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Vasculogenic Mimicry as an Important Marker is Related to Chemotherapy Response and Prognosis in Locally Advanced Triple Negative Breast Cancer

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Abstract

Background: It is important for locally advanced triple negative breast cancer (TNBC) patients to predict the response of neoadjuvant chemotherapy and prognosis. Vasculogenic mimicry (VM) is found to be closely related to the resistance of chemotherapy and prognosis. To evaluate if VM may be used as an important marker to predict chemotherapy response and prognosis in patients with locally advanced TNBC.

Methods: A total of 74 patients with locally advanced TNBC underwent biopsy, NAC, surgery, and systemic treatment; the microvessel density (MVD) and VM density (VMD) were assessed before and after NAC. Survival analysis was conducted on long-term follow-up data.

Results: The pCR rate was 25.7%. With a median follow-up time of 55 months, DFS rate was 94.7% in pCR group and 69.1% in non-pCR group (p=0.025). OS rate was 94.7% in pCR group and 72.7% in non-pCR group (p = 0.045). And patients with VM positive before NAC could not achieve pCR. The expression of VM after NAC was related with the response to NAC. DFS rate with less tumor neovascularization density (MVD+VMD, TNVD) after NAC was 91.9% and 22.2% in those with more TNVD (p < 0.001). OS rate was 94.6% in those with less TNVD and 27.8% in those with more TNVD (p < 0.001).

Conclusion: Positive VM before NAC may be a predictive factor for NAC efficacy and prognosis in locally advanced TNBC. Positive VM and high TNVD after NAC may be important prognostic factors for these patients with residual disease.

Keywords: Locally advanced breast cancer; Triple negative breast cancer; Neo-adjuvant chemotherapy; Vasculogenic mimicry; Chemotherapy response

Introduction

Neo-Adjuvant Chemotherapy (NAC) is the standard treatment for patients with Locally Advanced Breast Cancer (LABC) [1]. Achieving Pathological Complete Response (pCR) after NAC predicts favorable outcomes for Triple Negative Breast Cancer (TNBC) patients [2]. Patients with chemoresistance have higher inefficiency rate and poorer prognosis even if they change the regimen [3]. Studies suggest that the prognosis of TNBC patients who have Residual Disease (RD) is still poor [4]. RECIST 1.1 criteria are widely used in assessing the efficacy of clinical treatment [5], they are, however, dependent upon the morphological change of the tumor; errors occur when denaturation, necrosis or fibrous tissue hyperplasia take place within the tumor [6]. Therefore, for patients with locally advanced TNBC, there is an urgent need for clinical indicators that can assess the response to NAC before the treatment and predict the prognosis of patients who still have RD after NAC. For malignant tumors, blood supply contributes to cancer progression, recurrence and metastasis [7]. Angiogenesis and Vasculogenic Mimicry (VM) are the two ways in which malignant tumor tissues acquire oxygen and nutrients [8]. Folkman first proposed the hypothesis of tumor angiogenesis in 1971, where angiogenesis is the formation of capillaries from existing blood vessels that supply the tumor through budding [9,10]. Microvessel density (MVD), a derived marker of angiogenesis, has been shown to be associated with poor outcome in several cancers including breast cancer, lung cancer and colon cancer [11-13]. VM was first introduced by Maniotis et al. [14] in highly aggressive uveal melanomas, and is a vascular-like tubular channel formed directly by the tumor cells themselves. Previous studies suggested that the formation of VM might be a potent predictor of poor prognosis for patients with glioma [15,16]. We therefore proposed that angiogenesis and VM may be strongly associated with the NAC resistance and the poor prognosis in patients with locally advanced TNBC. In this study, by investigating patients with locally advanced TNBC, we examined MVD and Vasculogenic Mimicry Density (VMD) before and after NAC; explored the association between the change of parameters and chemotherapy responsiveness. Survival analysis was also conducted on long-term follow-up data. NAC reactivity, MVD and VMD were used to predict NAC tolerance and the long-term prognosis.

Materials and Method

Collection of patient samples

Between 1 June, 2014 and 31 May, 2016, a total of 74 patients with locally advanced TNBC who underwent biopsy, NAC, surgery and systemic treatment were recruited in the Department of Breast Surgery at Liaoning

Cancer Hospital & Institute, Shenyang, China. Inclusion criteria were: 1) no prior history of breast cancer or other malignancies; 2) invasive ductal carcinoma diagnosed by biopsy; 3) ER, PR and HER2 negative by immunohistochemistry (IHC) before NAC. Exclusion criteria were: 1) metastatic breast cancer. 2) pregnancy or lactation. The detailed procedure is shown in **Figure 1**. Anthropometric data (age at diagnosis, menstrual history, family history, surgery, chemotherapy) as well as tumor related variables (size, location, histological grade, tumor thrombosis, nodes, MVD and VMD by IHC before and after NAC) were collected.

