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Original Article

Clinical Significance of Receptor-Interacting Protein 3 and Parkin, Essential Molecules for Necroptosis, in Breast Cancer

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Conflict of Interest
The authors declare that they have no competing interests.

ABSTRACT

Purpose: Receptor-interacting protein 3 (RIP3) is the main initiator of necroptosis. Parkin prevents the formation of the RIP1–RIP3 complex by promoting polyubiquitination of RIP3. However, the mechanism by which necroptosis affects the clinical features of breast cancer and prognosis is not known. Here, we aimed to study the effect of necroptosis on the clinical features and prognosis of breast cancer by assessing the expression of RIP3 and Parkin.
Methods: Tissue microarrays (TMAs) were constructed from 257 cases of breast cancer. Immunohistochemistry was performed on 4-μm tissue sections from each TMA block. The χ^2 test, Kaplan-Meier survival analysis with log-rank test, and Cox regression proportional hazard model were used for statistical analysis.
Results: Low RIP3 expression resulted in a large tumor size and high nuclear grade. Low RIP3 expression was correlated with human epidermal growth factor receptor 2 positivity, short overall survival (OS), and short disease-free survival (DFS). The triple negative breast cancer group with low RIP3 expression and lymph node (LN) positive group with low RIP3 expression had the shortest OS. High Parkin expression was associated with high histological grade, estrogen and/or progesterone receptor negativity, and lymphatic emboli, but was not correlated with OS and DFS. OS was correlated with LN metastasis and RIP3 loss and DFS with large tumor size, LN metastasis, and RIP3 loss.
Conclusion: Low RIP3 and high Parkin expression are associated with aggressive clinical features in breast cancer. RIP3, a molecular marker of necroptosis, is an independent factor associated with survival in breast cancer. Further in-depth studies are needed to investigate the role of necroptosis in breast cancer development, metastasis, and treatment in the future.

Keywords: Breast neoplasms; Necroptosis; Parkinson disease associated proteins; Receptor interacting protein serine-threonine kinase 3

Author Contributions

Conceptualization: Han SA; Data curation: Won KY, Min SY, Song JY, Han SA; Formal analysis: Won KY, Min SY, Song JY, Han SA; Funding acquisition: Han SA; Investigation: Won KY, Song JY, Lim SJ, Han SA; Methodology: Won KY, Lim SJ, Han SA; Project administration: Song JY, Han SA; Supervision: Song JY, Lim SJ, Han SA; Writing - original draft: Won KY, Han SA; Writing - review & editing: Min SY, Song JY, Lim SJ.

INTRODUCTION

Necroptosis is a type of programmed cell death that is separate from apoptosis; it is characterized by plasma membrane rupture, resulting in the spilling of the cellular contents and triggering of the immune system [1]. Necroptosis during various biological processes, including inflammation, immune response, embryonic development, and metabolic abnormalities, results in the generation of a typical necrotic morphology [2,3]. Receptor-interacting protein 3 (RIP3) is the main initiator of necroptosis, while RIP1 promotes the formation of a functional complex called the necrosome [4,5]. RIP1/RIP3 kinases play essential roles in inflammatory response-linked necroptosis [3-5].

Parkin (PARK2), an E3 ubiquitin ligase implicated in Parkinson's disease and a tumor suppressor, regulates necroptosis and inflammation by regulating necrosome formation [6,7]. Parkin prevents RIP1–RIP3 complex formation by promoting RIP3 polyubiquitination. Parkin deficiency enhances inflammation and inflammation-associated tumorigenesis. The adenosine monophosphate-activated protein kinase (AMPK)-Parkin axis negatively regulates necroptosis by inhibiting the formation of the RIP1–RIP3 complex, possibly serving as an essential mechanism for fine-tuning necroptosis and inflammation [8].

Several studies have shown that necroptosis suppresses cancer development and facilitates cancer therapy [9]. The induced inflammatory response may also promote tumorigenesis and cancer metastasis [10]. However, some studies report that the expression of necroptosis mediators is increased in certain types of cancers; therefore, the mechanism by which necroptosis affects breast cancer development and prognosis remains unclear. Here, we aimed to investigate the effects of necroptosis on the clinical features and prognosis of breast cancer by assessing the expression of RIP3, a marker of necroptosis, and Parkin, a regulator of RIP3.

METHODS

Patients and tissue specimens

The inclusion criteria were stage I–IV breast cancer patients who underwent surgical breast resection with or without neoadjuvant chemotherapy from 2005 to 2010 at Kyung Hee University Hospital at Gandong and Kyung Hee University Medical Center. Patients whose final pathology revealed ypTis or ypTx were excluded from analysis because immunohistochemical evaluation of invasive breast cancer cells would be impossible. Tissue samples from 257 patients with breast carcinoma were obtained. Research protocols were approved by the Institutional Review Board (KHNMC 2019-05-014). Two investigators (W.K.Y. and L.S.J.) reviewed all the original hematoxylin and eosin (H&E)-stained sections. The mean patient follow-up period was 106.2 months (range, 2–172.8 months). Among the 257 patients, 45 showed disease recurrence. The median patient age was 52.3 years (range, 25–82 years), and the median tumor size was 2.49 cm (range, 0.5–11 cm). Cancer grade was classified according to the Nottingham Modification of the Bloom–Richardson system. The following are the number of patients with corresponding grades: 48 grade I (18.9%), 130 grade II (51.2%), and 76 grade III (29.9%).

