Role of RIP3 in DNA damaging agent-induced breast cancer cell death

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-ABSTRACT-

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Receptor-interacting protein kinase 3 (RIP3 or RIPK3) is a central player in “programmed” or “regulated” necrotic cell death pathway. Here we show that programmed necrosis is activated in response to chemotherapeutic agents and contributes to chemotherapy-induced breast cancer cell death. However, we also show that RIP3 expression is silenced in basal like breast cancer cell lines due to DNA methylation near its transcription start site. Due to this silencing mechanism, loss of RIP3 expression in these cells leads to resistance to DNA damaging-agents. Hypomethylating agents restore RIP3 expression and promote sensitivity not only to death receptor ligands, but also to a surprising diversity of standard chemotherapeutic agents in a RIP3-specific manner. Our data indicates that RIP3-dependent breast cancer cell death is activated in response to DNA damaging-agents. This suggests that RIP3 plays a greater role in response to DNA damaging-agents than has been previously appreciated, we propose that hypomethylating agents, in combination with standard chemotherapy, may be useful in treating breast cancers that lack RIP3 expression.

Keywords: RIP3, MLKL, Programmed necrosis, Hypomethylating agents, Chemotherapy

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I. INTRODUCTION

Resistance to cell death is one hallmark of cancer cells, and tumor cells evolve a variety of strategies to limit or elude apoptosis. (Hanahan D et al., 2011). Evasion of apoptosis may contribute to treatment resistance and also tumor progression, since most current anticancer therapies including chemotherapy and immunotherapy primarily act by activating cell death pathways including apoptosis in cancer cells. Hence, a better understanding of the molecular mechanisms underlying tumor resistance to apoptotic cell death is expected to provide the basis for a rational approach to develop molecular targeted therapies. However, in addition to apoptosis, other forms of cell death exist programmed necrosis.

Programmed necrosis is activated in response to death receptor ligands and other type of cellular stressors (Declercq et al., 2009; Vandenabeele et al., 2010; Vanlangenakker et al.., 2012b). Programmed or “regulated” necrotic cell death is alternate programmed cell death pathway that is different from apoptosis in that caspases are not essential for cell death to occur, but necrotic events lead to membrane rupture and cell lysis (Galluzzi et al.., 2012), thus spilling the intracellular contents and ensuing the inflammation and immune system (N Vanlangenakker at al. 2012b; Michael J. Morgan, at al 2013).

Programmed cell death can be induced by a several stimuli, including the proinflammatory cytokine TNF-a. Studies of this mode of cell death often utilize the murine fibrosarcoma cell line L929, or the human colon

adenocarcinoma cell line HT-29 as models. In the case of L929 cells, TNF-a prominently causes necrosis instead of apoptosis, while in the case of the HT-29 cell line, the addition of either SMAC mimetic or cycloheximide, in combination with the pancaspase inhibitor zVAD, are necessary to induce this type of death.

The kinase activity of Receptor-interacting protein kinase-3 (RIP3, or RIPK3) is an essential for the programmed necrotic cell death execution (Duan-Wu Zhang et al. 2009; Sudan He et al. 2009; YoungSik Cho etal. 2009). Activation of the canonical programmed necrosis includes the formation of a complex containing RIP3 and RIP1 (RIPK1) and recruitment of mixed lineage kinase domain-like protein (MLKL) (Zhao, J et al 2012; Sun, L et al 2012), which is essential for death to proceed (Wu, J et al 2013; Murphy, J et al 2013).

RIP3-dependent phosphorylation and plasma membrane localization of MLKL is necessary for programmed necrotic cell death to occur (Wang, H et al 2014; Zhenyu Cai et al 2014; Chen, X et al 2014). The translocation of MLKL to the membrane has been alternately reported to leads to plasma membrane disruption by through its activation of ion channels or by directly permeabilizing the plasma membrane (Wang, H et al 2014; Dondelinger, Y et al 2014).

In addition, programmed necrosis is now acknowledged to play roles in pathological processes, including a facilitative role in tissue damage, such as ischemia-reperfusion injury, and in host defense of viral infections. According to recent reports that programmed necrosis could contribute to inflammatory skin diseases, chronic inflammation and high expression of RIP3 functions in

inflammatory disease pathogenesis.

Here we show that effective cytotoxic chemotherapy is facilitated by RIP3-dependent programmed necrotic cell death; however, RIP3 expression is often silenced in cancer due to genomic methylation near the RIPK3 transcription start site. We show a majority of breast cancer cell lines and specially, basal like subtype lack RIP3 expression due to this silencing mechanism. Basal like subtype is only found in about 15~20 percent of breast cancer diagnoses. It has been shown to be aggressive, unresponsive to treatment and, ultimately, indicative of a poor prognosis.

Loss of RIP3 expression in basal like breast cancer cells leads to greater resistance not only to death receptor ligands, but also to a surprising diversity of chemotherapeutic agents. Treatment of cells with hypomethylating agents restore RIP3 expression and thereby promotes sensitivity to chemotherapeutics in a RIP3-specific manner, suggesting that deficiency of RIP3 in tumor cells is selected for during tumor development and/or growth. Since hypomethylating agents are considerably well-tolerated in patients, an implication of our study is that RIP3-deficient breast cancer subtype patients may benefit from receiving hypomethylating agents to induce RIP3 expression prior to treatment with conventional chemotherapeutic agents.

II. MATERIALS AND METHODS

A. Experimental reagents

TNF-α and zVAD were from R&D Systems (Minneapolis, MN). Anti-phopho-JNK(44682G), Anti-RIP3(72106) and phospho-MLKL(187091) antibodies were from Abcam (Cambridge, MA). Doxorubicin, etoposide, 5-fluorouracil, cisplatin, 5-AD and necrostatin-1 were purchased from Sigma-Aldrich (St. Louis, MO). cycloheximide were from Calbiochem (Danvers, MA). Dabrafenib was from Selleckchem (Houston, TX). SMAC mimetic (LCL-161) was from Adooq Bioscience (Irvine, CA).