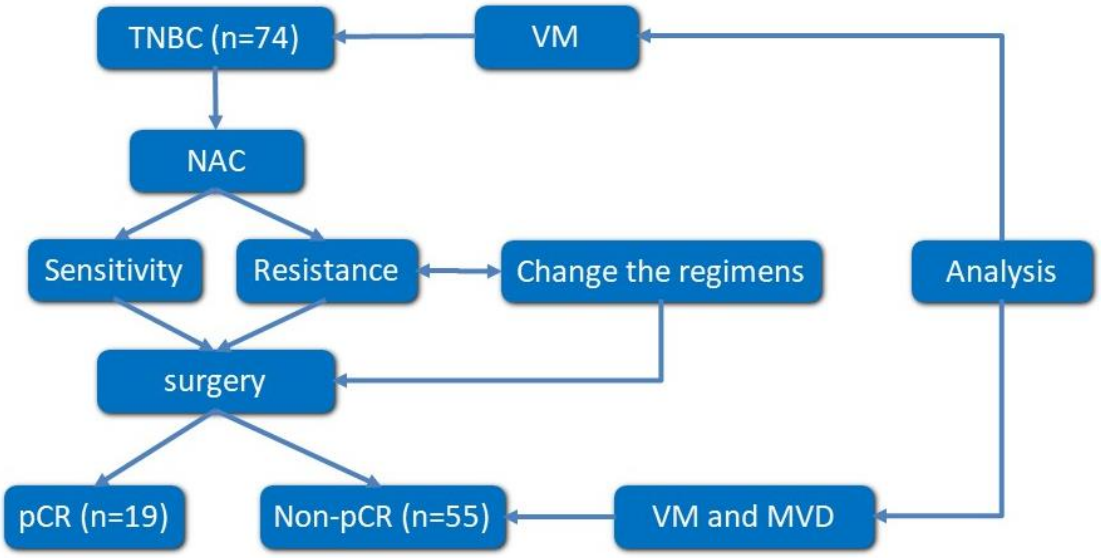

Figure 1: Flow chart of 74 locally advanced TNBC patients.

Immunohistochemistry (IHC) of tumor samples

IHC detecting ER, PR, HER2, Ki67, VM and blood microvessels was performed on formalin-fixed, paraffin-embedded tumor samples before and after NAC. The details of the operational procedures were consistent with the previous study [17]. Based on the St. Gallen Consensus 2013, the TNBC was negative for ER, PR and HER2 [18]. The pathological procedures and the antibodies used were the same for biopsy samples before NAC and samples obtained through open surgery after NAC.

CD31/PAS dual staining

CD31-PAS dual staining paraffin sections were cut at 5 μm. For demonstration of endothelial cells, the slides were incubated with rabbit polyclonal anti-human CD31 antibody (ab28364, abcam, UK, dilution 1:500), resulting in a brown product. To highlight the VM channels, slides were stained following the PAS staining procedures before counterstaining with Mayer's hematoxylin. To highlight the red blood cells in PAS positive patterns, the adjacent sections were stained with eosin after the above procedures. Microvessels were defined as any single CD31-stained cells or cluster of endothelial cells [11]. VM was determined as PAS-positive channels lined by tumor cells exclusively, rather than endothelial cells where RBCs were therein [16]. This part of the

study was completed by two additional independent pathologists who were unaware of the clinical outcome of patients. Tumor sections were examined under 200X magnification. The average numbers of the microvessels and VMs in up to five fields of view (for some biopsy specimens, only 1~2 fields were selected due to tissue size) represented the MVD and VMD of the tumor.

Clinical retrospective study

Participants were followed up with 3-month intervals in the first 2 years post-surgery; with 4~6-month intervals in 3~5 years after surgery; and with 6-month intervals after 5 years thereafter until 31 May, 2020. The median followed-up time was 55 months. The diagnosis of local recurrence or contralateral breast cancer was supported by biopsy; distant metastasis was diagnosed by biopsy and the Positron Emission Tomographic-Computer Tomography (PET-CT). Disease-Free Survival (DFS) was defined as the period between the first day after surgery and the date when first local recurrence or distant metastasis was confirmed. Overall Survival (OS) was calculated from the first day after surgery to death or 31 May, 2020. Anthropometric data and tumor related variables were collected. Tumor histological grades were classified as grades I–III according to the Nottingham combined histological grade **[19]**.

Statistical analysis

All statistical analyses were performed using SPSS software (version 17.0 for Windows). The differences in biological factors between groups were examined using student t test, chi-square test or rank-sum test where appropriate. For the survival analysis, Kaplan–Meier curves were built for OS and DFS analysis. ROC analysis was used to determine predictive values and determine cutoff value with optimal sensitivity and specificity and P values of < 0.05 were considered significant **[20]**. This cutoff value was used in the analysis. The log-rank test was used to compare survival differences among the groups. Cox proportional hazards models were established to calculate relative risk accounting for covariates.

Ethical approval

According to the Declaration of Helsinki, all the participants signed informed consent. The study protocol was approved by the ethics committee of Liaoning Cancer Hospital & Institute.

Results

Of the 74 patients, 19 were in the pCR group and 55 were in the non-pCR group. There were significant between-group differences in RECIST, preoperative Ki67, number of positive nodes after NAC, surgical approaches, cycle of NAC and VMD before NAC ($p < 0.05$). With a median follow-up time of 55 months, the DFS rate was 94.7% in the pCR group and 69.1% in non-pCR group ($p = 0.025$) with a significant difference in the curves for DFS ($p=0.028$) between the two groups **(Figure 2A)**. The OS rate were 94.7% in pCR group and 72.7% in non-pCR group ($p = 0.045$), the group-difference in the OS curves was also significant ($p = 0.047$) **(Figure 2B)**. Median DFS time were 64 months in pCR group and 63 months in non-pCR group ($p = 0.033$). There was no significant difference in the median OS time (63 months in the pCR group and 63 months in the non-pCR group, $p = 0.057$) **(Table 1)**.

