The role of reactive oxygen species production in activation of innate adjuvant receptor signals in thyroid carcinoma

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The role of reactive oxygen species production in activation of innate adjuvant receptor signals in thyroid carcinoma

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ABSTRACT

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Background/Aims: Innate adjuvant receptors recognize various pathogen associated molecules, and they have a major role in the innate and adaptive immunity through the production of type I interferon and other proinflammatory cytokines. Recently, the anti-tumor effects inducing apoptosis and growth arrest after cytoplasmic delivery of an innate adjuvant receptor ligand, polyinosinic-polycytidylic acid [poly(I:C)] has been reported in cancers. Intracellular reactive oxygen species have also been known to have regulatory function of cell proliferation, apoptosis involved in innate immune and proinflammatory responses. We hypothesized that reactive oxygen species production had the role influencing the innate adjuvant receptor signals in thyroid cancer cells. We aimed to validate the role of innate adjuvant receptors activation and intracellular reactive oxygen species generation and furthermore their correlation in the process of papillary thyroid cancer cell death.

Methods: Immunohistochemical analysis of innate adjuvant receptors including Toll-like receptor (TLR) 3, melanoma differentiation-associated gene (MDA) 5, and retinoic acid inducible gene-1 (RIG-I) was performed in 40 paraffin-embedded post-surgical papillary thyroid cancer tissues. The existence of functioning innate adjuvant receptors and the biological effects of their activation following poly(I:C) transfection on thyroid cancer cell deaths were evaluated in TPC-1 and 8505C cell lines. Detailed downstream molecular

signaling mediated by interferon-β and reactive oxygen species production were investigated in TPC-1, papillary thyroid cancer cells.

Results: TLR3, MDA5 and interferon-β were expressed in papillary thyroid cancer tissues with variable intensity, but RIG-I expression was not definite. Poly(I:C) transfection induced innate adjuvant receptors dependent apoptosis of papillary thyroid cancer cells which was revealed to be extrinsic caspase-3 and caspase-8 mediated. Increased mRNA expression of innate adjuvant receptors and downstream interferon-β were induced by poly(I:C) transfection. Intracellular reactive oxygen species production was also increased following activation of innate adjuvant receptors. Reactive oxygen species scavenging and interferon-β signal blocking both reduced the effect of poly(I:C) transfection. However, interferon-β expression was not attenuated by reactive oxygen species scavengers.

Conclusion: Innate adjuvant receptor signaling of TLR3 and MDA5 are functioning in papillary thyroid cancer cells and induce the cancer cell deaths. Both interferon-β signal and reactive oxygen species production contribute the innate adjuvant receptors mediated cell death, but intracellular ROS is not mandatory for IFN-β activation in papillary thyroid cancers.

Key words : innate adjuvant receptors, pattern recognition receptors, thyroid cancers, interferon, reactive oxygen species

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I. INTRODUCTION

Innate adjuvant receptors are variable receptor families capable of recognizing bacterial or viral pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides and viral double stranded ribonucleic acid (dsRNA), and also known as pattern-recognition receptors. Their expression mainly in the innate immune cells, such as dendritic cells has been first proposed in 1989 and thereafter, the innate immune signaling pathways of various innate adjuvant receptors have been discovered.[1,2]

Toll-like receptor-3 (TLR3) and the retinoid acid-inducible gene-1 (RIG-I) like receptors (RLRs) including RIG-I and melanoma differentiation-associated gene 5 (MDA5) are well known PRRs critically recognizing viral nucleic acids respectively in endosomal membrane and cytoplasm of infected cells.[2] Innate adjuvant receptors mediated anti-viral effects are mainly dependent on pro-inflammatory cytokine production. Among them, type I interferon (IFN) is well known anti-viral cytokine which have pleiotropic biologic effects on various target cells.[3]

These innate immune responses are usually tightly controlled because the over-activation of antiviral immune sensing signals can be deleterious to infected host cells and resulting in cell deaths.[4] In a sense, the apoptosis of host cell itself is

another way of inhibiting microbe spreading. With the similar manner, the activation of innate adjuvant receptors in cancer cells would be inducing anti-cancer effects. Recently, dsRNA or its synthetic analog, polyinosinic-polycytidylic acid [poly(I:C)] which is an innate adjuvant receptors ligand have been recently reported to trigger caspase-dependent apoptosis of melanoma, breast, prostate and hepatoma cell lines.[5-8] Type I IFNs are known to facilitate apoptosis in various cell types through the up-regulation of expression of proteins directly involved in cell death, including caspase, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and p53.[9,10] TLR3 dependent apoptotic effects of poly(I:C) involving IFN-β signaling, caspase activation have been well validated especially in breast cancer cells.[5,11,12]

Reactive oxygen species (ROS) are highly diffusible and reactive molecules containing oxygen, such as hydrogen peroxide, superoxide anion, and hydroxyl radicals.[13] In addition to the redox regulators of cellular signaling in biological system, intracellular ROS can facilitate the cellular damage and innate immune activation.[14] An excessive ROS generation also can induce apoptosis and growth arrest in many cancer models.[15-17]

It has been reported that thyrocytes express a functional TLR3 which is over-expressed especially in a papillary thyroid carcinoma cell line, and the highly expressed TLR3 signal has been suggested as the possible reason for the peritumoral immune cell infiltration of papillary thyroid carcinoma.[18,19] However, anti-tumor effects of innate adjuvant receptors signals and possible role of intracellular ROS generation through which thyroid cancer cell deaths might occur have not been fully elucidated. In this study, we hypothesized that intracellular ROS production had the essential role influencing the effects of innate adjuvant receptor signals to the thyroid cancer deaths. We aimed to validate the role of innate adjuvant receptors activation and intracellular ROS generation and furthermore their correlation in papillary thyroid cancer cell death.

