Breast Cancer: Targets and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Neuregulin-1 Secreted by Cancer-Associated Fibroblasts Promotes Growth of Triple-Negative Breast Cancer

Lu Xu*, Hongbo Xu*, Dengsheng Jiang*, Shilong Song, Gengming Wang, Xianwen Zhang, Yajun Zhang, Jing Qian, Jia Kang, Feng Cai

Department of Radiation Oncology, the First Affiliated Hospital of Bengbu Medical University, Bengbu, Anhui, 233000, People's Republic of China

*These authors contributed equally to this work

Correspondence: Feng Cai, Department of Radiation Oncology, the First Affiliated Hospital of Bengbu Medical University, Zhihuai Road, Bengbu, Anhui, 233000, People's Republic of China, Tel +86-13645529901, Email caifeng1225@bbmu.edu.cn

Background: Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer lacking estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, which limits targeted therapies. Cancer-associated fibroblasts (CAFs) in the tumor microenvironment are known to secrete factors that promote tumor progression. The role of neuregulin-1 (NRG1), a ligand of HER3 and HER4, in TNBC and its association with CAFs remains unclear.

Methods: We analyzed NRG1 mRNA and protein levels in TNBC tumor and adjacent tissues using qRT-PCR and ELISA, and evaluated their relationship with clinicopathological parameters and prognosis in 174 patients. Serum NRG1 levels were assessed via ELISA in TNBC patients and healthy controls. The effects of CAF-secreted NRG1 on TNBC cells were studied using conditioned medium, siRNA knockdown, and xenograft mouse models. Statistical analyses, including ROC curves and survival analyses, were performed to determine diagnostic and prognostic value.

Results: NRG1 expression was significantly upregulated in TNBC tissues and serum compared to controls, correlating with advanced TNM stages and poor prognosis. ROC analysis demonstrated diagnostic value of NRG1 in tissues and serum. CAFs secreted higher levels of NRG1 compared to normal fibroblasts. Conditioned medium from CAFs enhanced TNBC cell proliferation, migration, and MMP9 expression, effects reduced by NRG1 knockdown. In vivo, CAF-conditioned medium promoted tumor growth, while NRG1 knockdown reduced this effect.

Conclusion: Our findings suggest that CAF-secreted NRG1 is closely associated with TNBC progression and may represent a potential biomarker and therapeutic target, although further validation is needed to establish causality.

Keywords: triple-negative breast cancer, NRG1, cancer-associated fibroblasts, tumor microenvironment, biomarker

Introduction

Breast cancer is the most prevalent malignancy among women globally, with its incidence and mortality increasing with age.[1,2] Triple-negative breast cancer (TNBC), defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, accounts for 12–18% of breast cancer cases.[3,4] TNBC is associated with a high likelihood of recurrence, metastasis, and poor prognosis due to its aggressive nature and limited therapeutic options.[5] Despite the efficacy of chemotherapy regimens involving taxanes, anthracyclines, cyclophosphamide, cisplatin, and fluorouracil, TNBC often exhibits resistance, early metastasis, and frequent recurrence.[6,7] Consequently, identifying specific and accessible prognostic markers remains an urgent need in TNBC management.[8]

The tumor microenvironment (TME), comprising various stromal components, is increasingly recognized as a critical driver of cancer progression.[9,10] Among the cellular components of the TME, cancer-associated fibroblasts (CAFs) are the most abundant and are key regulators of tumor progression.[11] Unlike their quiescent counterparts in normal tissues,

Received: 12 July 2025
Accepted: 7 December 2025
Published: 26 December 2025

CAFs are activated fibroblasts characterized by elevated α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) expression. CAFs influence tumor growth, invasion, angiogenesis, and extracellular matrix remodeling.[12,13] Moreover, CAFs contribute to immune evasion by secreting immunosuppressive cytokines and enhancing immune checkpoint expression.[14]

Neuregulin-1 (NRG1), a membrane glycoprotein and member of the epidermal growth factor (EGF) family, plays essential roles in cell growth and differentiation by binding to HER3 and HER4 receptors and activating downstream ErbB signaling.[15,16] Although NRG1 has been implicated in resistance to HER2-targeted therapies in breast cancer, its role in TNBC remains poorly understood.[17] Given the heterogeneity of CAFs and their dynamic interactions with tumor cells, this study aimed to investigate the expression and functional role of NRG1 in TNBC, particularly its secretion by CAFs, and its impact on tumor progression and patient prognosis.

We hypothesized that CAF-secreted NRG1 drives TNBC progression and could serve as a biomarker and therapeutic target. To test this hypothesis, we analyzed NRG1 expression in TNBC tissues and serum, evaluated its diagnostic and prognostic significance, and assessed its functional role in vitro and in vivo.

Methods and Materials

Patient Enrollment and Criteria

A total of 174 patients with histopathologically confirmed triple-negative breast cancer (TNBC) were recruited at the First Affiliated Hospital of Bengbu Medical University. The study protocol was approved by the institutional ethics committee (#2024-JK-35), and written informed consent was obtained from all participants. Inclusion criteria were: (i) age 18–75 years; (ii) newly diagnosed, treatment-naïve TNBC confirmed by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH), showing ER, PR, and HER2 negativity; (iii) AJCC 8th edition stage I–III disease; and (iv) Eastern Cooperative Oncology Group (ECOG) performance status 0–1. Exclusion criteria included: (i) stage IV disease with distant metastasis; (ii) coexisting cancers or autoimmune diseases; (iii) severe organ dysfunction defined as ALT/AST >2.5 × ULN (≤5 × ULN in patients with liver metastasis), TBIL >1.5 × ULN, Scr >1.5 × ULN or Ccr <60 mL/min, NYHA class ≥III or LVEF <50%, ANC <1.5 × 10^9/L, PLT <100 × 10^9/L, or Hb <90 g/L. Comprehensive clinical, laboratory, imaging, and treatment data were available for all patients. For comparison, 100 age-matched healthy women were recruited as controls, and serum samples were collected for NRG1 analysis.

Sample Collection and Analysis

Blood samples were collected in EDTA-coated tubes and centrifuged at 1500 × g for 10 minutes at 4°C to separate the serum. The serum was aliquoted and stored at −80°C until further analysis. Serum NRG1 concentration was determined using Human NRG1 ELISA Kit (Cat# EH1972, Wuhan Fine Biotech Co., Ltd., Wuhan, China).

Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green Master Mix (Applied Biosystems, Waltham, MA) on a StepOnePlus Real-Time PCR System. Primers were designed for NRG1 and GAPDH (internal control) as follows:

- NRG1: Forward Primer CGGTGTCCATGCCTTCCAT, Reverse Primer GTGTCACGAGAAGTAGAGGTCT
- GAPDH: Forward Primer TGTGGGCATCAATGGATTTGG, Reverse Primer ACACCATGTATTCCGGGTCAAT

The relative expression levels of NRG1 were calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the normalization control.

Fibroblast Isolation and Culture

Primary cancer-associated fibroblasts (CAFs) were isolated from TNBC tumor tissues, while normal fibroblasts (NFs) were derived from paired adjacent normal tissues of the same patients. This paired design ensured that each NF/CAF pair originated from the same individual, thereby effectively eliminating confounding factors such as genetic background, age, lifestyle, and baseline physiological status. Thus, the NF source fully meets the recommendation of using paired

adjacent normal tissue as the optimal control. Tissues were rinsed with sterile PBS, minced into ~1 mm^3 fragments, and digested with 0.12% collagenase A at 37°C for 8 h. The digested samples were filtered, and cells were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. Fibroblasts at passages 2–6 were used for experiments. Conditioned medium (CM) was prepared by culturing fibroblasts in DMEM/F12 supplemented with 10% FBS and 1% L-glutamine. At ~95% confluency, media were replaced, and CM was collected after 48 h, centrifuged at 1500 × g for 10 min, filtered, and either used fresh or stored at –80°C.

