A Dissertation for the Degree of Doctor of Philosophy

Effect of Toll-Like Receptors 4, 5 and 7 during Prostate Tumor Progression in the Transgenic Adenocarcinoma of Mouse Prostate Model

전립선암 모델 마우스 (TRAMP) 의 전립선 종양 진행에서 Toll-Like receptors 4, 5, 7 의 영향

By

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ABSTRACT

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Toll-like receptors (TLRs), a family of transmembrane proteins that recognize highly conserved molecules in pathogens, have an important role on the triggering and promoting inflammation. Recent evidence showed that the stimulation of TLRs in cancer

cells can inhibit or promote tumorigenesis dependently on receptors or tumor cell type. TLR signaling may play an important role in tumor development and activation of TLRs might play a "double-edged sword" role in the influence of tumor progression. Moreover, *in vitro* and *in vivo* studies often lead to conflicting results depending on experimental conditions. Although various TLRs have been associated with immune response and tumorigenesis in the prostate cells, little is known about the role of TLR5 and 7. The TLR4 signaling and expression in prostate cancer cells are remained controversial. In this study, association of TLR4, 5, and 7 in the progression of prostate transformation was examined using *in vivo* and *in vitro* prostate model.

Chapter I study showed that expression of TLR4 and TLR5 was associated with progression of prostate transformation in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. The expression of TLR4 and TLR5 was evaluated by immunohistochemisty in formalin-fixed paraffin-embedded prostate tissue from wild-type (WT) and TRAMP mice. Normal prostate tissue from WT mice showed strong expression of TLR4 and TLR5. However, TLR4 expression in the prostate tissue from TRAMP mice gradually decreased as pathologic grade became more aggressive. TLR5 expression in the prostate tissue from TRAMP mice also decreased in low-grade prostate intraepithelial neoplasia (PIN), high-grade PIN and poorly differentiated adenocarcinoma. Overall, our results suggest that decreased expression of TLR4 and TLR5may contribute to prostate tumorigenesis.

TLR7 expression and function on tumorigenesis have been examined in several cancers including non-small cell lung cancer (NSCLC) and esophageal squamous cell

carcinoma (SCC). However, the role of TLR7 on prostatic tumor development has yet to be clarified. Chapter II examined the expression of TLR7 during tumor progression of TRMAP mice and its role on cell growth. Strong expression of TLR7 was detected in the normal prostate epithelia of WT mice, but not in TLR7-deficient mice. In contrast, TLR7 expression was weak in TRAMP-C2 cells, as compared with murine bone marrow derived macrophages (BMDMs). Moreover, TLR7 mRNA was markedly expressed in RWPE-1 cells (non-cancerous prostate epithelial cells), but not in PC3 and DU145 (prostate cancer cells). Immunohistochemically, TLR7 expression gradually decreased in TRAMP mice depending on the pathologic grade of the prostate cells. TLR7 agonists increased both the gene and protein expression of TLR7 and promoted production of proinflammatory cytokines/chemokines and IFN-β gene expression in prostate cancer cell lines. Moreover, TLR7 agonist, loxoribine inhibited the growth and colony formation of TRAMP-C2 cells dependent of TLR7.

In chapter III, anti-tumor effect of the small molecule imiquimod, also known as a TLR7 agonist, was examined in prostate cancer. Imiquimod inhibited the growth of mouse (TRAMP-C2) and human (PC-3) prostate cancer cells. Treatment with imiquimod induced cell cycle arrest at the G_2/M phase in TRMPA-C2 cells. Finally, imiquimod induced direct apoptosis in TRAMP-C2 cells via a mitochondrial-dependent pathway. Intratumoral injection with imiquimod reduced significantly tumor growth and increased apoptotic cells in mice subcutaneously implanted with TRAMP-C2 cells.

These findings suggest that TLRs (TLR4, 5, 7) may participate in tumor suppression and represent a promising class of immune response enhancers with the

potential to generate an effective antitumor immune response by create a tumor microenvironment in the *in vivo* TRAMP model. Moreover, the stimulation of TLR7 agonist appears to be augmented by suppression of a regulatory mechanism with pro-inflammatory responses in prostate cancer. Specially, loxoribine would result in a highly efficient immune system activation and give rise to an enhanced anti-tumor activity in *in vivo* and *in vitro* prostate cancer. TLR7 signaling pathway suggests that TLR7-mediated tumor suppression by TLR7 agonists for prostate cancer supports immune-based therapies.

Keywords : prostate cancer, Toll-like receptors, TRAMP, tumorigenesis

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LIST OF ABBREVIATION

AC	Adenocarcinoma
BMDM	Bone marrow-derived macrophages
CLQ	Chloroquine
ERK	Extracellular signal-regulated kinases
IFN	Interferon
IHC	Immunohistochemisty
IL	Interleukin
IMQ	Imiquimod
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
LOX	Loxoribine
MAPKs	Mitogen-activated protein kinases
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-κB
PBS	Phosphate-buffered saline

PIN	Prostate intraepithelial neoplasia
TIR	Toll-interleukin 1 receptor
PLC	Phylloides-like cancer
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAMP	Transgenic adenocarcinoma of mouse prostate
TRIF	TIR domain-containing adaptor proteins, thus inducing interferon β

TABLE OF CONTENTS

GENERAL INTRODUCTION

Prostate cancer is the most commonly diagnosed male malignancy and the first cause of malignant male death worldwide (1). It is widely accepted that environmental factors such as chronic inflammation and infection are important for development of prostate cancer. Although there is a lot of evidence that inflammation might be involved in the prostate cancer, the precise mechanisms of inflammatory involvement have not been fully investigated. Many pathogens, including bacterial species and viruses, have been detected in the prostate (2, 3), but only some of them are associated with inflammation. These observations open a new field of investigation aimed at clarifying the possible role of bacterial and viral sequences in the activation of Toll-like receptors (TLRs) that might positively or negatively influence tumorigenesis by triggering the innate and adaptive immune responses (4). TLRs may serve tumorigenesis by promoting malignant transformation of epithelial cells and tumor growth, or on the contrary, inhibiting tumor progression inducing apoptosis.

Growing reports have been done to investigate whether there is a connection between TLR and prostate cancer risks, the results are controversial and open up new avenues for understanding the role of TLR4 in tumor cells. For example, stimulation of TLR4 in DU145 by LPS activates NF-κB signaling pathway, which leads to production of pro-inflammatory cytokines such as IL-6 and IL-1β through MyD88 dependent pathway (5). In addition, TLR4 activation increases expression of VEGF and TGF-β_1 in PC3 cells, which promote tumor development (6). Knock down of TLR4 using siRNA in PC3 cells

reduces tumor cell migration and invasion (7). However, two independent studies showed that the percentage of epithelial cells expressing TLR4 is gradually reduced as pathologic grade increases in human and Copenhagen rats prostate tissues (5, 8). *In vitro* and *in vivo* studies often lead to conflicting results depending on experimental conditions. Moreover, diversity of *in vivo* microenvironment including stroma-derived factors and immune cells might be an important factor to be explored. Although a number of TLRs have been investigated a possible association between TLR and prostate cancer risk, the functional implications of the majority of *in vivo* variations are still unknown. Moreover, literature review showed that TLR5 and 7 within the prostate cancer microenvironment have yet to be determined.

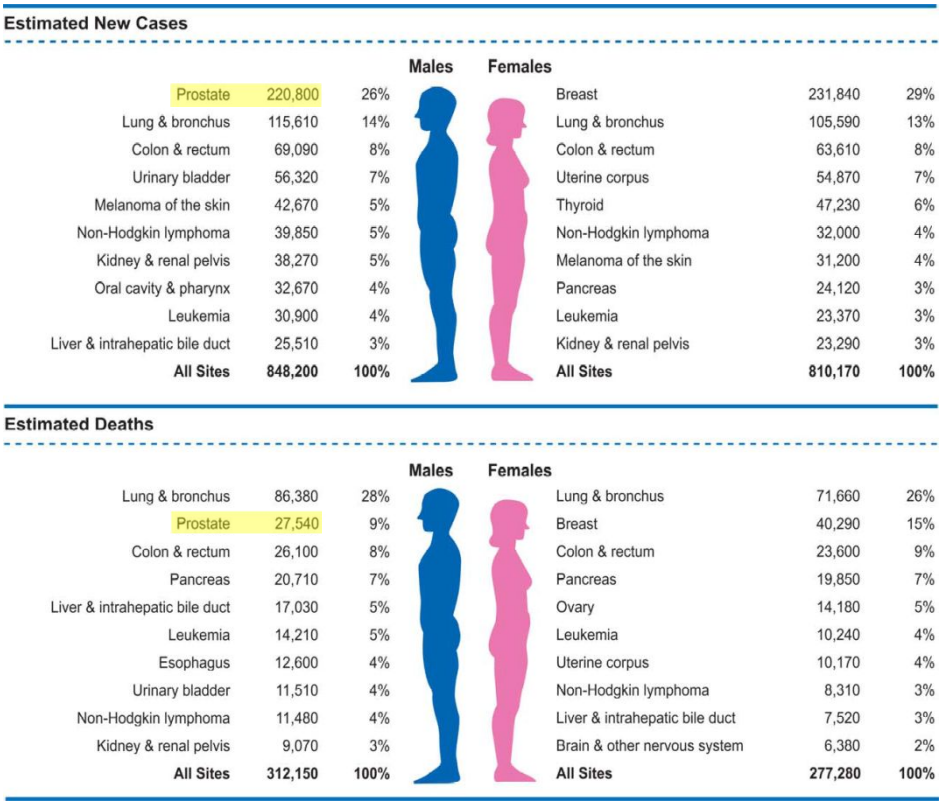
In this study, I evaluated the expression of TLR4, 5, and 7 during prostate cancer progression in TRAMP orthotopic and subcutaneous tumor models as a sensor in tumor immune surveillance.

LITERATURE REVIEW

Inflammation in prostate cancer

Prostate cancer is now the most frequently diagnosed malignancy in men and second leading cause of cancer-related male death (Figure I) (9-12). Despite this vast prevalence, the number of known risk factors is limited. Histologically, most lesions that contain either acute or chronic inflammatory infiltrates in the prostate are associated with atrophic epithelium or focal epithelial atrophy (13, 14). In morphological studies have observed transitions between atrophic epithelium and adenocarcinoma (15, 16) and frequent transitions between areas of proliferative inflammatory (PIA) and/or proliferative atrophy with high grade prostatic intraepithelial neoplasia (PIN) (13, 17).

Prostatic inflammation can be induced by various sources including by infection (for example, with sexually transmitted agents), cell injury (owing to exposure to chemical and physical trauma from urine reflux and prostatic calculi formation), hormonal variations and/or exposures, dietary factors such as charred meats or a combination of two or more of these factors (18). Inflammation can induce proliferative events and post-translational DNA modifications by enhancing the secretion of growth factors such as cytokines and chemokines (19). Emerging evidence now suggests that inflammation is closely associated with the development of prostate cancer. Despite rising epidemiological evidence linking inflammation and prostate cancer (20, 21), the precise mechanisms of inflammatory involvement are yet to be determined.



Estimated New Cases

Males				Females		
Prostate	220,800	26%		Breast	231,840	29%
Lung & bronchus	115,610	14%		Lung & bronchus	105,590	13%
Colon & rectum	69,090	8%		Colon & rectum	63,610	8%
Urinary bladder	56,320	7%		Uterine corpus	54,870	7%
Melanoma of the skin	42,670	5%		Thyroid	47,230	6%
Non-Hodgkin lymphoma	39,850	5%		Non-Hodgkin lymphoma	32,000	4%
Kidney & renal pelvis	38,270	5%		Melanoma of the skin	31,200	4%
Oral cavity & pharynx	32,670	4%		Pancreas	24,120	3%
Leukemia	30,900	4%		Leukemia	23,370	3%
Liver & intrahepatic bile duct	25,510	3%		Kidney & renal pelvis	23,290	3%
All Sites	**848,200**	**100%**		**All Sites**	**810,170**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	86,380	28%		Lung & bronchus	71,660	26%
Prostate	27,540	9%		Breast	40,290	15%
Colon & rectum	26,100	8%		Colon & rectum	23,600	9%
Pancreas	20,710	7%		Pancreas	19,850	7%
Liver & intrahepatic bile duct	17,030	5%		Ovary	14,180	5%
Leukemia	14,210	5%		Leukemia	10,240	4%
Esophagus	12,600	4%		Uterine corpus	10,170	4%
Urinary bladder	11,510	4%		Non-Hodgkin lymphoma	8,310	3%
Non-Hodgkin lymphoma	11,480	4%		Liver & intrahepatic bile duct	7,520	3%
Kidney & renal pelvis	9,070	3%		Brain & other nervous system	6,380	2%
All Sites	**312,150**	**100%**		**All Sites**	**277,280**	**100%**

Figure I. Prostate cancer is the most commonly diagnosed cancers in men (1). Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States, 2015.

Toll- like receptors and their role

Toll-like receptors (TLRs), a family of transmembrane proteins that recognize microbial moieties or endogenous molecules, have an important role on the triggering and promoting inflammation. During the past decade, there has been rapid progress in the understanding of innate immune recognition of microbial components and its critical role in host defense against infection. Currently, 13 mammalian-TLR analogs have been identified. TLR 1, 2, 4, 5 and 6 are expressed on the surface of cells, TLR 3, 7, 8 and 9 are detected in the cytosol of cells (Figure II). To date, 10 functional TLRs have been identified in humans and their ligands are bacterial lipoprotein (TLR1, TLR2), dsRNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), single-stranded RNA of bacterial or viral genome (ssRNA, TLR7/ 8), and unmethylated CpG-DNA (TLR9) to subsequently trigger inflammatory process (22) (Table I). The ligands for TLRs 10, 12 and 13 remain unidentified. TLR10 is expressed in humans but not in mice, TLR8 is not functional in mice and TLRs 11, 12 and 13 are expressed in mice but not in humans. TLRs are composed of three major domains; extracellular leucine rich repeats (LRRs) in the ectodomain that mediate the recognition of PAMPs, transmembrane domain and intracellular TIR domain, which initiates downstream signaling pathways.