II. MATERIALS AND METHODS

1. Cell cultures and reagents

The human thyroid cancer cell lines with various genetic abnormality (papillary thyroid carcinoma; TPC-1, anaplastic thyroid carcinoma; 8505C) were kindly provided by Dr. Woong Youn Chung (Yonsei University College of Medicine, Seoul, Republic of Korea). TPC-1 cell line was maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and gentamicin (20 μg/ml). 8505C cell line was maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 mM L-glutamine, 100 mM nonessential amino acids. Cell cultures were grown in a humidified atmosphere with 5% CO2 incubator at 37℃. To investigate the effect of cytoplasmic delivery of an innate adjuvant receptor ligand on two thyroid cancer cell lines, poly(I:C) (InvivoGen, San Diego, CA, USA) was added or transfected using X-treamGENE transfection reagent (4.2 μl/ml; Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Antibodies to cleaved caspase-3 and cleaved caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to caspase-8 and IFN-β were obtained from abcam (Cambridge, MA, USA). Antibodies to Bcl-2, Bcl-xL and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Neutralizing IFN-β antibody was obtained from R&D Systems (Minneapolis, MN, USA), and N-acetyl-L-cysteine (NAC) and mitoTEMPO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Immunohistochemistry analysis

Total 40 paraffin-embedded post-surgical thyroid cancer tissue samples including tumor and peritumoral normal thyroid tissues were collected from 1,879 papillary thyroid cancer patients who underwent thyroidectomy between January 2010 and May 2013 in Yonsei University Severance Hospital, Seoul, Republic of Korea and

whose serum anti-thyroid antibodies and BRAF mutation status had been preoperatively assessed. According to tumor size (smaller than 1 cm or not), the existence of histopathological lymphocytic thyroiditis and BRAF status (BRAFV600E or wild-type), five tissue samples in eight each assumed group were randomly selected. Immunohistochemical studies for tissue expression of innate adjuvant receptors were performed on formaldehyde-fixed, paraffin-embedded tissues. Briefly, 5-micron sections were deparaffinized. Endogenous peroxidase activity is quenched by incubation in 0.03% hydrogen peroxide, in 0.1 mol/l Tris-HCl buffer 1X (pH 7.6) for 5 min. Microwave/pressure cooker pretreatment is performed in 1 mmol/l EDTA buffer (pH 8.0). Sections are subsequently incubated at 4℃ overnight with the polyclonal rabbit antibody against TLR3 (abcam Inc., Cambridge, MA, USA), MDA5 (abcam Inc., Cambridge, MA, USA), and RIG-I (abcam Inc., Cambridge, MA, USA). Sections were then washed in Tris-HCl 1X buffer for 5 min and incubated with a peroxidase-conjugated goat anti-rabbit antibody for 30 min (ENVISION; DAKO Corp., Carpinteria, CA, USA). After further washes, peroxidase staining is revealed in diaminobenzidine tetrahydrochloride (Polysciences Inc., Warrington, PA, USA) with 0.1% (w/v) of hydrogen peroxide, in Tris buffer, 0.01 mol/l (pH 7.2). Sections were counterstained and mounted. The intensity of cytoplasmic innate adjuvant receptors was semi-quantitatively assessed as follows: score 1 (0-10% of cells stained with absent/faint intensity), score 2 (11-50% of cells stained with weak or moderate intensity), score 3 (>50% of cells stained with high intensity). The tissue expression of each innate adjuvant receptor was classified into two groups: weak staining group (score 1) and strong staining group (score 2 or 3).

3. Immunofluorescence analysis

Total 10 selected cases with various immunohistochemical staining intensity of innate adjuvant receptors among the above mentioned thyroid cancer tissue samples were incubated with an anti IFN-β antibody (abcam, Cambridge, MA, USA) and followed by incubation with Cy3-tagged secondary antibody. Nuclei

were counterstained with DAPI, and the protein expression and localization of IFN-β were examined by Olympus BX53 microscope.

4. Cell viability assays

Cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each thyroid cancer cells (TPC-1 and 8505C) were seeded in 96-well culture plates (1x103 cells/well) and a different concentration of poly(I:C) was added or transfected for 24 and 48 h. After treatment of poly(I:C), cells were incubated with 5 mg/ml MTT solution for 4 h at 37℃ and solubilized in ice-cold isopropanol and analyzed spectrophotometrically. Absorbance of the dye was measured at 560 nm with background subtraction at 630 nm with a microplate reader (EL 340 Biokinetics Reader; Bio-Tek Instruments, Winooski, VT, USA).

5. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated using the RNeasy mini kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instruction. DNA was removed from total RNA using the DNA-free kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. A total of 1 μg RNA was used to synthesize cDNA using the Advantage RT-for-PCR kit (BD biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. The resulting cDNA was amplified on the ABI 7300 real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA) using CYBER Green universal PCR master mix and recommended PCR conditions to assess gene transcript levels quantitatively in the cell samples. Primers for TLR3 were 5'-AGA GTT GTC ATC GAA TCA AAT TAA AG-3' (forward) and 5'-AAT CTT CCA ATT GCG TGA AAA-3' (reverse); for MDA5, 5'-GGG AGA ACC CTC TCC CTT CT-3' (forward) and 5'-GCT GAA GGA GGT TCA GCA GTT-3' (reverse); for RIG-I, 5'-CGT GAT CCA TCT GGC TCC TC-3' (forward) and 5'-CCC CAC AGC TGC TTC TAA CA-3' (reverse); for

IFN-β, 5'-ACC AAA CCT GTT CGA GGC AC-3' (forward) and 5'-CAT GGC CC AAC AAC TGA CG-3' (reverse). GAPDH primers were 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and 5'-GTC CAC CAC CCT GTT GCT GTA-3' (reverse). All samples were executed by duplicate real-time RT-PCR analysis for each sample, and the obtained threshold cycle values (Ct) were averaged. The gene expression level, normalized to the housekeeping gene GAPDH and relative to the control sample, was calculated.

6. Flow cytometry analysis and apoptosis measurements

Apoptosis is measured using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) and propidium iodide (PI). Analysis is performed using a FACSCalibur flow cytometer (Becton–Dickinson, Fullerton, CA, USA), according to the manufacturer's procedure.

7. Western blot assay

Differentiated cells were washed with ice-cold PBS and lysed on ice for 30 min in cell lysis buffer consisting of 20 mM HEPES (pH 7.2), 10% (v/v) glycerol, 10 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% (v/v) Triton X-100. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lysates were centrifuged for 10 min at 12,000x g, and cell homogenate fractions were stored at -70℃ before use. Protein concentrations in supernatant fractions were determined by the Bradford assay (BioRad). Equal amounts of protein (50 μg) were boiled in sample buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% (w/v) gels. The separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Billerica, MA, USA), probed overnight with primary antibodies in Tris Buffer Saline Tween 20, and washed three times with Tris Buffer Saline Tween 20. Immunoreactive bands were detected with horseradish peroxidase conjugated secondary antibody, and the

bound peroxidase was visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure to X-ray film (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The relative amount of each immunoreactive band was quantified by densitometry and normalized to the β-actin level in the same sample.