Tissue microarray (TMA) construction

The H&E-stained sections of formalin-fixed, paraffin-embedded tumor tissue blocks were screened to identify the viable and representative areas of invasive breast carcinomas.

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Corresponding areas on the block were marked for punching out the tissue cores. The TMAs were assembled using a commercially available manual TMA (Quick Ray; UNITMA Co., Ltd., Seoul, Korea). Briefly, 3 representative tumor cores with a diameter of 2.0 mm were punched out from each tumor tissue block and arrayed into 3 paraffin recipient blocks. We arrayed 3 cores per case to increase the concordance between the immunohistochemistry results of the TMAs and whole sections. Each TMA block also contained 4 normal breast tissue cores. H&E staining was performed for each block to verify the tumor cell content. Samples with only stromal tissue or insufficient carcinoma tissue in the cores were excluded from the analysis.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm tissue sections of each TMA block using the BOND Polymer Intense Detection System (Vision BioSystems, Victoria, Australia), according to the manufacturer's instructions with minor modifications. Briefly, 4-μm formalin-fixed, paraffin-embedded tissue sections were deparaffinized with BOND Dewax Solution (Vision BioSystems). Antigen retrieval was performed using the BOND Epitope Retrieval Solution (Vision BioSystems) at 100 °C for 30 min. Endogenous peroxidase activity was quenched by incubating the tissue with hydrogen peroxide for 5 min. The sections were incubated with primary antibodies against RIP3 (1:1,000; Abcam, Burlingame, USA), Parkin (1:200, Abcam), estrogen receptor (ER, 1:200, 6F11; Novocastra, Newcastle, UK), progesterone receptor (PR, 1:200, 16; Novocastra), Ki-67 (1:200, M 7240; Dako, Glostrup, Denmark), p53 (1:500, DO-7; Novocastra), and human epidermal growth factor receptor 2 (HER2)/ErbB2 (1:600, CB11; Novocastra) using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in a BOND-MAX automatic slide stainer (Vision BioSystems) at ambient temperature for 15 minutes. Nuclei were counterstained with hematoxylin. The negative control was treated identically, but mouse immunoglobulin G instead of the primary antibodies was used for incubation.

Evaluation of immunohistochemical staining

Immunohistochemistry results were semiquantitatively analyzed by calculating the ratio of positive tumor cells to the total number of tumor cells, which ranged from 0 to 100% in increments of 5%. We evaluated the immunohistochemistry results by calculating the average score for the 3 cores in each sample. The expression of RIP3 and Parkin was evaluated based on its intensity and proportion. The intensity score was defined as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The proportion score was calculated as the number of positive tumor cells over the total number of tumor cells, and was defined as 1 (< 30% tumor cells), 2 (30% ≤ tumor cells < 60%), and 3 (≥ 60% tumor cells). The intensity and proportion scores were multiplied to obtain the total score. Total scores were as follows: 0–1 (low) and 2–9 (high) for RIP3 expression; 0–4 (low) and 5–9 (high) for Parkin expression. All slides were evaluated independently by 2 investigators (W.K.Y. and L.S.J.) blinded to the patient's identity or clinical outcome. ER and PR were identified as positive if the proportion of positive cells was > 1% [11]. The Ki-67 index was considered "high" if there was ≥ 14% positive average nuclear staining of strong intensity [12]. Expression of p53 was considered positive if there was > 10% positive average nuclear staining with strong intensity [13]. *HER2/ErbB2* status was determined and fluorescence *in situ* hybridization (FISH) was performed in accordance with the guidelines proposed by the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) [14].

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FISH

The tumor-infiltrating region on the H&E-stained slides was reviewed and marked before FISH was performed. *HER2/ErbB2* amplification was evaluated using a PathVysion HER2 DNA Probe Kit (Abbott Molecular, Waukegan, USA), according to the manufacturer's instructions. This kit included dual-color fluorescence, that is, an orange spectrum for *HER2/ErbB2* and a green spectrum for *CEP17* (D17Z1 of the centromere on chromosome 17). The *HER2/ErbB2* status was determined according to the ASCO/CAP guidelines [14]. ISH results were considered positive in (1) cases with a *HER2/CEP17* ratio ≥ 2.0 and *HER2* copy number ≥ 4.0; (2) Cases with a *HER2/CEP17* ratio ≥ 2.0 and *HER2* copy number < 4.0 with concurrent IHC 3+; (3) Cases with a *HER2/CEP17* ratio < 2.0 and *HER2* copy number ≥ 6.0 with concurrent IHC 2+ or 3+ (4) cases with a *HER2/CEP17* ratio < 2.0 and *HER2* copy number ≥ 4.0 and < 6.0 with concurrent IHC 3+. The molecular subtypes of tumors were defined as follows: luminal A (ER+ and/or PR+ and HER2−); luminal B (ER+ and/or PR+ and high Ki-67 or HER2+), HER2+ (ER−, PR−, and HER2+); triple-negative breast cancer (TNBC) (ER−, PR−, and HER2−) [15].

