# Strong cytoplasmic expression of NF-κB/p65 correlates with good prognosis in triple-negative breast cancer

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## Abstract

*Purpose* Recent studies have indicated that constitutive NF-κB activity could be involved in the proliferation of triple-negative breast cancer.

*Methods* NF-κB/p65 expression and the effects of the NF-κB inhibitor, (−)-DHMEQ were examined in triple-negative MDA-MB-231 breast cancer cells. Women with triple-negative breast cancer treated with neoadjuvant chemotherapy between 2002 and 2012 were retrospectively analyzed. Expression of NF-κB/p65, Bcl2 and Ki67 was examined by immunohistochemistry in pre- and post-treatment specimens, and predictive factors for neoadjuvant chemotherapy and prognosis were analyzed.

*Results* NF-κB/p65 was dominantly expressed in the cytoplasm in MDA-MB-231 cells. Of 34 triple-negative breast cancer patients, positive staining for NF-κB/p65 expression was detected in the nuclei of a few cells in 7 tumors before neoadjuvant chemotherapy, although expression of NF-κB/p65 in the cytoplasm was detected in almost all tumor cells of 33 tumors. Expression levels of NF-κB/p65 were not associated with response to neoaduvant chemotherapy, although expression levels of cytoplasmic NF-κB/p65 intensity were significantly decreased in post-treatment tumor samples compared with those in pretreatment samples. All patients whose tumors showed strong cytoplasmic NF-κB/p65 expression before neoadjuvant chemotherapy are currently disease-free.

*Conclusion* Our results suggest that strong cytoplasmic NF-κB/p65 expression could be a prognostic marker in triple-negative breast cancer.

**Key words:** NF-κB/p65; triple-negative breast cancer; prognosis; neoadjuvant chemotherapy

## Introduction

Triple-negative breast cancers are defined as tumors that lack expression of estrogen receptor (ER), progesterone receptor (PgR), and HER2 [1]. Because of the lack of targeted therapies, chemotherapy is currently the only treatment option for triple-negative breast cancer [2]. Although some patients respond, a large percentage treated in the early stage relapse within five years. The median survival for women with metastatic triple-negative breast cancer is less than 1 year.

Neoadjuvant chemotherapy has been established as a standard treatment strategy for patients with early stage triple-negative breast cancer. A recent study indicated that pathological complete response (pCR) was a suitable surrogate prognostic factor for patients with triple-negative breast cancer who received neoadjuvant chemotherapy [3]. Unfortunately, extensive subsets of patients do not experience pCR, even when treated with anthracyclins and taxanes [4, 5]. Our previous study showed that the level of Ki67 labeling index (LI) in residual tumors after neoadjuvant chemotherapy was a strong predictor of outcome for patients not achieving pCR [6]. Minckwitz and colleagues also showed that patients with high posttreatment Ki67 levels (above 35%) showed higher risk of disease relapse in both ER-positive and ER-negative breast cancers [7]. It is therefore necessary to establish innovative postneoadjuvant treatment for patients who do not achieve pCR and whose residual tumors express high Ki67 levels especially in triple-negative breast cancer.

Nuclear factor (NF)-κB is a heterodimeric molecule made up of two of five possible subunits: RelA/p65, c-Rel, RelB, p105/p50 and p100/p52 [8]. NF-κB is a transcription regulator with a

specific motif for Bcl2 transcription. The components of NF-κB form heterodimers which are located in the cytoplasm and rendered inactive by binding to specific inhibitory molecules of the inhibitor of NF-κB (IκB) family. Under the influence of external stimuli, IκB proteins are degraded via the ubiquitin-proteasome pathway, leading to release of the active form of NF-κB which translocates to the nucleus, where it regulates the expression of multiple genes involved in proliferation, apoptosis, invasion, adhesion, angiogenesis and chemoresistance [9, 10]. We recently reported that (−)-DHMEQ, a specific NF-κB inhibitor, induced anoikis and inhibited peritoneal metastasis in pancreatic cancer cells [11]. It has been shown that constitutive NF-κB activity is detected in breast cancer cell lines, and is preferentially involved in the proliferation of the basal-like (triple-negative) subtype [12]. Previous studies showed that nuclear NF-κB/p65 expression was a predictive factor of resistance to neoadjuvant chemotherapy in breast cancer [13, 14]. In contrast, a recent study demonstrated that nuclear NF-κB/p65 expression was associated with high histological grade, ER negativity, higher Ki67 index and increased pCR after neoadjuvant chemotherapy [15]. Another study showed that the increase of nuclear expression of NF-κB/p65 correlated with a decrease of expression of ER, increase of p53 accumulation, and was associated with HER2-positive and basal-like breast cancers [16].

In this study, we examined nuclear and cytoplasmic expressions of NF-κB/p65 in triple-negative MDA-MB-231 cells. Furthermore, we retrospectively investigated expression of NF-κB/p65 and Bcl2 by immunohistochemistry (IHC) in pre- and post-treatment specimens from patients with triple-negative breast cancer who were treated with anthracycline and/or

taxane-containing neoadjuvant chemotherapy, and analyzed whether nuclear or cytoplasmic expressions of NF-κB/p65 and Bcl2 expression affect response to neoadjuvant chemotherapy or disease prognosis.

## Methods

### Cell culture and reagents

The human breast cancer cell line MDA-MB-231 was obtained from ATCC. Cells were maintained in RPMI-1640 culture medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified incubator in an atmosphere of 5% $CO_2$ in air at 37°C. (−)-DHMEQ, a specific NF-κB inhibitor, synthesized as previously described [11, 17, 18], was dissolved in DMSO and then mixed with diluents for each experiment. Mouse monoclonal anti-p65 antibody (L8F6, #6956, Cell Signaling Technology, Danvers, MA, USA) was used for western blot analysis.

### Western blot analysis

The activation of NF-κB/p65 caused by human TNF alpha and/or (−)-DHMEQ was assessed by western blot analysis. MDA-MB-231 cells were treated with TNFα (20 ng/mL) for 30 min with or without (−)-DHMEQ (10 μg/mL) for 2 hours. Nuclear and cytoplasmic extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were probed with primary antibodies overnight at 4 $^{o}$C and with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were developed using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare Life Sciences, Chalfont St Giles, UK), and chemiluminescence was measured using the LAS-3000 imager (Fujifilm, Tokyo, Japan). Densitometry of immunoblots was

performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

**Patients and treatment**

A total of 34 women with Stage II to III triple-negative breast cancer treated with anthracycline and/or taxane-based neoadjuvant chemotherapy between 2002 and 2012 at Hokkaido Cancer Center were retrospectively recruited (Table 1). Neoadjuvant chemotherapy regimens comprised four cycles of FEC (5-fluorouracil 500 mg/m$^2$, epirubicin 100 mg/m$^2$ and cyclophosphamide 500 mg/m$^2$, every 3 weeks) or four cycles of EC (epirubicin 90 mg/m$^2$ and cyclophosphamide 600 mg/m$^2$, every 3 weeks) followed by either four cycles of docetaxel 75 mg/m$^2$ every 3 weeks or 12 doses of paclitaxel 80 mg/m$^2$ weekly. Clinical measurements of tumor size and nodal status were performed monthly, and the final clinical, sonographic or CT measurements were performed prior to the planned surgical excision of the tumor. Clinical responses were defined as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumors (RECIST, 2000). Pretreatment specimens were taken by core needle biopsies. Post-treatment specimens were obtained during surgery. The pathological response was assessed as grades 1 to 3 according to the following criteria: 0 (no response), 1 (mild to moderate response), 2 (marked response), 3 (complete response) according to the histopathological criteria for assessment of therapeutic response in breast cancer by the Japanese Breast Cancer Society [19]. pCR was defined as no invasive and no *in situ* residuals in

breast and nodes [3]. The median follow-up period was 41 months (range, 11 to 139 months). The study protocol was approved by the institutional review boards and conformed with the guidelines of the 1996 Declaration of Helsinki.