Table 1: Patient characteristics and survival analysis (grouped by efficacy).

Characteristic	pCR (n=19)	non-pCR (n=55)	Statistics	P
Age(years)	52.58±9.84	53.38±9.82	0.094	0.760
Menopause			2.159	0.146
Premenopausal	12	24		
Postmenopausal	7	31		
Family History			0.431	0.514
No	15	47		
Yes	4	8		
Diameter before NAC (cm)	8.15±2.29	8.00±3.24	0.033	0.857
Resist			117.268	0.000
CR	19	0		
PR	0	30		
SD	0	22		
PD	0	3		
Ki67 before NAC	45.26±12.64	25.22±15.18	26.686	0.000
Quadrant			0.025	0.876
Areolar	0	3		
Inner upper	4	9		
Inner lower	2	4		
Outer lower	3	11		
Outer upper	10	28		
Number of metastatic lymph nodes	1.16±1.71	8.91±6.34	27.504	0.000

Number of Nodes	25.84±2.71	25.89±5.22	0.002	0.969
Operation			9.760	0.003
MRM	9	43		
BRS	8	12		
BCS	2	0		
BRCA			3.147	0.080
No	19	47		
Yes	0	8		
NAC Program			1.849	0.178
TEC/TAC	19	50		
TP	0	5		
Cycle of NAC	5.68±0.75	4.95±1.03	8.289	0.005
VMD before NAC	0	0.64±0.93	8.812	0.004
MVD before NAC	25.37±8.58	28.78±9.57	1.890	0.173
Overall Survival	94.7%	72.7%	4.154	0.045
Survival	18	40		
Dead	1	15		
Median Survival Time	63.00	63.00	3.731	0.057
Disease-Free Survival	94.7%	69.1%	5.269	0.025
Disease-Free Survival	18	38		
Metastasis	1	17		
Median Disease-Free Survival Time	63.00	63.00	4.748	0.033

NAC = Neoadjuvant Chemotherapy

CR = Complete Remission

PR = Partial Remission

SD = Stable Disease

PD = Progressive Disease

MRM = Modified Radical Mastectomy

BRS = Breast Reconstruction Surgery

BCS = Breast Conservation Surgery

VMD = Vasculogenic Mimicry Density

MVD = Microvessel Density

The expressions of VM before NAC are shown in **Figure 2C and D**. As demonstrated in **Figure 2E**, patients with positive VM before NAC were unable to achieve pCR. VMD pre-NAC was negatively correlated with Miller-Payne (MP) grade **(Figure 2F)**. The proportion of positive VM after NAC trended downward from MP 4 to MP 1 grade **(Figure 2G)**.