8. Measurement of intracellular ROS production

Intracellular ROS levels were measured by flow cytometry using an oxidant-sensitive fluorescent probe, 5-(and 6)-carboxy-2',7'-dichlorodihydro fluorescin diacetate (H_2DCFDA; Molecular Probes, Inc., Eugene, OR, USA) and MitoSOX Red mitochondrial superoxide indicator (Molecular Probes, Inc., Eugene, OR, USA) which was novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. TPC-1 cells were transfected with 100 ng/ml of poly(I:C) for 24 h. The culture medium was then removed and the cells were washed with PBS, cells were incubated with 5 mM H_2DCFDA for 10 minutes and with 5 mM MitoSOX Red for 2 minutes. The cells were then trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured with an IX71-F22PH inverted fluorescence microscope (Olympus, Japan) and a flow cytometric analysis was performed (ELITE flow cytometer, Coulter Cytometry, Inc., Hialeah, FL, USA). For each sample, ≥10,000 events were acquired. Cells were gated out and the analysis was performed using only live populations.

9. Transfection of small interfering RNA (siRNA)

Transfection of siRNA is performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. siRNA for TLR3, MDA5, RIG-I, and IFN-β (Dharmacon, Chicago, IL, USA) were transfected to TPC-1 cell lines. After 48 h of siRNA transfection, thyroid cancer cells are used for the subsequent experiments.

10. Statistical analysis

All statistical analyses are performed with IBM SPSS software package for Windows (Version 20.0; IBM Corp., New York, United States). All experiments were performed at least three times, and samples were assayed in duplicate each time. Value with normal distribution is expressed as means ± standard deviation (SD). Statistical comparison between two groups is performed using Student's T test for independent samples. Data with a P value < 0.05 are considered statistically significant.

III. RESULTS

1. TLR3 and MDA5 and downstream IFN-β were expressed in papillary thyroid cancer tissues with variable intensity.

The intensity and extent of immunohistochemical staining with three kinds of anti-innate adjuvant receptors (TLR3, MDA5 and RIG-I) antibodies for 40 paraffin embedded human tissue samples including papillary thyroid cancers and peritumoral normal thyroid parenchyma were assessed and categorized into weak or strong staining groups. Total 40 tissue samples included were obtained from the equal number of patients with or without the coexisting tissue lymphocytic infiltration, BRAFV600E somatic mutation. The number of patients with papillary thyroid micro carcinoma (tumor size < 10 mm) and the number of larger ones were also equal. Majority of histologic subtype were classical papillary thyroid carcinoma except six follicular variant and one solid variant of papillary thyroid carcinoma. The baseline clinicopathological characteristics are summarized in Table 1.

Table 1. Clinicopathologic characteristics of papillary thyroid cancer subjects and innate adjuvant receptor staining

Factors	The value of each parameter
Total patients	40
Age (median (range))	45.0 (37.0-56.3)
Sex	
Male (%)	8 (20.0)
Female (%)	32 (80.0)
Histologic subtype	
Classical	33
Follicular variant	6
Solid variant	1
Innate adjuvant receptor staining	
TLR3 (strong (%))	22 (55.0)
MDA5 (strong (%))	9 (22.5)
RIG-I (strong (%))	3 (7.5)

The immunohistochemical staining of all three innate adjuvant receptors were not found in peritumoral normal thyroid parenchyma (Figure 1). On the other hand, innate adjuvant receptors showed variable immunohistochemical staining pattern in tumor tissues as presented in Figure 2. TLR3 was stained in thyroid cancer tissues with strong intensity in 55.0% of subjects and MDA5 showed strong immunohistochemical staining in 22.5% of subjects. RIG-I was rarely stained in only 7.5% of subjects (Table 1).

Figure 1. Immunohistochemical analysis of normal thyroid tissues. Peritumoral normal thyroid parenchyma shows no immunohistochemical staining with (A) TLR3, (B) MDA5, and (C) RIG-I. (X40)

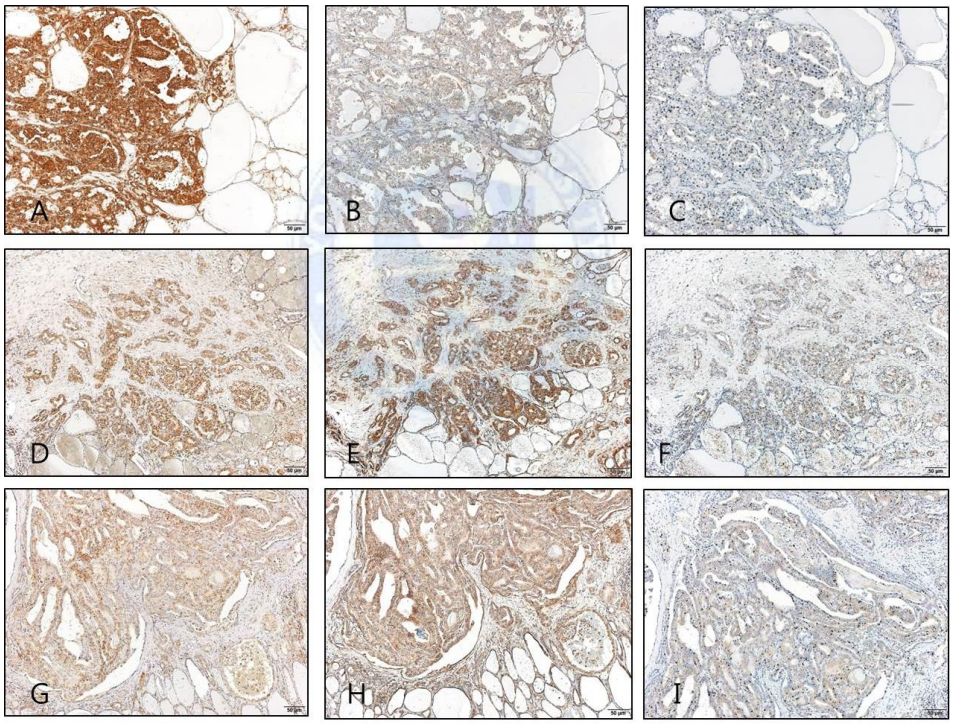

Figure 2. Variable intensity of innate adjuvant receptors expression in thyroid cancer tissues. The examples of three different papillary thyroid cancer tissues showing variable immunohistochemical staining intensity of TLR3 (A, D, G), MDA5 (B, E, H), and RIG-I (C, F, I). (X40)

All of cases with strong MDA5 staining except one also showed strong immunohistochemical staining with TLR3. The subjects with strong intensity of both TLR3 and MDA5 showed tendency of the smaller tumor size than the ones with a weak intensity as presented in Figure 3.