Statistical analysis

Pearson's χ^2 test and Fisher's exact test were used to evaluate the association between parkin and RIP3 expression and several clinicopathological variables. The Kaplan-Meier method was used to determine the probability of disease-free survival (DFS) and overall survival (OS), and the data were analyzed using the log-rank test. All statistical analyses were conducted using SPSS Statistics (version 18, IBM Corporation, Armonk, USA) for Windows. A p-value < 0.05 was considered significant.

RESULTS

The descriptive statistics for the patient population and clinicopathological parameters are shown in **Table 1**. Immunoreactivity of RIP3 and Parkin in human breast cancer tissue is shown in **Figure 1**. The cancer cells showed diffusely strong cytoplasmic Parkin and RIP3 expression. RIP3 expression appeared as fine granular and diffuse cytoplasmic staining, and Parkin expression appeared as granular cytoplasmic staining. There was no statistical correlation between Parkin and RIP3 expression (Spearman's rho correlation coefficient = 0.086, p = 0.166).

Table 1. General patient characteristics and clinicopathological factors

Characteristics	Value (n = 257)
Mean age (yr)	52.3 ± 10.7
Median follow-up (mon)	106.2 (2–172.8)
Median disease-free survival (mon)	146.5 (139.5–153.6)
Overall survival (mon)	161.4 (156.6–166.2)
Age (yr)	
< 50	113 (44)
≥ 50	144 (56)
T stage	
T1	119 (46.3)
T2	125 (48.6)
T3	12 (4.7)
T4	1 (0.4)

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Table 1. (Continued) General patient characteristics and clinicopathological factors

Characteristics	Value (n = 257)
N stage	
N0	146 (56.8)
N1	64 (24.9)
N2	19 (7.4)
N3	24 (9.3)
Nx	4 (1.6)
M stage	
M0	253 (98.4)
M1	4 (1.5)
Estrogen receptor	
Positive	164 (63.8)
Negative	93 (36.2)
Progesterone receptor	
Positive	136 (52.9)
Negative	121 (47.1)
HER2 receptor	
Positive	51 (19.8)
Negative	206 (80.2)
Ki-67 index	
High	33 (12.8)
Low	209 (81.3)
Missing	15 (5.8)
P53 expression	
High	62 (24.1)
Low	180 (70.1)
Missing	15 (5.8)
Intrinsic subtype	
Luminal A	130 (50.6)
Luminal B	34 (13.2)
HER2 enriched	23 (8.9)
Triple negative	70 (27.2)
Histological grade	
1	48 (18.9)
2	130 (51.2)
3	76 (29.9)
Missing	3
Nuclear grade	
1	26 (10.1)
2	148 (57.6)
3	80 (31.5)
Missing	3
Adjuvant treatment	
Chemotherapy	191 (74.3)
NAC	32/191 (16.8)
Hormonal therapy	157 (61.0)
Radiation	119 (46.3)
Trastuzumab	20 (7.8)
Recurrence	
No	212 (82.5)
Yes	45 (17.5)
Death	
No	237 (92.2)
Yes	20 (7.8)

Data are shown as mean ± standard deviation or number (%).
HER2 = human epidermal growth factor 2 receptor; NAC = neoadjuvant chemotherapy.

Clinical correlation of RIP3 and Parkin expression

RIP3 was expressed at high levels in 111 (43.2%) patients and at low levels in 146 (56.8%) patients. The low RIP3 group exhibited a large tumor size. The proportion of T3–4 was

