	50	IDC	II	–	+	–	+++	+
40	T240	49	IMC	II	–	+	–	–	+
41	T241	52	ISDC	II	–	+	++	–	+
42	T242	37	ISDC	I	–	++	+	–	+
43	T243	48	IDC	II	–	–	–	–	+
44	T244	62	IDC	III	+++	–	+	++	+

(*continued*)

Table 2. (*Continued*)

NO.	PATIENT CODE	AGE	CANCER HISTOLOGY	CANCER GRADE	HER2/neu EXPRESSION	ER EXPRESSION	PR EXPRESSION	*WBP2NL* OVER EXPRESSION (FIRST PCR)	NESTED PCR
45	T245	44	IDC	II	+	+	+	–	+
46	T246	57	ISDC	I	–	+	++	–	–
47	T247	48	ISDC	II	–	–	++	–	+
48	T248	35	ISLC	II	–	+	–	–	+
49	T249	39	IMC	II	–	++	+	–	+
50	T250	56	ISDC	II	–	+	++	–	+

Notes: +++ means similar band in Figure 1 Lane A5; ++ means similar band in Figure 1 Lane A8; + means similar band in Figure 1 Lane A12.
Abbreviations: IDC, infiltrating ductal carcinoma; IMC, infiltrating mucinus carcinoma; ISDC, in situ ductal carcinoma; ISLC, in situ lobular carcinoma.

Statistical analysis. Statistical analysis was carried out using the SPSS software (SPSS Inc.), and significance was defined as $P < 0.05$.

Results

Complementary DNA amplification obtained from breast tumor samples was carried out with specific primers for *WBP2NL* gene using RT-PCR. We carried out semi-nested RT-PCR method in order to analyze the semiquantitative expression level of *WBP2NL*.

We detected *WBP2NL* expression in 45 out of 50 (90%) breast cancer samples (Fig. 1). Fifteen (30%) cases expressed *WBP2NL* in the first round of RT-PCR, representing a high level of gene expression (Fig. 1A), and 30 (60%) cases expressed *WBP2NL* in the second amplification by semi-nested RT-PCR, representing a low level of *WBP2NL* expression in the samples (Fig. 1B). Negative results from semi-nested RT-PCR showed a nondetectable *WBP2NL* transcript in the breast cancer samples (Fig. 1B). No expression of *WBP2NL* was seen in ANCT samples (Fig. 2). The different levels of *WBP2NL* gene expression in normal and breast cancer tissues are summarized in Tables 2 and 3.

Analysis of the *WBP2NL* gene expression levels was examined in women of two different age groups with breast cancer: young (35–50 years) and elderly (51–65 years), but no significant association was detected between age and the gene expression.

For more information, we evaluated the correlation between the histological feature and grade status of tumor samples and *WBP2NL* expression. A significant difference was detected in *WBP2NL* gene expression levels and the type of infiltrating ductal carcinoma ($P < 0.01$). We also analyzed the correlation between *WBP2NL* gene expression levels and additional factors including ER/PR and HER2/neu expression of tumor samples. A significant difference was detected in *WBP2NL* gene expression levels and HER2/neu expression ($P < 0.05$).

Fifteen peripheral blood samples from breast cancer patients were examined for the expression of *WBP2NL*, but the transcript was not found in the peripheral blood samples from patients using RT-PCR (data not shown).

We also evaluated *WBP2NL* expression in the breast cancer cell lines MCF-7 and MDA-MB-231 (Table 3). The latter cell line showed expression of *WBP2NL* in the first round of RT-PCR, whereas the expression of *WBP2NL* was negative in RT-PCR and semi-nested RT-PCR (Fig. 1).

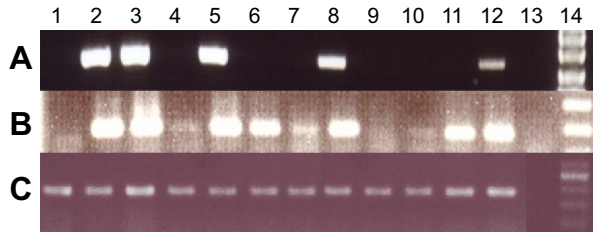

Figure 1. RT-PCR results from breast cancer samples, testis tissue, and breast cancer cell lines. (**A**) RT-PCR was performed to analyze of *WBP2NL* gene expression in breast cancer samples, testis tissue, and cell lines. Lane 1 and 2 are cDNA from MCF-7 and MDA-MB-231 cell line, respectively. Lane 3 is cDNA from an adult human testis as a positive control. Lanes 4–12 are cDNAs from breast cancer samples, Lane 13 is the negative control (water), and Lane 14 is 100 bp DNA marker. (**B**) Nested PCR was performed to analyze low expression of *WBP2NL* gene in breast cancer and cell lines. (**C**) All samples were controlled for presence of cDNA using the housekeeping gene *HPRT*.