B. Cell Culture

Cancer cell lines were cultured in recommended media from ATCC. All cells were cultured at 37 ℃ in 5% CO2. HT-29 and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. MDAMB231, HCC1937, SKBR3 and T47D cells were cultured in RPMI supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

C. Lentiviral shRNA experiments

Short-hairpin RNA (shRNA) plasmids targeting hRIP3 mRNA (NM_006871), and non-targeting control (NM_027088) were from Sigma-Aldrich. Lentiviral plasmids were transfected into 293T cells (System Biosciences, LV900A-1) using Lipofectamine 2000(Invitrogen). Pseudoviral

particles were collected 2 days after the lentiviral plasmid transfection and infected into cells with polybrene (10 μg/mL). Infected cells were puromycin selected 2 days after infection.

D. Reverse Transcription-PCR

Reverse Transcription-PCR RNA was extracted using RNeasy (Qiagen). 200 ng of total RNA from each sample was used for cDNA synthesis with reverse transcriptase (Invitrogen). Equal amounts of cDNA product were used for PCR with Taq DNA polymerase (Takara). Amplification was performed using the following primers: RIP3 sense (5′-CAAGGAGGGACAGAAATGGA-3′), RIP3 antisense (5′-GCCTTCTTGCGAACCTACTG-3′), actin sense (5′-CAGGTCATCACCATTGGCAATGAGC-3′), actin antisense (5′-GATGTCCACGTCACACTTCATGA-3′) Final PCR products were resolved in 1% agarose gel and stained with ethidium bromide.

E. Cell viability assay

Representative images were taken by a phase-contrast microscope. Cell viability was determined using tetrazolium dye colorimetric tests (the MTT assay) read at 570 nm. Lactate dehydrogenase leakage was quantified using a cytotoxicity detection kit (Promega, Madison, WI).

F. Western blot analysis

Upon treatment, Cells were lysed in M2 buffer (20mM Tris at pH 7, 0.5% NP-40, 250mM NaCl, 3mM EDTA, 3mM EGTA, 2mM DTT, 0.5mM PMSF, 20mM β-glycerol phosphate, 1mM sodium vanadate, and 1 μg/ml leupeptin).

Equal cell extracts were resolved by 10% SDS-PAGE and analyzed by western blot using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ).

G. Immunoprecipitation

Cells lysis was in M2 buffer. For immunoprecipitation, lysates were mixed and precipitated with antibody and protein G-agarose beads overnight at 4℃. Bound proteins were removed by boiling in SDS and resolved by SDS-PAGE for immunoblotting.

H. Pyrosequencing of Bisulfate converted genomic DNA.

Pyrosequencing of RIPK3 Genomic DNA from cells was performed by Genomictree, Inc. Daejeon, South Korea using standard protocols. Briefly, Bisulfite-treated DNA samples were prepared using EZDNA Methylation-Lightning kit (Zymo Research) and then amplified by PCR. The reaction conditions were as follows : 95℃ for 10min → [95℃ for 30sec → 56℃ for 30sec → 72℃ for 30sec] 45cycles → 72℃ for 5min. From this reaction, 2 μL of the PCR product was visualized by ethidium bromide staining on a 2% agarose gel. The remainder of the product underwent pyrosequencing performed on a PyroMark ID system (Qiagen) using a PyroGold reagent kit for quantification of the methylation level.

I. Colony formation assay

T47D, SKBR3 cells were seeded in 6-well plates with a density of 100-500 cells per well for 2 - 3 weeks. The medium was discarded and each

well was washed twice with PBS carefully. The colonies were fixed in methanol for 10 min and then stained with Crystal Violet solution. Experiments were repeated three times.

J. Invasion assay

Matrigel transwell invasion assay(BD Falcon #0006). Transparent PET membrane were coated with matrigel, and MDAMB231 cells were plated ($2.5x10^5$ cell/well) onto upper chamber in serum-free media and than put serum-add media into low chamber. Filters were excised, and migrated cells were stained with crystal violet.

III. RESULTS

A. RIP3 expression is often silenced in breast cancer cells due to genomic methylation near its transcriptional start site

RIP3 is essential for programmed necrosis (Duan-Wu Zhang et al. 2009; Sudan He et al. 2009; YoungSik Cho etal. 2009). In the present study, we examined RIP3 expression in subtypes of breast cancer by immunoblotting and RIP3 protein is clearly absent in basal like breast cancer, but other subtype or normal cells express RIP3 (Figure 1). To further test this hypothesis, RIP3 expression is adjusted in breast cancer cells due to genomic methylation so, we examined whether DNA methylation was responsible for RIP3 deficiency. Treatment with the hypomethylating agent (5-aza-2′-deoxycytidine, abbreviated: 5-AD) restored RIP3 expression. In several breast cancer cells, 5-AD induced RIP3 expression in a time or dose-dependent manner (Figure 2A-B).

We further examined whether DNA methylation was responsible for RIP3 deficiency. First, we checked RIP3 expression cancer cells or loss of RIP3 cancer cells by western blot (Figure 3A). To verify that the expression of RIP3 was associated with methylation of genomic DNA near the transcription start site of RIP3 we conducted pyrosequencing of individual CpGs in bisulfate converted DNA within this region in RIP3 expression cells or unexpression cells. HT-29 and T47D cells, which express RIP3 highly, had very low methylation of the entire region. On the other hand, had very low

methylation of the entire region (i.e. HCC1937, MDAMB231, HeLa) (Figure 3B).

These data collectively demonstrate that the RIP3 transcription start site is methylated in breast cancer cell lines and is negatively associated with RIP3 expression.