TLRs recognize conserved microbe-derived molecular patterns at the cell surface and/or the endosomal membrane, subsequently triggering the host innate immune response TLR ligation results in activation of a variety of downstream signal that are used for IL-1R signaling (23). TLR signaling pathways originate from the cytoplasmic toll-interleukin 1 receptor (TIR) domain. A TIR domain-containing adaptor, myeloid

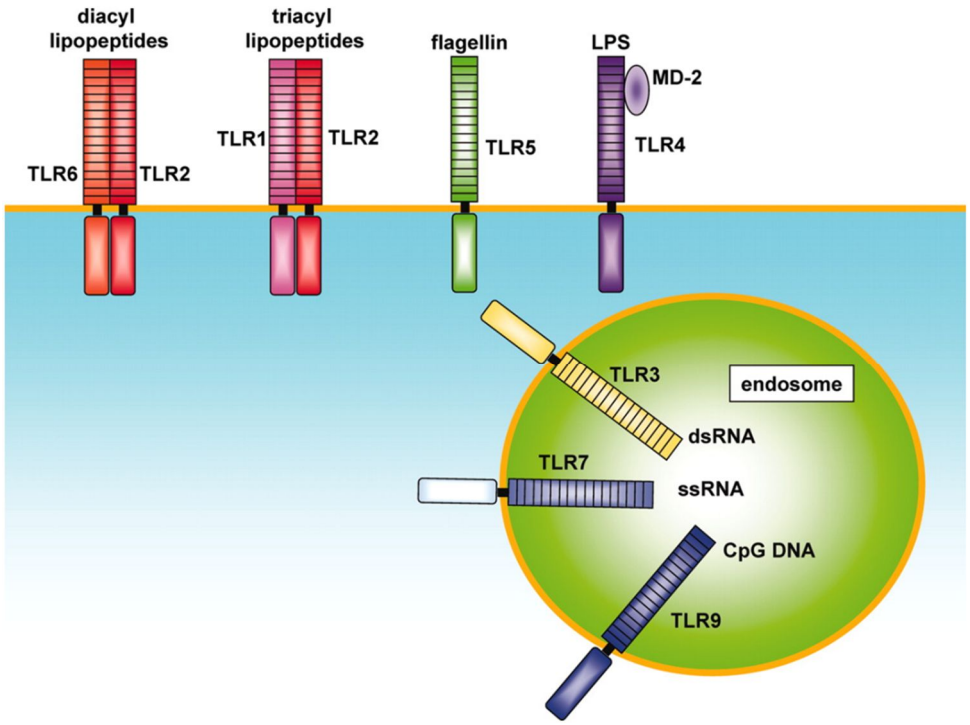


Figure II. TLRs and their ligands (22). Extracellular TLRs – including TLR2, TLR4, and TLR5 – recognize bacterial molecules such as lipoprotein, lipopolysaccharide (LPS), and flagellin. In contrast, endosomal TLRs – composed of TLR3, TLR7/8, and TLR9 – are primarily responsible for sensing viral nucleic acids.

Table I. Ligands recognized by TLRs

Proteins	Major ligands (or activators)	Major cell types
TLR1	Triacyl lipopeptides from bacteria and mycobacteria	MΦ, cDC, neutrophil, mast cells
TLR2	LTA from gram-positive bacteria, yeast zymosan, lipopeptides (Pam_3CSK_4, MALP2), lipoarabinomannan from mycobacteria	MΦ, cDC, neutrophil, mast cell
TLR3	Viral dsRNA, poly(I:C)	cDC, MΦ (mouse), endo/epithelial cells
TLR4	LPS from gram-negative bacteria, mannan from *Candida albicans*, GIPLs from *Trypanosoma*, viral envelope proteins from RSV and MMTV	MΦ, cDC, neutrophil, mast cell, eosinophil
TLR5	Bacterial flagellin	Monocyte, cDC, iEC
TLR6	Diacyl lipopeptides from *Mycoplasma*, LTA from gram-positive bacteria, yeast zymosan	Monocyte, mast cell, cDC, neutrophil
TLR7	ssRNA from RNA viruses, imiquimod, resiquimod (R848), synthetic polyU RNA, certain siRNAs	pDC, neutrophil, eosinophil
TLR8	Resiquimod (R848), viral ssRNA	Monocyte, cDC, mast cell, neutrophil
TLR9	Bacterial and viral CpG DNA, hemozoin from *Plasmodium*	pDC, NK cell, eosinophil, neutrophil
TLR10	–	pDC, B cell
TLR11	Profilin-like molecule from *Toxoplasma gondii,* unknown ligand(s) from uropathogenic bacteria	MΦ, epithelial

differentiation factor 88 (MyD88), associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. MyD88-adapter protein recruits IRAKs and TRAF6. The TRAF6 in turn activates TAK1 that phosphorylates and activates the IKK complex culminating in the release and translocation of nuclear factor-kappa B (NF-κB) to the nucleus which induces expression of inflammatory cytokines. TAK1 also activates mitogen-activated protein kinases (MAPKs). In TLR3 mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. TIR-domain-containing adapter-inducing interferon-β (TRIF) is essential for the MyD88-independent pathway. TLR4 is unique in that it activates both MyD88-dependent and TRIF-dependent pathways (22, 24) (Figure III). TLR signaling also activates DCs and macrophages to secrete IL-12, a cytokine that directs the adaptive immune response toward a Th1 phenotype. TLRs play a major role as the initiator of the innate immune responses to defend against bacteria, viruses and other pathogens and also required for an effective secondary immune response.

TLRs in cancer

TLRs are expressed on the surface of monocytes, macrophages, DCs and epithelial cells or in the cytoplasm of cells from different tissues (26). Recently, several studies have shown that functional TLRs are expressed not only on immune cells, but are also expressed on tumor cells (Table II). It is believed that TLR stimulation not only depends on receptors, but also according to different tumor types. Moreover, various studies have shown the role of TLRs in variety of tumor cells/cell lines, and TLR-

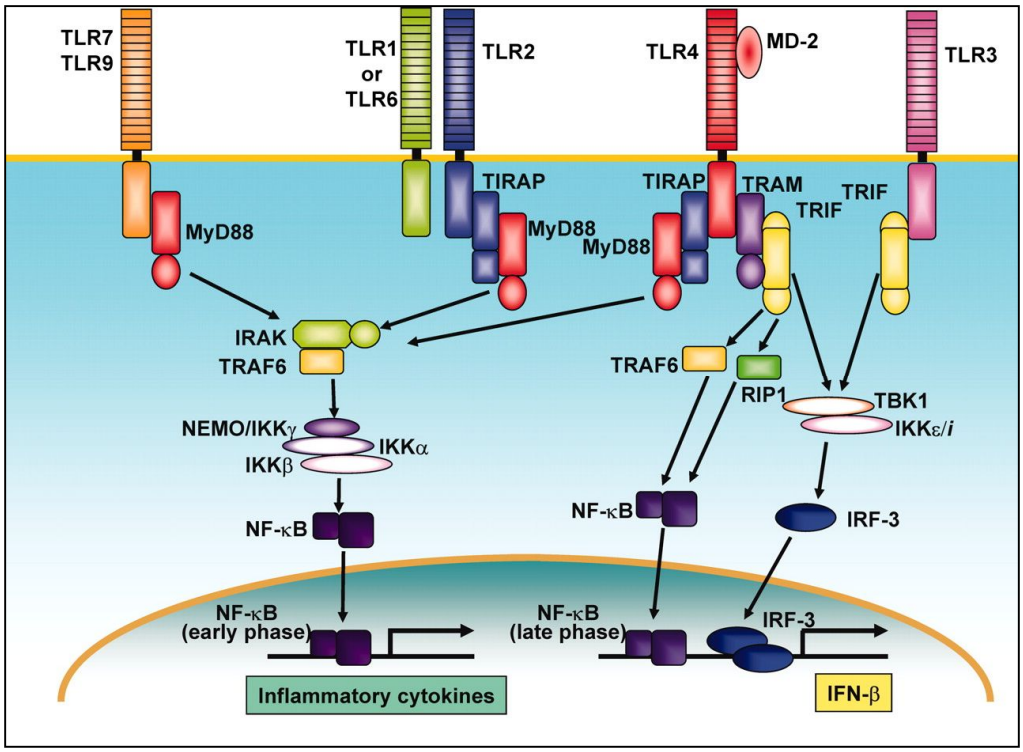


Figure III. TLR signaling pathway (25). Stimulated TLR with ligand recruits adaptor proteins containing a TIR domain, including MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 and TRIF are responsible for the activation of distinct signaling pathways and leading to the production of proinflammatory cytokines NF-κB, and IRFs, respectively.

variants are associated with cancer risk. Zheng *et al.* first reported that sequence variants of TLR 4 are associated with prostate cancer (27). Sequence variants of TLR4 and TLR10 are associated with nasopharyngeal carcinoma risk (28, 29). TLRs are expressed on tumor cell lines and showed that the activation of TLRs influence tumor growth and host immune responses (30). Activation of TLRs in cancer cells leads to the activation in tumor microenvironment such as production of pro-inflammatory cytokines, chemokines, as well as adhesion molecules, and activation of adaptive immunity.

TLR as negative regulator of cancer: In the early 18th century, Deidier reported that infection in cancer patients could be correlated with the remission of malignant diseases (31). William Coley observed that repeated injections of a mixture of bacterial toxins from the Gram-positive bacterium *Streptococcus pneumoniae* and the Gram-negative bacterium *Serratia marcescens* served as efficient anti-tumor therapeutic agents, providing evidence that microbial products may mediate an anti-tumor effect (32). It was later discovered detecting distinctive molecular patterns (LPS) present the outer membranes of bacteria that accounted for its anti-tumor effect (31, 33). Another bacterial strain, *Myco bacterium bovis* BCG, which acts as a TLR2/4 agonist, is effective against superficial bladder tumors (72). Recently, potent anti-cancer effects against established tumors in both mice and humans by TLRs ligands have been demonstrated through a multitude of mechanisms. Triggering of TLR3 on human tumor cells with poly (I:C) inhibits tumor cell proliferation and triggers apoptosis (73). Triggering of TLR3 induced apoptosis in human pharynx carcinoma cells and head and neck cancer (73, 74) and TLR9 agonist inhibited tumor growth by sensitizing lung cancer cell to apoptosis (75).

Table II. Expression of TLRs in various tumor cells/cell lines (34).

TLRs expressed	Tumor cells/cell lines	References
TLR1, 7, and 9	Multiple myeloma cells	(35)
TLR2	Oral squamous cell carcinoma	(36)
TLR2, 3, and 4	Laryngeal carcinoma	(37)
TLR2, 4, and 5	Intestinal adenocarcinoma	(38)
TLR2, 3, 4, and 5	Ovarian carcinoma	(39)
TLR3	Human neuroblastoma (NB) cells, breast adenocarcinoma, cervical, hepatocellular, papillary thyroid, nasopharyngeal and lung carcinomas, and murine colon carcinoma	(40-47)
TLR3, 4, and 9	Breast and prostate carcinomas	(48)
TLR3, 4, 7, and 9	Esophageal squamous cell carcinoma	(49)
TLR4	Colon carcinoma, human head and neck squamous cell carcinoma, melanoma cell lines, NB-1 neuroblastoma, lung carcinoma, pancreatic ductal adenocarcinoma, colorectal carcinoma, adrenocortical carcinoma, ovarian cancer cell lines, bladder cancer cells	(50-59)
TLR4 and 9	Lung carcinoma	(60)
TLR4, 5, and 9	Gastric carcinoma	(61)
TLR5	Cervical tumor cells, breast cancer cells, colon carcinoma, gastric cancer cells	(62-65)
TLR7 and 8	Lung carcinoma, colorectal carcinoma	(66, 67)
TLR9	Lung carcinoma, cervical cancer cells, prostate carcinoma, renal cell carcinoma, breast cancer cells, ovarian cancer cells	(68-70)
Multiple TLRs	Human breast cancer cell line MDA-MB-231, murine tumor cell lines (MC26, 4T1, RM1, B16, LLC1), human tumor cell lines (HCT15, SW620, MCF7, UACC-62, MDA-MB435)	(30, 71)

The TLR9 activator, CpG, which is being studied for the treatment of breast cancer, colorectal cancer, lung cancer, melanoma, glioblastoma, and some lymphomas and leukemias (77). Wang *et al.* showed that stimulation of TLR9 sensitized lung cancer cells to apoptosis, leading to the arrest of tumor growth (75). CpG-containing oligodeoxynucleotide (CpG ODN) agonists directly induce the activation and maturation of DCs, enhance the differentiation of B cells into antibody secreting plasma cells, and promote the development of antitumor T-cell responses (77). HMGB1 binds to TLR4 and that HMGB1, which is released by chemotherapy-induced cell death, can activate TLR4 and induce anti-tumor T-cell immunity (78). However, it should be remembered that not all TLR agonists and TLR signaling pathways lead to relevant antitumor activity.

TLR as a positive regulator of cancer: Several studies have demonstrated that activation of TLR expressed on tumor cells may contribute to tumorigenesis in numerous organs. Huang *et al*. showed that *Listeria monocytogenes* infection of a local tumor can accelerates tumor growth in hepatocellular carcinoma cell line through TLR2 signaling, which leads to the production of immunosuppressive molecules (79). Triggering of TLR4 expressed in human head and neck squamous cell carcinoma promotes tumor development by the increased production of IL-6, IL-8, VEGF and granulocyte macrophage colony stimulating factor and promotes the immune escape of tumor cells (55). Recent studies have shown that TLR4 is an important TLR in ovarian cancer, prostate cancer and colorectal cancer (7, 80, 81). Activation of TLR4 signaling promotes the growth and chemoresistance of epithelial ovarian cancer cells (81, 82). Another study demonstrated that LPS may also promote tumor invasion through upregulation of iNOS

and matrix metalloproteinase 2 (MMP2) and the β1 integrin subunit (83, 84). TLR4 stimulation promoted the survival and proliferation of hepatocarcinoma cells (85) and TLR5 stimulation promotes the migration and invasion of salivary gland adenocarcinoma (86) and the proliferation of gastric cancer cells (87). In addition, TLR7 and 9 induces tumor cell growth and prevents chemotherapy (35, 88). *In vitro* study indicates that TLR9 agonists can stimulate prostate cancer and breast cancer cell invasion by increasing the activity of MMP13 activity (88, 89). Furthermore, several studies have also shown that TLR deficiency in cancer cells/cell lines may lead to cancer regression (90, 91).