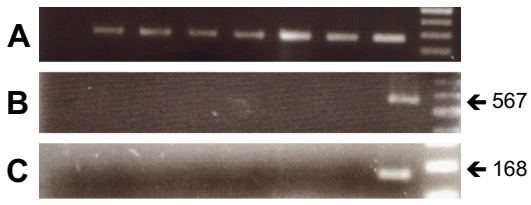

Figure 2. RT-PCR results from ACNT samples. (**A**) Presence of cDNA was checked in all samples using the housekeeping gene *HPRT*. (**B**) Lanes 2–7 are results of RT-PCR from some ACNT samples which showed no *WBP2NL* gene expression. (**C**) Nested PCR was carried out, which showed no expression of *WBP2NL* gene in ACNT samples (Lanes 2–7). Lane 1 was a negative control (water) and Lane 8 was RT-PCR from an adult human testis as a positive control. Lane 9 is a 100-bp DNA marker.

Table 3. *WBP2NL* gene expression in all samples after two rounds amplification (first PCR and semi-nested PCR).

WBP2NL GENE EXPRESSION	SAMPLE TYPE			
	ANCT	BC	MDA-MB-231	MCF-7
Positive in the first PCR (high expression)	0 (0%)	15 (30%)	2 (100%)	0 (0%)
Positive in the semi-nested PCR out of negatives in the first PCR (low expression)	0 (0%)	30 (60%)	2 (100%)	0 (0%)
Negative in the semi-nested PCR of negatives in the first PCR (no expression)	20 (100%)	5 (10%)	0 (0%)	2 (100%)
Total positive expression	0 (0%)	45 (90%)	2 (100%)	0 (0%)
Total samples	20	50	2	2

Discussion

Breast cancer is the most frequent cause of cancer death in women worldwide.[1] Nowadays, progressive studies have been developed to find the genes involved in the disease as well as new biomarkers for early diagnosis and treatment of the patients. In this respect, CT genes are among the best candidates for new therapeutics for breast cancer.

The CT genes are a group of tumor antigens that are normally expressed during spermatogenesis, usually in spermatocytes, but absent in normal somatic cells.[2,3] This special feature makes CT antigens a promising tumor-specific marker and very good candidate for cancer vaccines and immunotherapy approaches.[4,5,19] Previous studies have shown the expression of some CT antigens in breast cancer tumors.[20–23]

The testis-specific protein *WBP2NL* is a signaling molecule expressed in spermatocytes and localized in the PAS of elongated spermatocytes.[12,13] The *WBP2NL* protein enters in the oocyte during fertilization, and induces meiotic resumption and oocyte activation events.[14–16] The N-terminal part of *WBP2NL* has homology with WBP2, whereas its C-terminal contains a functional proline-rich (PPXY) motif that allows it to interact with group I WW domain proteins such as TAZ and YAP.[24–26] Both transcription coactivators YAP and TAZ are the key downstream effectors of the mammalian Hippo pathway,[27] which plays important roles in cell proliferation and tumorigenesis processes.[28–30] Recent studies have revealed that the interaction of WBP2 via PPxY-containing C-terminal region with WW domain in TAZ is important for the oncogenic properties of TAZ.[9–11] It has been reported that TAZ is also overexpressed in other human cancers and promotes malignant cell growth.[31–34] Some recent studies have suggested WBP2 as a target of cancer therapy, and proposed that either inhibition of WBP2 expression or interfering in its relation with YAP and TAZ could suppress tumor cell proliferation.[35,36]

Previous study by Aarabi et al revealed that *WBP2NL* contributes to sperm-induced calcium release and thereby initiates oocyte activation events.[17] Our recent study has shown that *WBP2NL* is elevated in the embryonic stem cells and actively dividing cancerous cell lines.[18] The *WBP2NL* structural considerations, together with previously published data, leads us to suggest that *WBP2NL* may have a potential role in the proliferation and activation of breast cancer cells maybe through binding to a group I WW domain protein.

Conclusion

In conclusion, elevated expression of the *WBP2NL* gene in breast cancer cells and MDA-MB-231 cell line leads us to suggest that *WBP2NL* might be classified as a CT antigen that is implicated in tumorigenesis. Therefore, it might be considered as a novel prognostic marker for early diagnosis of breast cancer.

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Author Contributions

Conceived and designed the experiments: SN, MD, MA, MHM. Analyzed the data: SN, MD, MBM, MA. Wrote the first draft of the manuscript: SN, MD, MBM. Contributed to the writing of the manuscript: SN, MD, MBM, MA, MHM. Agreed with the manuscript, results, and conclusions: SN, MD, MBM, GK, MA, MHM. Jointly developed the structure and arguments for the paper: SN, MD, MBM, GK, MA, MHM. Made critical revisions and approved the final version: SN, MD, MBM, GK, MA, MHM. All authors reviewed and approved of the final manuscript.

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