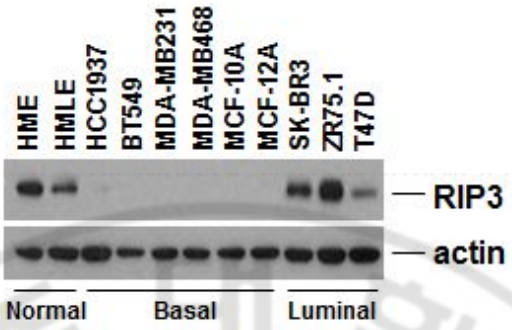
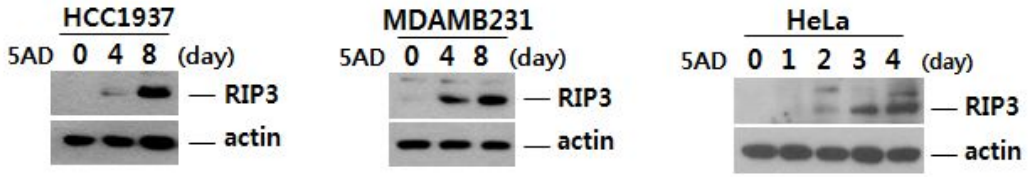


Fig. 1. RIP3 is silenced in many breast cancer cell lines.

Western blotting analysis of lysates from various breast cancer cell lines showing RIP3 expression.

A



B

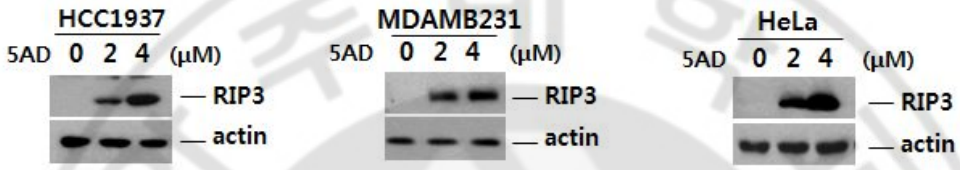
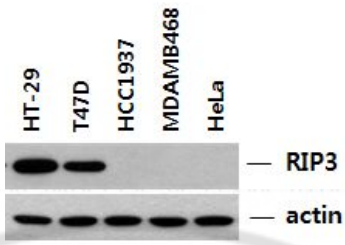


Fig. 2. Hypomethylating agent restores RIP3 expression in cancer cells.

(A) Western blotting analysis of RIP3 expression in HCC1937, MDAMB231, and HeLa cells treated with 2μM 5-AD for indicated time points.

(B) Western blotting analysis of RIP3 expression in HCC1937, MDAMB231, and HeLa cell treated with indicated concentrations (MDAMB231 and HeLa cells for 4 days, HCC1937 cell for 8 days.)

A



B

Methylation level 0 50 100

RIPK3:	CpG_1 3	CpG_2 17	CpG_3 46	CpG_4 65	CpG_5 170	CpG_6 175	CpG_7 216	CpG_8 220	CpG_9 252	CpG_10 256	CpG_11 265
T470	9.67	8.74	8.26	5.44	11.98	10.83	8.27	8.86	7.08	8.82	34.26
HT-29	2.77	2.98	3.29	2.41	3.29	2.72	5.03	2.83	2.37	2.84	3.6
HCC1937	95.99	97.38	89.92	91.2	93.78	92.31	95.63	95.91	96.91	93.56	96.66
MDAMB468	53.92	85.98	failed	failed	93.94	94.13	94.21	94.43	96.65	100	91.54
HeLa	92.86	97.94	77.68	92.31	92.73	93.09	94.66	95.19	93.68	96.38	94.07
Methylated DNA	95.38	98.32	89.69	90.46	88.82	95.08	99.33	96.91	96.78	92.12	100
Unmethylated DNA	2.36	3.13	3.27	2.92	3.17	3.64	3.59	1.87	1.55	2.86	5.63

Fig. 3. RIP3 is silenced by methylation in cancer cell lines.

(A) Western blotting analysis of RIP3 expression in HT-29, T47D, HCC1937, MDAMB231 and HeLa cells. (B) Summary of pyrosequencing analysis of bisulfite-converted genomic DNA from T47D, HT-29, HCC1937, MDAMB468, and HeLa cells.

B. Expression of RIP3 is essential for TNF-induced necrotic cell death in cancer cells.

RIP3 is essential molecular for programmed necrosis (Duan-Wu Zhang et al. 2009; Sudan He et al. 2009; YoungSik Cho etal. 2009). First, we experimented with HT-29 cell and HeLa cell which were researched about necroptosis. Consistent with the many paper, cells deficient RIP3 expression are completely resistant to prototypical programmed necrotic stimuli(TNF-α+ zVAD + either SMAC mimetic or cycloheximide; also referred to herein as TSZ or TCZ), so become sensitive when RIP3 is ectopically expressed (Figure 4A) and then cell viability was analyzed by phase-contrast microscopy (Figure 4B).

We next sought to test whether hypomethylating agents would sensitize RIP3-deficient cells to necroptic condition (TSZ, TCZ) via restoration of RIP3 expression. Treatment with the hypomethylating agent (5-AD) restored RIP3 expression in HeLa cells (Figure 5A). TNF-α induced necrosis become sensitive after 5-AD treatment (Figure 5B). RIPK1 inhibitor necrostatin-1 blocks cell death upon 5-AD treatment followed by necrotic stimulus, indicating the canonical regulated necrotic death pathway is activated (Figure 5C). Importantly, 5-AD does not affect necrotic cell death in HT-29 cells (Figure 6), suggesting the sensitizing effect of 5-AD is mediated though induction of RIP3 expression.