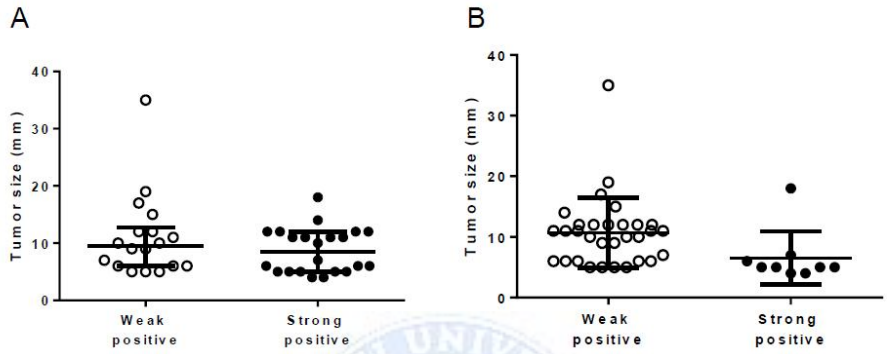

Figure 3. The distribution of tumor size of the papillary thyroid cancer subjects according to the immunohistochemical staining intensity of TLR3 (A) and MDA5 (B).

The immunofluorescence analysis for IFN-β, known as a common downstream signal of innate adjuvant receptors was performed to five selected papillary thyroid cancer tissues among the each group with strong and weak staining intensity of TLR3 and MDA5. The definite expression of cytoplasmic IFN-β was only found in tumor tissues showing the high immunohistochemical staining intensity of TLR3 and MDA5 as presented in Figure 4.

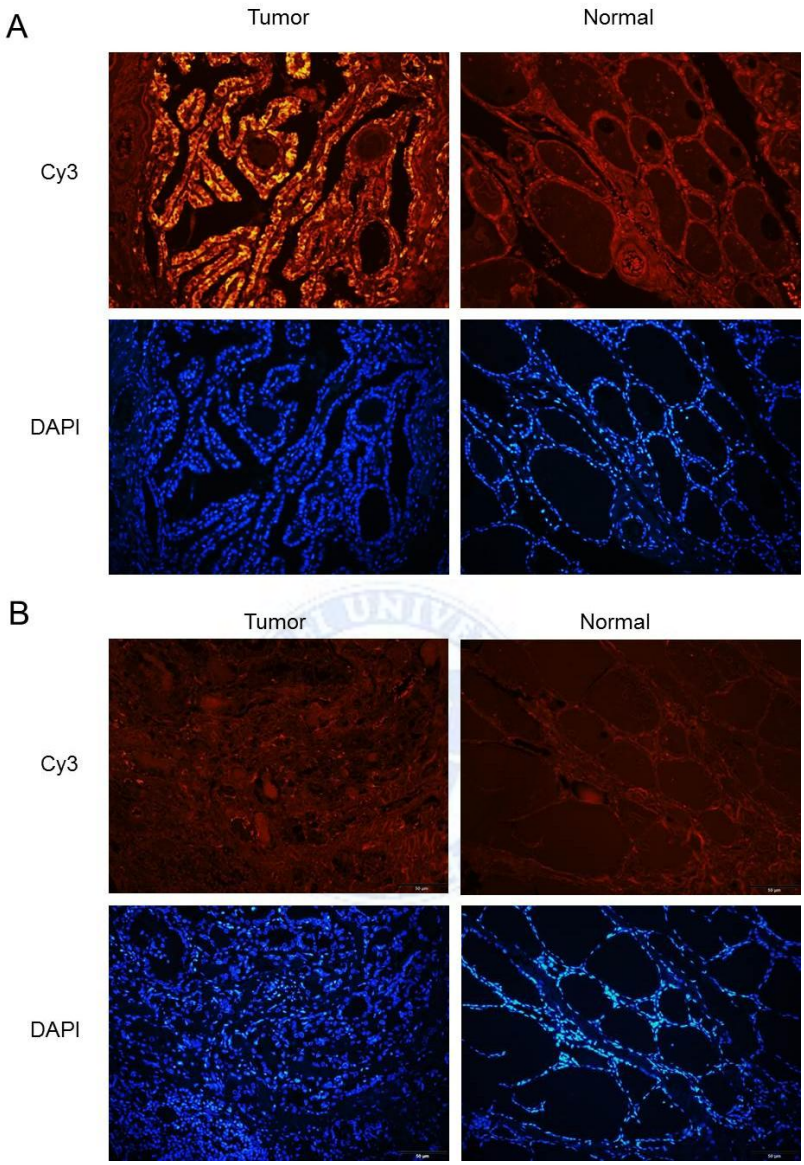

Figure 4. Immunofluorescence detection of IFN-β in thyroid cancers. IFN-β expression is found only in papillary thyroid cancer tissues showing the high intensity of TLR3 and MDA5 (A) but not in the tumors with low intensity of TLR3 and MDA5 (B). (X200)

2. Poly(I:C) transfection reduces the viability of papillary thyroid cancer cell line.

The expression of mRNA expression of three innate adjuvant receptors were examined with RT-PCR in papillary thyroid cancer cell line (TPC-1) and anaplastic thyroid cancer cell line (8505C). In line with the immunohistochemistry data, only TLR3 and MDA5 showed definite mRNA expression in TPC-1 cell line (Figure 5A). Thereafter, the synthetic dsRNA analog poly(I:C) was added on or transfected into TPC-1 and 8505C cell lines to assess the effects of innate adjuvant receptor activation on thyroid cancer cell viability. TPC-1 cell viability was reduced dose-dependently to 52.8% after 24 h of poly(I:C) 100 ng/ml transfection (Figure 5B). This anti-tumor effects of poly(I:C) transfection was not definite when TPC-1 cell line was cultured with poly(I:C) for 24 h. Even after high dose treatment of poly(I:C) of 50 μg/ml for 24-48 h, TPC-1 cell viability was stably maintained (Figure 5C). Neither mRNA expression of innate adjuvant receptors nor the anti-tumor effects of poly(I:C) transfection for 24-48 h was definite in 8505C cell line (Figure 5D).

