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Original Article

Up-regulation of Wnt7b rather than Wnt1, Wnt7a, and Wnt9a indicates poor prognosis in breast cancer

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Abstract: Aberrant activation of Wnt/β-catenin signaling is one of the most frequent abnormalities in human cancer, including breast cancer. The prognostic value of Wnt ligands has never been fully characterized. In this study, we focused on four Wnt ligands, namely Wnt1, Wnt7a, Wnt7b and Wnt9a, which were commonly studied and found pivotal in Wnt/β-catenin signaling, but seldom explored for their prognostic value. We investigated the expression of Wnt1, Wnt7a, Wnt7b and Wnt9a in breast cancer tissues by using real-time PCR and immunohistochemical analysis, and further identified their prognostic significance. Results demonstrated that only Wnt7b expression level in breast cancer was significantly higher than that of benign breast. Spearman rank-correlation analysis revealed the expression level of Wnt1, Wnt7b and Wnt9a, but not Wnt7a, were all significantly associated with positive lymph nodes. The Kaplan-Meier survival curve demonstrated that patients with high Wnt7b expression had a shorter overall survival (OS) and recurrence-free survival (RFS) than those with low Wnt7b expression. Moreover, the univariate and multivariate analysis revealed that Wnt7b expression was an independent prognostic factor for both OS and RFS of breast cancer patients. In addition, the high expression of Wnt7b in breast cancer and its prognostic role were further validated by GENT (Gene Expression database of Normal and Tumor tissues) database and the Kaplan-Meier plotter database. Taken together, we identified that high expression of Wnt7b, rather than Wnt1, Wnt7a and Wnt9a, may serve as a prognostic biomarker for breast cancer.

Keywords: Breast cancer, prognosis, Wnt1, Wnt7a, Wnt7b, Wnt9a

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and a leading cause of cancer death, accounting for 25% of cancer cases and 15% of cancer deaths among females in less developed countries [1, 2]. Despite improvement in early clinical detection and therapy strategies of breast cancer, the prognosis of breast cancer patients still remains unsatisfying because of metastasis and recurrence [3]. Therefore, it is urgently necessary to identify more novel prognostic biomarkers and develop new precise therapeutic strategies.

Wingless/integrase-1 (Wnt) signaling plays a critical role in a variety of biological processes, including cell proliferation, migration, polarity establishment, and stem cell self-renewal [4]. Deregulation of Wnt signaling is associated with oncogenesis and development of various cancers, including breast cancer [5, 6]. Wnt ligands, such as Wnt1 [7], Wnt7a [8], Wnt7b [9, 10], Wnt9a [11], can initiate canonical β-catenin signaling by interacting with cell surface receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Subsequent recruitment of downstream signal mediators results in disassembly of the β-catenin destruction complex, leading to the nuclear translocation of β-catenin. Binding of β-catenin with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) activates the transcription of Wnt target genes, ultimately initiating cell proliferation, invasion, and migration [12, 13]. In addition to the canonical Wnt pathway, Wnt

can also signal to activate the non-canonical Wnt pathways, namely Rho/JNK planar cell polarity and Ca^{2+}-dependent pathways [4]. However, the prognostic value of these Wnt ligands in breast cancer remains unclear.

In this study, we investigated the expression of Wnt1, Wnt7a, Wnt7b, and Wnt9a in breast cancer tissues by using real-time PCR and immunohistochemical analysis, and further identified their prognostic significance. We found high expression of Wnt7b was associated with aggressive clinicopathologic features and poor clinical outcome of breast cancer patients. Moreover, we validated these results with bioinformatic analysis.

Materials and methods

Patients and tissue samples

Primary invasive ductal carcinomas of breast were obtained from 106 female patients at the Department of Breast and Thyroid Surgery, the Eastern Hospital of First Affiliated Hospital, Sun Yat-sen University, from June 2004 to August 2009. Pathologic diagnosis, as well as ER, PR, and Her2 status, were verified by two different pathologists. Patients with invasive carcinomas, other than DCIS, underwent six cycles of postoperative adjuvant chemotherapy with FAC regimen (5-fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, and cyclophosphamide 500 mg/m^2). Subsequently, patients with ER (+) tumors underwent endocrine therapy according to NCCN guidelines. No distant metastasis was identified in the patients upon diagnosis. In addition, fresh samples of normal breast tissue, benign breast tumor tissues, and invasive ductal carcinoma tissues were collected from patients who underwent mastectomy or lumpectomy for benign or malignant breast disease. All samples were snap-frozen for mRNA assessment and were collected with informed written consent from patients. The complete clinical and pathologic features of these patients were collected and stored in our database by a researcher fellow. The study protocol followed the Ethical Guidelines of the 1975 Declaration of Helsinki, revised in 2000. All related procedures were performed with the approval of the Internal Review and the Ethics Boards of the First Affiliated Hospital, Sun Yat-sen University. All research protocols strictly complied with REMARK guidelines for reporting prognostic biomarkers in cancer [14].