**IHC analysis**

One 4-μm section of each submitted paraffin block was stained first with hematoxylin-eosin to verify that an adequate number of carcinoma cells were present and that the fixation quality was adequate for IHC analysis. Serial sections (4μm) were prepared from selected blocks and float-mounted on adhesive-coated glass slides for ER, PgR, HER2, Ki67, NF-κB/p65 and Bcl2 staining. To determine the level of HER2 expression, tumors with a score of 2+ were tested for gene amplification by FISH. Tumors were considered HER2-positive if IHC staining was 3+ or FISH positive [20]. Primary antibodies included mouse monoclonal anti-human Ki67 antibody (clone 30-9, Ventana Medical Systems, Tucson, AZ, USA) for Ki67, rabbit polyclonal anti-NFκB p65 antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:600 dilution for NF-κB/p65 and mouse monoclonal anti-human BCL2 antibody (clone 124, Dako, Glostrup, Denmark) at 1:200 dilution for Bcl2. The iVIEW DAB detection kit (Ventana Medical Systems) was used as the detection system. The Ki67 labeling index (LI) was assessed as the percentage of tumor cells showing definite nuclear staining among 1,000 invasive tumor cells in randomly selected high-power (Magnification 400x) fields. NF-κB/p65 staining was assessed based on the percentage of cells showing positive nuclear

and/or cytoplasmic staining and the average cytoplasmic staining intensity of positive tumor cells rated as 0 (none) 1 (weak), 2 and 3 (strong), as compared with the staining intensity of splenic cells used as an external control. When the staining level was the same as the external control, the intensity was set at 2. Bcl2 staining was assessed as the percentage of tumor cells showing definite cytoplasmic staining among 1,000 invasive tumor cells.

**Statistical analysis**

The paired *t*-test was used to compare biological markers in tumors before and after neoadjuvant chemotherapy. Spearman's rank correlation test was used to study relationships between expression levels of clinicopathological factors and biological markers. Spearman's correlation coefficient > +0.40 or < -0.40 and $P$ <0.05 in Spearman's rank correlation test were considered significant. The Mann-Whitney *U* test was used to compare the IHC scores of biological markers with the response to neoadjuvant chemotherapy. Cox's proportional hazards model was used for univariate and multivariate analyses of predictive values for neoadjuvant chemotherapy and prognostic values for disease-free and overall survival. Estimation of survival was performed using the Kaplan-Meier method, and differences between survival curves were assessed with the log-rank test.

## Results

### NF-κB/p65 expression in triple-negative MDA-MB-231 breast cancer cells

We first examined NF-κB/p65 expression in the cytoplasm and the nuclei in triple-negative MDA-MB-231 breast cancer cells by immunoblotting. Cells were grown in culture medium and treated with (Fig. 1, lanes 3 and 4) or without (Fig. 1, lanes 1 and 2) TNFα (20 ng/mL) for 30 min. Immunoblotting with anti-NF-κB/p65 antibodies showed that NF-κB/p65 was dominantly expressed in the cytoplasm (Fig. 1, lanes 1 and 2), and its expression in the cytoplasm was transferred into the nuclei following stimulation with TNFα (Fig. 1, lanes 3 and 4). The NF-κB inhibitor, (−)-DHMEQ inhibited NF-κB/p65 expression in the cytoplasm (Fig. 1, lane 6), and also inhibited NF-κB/p65 expression in nuclei stimulated with TNFα (Fig. 1, lane 7). We conclude from these experiments that NF-κB/p65 is dominantly expressed in the cytoplasm in triple-negative MDA-MB-231 cells.

### IHC staining for expression of NF-κB/p65, Bcl2 and Ki67 in triple-negative breast cancer

We next examined expression of NF-κB/p65, Bcl2 and Ki67 in triple-negative breast cancer specimens before and after neoadjuvant chemotherapy by IHC. Of the 34 patients in the study, positive staining of NF-κB/p65 expression was present in less than 2% of cells in the nuclei of 7 tumors before neoadjuvant chemotherapy, and in less than 10% of cells in the nuclei of 6 tumors after treatment (Fig. 2a). On the other hand, expression of NF-κB/p65 in the cytoplasm was detected in more than 85% of cells in 33 tumors, and in more than 98% of cells in 30 tumors (Fig. 2b-d). There were 6 tumors in

which the staining intensity showed strong expression of cytoplasmic NF-κB/p65 (score 3). Expression levels of NF-κB/p65, Bcl2 and Ki67 were compared between samples before and after neoadjuvant chemotherapy (Table 2). Expression levels of cytoplasmic NF-κB/p65 intensity and Ki67 were significantly lower in post-treatment tumors compared with those in pretreatment samples ($p$ = 0.004, $p$ = 0.004 and $p$ = 0.0002, respectively). In contrast, Bcl2 expression was significantly higher in post-treatment tumors compared with those in pretreatment samples ($p$ = 0.004). Cytoplasmic NF-κB/p65 expression was negatively correlated with lymph node status (Spearman's correlation coefficient -0.465, $p$ = 0.006 by Spearman's rank correlation test, Table 3). Bcl2 expression was not significantly correlated with clinicopathological factors (Table 3). Expression levels of NF-κB/p65, Bcl2 and Ki67 before neoadjuvant chemotherapy showed no significant correlation (data not shown).

**Expression of NF-κB/p65, Bcl2 and Ki67 is not associated with response to neoaduvant chemotherapy**

We then examined whether expression of NF-κB/p65, Bcl2 and Ki67 affected the response to neoadjuvant chemotherapy. Expression levels of nuclear or cytoplasmic NF-κB/p65, Bcl2 and Ki67 in the pretreatment samples were not associated with clinical response to neoadjuvant chemotherapy (Table 4). There were six patients with tumors which achieved a pathological complete response (pCR) after the treatment. Expression levels of NF-κB/p65, Bcl2 and Ki67 before neoadjuvant chemotherapy were compared between the tumors with grade 0-2b and those with pCR. Expression

levels of these three genes were not associated with pCR (Table 5). The cytoplasmic NF-κB/p65 intensity score before neoadjuvant chemotherapy was 3 in 2 tumors and 2 in 4 tumors of the 6 tumors that achieved pCR. There were no clinicopathological nor biological factors that could predict pCR according univariate analysis (Table 6).