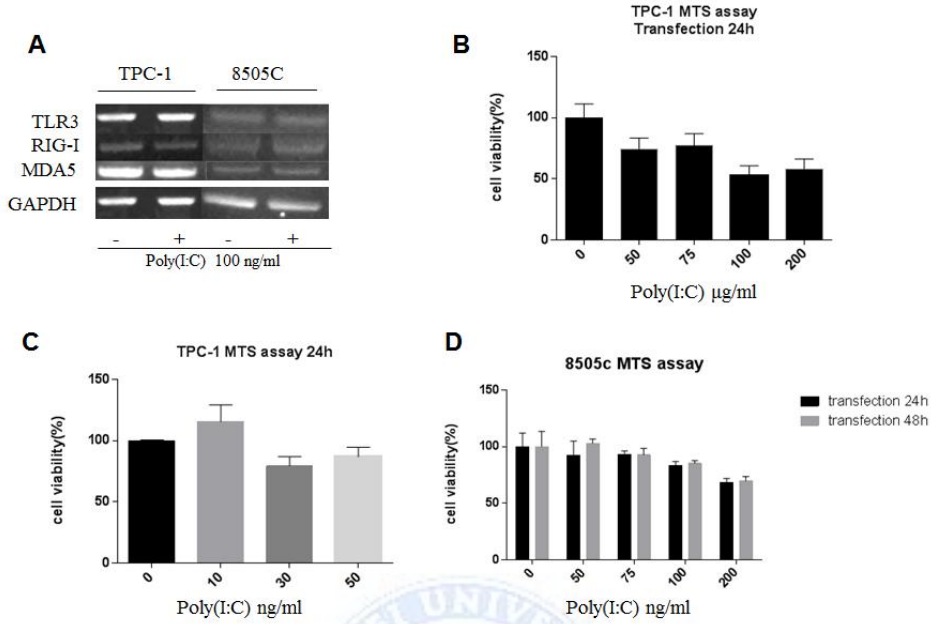

Figure 5. Cell line expression of innate adjuvant receptors and biological effect of poly(I:C) transfection. A. mRNA expression of TLR3 and MDA5 are definite in TPC-1 cell line, but not in 8505C. B. Transfected synthetic dsRNA decreases cell viability of TPC-1 dose-dependently after 24 h. C. Simple co-culture with poly(I:C) does not influence TPC-1 cell viability. D. The effect of poly(I:C) transfection is lower in 8505C cells compared to TPC-1 cell line.

3. The cytosolic transfection of poly(I:C) triggers caspase dependent apoptosis in papillary thyroid cancer cell line.

Pro-apoptotic effects of innate adjuvant receptor activation after poly(I:C) transfection was validated using flow cytometry analysis. The percentage of FITC-conjugated Annexin V positive TPC-1 cells increased after 24 h of poly(I:C) 100 ng/ml transfection, which indicated an early or late apoptosis (Fig. 6A). Poly(I:C) transfection also induced the cleavages of caspase-3 and caspase-8, but

not of caspase-9 in TPC-1 cell line. Furthermore, the expression of anti-apoptotic regulator proteins such as Bcl-2 and Bcl-xL were found to be decreased after poly(I:C) transfection into TPC-1 cells (Figure 6B). These results suggest that the activation of innate adjuvant receptors with poly(I:C) transfection induces papillary thyroid cancer cell deaths involving the extrinsic apoptosis pathway.

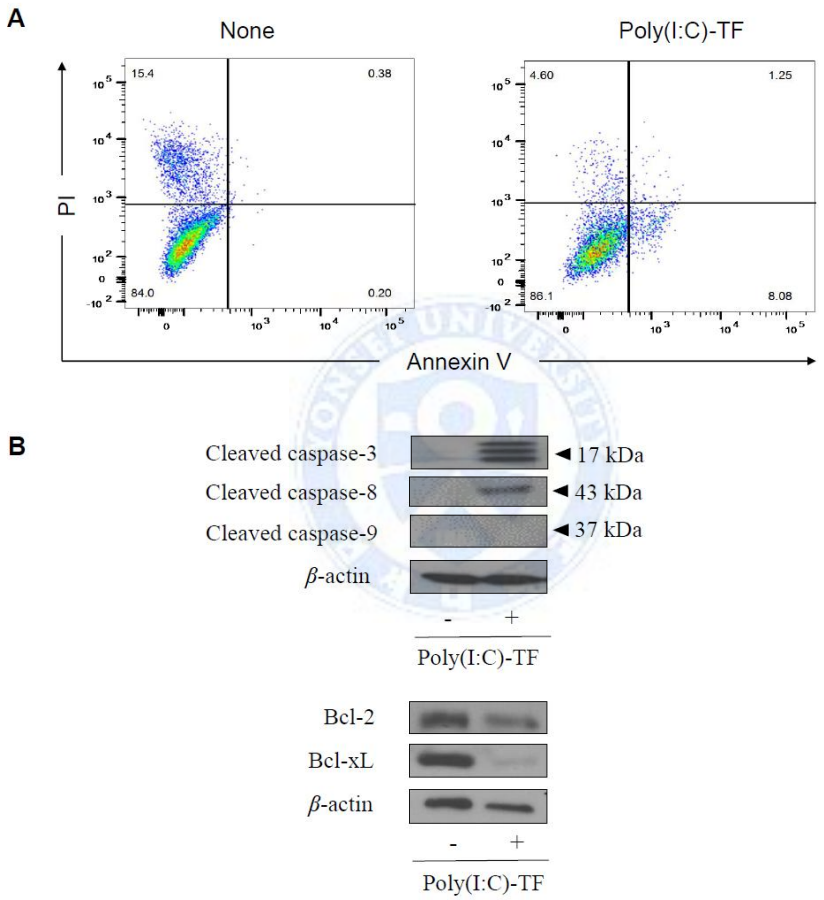

Figure 6. Apoptotic effects of poly(I:C) transfection. A. Flow cytometry analysis was performed for TPC-1 cells stained with FITC-conjugated Annexin V and propidium iodide (PI) after 24 h after poly(I:C) 100 ng/ml transfection (TF). Numbers represent the percentages of cells in each subset. B. Poly(I:C) transfection induces the cleavages of caspase-3 and caspase-8, but not of caspase-9

in TPC-1 cell line. The expression of Bcl-2 and Bcl-xL decrease after poly(I:C) transfection into TPC-1 cells.

4. Transfection of poly(I:C) increases IFN-β mRNA expression and intracellular ROS production.

In TPC-cell line, the transfection of poly(I:C) also triggered dramatic increase of IFN-β mRNA expression as well as innate adjuvant receptors mRNA expression, indicating the working downstream signals of activated innate adjuvant receptors (Figure 7A). The increased ROS production was also measured after 24 h of poly(I:C) 100 ng/ml transfection into TPC-1 cell line by flow cytometry using both H_2DCFDA and MitoSOX Red (Figure 7B). The results suggest that the intracellular ROS generation is a downstream phenomenon of innate adjuvant receptor activation and mitochondria is also involved in poly(I:C) induced ROS generation in TPC-1 cells.