Functional Analysis of NRG1 in CAFs

NRG1 knockdown in CAFs was achieved using siRNA (GenePharma, Shanghai, China) transfected with Lipofectamine 2000 (Thermo Fisher Scientific). Cells were incubated with siRNA for 48 h before use. The efficiency of knockdown was verified by qRT-PCR, Western blot, and ELISA of secreted NRG1. To evaluate functional effects, MDA-MB-231 cells were cultured with CM from NFs (NF-CM), CAFs (CAF-CM), or NRG1-knockdown CAFs (siNRG1-CAF-CM). Rescue experiments were conducted by supplementing siNRG1-CAF-CM with recombinant human NRG1 (10 ng/mL; Cell Signaling Technology).

Cell viability was assessed using the CCK-8 assay (Dojindo, Japan). For colony formation assays, cells were seeded in 6-well plates and cultured with CM for 10 days. Colonies were fixed in methanol, stained with 0.1% crystal violet, and quantified.

Animal Experiments

Female BALB/c nude mice (6–7 weeks old, SPF grade) were obtained from an accredited facility. Subcutaneous xenografts were generated by injecting 2 × 10^6 MDA-MB-231 cells mixed with 50% Matrigel into the left flank. When tumors reached ~100 mm^3, mice were randomized into three groups (n = 6/group): control, CAF-CM (intratumoral injection of 10 μL 50× concentrated CAF-CM every three days), and siNRG1-CAF-CM (intratumoral injection of 10 μL 50× concentrated siNRG1-CAF-CM every three days). Tumor volumes were measured every three days using calipers (volume = 0.5 × length × $width^2$). After 35 days, tumors were excised, weighed, photographed, and processed for IHC analysis of Ki67 and MMP9. All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) (#2024-DWSY-208).

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0. Results are expressed as mean ± standard deviation (SD). Comparisons between two groups were made using unpaired or paired *t*-tests as appropriate. For multiple group comparisons, one-way or two-way ANOVA followed by Tukey's post hoc test was used. Kaplan–Meier survival curves were analyzed with the Log rank test. ROC curves were constructed to evaluate diagnostic performance. A p-value < 0.05 was considered statistically significant.

Results

NRG1 mRNA Expression and Clinicopathological Features of TNBC Patients

Based on the median value, patients were stratified into high and low NRG1 mRNA expression groups, and the associations with clinicopathological characteristics were analyzed (Table 1). High NRG1 expression was significantly correlated with more advanced T stage (T3–T4, p = 0.021), higher pathological stage (stage III, p = 0.033), and more severe N stage (N2–N3, p = 0.009). No significant associations were observed with age, menstrual status, or tumor laterality.

NRG1 mRNA Expression in TNBC Tumor Tissues

qRT-PCR analysis showed that NRG1 mRNA expression was markedly higher in TNBC tumor tissues compared with adjacent normal tissues (p < 0.001; Figure 1a). Paired analysis from the same patients confirmed this upregulation (p < 0.001; Figure 1b). ROC curve analysis further demonstrated the diagnostic potential of NRG1 mRNA, with an AUC of

Table 1 *NRG1* mRNA Expression and Clinicopathological Characterization in Triple-Negative Breast Cancer (TNBC) Patients (n = 174)

Characterization	*NRG1* mRNA		p value
	Low Expression (n = 87)	High Expression (n = 87)	
Age (years)			
< 50	38 (43.7%)	32 (36.8%)	0.439
≥ 50	49 (56.3%)	55 (63.2%)	
Menstrual state			
Menopause	52 (59.8%)	56 (64.4%)	0.639
Non-menopause	35 (40.2%)	31 (35.6%)	
Tumor location			
Left	43 (49.4%)	41 (47.1%)	0.880
Right	44 (50.6%)	46 (52.9%)	
T stage			
T1–T2	68 (78.2%)	53 (60.9%)	0.021
T3-T4	19 (21.8%)	34 (39.1%)	
Pathological stage			
I–II	50 (57.5%)	35 (40.2%)	0.033
III	37 (42.5%)	52 (59.8%)	
N stage			
N0-N1	54 (62.1%)	36 (41.4%)	0.009
N2-N3	33 (37.9%)	51 (58.6%)	

Notes: The data were shown with n (%). Fisher's exact test.
Abbreviation: TNM, tumor-node-metastasis.

0.76 and sensitivity and specificity of 66.67% and 76.44%, respectively (Figure 1c). Subgroup analysis revealed that elevated NRG1 expression was significantly associated with advanced T stage (T3–T4, $p < 0.001$; Figure 1d), higher pathological stage (stage III, $p < 0.01$; Figure 1e), and more severe N stage (N2–N3, $p < 0.01$; Figure 1f). These results indicate that NRG1 mRNA is consistently upregulated in TNBC tissues and correlates with disease progression.

NRG1 Protein Concentration in TNBC Tumor Tissues

ELISA analysis revealed that NRG1 protein concentrations were significantly higher in tumor tissues than in adjacent normal tissues ($p < 0.001$; Figure 2a). This finding was validated in paired analyses of tumor and adjacent tissues from the same patients ($p < 0.001$; Figure 2b). ROC curve analysis demonstrated strong diagnostic value, with an AUC of 0.79, and sensitivity and specificity of 62.64% and 85.63%, respectively (Figure 2c). Subgroup analyses showed that higher NRG1 protein levels were significantly associated with advanced T stage (T3–T4, $p < 0.01$; Figure 2d), pathological stage (stage III, $p < 0.001$; Figure 2e), and severe N stage (N2–N3, $p < 0.01$; Figure 2f). Collectively, these findings highlight NRG1 protein as a reliable diagnostic and progression marker in TNBC.