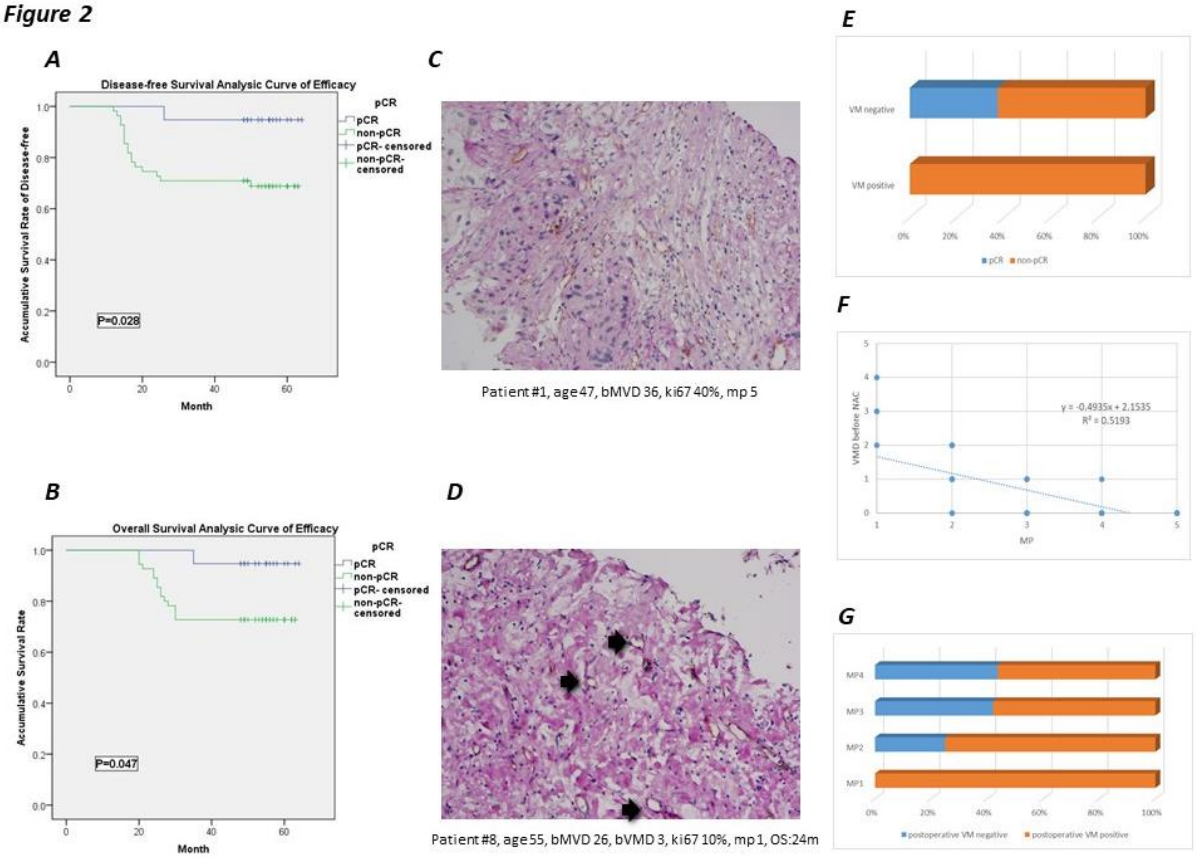

Figure 2: A) The disease-free survival (DFS) analytic curve of efficacy; B) The overall survival (OS) analytic curve of efficacy; C) The brown were CD31-stained cells or cluster of endothelial cells in biopsy; D) The black arrows pointed the structure of Vasculogenic Mimicry (VM); E) The proportion of patients with VM negative or

positive before NAC in pCR or non-pCR group; F) The correlation between VM density and MP grades; G) The proportion of patients with different MP grades in VM disappear or continue after NAC group.

Of the 74 patients, 51 were classified into the VM negative before NAC group and 23 patients into the VM positive group before NAC **(Table 2)**. There were significant differences in post-NAC diameter, Ki67 before NAC, RECIST, number of positive nodes, MP between the two groups ($p < 0.05$). With a median follow-up time of 55 months, the DFS rate in the VM negative before NAC group and the VM positive before NAC group were 88.2% and 47.8%, respectively ($p < 0.001$); significant difference was observed in DSF surviving curves between groups ($p < 0.001$) **(Figure 3A)**. The OS rates in the VM negative before NAC group and in the VM positive before NAC group groups were 90.2% and 52.2%, respectively ($p < 0.001$); significant difference was found in OS surviving curves ($p < 0.001$) **(Figure 3B)**. The median DFS time were 64 months in the VM negative before NAC group and 25.5 months in the VM positive before NAC group ($p < 0.001$). The median OS time were 64 months in the VM negative before NAC and 62 months in the VM positive before NAC group ($p = 0.001$).

Table 2: Patient characteristics and survival analysis (grouped by VM before NAC).

Characteristic	**VM negative before NAC (n=51)**	**VM positive before NAC (n=23)**	**Statistics**	**P**
Age(years)	53.43±9.72	52.61±10.05	0.111	0.740
Menopause			0.162	0.689
Postmenopausal	24	12		
Premenopausal	27	11		
Family History			0.736	0.394
No	44	28		
Yes	7	5		
Diameter before NAC (cm)	7.90±2.82	8.34±3.45	0.328	0.568
Diameter after NAC (cm)	4.17±2.54	5.92±3.19	5.172	0.027
Resist			19.354	0.000
CR	19	0		
PR	22	8		

SD	8	14		
PD	2	1		
Ki67 before NAC	33.35±16.26	23.74±16.92	5.404	0.023
Quadrant			2.489	0.119
Areolar	1	2		
Inner upper	7	6		
Inner lower	6	0		
Outer lower	8	6		
Outer upper	29	9		
Histological Grade after NAC			0.211	0.648
I	2	0		
II	9	11		
III	21	12		
Cancer Thrombosis after NAC			0.457	0.502
Negative	21	13		
Positive	11	10		
Number of metastatic lymph nodes	4.57±4.82	12.13±6.74	30.170	0.000
Number of Nodes	26.47±4.59	24.57±4.74	2.679	0.106
Operation			0.168	0.683
MRM	35	17		
BRS	14	6		
BCS	2	0		
BRCA			0.168	0.683
No	46	20		

Yes	5	3		
NAC Program			0.194	0.661
TEC/TAC	48	21		
TP	3	2		
Cycle of NAC	5.16±1.03	5.09±1.00	0.075	0.785
MP			77.599	0.000
1	0	4		
2	2	14		
3	15	4		
4	15	1		
pCR	19	0		
Overall Survival	90.2%	52.2%	16.099	0.000
Survival	46	12		
Dead	5	11		
Median Survival Time	64	63	11.429	0.001
Disease-Free Survival	88.2%	47.8%	16.891	0.000
Disease-Free Survival	45	11		
Metastasis	6	12		
Median Disease-Free Survival Time	64	25.5	14.419	0.000