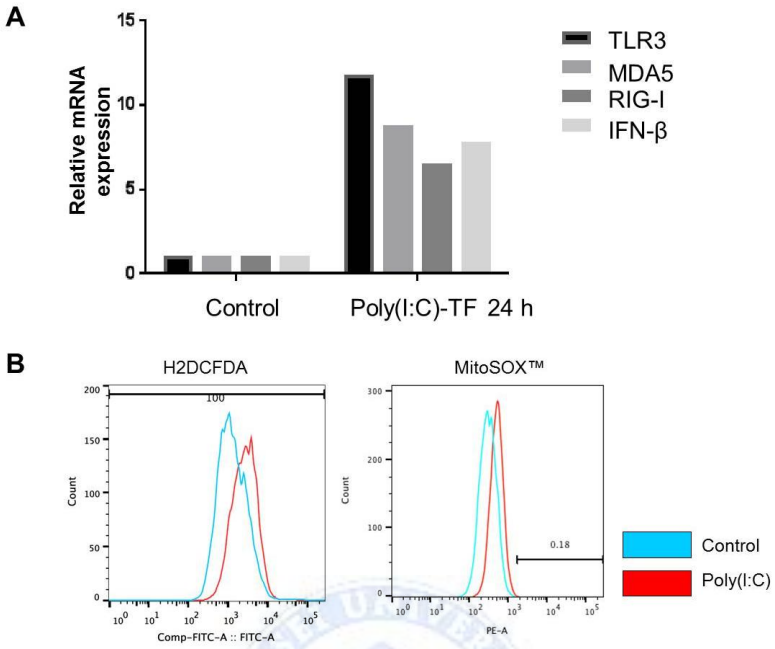

Figure 7. Increased IFN-β gene expression and intracellular ROS following poly(I:C) transfection. A. Transfection of poly(I:C) 100 ng/mL induces innate adjuvant receptors and IFN-β mRNA expression in TPC-1 cells. B. Intracellular ROS production is increased after 24 h after poly(I:C) 100 ng/ml transfection into TPC-1 cells.

5. Innate adjuvant receptor mediated apoptosis in TPC-1 cell line requires IFN-β and intracellular ROS production.

The functional roles of IFN-β signal and intracellular ROS generation as well as innate adjuvant receptors including TLR3 and MDA5 were further validated using RNA interference. The decreased viability of TPC-1 cell line following poly(I:C) transfection was partially restored by knockdown of TLR3 and MDA5 respectively or simultaneously (Figure 8).

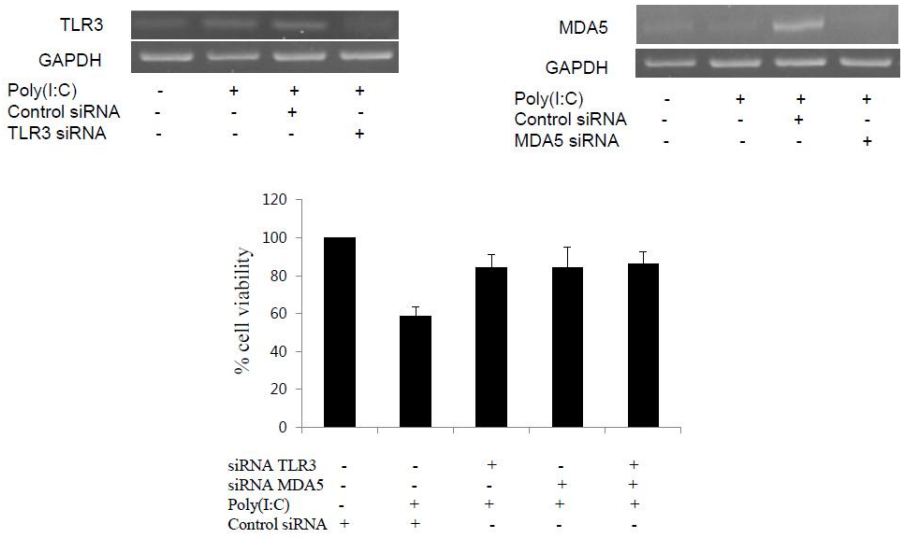

Figure 8. The cell viability effects of RNA interference of innate adjuvant receptors. The decreased viability of TPC-1 cell line following poly(I:C) transfection is partially restored by knockdown of TLR3 or MDA5 through RNA interference.

The partial restoration of poly(I:C) induced TPC-1 cell apoptosis was found when ROS scavenger, NAC or the inhibitor of the mitochondrial respiratory chain reaction, mitoTEMPO was pretreated. The blocking of IFN-β signal using RNA interference or anti IFN-β neutralizing antibody also inhibited the apoptotic effects of poly(I:C) transfection into TPC-1 cell line (Figure 9).

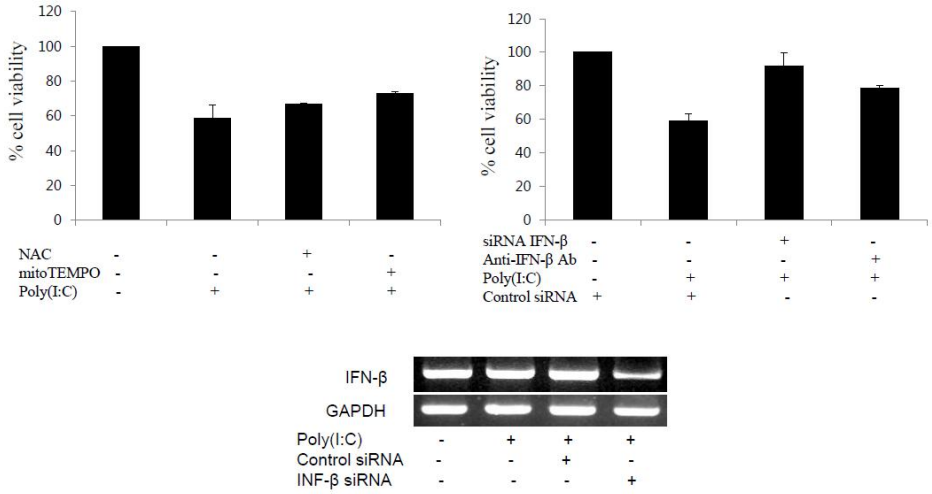

Figure 9. The cell viability effects of ROS scavenging and IFN-β blocking. Poly(I:C) induced TPC-1 cell apoptosis is partially restored by ROS scavenging through pretreatment of NAC or mitoTEMPO, or by blocking of IFN-β signal using RNA interference or anti-IFN- β neutralizing antibody.