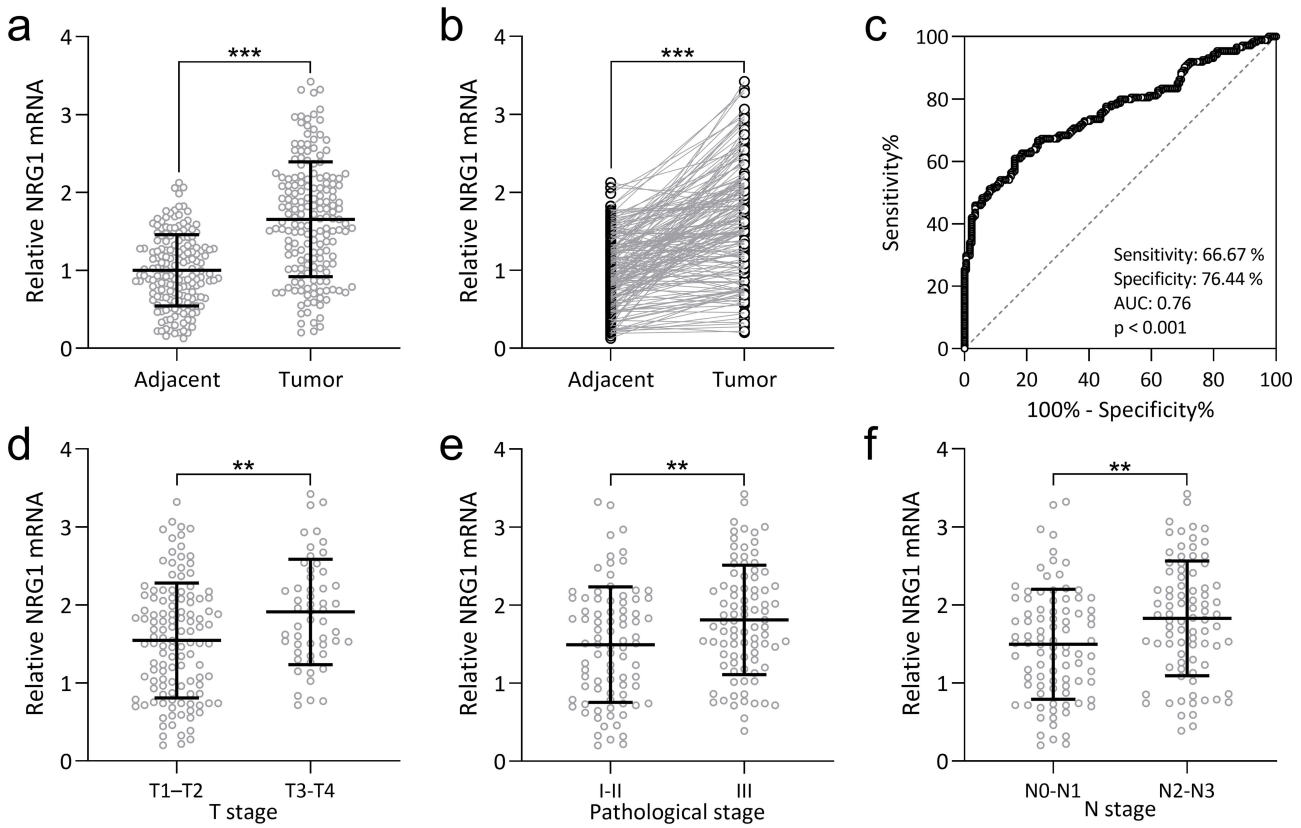

Figure 1 NRG1 mRNA expression in TNBC tissues. (**a**) NRG1 mRNA expression was significantly higher in TNBC tumor tissues compared to adjacent normal tissues, as determined by qRT-PCR. (**b**) Paired t-tests confirmed elevated NRG1 mRNA levels in tumor tissues compared to adjacent normal tissues from the same patients (n = 174). (**c**) ROC curve analysis demonstrated the diagnostic potential of NRG1 mRNA expression, with an AUC of 0.76, sensitivity of 66.67%, and specificity of 76.44%. (**d–f**) Elevated NRG1 mRNA expression was significantly associated with advanced T stage (**d**), pathological stage (**e**), and N stage (**f**). Data are presented as mean ± SD. **p < 0.01, ***p < 0.001.

Serum NRG1 as a Biomarker for TNBC

Compared with healthy controls, serum NRG1 levels in TNBC patients were significantly elevated ($p < 0.001$; Figure 3a). ROC analysis confirmed the diagnostic potential of serum NRG1, yielding an AUC of 0.72 with sensitivity and specificity of 59.2% and 76.0%, respectively (Figure 3b). Subgroup analysis further demonstrated that increased serum NRG1 was associated with advanced T stage (T3–T4, $p < 0.05$; Figure 3c), pathological stage (stage III, $p < 0.01$; Figure 3d), and severe N stage (N2–N3, $p < 0.01$; Figure 3e). These data suggest that serum NRG1 may serve as a non-invasive biomarker reflecting TNBC progression.

Prognostic Value of NRG1 in TNBC

Kaplan–Meier survival analysis demonstrated that patients with high NRG1 mRNA expression in tumor tissues had significantly poorer 5-year overall survival compared with those with low expression ($p < 0.05$; Figure 4a). Similarly, higher NRG1 protein concentrations in tumor tissues were associated with reduced survival ($p < 0.05$; Figure 4b). Elevated serum NRG1 levels also predicted worse overall survival ($p < 0.05$; Figure 4c). These findings collectively underscore the prognostic significance of NRG1 expression at both tissue and serum levels in TNBC.

NRG1 Secretion by CAFs

To verify the successful isolation and characterization of CAFs, we first examined the expression of classical CAF markers. Western blot analysis showed that CAFs expressed significantly higher levels of α-SMA and FAP compared with NFs, confirming their identity as activated fibroblasts (Figure 5a). We next investigated the expression of NRG1 in these cells at both the mRNA and protein levels. RT-qPCR analysis revealed that CAFs exhibited a marked increase in

https://doi.org/10.2147/BCTT.S549363

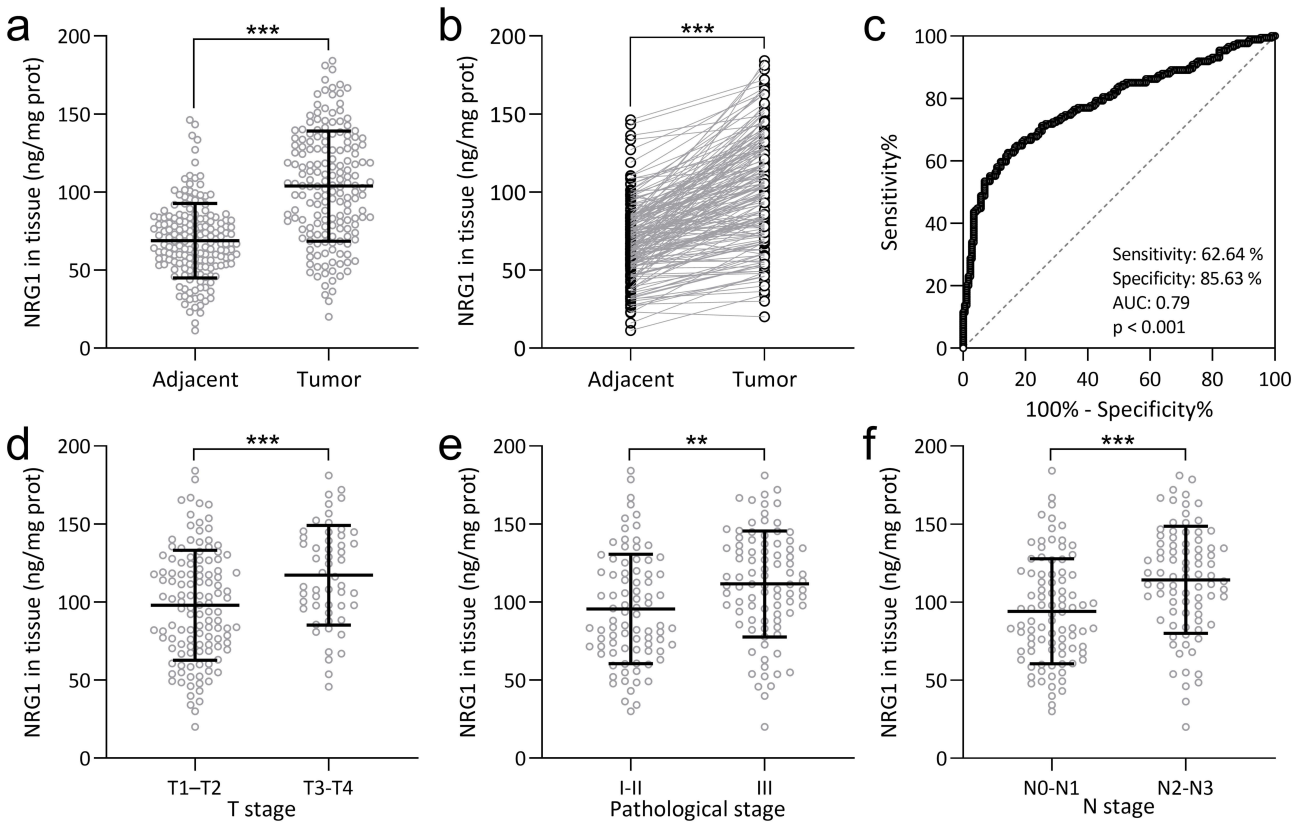

Figure 2 NRG1 protein concentration in TNBC tissues. (**a**) NRG1 protein concentration was significantly higher in TNBC tumor tissues compared to adjacent normal tissues, as determined by ELISA. (**b**) Paired t-tests confirmed higher protein levels in tumor tissues compared to adjacent normal tissues from the same patients (n = 174). (**c**) ROC analysis revealed an AUC of 0.79, sensitivity of 62.64%, and specificity of 85.63%, indicating strong diagnostic value. (**d–f**) Elevated NRG1 protein levels were significantly associated with advanced T stage (**d**), pathological stage (**e**), and N stage (**f**). Data are presented as mean ± SD. **p < 0.01, ***p < 0.001.