Recent reports showed that the stimulation of TLRs in cancer cells can inhibit or promote tumorigenesis dependently on receptors or tumor cell type. TLR signaling may play an important role in tumor development and activation of TLRs might play a "double-edged sword" role in the influence of tumor progression.

Contribution of TLRs in prostate cancer

TLRs are expressed in various cancer cells and function as modulators of tumor development and progression. TLR stimulation drives tumorigenesis by promoting cell proliferation, invasion, and migration, as well as by inducing an immunosuppressive microenvironment (92). Conversely, TLRs can also act as negative regulators of cancer by inducing apoptosis, activating the immune system, and enhancing chemo-sensitivity (92, 93). In combination, these seemingly opposite effects are accounted for by the differences in TLR type and tumor origin. Accordingly, TLRs have an important role in the host defense against prostate infection (Table III). TLR2 mediated *Mycoplasma*

Table III. Contribution of TLRs in prostate cancer

Targeting TLR	Effect	References
TLR2	Inflammation	(95)
TLR 3	Apoptosis, immune surveillance	(99)
TLR4	controversial	(6, 7, 97, 100)
TLR5	Inflammation	(96)
TLR7	Unknown	-
TLR9	Proliferation, invasion	(89, 97, 98)

hominis induced IL-8 production in prostate epithelial cells (94) and TLR5 induced early expression of proinflammatory gene against *Escherichia coli* infection and deficiency in TLR5 led to overwhelming bacterial growth (95). TLR9 agonists promote cell proliferation and the production of IL-8 and TGF-β_1 via NF-κB activation in prostate cancer cells (96, 97). In addition, It can stimulate prostate cancer invasion by increasing MMP13 activity (88). Both studies suggest that TLR9 signaling plays a role in cancer progression and metastasis. In contrast, TLR3 stimulation by poly(I:C) induces apoptosis of prostate cancer cells through a PKC-α-dependent pathway (98). TLR4 stimulation by LPS is shown to contribute to chemoresistance to docetaxel in prostate cancer cells (73). TLR4 signaling is also associated with the proliferation, invasion, and survival of prostate cancer cells and also promotes tumor-relating gene expression, including that of VEGF and TGF-β_1 (6, 7, 96). Moreover, peroxiredoxin 1 (Prx1) appears to be an agonist of TLR4 in prostate cancer development (99). A lot of evidence suggest that whether there is a connection between TLR gene polymorphisms and prostate cancer risk with chronic intra-prostatic inflammation (100).

CHAPTER I

Decreased Expression of TLR 4 and 5 during Progression of Prostate Transformation in TRAMP Mice

1.1 INTRODUCTION

Chronic prostate inflammation can be induced by various sources including infection, urine reflux, dietary factors, or a combination of two or more of these factors (14). Sexually transmitted agents, such as *Neisseria gonorrhea*, *Chlamydia trachomatitis*, *Trichomonas vaginalis* and *Escherichia coli* have been known to cause acute and chronic bacterial prostatitis (101, 102). In addition, uric acid, a chemical compound in the urine (103) and dietary 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) treatment has been known to induce chronic prostatitis.

Although TLRs play a central role in the host defense response against microbial infection, recent reports underlines these receptors as crucial mediators involved in tumor growth and progression (62, 100). Several epidemiological studies demonstrated a significant decreased expression of TLR4, 5, 7 and 9 in prostate carcinoma compared to benign prostate hyperplasia (104). However, Vaisnen *et al.* showed the increased expression of TLR9 in prostatic carcinoma compared to benign prostate hyperplasia (105) and high expression of TLR3, 4, and 9 in prostate carcinoma recurrence (106). These controversial data may come from individual difference in genetic and environmental background even in the same group. For example, micro-organisms colonized in tissue, which may possibly lead to up-regulation of TLR expression, was not taken into consideration.

There are several mouse models to study carcinogenesis of prostate cancer. The transgenic adenocarcinoma mouse prostate (TRAMP) model is one of the widely used *in vivo* system (107). In this model, the SV40 large and small T antigens are expressed

under the androgen-dependent control of the rat probasin promoter in the prostatic epithelium (107, 108). It provides a convincing animal model system to study the progression of prostate cancer. TRAMP mice progressively develop intraepithelial neoplasia (PIN), adenocarcinoma and subsequent metastasis with similar pathology of human prostate cancer (109). In this study, I evaluated the expression of TLR4 and TLR5 during prostate cancer progression in TRAMP model with same genetic and environmental background.

1.2 MATERIALS AND METHODS

1.2.1 Mice

TRAMP mice, a well characterized transgenic mouse model of prostate cancer are well described in the literature (107). TRAMP mice with a C57BL/6 background and wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and Koatech (Pyeongtaek, Kyounggi-do, Korea) respectively. Mice were housed in a temperature-controlled room with a 12 h light:dark cycle and maintained at a constant temperature of 24 ± 1°C and humidity of 55 ± 10%. All animal studies were approved by the Institutional Animal Care and Use Committee in Seoul National University, and all associated regulations were closely adhered to.

1.2.2 Histology

Age-matched WT C57BL/6 and TRAMP mice were sacrificed at 28-32 weeks of age. Urogenital tissues including the urinary bladder, seminal vesicle, and prostate were obstained, fixed in 10% formalin for 24 h, and processed in a standard alcohol-xylene series. The tissues were then embedded in paraffin, and 3 μm sections were prepared and stained with hematoxylin and eosin (H&E, Sigma-Aldrich Co, St. Louis, MO, USA). Prostate samples classified as normal, low-grade prostate intraepithelial neoplasia (PIN), high-grade PIN, adenocarcinoma, and phylloides-like cancer according to Gleason grades (106).

1.2.3 Immunohistochemistry

Paraffin sections were blocked with 3% hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) for 10 min to remove endogenous peroxidase and blocked with 10% normal goat serum. Paraffin sections were blocked with 3% H_2O_2 in PBS for 10 min to remove endogenous peroxidase, then blocked with 10% normal goat serum in PBS for 30 min. Next, the sections were incubated overnight in a solution containing diluted rabbit or mouse IgG isotype, anti-cytokeratin 8 (1：200 dilution; Covance, USA), anti-synaptophysin (1：200 dilution; Abcam, UK), anti-TLR4 (1：200 dilution; Imgenex, USA) or anti-TLR5 antibody (1：200 dilution; Imgenex) at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG or anti-mouse IgG and streptavidin peroxidase complex (Vector Laboratories, USA). These sections were then visualized with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer and counterstained with methyl green (Sigma-Aldrich Co). Three fields of epithelial area from each view image at magnifications of 100 and 400× were selected at random, and the intensity was determined using the ImageJ software (ver. 1.45p; NIH, USA).

1.2.4 Statistical analysis

The differences among the mean values of the different groups were assessed, and all data are expressed as the mean ± SD. Statistical calculations consisted of Pearson's Chi-squared test and one-way ANOVA followed by Tukey's multiple comparison test for multi-group comparisons using GraphPad Prism (ver. 5.01; GraphPad Software, USA). *P* values < 0.05 were considered statistically significant.

1.3 RESULTS

1.3.1 Pathologic grade of TRAMP prostate

I first determined the pathologic grade of prostate from WT and TRAMP mice as described previously (109-111). Normal prostate glands of WT C57BL/6 mice were lined by a monolayer of cuboidal to columnar epithelial cells with basally oriented nuclei (Figure 1-1a). However, the prostate glands of TRAMP mice consisted of foci with multilayer of atypical cells in the epithelium or in the lumen of the duct (Figure 1-1b-e). The prostate glands showing large foci with two or three layers of atypical cells without luminal involvement were classified as low-grade intraepithelial neoplasia (PIN) and those showing foci with multi layers of atypical cells without luminal involvement were classified as high-grade PIN. In high-grade PIN, prostate gland showed epithelial crowing and stratification including variation of nuclear shape, chromatin condensation, and an increase of nuclear-to-cytoplasmic ratio (Figure 1-1c). Poorly differentiated adenocarcinoma (AC) was characterized by anaplastic sheets of cells containing pleomorphic cells with irregular nuclei (Figure 1-1d). In addition to pathologic grade of prostate, TRAMP mice developed phylloides-like cancer (PLC) in seminal vesicles. The neoplastic stromal cells of PLC emerged multicentrically just beneath the cuboidal to columnar epithelium (Figure 1-1e).

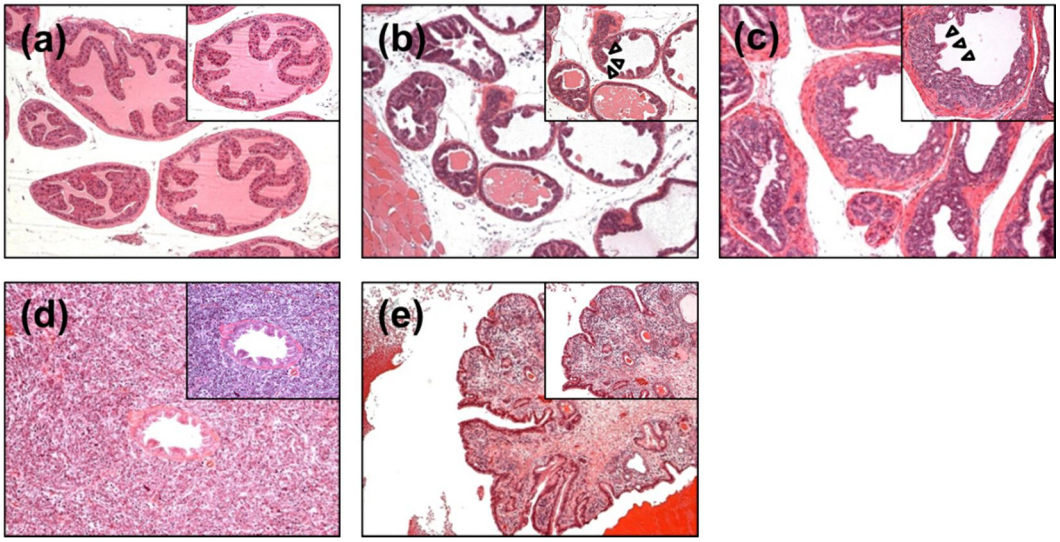


Figure 1-1. Pathologic grade of transgenic adenocarcinoma of mouse prostate (TRAMP). Prostate tissues from wild-type (WT) and TRAMP mice were fixed in 10% formalin for 24 h, then processed in a standard alcohol-xylene series. The tissues were subsequently embedded in paraffin, after which 3μm sections were prepared and stained with hematoxylin and eosin. Images of normal prostate tissue from WT mice (a) and low-grade prostate intraepithelial neoplasia (PIN) (b), high-grade PIN (c), poorly differentiated adenocarcinoma (d) and phylloides-like cancer (e) from TRAMP mice. Magnification: 400× (a~e), 100× (inset).

1.3.2 Expression of cytokeratin 8 and synaptophysin in TRAMP prostate

As recent consensus report on mouse models of human prostate cancer demonstrated that TRAMP mice have a high incidence of neuroendocrine phenotype arising in the prostate (112), I determined the expression of cytokeratin 8 (marker of epithelial differentiation) and synaptophysin (marker of neuroendocrine differentiation) in TRAMP prostate. Normal and TRAMP prostate epithelium from all pathologic grade showed strong expression of cytokeratin 8 (Figure 1-2a~d). However, synaptophysin was occasionally detected only in single cells located on prostate epithelium of high-grade PIN (Figure 1-3c) and on normal prostate epithelium in poorly differentiated adenocarcinoma tissue (Figure 1-3d). These IHC data showed that prostate tissues from all pathologic grade examined show epithelial phenotype.

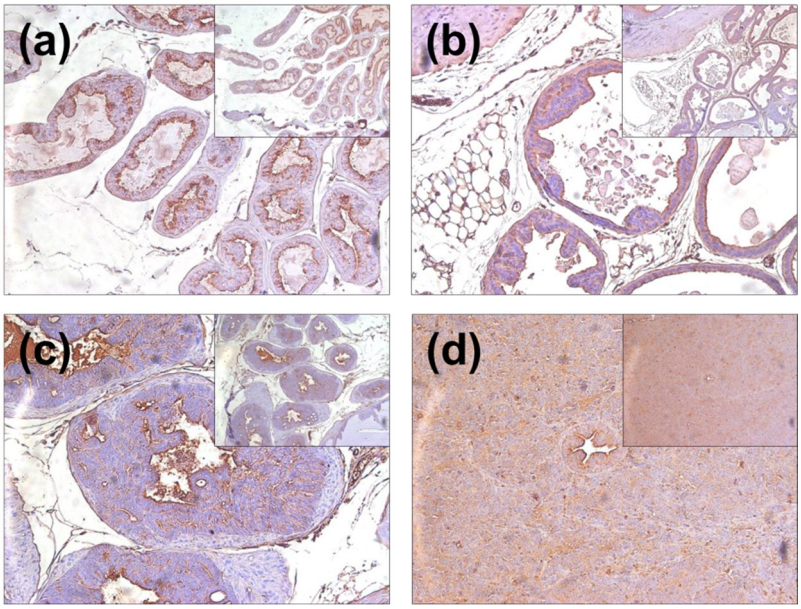


Figure 1-2. Cytokeratin 8 expression in pathologic grades of TRAMP. Sections from Figure 1-1 were incubated with anti-cytokeratin 8 antibody. (a) Images of normal prostate. (b) Low-grade PIN. (c) High-grade PIN. (d) Poorly differentiated adenocarcinoma. Magnification: 400× (a~d), 100× (inset).