Immunohistochemistry

Archived paraffin-embedded tumor tissues collected from 106 consecutive patients with breast cancer and 34 consecutive patients with benign breast tumor treated in our hospital between 2004 and 2009 were used for tissue microarray construction and immunohistochemistry (IHC). The IHC was performed using the polymer HRP detection system (Zhongshan Goldenbridge Biotechnology, Beijing, China) as the protocol described. Wnt1, Wnt7a, Wnt7b, and Wnt9a expression level were scored semiquantitatively using the IRS (immunoreactive score) = SI (staining intensity) × PP (percentage of positive cells) as described [15, 16]. Briefly, SI was determined as 0, negative; 1, weak; 2, moderate and 3, strong. PP was defined as 0, <1%; 1, 1-10%; 2, 11-50%; 3, 51-80% and 4, >80% positive cells. Five visual fields from different areas of each tumor were used for the IRS evaluation. IRS≤4 was defined as low expression and IRS>4 were defined as high expression. The antibodies and reagents are listed in the Tables S1 and S2.

RNA extraction and real-time quantitative polymerase chain reaction

TRIzol® Reagent (Life Technologies, Carlsbad, CA) was used to isolate total RNA from frozen tissue samples and cell lines according to the manufacturer's protocol. cDNA was synthesized using the universal cDNA synthesis kit (Toyobo, Tokyo, JP). The RNA was then reverse-transcribed to obtain cDNA by the universal cDNA synthesis kit (Toyobo, Tokyo, JP) at 37°C for 50 min. The cDNA was subjected to quantitative real-time PCR (qRT-PCR) using the SYBR Green PCR Kit (Roche Life Sciences, Switzerland) and the assay was performed on a PRISM 7300 Sequence Detection System (Applied Biosystems, CA). Each sample was prepared in triplicate. The mean values were used for calculation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control to normalize gene expression. The experiments were done in triplicate. The primers were all synthesized and bought from Sangon Biotech (Shanghai, China). The primer sequences are listed in the Table S3.

