Journal of Cancer Research and Practice

journal homepage: www.ejcrp.org

Original Article

Netrin-1 Expression in Breast Cancer

Shyr-Ming Sheen-Chen[1]*, Ching-Hua Tsai[2], Yueh-Wei Liu[2], Chao-Cheng Huang[3]

[1]Department of Surgery, Taipei Tzu Chi Hospital, Taipei, Taiwan
[2]Department of Surgery, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University, Kaohsiung, Taiwan
[3]Department of Pathology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University, Kaohsiung, Taiwan

Abstract

Background: Breast cancer is a collection of molecularly and clinically distinct neoplastic disease. Recent research has shown that the gene expression in breast cancer can be useful when designing an optimal treatment plan and may also provide with prognostic information. The development of tissue microarrays (TMAs) has allowed for rapid immunohistochemical analysis of thousands of tissue samples in parallel with minimal damage to the original blocks. The aim of this study was to use TMAs to analyze the netrin-1 (NTN 1) status in patients with breast cancer with the hope of elucidating the possible relationship between NTN 1 expressions and breast cancer. **Materials and Methods:** Archival tissue specimens from 106 patients with primary invasive breast cancer were analyzed for NTN 1 expression using immunohistochemical staining with TMAs. Results were compared to clinicopathological data using multivariate analysis. **Results:** Tumor-node-metastasis stage was significantly related to the overall 5-year survival rate; however, NTN 1 expression was not significantly associated with overall 5-year survival. **Conclusion:** Immunohistochemical staining with TMAs was convenient and feasible to analyze the expression of NTN 1, in patients with breast cancer. Our preliminary results showed that NTN 1 expression had no significant prognostic value in breast cancer.

Keywords: Biomarker, breast cancer, netrin-1, tissue microarray

Introduction

Breast cancer is a complex disease entity with different biological characteristics and clinical behaviors.[1-3] A variety of clinical features, pathological findings, and genetic variant have been identified and characterized for the prediction of responses to treatment and outcomes in patients with breast cancer.[3,4]

Netrin-1 (NTN 1) is a diffusible laminin-related protein that has been shown to play a major role in the control of neural navigation in the developing nervous system.[5,6] Recently, NTN 1 has been proposed to play crucial roles in tumorigenesis as it is involved in the regulation of apoptosis by binding to deleted in colorectal cancer (DCC) and UNC5H families of dependence receptors that share the ability to induce apoptosis in the absence of their ligands, a trait that confers tumor suppressor activity on these receptors.[7] The expression of one of these dependence receptors at the surface of a tumor cell is considered to render it dependent on ligand availability for survival, thus inhibiting uncontrolled tumor cell proliferation and/or metastasis.

Received: 06-Jan-2019 **Revised:** 16-Jan-2019 **Accepted:** 08-Mar-2019

Access this article online

Quick Response Code:

Website: www.ejcrp.org

DOI: 10.4103/JCRP.JCRP_8_19

Address for correspondence: Dr. Shyr-Ming Sheen-Chen, Department of Surgery, Taipei Tzu Chi Hospital, No. 289, Jianguo Road, Xindian District, New Taipei City 23142, Taiwan.
E-mail: smsheen@yahoo.com

How to cite this article: Sheen-Chen SM, Tsai CH, Liu YW, Huang CC. Netrin-1 expression in breast cancer. J Cancer Res Pract 2019;6:124-7.

The development of tissue microarrays (TMAs) has allowed for the rapid immunohistochemical analysis of thousands of tissue samples in parallel with minimal damage to the original blocks.[8,9] The aim of this study was to evaluate the use of TMAs to analyze the expression of NTN 1 in patients with breast cancer and to explore its potential role in the management of breast cancer.

Materials and Methods

Specimen selection and data collection

Archival tissue specimens from 106 patients with primary invasive breast cancer were selected from pathology files at Kaohsiung Chang Gung Memorial Hospital between January 1994 and December 1998. All patients had been undergone treatment at this institution due to invasive breast cancer, defined as carcinoma with invasion to or beyond the basement membrane regardless of histological classification (ductal or lobular).[10] Data regarding primary tumor staging, age, estrogen receptor status,[11-13] lymph node status, histological grading, and tumor-node-metastasis (TNM) staging were also collected. Hematoxylin and eosin-stained slides of paraffin-embedded tumor specimens were reviewed by our pathologists to confirm the accuracy of the histological diagnoses and lymph node status.