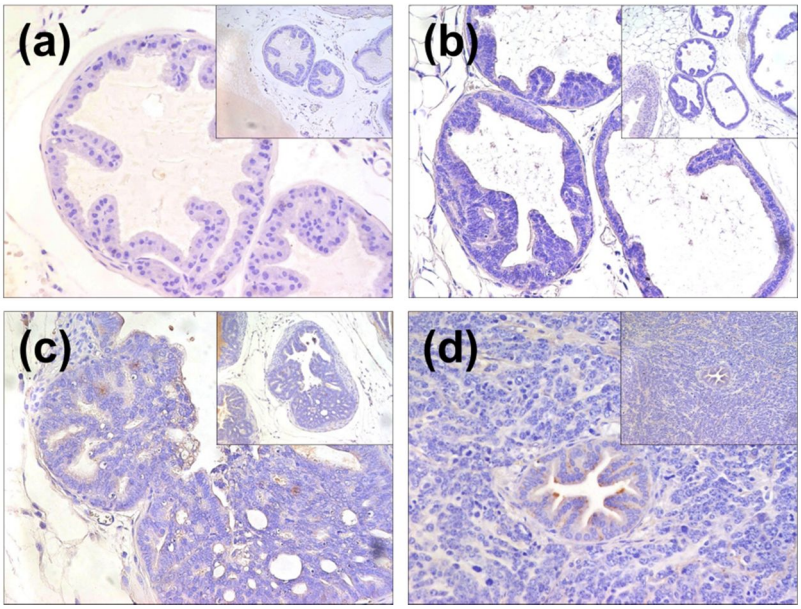


Figure 1-3. Synaptophysin expression in pathologic grades of TRAMP prostate. Sections from Figure 1-1 were incubated with antisynaptophysin antibody. (a) Images of normal prostate. (b) Low-grade PIN. (c) High-grade PIN. (d) Poorly differentiated adenocarcinoma. Magnification: 400× (a~d), 100× (inset).

1.3.3 Expression of TLR4 and TLR5 in TRAMP prostate

Next, I evaluated the expression of TLR4 and TLR5 in prostate tissues from WT and TRAMP mice with various pathologic grades. TLR4 was strongly expressed in the normal prostate epithelia of WT mice (Figure 1-4a and e). However, intensity of TLR4 immunostaining in the prostate tissue from TRAMP mice was gradually decreased as pathologic grade becomes more aggressive ($P < 0.001$) (Figure 1-4b~e). Both low- and high-grade PIN tissues showed reduced expression of TLR4 and TLR4 expression of AC was very weak (Figure 1-4b~e).

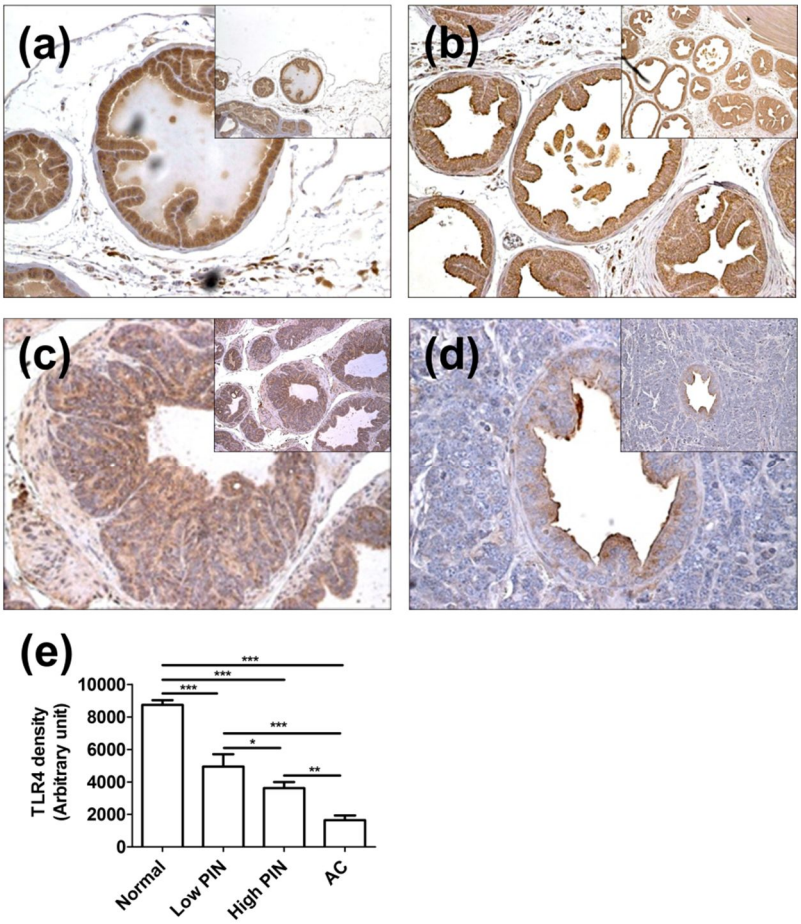


Figure 1-4. TLR4 expression in pathologic grades of TRAMP prostate. Sections from Figure 1-1 were incubated with anti-TLR4 antibody. (a) Images of normal prostate. (b) Low-grade PIN. (c) High-grade PIN. (d) Poorly differentiated adenocarcinoma. The intensity of TLR4 immunostaining in each pathologic grade is shown (e). Intensity of TLR4 immunostaining was measured in an average of three fields and is presented as the mean ± SD (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Normal, normal prostate; Low PIN, low-grade PIN; High PIN, high-grade PIN; AC, poorly differentiated adenocarcinoma. Magnification: 400× (a~d), 100× (inset).

TLR5 was moderately expressed in normal prostate (Figure 1-5a) but decreased during progression of prostate transformation ($P < 0.001$). Low-grade PIN, high-grade PIN and adenocarcinoma from TRAMP mice showed reduced expression of TLR5 compared with normal prostate tissue although there was no significant difference between low-grade PIN, high-grade PIN and AC (Figure 1-5b~e). A comparison of TLR4 and TLR5 expression by measuring intensity of immunostaining using an image analyzer is shown (Figure 1-4e and 1-5e). Staining with IgG antibody was not detected in prostate epithelial cells (data not shown).

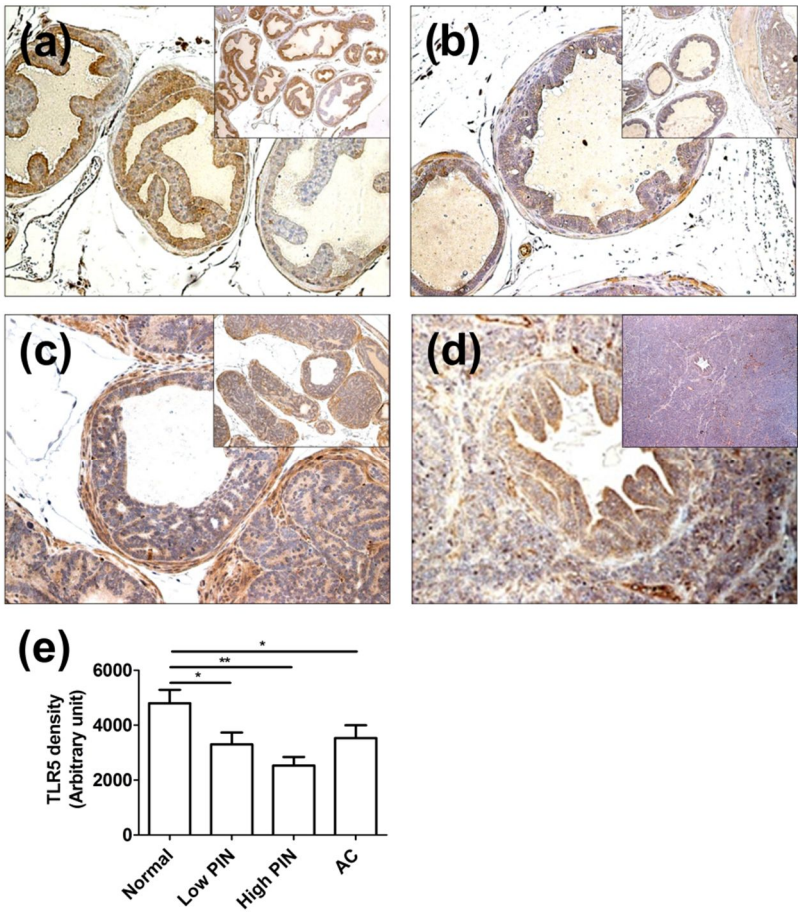


Figure 1-5. **TLR5 expression in pathologic grades of TRAMP prostate.** Sections from Figure 1-1 were incubated with anti-TLR5 antibody. (a) Images of normal prostate. (b) Low-grade PIN. (c) High-grade PIN. (d) Adenocarcinoma. The intensity of TLR5 immunostaining in each pathologic grade is shown (e). Intensity of TLR5 immunostaining was measured in an average of three fields and is presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$. (a~d) 400× magnification, (inset) 100× magnification.

1.4 DISCUSSION

My IHC results showed that the expression of TLR4 and TLR5 was decreased as the tumor progresses in TRAMP prostate. This reduced expression of TLR4 and TLR5 in transformed prostate may provide tumor cells with one strategy to escape host immune attack because TLRs on tumor cells can induce antitumor immune response as mentioned above. On the other hand, the loss of differentiation in aggressive cells during tumorigenesis might lead to reduced expression of TLR4 and TLR5.

In human prostate cancer cells, TLR4 promoted an immune escape, survival and progression of malignancy. LPS treatment enhance metastasis of human prostate cancer cell line PC3 by promoting VEGF and TGF-β_1 expression (113) and tumor-derived peroxiredoxin 1 mediates tumor metastasis via TLR4 providing a mechanistic link between inflammation and TLR4 in prostate carcinogenesis (99). However, in accordance with my results, two independent studies showed that the percentage of epithelial cells expressing TLR4 is gradually reduced as pathologic grade increases in human prostate tissues (5) and that stimulation of prostate cancer cell MAT-LU with LPS *in vitro*, before inoculation, produces significant inhibition of tumor growth in Copenhagen rats (8). Although these discrepancies among studies could not be explained clearly, diversity of *in vivo* microenvironment including stroma-derived factors and immune cells might be an important factor to be explored.

The effect of TLR5 stimulation on prostate tumorigenesis has not been well studied. Galli *et al*. showed that TLR5 stimulation on human prostate cancer cell triggered the production of inflammatory cytokines and chemokines, which mediate

recruitment of inflammatory cells (4) and Konig *et al.* showed the decreased level of TLR5 mRNA in human prostate cancer tissue compared with that of benign prostate hyperplasia (104). Although the prostate epithelial expression of TLR5 was not defined in the latter because they showed TLR5 mRNA expression in crude prostate tissues, this result is in the line with my IHC results. Regarding the possible inhibitory function of TLR5 in cancer cells, Rhee *et al.* showed that blocking of TLR5-mediated signaling in human colon cancer xenografts was associated with enhanced tumor growth (63) and activation of TLR5 in breast cancer cells inhibited cell proliferation (64).

Recent consensus report on mouse models of human prostate cancer demonstrated that TRAMP mice have a high incidence of neuroendocrine tumor arising in the prostate that are high metastatic to lung, liver and other tissues (112). However, the origin of neuroendocrine malignancy remains controversial. Kaplan-Lefko *et al.* concluded that the poorly differentiated adenocarcinoma expressed neuroendocrine features as a consequence of a phenotypic switch as a function of cancer progression (109). In addition, Yuan *et al.* suggested that prostate cancer cells undergo a transdifferentiation process to become neuroendocrine-like cells, which acquire the neuroendocrine phenotype and express neuroendocrine markers in review paper (114). However, Chiaverotti *et al.* suggested that neuroendocrine carcinomas arise independently from atypical hyperplasias and may develop from bipotential progenitor cells at an early stage of prostate tumorigenesis by transplantation studies using TRAMP prostatic ducts (115). My IHC data showed that normal prostate, low-grad PIN, high-grad PIN and poorly differentiated adenocarcinoma tissue expressed cytokeratin 8 but not

synaptophysin demonstrating epithelial origin of poorly differentiated adenocarcinoma cells. Because epithelial to neuroendocrine transdifferentiation is thought to be a stochastic event related to prostate cancer progression in TRAMP, the absence of synaptophysin expression on poorly differentiated adenocarcinoma cells may explained by insufficient progression of prostate epithelial malignancy to show neuendocrine phenotype.

In my study, the expression of TLR4 and TLR5 in TRAMP prostate was decreased as pathologic grade became more aggressive, which could be due to loss of differentiation during tumor progression and may provide tumor cells with a strategy to escape host immune surveillance suggesting that TLR4 and TLR5 may contribute to antitumor immune response.

CHAPTER II

TLR7 Expression is Decreased during Tumor Progression in TRAMP Mice and Its Activation Inhibits Growth of Prostate Cancer Cells

2.1 INTRODUCTION

TLR7 has been well-described as being able to recognize both synthetic imidazoquinoline components (e.g., imiquimod, resiquimod) and viral ssRNA (116). Furthermore, TLR7 is highly expressed in plasmocytoid dendritic cells (pDCs), and its agonists lead to the induction of type I interferons but not conventional DCs in these cells (116, 117). Moreover, TLR7 agonists exert a potent antitumor activity by inducing immune activation *in vivo* and apoptotic cell death in various cell types, including cancer cells (116). TLR7 expression and function on tumorigenesis have been examined in several cancers including non-small cell lung cancer (NSCLC) and esophageal squamous cell carcinoma (SCC). TLR7 expression significantly increases in NSCLCs, as compared with normal bronchoscopic controls (118). TLR7 ligation with agonists promotes the survival of human lung cancer cells and induces chemoresistance (66). In one esophageal SCC study, only 9.2% of normal controls were TLR7 positive, in contrast to greater than 50% of cases with esophageal SCC (119). Moreover, the percentage of TLR7 positivity gradually increases according to pathologic grade, with 46.7% of well-differentiated SCCs, 65.6% of moderate-differentiated SCCs, and 89.3% of poorly-differentiated SCCs found to be TLR7 positive (119). In human pancreatic cancers, TLR7 is highly expressed in inflammatory and epithelial cells, as compared with normal human pancreas (120). TLR7 ligation accelerates pancreatic tumorigenesis in mice and leads to altered expression of tumor suppressor and oncogenic protein (120). Conversely, TLR7 expression is lower in hyperplastic or tubulovillous polyps from patients who developed colorectal cancer (CC), as compared with those from patients who did not develop CC

(121).