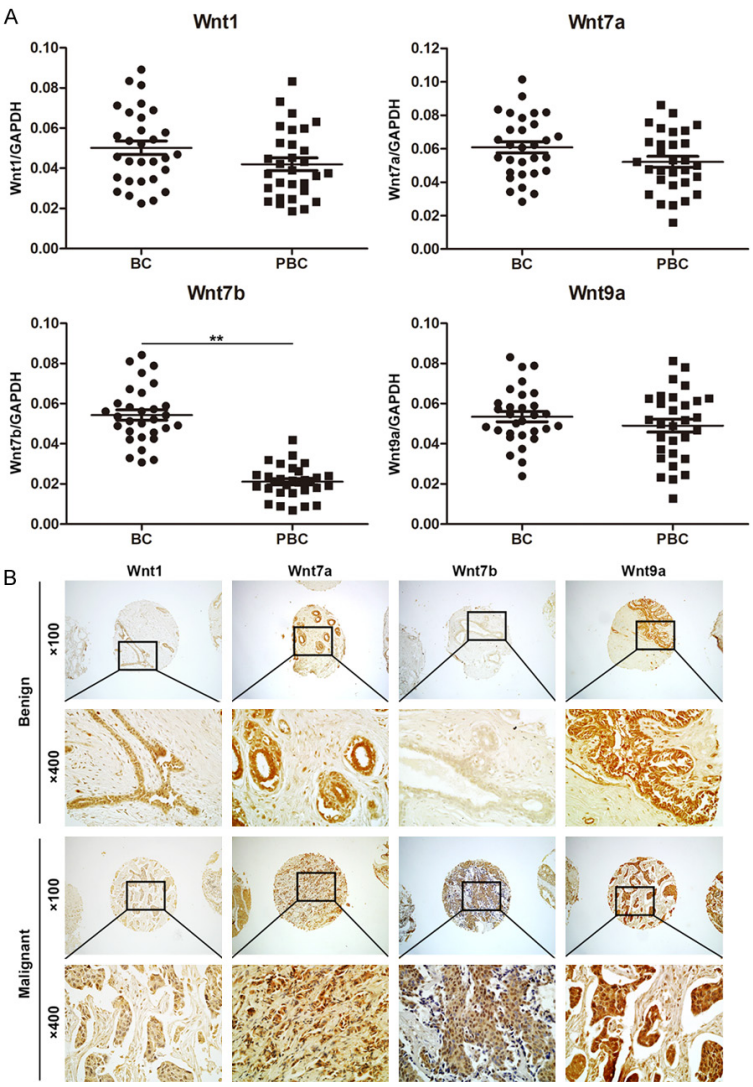

Figure 1. Expression traits of Wnt1, Wnt7a, Wnt7b, and Wnt9a in human breast cancer tissues. A. mRNA level of Wnt1, Wnt7a, Wnt7b, and Wnt9a in 30 paired surgical specimens of breast cancer (BC) and peri-tumor tissue (PBC) detected by real-time PCR. $**P<0.01$. B. Immunohistochemical staining of Wnt1, Wnt7a, Wnt7b, and Wnt9a on tissue microarray, which was constructed with 106 consecutive patients with breast cancer and 34 consecutive patients with benign breast lesion.

Statistical and bioinformatics analysis

The correlation between Wnt ligands expression and clinicopathological parameters was analyzed by Spearman rank-correlation analysis. Kaplan-Meier method was employed to construct survival curves and evaluate the difference of these groups by using the log-rank test. The Cox proportional hazard regression model was used to identify factors that were independently associated with overall survival and recurrence-free survival. Only factors that had $P<0.05$ in univariate analysis could be analyzed in multivariate analysis. Continuous data in this study were presented as mean ± standard deviation (SD) from at least three independent experiments. Categorical data were analyzed with X^2 test or Fisher's exact test. SPSS 17 software and GraphPad Prism 5 was used for performing all statistical analyses. Wnt7b expression patterns across diverse human cancer and normal tissues were analyzed by web-accessible GENT (Gene Expression database of Normal and Tumor tissues) database (medicalgenome.kribb.re.kr/GENT/). The prognostic significance of the mRNA expression of Wnt7b in breast cancer was also evaluated using the Kaplan-Meier plotter (www.kmplot.com), an online database including gene expression data and clinical data. In this study, P value <0.05 was considered significant.

Results

Expression traits of Wnt1, Wnt7a, Wnt7b and Wnt9a in human breast cancer tissues

To investigate expression traits of Wnt1, Wnt7a, Wnt7b and Wnt9a in breast cancer, we comparatively analyzed their mRNA level in 30 paired surgical specimens of primary invasive ductal carcinoma (IDC) and peri-tumor tissue. Real-time PCR analysis revealed that, compared with matched peri-tumor tissue of breast cancer (PBC), only Wnt7b mRNA in breast cancer tissue (BC) was significantly up-regulated, whereas mRNA levels of Wnt1, Wnt7a and Wnt9a between BC and PBC showed no difference (**Figure 1A**). Further we performed immunohistochemical staining of Wnt1, Wnt7a, Wnt7b and Wnt9a on a tissue microarray, which was constructed with 106 consecutive patients with breast cancer and 34 consecutive patients

Table 1. Correlations of Wnt ligands with characteristics of breast tumor

Wnt ligands	n	Tumor characteristic		*P*
		Benign	Malignant	
Wnt1				
Low	51	11	40	
High	89	23	66	0.570
Wnt7a				
Low	19	8	11	
High	121	26	95	0.051
Wnt7b				
Low	74	25	49	
High	66	9	57	*0.006*
Wnt9a				
Low	43	15	28	
High	97	19	78	0.052

with benign breast tumor (**Figure 1B**). Results demonstrated that only Wnt7b expression level in breast cancer was significantly higher than that in benign breast tumor, whereas expression levels of Wnt1, Wnt7a, and Wnt9a between breast cancer and benign breast tumor showed no difference (**Table 1**). With these findings, our results indicate that Wnt7b is markedly up-regulated in breast cancer.