6. Intracellular ROS generation following poly(I:C) transfection requires IFN-β signaling.

To elucidate the correlation between increased IFN-β signaling and ROS generation after poly(I:C) transfection into TPC-1 cell line, the effects of IFN-β signal blocking and ROS scavenging were examined. The increased intracellular ROS generation following poly(I:C) transfection into TPC-1 cell line disappeared when IFN-β signaling was blocked using RNA interference or anti IFN-β neutralizing antibody treatment (Figure 10A). In contrast, ROS scavenging with NAC or mitoTEMPO pre-treatment did not attenuate the increased IFN-β mRNA expression following poly(I:C) transfection into TPC-1 cell line (Figure 10B). These results suggest that ROS generation required for innate adjuvant receptor mediated TPC-1 cell apoptosis follows IFN-β signaling activation.

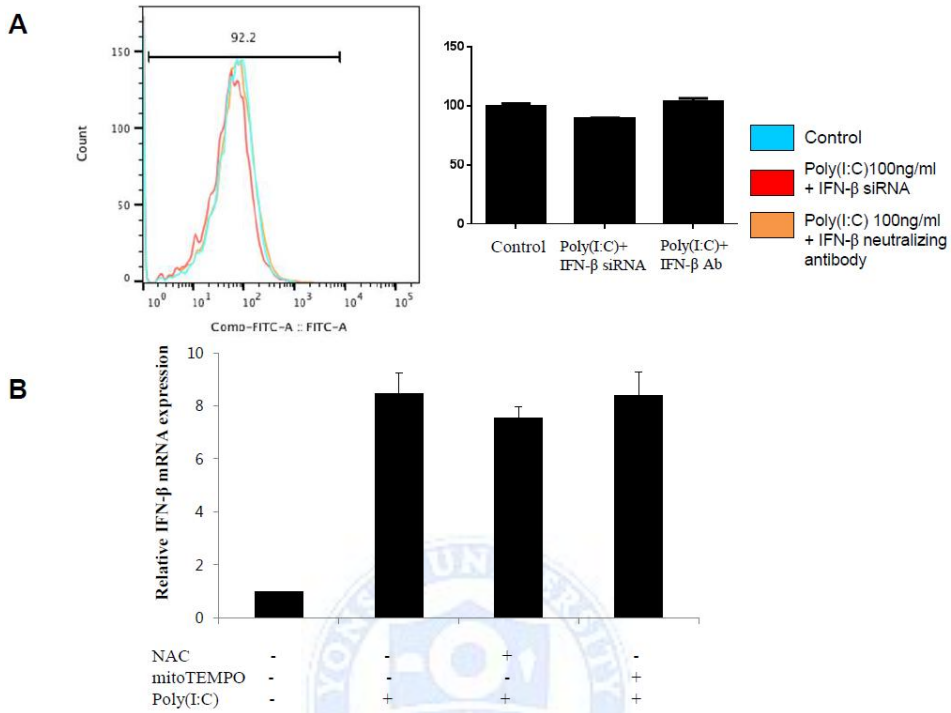

Figure 10. The correlation between increased IFN-β signaling and ROS generation after poly(I:C) transfection. A. The blocking IFN-β signaling through RNA interference or anti- IFN-β neutralizing antibody prevents intracellular ROS generation following poly(I:C) transfection into TPC-1 cells. B. The pretreatment ROS scavengers including NAC and mitoTEMPO do not influence an increase of IFN-β mRNA expression following poly(I:C) transfection into TPC-1 cells.

IV. DISCUSSION

Innate adjuvant receptors mediated immune response allows for the rapid induction of various cytokines and chemokines facilitating in the eradication of pathogens, and further promotes adaptive immune system.[20] These receptor signals activate various downstream effectors including nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs), resulting in the production of type I IFNs.[21]

Type I IFNs are well known cytokines that induce antiviral and immunomodulatory effects through the coordination of signaling cascades including the classical Janus activated kinase (JAK) – signaling transducer and activator of transcription (STAT) pathway.[22] Among many kinds of type I IFNs in humans (IFN-α,β,ε,κ,ω), IFN-β has been well known to have the antiproliferative effects on some cancer models. However, type I IFNs show the pleiotropic biological effects on different target cells and disease models due to diversity of downstream signaling cascades that are regulated directly or indirectly by IFNs.[23]

The biological roll of various innate adjuvant receptors and downstream type I IFN signals in thyroid cancer cell death has not been fully evaluated so far. In this study, we focused on elucidating the existence of working innate immune receptors including TLR3, MDA5, RIG-I and IFN-β in papillary thyroid cancer cell model. We also validated the anti-tumor effects of these signals activated by a transfection of synthetic dsRNA analog, poly(I:C). TLR3 and MDA5 were revealed to be major forms of innate adjuvant receptors functioning in a papillary thyroid carcinoma cell line, TPC-1 in our experiments. The activation of these receptors through poly(I:C) transfection dramatically increased mRNA expression of IFN-β and innate adjuvant receptors themselves, followed by a significant decrease in viability of TPC-1 cells. Decreased cell viability induced by poly(I:C) transfection was at least partially restored when TLR3, MDA5 and IFN-β signal were knockdown by RNA interference. Therefore, functioning innate adjuvant receptors and downstream signaling mediated by IFN-β were turned out to be mandatory for poly(I:C) induced TPC-1 cell death. These biological effects of poly(I:C) were not definite in anaplastic thyroid carcinoma cell line, 8505C which did not show a definite mRNA expression of innate adjuvant receptors or when poly(I:C) was simply added on TPC-1 cells. These finding suggest the working functionality of innate adjuvant receptors in thyroid cancer models and the low probability of direct non-specific cytotoxicity of poly(I:C) itself.

As a preliminary experiment to anticipate the clinical implication of innate immune signaling in papillary thyroid cancer that was the most common histologic subtype of malignancy occurred in thyroid gland, we performed

immunohistochemistry analysis of three kinds of innate adjuvant receptors on selected postoperative paraffin embedded human papillary thyroid cancer tissues including normal peritumoral thyroid tissues. Although a precise conclusion about the significant correlation between clinicopathological characteristics and innate adjuvant receptors expression was not feasible yet due to the small volume of selected cases in this study, we observed the variability of TLR3 and MDA5 expression and accordingly increased IFN-β protein expression in papillary thyroid cancer tissues. Collectively considering a functional relevance of innate adjuvant receptors that was found only in papillary thyroid cancer cell line, but not in the more aggressive anaplastic thyroid cancer cells, innate immune response signals can be the possible candidate of prognostic marker and therapeutic target for thyroid cancers, while a further clinical validation on the larger sized subjects is indeed required.