**Patients whose tumors show high cytoplasmic NF-κB/p65 expression before neoadjuvant chemotherapy have good prognosis**

To analyze factors that affected the prognosis of patients who received neoadjuvant chemotherapy, clinicopathological factors and expression of NF-κB/p65, Bcl2 and Ki67 in tumors both in pre- and post-treatment specimens were estimated. Lymph node status, nuclear grade, and Ki67 LI before neoadjuvant chemotherapy were significantly associated with disease-free survival by univariate analysis ($p = 0.02$, $p = 0.04$ and $p = 0.04$, respectively, Table 7). Lymph node status was the only factor that was significantly associated with disease-free survival by multivariate analysis ($p = 0.03$, Table 7).

All patients whose tumors showed high cytoplasmic NF-κB/p65 expression (score 3) in pretreatment samples are currently disease-free (Fig. 3a). Moreover, all patients whose tumors achieved pCR in this analysis are currently disease-free. A Kaplan-Meier analysis showed that high cytoplasmic NF-κB/p65 expression (intensity score 3) (Fig. 3a and b) was correlated with improved disease-free and overall survival. Although nuclear NF-κB/p65 expression was detected in less than

2% of cells in 7 pre-treatment tumors, and less than 10% of cells in 6 post-treatment tumors, no correlation was observed between nuclear NF-κB/p65 expression and prognosis.

## Discussion

We analyzed expression of NF-κB/p65, Bcl2 and Ki67 in pre- and post-treatment samples in order to investigate their prognostic and predictive potential in women with triple-negative breast cancer who had been treated with neoadjuvant chemotherapy. All patients whose tumors showed high cytoplasmic NF-κB/p65 expression (intensity score 3) before neoadjuvant chemotherapy are currently disease-free. Furthermore, the cytoplasmic NF-κB/p65 intensity score before neoadjuvant chemotherapy was 3 in 2 tumors and 2 in 4 tumors of the 6 tumors that achieved pCR, and all 6 patients whose tumors achieved pCR are currently disease-free. Our results suggest that high cytoplasmic NF-κB/p65 expression might be a prognostic marker in triple-negative breast cancer.

Our previous study demonstrated that patients whose tumors contained high levels of Ki67 responded effectively to anthracycline and taxane-containing neoadjuvant chemotherapy [6]. Furthermore, a high Ki67 expression level in post-treatment tumors was strongly correlated with poor disease-free and overall survival. Jones and colleagues also demonstrated that post-chemotherapy Ki67 level was a strong predictor of outcome for patients not achieving pCR [21]. Our present study demonstrated that expression of Ki67 in pre-treatment tumors was not associated with improved pCR rates, and that high Ki67 expression levels in post-treatment tumors were not correlated with poor prognosis in triple-negative breast cancer, although Ki67 expression levels were significantly decreased in post-treatment tumors compared with those in pretreatment samples.

Recent analyses have shown that the prognostic value of pCR after neoadjuvant

chemotherapy must be rated differently according to subtype [22], and that patients with triple-negative, ER- HER2+, and luminal B tumors who achieve pCR after neoadjuvant chemotherapy showed a significantly better outcome than did patients who did not achieve pCR [3]. Our previous study showed that all patients whose tumors achieved pCR had good prognosis regardless of the breast cancer subtypes [6]. Our present study also demonstrates that all 6 patients whose tumors achieved pCR are currently disease-free. Furthermore, we show that all patients whose tumors showed high NF-κB/p65 expression (intensity score 3) in pretreatment tumors are currently disease-free.

Previous studies demonstrated that nuclear NF-κB/p65 staining in pre-treatment samples was linked to resistance to neoadjuvant chemotherapy [13, 14]. In contrast, Jones and colleagues reported that nuclear NF-κB/p65 staining was associated with high histological grade, ER negativity and higher grade, and that patients with nuclear NF-κB/p65 staining had a higher pCR rate than those without [15]. Furthermore, NF-κB/p65 staining was evident in the nuclei of only a few cells of 7 tumors before neoadjuvant chemotherapy in our analysis, although the antibodies for NF-κB/p65 we used were the same as those used in the previous studies. Moreover, nuclear NF-κB/p65 expression was detected in only a few cells in 6 post-treatment tumors, and no correlation was observed between nuclear NF-κB/p65 expression and response to neoadjuvant chemotherapy nor to prognosis. Furthermore, Bcl2 expression as well as nuclear NF-κB/p65 expression was detected in few cells, and there was no correlation between expression of NF-κB/p65 and Bcl2 in our analysis. Little NF-κB/p65 was present in the nuclei of triple-negative MDA-MB-231 cells, and NF-κB/p65 expression in the cytoplasm was

transferred into the nuclei following stimulation with TNFα. Thus, constitutive activation of NF-κB/p65 might not be frequent in triple-negative breast cancer. Because ER or HER2-positive breast cancers were included in the previous studies, the status of NF-κB/p65 expression or activation might differ among breast cancer subtypes.

Our results indicate that strong cytoplasmic NF-κB/p65 expression correlates with good prognosis in triple-negative breast cancer. The role of cytoplasmic NF-κB/p65 has not yet been identified, although several studies have demonstrated a correlation between nuclear expression of NF-κB/p65 and response to chemotherapy. NF-κB in the cytoplasm forms dimers which bind to specific inhibitors (IκBs), and cell stimulation activates the IκB kinase (IKK) complex. Activated IKK then phosphorylates NF-κB-bound IκB proteins, targeting them for polyubiquitination and rapid degradation. The freed NF-κB dimers translocate to the nucleus where they coordinate the transcriptional activation of several hundred target genes [9]. It is suggested that some mechanisms of inactivation of NF-κB/p65 retain NF-κB/p65 in the cytoplasm, and lead to a good prognosis in triple-negative breast cancer.

In conclusion, the present data indicate that cytoplasmic NF-κB/p65 expression is negatively correlated with lymph node status, and that patients whose tumors contain high NF-κB/p65 expression before neoadjuvant chemotherapy have good prognosis regardless of response to the treatment. Our results suggest that high cytoplasmic NF-κB/p65 expression could be a prognostic marker in triple-negative breast cancer. Investigation of the mechanisms of NF-κB/p65 accumulation in the

cytoplasm could lead to the identification of the characteristics of triple-negative breast cancer.

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**Conflicts of interest**

None of the authors has any conflict of interest to declare in association with this study.