However, the role of TLR7 on prostatic tumor development has yet to be clarified. Accordingly, this study examines TLR7 expression in prostate cancer cells and during tumor progression in TRAMP (the transgenic adenocarcinoma of mouse prostate) mice and its role on cell growth.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture and reagents

The nontumorigenic human prostate epithelial cell line RWPE-1, the metastasized human prostate cancer cell lines PC3 and DU145, and transgenic adenocarcinoma of mouse prostate (TRAMP) model-derived prostatic epithelial cell line TRAMP-C2 were purchased from the American Type Culture Collection (Manassas**,** VA, USA). The RWPE-1 cells were cultured in keratinocyte serum-free medium (KSFM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with 50 mg/L bovine pituitary extract, 5% L-glutamine and 5 μg/l epidermal growth factor (EGF, GIBCO). The PC3 and DU145 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin. The TRAMP-C2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) with 10% FBS, 1% penicillin-streptomycin, 5% Nu-serum IV (Collaborative Biomedical Products), 5 μg/ml insulin (Sigma-Aldrich Co., St. Louis, MO, USA), and 10^{-8} M dihydrotestosterone (Sigma-Aldrich Co.). For bone marrow-derived macrophages (BMDMs) isolation, bone marrow from femur and tibia was extracted and dispersed in complete Iscove's Modified Dulbecco's Medium (IMDM) including 30% L929 cell culture supernatant, 10% FBS, 1 mM sodium pyruvate, 1× MEM non-essential amino acids, and 1% penicillin/ streptomycin. Bone marrow cells were cultured in 20 ml of complete IMDM in 150 mm culture dish in a 5% CO_2 incubator at 37 °C. At day 3, 10 ml of fresh media was added and the cells were incubated for additional 3 days.

Imiquimod was dissolved in ultrapure water as a stock solution at a concentration of 5 mg/ml. The dose of imiquimod was chosen on the basis of previously published studies (122, 123). Loxoribine was dissolved in DMSO as a stock solution at a concentration of 100 mM and its dose chosen on the basis of previously published studies (124, 125).

2.2.2 Mice

TRAMP mice are well described in the literature (107). TRAMP mice with a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). TLR7-deficient mice with a BALB/c background were a gift from S. Akira (Osaka University, Osaka, Japan). Wild-type (WT) C57BL/6 and BALB/c mice were obtained from Koatech (Pyeongtaek, Korea). All animal studies were approved by the Institutional Animal Care and Use Committee in Konyang University (Approval No. 1-2011-04-01), and all associated regulations were closely adhered to.

2.2.3 RT-PCR analysis

TRAMP-C2, PC3, and DU145 cells were treated imiquimod at a concentration of 10 μg/ml for 2, 4, and 8 h. Total RNA was then extracted from each cell using easy-BLUE (Intron biotechnology, Daejeon, Korea) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA for PCR analysis using the Power cDNA Synthesis Kit (Intron biotechnology) and target genes were amplified using AccuPower® HotStart PCR PreMix (Bioneer, Daejeon, KOREA)

with the following primer sets;

mouse TLR7;	F: 5'-CAAACTTCTGTAGACCGTCATGGG-3' R: 5'-AAGTACCGCAACTCTCTCAACGG-3'
mouse IFN-β;	F: 5'-ATGAACTCCACCAGCAGACAG-3' R: 5'-ACCACCATCCAGGCGTAG-3'
mouse β-actin;	F: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' R: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'
human TLR7;	F: 5'-AAA CGA TAG GGA CGG CTG TGA CAT-3' R: ACA ACA TGT GCA TCA AGA GGC TGC-3'
human GAPDH;	F: 5'-GTCGGAGTCAACGGATT-3' R: 5'-AAGCTTCCCGTTCTCAG-3'

The PCR conditions were as follows: 1 cycle of 94°C for 5 min; 30-35 cycles of 94°C for 30 sec, 56-60°C for 30 sec, and 72°C for 30 sec; and 1 cycle of 72°C for 10 min. All PCR products were then electrophoresed on a 1.5% agarose gel and visualized using a gel documentation system.

2.2.4 Immunohistochemistry

Six-week-old WT and TLR7-deficient BALB/c mice were sacrificed, while WT C57BL/6 and age-matched TRAMP mice were sacrificed at 28-32 weeks of age. Urogenital tissues including the urinary bladder, seminal vesicle, and prostate were removed, fixed in 10% formalin for 24 h, and processed in a standard alcohol-xylene series. The tissues were then embedded in paraffin, and 3 μm sections were prepared, each of which was treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min to

remove endogenous peroxidase and blocked with 10% normal goat serum in PBS for 30 min. Next, the sections were incubated overnight in a solution containing diluted rabbit isotype IgG anti-TLR7 (1:200 dilution) at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (Vector, Burlingame, CA, USA) (128). These sections were then visualized with 3,3′-diaminobenzidine in 0.1 M Tris–HCl buffer and counterstained with hematoxylin (Sigma-Aldrich Co.). Three field of view images (×200 magnification) were stitched grey scale images. For determination of the DAB staining density, stained tissue was outlined and exported to individual images using ImageJ (1.45p, NIH, Bethesda)

2.2.5 Western blot analysis

TRAMP-C2 cells (5×10^4 cells/ml) were plated into 60 mm-cultures dish and incubated overnight. The cells were then stimulated with imiquimod (20 μg/ml), harvested, and lysed in a buffer containing 1% Nonidet-P40 supplemented with complete protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol. The resulting lysates were resolved by SDS-PAGE, transferred to polyvinyldifluoride membranes, and immunoblotted with primary antibodies. Antibodies to IκB-α, p38, ERK1/2, and JNK (phosphorylated and unphosphorylated forms) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies to TLR7 (Imgenex, San Diego, CA, USA), IRF-7 (abcam, Cambridge, MA, USA), and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were also used. After immunoblotting with secondary antibodies, proteins were visualized via an enhanced chemiluminescence (ECL) reagent (Intron

Biotechnology, Seongnam, Korea).

2.2.6 Measurement of cytokines and chemokines

For all experiments pertaining to cytokines/chemokines production, cells were seeded into 48-well culture plates and incubated overnight. The cells were then treated with the indicated doses of imiquimod or loxoribine for 24 h, after which the culture supernatants were collected for further analysis. The culture supernatant concentrations of IL-6, CXCL1, CCL2, and CXCL2 were determined using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA).

2.2.7 MTT assay

The effects of TLR7 ligand loxoribine (500-1000 nM) on TRAMP-C2 cells growth were determined by MTT (3-[4,5-dimethylhiazol-2-yl]- 2,5-diphenyltetrazolium bromide, AMRESCO®, solon, OH, USA) assay. TRAMP-C2 cells were plated into 48-well tissue culture plates at concentration of 1×10^4 cells/well. After 12 h, TRAMP-C2 cells were pretreated with 100 nM of chloroquine (Sigma-Aldrich Co.) for 2 h and then loxoribine (1000nM) was added to the medium with chloroquine for 72 h. The cell culture supernatant was removed and cells were incubated with 5 mg/ml MTT solution for 4h. At the end of incubation, MTT solution was aspirated off and then cells were mixed with dimethyl sulfoxide (DMSO, AMRESCO®). The dye absorption was quantified using an automatic microplate spectrophotometer (BERTHOLD technologies GmbH, Vienna, Austria) at 540 nm.

2.2.8 Crystal violet assay

The intensity of crystal violet staining is directly proportional to the numbe r of adherent cells. In order to observe the long-term anti-proliferative effect, TRAMP-C2 cells were seeded in flat-bottom six-well plates at 10^4 cells/well. After 12 h, TRAMP-C2 cells were treated with loxoribine and incubated for 9 days at 37 °C, with a media change at day 4. At day 9, the medium was removed carefully, 2 ml/well of 4% neutral formalin solution was added to fix the cells. And then, cell colonies were stain with crystal violet solution. After incubation at room temperature for 15 min, the crystal violet solution was poured off and the plates were washed with str eam of water. For TLR7 inhibitor treatment, TRAMP-C2 cells were pretreated with 100 nM of chloroquine for 2 h and then loxoribine (1000nM) was added to the medium with chloroquine for 9 days. Colony formation of cell was determined by crystal violet assay as described above.

2.2.9 Statistical analysis

The differences between the relative mean values of the different groups were derived and expressed as mean ± SD. All of the statistical calculations were performed by one-way ANOVA using GraphPad Prism version 5.01 (GraphPad Software, San Diego, California, USA). In all cases, values of $P < 0.05$ were considered statistically significant.

2.3 RESULTS

2.3.1 *In vivo* TLR7 expression in mouse prostate tissue and prostate epithelial cells

I first examined *in vivo* TLR7 expression in mouse prostate tissue using immunohistochemistry. No positive signal was seen in prostate sections exposed to isotype IgG in either WT or TLR7-deficient mice (Figure 2-1a; left and right upper panels). TLR7 protein was strongly expressed in normal prostate epithelia of WT mice but not in stroma (Figure 2-1a; left lower panel). A very weak positive signal was also seen in prostate epithelia of TLR7-deficient mice, though this was considered to represent a non-specific reaction (Figure 2-1a; right lower panel). Next, the gene expression of TLR7 was assessed in prostate epithelial cells via RT-PCR. TLR7 mRNA was highly expressed in mouse BMDMs (Figure 2-1b), which were used as a positive control. In contrast, TLR7 mRNA was only weakly expressed in the mouse prostate cancer cell line, TRAMP-C2 cells (Figure 2-1a). To determine whether TLR7 expression differs between tumorigenic and non-tumorigenic epithelial prostate cells, I compared gene expression between RWPE-1 cells (non-tumor) – a primary immortalized prostate epithelial cell line – and PC3 and DU145 cells, two prostate cancer cell lines. As displayed in Figure 2-1c, TLR7 mRNA was highly expressed in RWPE-1 cells but not in PC3 and DU145 cells. These results suggest that TLR7 is highly expressed in normal prostatic tissue but may be decreased in prostate cancer.

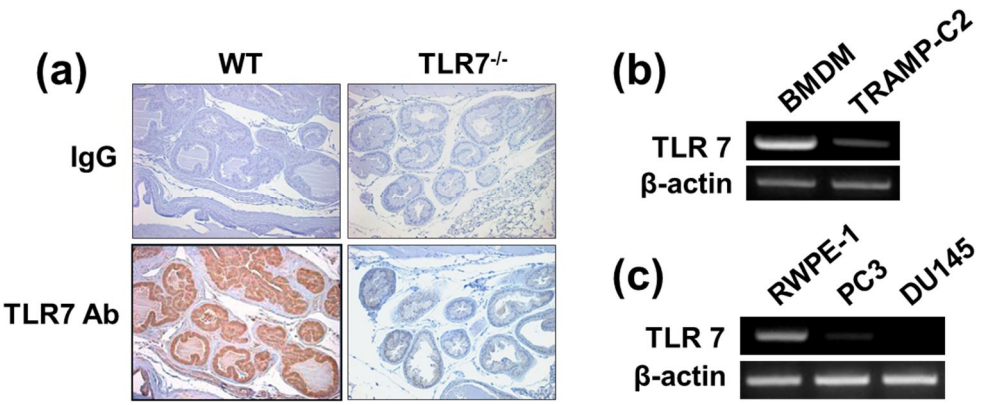


Figure 2-1. TLR7 expression in mouse prostate and prostate epithelial cells. Sections were prepared from the prostates of WT and TLR7-deficient mice and exposed to isotype IgG or TLR7 antibodies (a; 200×). RNA was extracted from mouse BMDMs and TRAMP-C2 cells, and TLR7 mRNA was assessed by RT-PCR (b). TLR7 gene expression was compared between a human primary and immortalized prostate epithelial cell line RWPE-1 and prostate cancer cell lines PC3 and DU145 (c). β-actin was used as a control for RNA loading (b and c).

2.3.2 TLR7 expression in TRAMP mice is dependent on pathological prostatic grade

Next, I attempted to determine TLR7 expression in prostatic tissue of TRAMP mice with various pathological grades. TLR7 was highly expressed in prostate epithelia of age-matched WT mice (Figure 2-2a), as well as in low grade of prostate intraepithelial neoplasia (PIN) (Figure 2-2b). TLR7 density was slightly decreased in high grade of PIN (Figure 2-2c). In phyllodes-like tumors, TLR7 was moderately expressed in marginal epithelia, though only weakly expressed in stroma with loosely arranged mesenchymal cells (Figure 2-2d). Conversely, the expression level of TLR7 was dramatically reduced in adenocarcinoma, where only very weak positive signal was detected (Figure 2-2e and f). Interestingly, TLR7 was moderately detectable in well-structured glands, even within adenocarcinoma lesions (Figure 2-2f). Degree of TLR7 expression was evaluated by measuring density by an image analyzer (Figure 2-2g). My findings suggest that TLR7 expression not only gradually decreases during prostate cancer progression, but also may reflect the pathologic severity.

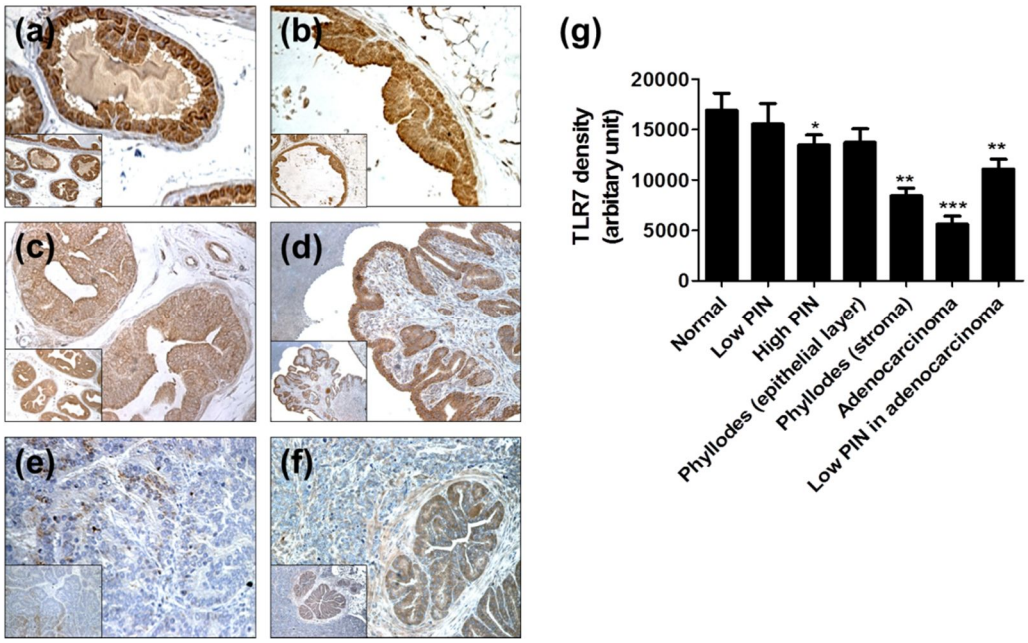


Figure 2-2. TLR7 expression in TRAMP mice is dependent on prostatic pathologic grade. WT and TRAMP mice were sacrificed at 28-32 weeks of age, and histological sections for prostatic tissues were prepared and exposed to TLR7 antibodies. Photographs of normal prostate (a), low-grade PIN (b), high-grade PIN (c), phyllodes-like tumor (d), and adenocarcinoma (e and f) were taken at a magnification of 200×. Small quadrangles within each photograph are at low magnification (100×). The density of TLR7 in each prostatic pathologic grade is shown (g). Density of TLR7 were measured in an average of tree fields and are presented as mean ± SD. *P<0.05, ** P <0.01, *** P <0.001. Normal; normal prostate, Low PIN; low-grade PIN, High PIN; high-grade PIN, PLT; phyllodes-like tumor. AG; adenocarcinoma.