As previously mentioned, the anti-tumor effects of poly(I:C) have been reported in some other *in-vitro* cancer models. However, compared to the proinflammatory reaction induced by innate adjuvant receptors following viral infection, the molecular signaling of innate adjuvant receptors mediated direct cancer cell death has not been fully elucidated and found to be somewhat variable according to a type of cancer model. TLR3 mediated apoptosis of breast cancer cells has been reported to be dependent on caspase-3 and caspase-8 activation.[5] Caspase-8 dependent apoptotic pathways induced by poly(I:C) have been also implicated in melanoma cells.[24] The activation of caspase-8, -9, and caspase-2 have been reported in renal cell carcinoma cells transfected by poly(I:C).[25] In this study, we validated the apoptosis of TPC-1 cells following poly(I:C) transfection through an Annexin V staining and flow cytometry analysis and confirmation of caspase activation and decreased prosurvival factors such as Bcl-2 and Bcl-xL. Caspase-3 and caspase-8 that were observed to be activated in our experiments were known to be involved in extrinsic apoptosis pathway. Caspase-9 mainly responsible for intrinsic apoptosis was not been activated in TPC-1 cells following poly(I:C) transfection in this study. Collectively, extrinsic caspase dependent apoptosis was suggested to be the major molecular mechanism inducing innate adjuvant receptor

mediated papillary thyroid cancer cell death.

The role of intracellular ROS as signaling molecules regulating cell proliferation and apoptosis in immune and inflammatory process has been well defined along with the ROS producing NADPH oxidase (NOX)/dual oxidase (DUOX) systems involved in TLR mediated innate immune defense mechanism.[26,27] In many aspects, the downstream adaptor molecules are cross talking with mitochondrial membrane proteins involved in mitochondrial ROS production for example through TNF receptor-associated factor (TRAF) 6 or mitochondrial antiviral-signaling protein (MAVS).[28] In our experiment, intracellular ROS was also increased following innate adjuvant receptor activation and ROS scavenging partially protected poly(I:C) induced cancer cell death. However, IFN-β activation was not attenuated following pretreatment of ROS scavengers. Although intracellular ROS including mitochondrial ROS had some contribution to innate adjuvant receptor mediated apoptosis in TPC-1 cells, it seemed to be not solely mandatory for IFN-β signaling activation. The correlation between innate adjuvant receptors signaling and mitochondrial dysfunction associated ROS production has been reported with complexed fashion in other disease models. Mitochondrial dysfunction induced by TLR3 was not found to be involved in NF-κb signaling pathway which was downstream to TLR3 in hepatocytes cell death model.[29] ROS dependency of innate immune signaling and IFN-β signals might has the cancer cell-type specific patterns. DUOX gene expression showed wide range of variation according to different subjects and tumor differentiation of thyroid cancers.[30] The exact molecular mechanism explaining the reduced ROS production following IFN-β signal blocking in this study also has not been reported. Whether this correlation was an indirect phenomenon merely induced by increased cell death itself, and the functionality of intracellular ROS including a cross linkage between ROS and innate adjuvant receptors signaling in thyroid cancer cells also need to further elucidated through studies about various ROS producing enzymes and related signaling molecules.

V. CONCLUSION

Innate adjuvant receptor signaling of TLR3 and MDA5 are functioning in papillary thyroid cancer cells and induce the cancer cell deaths. Both IFN-β signal and intracellular ROS production contribute the innate adjuvant receptors mediated cell death, but intracellular ROS is not mandatory for IFN-β activation in papillary thyroid cancers. Innate adjuvant receptor signaling is the significant candidate for prognostic factors and possible therapeutic target of papillary thyroid cancers.

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ABSTRACT(IN KOREAN)

갑상선암종 내 innate adjuvant receptor 신호 활성화에 있어 세포 활성산소 생성의 역할

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서론: Innate adjuvant receptor는 병원체 연관 분자 패턴을 인식하여 선천면역과 적응면역반응 모두에서 중요한 역할을 담당하며 주로 제1형 인터페론 등의 염증성 사이토카인 발현을 활성화시킴으로써 작용한다. 최근 몇몇 암종에서는 innate adjuvant receptor의 리간드인 polyinosinic-polycytidylic acid [poly(I:C)]의 세포질 내 전달 후 세포자가사멸과 성장 정지를 통한 항 종양 효과가 보고되었다. 세포 내 활성산소 또한 세포 증식과 자가사멸을 조절하여 선천면역과 염증촉진 반응에 관여하는 것으로 알려져 있다. 본 연구에서는 갑상선암에서의 innate adjuvant receptor 신호에 활성산소 생성이 영향을 줄 것이라는 가설 하에 갑상선유두암 세포사멸 과정에서의 innate adjuvant receptor 및 활성산소의 역할 및 상호관계를 규명하고자 하였다.

대상 및 방법: Toll-like receptor (TLR) 3, melanoma differentiation-associated gene (MDA) 5, retinoic acid inducible gene-1 (RIG-I) 등 innate adjuvant receptor 발현에 대한 면역조직화학염색 분석을 총 40례의 갑상선유두암 수술 후 조직을 대상으로 시행하였다. TPC-1과 8505C 세포주를 대상으로 기능하는 innate adjuvant receptor의 존재 여부 및 poly(I:C) 세포감염에 의한 세포생물학적 효과를 검증하였으며, 갑상선유두암 세포주인 TPC-1을

대상으로 인터페론-베타와 활성산소 생성을 매개로 하는 하위 분자 신호를 규명하였다.
결과: 갑상선유두암 조직에서 TLR3, MDA5 및 인터페론-베타는 다양한 정도로 발현되었으나, RIG-I 발현은 미미하였다. Poly(I:C) 세포감염에 의한 갑상선유두암 세포의 innate adjuvant receptor 의존적인 세포자가사멸은 caspase-3와 caspase-8 활성화를 매개로 하는 외인성 경로를 통하여 유발되는 것으로 규명되었다. Poly(I:C) 세포감염은 또한 innate adjuvant receptor와 인터페론-베타의 mRNA 발현을 유발하였으며 poly(I:C)에 의한 innate adjuvant receptor 활성화 후 세포 내 활성산소가 증가하는 것 또한 입증되었다. 활성산소의 포착과 인터페론-베타 신호의 차단은 모두 poly(I:C) 세포감염의 효과를 감소시켰으나, 활성산소 포착제 처리에 의하여 인터페론-베타 발현이 약화되지는 않았다.
결론: TLR3와 MDA5를 매개로 하는 innate adjuvant receptor 신호는 갑상선유두암 세포에서 기능하며 세포사멸을 유도한다. 인터페론 베타 신호와 활성산소 생성은 모두 innate adjuvant receptor 매개 세포 사멸에 기여하나 세포 내 활성산소가 갑상선유두암에서 인터페론 베타 활성화에 필수적인 것은 아니다.

핵심되는 말 : innate adjuvant receptors, pattern recognition receptors, 갑상선암, 인터페론, 활성산소