## References


1. Foulkes WD, Smith IE, Reis-Filho JS Triple-negative breast cancer. N Engl J Med 2010; 363: 1938-48.
2. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 2011; 121: 2750-67.
3. von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol 2012; 30: 1796-804.
4. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS, et al. Relationship between oestrogen receptor status and proliferation in predicting response and long-term outcome to neoadjuvant chemotherapy for breast cancer. Breast Cancer Res Treat 2010; 119: 315-23.
5. Lips EH, Mulder L, de Ronde JJ, Mandjes IA, Vincent A, Vrancken Peeters MT, et al. Neoadjuvant chemotherapy in ER+ HER2- breast cancer: response prediction based on immunohistochemical and molecular characteristics. Breast Cancer Res Treat 2012; 131: 827-36.
6. Yoshioka T, Hosoda M, Yamamoto M, Taguchi K, Hatanaka KC, Takakuwa E, et al. Prognostic significance of pathologic complete response and Ki67 expression after neoadjuvant chemotherapy in breast cancer. Breast Cancer 2013; doi: 10.1007/s12282-013-0474-2.
7. von Minckwitz G, Schmitt WD, Loibl S, Muller BM, Blohmer JU, Sinn BV, et al. Ki67 measured after neoadjuvant chemotherapy for primary breast cancer. Clin Cancer Res 2013; 19: 4521-31.
8. Karin M, Lin A NF-kappaB at the crossroads of life and death. Nat Immunol 2002; 3: 221-7.
9. Karin M Nuclear factor-kappaB in cancer development and progression. Nature 2006; 441: 431-6.
10. Li N, Karin M Signaling pathways leading to nuclear factor-kappa B activation. Methods Enzymol 2000; 319: 273-9.
11. Sato M, Nakanishi K, Haga S, Fujiyoshi M, Baba M, Mino K, et al. Anoikis induction and inhibition of peritoneal metastasis of pancreatic cancer cells by a nuclear factor-kappaB inhibitor, (-)-DHMEQ. Oncol Res 2013; 21: 333-43.
12. Yamaguchi N, Ito T, Azuma S, Ito E, Honma R, Yanagisawa Y, et al. Constitutive activation of nuclear factor-kappaB is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines. Cancer Sci 2009; 100: 1668-74.
13. Buchholz TA, Garg AK, Chakravarti N, Aggarwal BB, Esteva FJ, Kuerer HM, et al. The nuclear transcription factor kappaB/bcl-2 pathway correlates with pathologic complete response to doxorubicin-based neoadjuvant chemotherapy in human breast cancer. Clin Cancer Res 2005; 11: 8398-402.
14. Montagut C, Tusquets I, Ferrer B, Corominas JM, Bellosillo B, Campas C, et al. Activation of nuclear factor-kappa B is linked to resistance to neoadjuvant chemotherapy in breast cancer patients. Endocr Relat Cancer 2006; 13: 607-16.
15. Jones RL, Rojo F, A'Hern R, Villena N, Salter J, Corominas JM, et al. Nuclear NF-kappaB/p65

expression and response to neoadjuvant chemotherapy in breast cancer. J Clin Pathol 2011; 64: 130-5.

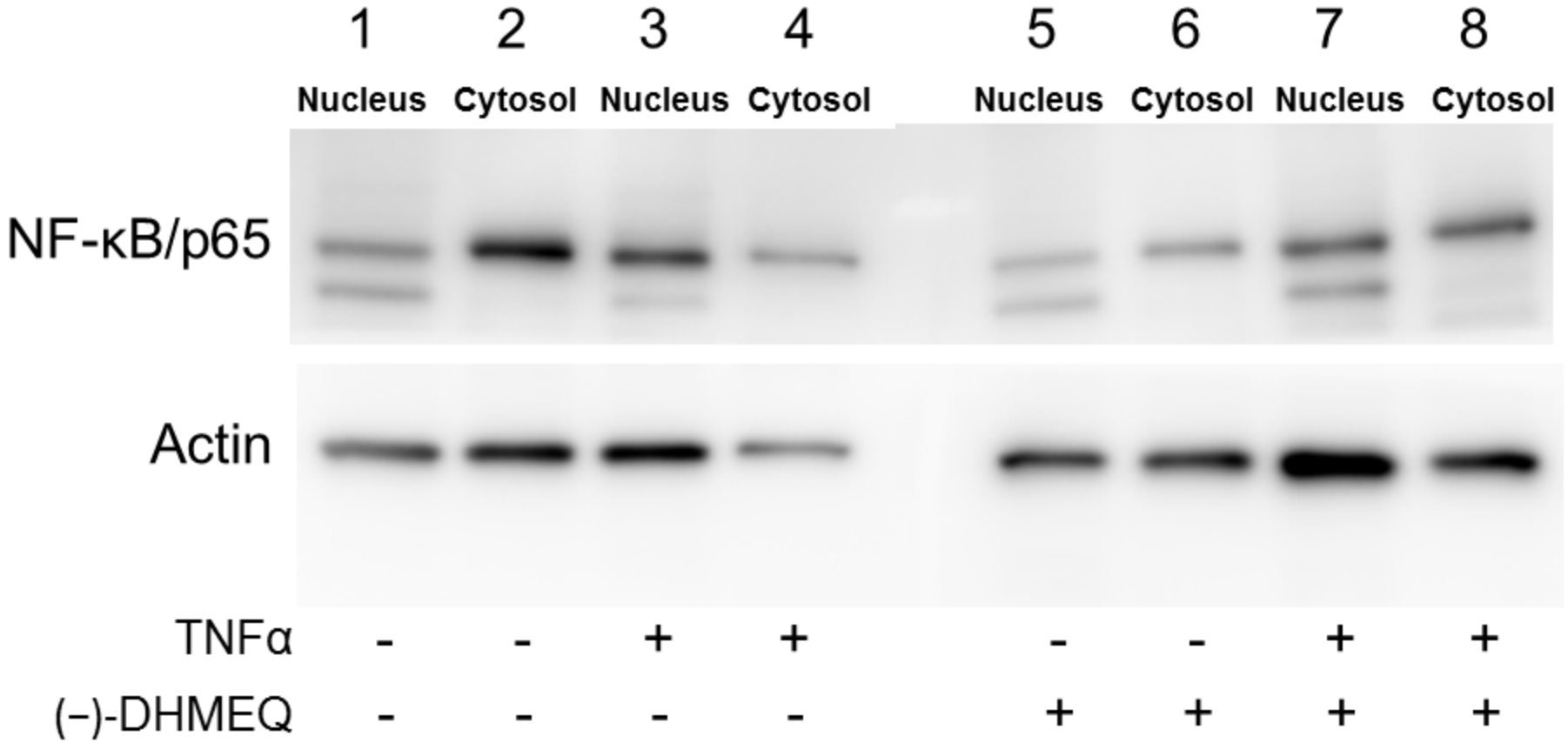
16. Shapochka DO, Zaletok SP, Gnidyuk MI Relationship between NF-kappaB, ER, PR, Her2/neu, Ki67, p53 expression in human breast cancer. Exp Oncol 2012; 34: 358-63.
17. Watanabe M, Dewan MZ, Okamura T, Sasaki M, Itoh K, Higashihara M, et al. A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. Int J Cancer 2005; 114: 32-8.
18. Umezawa K Possible role of peritoneal NF-kappaB in peripheral inflammation and cancer: lessons from the inhibitor DHMEQ. Biomed Pharmacother 2011; 65: 252-9.
19. Kurosumi M, Akashi-Tanaka S, Akiyama F, Komoike Y, Mukai H, Nakamura S, et al. Histopathological criteria for assessment of therapeutic response in breast cancer (2007 version). Breast Cancer 2008; 15: 5-7.
20. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007; 25: 118-45.
21. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS, et al. The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer. Breast Cancer Res Treat 2009; 116: 53-68.
22. von Minckwitz G Neoadjuvant chemotherapy in breast cancer-insights from the German experience. Breast Cancer 2012; 19: 282-8.