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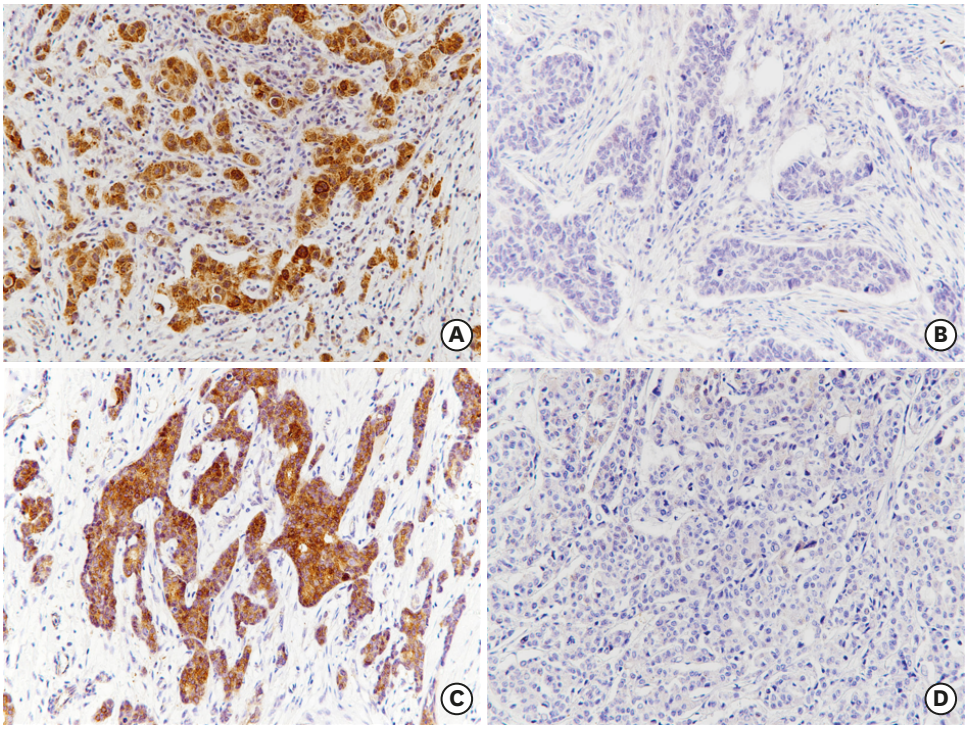

Figure 1. Investigation of the expression of parkin and RIP3 using immunochemistry.
Representative photographs of high parkin (A) and RIP3 (C) expression in invasive ductal breast carcinoma. The cancer cells show diffusely strong cytoplasmic parkin (A) and RIP3 (C) expression. Representative photographs of low parkin (B) and RIP3 (D) expression in invasive ductal breast carcinoma.
RIP3 = receptor-interacting protein 3.

7.5% in the low RIP3 group and 1.8% in the high RIP3 group (p = 0.032). The low RIP3 group presented with high nuclear grade (36.3% vs. 25.0%, p = 0.037) and tended to have more lymph node (LN) metastasis (47.9% vs. 36.9%, p = 0.051). Low RIP3 expression was strongly correlated with HER2 positivity (24.7 vs. 13.5%, p = 0.019) (**Table 2**). No significant correlation was observed between the expression of RIP3 and the hormone receptor and between Ki-67 index and p53 expression.

Parkin was expressed at high levels in 101 (39.3%) patients and at low levels in 156 (60.7%) patients. The group with high Parkin expression exhibited high histological grade (35.6% vs. 25.6%, p = 0.05), ER negativity (53.5 vs 32.1%, p = 0.001) and/or PR negativity (59.4% vs. 39.1%, p = 0.001), and lymphatic emboli (14.9% vs. 4.5%, p = 0.004) (**Table 3**). High Ki-67 index (p = 0.005) and high p53 expression (p = 0 011) were significantly correlated with high Parkin expression. TNBC was significantly more frequent in the high Parkin expression group (37.6% vs. 21.8%, p = 0.005, **Table 3**). Correlation analysis between clinical factors and RIP3/Parkin expression in the TNBC group (**Appendices 1** and **2**) revealed a significant correlation only between lymphatic emboli and RIP3 expression (p = 0.013).

Survival outcome analysis

In survival outcome and RIP3 expression analysis, RIP3 loss consistently and significantly affected OS and DFS. Patients with low RIP3 expression had a worse OS than those with high RIP3 expression (**Figure 2A**). The OS of the group with low RIP3 expression (155.6 ± 3.9 months; 95% confidence interval [CI], 147.9–163.2) was significantly shorter than that of the high RIP3 group (165.8 ± 2.0 months; 95% CI, 161.8–169.8) (p = 0.007). These findings were

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Table 2. Correlation between clinicopathological factors and RIP3 expression

Characteristics	High expression (n = 111)	Low expression (n = 146)	*p*-value
Age (yr)			
< 50	50 (45.0)	63 (43.2)	
≥ 50	61 (55.0)	83 (56.8)	0.430
T			
T1–2	109 (98.2)	135 (92.5)	
T3–4	2 (1.8)	11 (7.5)	0.032
N			
N0	70 (63.1)	76 (52.1)	
N1–3	41 (36.9)	70 (47.9)	0.051
M			
M0	111 (100.0)	142 (97.3)	
M1	0 (0.0)	4 (2.7)	0.102
Lymphatic emboli			
Positive	6 (8.1)	13 (8.9)	
Negative	102 (91.9)	133 (91.1)	0.503
Nuclear grade			
G1,2	81 (75.0)	93 (63.7)	
G3	27 (25.0)	53 (36.3)	0.037
Histological grade			
G1,2	75 (69.4)	103 (70.5)	
G3	33 (30.6)	43 (29.5)	0.478
Estrogen receptor			
Positive	76 (68.5)	88 (60.3)	
Negative	35 (31.5)	58 (39.7)	0.110
Progesterone receptor			
Positive	64 (57.7)	72 (49.3)	
Negative	47 (42.3)	74 (50.7)	0.115
HER2 status			
Positive	15 (13.5)	36 (24.7)	
Negative	96 (86.5)	110 (75.3)	0.019
Triple negative breast cancer			
No	83 (74.8)	104 (71.2)	
Yes	28 (25.2)	42 (28.8)	0.313
Ki-67 index (n = 244, missing 13)			
High*	19 (17.9)	14 (10.3)	
Low	87 (82.1)	122 (89.7)	0.064
p53 (n = 244, missing 13)			
High†	27 (25.5)	35 (25.4)	
Low	79 (74.5)	103 (74.6)	0.550