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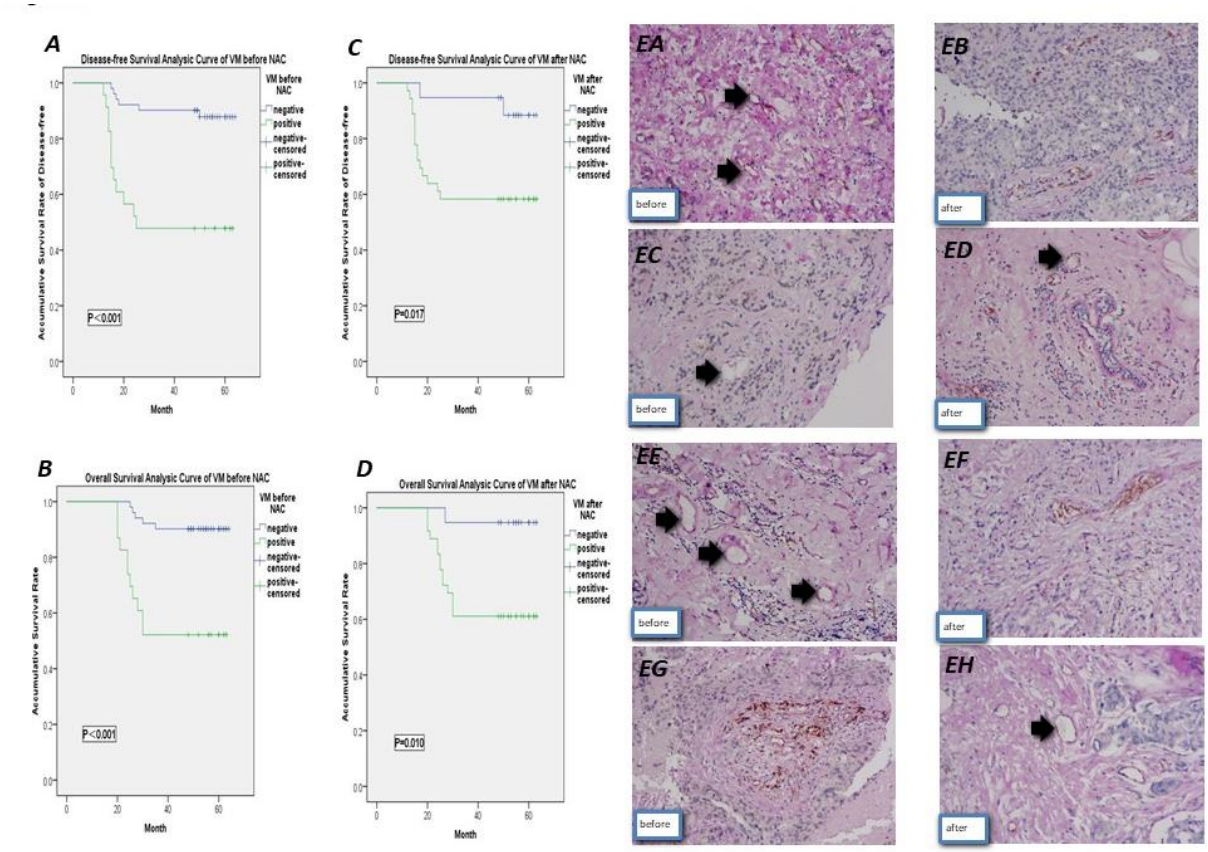

Figure 3: (A-D) the survival analytic curve; (E) the IHC before and after NAC, the brown was CD31-stained cells or cluster of endothelial cells in biopsy, the black arrows pointed the structure of VM. Of the 55 patients with non-pCR, 19 were classified into the postoperative VM negative group and 36 patients into the postoperative VM positive group **(Table 3)**. There were significant differences in post-NAC diameter, RECIST, number of positive nodes between the two groups ($p < 0.05$). With a median follow-up time of 55 months, the DFS rate in the postoperative VM negative group and the postoperative VM positive group were 89.5% and 58.3%, respectively ($p = 0.017$); significant difference was observed in DSF surviving curves between groups ($p = 0.019$) **(Figure 3C)**. The OS rates in the postoperative VM negative group and in the postoperative VM positive group groups were 94.7% and 61.1%, respectively ($p = 0.007$); significant difference was found in OS surviving curves ($p=0.010$) **(Figure 3D)**. The median DFS time were 63 months in the postoperative VM negative group and the postoperative VM positive group ($p = 0.023$). The median OS time were 63 months in the postoperative VM negative and postoperative VM positive group ($p = 0.037$). The survival times were identical because of the small sample size.

Table 3: Patient characteristics and survival analysis (grouped by VM after NAC).

Characteristic	Postoperative VM negative (n=19)	Postoperative VM positive (n=36)	Statistics	P
Age (years)	55.58±7.426	52.22±10.79	1.466	0.231
Menopause			0.159	0.692
Postmenopausal	9	15		
Premenopausal	10	21		
Family History			2.013	0.162
No	18	29		
Yes	1	7		
Diameter before NAC (cm)	7.04±2.59	8.51±3.46	2.653	0.109
Diameter after NAC (cm)	3.24±2.07	5.78±2.96	11.085	0.002
Resist			11.738	0.001
PR	16	14		
SD	3	19		
PD	0	3		
Ki67 before NAC	28.74±15.87	23.36±14.68	1.577	0.215
Quadrant			0.716	0.401
Areolar	2	1		
Inner upper	3	6		
Inner lower	1	3		
Outer lower	5	6		
Outer upper	8	20		
Histological Grade after NAC			0.720	0.400

I	2	0		
II	6	14		
III	11	22		
Cancer Thrombosis after NAC			0.183	0.670
Negative	11	23		
Positive	8	13		
Number of metastatic lymph nodes	6.26±4.34	10.31±6.82	5.483	0.023
Number of Nodes	26.74±5.11	25.44±5.29	0.759	0.388
Operation			0.010	0.922
MRM	15	28		
BRS	4	8		
BCS	0	0		
BRCA			0.035	0.853
No	16	31		
Yes	3	5		
NAC Program			0.070	0.793
TEC/TAC	17	33		
TP	2	3		
Cycle of NAC	4.84±1.07	5.00±1.01	0.291	0.592
MP			3.205	0.079
1	0	4		
2	4	12		
3	8	11		
4	7	9		
Overall Survival	94.7%	61.1%	7.843	0.007