A B

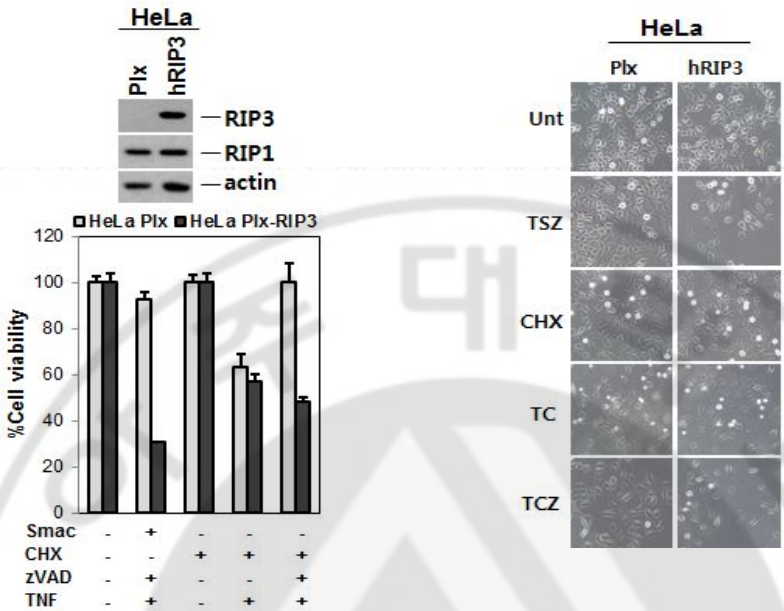
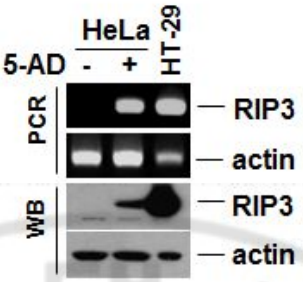


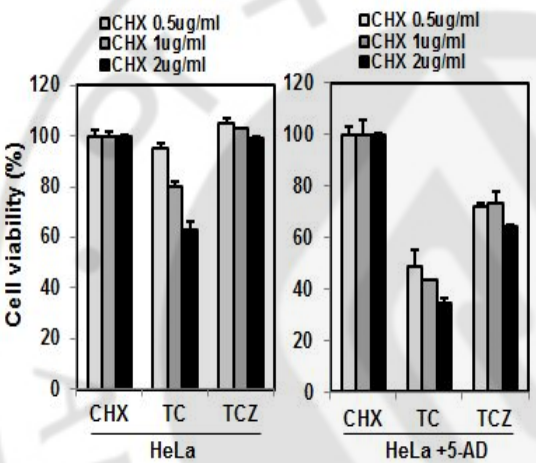
Fig. 4. Expression of RIP3 is essential for TNF-induced necrotic cell death.

(A) Viability (MTT) of HeLa cell ectopically expressing RIP3 treated 36 hrs with TCZ [TNF (30 ng/mL), cycloheximide (CHX, 2 μg/mL), and zVAD (20 μM)] or TSZ [TNF, SMAC mimetic (100 nM), and zVAD]. (B) Cell viability was analyzed by phase-contrast microscopy.

A


HeLa
5-AD - +
HT-29
PCR
RIP3
actin
WB
RIP3
actin

B


CHX 0.5ug/ml
CHX 1ug/ml
CHX 2ug/ml
Cell viability (%)
120
100
80
60
40
20
0
CHX
TC
TCZ
HeLa
HeLa +5-AD

C

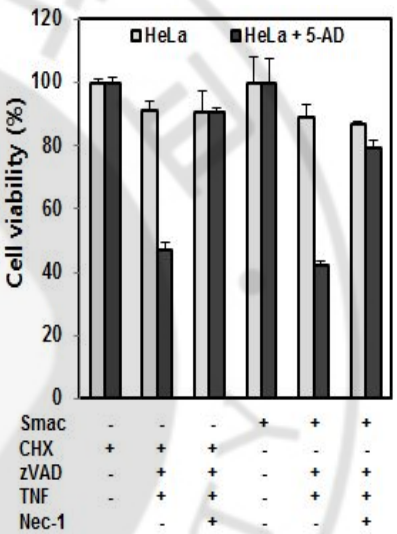

HeLa
HeLa + 5-AD
Cell viability (%)
120
100
80
60
40
20
0
Smac
CHX
zVAD
TNF
Nec-1

Fig. 5. Hypomethylating agent restores TNF-induced necrotic cell death in RIP3-deficient cells.

(A) HeLa cells were treated with 5-AD (2 μM, 4 day) and cell lysates were analyzed by immunoblotting and reverse transcription-PCR. (B) HeLa cells were pretreated with 5-AD (2 μM, 4 day) and then further treated with TCZ [TNF (30 ng/mL), cycloheximide (CHX, 0.5, 1, 2 μg/mL), and zVAD (20 μM)] for 48 hrs. (C) TNF-induced necrotic cell death in RIP3-restored HeLa cells is inhibited by necrostatin-1. Cells were pretreated with necrostatin-1 (40 μM) for 1 h before TCZ or TSZ treatment for 48 hrs and cell viability analyzed by MTT assay.

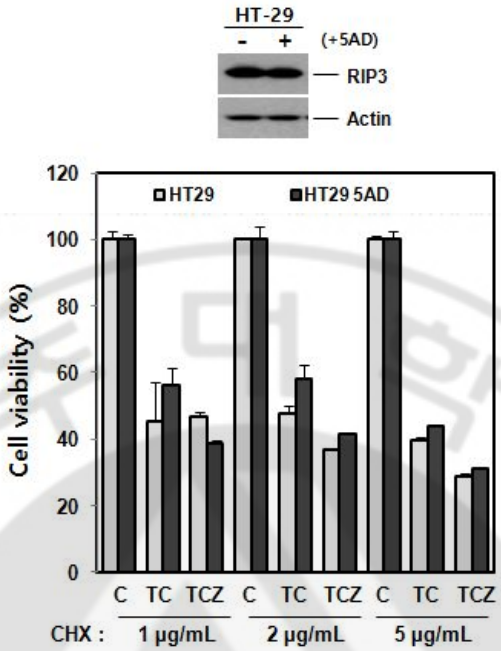


Fig. 6. Hypomethylating agent dose not affect RIP3 expression in HT-29 cells.

HT-29 cells were pretreated with 5-AD (2 μM, 4 day) and then further treated with TCZ [TNF (30 ng/mL), cycloheximide (CHX, 1, 2, 5 μg/mL), and zVAD (20 μM)] for 48 hrs and cell viability analyzed by MTT assay.