Values are presented as number (%).
HER2 = human epidermal growth factor 2 receptor.
*Ki-67 index high > 14% (reference); †p53 expression high > 10% (reference).

maintained even when age (50 < vs. ≥ 50), tumor size (T1–2 vs. T3–4), LN metastasis (N0 vs. N1–3), and ER expression (positive vs. negative) were corrected (data not shown).

The DFS of the low RIP3 expression group (137.3 ± 5.5 months; 95% CI, 126.5–148.1) was significantly worse than that of the high RIP3 expression group (154.7 ± 4.0 months; 95% CI, 146.8–162.6) ($p = 0.007$) (**Figure 2B**). The TNBC group exhibited significantly worse OS than the non-TNBC group (139.1 ± 5.3 months; 95% CI, 128.6–149.6 vs. 164.7 ± 2.4 months; 95% CI, 160.0–169.4; $p = 0.039$). The low RIP3-expressing TNBC group had the shortest OS (**Figure 2C**, $p = 0.009$). In the TNBC group, the low RIP3-expressing group tended to have shorter OS than the high RIP3-expressing group, but the difference was not significant (**Figure 2C**, $p = 0.061$). Among the patients in the non-TNBC group, OS significantly differed based on RIP3 expression. (**Figure 2C**, **Table 4**). Among the patients in the high RIP3 expression group, OS was not significantly different between the TNBC and non-TNBC

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Table 3. Correlation between clinicopathological factors and Parkin expression

Characteristics	High expression (n = 101)	Low expression (n = 156)	*p*-value
Age (yr)			
< 50	44 (43.6)	69 (44.2)	
≥ 50	57 (56.4)	87 (55.8)	0.510
T			
T1–2	97 (96.0)	147 (94.2)	
T3–4	4 (4.0)	9 (5.8)	0.369
N			
N0	56 (55.4)	90 (57.7)	
N1–3	45 (44.6)	66 (42.3)	0.410
M			
M0	100 (99.0)	153 (98.1)	
M1	1 (1.0)	3 (1.9)	0.487
Lymphatic emboli			
Positive	15 (14.9)	7 (4.5)	
Negative	86 (85.1)	149 (95.5)	0.004
Nuclear grade			
G1,2	66 (66.7)	108 (69.7)	
G3	33 (33.3)	47 (30.3)	0.356
Histological grade			
G1,2	63 (63.6)	115 (74.2)	
G3	36 (36.4)	40 (25.8)	0.050
Estrogen receptor			
Positive	47 (46.5)	106 (67.9)	
Negative	54 (53.5)	50 (32.1)	0.001
Progesterone receptor			
Positive	41 (40.6)	95 (60.9)	
Negative	60 (59.4)	61 (39.1)	0.001
HER2 status			
Positive	18 (17.8)	28 (17.9)	
Negative	83 (82.2)	128 (82.1)	0.559
Triple negative breast cancer			
No	63 (62.4)	122 (78.2)	
Yes	38 (37.6)	34 (21.8)	0.005
Ki-67* (n = 244, missing 13)			
High	20 (21.5)	13 (8.7)	
Low	73 (78.5)	136 (91.3)	0.005
p53† (n = 244, missing 13)			
High	32 (34.4)	30 (20.1)	
Low	61 (65.6)	119 (79.9)	0.011

Values are presented as number (%).
HER2 = human epidermal growth factor 2 receptor.
*Ki-67 index high> 14% (reference); †p53 expression high > 10% (reference).

samples (**Figure 2C**, **Table 4**). The group with LN metastasis had significantly shorter OS than the group without LN metastasis ($p < 0.001$). The LN metastasis group with low RIP3 expression had the shortest OS (**Figure 2D**, $p < 0.001$); OS and DFS were not correlated with Parkin expression.

Additionally, multivariate analysis was performed to investigate the correlation between OS and DFS and clinicopathological features, such as age, tumor size, LN metastasis, lymphatic emboli, nuclear grade, histological grade, expression of RIP3, Parkin, Ki-67, and p53, and TNBC were performed for all subjects. Factors that significantly affected OS included LN metastasis (OR, 7.978; $p = 0.001$) and RIP3 loss (OR, 4.566; $p = 0.015$). Factors that significantly affected DFS included large tumor size (OR, 2.608; $p = 0.034$), LN metastasis (OR, 2.242; $p = 0.011$), and RIP3 loss (OR, 2.044; $p = 0.040$).