Survival	18	22		
Dead	1	14		
Median Survival Time	63	63	4.563	0.037
Disease-Free Survival	89.5%	58.3%	6.065	0.017
Disease-Free Survival	17	21		
Metastasis	2	15		
Median Disease-Free Survival Time	63	63	5.483	0.023

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Tumor Neovascularization Density (TNVD) (AUC=0.943, $P < 0.05$, **Figure 4A**) ovaries significantly predicted poor prognosis. And according to the ROC curve, the optimal cutoff value for mean intensity of TNVD was 30/x20 for DFS. There were 37 patients with less TNVD and 18 patients with more TNVD after NAC **(Table 4)**. Difference was significant in diameter before and after NAC, Ki67 before NAC, number of positive nodes after NAC and MP between two groups ($p < 0.05$). With a median follow-up time of 55 months, the DFS rate was 91.9% in those with less TNVD and 22.2% in those with more TNVD ($p < 0.001$), with significant difference in DFS curves ($p < 0.001$) between two groups **(Figure 4B)**. The OS rate was 94.6% in those with less TNVD and 27.8% in those with more TNVD ($p < 0.001$), the difference was also significant in OS curves ($p < 0.001$) **(Figure 4C)**. The median OS time for patients with less TNVD was 63 months, and 26.5 months for patients with more TNVD ($p < 0.001$). Those with less TNVD had a median DFS time of 63 months, compared with 16.5 months for those with more TNVD ($p = 0.006$) **(Table 4)**. The expressions of VM and angiogenesis are shown in **Figure 4F and G**.

Table 4: Patient characteristics and survival analysis (grouped by TNVD after NAC).

Characteristic	Less TNVD after NAC (n=37)	More TNVD after NAC (n=18)	Statistics	P
Age(years)	53.81±9.64	52.50±10.40	0.213	0.647
Menopause			0.007	0.934
Postmenopausal	16	8		
Premenopausal	21	10		
Family History			0.246	0.622
No	31	16		
Yes	6	2		
Diameter before NAC (cm)	6.70±2.18	10.68±3.47	27.048	0.000
Diameter after NAC (cm)	4.02±2.61	6.70±2.78	12.264	0.001
Resist			1.845	0.180
CR				
PR	23	7		
SD	12	10		
PD	2	1		
Ki67 before NAC	30.54±14.47	14.28±9.98	18.384	0.000
Quadrant			2.375	0.129
Areolar	2	1		
Inner upper	4	5		
Inner lower	2	2		
Outer lower	8	3		
Outer upper	21	7		

Histological Grade after NAC			2.116	0.152
I	2	0		
II	15	5		
III	20	13		
Cancer Thrombosis after NAC			1.571	0.216
Negative	25	9		
Positive	12	9		
Number of metastatic lymph nodes	5.92±4.33	15.06±5.33	46.277	0.000
Number of Nodes	25.84±5.06	26.00±5.68	0.011	0.915
Operation			0.404	0.528
MRM	28	15		
BRS	9	3		
BRCA			0.001	0.194
No	30	17		
Yes	7	1		
NAC Program			0.001	0.534
TEC/TAC	33	17		
TP	4	1		
Cycle of NAC	4.78±1.03	5.28±0.96	2.907	0.094
MP			19.634	0.000
1	0	4		
2	8	8		
3	14	5		
4	15	1		

Overall Survival	94.6%	27.8%	52.067	0.000
Survival	35	5		
Dead	2	13		
Median Survival Time	63.0	26.5	52.894	0.000
Disease-Free Survival	91.9%	22.2%	53.088	0.000
Disease-Free Survival	34	4		
Metastasis	3	14		
Median Disease-Free Survival Time	63	16.5	56.083	0.000

TNVD = Tumor Neovascularization Density

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BRS = Breast Reconstruction Surgery