Tissue microarray assembly

Representative areas of both tumor and nontumor tissues from each case were selected and circled to match the blocks for the TMA. Blocks matching the circled slides were retrieved and prepared for the microarray. Three areas each for both tumor and nontumor parts per case were used to assemble the recipient blocks. Each target area on the selected blocks was punched to form a 0.6-mm diameter tissue core and placed consecutively on the recipient blocks approximately 3 cm × 2 cm in size with a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as described elsewhere.[14]

Immunohistochemical analysis

Goat anti-NTN 1 polyclonal antibodies (Abcam) were diluted 1:400 in phosphate-buffered saline (PBS). Five-micrometer sections were cut from the recipient blocks of the TMA, incubated overnight in a 37°C oven, dewaxed in xylene, and dehydrated in a series of graded alcohols. The sections were then treated with 3% hydrogen peroxide for 10 min to exhaust the endogenous peroxidase activity and microwaved in 10 mM citrate buffer pH 6.0 to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted anti-NTN 1 antibodies for 1 h followed by washing with PBS wash. Horseradish peroxidase (HRP)/Fab polymer conjugate (UltraTek HRP anti-polyvalent kit; ScyTek, Utah, USA) was then applied to the sections for 30 min. After washing, the sections were incubated with peroxidase substrate diaminobenzidine for 5 min and counterstained with hematoxylin.

Grading of netrin-1 expression by immunohistochemical analysis

The expression of NTN 1 in immunohistochemical analysis was graded as: 0, no staining in tumor cells; 1+, weak cytoplasmic staining in tumor cells; 2+, moderate cytoplasmic staining in tumor cells; and 3+, strong cytoplasmic staining in tumor cells [Figure 1].

Patients and follow-up

All of the patients were women with a mean age of 48.8 ± 10.2 years (range, 26–76 years). The mean follow-up was 69.0 ± 27.2 months (range, 5–98 months). Follow-up was usually performed every 3 months for the first 2 years and then every 6 months for the next 3 years. After 5 years, follow-up became annual. Chest radiography, serum alkaline phosphatase level, and detailed physical examination were usually performed during follow-up visits. Annual mammography or breast sonography (for the younger patient) was also performed. Radionuclide bone scan, abdominal sonography, or other image studies were performed if specific symptoms, signs, or elevated serum alkaline phosphatase levels were noted. Data regarding patient survival, clinical status, and clinicopathological factors were obtained from medical records, contact with the patients at the outpatient clinics or by telephone, or both.

Statistical analysis

Comparisons between groups were performed using Fisher's test or the Chi-squared test as appropriate. For survival analysis, the end point was overall survival. Survival differences were compared using the log-rank test. To assess the relative influence of the potential prognostic variables on survival, all clinicopathological and genetic variables were entered into a final Cox's proportional hazards model for multivariate analysis. Statistical analyses were conducted using SPSS software (version 17.0; SPSS, Chicago, IL, USA). Statistical significance was set at $P < 0.05$. All P values were estimated from two-sided tests.

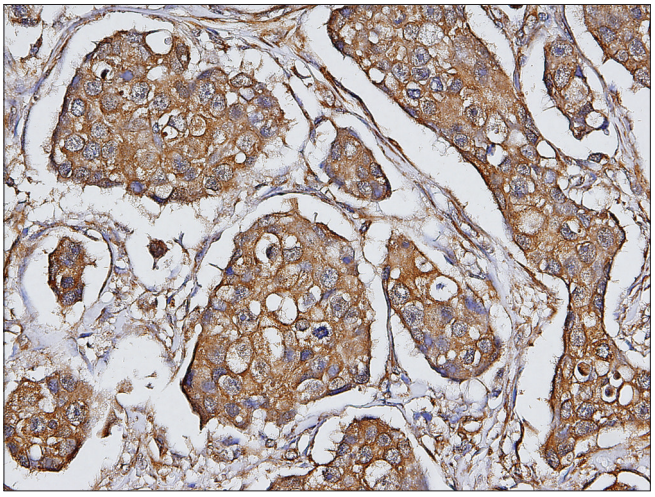

Figure 1: Representative case of breast cancer with netrin-1 immunostaining on tissue microarray. The case showed strong cytoplasmic staining in tumor cells and was graded as 3+, (×200)

Results

Fourteen patients (13.2%) had a score of 1, 46 patients (43.4%) had a score of 2, and 46 patients (43.4%) had a score of 3 [Table 1]. There were no significant relationship between NTN 1 expression and age ($P = 0.609$), estrogen receptor status ($P = 0.691$), histological grading ($P = 0.634$), primary tumor staging ($P = 0.342$), lymph node status ($P = 0.224$), or TNM stage [$P = 0.910$, Table 1].

For survival analysis, the end point was overall survival. The overall 5-year survival rates for different categories are listed in Table 2. By multivariate analysis, TNM stage was significantly associated with the overall 5-year survival rate [Table 3, $P < 0.001$]. However, NTN 1 expression was not significantly associated to the overall 5-year survival ($P = 0.111$).