## Figure legends

**Figure 1:** NF-κB/p65 expression in triple-negative MDA-MB-231 breast cancer cells analyzed by western blot.

**Figure 2:** NF-κB/p65 immunostaining in triple-negative breast cancer. Representative figures are shown (x400). (A) Breast cancer specimen with nuclear NF-κB/p65 immunoreactivity detected in tumor cells. (B) Breast cancer specimen with a staining intensity score of 1 for cytoplasmic NF-κB/p65 (less than the external positive control). (C) Breast cancer specimen with a staining intensity score of 2 for cytoplasmic NF-κB/p65 (the same as the external positive control). (D) Breast cancer specimen with a staining intensity score of 3 for cytoplasmic NF-κB/p65 (much more than the external positive control).

**Figure 3:** Disease-free (A) and overall (B) survival according to the intensity score of cytoplasmic NF-κB/p65 expression before neoadjuvant chemotherapy. All patients with a score of 3 were disease-free.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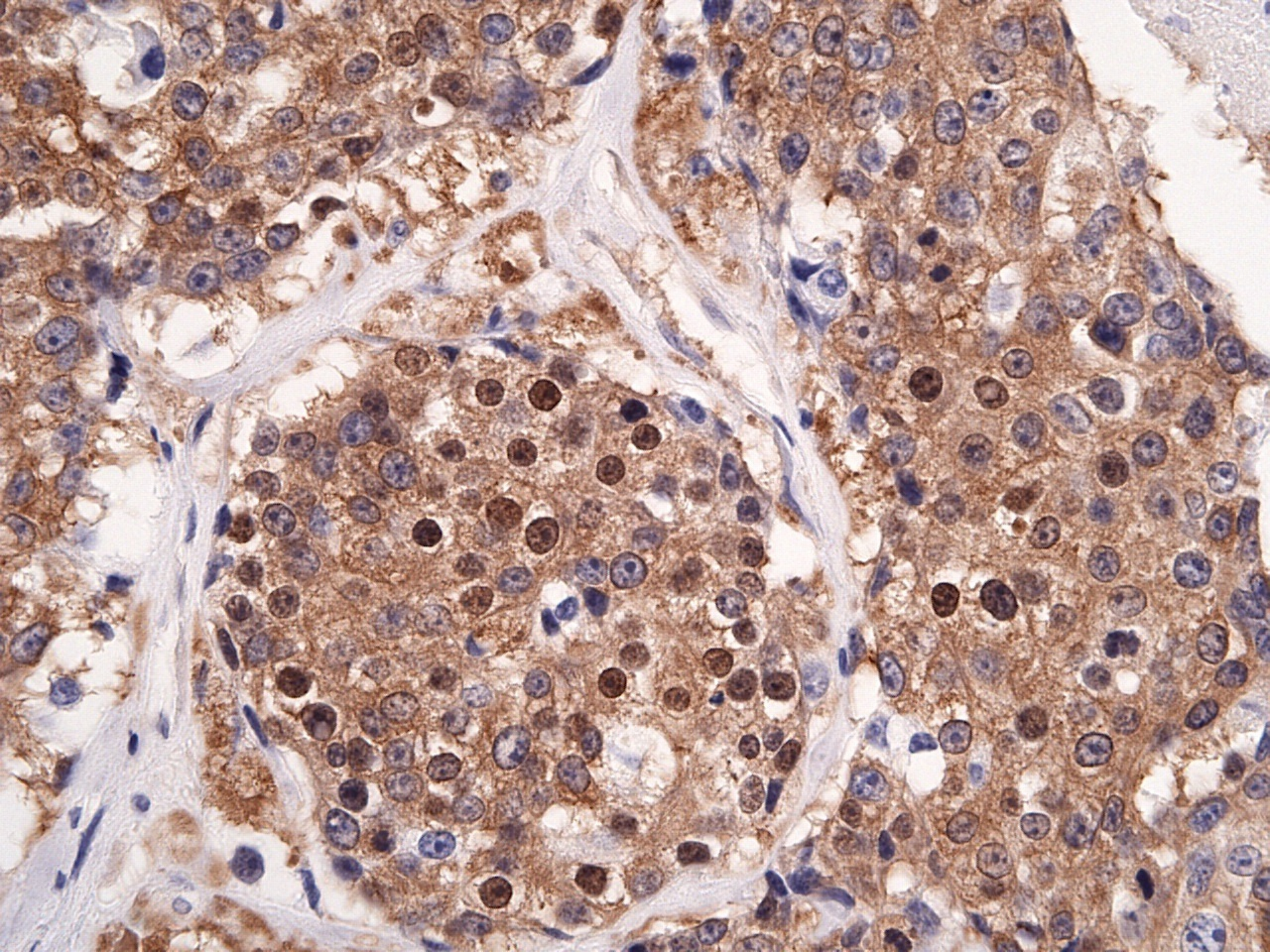