2.3.3 TLR7 gene and protein expression is upregulated by its agonists in prostate cancer cells

I also assessed whether the gene and protein expression of TLR7 was affected by its agonists (imiquimod and loxoribine) in prostate cancer cells. Treatment with imiquimod increased the gene expression of TLR7 in TRAMP-C2 cells in a time-dependent fashion (Figure 2-3a). The associated gene expression in two human prostate cancer cell lines (PC3 and DU145 cells) was also assessed. Although TLR7 mRNA was not detected in intact PC3 and DU145 cells, it was shown to be upregulated by imiquimod in both cell types 2 to 4 h after treatment (Figure 2-3b and c, respectively). TLR7 protein expression was also enhanced by imiquimod, although its level was very weak in intact TRAMP-C2 cells (Figure 2-3d). Protein from spleen of WT mice was used as positive control for TLR7 expression. In addition, loxoribine increased the gene and protein expression of TLR7 in TRAMP-C2 cells (Figure 2-3e and f). These results indicate that TLR7 expression can be upregulated in prostate cancer cells via agonist stimulation.

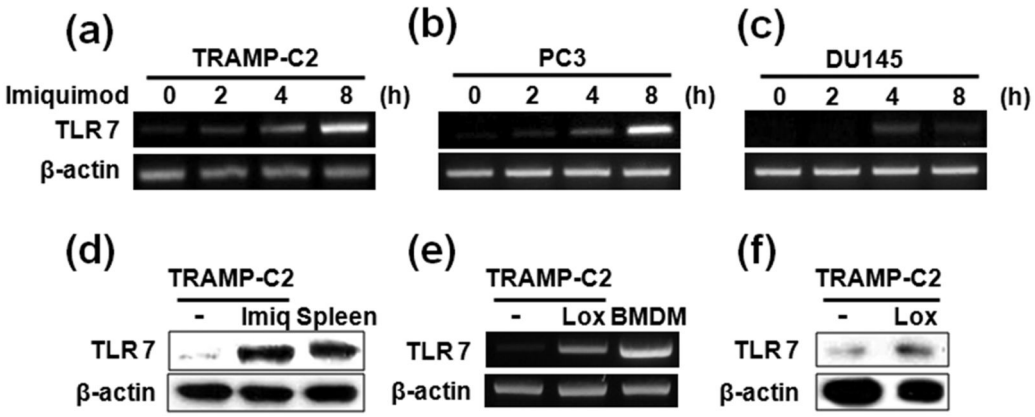


Figure 2-3. TLR7 ligands enhance the gene and protein expression of TLR7 in prostate cancer cells. Mouse or human prostate cancer cells were stimulated with TLR7 liands, imiquimod (10 μg/ml) and loxoribine (1000nM) (a to f). The mRNA expression of TLR7 was examined by RT-PCR (a to c, and e) and protein levels were determined using TLR7 Antibody (d and f). Protein extracted from spleen (d) and mRNA from BMDM (e) were used as a positive control.

2.3.4 TLR7 agonists enhance proinflammatory cytokine and chemokine production and gene expression of IFN-β in prostate cancer cells

To assess whether the production of these proinflammatory cytokines and chemokines in prostate cancer cells is related to TLR7 stimulation, cells were treated with various doses of imiquimod or loxoribine for 24 h. After this period, the amounts of the previously specified cytokines and chemokines in the culture supernatant were quantified via ELISA. My results show that imiquimod enhances the production of IL-6, CXCL1, CXCL2, and CCL2 in TRAMP-C2 cells at doses greater than 5 μg/ml (Figure 2-4a~d). Loxoribine also increased the production of IL-6, CXCL1, and CXCL2 in TRAMP-C2 cells in a dose-dependent manner (Figure 2-4e~g). Imiquimod was able to increase IL-6 production in human prostate cancer cells (Figure 2-4h). Moreover, imiquimod induces the gene expression of IFN-β in TRAMP-C2 cells (Figure 2-4i). It seems likely that TLR7 activation induces the immune response in prostate epithelial cells.

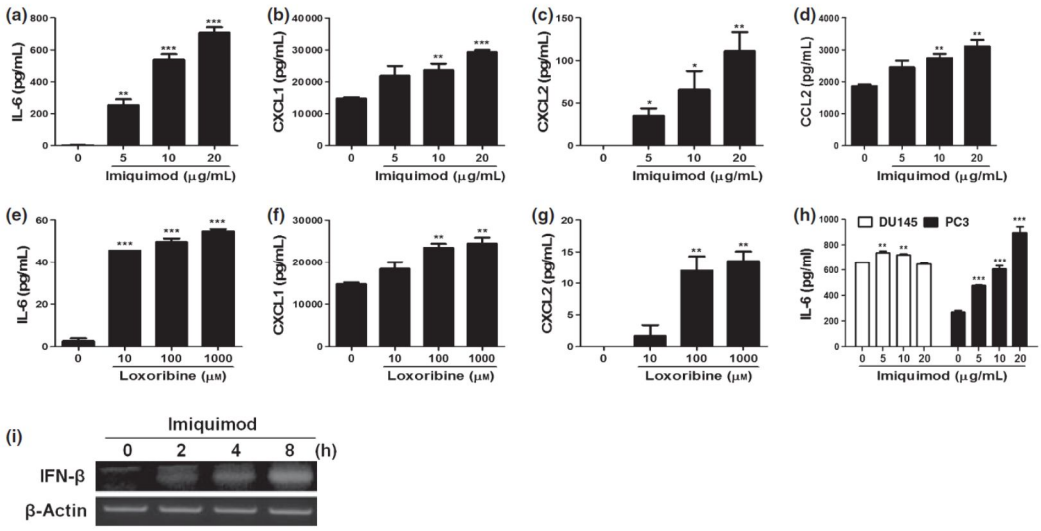


Figure 2-4. TLR7 agonists augment proinflammatory cytokine and chemokine production in prostate cancer cells. TRAMP-C2 cells were treated with the indicated doses of imiquimod or loxoribine for 24 hr (a~g). PC3 and DU145 cells were also stimulated with imiquimod at various doses (h). The production of IL-6, CXCL1, CXCL2, and CCL2 was assessed by ELISA in the culture supernatant. The results presented here are from one representative experiment of three independent experiments and are presented as mean ± SD. The cells were also treated with 10 μg/ml of imiquimod, and RNA was extracted at indicated times after treatment (i). The gene expression of IFN-β was determined by RT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001.

2.3.5 Loxoribine inhibits growth and colony formation of TRAMP-C2 cells via TLR7-dependent pathway

I finally examined whether TLR7 stimulation affects growth and colony formation of prostate cancer cells. TRAMP-C2 cells were seeded in a 48-well plate (1×10^4 cells/well) and cultured for 3 days at the absence or presence of loxoribine. MTT assay showed that treatment with loxoribine reduced growth of TRAMP-C2 cells dose-dependently, which was restored by an endosomal TLRs inhibitor, chloroquine (Figure 2-5). Moreover, the ability of colony formation was decreased by loxoribine in a dose-dependent manner (Figure 2-6a and b). Chloroquine also restored loxoribine-induced inhibition of colony formation (Figure 2-6c). These results indicate that TLR7 activation may inhibit growth and colony formation of prostate cancer cells.

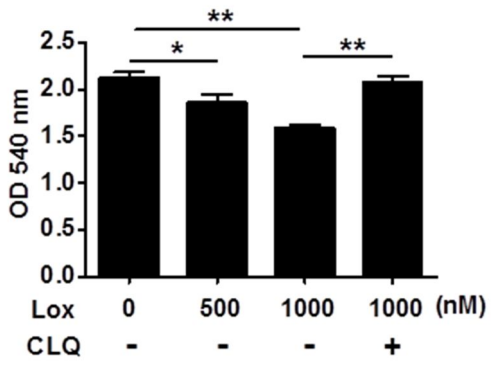


Figure 2-5. Loxoribine inhibits growth of TRAMP-C2 cells via TLR7 dependent pathway. The effect of loxoribine on TRAMP-C2 cell growth was determined by MTT assay with or without chloroquine pre-treatment. The results presented here are from one representative experiment of three independent experiments and are presented as mean ± SD. *P < 0.05, **P < 0.01.

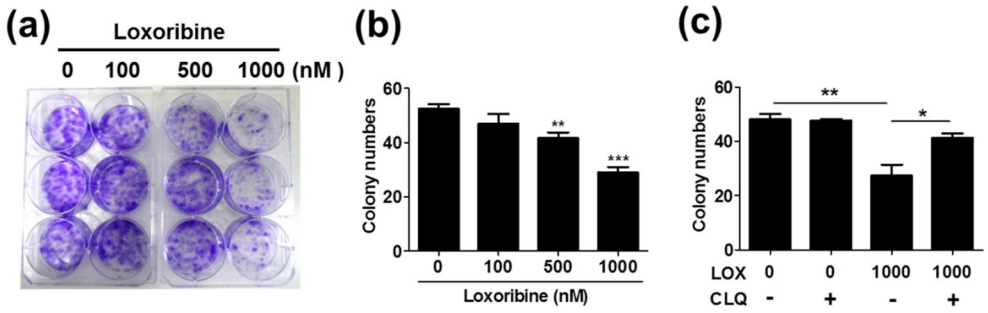


Figure 2-6. Loxoribine inhibits colony formation of TRAMP-C2 cells via TLR7-dependent pathway. The effect of loxoribine on TRAMP-C2 cell colony formation was determined by crystal violet assay with (c) or without (a and b) chloroquine pretreatment. The results presented here are from one representative experiment of three independent experiments and are presented as mean ± SD. **P*<0.05, ** *P* <0.01, *** *P* <0.001.

2.4 DISCUSSION

Previous studies have reported the expression of TLRs in prostate epithelial cells and tissues. Specifically, Galli *et al*. screened the gene expression of various TLRs in DU145, PC3, and LNCaP cells, the three most well-studied prostate cancer cell lines (4). Their results showed that the DU145 and LNCaP cells expressed high levels of TLR3 and TLR5, whereas the other TLRs – including TLR2, 4, 6, 7, 8, 9 – were either only minimally expressed or completely absent (4). In contrast, PC3 cells demonstrated strong expression of TLR2, 3, 4, 5, 6 but no expression of TLR7, 8, or 9 (4). Interestingly, TLR7 expression was not identified in any of the three prostate cancer cell lines. In the present study, TLR7 was highly expressed in RWPE-1 cells, a non-tumor prostate epithelial cell line. Conversely, TLR7 expression was completely undetectable in prostate cancer PC3 and DU145 cells, which was in accordance with the previous study (4). Moreover, TLR7 was only weakly expressed among TRAMP-C2 cells, even though high levels of TLR7 are expressed in normal prostate epithelia *in vivo*. These findings suggest that TLR7 expression may vary by cellular condition (normal *vs* cancer). In fact, a separate study by König *et al*. (2004) revealed that TLR7 gene expression was almost ubiquitous in most benign prostate hyperplasia (100%, 18/18), though relatively rare in prostate cancer specimens (5.6%, 1/18) (127). Similarly, my results show that TLR7 protein expression was relatively high in normal prostate epithelia or low grade of PIN and decreased in prostate adenocarcinomas from TRAMP mice. Taken together, it seems likely that TLR7 expression gradually reduces during prostatic tumor development, with the expression level dependent on prostate cancer malignancy.

Inflammation induced by microorganisms such as bacteria and viruses is considered a risk factor for prostate cancer, with evidence now showing that chronic infection with uropathogenic *E. coli* induces prostatic intraepithelial neoplasia or high-grade dysplasia in TLR4-defective C3H/HeOuJ mice (128, 129). Regarding virus-associated disease, the most common that infect human prostate tissue are polyomavirus, human papilloma virus (HPV), and cytomegalovirus. Viral nucleic acids are detected by endosomal TLRs – including TLR3, TLR7, and TLR9 – and have been associated with prostate cancer pathogenesis in several previous studies. The TLR3 agonist poly I:C has also been shown to induce prostate cancer cell apoptosis via a PKC-α-dependent pathway (98). A separate *in vivo* study showed that tumor growth resulting from s.c. injections of syngenic tumor cells (TRAMP-C2 cells) was upregulated in TLR3-deficient mice when compared to WT mice mice (130), while type I IFN was required for poly I:C-induced tumor growth suppression (130). König *et al*. (2003) showed that the expression of the cytokines and chemokine, such as Rantes, IL-8, MMP-2, MMP-9, and CCR-5, more pronounced in prostate cancer as compared to BPHb tissue (131). In the present study, TLR7 stimulation was shown to induce the production of various cytokines and chemokines, and upregulate INF-β gene expression. These findings suggest that TLR7 signaling may be associated with both prostate cancer pathogenesis as well as the host immune response to viral infection.

In my current study, as well as lower expression of TLR7 in adenocarcinoma than normal or various grades of PIN, TLR7 stimulation inhibited growth and colony formation of prostate cancer cells. It is likely that TLR7 may differently act on

tumorigenesis depending on tumor origin and further studies are clearly needed to better define the distinct role of TLR7 in prostatic tumorigenesis.