Table 1: Netrin-1 in relation to clinicopathological variables

	1 (%)	2 (%)	3 (%)	*P*
Age				
≥50	5 (36)	23 (50)	20 (43)	0.609
<50	9 (64)	23 (50)	26 (57)	
ER status				
Negative	10 (71)	27 (59)	28 (61)	0.691
Positive	4 (29)	19 (41)	18 (39)	
Histologic grading				
1	1 (7)	6 (13)	7 (15)	0.634
2	11 (79)	26 (57)	28 (61)	
3	2 (14)	14 (30)	11 (24)	
Primary tumor staging				
T1	3 (21)	12 (26)	6 (13)	0.342
T2	10 (71)	22 (48)	24 (52)	
T3	0 (0)	8 (17)	11 (24)	
T4	1 (7)	4 (9)	5 (11)	
N status				
N0	9 (64)	19 (22)	23 (50)	0.224
N1	1 (7)	8 (17)	11 (24)	
N2	4 (29)	10 (22)	5 (11)	
N3	0 (0)	9 (20)	7 (15)	
TNM stage				
I	2 (14)	7 (15)	5 (11)	0.910
II	7 (50)	19 (41)	24 (52)	
III	5 (36)	18 (39)	15 (33)	
IV	0 (0)	2 (4)	2 (4)	

ER: Estrogen receptor, TNM: Tumor, node, and metastasis

Table 2: Overall 5-year survival rate for each category of breast cancer

Variable	Category	5-year survival rate (%)	*P*
Age (years)	≥50	64.6	0.195
	<50	77.4	
TNM stage	I	100.0	<0.00001
	II	89.9	
	III	42.1	
	IV	0	
ER status	Negative	64.6	0.063
	Positive	80.1	
Histologic grading	1	57.1	0.411
	2	75.4	
	3	66.7	
Netrin-1	1	71.4	0.553
	2	76.0	
	3	65.0	

ER: Estrogen receptor, TNM: Tumor, node, and metastasis

Discussion

NTN 1 has been reported to play a crucial role in tumorigenesis as it is involved in the regulation of apoptosis by binding to the DCC and UNC5H families of dependence receptors that share the ability to induce apoptosis in the absence of their ligands, a trait that confers tumor suppressor activity on these receptors.[7] Accumulating evidence implies that this positive signaling pathway is frequently inactivated in human cancers as it has been shown that the expression of DCC and UNC5H is lost during tumor progression, thus conferring a selective advantage to the tumor cell for survival.[7] In addition, this model predicted that a similar advantage may be obtained by gaining autocrine expression of the NTN 1 ligand in tumor cells. Indeed, a high percentage of colorectal[15] and metastatic breast[16] cancers have been shown to overexpress NTN 1. However, to the best of our knowledge, little information about the expression of NTN 1 in breast cancer has been reported.

Therefore, this study was designed to evaluate the use of TMA to analyze the expression of NTN 1 in patients with breast cancer and to explore its potential in the management of breast cancer.

An array-based high-throughput technique has been reported to allow analysis of very large numbers of tumors simultaneously at the DNA, RNA, or protein level.[17] With minimal damage to the origin blocks, as many as 1000 cylindrical tissue biopsy specimens from individual tumor can be arrayed in a TMA block in parallel fashion.[8,9,17] TMA provides a higher level of standardization for immunohistochemical staining owing to the pretreatment and staining of the tumors under exactly the same conditions as compared to immunohistochemical analyses of large tissue section. Moreover, analysis using large sections requires the integration of observations from multiple regions of a tissue section. In contrast, morphologic classification and interpretation of immunoreactivity in TMA are based on the findings within one small, highly defined tissue area. The diagnostic criteria are therefore much easier to establish between individual samples in the array allowing for comparisons between different observers.[8,9,17]

Whether these small specimens (diameter 0.6 mm) are really representative of their donor tumors is the major concern about the use of TMA. The TMA approach was designed to examine tumor populations rather than individual tumors.[8] Although heterogeneity within tumors has been reported, it has also been suggested that this heterogeneity did not influence the identification of prognostic parameters.[8] In a series of

Table 3: Multivariate analysis for overall 5-year survival rate

Variable	*P*	OR	95% CI
Age (≥50 versus<50)	0.641	1.2	0.5-2.7
TNM stage (I, II, III, IV)	0.000	11.1	5.1-24.2
ER status (positive vs. negative)	0.077	0.5	0.2-1.1
Histologic grading (I, 2, 3)	0.252	1.4	0.8-2.4
Netri-1 (1, 2, 3)	0.111	1.6	0.9-2.8

OR: Odds ratio; CI: Confidence interval, ER: Estrogen receptor, TNM: Tumor, node, and metastasis