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Figure 4

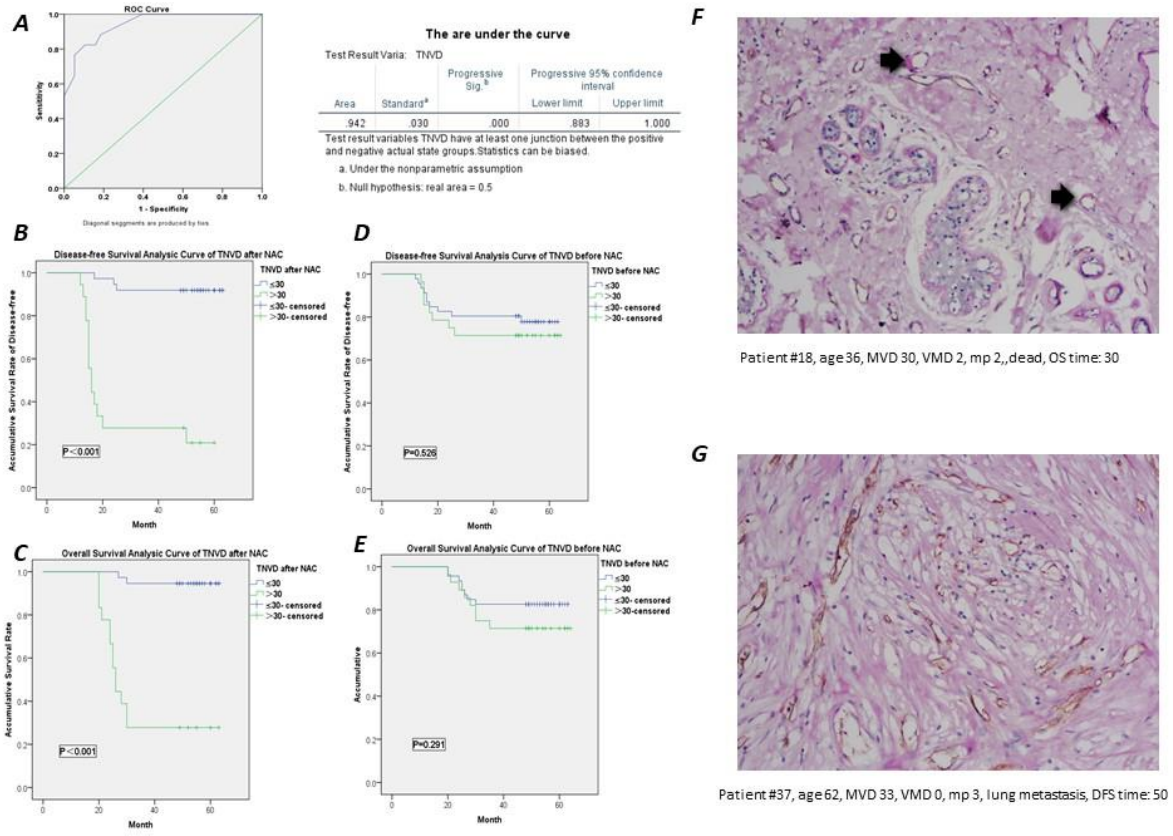

Figure 4: (A) The ROC curve; (B-E) the survival analytic curve; (F-G) the IHC after NAC, the brown was CD31-stained cells or cluster of endothelial cells in biopsy, the black arrows pointed the structure of VM.

Among the total of 74 patients, 46 had less TNVD and 28 had more TNVD before NAC, the difference was statistically significant. With a median follow-up time of 55 months, the DFS rate was 78.3% in those with less TNVD and 71.4% in those with more TNVD (p = 0.513), with no significant difference in the curves for DFS (p = 0.526) between the two groups **(Figure 4D)**. The OS rate was 82.6% in those with less TNVD and 71.4% in those with more (p = 0.263), with no significant difference in the curves for OS (p = 0.291) **(Figure 4E)**. No statistical difference was found in OS rate or median OS time (63 months for those with less TNVD and 64 months for those with more TNVD, p = 0.383). There was no significant difference in median DFS time (63 months for those with less TNVD and 64 months for those with more TNVD, p = 0.405) **(Table 5)**.

Table 5: Patient characteristics and survival analysis (grouped by TNVD before NAC).

Characteristic	**Less TNVD before NAC (n=46)**	**More TNVD before NAC (n=28)**	**Statistics**	**P**
Age(years)	54.78±8.81	50.54±10.80	3.403	0.069
Menopause			2.648	0.108

Postmenopausal	19	17		
Premenopausal	27	11		
Family History			0.087	0.769
No	39	23		
Yes	7	5		
Diameter before NAC (cm)	8.00±2.90	8.11±3.23	0.025	0.875
Diameter after NAC (cm)	4.44±2.97	5.54±2.80	1.910	0.173
Resist			2.584	0.112
CR	14	5		
PR	20	10		
SD	10	12		
PD	2	1		
Ki67 before NAC	32.33±17.33	27.14±16.11	1.641	0.204
Quadrant			0.277	0.600
Areolar	2	1		
Inner upper	7	6		
Inner lower	3	3		
Outer lower	10	4		
Outer upper	21	14		
Histological Grade after NAC			0.000	0.986
I	0	2		
II	14	6		
III	18	15		
Cancer Thrombosis after			0.987	0.325

NAC				
Negative	18	16		
Positive	14	7		
Number of metastatic lymph nodes	6.20±6.66	8.11±6.11	1.523	0.221
Number of Nodes	25.52±4.26	26.46±5.34	0.701	0.405
Operation			0.173	0.679
MRM	33	19		
BRS	12	8		
BCS	1	1		
BRCA			0.010	0.459
No	42	24		
Yes	4	4		
NAC Program			0.010	0.919
TEC/TAC	43	26		
TP	3	2		
Cycle of NAC	5.11±1.03	5.18±0.98	0.082	0.775
MP			1.524	0.221
1	4	0		
2	5	11		
3	13	6		
4	10	6		
pCR	14	5		
Overall Survival	82.6%	71.4%	1.271	0.263
Survival	38	20		
Dead	8	8		
Median Survival Time	63.0	64	0.771	0.383

Disease-Free Survival	78.3%	71.4%	0.432	0.513
Disease-Free Survival	36	20		
Metastasis	10	8		
Median Disease-Free Survival Time	63	64	0.447	0.506

TNVD = Tumor Neovascularization Density

NAC = Neoadjuvant Chemotherapy

CR = Complete Remission

PR = Partial Remission

SD = Stable Disease

PD = Progressive Disease

MRM = Modified Radical Mastectomy

BRS = Breast Reconstruction Surgery

BCS = Breast Conservation Surgery

In the COX proportional hazard model of death and tumor progression, TNVD had a significant effect on death, histological grade, number of metastatic lymph nodes and tumor progression ($p < 0.05$) **(Table 6)**.