NRG1 mRNA expression relative to NFs, with differences reaching high statistical significance ($p < 0.001$; Figure 5b). Consistently, Western blot analysis demonstrated that NRG1 protein levels were also elevated in CAFs compared with NFs, using GAPDH as a loading control (Figure 5c). Quantitative densitometric analysis of the Western blot bands further confirmed that the NRG1 protein was significantly upregulated in CAFs, reinforcing the transcriptional findings ($p < 0.001$; Figure 5d). In addition to intracellular expression, we assessed whether CAFs secreted higher levels of NRG1 into the extracellular milieu. ELISA performed on conditioned media revealed that CAF-CM contained markedly higher concentrations of NRG1 compared with NF-CM ($p < 0.001$; Figure 5e). This observation is consistent with the role of CAFs as a key stromal source of tumor-promoting cytokines and growth factors. Representative blots and quantitative analyses are provided in Figure 5f and g, showing reproducible differences across multiple independent experiments. Taken together, these results consistently demonstrate from multiple angles—CAF marker expression, mRNA and protein quantification, and secreted NRG1 detection—that CAFs serve as a major source of NRG1 in the TNBC tumor microenvironment, thereby supporting their potential role in driving tumor progression.

Effect of CAF-Secreted NRG1 on TNBC Cell Growth

To explore the functional role of CAF-secreted NRG1 in regulating TNBC cell growth, we first established an siRNA-mediated knockdown model in CAFs. qRT-PCR analysis confirmed that NRG1 mRNA expression was markedly reduced following siNRG1 transfection ($p < 0.001$; Figure 6a), and consistent results were obtained at the protein level, where Western blotting showed a significant decrease in NRG1 protein expression ($p < 0.001$; Figure 6b). Correspondingly, ELISA analysis of conditioned medium demonstrated a substantial reduction in secreted NRG1 concentrations after siRNA knockdown compared with untreated CAFs ($p < 0.001$; Figure 6c), confirming that the knockdown effectively suppressed both intracellular expression and extracellular secretion of NRG1. Functionally, TNBC cells cultured with

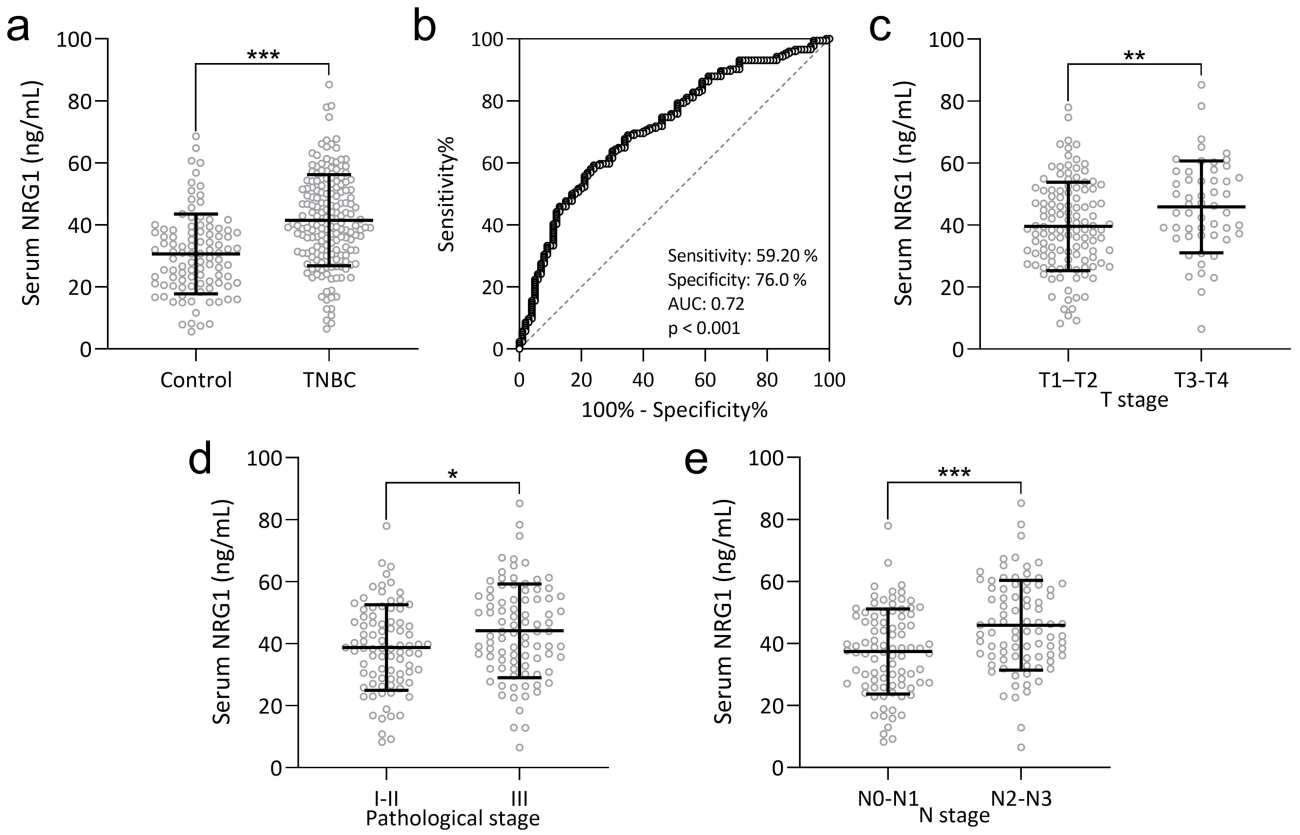

Figure 3 Serum NRG1 as a biomarker for TNBC. (**a**) Serum NRG1 levels were significantly elevated in TNBC patients (n = 174) compared to healthy controls (n = 100). (**b**) ROC analysis demonstrated an AUC of 0.72, with sensitivity of 59.2% and specificity of 76%, indicating the diagnostic potential of serum NRG1. (**c–e**) Elevated serum NRG1 levels were associated with advanced T stage (**c**), pathological stage (**d**), and N stage (**e**). Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