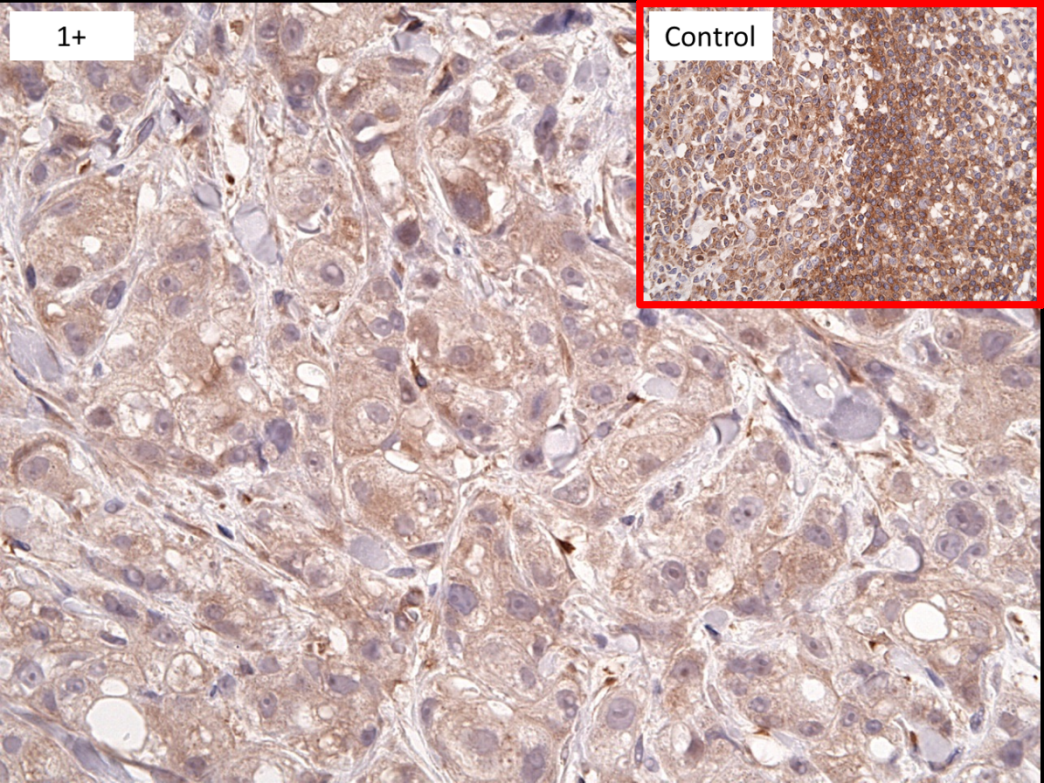
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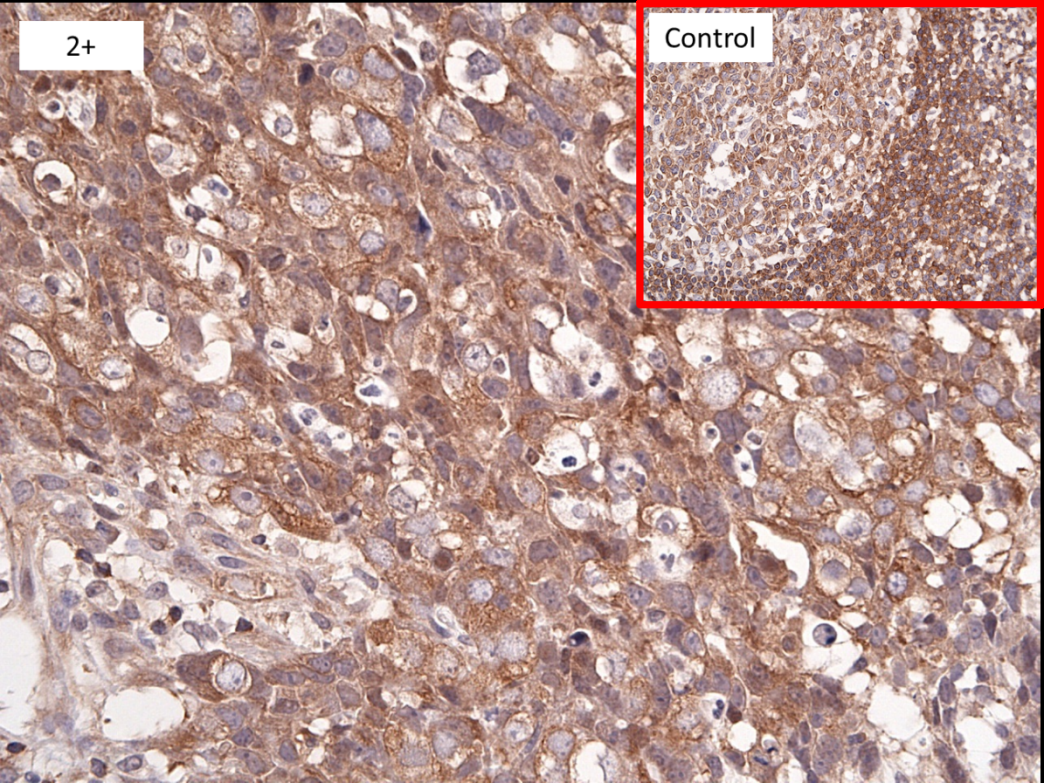
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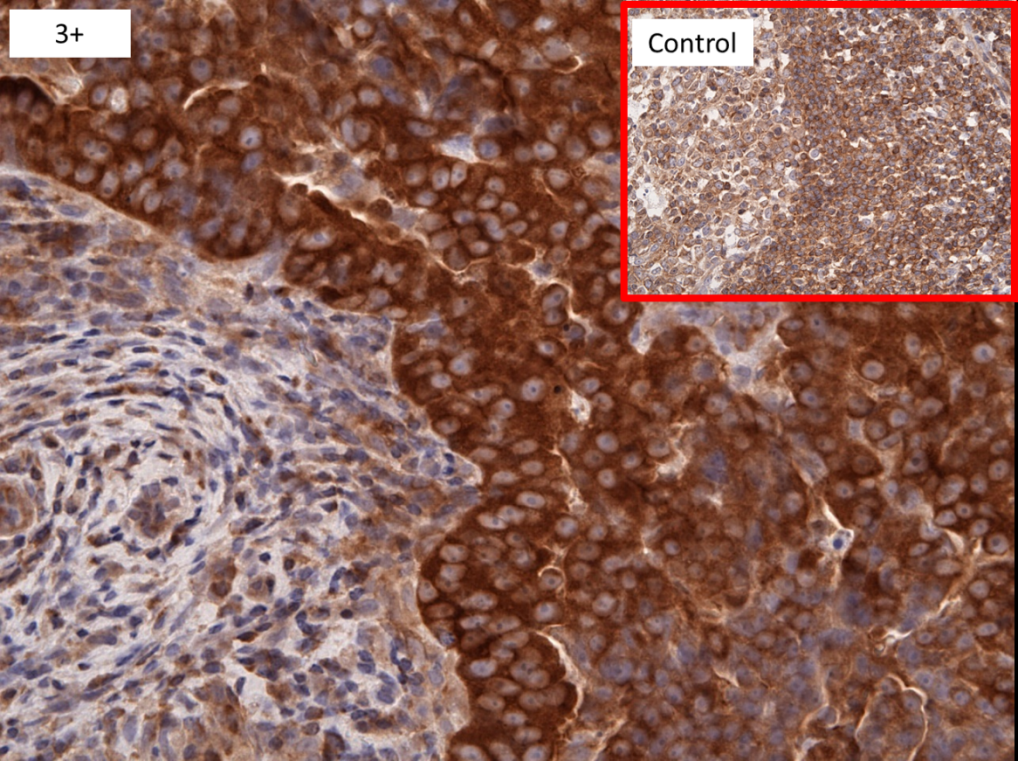
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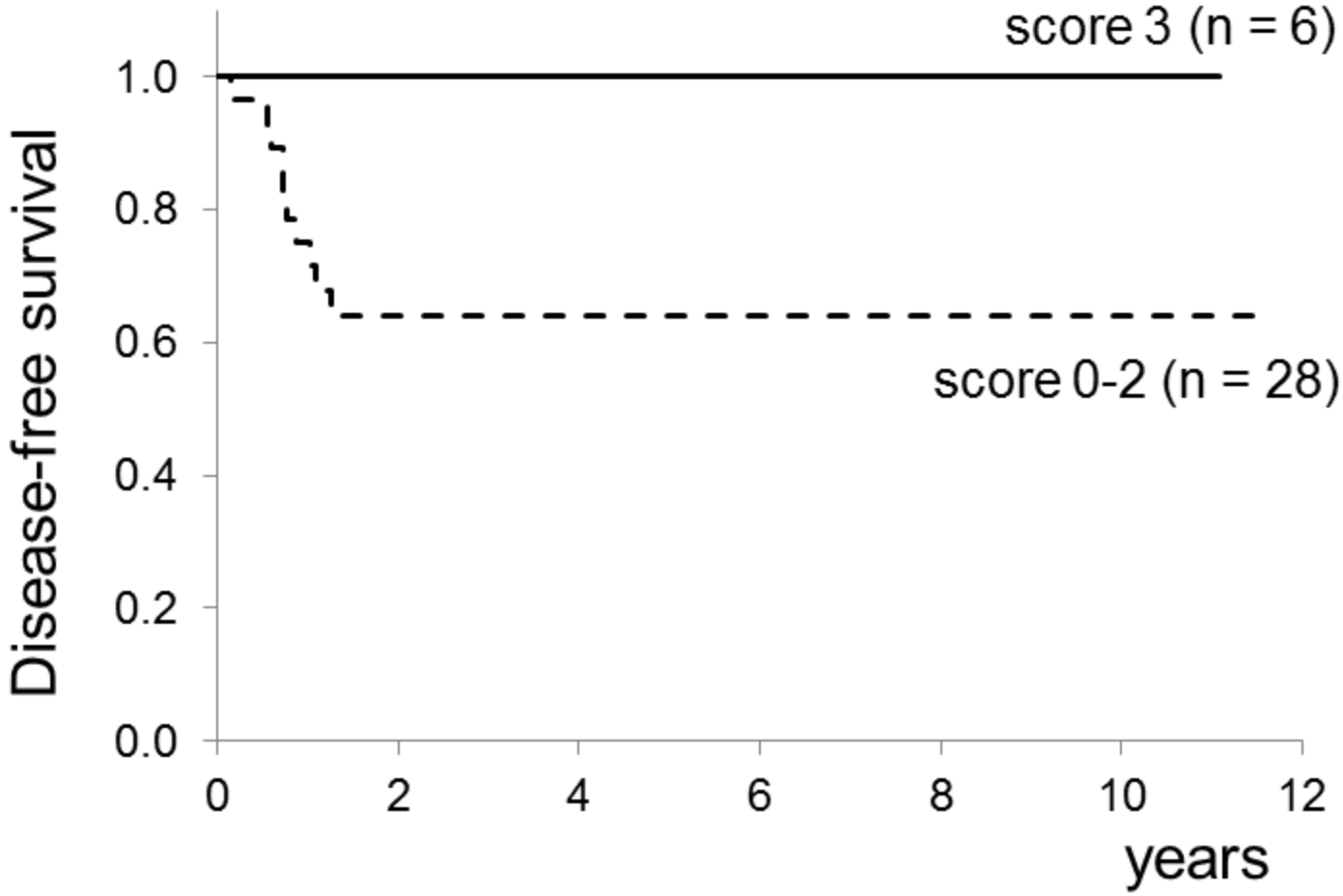
score 3 (n = 6)
score 0-2 (n = 28)
Disease-free survival
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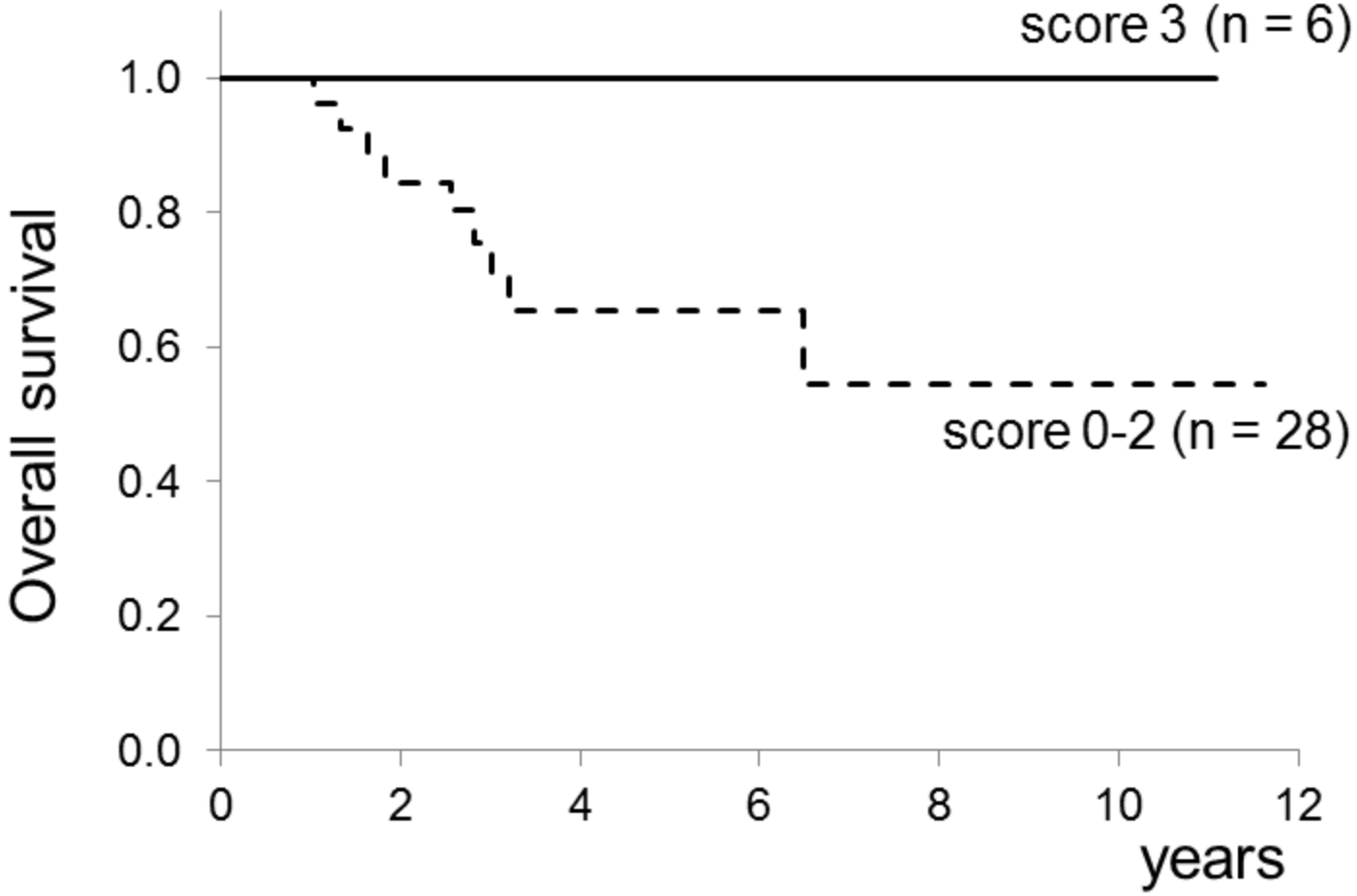
score 3 (n = 6)
score 0-2 (n = 28)
Overall survival
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
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8
10
12
years