In vitro and *in vivo* studies often lead to conflict results depending on experimental conditions. For instance, several *in vitro* studies revealed that TLR4 signaling promotes tumor development and progression (6, 7, 96). In contrast, an *in vivo* study showed that TLR4 is associated with inhibition of tumor growth in a syngeneic rat model of prostate cancer (8). Tumor growth was decreased in rats s.c injected with LPS-stimulated prostate cancer cells (MAT-LU), as compared with animals injected with non-stimulated cells. They revealed that this effect is not due to a direct consequence of TLR4 signaling on the proliferation/apoptosis balance of the tumor cells (8). It seems that LPS stimulation induces chemokines production in prostate cancer cells, which result in alteration of phenotypic/functional pattern of tumor infiltrating lymphocytes *in vivo* (8). Therefore, *in vivo* study should be considered to determine the role of TLR7 on tumorigenesis of prostate cancer.

In conclusion, my results demonstrate that, while TLR7 is strongly expressed in normal prostate epithelia, its expression decreases during prostatic tumorigenesis. Additionally, I showed that TLR7 agonists enhances the gene expression and inhibits tumor cell growth and colony formation. My findings suggest that TLR7 may suppress prostate cancer cell proliferation.

CHAPTER III

In vitro and *in vivo* growth inhibition of prostate cancer by a small molecule imiquimod

3.1 INTRODUCTION

Prostate cancer is the most commonly diagnosed male malignancy and the second malignant cause of male death worldwide after lung cancer (9). Infectious agents, physical trauma, hormones, and a break of immune tolerance to prostate antigen are considered as the cause of prostate cancer (132). There are several therapeutic options such as radical prostatectomy, radiation, and hormonal therapy for prostate cancer (133). However, these protocols have a limitation for metastatic and hormone refractory prostate cancer. Hormonal therapy in the form of medical or surgical castration can induce significant long-term remissions, but androgen independent state patients are ultimately developed metastatic prostate cancer resulting in death due to widespread metastases (133-135). Chemotherapy is also effective but long term use is not feasible due to its toxicity (136). Therefore, the development of alternative therapeutics has been required for prostate cancer.

Imiquimod is a low-molecular-weight compound belonging to the imidazo-quinolines family. It was first identified as a compound that has anti-viral activity in guinea pigs infected with herpes simplex virus, and has been successfully used for the treatment of genital warts caused by human papilloma virus in the clinic (122, 137). In addition, recent studies have attracted considerable interest owing to their profound antitumoral activities (138-140). Imiquimod exerts antitumor effect by activating immune response to suppress tumor growth in a variety of transplantable tumors including (138). As well, it also has direct proapoptotic activity against various tumor cell populations *in vitro* and *in vivo* (141, 142). The 5% imiquimod cream (Aldara) that is commercially

available has been successfully used for the treatment of several cancers including basal cell carcinomas and melanoma (140, 143-145).

Antitumor effects of the imidazoquinolines family have been demonstrated in urogenital cancers including bladder cancer and renal cell carcinoma (126, 146-149). Treatment with an imidazoquinoline (3M-011) down-regulated c-Myc expression in bladder cancer cells and reduced its transcriptional activity (149). It also significantly suppressed *in vivo* tumor growth in mice model for orthotopic bladder cancer (149). Imiquimod also induced apoptosis and cytokines production in various bladder cancer cell lines and effectively inhibited *in vivo* tumor growth (126). An imidazoquinoline also enhanced *in vivo* apoptosis and increased lymphocytic infiltration and proinflammatory cytokines production in mice model of renal cell carcinoma (150). However, the effect of imidazoquinolines on the growth of prostate cancer has not been studied. Therefore, in this study, I evaluated *in vitro* and *in vivo* antitumor effect of an imidazoquinoline, imiquimod, against prostate cancer.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Specific pathogen-free (SPF) C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). All animal studies were approved and followed by the regulations of the Institutional Animal Care and Use Committee in Konyang University.

3.2.2 Cell lines and reagents

Transgenic adenocarcinoma of the mouse prostate (TRAMP) model-derived prostatic epithelial cell line (TRAMP-C2) (108) and the metastatic human prostate cancer cell line (PC3) were purchased from the American Type Culture Collection (Manassas, VA, USA). TRAMP-C2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, San Diego, CA, USA) with 10% fetal bovine serum, 1% penicillin-streptomycin, 5% Nu-serum IV (Collaborative Biomedical Products, Bedford, MA, USA), 5 μg/ml insulin (Sigma-Aldrich Co., St. Louis, MO) and 10 nM dihydrotestosterone (Sigma-Aldrich Co.) (151). PC3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin. Imiquimod was purchased from InvivoGen (San Diego, CA, USA) and dissolved in ultrapure water as a stock solution at a concentration of 5 mg/ml.

3.2.3 MTT assay

The effects of imiquimod (1-10 μg/ml) on TRAMP-C2 cells growth were

determined by MTT (3-[4,5-dimethylhiazol-2-yl]- 2,5-diphenyltetrazolium bromide, AMRESCO®, solon, OH, USA) assay. TRAMP-C2 cells were plated into 48-well tissue culture plates at concentration of 5×10^4 cells/well. After 12 h, TRAMP-C2 cells were treated with various concentration of imiquimod for 24, 48, and 72 h. The cell culture supernatant was removed and cells were incubated with 5 mg/ml MTT solution for 4h. At the end of incubation, MTT solution was aspirated off and then cells were mixed with dimethyl sulfoxide (DMSO, AMRESCO®). The dye absorption was quantified using an automatic microplate spectrophotometer (BERTHOLD technologies GmbH, Vienna, Austria) at 540 nm. For TLR7 inhibitor treatment, TRAMP-C2 cells were pretreated with various concentration chloroquine for 2 h and then imiquimod (10 μg/ml) was added to the medium with chloroquine for 72 h. Cell growth was determined by MTT assay as described above.

3.2.4 Flow cytometry

Imiquimod-treated TRAMP-C2 cells were stained with PI (Sigma-Aldrich Co.) or PI/Annexin-V-FITC (BD Bioscience, Franklin Lakes, NJ) and analyzed by flow cytometry (BD LSR flow cytometer, San Jose, CA, USA).

3.2.5 Western blot analysis

TRAMP-C2 cells were plated into 60 mm-culture dish about 2×10^5 cells/well. After overnight incubation, the culture medium was replaced with fresh media and cells were treated with imiquimod (20 μg/ml). The cells were lysed 0, 12, 24 and 48 h after

stimulation using lysis buffer with 1% Nonidet-P40, complete protease inhibitor cocktail (Roche, Basel, Swiss) and 2 mM dithiothreitol. Cell lysates were incubated on ice for 30 min and centrifuged for 15 min at 13,000 rpm. The protein-containing supernatant was harvested and the total protein amount was quantified using Bradford assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates were added with sample buffer and loaded onto a 10 or 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane and detected with the following anti-bodies: cleaved- PARP, caspase-3, caspase-7, caspase-9, cyclinB1 and phospo-CDC2 (Abcam, Cambridge, UK), p21 (Cell signaling, Beverly, MA, USA), and anti–β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology). The blots were developed using ECL substrate (Thermo Scientific, Waltham, MA, USA).

3.2.6 Cytokines production

The culture supernatants of imiquimod-treated TRAMP-C2 cells with or without cloroquine pretreatment at the concentration of 1, 10, and 50 μg/ml were obtained and kept at -20 °C until cytokine measurement. The concentration of IL-6 in culture supernatants was determined using a commercial DuoSet ELISA Development kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions

3.2.7 *In vivo* antitumor efficacy

C57BL/6 mice received subcutaneous (SC) single injection of TRAMP-C2 cells (1×10^6 cells/mouse in 100 μl of injectable saline) into the shaven right flank, and tumor

growth was monitored. On day 10 after tumor implantation, imiquimod was injected intratumorally at 50 μg daily for 9 days. Tumors were injected in a different site for each treatment day. Tumor length (L) and width (W) were measured and tumor weight (WR) was calculated twice a week as follows: $WR = 1/2 \times L \times W^2$

3.2.8 Histological analysis

Tumor mass were removed, fixed in 10% formalin for 24 h, and processed in a standard alcohol-xylene series. The tissues were then embedded in paraffin, and 3 μm sections were prepared, each of which was stained with H&E (Sigma-Aldrich Co.).

3.2.9 TUNEL assay

Sections were stained for apoptotic cells by a modified terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) assay using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. And then sections were counterstained with Methyl Green (Sigma-Aldrich Co.).

2.2.10 Statistical analysis

All assays were derived from at least three independent experiments perform statistical comparisons among the different values using the Prism 5 GraphPad Prism software (San Diego, CA, USA). And data are presented as mean ± SD.

3.3 RESULTS

3.3.1 Imiquimod inhibits the growth of TRAMP-C2 cells

Inhibitory effect of imiquimod on the growth of TRAMP-C2 cells was examined by MTT assay. The growth curve of untreated cells increased rapidly by 48 h and slightly decreased at 72 h. Treatment of over 5 μg/ml of imiquimod delayed the cell growth by 48 h (Figure 3-1a). To determine more definite dose-dependent effect of imiquimod on the cell growth, the experiment was repeated with a narrow dose range of imiquimod. 1 and 2.5 μg/ml of imiquimod did not affect the cell growth by 48 h post-treatment (Figure 3-1b). However, the cell growth was more reduced by the treatment with 2.5 μg/ml of imiquimod at 72 h post-treatment, as compared with that in untreated cells (Figure 3-1b). In addition, the growth of PC3 cells, a human prostate cancer cell line, was inhibited by imiquimod at 72 h post-treatment in a dose-dependent manner (Figure 3-1c).

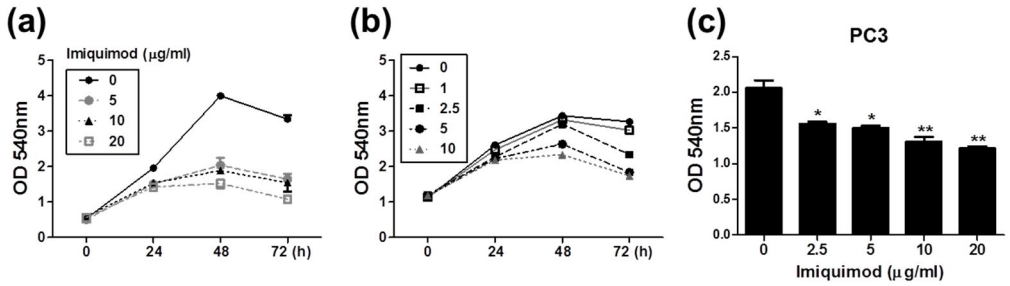


Figure 3-1. Imiquimod inhibits cell proliferation in prostate cancer cell lines. TRAMP-C2 (a, b) or PC3 (c) cells were treated with the indicated concentration of imiquimod for 24, 48, and 72 h. Cell growth inhibition was assessed by MTT assay at high dose (a) and low dose (b) of imiquimod treatment. The results presented here are from one representative experiment of three independent experiments and are presented as mean ± SD. **P*<0.05, ** *P* <0.01.

3.3.2 Imiquimod leads to G_2/M cell cycle arrest in TRAMP-C2 cells

I next investigated the effect of imiquimod on the cell cycle arrest in TRAMP-C2 cells. For this, the cells were treated with various doses of imiquimod for 48 h and DNA contents for cell cycle arrest were determined by FACS analysis. The cell percentage in G_1 phase was reduced in imiquimod-treated TRAMP-C2 cells (Figure 3-2a and b). In contrast, imiquimod increased the cell percentage in G_2/M phase dose-dependently, as compared with untreated cells (Figure 3-2a and b). In addition, Western blot analysis was performed to determine the changes of expression or activation of G_2/M cell cycle-specific markers. Results showed that the expression of cyclin B1, which promotes nuclear accumulation and initiation of mitosis (152), was decreased in TRAMP-C2 cells by imiquimod at 48 h post-treatment (Figure 3-2c). Moreover, imiquimod enhanced the phosphorylation of CDC2, which is a master regulatory kinase on the control of the G_2/M transition (153), from 6 h after treatment (Figure 3-2c). The expression of cell cycle regulator p21 was also increased by imiquimod from 12 h after treatment (Figure 3-2c). These findings suggest that imiquimod may suppress the growth of prostate cancer cells via G_2/M cell cycle arrest.

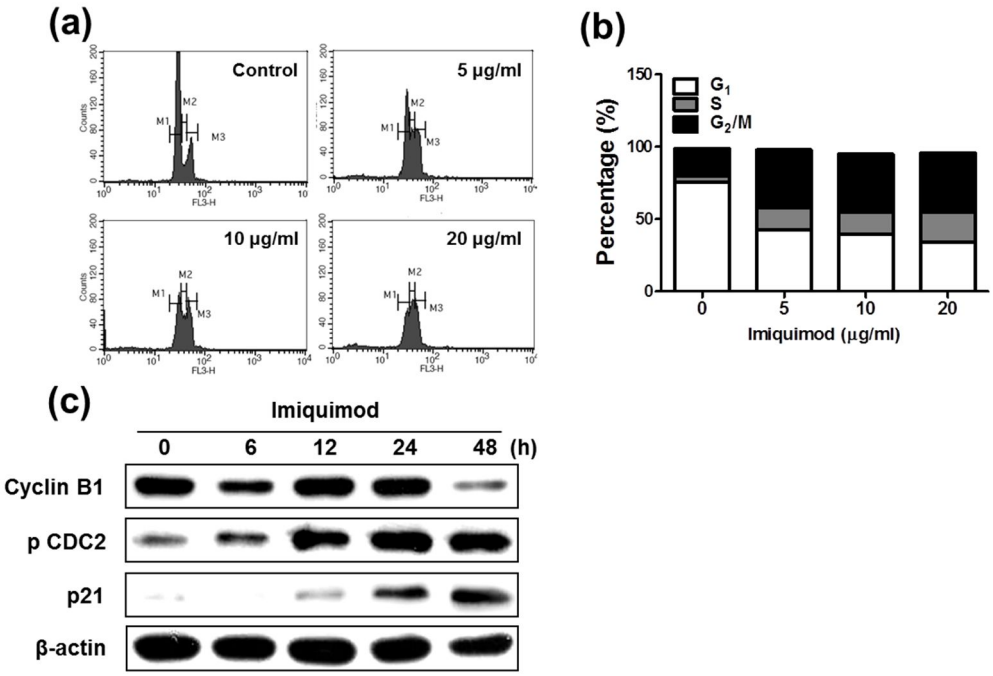


Figure 3-2. Imiquimod leads to G_2/M cell cycle arrest in TRAMP-C2 cells. TRAMP-C2 cells with or without imiquimod treatment were stained with PI and DNA contents were analyzed by flow cytometry (a). The percentage of cells in each phase of the cell cycle is shown (b). The protein level of cell cycle-related genes in the imiquimod-treated TRAMP-C2 cells was evaluated by Western blot (c).