C. RIP3 expression sensitizes to DNA-damaging agents in breast cancer cells

We therefore sought to determine whether hypomethylating agents promote chemotherapeutic cell death by via enhancing RIP3 expression. First, hypomethylating agent would sensitize RIP3-deficient cells to necroptic condition (TSZ, TCZ) via restoration of RIP3 expression. Treatment with the hypomethylating agent (5-AD) restored RIP3 expression in MDAMB231 cells (Figure 7A-B)

Indeed, 5-AD treatment sensitizes MDAMB231 cells to doxorubicin (Doxo), etoposide (Etopo), 5-Fluorouracil (5-FU), Camptothecin (CPT), and Cisplatin (Figure 7C), suggesting induction of RIP3 may contribute to cell death from a wide range of chemotherapeutic drugs with different mechanisms of action. In the absence of RIP3, hypomethylating agents lose their ability to sensitize cells to chemotherapeutic agents (Figure 8), confirming that their sensitization is dependent on increased RIP3 expression. While the above data indicate that programmed necrosis contributes to chemotherapeutic cytotoxicity.

We examined whether RIP3 contributed to death from chemotherapeutic agents, a role for RIP3 in cell death induced by standard chemotherapeutic cytotoxic agents has never been reported. In MDAMB231 cells (which lack endogenous RIP3 expression), MDAMB231 cell ectopically expressing RIP3 treated necroptic condition (TSZ, TCZ) and cell viability was analyzed by immunoblotting (Frigure 9A-B). The ectopic expression of RIP3 bestowed additional sensitivity both to etoposide , doxorubicin and CPT as measured by

MTT assays (Figure 9C). suggesting that RIP3 can contribute to the cytotoxicity of multiple drugs with diverse mechanisms of action.

We sought to investigate the mechanism by which RIP3 was sensitizing to chemotherapeutics. We examined whether RIP3 was in the same complex as caspase-8 upon treatment of cells with doxorubicin. Doxorubicin led to interaction of caspase-8 with RIP1 and RIP3, along with FADD, though no interaction was detected in RIP3 knockdown HT-29 cells (Figure 10). this data suggested to us that programmed necrosis might be important for RIP3-dependent chemocytotoxicity.

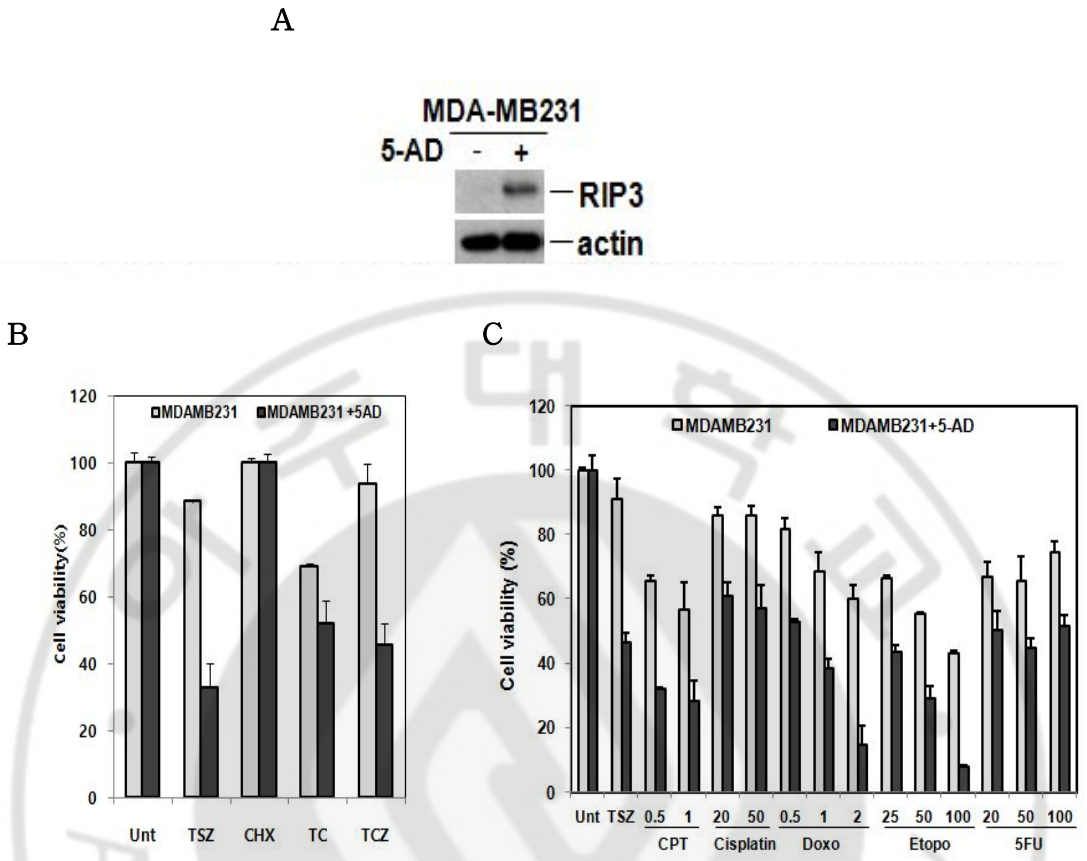


Fig. 7. Restoration of RIP3 by hypomethylating agent enhances sensitivity to chemotherapeutic agents.

(A) MDAMB231 cells were treated with the hypomethylating agent, 5-AD (2 μM, 4day) and then analyzed RIP3 expression by western blotting. (B) Cells from (A) were treated with TCZ [TNF (30 ng/mL), cycloheximide (CHX, 2 μg/mL), and zVAD (20 μM)] for 48 hrs and cell viability analyzed by MTT assay. (C) MDAMB231 cells were treated with 5-AD (2 μM, 4 day) and then further treated with camptothecin (CPT μg/mL), cisplatin (μM), doxorubicin (Doxo μM), etoposide (Etopo μM), or 5-fluorouracil (5-FU, μM) for 36 hours. Cell viability was analyzed by MTT assay.