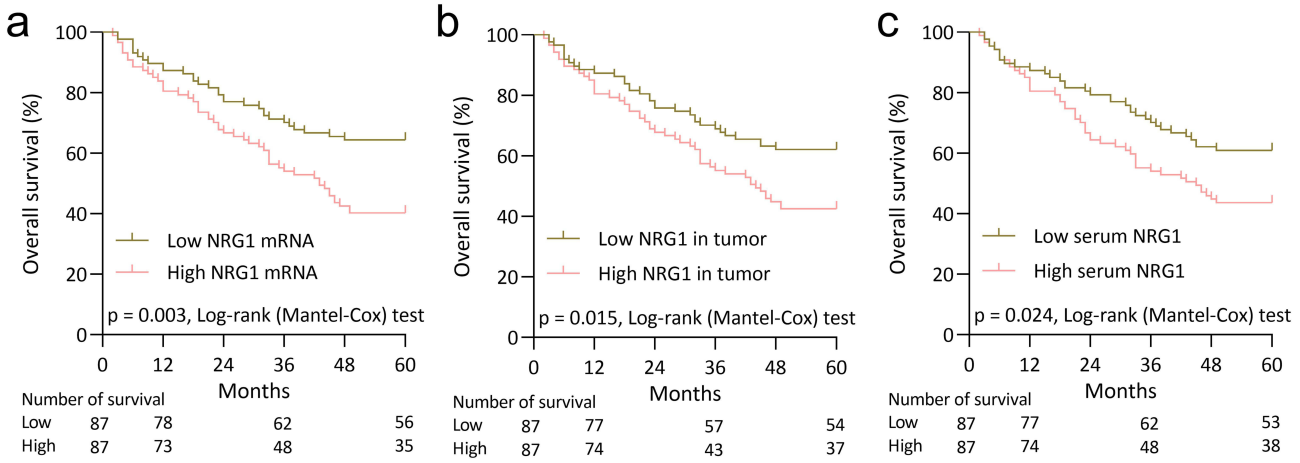

Figure 4 Prognostic value of NRG1 in TNBC patients. Kaplan-Meier survival curves demonstrate the impact of NRG1 levels on the 5-year overall survival of TNBC patients. (**a**) High tumor NRG1 mRNA expression was associated with poorer survival compared to low expression. (**b**) Similarly, higher NRG1 protein concentration in tumor tissues correlated with reduced survival. (**c**) Elevated serum NRG1 levels were also linked to worse survival outcomes. Log rank test was used for statistical analysis.

CAF-CM exhibited significantly enhanced viability compared to those treated with NF-CM, whereas exposure to siNRG1-CAF-CM attenuated this growth-promoting effect ($p < 0.05$; Figure 6d). Consistent with this observation, colony formation assays revealed that CAF-CM robustly promoted TNBC cell proliferation, as evidenced by an increased number and size of colonies. In contrast, siNRG1-CAF-CM significantly reduced both colony size and colony formation efficiency ($p < 0.05$; Figure 6e and f), suggesting that NRG1 is required for the full proliferative impact of

https://doi.org/10.2147/BCTT.S549363

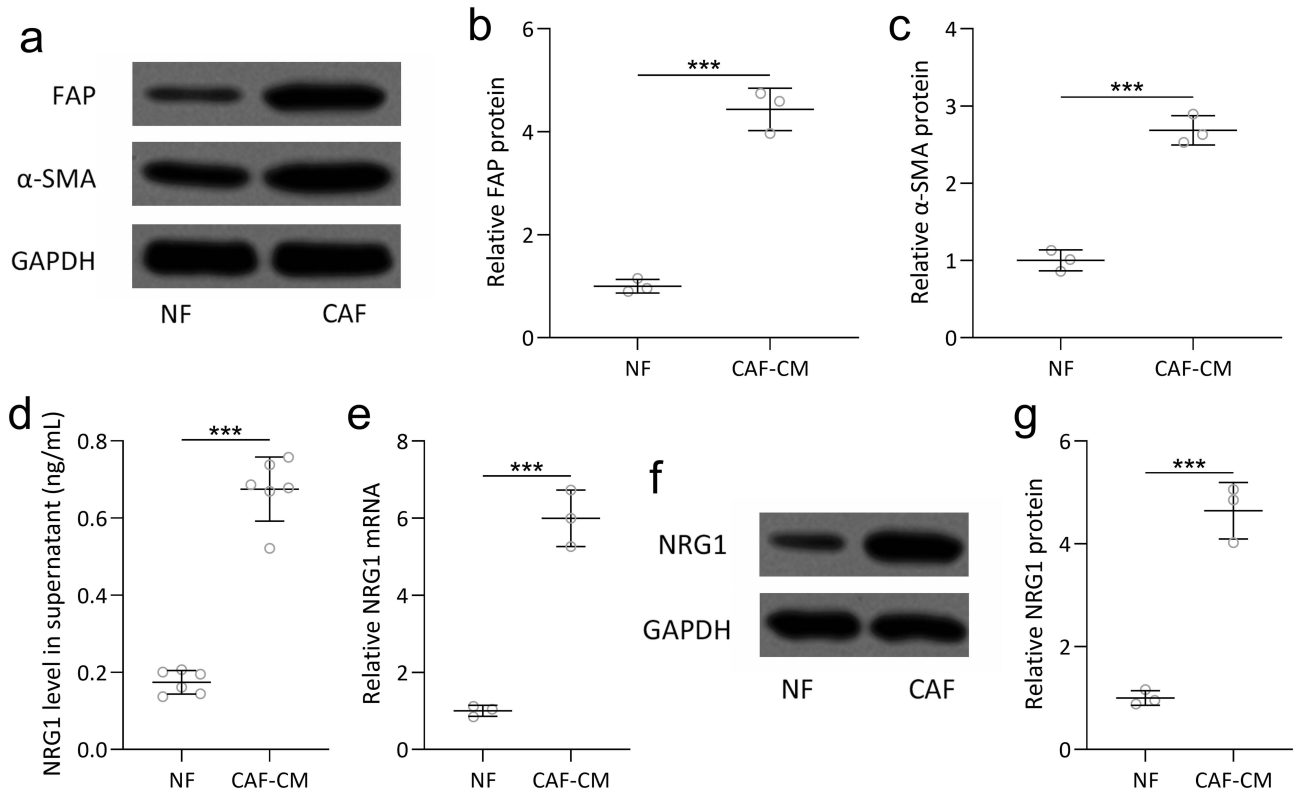

Figure 5 NRG1 secretion by CAFs in TNBC. (**a**) Western blot analysis confirmed elevated expression of CAF markers α-SMA and FAP compared to NFs. CAFs exhibited significantly higher (**b**) FAP protein levels and (**c**) α-SMA protein levels than NFs. (**d**) ELISA analysis of conditioned medium showed that CAFs secreted significantly more NRG1 compared to NFs. (**e**) RT-qPCR was used to measure the mRNA expressions of NRG1 from NF and CAF. (**f**) Western blot analysis was used to measure the protein expressions of NRG1 from NF and CAF. GAPDH was used as a loading control and the expressions were normalized to NF (**g**). n = 3 for Western blot analysis and RT-qPCR, n = 6 for ELISA. The experiments have been repeated for 3 times to confirm the results. Data are presented as mean ± SD. ***p < 0.001 compared to NFs.

CAF-CM on TNBC cells. We further examined molecular markers associated with invasion. Western blot analysis demonstrated that TNBC cells exposed to CAF-CM displayed markedly upregulated expression of MMP9 compared with control medium ($p < 0.01$; Figure 6g). Importantly, this induction was significantly reduced when the cells were treated with siNRG1-CAF-CM ($p < 0.05$). Quantitative densitometry of MMP9 protein levels corroborated this finding, showing that silencing NRG1 in CAFs directly diminished CAF-CM–induced MMP9 upregulation in TNBC cells (Figure 6h).

Supplementary rescue experiments were conducted to further verify the specificity of NRG1 knockdown. Addition of recombinant human NRG1 (10 ng/mL; Cell Signaling Technology) to the conditioned medium from NRG1-silenced CAFs (siNRG1-CAF-CM) significantly reversed the inhibitory effects observed in TNBC cells. As shown in Figure S1, MDA-MB-231 cells cultured with siNRG1-CAF-CM supplemented with recombinant NRG1 (siNRG1-CAF-CM-rNRG1) exhibited restored cell viability (Figure S1a) and increased MMP9 protein expression compared to the siNRG1-CAF-CM group (Figure S1b and c). These findings confirm that the tumor-promoting activity of CAF-CM is specifically mediated by NRG1 rather than off-target effects of siRNA.