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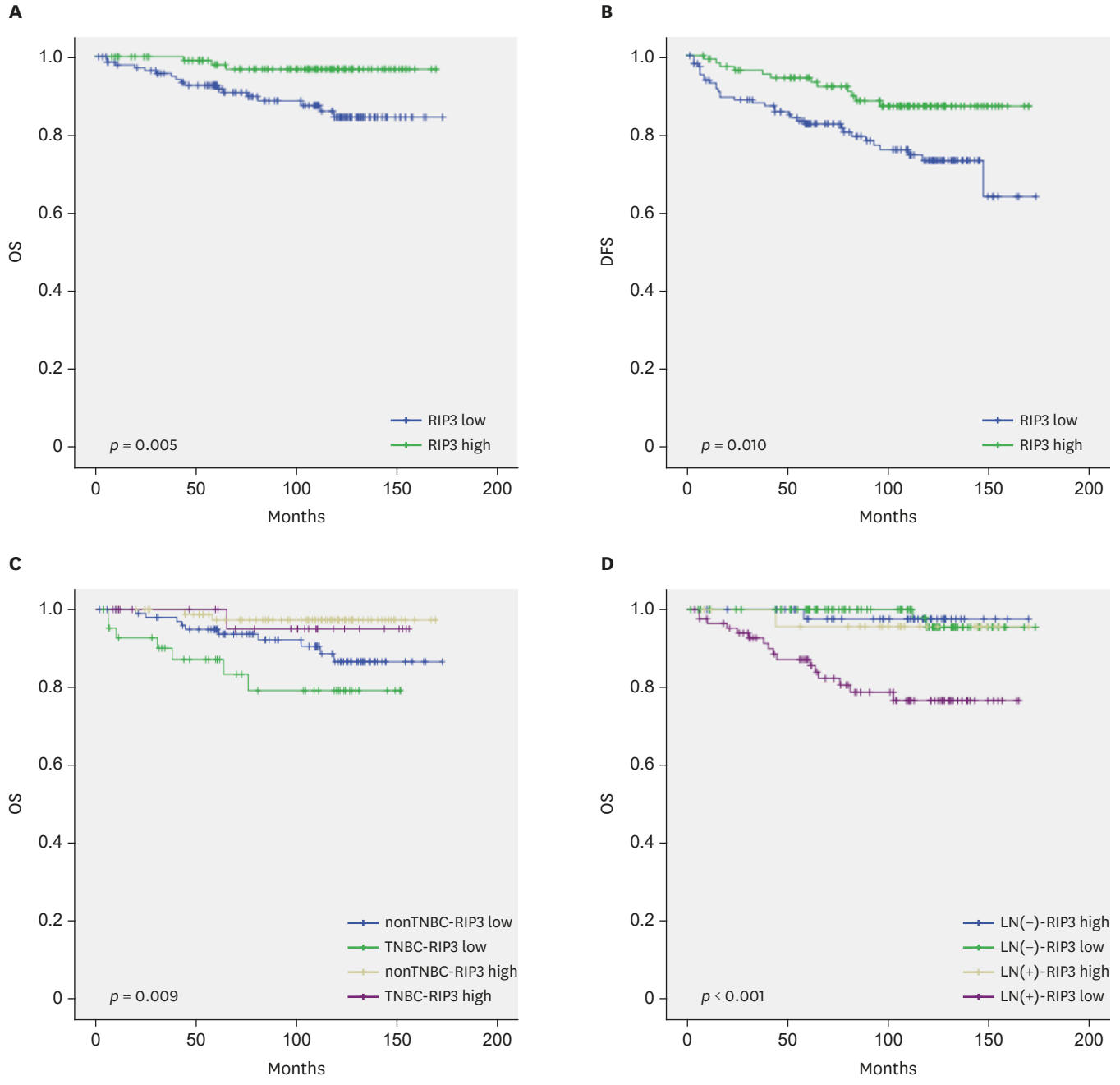

Figure 2. (A) OS and (B) DFS, according to RIP3 expression. (A, B) OS and DFS of the low RIP3 group were significantly worse than those of the high RIP3 group. (C) TNBC-RIP3 low group had the worst OS. Among RIP3 high group, OS was not statistically different between TNBC and non-TNBC. (D) LN metastasis group with low RIP3 had the worst OS ($p < 0.001$).
OS = overall survival; DFS = disease-free survival; RIP3 = receptor-interacting protein 3; TNBC = triple-negative breast cancer; LN = lymph node.