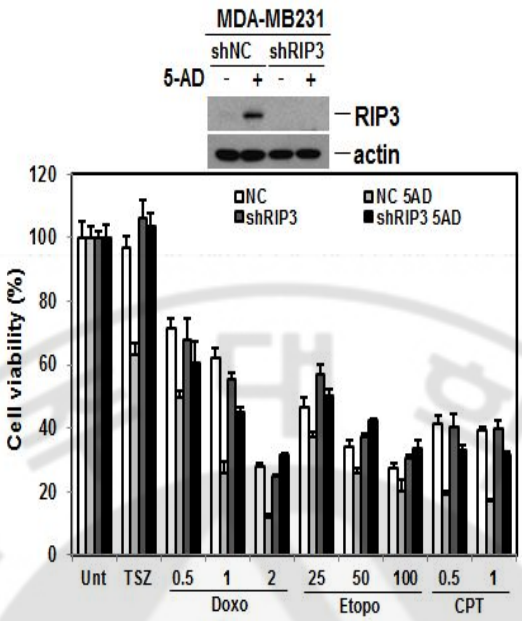


Fig.8. Hypomethylating agent-induced sensitization to chemotherapeutic induced necrotic cell death is dependent on the induction of RIP3 expression.

MDAMB231 cells were infected with lentivirus encoding RIP3 shRNA or a non-silencing control. After selection with puromycin, cells were pretreated with 5-AD (2 μM) for 4 day and then analyzed for knockdown by western blotting or treated with doxorubicin (μM), etoposide (μM), camptothecin (μg/mL) for 48 hrs and cell viability then analyzed by MTT assay.

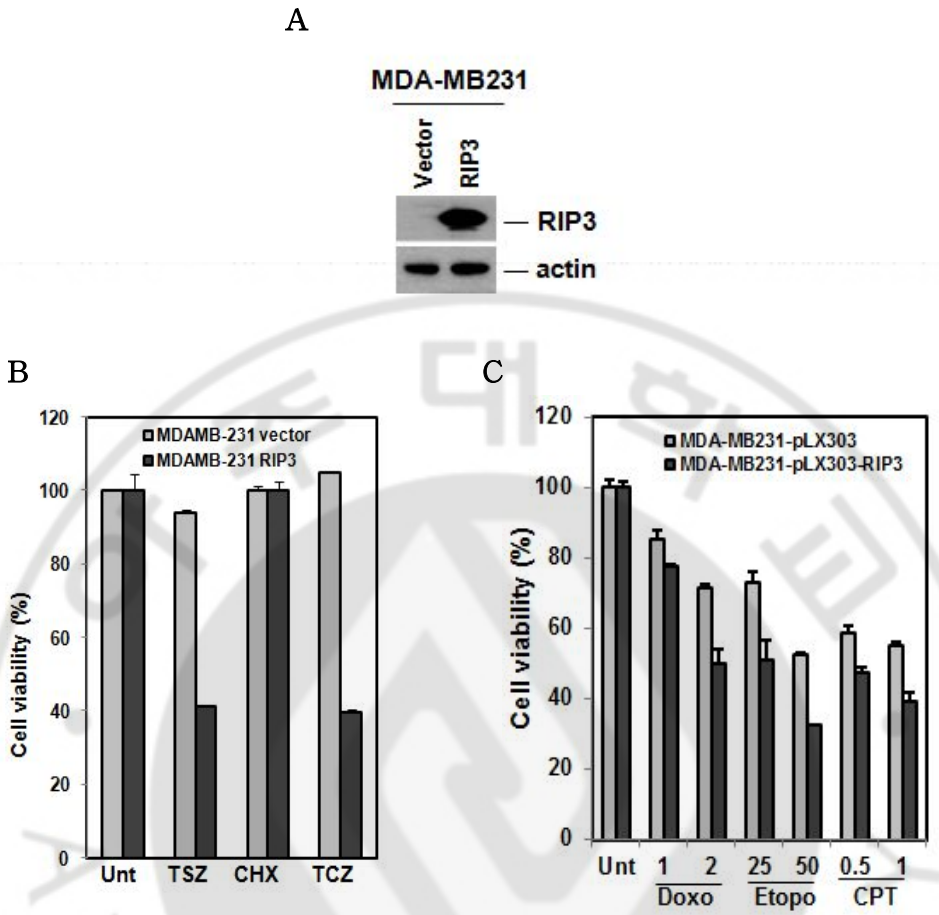


Fig. 9. Expression of RIP3 contributes to sensitivity to DNA-damaging agents.

(A) MDAMD231 cells were infected with pLX303-hRIP3 lentiviral plasmid to establish stable RIP3-expressing cells. RIP3 expression was confirmed by western blot. (B-C) MDAMB231 cells ectopically expressing RIP3 were treated with TCZ [TNF (30 ng/mL), cycloheximide (CHX, 2 μg/mL), and zVAD (20 μM)] for 48 hrs and cell viability was analyzed by MTT assay. (C) Cells were treated with indicated concentration of doxorubicin, etoposide or CPT for 2 day and cell viability was analyzed by MTT assay.

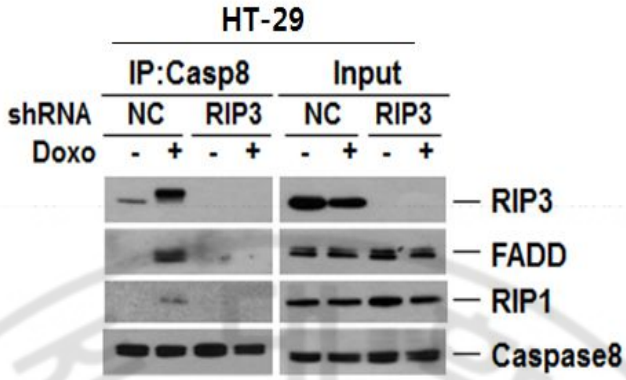


Fig. 10. Doxorubicin induces necrosome complex formation.

Western blotting of Caspase-8 immunoprecipitates from HT-29 and RIP3 knockdown HT-29 cells pretreated with zVAD (20 μM) for 1 hr, and then treated with doxorubicin(5 μM) for 18 hrs.

D. DNA-damaging agents activate RIP3-dependent MLKL phosphorylation

RIP3-dependent phosphorylation and subsequent plasma membrane localization of MLKL is necessary for programmed necrotic cell death (Cai et al., 2014; Chen et al., 2014). We examined whether RIP3 dependent of MLKL activation by doxorubicin. Doxorubicin induced time or dose dependent MLKL phosphorylation in endogenous RIP3 expression T47D cells (Fig 11A-B).