Taken together, these results provide compelling evidence that CAF-derived NRG1 enhances TNBC cell growth and invasive potential. Mechanistically, this effect appears to be mediated at least in part through the regulation of MMP9 expression, thereby linking stromal NRG1 secretion to tumor cell proliferation and invasion.

In vivo Effects of NRG1 in a TNBC Xenograft Model

To investigate the in vivo role of CAF-secreted NRG1, we established a TNBC xenograft model with MDA-MB-231 cells. Tumors treated with CAF-conditioned medium (CAF-CM) exhibited significantly accelerated growth compared with controls, reflected by larger tumor volumes over time ($p < 0.001$; Figure 7a). At the study endpoint, both tumor size

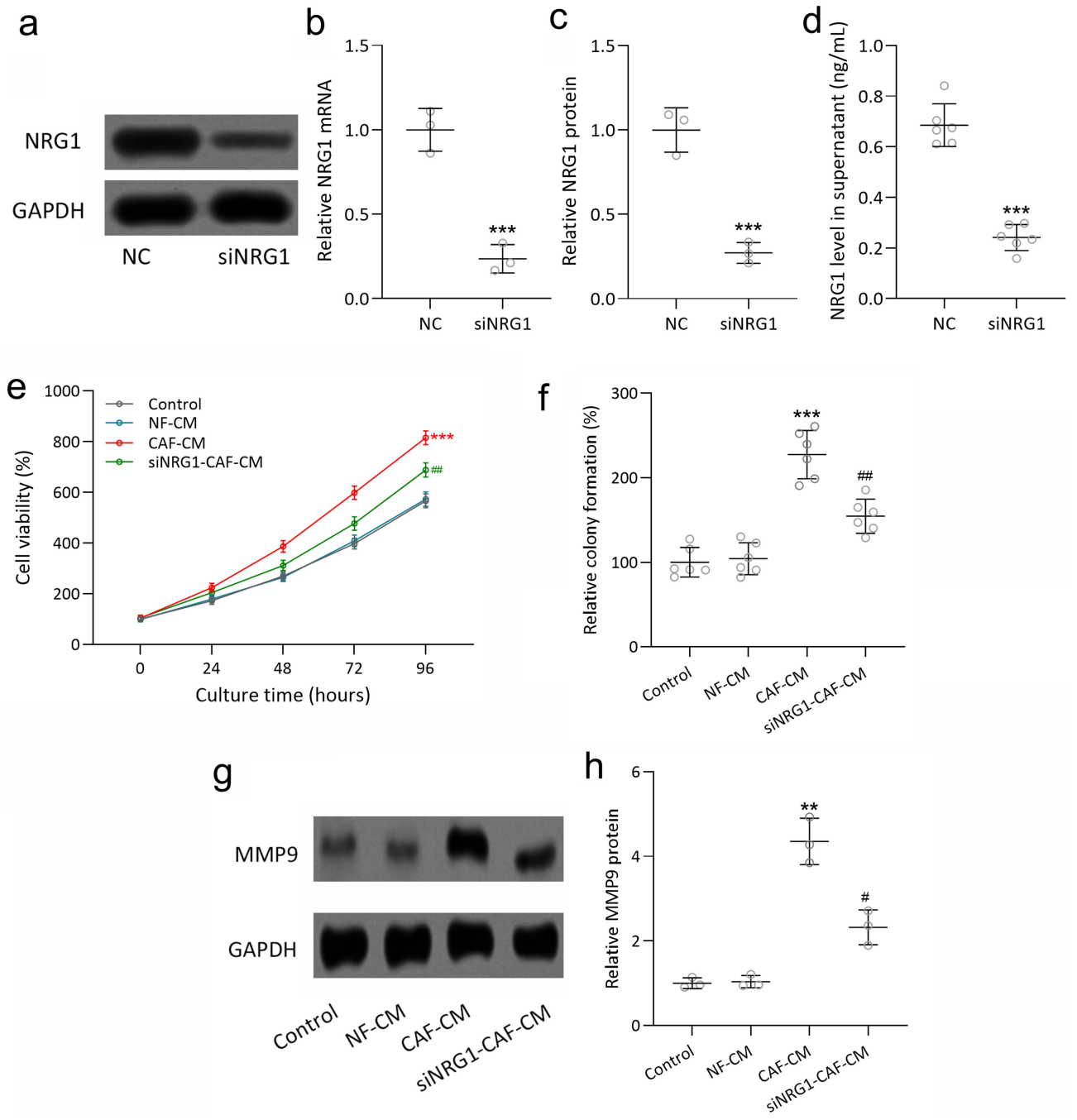

Figure 6 Effect of CAF-secreted NRG1 on TNBC cell growth. CAFs were transfected with siNRG1 for 48 hours. (**a**) Western blotting were used to measure the protein expressions of NRG1 in CAFs. (**b**) mRNA expression of NRG1 in CAFs, determined by qRT-PCR. (**c**) NRG1 protein expression in CAFs. (**d**) The NRG1 concentrations from the cell supernatant were measured by ELISA. MDA-MB-231 cells were cultured with 50% conditioned medium from NF or CAF or CAF transfected with siNRG1. (**e**) The cell viabilities were measured at different time points. After 10 days of culture, the colony formation assay was conducted. The relative colony formation was compared (**f**). After 2 days of culture, Western blotting was used to measure the protein expressions of MMP9 (**g**). GAPDH was used as a loading control and the expressions were normalized to control (**h**). n = 3 for Western blot analysis and RT-qPCR, n = 6 for cell viability and colony formation assay. The experiments have been repeated for 3 times to confirm the results. Data are presented as mean ± SD. **p < 0.01, ***p < 0.001 compared to control; #p < 0.05, ##p < 0.01 compared to CAF-CM.

and tumor weight were markedly increased in the CAF-CM group relative to controls ($p < 0.01$; Figure 7a and b). Conversely, tumors treated with conditioned medium derived from NRG1-knockdown CAFs (siNRG1-CAF-CM) showed significantly reduced growth compared with CAF-CM–treated tumors ($p < 0.01$), indicating that NRG1 is required for the full tumor-promoting effect of CAFs.