Correlations of expression levels of Wnt1, Wnt7a, Wnt7b, and Wnt9a with clinicopathological features and postoperative survival of breast cancer

To clarify the clinical relevance of expression levels of Wnt1, Wnt7a, Wnt7b, and Wnt9a in breast cancer, we further analyzed the above IHC results of the breast cancer tissue microarray of 106 breast cancer patients. Patients were dichotomized according to low (IRS≤4) or high (IRS>4) expression of Wnt1, Wnt7a, Wnt7b, and Wnt9a. We included the following clinicopathological features: age, positive lymph node, tumor size, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (Her2), and TNM stage. We showed that the expression levels of Wnt1, Wnt7b and Wnt9a were all significantly associated with positive lymph nodes; and interestingly, Wnt7b expression was also markedly correlated with age; whereas Wnt7a expression showed no relationship with the above clinicopathologic parameters (**Table 2**).

We next analyzed the correlation between the expression level of Wnt1, Wnt7a, Wnt7b, and Wnt9a and the patients' prognosis. The Kaplan-Meier survival curve demonstrated that patients with high Wnt7b expression had a shorter overall survival (OS) (*P*=0.005) and recurrence-free survival (RFS) (*P*=0.013) than those with low Wnt7b expression, whereas no significant difference was found between patients with high/low expression of Wnt1, Wnt7a, and Wnt9a in OS and RFS, respectively (**Figure 2**). Moreover, the univariate and multivariate analysis revealed that Wnt7b expression was an independent prognostic factor for both OS and RFS of breast cancer patients (**Tables 3**, **4**), whereas Wnt1, Wnt7a and Wnt9a were not. The above results indicated that Wnt7b was closely correlated with poor survival and may be a novel independent prognostic biomarker for breast cancer.

Validation of prognostic value of Wnt7b in breast cancer by bioinformatics analysis

To further validate the prognostic value of Wnt7b in breast cancer, we first evaluated its expression patterns across diverse human cancer and normal tissues by using GENT database (medicalgenome.kribb.re.kr/GENT/), which included more than 40000 samples collected from public resources, and profiled by Affymetrix U133A (sample size >16400) or U133plus2 (sample size >24300) platforms in many different laboratories across the world [17]. Results demonstrated that Wnt7b expression was significantly higher in breast cancer than that in normal tissue (**Figure 3A**). Next, we studied the relationship between mRNA expression of Wnt7b and clinical outcome using a Kaplan-Meier plotter (www.kmplot.com). In this database, data of lung cancer, ovarian cancer, gastric cancer, and breast cancer are available. With the purpose to assess prognostic value of a specific gene, the patient samples were divided into two cohorts according to the median expression of the gene (high vs. low expression) [18]. Briefly, Wnt7b was uploaded into the database to obtain the Kaplan-Meier survival plots, in which the number-at-risk was shown below the main plot. Log rank *P*-value and hazard ratio (HR) with 95% confidence intervals were calculated and displayed on the webpage. Results showed that Wnt7b high expression

Table 2. Correlations of Wnt ligands with clinicopathologic features of breast cancer patients

Clinicopathologic Variable	n	Wnt1 expression			Wnt7a expression			Wnt7b expression			Wnt9a expression		
		Low	High	*P*	Low	High	*P*	Low	High	*P*	Low	High	*P*
Age (year)													
≤45	35	13	22		5	30		23	12		10	25	
>45	71	27	44	0.930	6	65	0.499	26	45	*0.007*	18	53	0.816
Positive lymph node													
≤3	78	34	44		8	70		42	36		25	53	
>3	28	6	22	*0.043*	3	25	0.946	7	21	*0.014*	3	25	*0.044*
Tumor size (cm)													
≤2	29	13	16		3	26		16	13		8	21	
>2	77	27	50	0.377	8	69	0.995	33	44	0.282	20	57	0.867
ER													
Positive	54	19	35		7	47		28	26		13	41	
Negative	52	21	31	0.689	4	48	0.527	21	31	0.250	15	37	0.662
PR													
Positive	39	13	26		6	33		20	19		9	30	
Negative	67	27	40	0.537	5	62	0.208	29	38	0.545	19	48	0.650
Her2													
Negative	67	30	37		5	62		34	33		20	47	
Positive	39	10	29	0.062	6	33	0.208	15	24	0.234	8	31	0.364
TNM stage													
I-II	69	28	41		7	62		33	36		15	54	
III	37	12	25	0.529	4	33	0.915	16	21	0.687	13	24	0.167

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; Her2, Human Epidermal Growth Factor Receptor type 2; TNM, tumor node metastasis.