Table 6: Cox proportional hazards model of biological factors.

Parameters	**Death**		**Tumor Progression**	
	Sig.	**EXP(B)**	**Sig.**	**EXP(B)**
Age	0.160	0.910	0.192	0.918
Menopause	0.188	5.786	0.109	7.854
Family History	0.286	2.792	0.203	3.270
Tumor Size after NAC	0.717	1.061	0.564	1.087
Tumor Location	0.255	0.759	0.113	0.683
Histological Grade	0.240	0.377	0.044	0.239

Cancer Thrombosis	0.918	0.913	0.353	2.175
Number of metastasis Lymph Nodes	0.091	1.186	0.016	1.305
TNVD	0.027	13.621	0.007	17.175
MP	0.942	1.056	0.834	0.855
Ki67	0.498	1.056	0.214	1.047

NAC = Neoadjuvant Chemotherapy

TNVD = Tumor Neovascularization Density

Discussion

Similar to previous studies, patients with TNBC who achieved pCR had a significantly better prognosis than those who did not achieve pCR after NAC **(Figure 2A and B)** **[21]**. In Zheng's study on the efficiency of NAC in patients with locally advanced breast cancer, the concurrence of osteopontin expression and positive VM predicted a failure achieving pCR **[22]**. In our study, none of the patients with observable VM structures prior to treatment achieved pCR after NAC **(Figure 2C-E)**. The formation of VM was reported to be closely associated with the mode of drug resistance in studies including melanoma and breast cancer **[23,24]**. Further analysis revealed that pre-treatment VMD was negatively correlated with MP grading before NAC, and whether VM was expressed after NAC was also correlated with MP grading **(Figure 2F and G)**. There are also studies showed that tumor cells with a stem cell-like phenotype were involved in VM formation, which may be closely related to chemoresistance **[24,25]**. The observation of VM structure pre- or post- NAC is suggestive of tumor insensitivity to NAC. The main cause of death in malignant solid tumors, including breast cancer, is metastasis **[26]**. VM is widely present in highly malignant tumors, and is strongly related to metastasis **[27,28]**. The present study demonstrates that the presence of VM both before and after NAC suggests increased risk of metastasis and poor prognosis **(Figure 3A-D)**. Indeed, the hypoxic tumor microenvironment in tumor center induces VM formation, also enhances tumor aggressiveness and increases the probability of distant metastasis through inducing an epithelial-mesenchymal-transition-like phenotype **[29]**. Specifically, in the early stage of VM in nearly all cases, planar-like pattern was predominant; cytokinesis was parallel to the VM channel. In contrast, in the late stage, mitotic spindle orientation was perpendicular to the VM channel wall, one daughter cell would stay in the VM channel and the other daughter cell would be detached from the VM channel wall and flow into the lumen of VM **[30]**. Consequently, VM not only serves as a blood supply mechanism spontaneously generated by tumor cells, but also is closely related to its invasive metastasis and affects the prognosis.

The formation of VM is considered to be the main reason for the resistance to antiangiogenic therapy in clinical practice **[31]**. The formation of VM was promoted by bevacizumab-induced macroautophagy/autophagy in glioma stem cells, which was associated with tumor resistance to antiangiogenic therapy **[16]**. Both angiogenesis and vasculogenic mimicry were ways for tumors to obtain blood supply, leading to poor prognosis **[32]**. These indicate that the molecular mechanisms of VM formation are complex and the relationship between

VM and angiogenesis is still unclear. However, previous studies suggested that MVD and VM could be independently important predictors of malignancy prognosis **[11-13,16]**. We considered both VM and angiogenesis as measures for tumor neovascularization, the high ROC curve of TNVD represented high prediction accuracy, and we determined a cutoff value of 30 for TNVD **(Figure 4A)**. TNVD more than 30/x200 after NAC suggests severe poor prognosis **(Figure 4B and C)**. But when using 30/x200 for verification before NAC, TNVD was not associated with prognosis **(Figure 4D and E)**. It might be because that the size of biopsy pathology was generally too small, and the pathology after NAC could be more representative of the intratumoral neovascular status than the biopsy pathology. Intratumoral neovascularization is linked to the possibility for the tumor to metastasize, thus is a key determinant of tumor overall aggressiveness and the prognosis **[33]**. The COX proportional hazard model suggested that TNVD after NAC had a significant effect on death, in addition to histological grade and number of metastatic lymph nodes, and a significant effect on tumor progression. Both angiogenesis and VM were means for tumor to obtain nutrition, and high TNVD after NAC indicated poor prognosis. In summary, we have found that, a positive VM before NAC suggested NAC resistance and poor prognosis in locally advanced TNBC; a positive VM after NAC indicated poor prognosis; a higher TNVD (30/x200) after NAC indicated a severe poor prognosis.

Conclusion

The formation of VM before NAC may be an important predictive factor for NAC efficacy and prognosis in locally advanced TNBC, the VM and TNVD after NAC may be important prognostic factors. However, the relationship between angiogenesis and VM needs to be further investigated.

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