https://doi.org/10.2147/BCTT.S549363

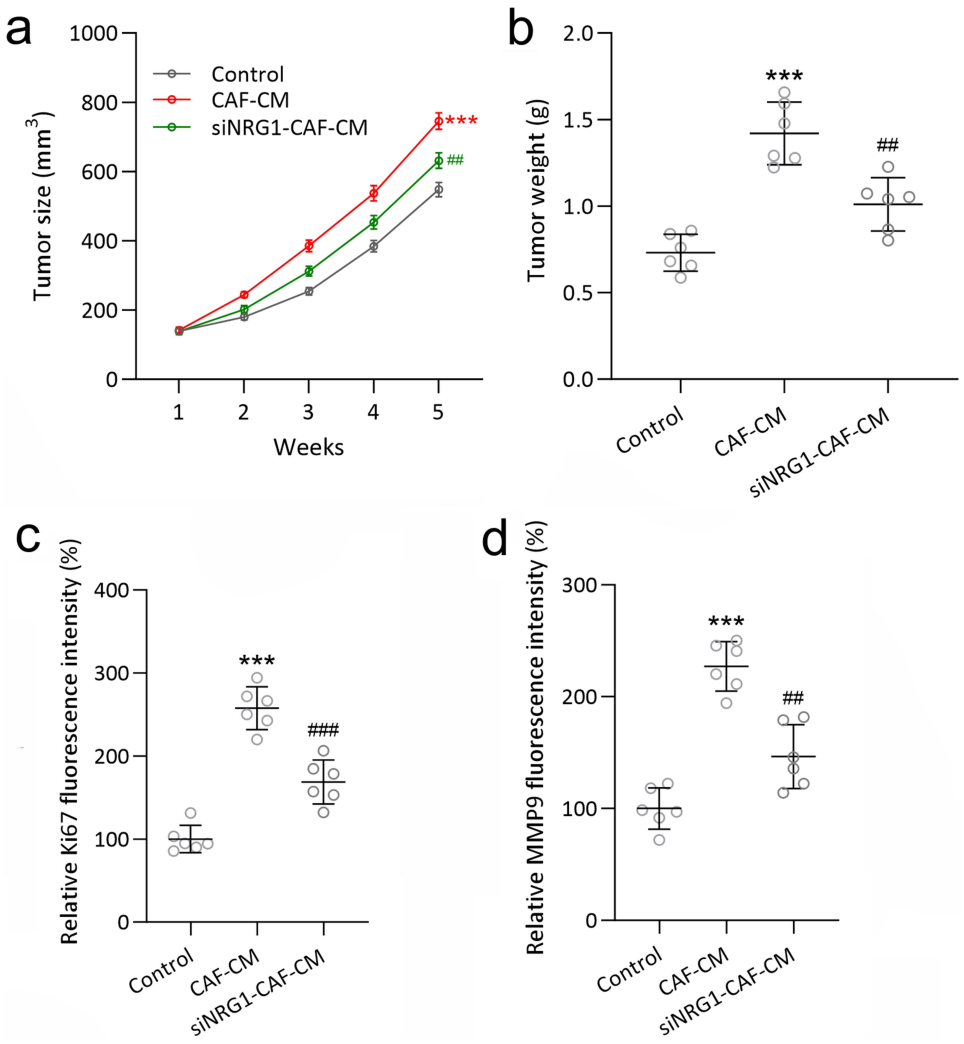

Figure 7 In Vivo effects of NRG1 in a TNBC xenograft model. (**a**) The tumor size and (**b**) tumor weight were recorded and calculated at indicated time points. IHC was used to measure the (**c**) Ki67 and (**d**) MMP9 expressions from the tumors and the comparisons of Ki67 and MMP9 intensity. 6 mice were used for each group. Data are presented as mean ± SD. ***p < 0.001 compared to control; ##p < 0.01, ###p < 0.001 compared to CAF-CM.

Furthermore, Ki67 (a marker of cell proliferation) staining intensity was significantly diminished in tumors treated with siNRG1-CAF-CM ($p < 0.01$; Figure 7c). Increased Ki67 expression was also revealed in tumors treated with CAF-CM compared to controls ($p < 0.001$; Figure 7d). Similarly, immunohistochemistry demonstrated that CAF-CM robustly upregulated MMP9 expression in tumor tissues ($p < 0.001$; Figure 7d), whereas this effect was markedly attenuated when CAFs were depleted of NRG1 ($p < 0.01$). Together, these results provide in vivo evidence that CAF-secreted NRG1 promotes TNBC tumor growth and proliferation, at least in part through the upregulation of MMP9.

Discussion

This study comprehensively investigated the role of NRG1 in TNBC, focusing on its expression, diagnostic and prognostic value, and its mechanistic role through CAFs. Our findings demonstrated that NRG1 is significantly upregulated in TNBC tumor tissues and serum, correlates with disease progression, and is secreted by CAFs to promote tumor growth and invasion. These results provide novel insights into the tumor microenvironment of TNBC and suggest potential therapeutic implications.

NRG1 mRNA and protein expression were significantly higher in TNBC tissues compared to adjacent normal tissues, and elevated levels were associated with advanced T stage, pathological stage, and N stage. These findings align with

previous studies suggesting that NRG1 plays a role in tumorigenesis across various cancer types, including HER2-positive breast cancer, lung adenocarcinoma, and pancreatic cancer.[15,18] However, in contrast to these studies that focused on other breast cancer subtypes, our results specifically highlight the involvement of NRG1 in the aggressive TNBC subtype, a cancer type characterized by limited therapeutic options. The diagnostic potential of NRG1, reflected by its ROC curve with an AUC of 0.76 for mRNA and 0.79 for protein levels, supports its use as a biomarker for TNBC detection. Previous studies on HER3/NRG1 signaling in HER2-positive breast cancer reported a similar diagnostic potential,[19] but our study is the first to extend this analysis to TNBC, providing a broader understanding of NRG1's role.

In the serum, NRG1 levels were significantly higher in TNBC patients compared to healthy controls. This non-invasive biomarker demonstrated a diagnostic AUC of 0.72, with sensitivity and specificity of 59.2% and 76%, respectively. These findings corroborate previous reports of elevated serum NRG1 in other cancers, such as prostate cancer and gastric cancer.[20–22] However, while those studies did not explore the correlation of serum NRG1 with disease stages, our results revealed that serum NRG1 levels increase with tumor progression, linking higher levels to advanced T, N, and pathological stages. This novel insight highlights the utility of serum NRG1 as a real-time indicator of tumor burden and progression in TNBC.

The survival analysis demonstrated that high NRG1 mRNA, protein, and serum levels were associated with significantly poorer 5-year overall survival. This observation is consistent with findings in HER2-positive breast cancer, where NRG1 expression correlated with trastuzumab resistance and worse prognosis.[23,24] However, our study reveals that NRG1's prognostic significance extends beyond HER2-positive cancers, establishing it as a robust prognostic marker for TNBC. The findings also highlight that targeting NRG1 or its downstream pathways may improve clinical outcomes in TNBC patients.

Mechanistically, our study demonstrated that NRG1 is secreted by CAFs, as evidenced by higher NRG1 mRNA and protein levels in CAFs compared to normal fibroblasts. Moreover, conditioned medium from CAFs significantly enhanced TNBC cell viability, colony formation, and MMP9 expression, effects that were attenuated upon NRG1 knockdown. These results align with previous studies indicating that CAFs promote tumorigenesis through the secretion of growth factors such as TGF-β, VEGF, and HGF. However, our study provides the first evidence that NRG1 is a critical CAF-secreted factor in TNBC, underscoring its role in the tumor microenvironment. In vivo experiments further validated this mechanism, showing that conditioned medium from CAFs significantly promoted tumor growth in xenograft models, while NRG1 knockdown abrogated these effects. This finding highlights NRG1 as a potential therapeutic target, especially in CAF-rich TNBC tumors.

Compared to previous studies on TNBC, our research offers several advancements. While earlier studies focused on immune checkpoint inhibitors and chemotherapy resistance,[25] our study identifies a novel stromal target, NRG1, expanding the therapeutic landscape for TNBC. Furthermore, the dual evaluation of tissue and serum NRG1 provides a comprehensive perspective, bridging biomarker discovery with potential clinical applications.

Despite these significant findings, several limitations should be acknowledged. First, the strength of causal inference in our study was initially limited by the absence of rescue experiments and key control groups. In the revised work, we addressed these issues by performing in vitro rescue experiments with recombinant NRG1 and by clarifying that NFs were derived from paired adjacent normal tissues of the same patients, thereby ensuring rigorous controls. Second, although our xenograft models confirmed the tumor-promoting role of CAF-secreted NRG1, they do not fully capture the complexity of human TNBC. Future studies incorporating patient-derived xenografts or organoid models may better reflect clinical reality. Third, while our data strongly suggest that NRG1 is a critical CAF-derived factor, contributions from other stromal and immune cell types cannot be excluded and warrant further investigation. Finally, our approach focused primarily on gene silencing, and did not evaluate pharmacotherapeutic interventions. Future studies should assess NRG1-targeted strategies, such as neutralizing antibodies or ErbB receptor inhibitors, and explore potential combination regimens with chemotherapy or immune checkpoint inhibitors. These directions will be essential for establishing causality and translating our findings into clinically applicable therapies.