Table 1 Clinicopathological characteristics of patients and tumors with triple-negative breast cancer

| Factor | | Number of patients (%) |
|---|---|---|
| Total number of patients | | 34 |
| Age at neoadjuvant chemotherapy (years) | Mean ± SD | 51.4 ± 13.8 |
| | Range | 24-79 |
| Menopausal status | Premenopausal | 13 (38.2%) |
| | Postmenopausal | 21 (61.4%) |
| Tumor size (cm) | < 2 | 2 (5.9%) |
| | 2.1- 5 | 21 (61.8%) |
| | 5 < | 11 (32.3%) |
| Lymph nodes status | N0 | 10 (29.4%) |
| | N1 | 13 (38.2%) |
| | N2 | 5 (14.7%) |
| | N3 | 6 (17.6%) |
| Stage | II | 16 (47.1%) |
| | III | 18 (52.9%) |
| Nuclear grade | 1 | 8 (23.5%) |
| | 2 | 7 (20.6%) |
| | 3 | 19 (55.9%) |
| Ki67 labeling index (%) | Mean ± SD | 73.2 ±19.8 |

Table 2 Comparison of expression levels of biological markers in tumors before and after neoadjuvant chemotherapy

| | Before<br>Mean ± SD | After<br>Mean ± SD | $p^{††}$ |
|---|---|---|---|
| nuclear NF-κB/p65† | 0.19 ± 0.43 | 0.64 ± 2.14 | 0.3 |
| cytoplasmic NF-κB/p65† | 96.12 ± 17.23 | 91.09 ± 26.31 | 0.5 |
| cytoplasmic NF-κB/p65 intensity | 2.00 ± 0.65 | 1.43 ± 0.63 | 0.004* |
| Bcl2 | 2.76 ± 10.93 | 10.03 ± 17.42 | 0.004* |
| Ki67 LI | 73.24 ± 19.84 | 45.83 ± 33.89 | 0.0002* |

† percentage of cells

†† paired *t*-test.