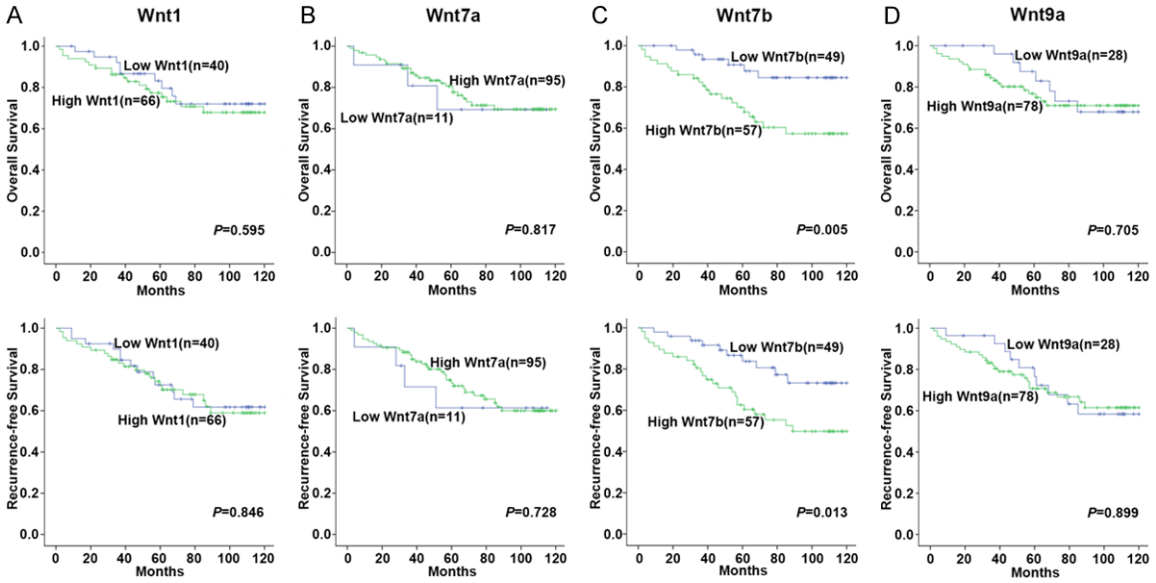

Figure 2. The Kaplan-Meier curves showed overall survival (OS) and recurrence-free survival (RFS) of breast cancer patients with low/high expression of Wnt1, Wnt7a, Wnt7b, and Wnt9a. (A) Wnt1, (B) Wnt7a, (C) Wnt7b, (D) Wnt9a. *P* value is shown in each panel respectively.

was found to be correlated to significantly worse OS (HR=1.45 [1.15-1.82], *P*=0.0014) and RFS (HR=1.3 [1.1-1.54], *P*=0.0017) for breast cancer patients (**Figure 3B**).

Table 3. Univariate and multivariate analysis of factors associated with overall survival in breast cancer patients

Variable	n	Univariate Analysis		Multivariate Analysis	
		RR (95% CI)	*P*	RR (95% CI)	*P*
Age (year)					
≤45	35	1			
>45	71	1.532 (0.648-3.623)	0.332	n.a.	n.a.
Positive lymph node					
≤3	78	1		1	
>3	28	2.584 (1.197-5.579)	*0.016*	1.015 (0.324-3.174)	0.980
Tumor size (cm)					
≤2	29	1			
>2	77	1.515 (0.611-3.759)	0.370	n.a.	n.a.
ER					
Positive	54	1			
Negative	52	1.463 (0.678-3.153)	0.332	n.a.	n.a.
PR					
Positive	39	1			
Negative	67	1.174 (0.527-2.615)	0.694	n.a.	n.a.
Her2					
Negative	67	1		1	
Positive	39	2.259 (1.061-4.812)	*0.035*	2.004 (0.931-4.313)	0.076
TNM stage					
I-II	69	1		1	
III	37	2.473 (1.162-5.267)	*0.019*	2.318 (1.079-4.978)	*0.031*
Wnt1 expression					
Low	40	1			
High	66	1.242 (0.558-2.764)	0.596	n.a.	n.a.
Wnt7a expression					
Low	11	1			
High	95	0.868 (0.261-2.883)	0.817	n.a.	n.a.
Wnt7b expression					
Low	49	1		1	
High	57	3.358 (1.355-8.323)	*0.009*	3.477 (1.400-8.637)	*0.007*
Wnt9a expression					
Low	28	1			
High	78	1.181 (0.499-2.795)	0.706	n.a.	n.a.