553 cases of breast cancer, the prognostic values of estrogen receptor, progesterone receptor, and p53 were found to be completely reproducible using the TMA method.[18] Even though prostate cancer is well known for its heterogeneity, TMA has still been shown to be a reliable tool evaluating biomarkers as prognostic indicator in prostate cancer.[19] In addition, TMAs have been reported to be reliable tools for the clinicopathological characterization of lung cancer tissue.[20] In this study, the results of NTN 1 expression in breast cancer using TMA were achieved smoothly. To the best of our knowledge, this is the first report with long-term follow-up regarding the use of TMAs to evaluate NTN 1 expression in invasive breast cancer. In this study, TMAs were used to analyze the expression of NTN 1 in 106 patients with a mean follow-up period of 69.0 ± 27.2 months (range, 5–98 months). We found that there was no significant relationship between NTN 1 and other basic clinicopathological parameters [Table 1]. By multivariate analysis, NTN 1 did not have any meaningful prognostic value and was not significantly related to the overall 5-year survival rate [$P = 0.111$, Table 3]. This is probably due to the retrospective nature of the study and the relatively small number of patients in this study. A prospective study with a larger number of patients is warranted to further investigate the prognostic value of NTN 1.

Conclusion

Immunohistochemical staining with TMAs was convenient and feasible to analyze the expression of NTN 1, in patients with breast cancer. Our preliminary results showed that NTN 1 expression had no signi cant prognostic value in breast cancer.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Dowsett M, Dunbier AK. Emerging biomarkers and new understanding of traditional markers in personalized therapy for breast cancer. Clin Cancer Res 2008;14:8019-26.
2. Pusztai L. Current status of prognostic profiling in breast cancer. Oncologist 2008;13:350-60.
3. Mook S, Schmidt MK, Rutgers EJ, van de Velde AO, Visser O, Rutgers SM, *et al*. Calbration and discriminatory accuracy of prognostic calculation for breast cancer with the online adjuvant program: A hospital-based retrospective cohort study. Lancet Oncol 2009;10:1070-6.
4. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, *et al.* Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A 2003;100:10393-8.
5. Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, *et al.* Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 1996;87:1001-14.
6. Forcet C, Stein E, Pays L, Corset V, Llambi F, Tessier-Lavigne M, *et al.* Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature 2002;417:443-7.
7. Arakawa H. Netrin-1 and its receptors in tumorigenesis. Nat Rev Cancer 2004;4:978-87.
8. Moch H, Kononen J, Kallioniemi OP, Sauter G. Tissue Microarrays: What will they bring to molecular and anatomic pathology. Adv Anat Pathol 2001;8:14-20.
9. van de Rijn M, Gilks CB. Applications of microarrays to histopathology. Histopathology 2004;44:97-108.
10. The World Health Organization histological typing of breast tumor – Second edition. Am J Clin Pathol 1982;78:808-16.
11. Sheen-Chen SM, Chen WJ, Eng HL, Chou FF. Serum concentration of tumor necrosis factor in patients with breast cancer. Breast Cancer Res Treat 1997;43:211-5.
12. Sheen-Chen SM, Eng HL, Chou FF, Chen WJ. The prognostic significance of proliferating cell nuclear antigen in patients with lymph node-positive breast cancer. Arch Surg 1997;132:264-7.
13. Sheen-Chen SM, Chen WJ, Eng HL, Chou FF. Serum-soluble interleukin-2 receptor concentrations in patients with breast cancer: A preliminary report. Changgeng Yi Xue Za Zhi 1998;21:133-8.
14. Perrone EE, Theoharis C, Mucci NR, Hayasaka S, Taylor JM, Cooney KA, *et al.* Tissue microarray assessment of prostate cancer tumor proliferation in African- American and White men. J Natl Cancer Inst 2000;92:937-9.
15. Paradisi A, Maisse C, Coissieux MM, Gadot N, Lépinasse F, Delloye-Bourgeois C, *et al.* Netrin-1 up-regulation in inflammatory bowel diseases is required for colorectal cancer progression. Proc Natl Acad Sci U S A 2009;106:17146-51.
16. Fitamant J, Guenebeaud C, Coissieux MM, Guix C, Treilleux I, Scoazec JY, *et al.* Netrin-1 expression confers a selective advantage for tumor cell survival in metastatic breast cancer. Proc Natl Acad Sci USA 2009;105:4850-5.
17. Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, *et al.* Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-7.
18. Torhortst J, Bucher C, Kononen J, Haas P, Zuber M, Köchli OR, *et al.* Tissue microarray for rapid liking of molecular changes to clinical endpoints. Am J Pathol 2001;159:2249-56.
19. Rubin MA, Dunn R, Strawderman M. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 2002;26:312-9.
20. Lars HS, Stefan B, Andreas F. Tissue microarray are reliable tools for the clinicopathological characterization of lung cancer tissue. Anticancer Res 2009;29:201-10.