Conclusions

In conclusion, this study highlights the critical role of NRG1 in TNBC progression and its potential as a diagnostic and prognostic biomarker. Our findings establish CAFs as a key source of NRG1, elucidating a novel mechanism through which the tumor microenvironment promotes TNBC aggressiveness. Compared to previous research, this study extends the understanding of NRG1 beyond HER2-positive cancers, providing novel insights into its involvement in TNBC. However, our results currently demonstrate a strong correlation rather than definitive causation, as certain methodological gaps such as in vivo NF-CM controls and comprehensive rescue experiments remain. Thus, CAF-derived NRG1 should be viewed as a promising mechanistic link and potential therapeutic target in TNBC, pending further validation. Future research focusing on therapeutic inhibition of NRG1, coupled with validation in larger clinical cohorts and additional functional experiments, will be essential to translate these findings into clinical benefits for TNBC patients.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of First Affiliated Hospital of Bengbu Medical University, and followed the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985). The human studies were approved by the Institutional Ethics Committee of First Affiliated Hospital of Bengbu Medical University. Written informed consent was obtained from the patients. The human studies were performed in strict accordance with the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

Informed Consent

All patients signed the written informed consent.

Funding

The study was supported by the Key Project of Natural Science Research in Universities of Anhui Province (2024AH051252).

Disclosure

The authors declare they have no conflict of interest regarding this research study.

References

1. Katsura C, Ogunmwonyi I, Kankam HK, Saha S. Breast cancer: presentation, investigation and management. *Br J Hosp Med*. 2022;83:1–7. doi:10.12968/hmed.2021.0459
2. Barzaman K, Karami J, Zarei Z, et al. Biology, biomarkers, and treatments. *Int Immunopharmacol*. 2020;84:106535. doi:10.1016/j.intimp.2020.106535
3. Harbeck N, Gnant M. Breast cancer. *Lancet*. 2017;389(10074):1134–1150. doi:10.1016/S0140-6736(16)31891-8
4. Kolak A, Kaminska M, Sygit K, Budny A, Surdyka D, Kukielka-Budny B. Primary and secondary prevention of breast cancer. *Ann Agric Environ Med*. 2017;24:549–553. doi:10.26444/aaem/75943
5. Li Z, Wei H, Li S, Wu P, Mao X. The role of progesterone receptors in breast cancer. *Drug Des Devel Ther*. 2022;16:305–314. doi:10.2147/DDDT.S336643
6. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. *Am Fam Physician*. 2010;81:1339–1346.
7. Fahad Ullah M. Breast cancer: current perspectives on the disease status. *Adv Exp Med Biol*. 2019;1152:51–64. doi:10.1007/978-3-030-20301-6_4
8. Thomas HR, Hu B, Boyraz B, et al. Metaplastic breast cancer: a review. *Crit Rev Oncol Hematol*. 2023;182:103924. doi:10.1016/j.critrevonc.2023.103924
9. So JY, Ohm J, Lipkowitz S. Yang L Triple negative breast cancer (TNBC): non-genetic tumor heterogeneity and immune microenvironment: emerging treatment options. *Pharmacol Ther*. 2022;237:108253. doi:10.1016/j.pharmthera.2022.108253
10. Mehraj U, Dar AH, Wani NA, Mir MA. Tumor microenvironment promotes breast cancer chemoresistance. *Cancer Chemother Pharmacol*. 2021;87:147–158. doi:10.1007/s00280-020-04222-w
11. Giorello MB, Borzone FR, Labovsky V, Piccioni FV, Chasseing NA. Cancer-associated fibroblasts in the breast tumor microenvironment. *J Mammary Gland Biol Neoplasia*. 2021;26:135–155. doi:10.1007/s10911-020-09475-y

12. Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. *Nat Rev Drug Discov*. 2019;18:99–115. doi:10.1038/s41573-018-0004-1
13. Deepak KGK, Vempati R, Nagaraju GP, Dasari VR, N S, Rao DN. Tumor microenvironment: challenges and opportunities in targeting metastasis of triple negative breast cancer. *Pharmacol Res*. 2020;153:104683. doi:10.1016/j.phrs.2020.104683
14. Timperi E, Gueguen P, Molgora M, et al. Lipid-associated macrophages are induced by cancer-associated fibroblasts and mediate immune suppression in breast cancer. *Cancer Res*. 2022;82:3291–3306. doi:10.1158/0008-5472.CAN-22-1427
15. Jarde T, Chan WH, Rossello FJ, et al. Mesenchymal niche-derived neuregulin-1 drives intestinal stem cell proliferation and regeneration of damaged epithelium. *Cell Stem Cell*. 2020;27:646–662e647. doi:10.1016/j.stem.2020.06.021
16. Zhang C, Mei W, Zeng C. Zeng C Oncogenic Neuregulin 1 gene (NRG1) fusions in cancer: a potential new therapeutic opportunities. *Biochim Biophys Acta Rev Cancer*. 2022;1877:188707. doi:10.1016/j.bbcan.2022.188707
17. Lin Y, Liu H, Wang X. Neuregulin-1, a microvascular endothelial-derived protein, protects against myocardial ischemia-reperfusion injury (Review). *Int J Mol Med*. 2020;46:925–935. doi:10.3892/ijmm.2020.4662
18. Adashek JJ, Pandya C, Maragakis NJ, De P, Cohen PR, Kato S. Neuregulin-1 and ALS19 (ERBB4): at the crossroads of amyotrophic lateral sclerosis and cancer. *BMC Med*. 2024;22:74. doi:10.1186/s12916-024-03293-3
19. Li Q, Zhu B, Chen J, Wang H, Wu Y, Chen H. Effects of oncogene neuregulin 1 on breast cancer cells. *Pak J Biol Sci*. 2022;25:345–352. doi:10.3923/pjbs.2022.345.352
20. Segers VFM, Dugaucquier L, Feyen E, Shakeri H, De Keulenaer GW. The role of ErbB4 in cancer. *Cell Oncol*. 2020;43:335–352. doi:10.1007/s13402-020-00499-4
21. Zhang Z, Karthaus WR, Lee YS, et al. Sawyers CL tumor microenvironment-derived NRG1 promotes antiandrogen resistance in prostate cancer. *Cancer Cell*. 2020;38:279–296e279. doi:10.1016/j.ccell.2020.06.005
22. Gil V, Miranda S, Riisnaes R, et al. Team PSCIPCD HER3 Is an actionable target in advanced prostate cancer. *Cancer Res*. 2021;81:6207–6218. doi:10.1158/0008-5472.CAN-21-3360
23. Guardia C, Bianchini G, Arpi LO, et al. Preclinical and clinical characterization of fibroblast-derived neuregulin-1 on trastuzumab and pertuzumab activity in HER2-positive breast cancer. *Clin Cancer Res*. 2021;27:5096–5108. doi:10.1158/1078-0432.CCR-20-2915
24. Koninki K, Barok M, Tanner M, et al. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. *Cancer Lett*. 2010;294:211–219. doi:10.1016/j.canlet.2010.02.002
25. Bagchi S, Yuan R, Engleman EG. Engleman EG immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. *Annu Rev Pathol*. 2021;16:223–249. doi:10.1146/annurev-pathol-042020-042741