DISCUSSION

Resisting cell death is one of the hallmarks of cancer, with sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [16]. Many studies have shown that necroptosis, another form of apoptosis, prevents cancer initiation, growth, and metastasis [16]. The main method

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Table 4. Overall survival with 95% CI according to RIP3 expression and triple negative breast cancer

Subgroup	Mean for survival time			
	Estimate	SE	95% CI	
			Lower bound	Upper bound
NonTNBC low (blue)	159.6	3.9	151.903	167.390
TNBC low (green)	128.6	8.0	112.791	144.327
NonTNBC high (gold)	166.3	2.2	161.892	170.643
TNBC high (purple)	151.9	4.4	143.091	160.476
Overall	161.4	2.4	156.651	166.231

Data are represented as below: 1) TNBC low < nonTNBC low: $p = 0.048$; 2) TNBC low < TNBC high: $p = 0.061$(there is a trend, but the difference is not significant); 3) TNBC low < nonTNBC high: $p = 0.001$; 4) NonTNBC low < nonTNBC high: $p = 0.045$; 5) NonTNBC low = TNBC high: $p = 0.631$; 6) NonTNBC high = TNBC high: $p = 0.758$.
CI = confidential interval; RIP3 = receptor-interacting protein 3; SE = standard error; TNBC = triple-negative breast cancer.

to identify necroptosis includes detecting the expression of critical molecules, such as RIP1 and RIP3, which control necroptosis at the messenger RNA and protein levels. The degree of necroptosis can be confirmed by measuring the expression of major proteins activated through phosphorylation via immunoblotting or immunohistochemistry [17,18]. In this study, we determined RIP3 expression via immunohistochemistry in human breast cancer tissues and investigated the relationship between necroptosis and the clinical features of breast cancer. Our study showed that the high RIP3 group tended to be small and associated with low-grade tumor clinical features. The low RIP3 group was associated with a large tumor size, high-grade features, and HER2 positivity. Besides, worse OS and DFS of the low RIP3 group supported this hypothesis.

Studies have shown that reduced RIP3 expression promotes cancer development. RIP3 expression is significantly reduced in most acute myeloid leukemia (AML) cases, resulting in a decrease in apoptosis/necroptosis and promotion of nuclear factor-κB-mediated survival [19]. Genetic loss of RIP3 promotes AML development by enhancing leukemia-initiating cell accumulation in mice; the link between RIP3 suppression and cell death blockage has been validated in primary AML patient cohorts [20]. The expression of RIP1 and RIP3 is also significantly decreased in colon cancer tissues compared to that in adjacent normal colon tissues, thereby impairing the response of cancer cells to necroptosis triggers [21]. RIP1/3 may repress cancer metastasis by regulating oxidative stress to kill metastatic tumor cells [22]. RIP3 stimulates reactive oxygen species (ROS) production, and high ROS levels inhibit cancer cell metastasis [23]. Shikonin inhibits cancer cell metastasis by inducing RIP1/3 expression and promoting necroptosis [24]. These studies have been conducted on different substances involved in necroptosis in various carcinomas, but in common, it is known that silenced necroptosis promotes cancer initiation and growth. While studies have shown that a decrease in RIP3 expression is associated with a poor prognosis, other studies have reported that an increase in RIP3 expression is associated with a poor prognosis. Notably, the expression of RIP1, RIP3, FADD, and MLKL is elevated in pancreatic ductal adenocarcinoma [12], and this phenomenon is accompanied by the promotion of oncogenesis. The underlying mechanisms are angiogenesis and cancer cell proliferation, which are stimulated by cytokines produced as a result of the inflammatory reaction accompanying necroptosis, or genomic instability due to accumulation of ROS, a by-product of the inflammatory reaction, which eventually leads to cancer progression.

Few studies have examined RIP3 and Parkin expression in breast cancer tissue. One study reported that breast cancer tissues express lower levels of RIP3 than normal and benign breast tumor tissues by western blot [25]. In the cited study, RIP3 expression was significantly

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lower in the pre-menopauses, grade III, ER-negative, and c-erbB2-negative malignant tumors, but no correlation was detected between tumor size, PR, and P53 status among 30 breast cancer tissues. The results of this study show RIP3 suppression in high-grade tumors, which is consistent with our study, but the association with ER-negative and c-erbB2-negative malignant tumors and tumor size is contrary to our study.

Loss of the E3-ubiquitin ligase function of Parkin is common in juvenile Parkinson's disease [7,26] and in a variety of human cancers [6,27,28]. Parkin has been considered a tumor suppressor based on research results that inhibit breast cancer cell proliferation [28]. However, as a result of our study, high Parkin expression was associated with lymphatic emboli, TNBC subtype, Ki-67 high index, and high p53 expression, Parkin did not appear to act as a tumor suppressor. One research team found that 68% of patients with breast cancer had low Parkin expression, and that patients with low Parkin expression had poor OS [29]. Research results on the role of Parkin in breast cancer are inconsistent, and in-depth studies are needed. Our findings suggest that the role of Parkin in coordinating necroptosis might not be direct because the group with high Parkin expression showed an aggressive clinical pattern. According to Lee et al., [8] Parkin prevents the formation of the RIP1–RIP3 complex by promoting polyubiquitination of RIP3. Parkin is phosphorylated and activated by the cellular energy sensor AMPK. Parkin-deficiency potentiates RIP1–RIP3 interaction, RIP3 phosphorylation, and necroptosis. These findings demonstrate that the AMPK-Parkin axis negatively regulates necroptosis by inhibiting RIP1–RIP3 complex formation, and this regulation may serve as an important mechanism for fine-tuning necroptosis and inflammation [8]. Conversely, Parkin overexpression inhibits necroptosis by inhibiting RIP3 formation.