We examined whether RIP3 contributed to death from chemotherapeutic agents in ectopically expressing RIP3 MDAMB231 and HCC1937 cells. Phosphorylated MLKL was detected in MDAMB231 and HCC1937 cells treated with doxorubicin (Figure 12A-B), suggesting that RIP3 potentiates these death processes through activation of MLKL-dependent programmed necrosis. indicating that MLKL activation contributed to doxorubicin-mediated cell death.

A B

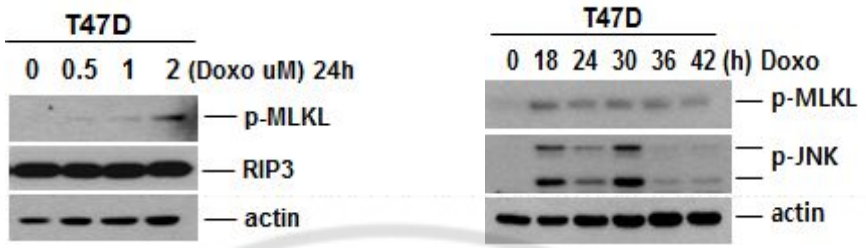
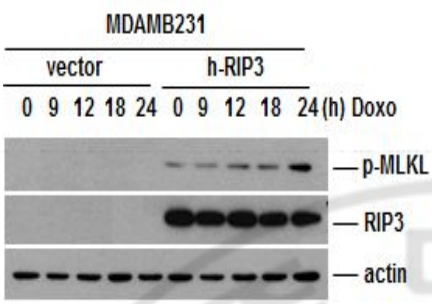


Fig. 11. DNA-damaging agent induced MLKL phosphorylation in RIP3-expressing cells.

(A) T47D cells were treated with doxorubicin (μM) for 24 hrs and cell lysates were analyzed by western blotting. (B) Western blots of lysates from T47D cells treated with Doxorubicin (2μM) at indicated time points.

A



B

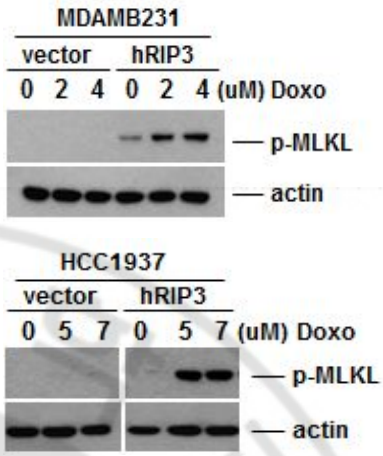


Fig. 12. MLKL phosphorylation induced by DNA-damaging agent is dependent on RIP3.

(A) Western blots of lysates from Mock vector and RIP3 overexpressing MDAMB231 cells treated with doxorubicin (2μM) at indicated time points. (B) RIP3 expressing cells were treated with different concentrations of doxorubicin for 24 hrs and cell lysates were analyzed by western blotting.

E. RIP3 acts as a tumor suppressor

While loss of genes involved in DNA repair can render cancer cells more susceptible to DNA-damaging chemotherapies, many are also tumor suppressors because their loss results in an increase in genomic instability and oncogenic growth. Therefore, we tested the hypothesis that RIP3 is acting as a tumor suppressor, and its loss will promote malignant phenotypes, including increased growth rates, invasive potential. We performed these experiments in MDAMB231, T47D and SKBR3 cells. These different cell lines were chosen because they represent a sampling of different breast cancer types. MDAMB231 cells are triple negative (ER-, PR-, HER2) and have completed epithelial to mesenchymal transition (EMT) (Dawn A et al, 1999, Honor Hugo et al, 2007), T47D and SKBR-3 cells overexpress HER2 and do not express ER or PR.

To test whether expression of RIP3 could increase breast cancer cell migration, we performed scratch-wound migration assays. MDAMB231 cell were infected with RIP3 or control lentivirus and assayed for their ability to fill a scratch wound. As in the wound healing assays, the MDAMB231 cell more slowly filled the wound when increased for RIP3-overexpressing (Figure 13A). However, the results from the scratch-wound assay could also reflect increased cell proliferation, so we additionally measured the effect of RIP3-overexpressing on cell invasion. In contrast to the results observed above, RIP3-overexpressing significantly decreased the invasion of MDAMB231 cell (Figure 13B).

To confirm that RIP3 depletion breast cancer growth, we tested colony

formation assay. T47D and SKBR3 cell lines were infected with RIP3 KD or control lentivirus. RIP3 knockdown cells more quickly filled the colony formation (Figure 13C), and in another cell lines also showed same results (Figure 13D). These data suggest that RIP3 have a tumor suppressor function and will play a key role in tumorigenesis.