Abbreviations: n.a., Not applicable; ER, estrogen receptor; PR, progesterone receptor; Her2, Human Epidermal Growth Factor Receptor type 2; TNM, tumor node metastasis.

Taken together, these data fully demonstrated that Wnt7b was closely correlated with poor survival and could be a novel independent prognostic biomarker for breast cancer.

Discussion

About 15% of breast cancer patients develop disseminated metastasis before or after diagnosis, and distant metastasis is responsible for approximately 90% of breast cancer-associated mortality [19]. Though there have been major breakthroughs in the targeted therapy of breast cancer, its prognosis still remains unsatisfying. Therefore, it is increasingly important to understand the mechanisms that underlie breast cancer progression and identify new prognostic markers for precise therapeutic strategies [20]. In our previous studies, we found Wnt/β-catenin signaling, which could be activated by Collagen triple helix repeat containing-1 (CTHRC1), played a vital role in breast cancer progression [21]. Thus we continued to focus on Wnt signaling.

Wnt signaling, activated by corresponding Wnt ligands, participates in a large set of cellular processes, including proliferation, differentiation, migration, and apoptosis. Canonical Wnt/β-catenin pathway is involved in cell fate choices and stem-cell renewal and differentiation, whereas non-canonical Wnt/Ca^{2+} and Wnt/planar cell polarity pathways deal with morphological changes and tissue organization [5, 22]. Aberrant activation of Wnt/β-catenin signaling is one of the most frequent abnormalities in human cancer, including breast cancer [23]. Huguet et al. [24] explored differential expression of human Wnt Genes 2, 3, 4, and 7B in

Table 4. Univariate and multivariate analysis of factors associated with recurrence-free survival in breast cancer patients

Variable	n	Univariate Analysis		Multivariate Analysis	
		RR (95% CI)	*P*	RR (95% CI)	*P*
Age (year)					
≤45	35	1			
>45	71	1.251 (0.595-2.630)	0.554	n.a.	n.a.
Positive lymph node					
≤3	78	1		1	
>3	28	3.629 (1.867-7.055)	*<0.001*	1.720 (0.586-5.047)	0.323
Tumor size (cm)					
≤2	29	1			
>2	77	2.077 (0.855-5.046)	0.106	n.a.	n.a.
ER					
Positive	54	1			
Negative	52	1.594 (0.792-3.207)	0.191	n.a.	n.a.
PR					
Positive	39	1			
Negative	67	1.384 (0.658-2.909)	0.392	n.a.	n.a.
Her2					
Negative	67	1		1	
Positive	39	2.011 (1.035-3.907)	*0.039*	1.753 (0.894-3.437)	0.103
TNM stage					
I-II	69	1		1	
III	37	3.092 (1.582-6.044)	*0.001*	3.123 (1.597-6.109)	*0.001*
Wnt1 expression					
Low	40	1			
High	66	1.070 (0.539-2.125)	0.847	n.a.	n.a.
Wnt7a expression					
Low	11	1			
High	95	0.831 (0.293-2.356)	0.831	n.a.	n.a.
Wnt7b expression					
Low	49	1		1	
High	57	2.463 (1.182-5.130)	*0.016*	2.494 (1.197-5.196)	*0.015*
Wnt9a expression					
Low	28	1			
High	78	1.048 (0.503-2.185)	0.899	n.a.	n.a.