Our study is one of the few achievements that revealed that the IHC expression of RIP3 and Parkin is statistically related to the clinical features of breast cancer. Multivariate analysis confirmed that RIP3 expression was significantly associated with OS and DFS in breast cancer patients. Significant factors for OS were LN metastasis (OR, 7.978; $p = 0.001$) and RIP3 loss (OR, 4.566; $p = 0.015$). Significant factors for DFS were large tumor size (OR, 2.608; $p = 0.034$), LN metastasis (OR, 2.242; $p = 0.011$), and RIP3 loss (OR, 2.044; $p = 0.040$). Although our study has limitations as we did not experimentally confirm the expression at the gene or protein level, it is still useful to confirm major mechanisms of cancer that are extracted from actual patient survival data. The finding that RIP3 expression is an independent survival factor in breast cancer is of significance. Among the studies that applied the clinical significance of RIP3, there was an attempt to improve the response to breast cancer treatment by restoring RIP3 expression. The study reported that RIP3 expression was frequently silenced via methylation in cancer. Since necroptosis contributes to chemotherapy-induced cell death, it has been reported that when RIP3 is suppressed, the response to chemotherapy might be lowered [30]. This study predicted that treatment sensitivity could be improved through RIP3 demethylation.

In our study, no statistical correlation between Parkin and RIP3 expression patterns was observed, although the 2 substances were functionally related. It can be assumed that the expression of RIP3, which is directly involved in necroptosis, is not regulated by Parkin alone, but by a response involving various regulators in different pathways. Hence, a more sophisticated molecular study is needed to elucidate the mechanism of action of Parkin and RIP3 in necroptosis in breast cancer.

In conclusion, low necroptotic expression was associated with ER-negative, high-grade tumors, more LN metastasis, and worse OS and DFS. Necroptosis is emerging as an essential

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concept in understanding and treating cancer. Further in-depth studies are needed to investigate the role of necroptosis in breast cancer development, metastasis, and treatment.

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Appendix 1. Correlation between clinicopathological factors and RIP3 expression among Triple negative breast cancer

Characteristics	High expression (n = 28)	Low expression (n = 42)	p-value
Age (yr)			
< 50	11 (39.3)	15 (35.7)	
≥ 50	17 (60.7)	27 (64.3)	0.433
T			
T1–2	28 (100.0)	40 (95.2)	
T3–4	0 (0.0)	2 (4.8)	0.357
N			
N0	17 (60.7)	18 (42.9)	
N1–3	11 (39.3)	24 (57.1)	0.111
M			
M0	28 (100.0)	42 (100.0)	
M1	0 (0.0)	0 (0.0)	NA
Lymphatic emboli			
Positive	0 (0.0)	8 (13.8)	
Negative	28 (100.0)	34 (81.0)	0.013
Nuclear grade			
G1,2	10 (35.7)	20 (47.6)	
G3	18 (64.3)	22 (52.4)	0.230
Histological grade			
G1,2	7 (25.0)	20 (47.6)	
G3	21 (75.0)	22 (52.4)	0.080
Ki-67*			
High	10 (35.7)	8 (19.0)	
Low	17 (60.7)	33 (78.6)	0.266
p53†			
High	17 (63.0)	21 (50.0)	
Low	10 (37.0)	21 (50.0)	0.210

Values are presented as number (%).
NA = not applicable.
*Ki-67 index high > 14% (reference); †p53 expression high > 10% (reference).

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Appendix 2. Correlation between clinicopathological factors and parkin expression among triple negative breast cancer

Characteristics	High expression (n = 36)	Low expression (n = 34)	p-value
Age (yr)			
< 50	11 (30.6)	15 (44.1)	
≥ 50	25 (69.4)	19 (55.9)	0.177
T			
T1–2	34 (94.4)	34 (100.0)	
T3–4	2 (5.3)	0 (0.0)	0.261
N			
N0	17 (47.2)	18 (52.7)	
N1–3	19 (52.8)	16 (47.1)	0.406
M			
M0	36 (100.0)	34 (100.0)	
M1	0 (0.0)	0 (0.0)	NA
Lymphatic emboli			
Positive	6 (16.7)	2 (5.9)	
Negative	30 (83.3)	32 (94.1)	0.149
Nuclear grade			
G1,2	15 (41.7)	15 (44.1)	
G3	21 (58.3)	19 (55.9)	0.514
Histological grade			
G1,2	17 (47.2)	10 (29.4)	
G3	19 (52.8)	24 (70.6)	0.099
Ki-67*			
High	12 (35.3)	6 (17.6)	
Low	22 (64.7)	28 (82.4)	0.277
p53†			
High	21 (60.0)	17 (50.0)	
Low	14 (40.0)	17 (50.0)	0.323

Values are presented as number (%).
NA = not applicable.
*Ki-67 index high > 14% (reference); †p53 expression high > 10% (reference).

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