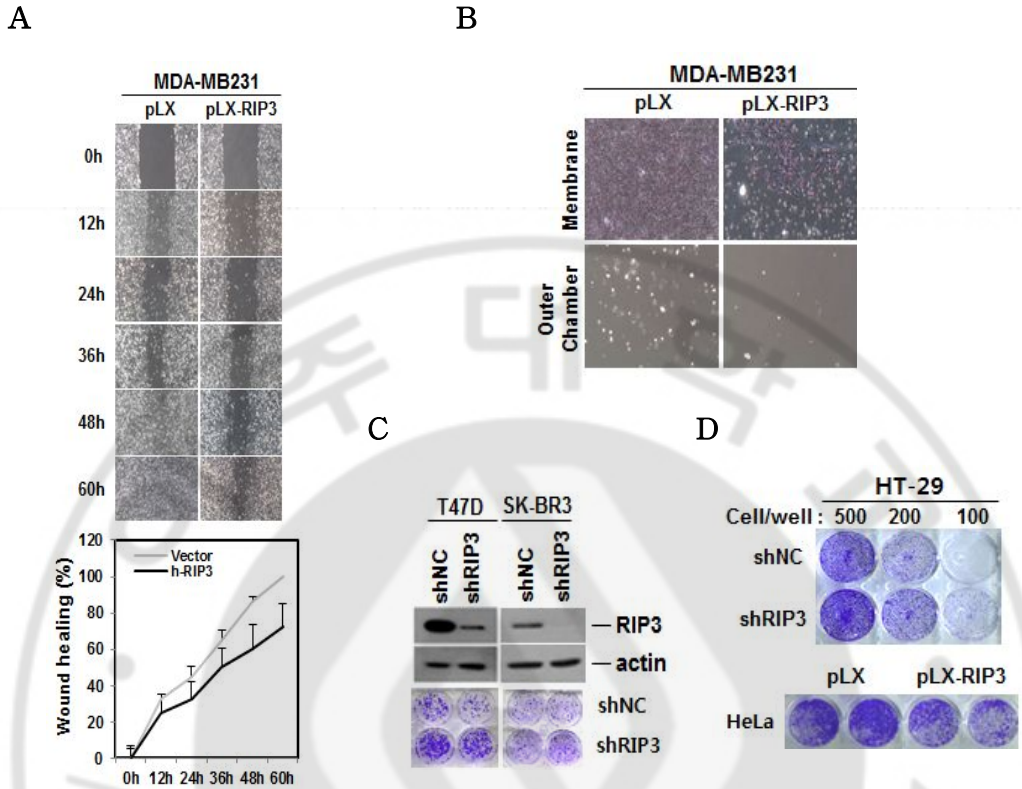

A
MDA-MB231
pLX
pLX-RIP3
0h
12h
24h
36h
48h
60h
Wound healing (%)
Vector
h-RIP3
0h 12h 24h 36h 48h 60h
B
MDA-MB231
pLX
pLX-RIP3
Membrane
Outer Chamber
C
T47D
SK-BR3
shNC
shRIP3
RIP3
actin
D
HT-29
Cell/well : 500 200 100
shNC
shRIP3
pLX
pLX-RIP3
HeLa

Fig. 13. RIP3 suppresses migration and invasion in breast cancer cells.

(A) Scratch wound healing assay in mock vector MDAMB231 and RIP3 overexpressing MDAMB231 cells. Wound healing was analyzed by phase-contrast microscopy. (B) Matrigel transwell invasion assay. Transwell filters were coated with matrigel and MDAMB231 cells were plated ($2.5x10^5$ cell/well) onto upper chamber in serum-free media and than put serum-added media into low chamber. Filters were stained with crystal violet. (C) T47D and SKBR3 cells were infected with a lentivirus encoding RIP3 shRNAs or non-silencing control vector. After selection with puromycin, cells were analyzed for knockdown by western blotting. Cell growth was analyzed by colony formation assay after 15 days. (D) RIP3 knock-downed HT-29 cell or RIP3 overexpressing HeLa cell growth was analyzed by colony formation assay after 15 days.

IV. DISCUSSION

Programmed necrosis has been identified as an important mechanism underlying multiple physiological and pathological processes (Vanlangenakker et al., 2012a; Kaczmarek et al., 2013). Here we have discussed data that indicates that the expression of RIP3 is lost in breast cancer cells, and that the mechanism for this silencing is largely due to DNA methylation of the RIPK3 genomic sequence. RIP3 expression may be restored in a majority of cells with RIP3 deficiency by treating them with hypomethylating agents, thus restoring sensitivity of these cells to programmed necrosis.

Our data indicate that RIP3 expression is likely selected against during cancer growth or progression, with methylation of the genomic region near the RIPK3 transcription start site leading to RIP3 silencing. Since RIP3 plays a greater role in response to chemotherapeutics than has been previously appreciated. The response of cells to 5-AD suggests the exciting possibility that RIP3 expression may be manipulated in cancer cells to make them sensitive to regulated necrosis, we propose that hypomethylation agents, in combination with standard chemotherapy, may be useful in treating cancers that lack RIP3 expression. RIP3 deficiency is likely to have important biological and therapeutic consequences, including possible effects on the responses of tumor cells to chemotherapy. How RIP3 is activated by from drugs with such mechanistic differences is an important matter for future investigation and we will investigate how the tumor suppressor role in RIP3 have any mechanism.

This fact also potentially opens up new therapeutic possibilities for the

treatment of cancer by potentially restoring RIP3 expression in human patients.

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- 국문요약 -

DNA damaging agents에 의한 유방암 세포 사멸에서 RIP3의 역할에 관한 연구

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수용체- 상호 작용 단백질 키나제 3 (Receptor-interacting protein kinase-3)는 '프로그램' 또는 '조절되는' 괴사 세포사멸에서 중요한 역할을 하는 단백질이다. 본 연구에서, 화학치료제가 프로그램된 괴사를 활성화시키고, 화학치료에 의한 유방암세포 사멸에 기여함을 보여주었다. 하지만, RIP3 발현이 전사 시작 지점 근처의 DNA 메틸화에 의해 basal like 유방암세포에서 억제되어 있음을 확인하였다. 이러한 억제 기전을 통한, basal like 유방암 세포에서의 RIP3 발현 손실은 DNA 손상 약물에 대한 저항성을 야기한다. 반면, 탈메틸화제는 RIP3의 발현을 회복시키고 사멸 수용체 리간드에 대한 감수성 뿐만 아니라 놀랍도록 다양한 표준 화학치료 약물에 대한 감수성 역시 RIP3 특이적으로 촉진하였다. 본 결과에서는 RIP3 의존적 유방암 세포사멸이 DNA 손상 약물에 반응하여 활성화됨을 확인하였다. 이러한 결과는 RIP3가 이전에 인식 된 것보다 DNA 손상 약물에 대한 반응에서 중요한 역할을 함을 보여주었으며, 표준 화학치료와 함께 탈메틸화제를 병합 처리하는 것이 RIP3가 발현하지 않는 유방암 치료에 유용한 치료법이 될 것이라고 제안한다.

핵심어: RIP3, MLKL, 프로그램된 괴사, 탈메틸화 작용제, 화학요법