Abbreviations: n.a., Not applicable; ER, estrogen receptor; PR, progesterone receptor; Her2, Human Epidermal Growth Factor Receptor type 2; TNM, tumor node metastasis.

human breast cell lines and normal and disease states of human breast tissue, and found the level of expression of Wnt2 and Wnt4 was 10 to 20-fold higher in fibroadenomas than it was in normal or malignant breast tissue, and in 10% of tumors Wnt7b expression was 30-fold higher than in normal or benign breast tissues. Benhaj et al. [25] analyzed the expression profiles of 19 known Wnt ligands, 10 known Frizzled receptors, two LRP co-receptors and four TCF/LEF transcription factor genes, in a panel of six breast cancer cell lines. They found that the expression of canonical Wnt ligands was up-regulated, whereas non-canonical WNT5A and WNT5B expression was down-regulated in breast cancer cell lines. But the prognostic value of Wnt ligands has never been fully characterized. Wnt ligands are encoded evolutionarily conserved secreted glycoproteins that act as signaling molecules essential for a variety of fundamental processes. Currently, their family in humans comprises 19 different ligands, which are historically defined by their amino-acid sequence rather than by their functional properties [4, 12]. In this study, we focused on four Wnt ligands, namely Wnt1, Wnt7a, Wnt7b and Wnt9a, which were commonly studied and found pivotal in Wnt/β-catenin signaling, but seldom explored in their prognostic value. We found only Wnt7b was up-regulated and closely correlated with poor survival, and could be used as a novel independent prognostic biomarker for breast cancer.

Wnt7b has been implicated in oncogenesis and in several developmental processes, including