* $p < 0.05$ is considered significant.

Table 3 Correlation between expression levels of biological markers and clinicopathological factors in pre-neoadjuvant chemotherapy specimens

| | Age | Tumor size | Lymph node status | Nuclear grade |
|---|---|---|---|---|
| pre nuclear NF-κB/p65[†] | -0.505[a] | 0.097 | 0.102 | -0.166 |
| | 0.78[b] | 0.59 | 0.57 | 0.36 |
| pre cytoplasmic NF-κB/p65[†] | 0.010 | -0.230 | -0.221 | 0.192 |
| | 0.96 | 0.20 | 0.22 | 0.29 |
| pre cytoplasmic NF-κB/p65 intensity | -0.0089 | -0.012 | -0.465* | 0.128 |
| | 0.96 | 0.95 | 0.006 | 0.48 |
| pre Bcl2 | -0.0036 | -0.0397 | 0.143 | -0.278 |
| | 0.98 | 0.83 | 0.43 | 0.12 |
| pre Ki67 LI | -0.238 | -0.0805 | 0.0741 | 0.336 |
| | 0.18 | 0.66 | 0.68 | 0.056 |

[†] percentage of cells

[a]Spearman's correlation coefficient.

[b]Spearman's rank correlation test.

*Spearman's correlation coefficient < -0.4 or 0.4 < is considered significant.

Table 4 Correlation between clinical response and expression levels of biological markers in specimens before neoadjuvant chemotherapy

| | PD (n=2) Mean ± SD | SD (n=4) Mean ± SD | PR (n=22) Mean ± SD | CR (n=6) Mean ± SD |
|---|---|---|---|---|
| pre nuclear NF-κB/p65† | 0.7 ± 0.9 | 0.5 ± 1.0 | 0.1 ± 0.3 | 0.2 ± 0.2 |
| pre cytoplasmic NF-κB/p65† | 99.4 ± 0.9 | 99.5 ± 1 | 94.2 ± 21.3 | 99.9 ± 0.2 |
| pre cytoplasmic NF-κB/p65 intensity | 2.0 ± 0 | 1.8 ± 0.5 | 2.1 ± 0.7 | 2.0 ± 0.6 |
| pre Bcl2 | 0.5 ±0.6 | 16.4 ± 31.5 | 1.2 ± 2.3 | 0.03 ± 0.1 |
| pre Ki67 LI | 54.9 ±40.9 | 72.6 ± 7.7 | 80 ± 11.8 | 55.2 ± 30.4 |

† percentage of cells

Table 5 Correlation between pathological response and expression levels of biological markers before neoadjuvant chemotherapy

| | Grade 0-2b (n = 28) Mean ± SD | Grade 3 (pCR) (n = 6) Mean ± SD | $p^{††}$ |
|---|---|---|---|
| pre nuclear NF-κB/p65[†] | 0.2 ± 0.5 | 0.1 ± 0.2 | 0.75 |
| pre cytoplasmic NF-κB/p65[†] | 95.4 ± 19.0 | 99.4 ± 1.4 | 0.80 |
| pre cytoplasmic NF-κB/p65 intensity | 1.9 ± 0.7 | 2.3 ± 0.5 | 0.45 |
| pre Bcl2 | 3.3 ± 12.0 | 0.4 ± 0.8 | 0.58 |
| pre Ki67 LI | 73.1 ± 21.7 | 74.0 ± 7.5 | 0.41 |

[†] percentage of cells

[††]Mann-Whitney *U*-test.

*$p < 0.05$ is considered significant.

Table 6 Univariate analysis of factors predictive of pCR

| | Univariate | | |
|---|---|---|---|
| Factor | RR | 95%CI | *p* |
| Age | 0.931 | 0.745-1.162 | 0.53 |
| Menopausal status | 4.005 | 0.009-1836.4 | 0.66 |
| Tumor size | 3.168 | 0.456-22.004 | 0.24 |
| Lymph node status | 0.370 | 0.081-1.683 | 0.20 |
| Nuclear grade | 0.276 | 0.016-4.798 | 0.38 |
| pre nuclear NF-κB/p65† | 1.539 | 0.027-89.33 | 0.84 |
| pre cytoplasmic NF-κB/p65† | 0.998 | 0.785-1.270 | 0.99 |
| pre cytoplasmic NF-κB/p65 intensity | 0.160 | 0.010-2.433 | 0.19 |
| pre Bcl2 | 1.802 | 0.3770-8.619 | 0.46 |
| pre Ki67 LI | 1.014 | 0.952-1.081 | 0.66 |

† percentage of cells

RR, relative risk; CI, confidence interval.

*$p < 0.05$ is considered significant.

Table 7 Univariate and multivariate analyses of factors predictive of disease-free survival

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| Factor | RR | 95%CI | *p* | RR | 95%CI | *p* |
| Age | 1.018 | 0.972-1.065 | 0.45 | | | |
| Menopausal status | 0.616 | 0.159-2.384 | 0.48 | | | |
| Tumor size | 1.145 | 0.387-3.389 | 0.81 | | | |
| Lymph node status | 1.920 | 1.098-3.359 | 0.02* | 1.997 | 1.063-3.751 | 0.03* |
| Nuclear grade | 0.476 | 0.234-0.966 | 0.04* | 0.439 | 0.182-1.059 | 0.07 |
| pre nuclear NF-κB/p65† | 1.650 | 0570-4.771 | 0.36 | | | |
| pre cytoplasmic NF-κB/p65† | 1.195 | 0.711-2.007 | 0.50 | | | |
| pre cytoplasmic NF-κB/p65 intensity | 0.532 | 0.745-1.153 | 0.11 | | | |
| pre Bcl2 | 0.983 | 0.900-1.073 | 0.70 | | | |
| pre Ki67 LI | 0.976 | 0.954-0.999 | 0.04* | 0.992 | 0.968-1.018 | 0.54 |
| post nuclear NF-κB/p65† | 1.093 | 0.857-1.394 | 0.47 | | | |
| post cytoplasmic NF-κB/p65† | 0.994 | 0.972-1.016 | 0.58 | | | |
| post cytoplasmic NF-κB/p65 intensity | 0.665 | 0.246-1.798 | 0.42 | | | |
| post Bcl2 | 1.000 | 0.966-1.036 | 0.98 | | | |
| post Ki67 LI | 0.988 | 0.969-1.007 | 0.21 | | | |
| pCR | 0.712 | 0.402-1.260 | 0.24 | | | |

† percentage of cells

RR, relative risk; CI, confidence interval.

*$p < 0.05$ is considered significant.