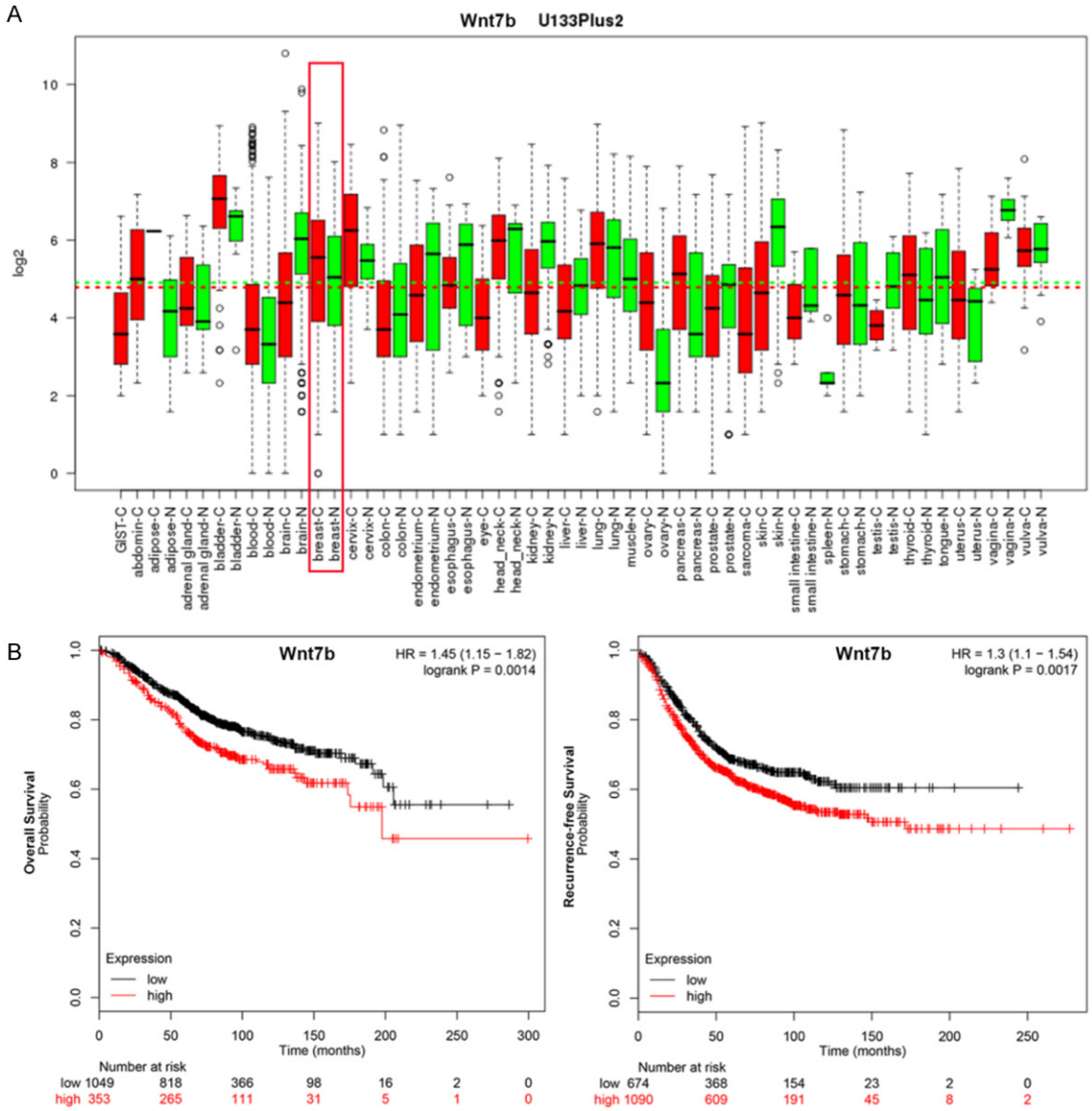

Figure 3. Validation of prognostic value of Wnt7b in breast cancer by bioinformatics analysis. A. Wnt7b expression patterns across diverse human cancer and normal tissues evaluated by GENT database. B. The relationship between mRNA expression of Wnt7b and clinical outcome using Kaplan-Meier plots.

regulation of cell fate and patterning during embryogenesis [26]. The role of Wnt7b in cancer progression has also been increasingly reported. Zheng et al. [27] found Wnt7b is necessary for the growth of prostate cancer cells and that this effect is enhanced under androgen-deprived conditions. Their further analyses revealed Wnt7b promoted androgen-independent growth of castration-resistant prostate cancer cells likely through the activation of protein kinase C isozymes; and prostate cancer-produced Wnt7b induced osteoblast differentiation both in vitro and in vivo. Arensman et al. [10] confirmed autocrine Wnt/β-catenin signaling in pancreatic adenocarcinoma can be primarily initiated and regulated by a single Wnt ligand, Wnt7b, acting alone or in conjunction with other Wnt ligands. They further supposed disrupting the interaction between Wnt ligands and their receptors might be a suitable approach for therapeutic modulation of Wnt/β-catenin signaling in pancreatic adenocarcinoma and other cancer contexts where this signaling pathway activation was mediated by

ligand expression rather than mutations. As for breast cancer, Yeo et al. [28] found Wnt7b was highly up-regulated in breast cancer through analysis of the Tissue Cancer Genome Atlas data sets, which also coincided with our results, and illustrated a critical role of myeloid Wnt7b in breast cancer progression, acting at the levels of angiogenesis, invasion, and metastasis. Moreover, consistent with our findings, Ojalvo et al. [23] also validated Wnt7b expression correlated with markers of poor prognosis such as lymph node positivity in human breast cancer. However, interestingly, our study indicated the Wnt7b expression level was also correlated with patient age. We supposed the activation level of Wnt signaling in breast cancer patients of different age might be different, as the morbidity of breast cancer increased with age, especially females older than 40 years [1], thus causing this difference. Alternatively, it perhaps was a pure coincidence. Therefore, this remains to be further examined in a cohort of larger sample size.

In summary, our study demonstrated that high expression levels of Wnt7b, rather than Wnt1, Wnt7a, and Wnt9a, could discriminate malignant from benign tumors in breast cancer and show a worse prognosis than low levels of Wnt7b. Wnt7b was an independent prognostic indicator for breast cancer patients. Therefore, we suggest strategies designed to down-regulate Wnt7b or disrupt the interaction between Wnt7b and its receptors may provide a promising method to alleviate breast cancer progression.

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Disclosure of conflict of interest

None.

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Table S1. List of the antibodies used in this study

Antibody name	Source
Wnt1	SIGMA (SAB5300004)
Wnt7a	SIGMA (SAB2700308)
Wnt7b	SIGMA (SAB2701193)
Wnt9a	SIGMA (SAB2900706)

Table S2. List of the reagents used in this study

Reagent name	Source
Polymer HRP Detection System	ZSGB-BIO (PV-9000)
DAB Kit	ZSGB-BIO (ZLI-9018)

Table S3. The sequences of qRT-PCR primers used in this study

Target gene	Sequence
Wnt1	F: CAGAGCCACGAGTTTGGATG
	R: AGTGGAGAGGGATTGGGTTG
Wnt7a	F: CCCACCTTCCTGAAGATCAA
	R: ACAGCACATGAGGTCACAGC
Wnt7b	F: ATGCACAGAAACTTTCGCAA
	R: TGCATCCGGTCCTCTAGAAC
Wnt9a	F: TGGAGGCCGTGAGCATGAGT
	R: CTTAAGGTTGTCTCCGCAGC