3.3.3 Imiquimod induces mitochondria-dependent apoptosis in prostate cancer cells

Previous studies showed that imiquimod can induce direct apoptosis in various cancer cells (126, 142, 146, 148). To investigate whether imiquimod induces apoptosis in prostate cancer cells, TRAMP-C2 cells were treated with 10 or 20 μg/ml of imiquimod for 48 h, stained with PI and annexin V, and analyzed by flow cytometry. The percentage of annexin V-positive cells was increased by the treatment of imiquimod dose-dependently (Fig 3-3a). In addition, I examined the change of molecules associating with mitochondrial-dependent apoptosis pathway by Western blot analysis. An anti-apoptotic molecule, bcl-2 expression in TRAMP-C2 cells was reduced by imiquimod in a time-dependent manner (Figure 3-3b). Imiquimod also led to cleavage of caspase-9, caspase-3, and caspase-7 by 48 h (Figure 3-3b). Moreover, cleaved form of PARP was also increased by imiquimod in TRAMP-C2 cells (Figure 3-3b). These findings indicate that imiquimod may lead to apoptosis in prostate cancer cells via intrinsic pathway.

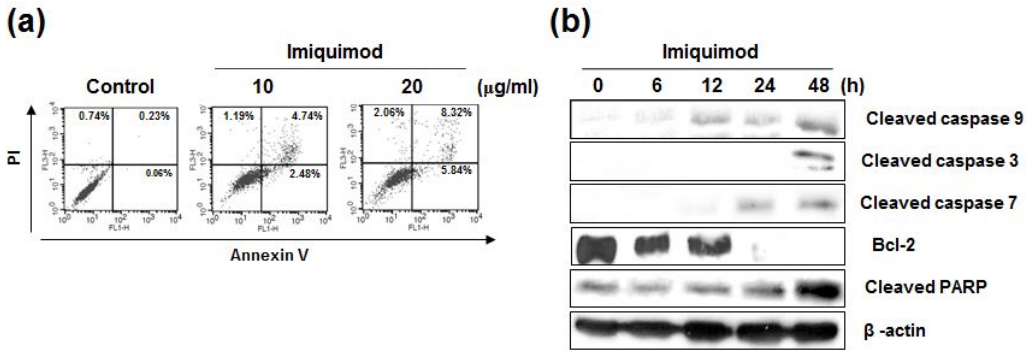


Figure 3-3. Imiquimod induces apoptosis in TRAMPC-2 cells. TRAMP-C2 cells with or without imiquimod treatment were stained with PI and Annexin-V-FITC and analyzed by flow cytometry (a). The protein level of cell apoptosis-related genes in the imiquimod-treated TRAMP-C2 cells was evaluated by Western blot (b).

3.3.4 A TLR7 inhibitor chloroquine did not restore the growth inhibition by imiquimod in prostate cancer cells

Although imiquimod has been well-described as TLR7 agonist, it is still unclear whether TLR7 is required for its antitumor effect. To clarify this, I performed an inhibitor assay using chloroquine, which is an anti-malarial drug to block activation of endosomal TLRs such as TLR3, 7, and 9 by inhibiting endosomal acidification (154). My preliminary experiment showed that imiquimod enhanced the gene expression of TLR7 and induced IL-6 production in TRAMP-C2 cells (data not shown). As shown in Fig 3-4a, chloroquine inhibited imiquimod-induced production of IL-6 in the cells in a dose-dependent manner. Even though there was significance, the restorative effect of chloroquine on imiquimod-induced inhibition of cell growth was minor (Figure 3-4b). In addition, in an experiment with more long time of incubation (9 days), chloroquine did not restore the growth inhibition by imiquimod in TRAMP-C2 cells (Figure 4c).

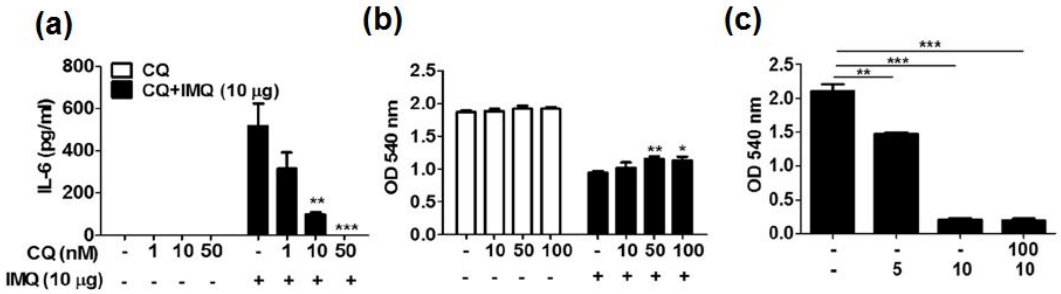


Figure 3-4. TLR7 is not required for the growth inhibition by imiquidmod in TRAMP-C2 cells. Imiquimod-induced IL-6 production (a) and cell growth inhibition (b and c) were evaluated with or without chloroquine pretreatment. Culture supernatants from imiquimond-treated TRAMP-C2 cells were collected and IL-6 levels were measured by ELISA (a). Growth inhibition was measured 3 days (b) or 9 days (c) after imiquimod treatment by MTT assay (b). The results presented here are from one representative experiment of two.

3.3.5 Imiquimod inhibits *in vivo* growth of prostate cancer in mice

In vivo antitumor efficacy of imiquimod was evaluated in mice model s.c. implanted with TRAMP-C2 cells. On day 10 after tumor implantation, mice were daily treated with PBS or imiquimod (50 μg) by intratumoral injection for 9 days. Tumor size was significantly reduced in imiquimod-treated mice, as compared with PBS-treated group (Figure 3-5a and b). Histologically, live tumor cells were compactly grown in PBS-treated mice (Figure 3-5c). In contrast, in imiquimod-treated mice, necrotic area was broadly observed and cellular components were mostly dead cells (Figure 3-5c). TUNEL staining revealed that apoptotic cells were increased by intratumoral injection of imiquimod in TRAMP-C2 cells-implanted mice (Figure 3-5d).

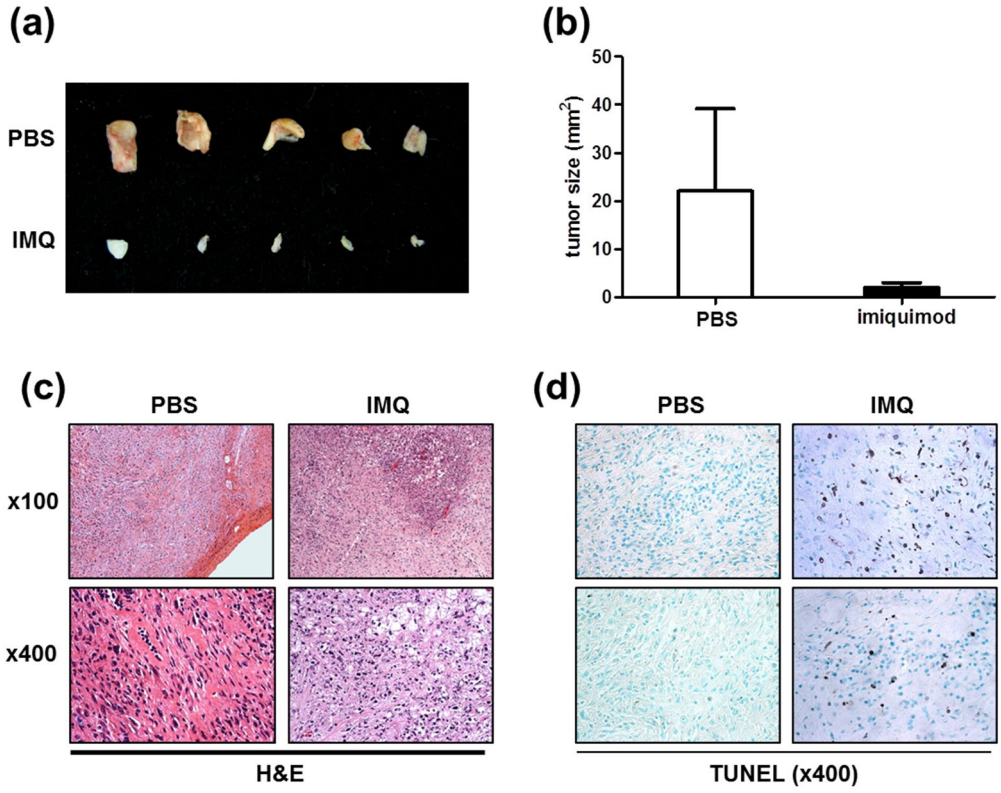


Figure 3-5. Imiquimod inhibits *in vivo* growth of prostate cancer in mice. After cancer cell implantation, mice were intratumorally injected with PBS (upper) or imiquimod (lower) (a). The mean size of prostate tumor in each group was shown (b). Tumor mass section was evaluated histologically by H&E staining (c) or TUNEL staining (d). Statistical comparisons are made between the treated groups and the control groups injected with the same volume of the saline: ** P <0.01.

3.4 DISCUSSION

Although imiquimod is known as a TLR7 agonist, it can also induce various cellular signaling TLR7-independently. Imiquimod can induce transcriptional activation of proinflammatory factors through adenosine receptor signaling (155). In addition, it activates p38, ERK, and JNK MAPKs and induces apoptosis in primary keratinocytes independently of TLR7 and MyD88 (156). Schön *et al*. suggest that imiquimod triggers inflammatory response via TLR7/8 or adenosine receptor signaling, whereas it induces direct apoptosis independently of these signalings (155). In this study, an endosomal TLRs inhibitor chloroquine inhibited imiquimod-induced production of IL-6 in TRAMP-C2 cells, whereas it did not affect cell growth inhibition by imiquimod. These results suggest that imiquimod may primarily induce growth inhibition in prostate cancer cells through a TLR7-independent mechanism.

Regulation of cell cycle and apoptosis can be important targets for cancer chemotherapy (157). The anti-proliferative effects of imidazoquinolines family are mediated by cell cycle arrest and/or apoptosis in several cancer cells (158, 159). However, the pattern of cell cycle arrest caused by imidazoquimolines is different between studies. An imidazoquinoline derivative NVP-BEZ235 inhibits the growth of T-cell acute lymphoblastic leukemia in the G_0/G_1 phase of cell cycle (160). In addition, imiquimod and resiquimod treatment of cancer cells induced decreasing cell proliferation through G_1/S phase arrest of the cell cycle through opioid growth factor receptor (OGFr) pathway (159). In contrast, treatment of EAPB0203, a member of the imidazo[1,2-a]quinoxalines, inhibited cell growth through G_2/M cell cycle arrest and apoptosis in HTLV-I–

transformed and HTLV-I–negative malignant T cells and fresh ATL cells (158). In the present study, flow cytometry and Western blot analysis revealed that imiquimod induces cell cycle arrest at G_2/M phase in TRAMP-C2 cells. It remains to be clarified whether different pattern of imidazoquinolines-induced cell cycle arrest is due to difference of cancer cell types or structural difference of imidazoquinolines.

It is well known that imiquimod induces direct apoptosis in various cancer cells (141, 143, 145, 161). The mechanism of imiquimod-induced apoptosis in tumor cells seems to be mitochondria-dependent pathway. In melanoma cells, the pro-apoptotic activity of imiquimod was independent of cell surface death receptors including CD95, TNF receptors or TRAIL (TNF-related apoptosis-inducing ligand) receptors (141, 142). Rather, it was depended on Bcl-2 degradation because melanoma cells overexpressing Bcl-2 were relatively resistant against imiquimod-induced apoptosis as compared with their sham-transfected control cells (142). The apoptosis of tumor cells was abrogated by inhibition of caspase activation (142). Moreover, blocking the functions of membrane-bound death receptors did not affect the pro-apoptotic activity of imiquimod (141). In addition, imiquimod resulted in release of mitochondrial cytochrome C into the cytosol, which is a process that eventually leads to activation of caspase-9 and caspase-3 by proteolytic cleavage (162). In this study, imiquimod led to Bcl-2 degradation and cleavage of caspase-9, which are critical for intrinsic apoptosis. Therefore, likely in other types of cancer, imiquimod seems to induce apoptosis in prostate cancer cells via mitochondria-dependent pathway.

In this study, intratumoral injection of imiquimod effectively inhibited *in vivo* tumor growth in mice s.c implanted with TRAMP-C2 cells. Although the number of apoptotic cells was increased in imiquimod-treated mice, *in vivo* antitumor action mechanism of imiquimod seems to be more complex. Several studies have demonstrated that cytokines such as type I IFN or cytotoxic T cells could participate in *in vivo* tumor destruction induced by imiquimod. Imiquimod can induce a profound tumor-directed cellular immune response (126, 163). Sullivan *et al.* revealed that anti-tumorigenic effect of imiquimod is mediated by upregulation of local IFN-α levels (145). Moreover, a recent *in vivo* study showed that imiquimod strongly enhances antigen-specific activation of anti-tumoral $CD8^+$ T cells (164). Take together, it is likely that *in vivo* antitumor effect of imiquimod is achieved by direct apoptosis and enhancement of immune responses.

To my knowledge, this study is the first attempt to demonstrate that a direct treatment of imiquimod can significantly inhibit the growth of prostate cancer. My current study revealed that imiquimod suppressed the proliferation of both mouse (TRAMP-C2) and human (PC3) prostate cancer cells via a TLR7-independent manner. Treatment of imiquimod resulted in G_2/M phase cell cycle arrest and intrinsic apoptosis. Finally, I showed that imiquimod effectively inhibited tumor growth in mice s.c. implanted with TRAMP-C2 cells. These results suggest that imiquimod can be an effective therapeutic against locally generated prostate cancer.

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GENERAL CONCLUSION

Toll-like receptors (TLRs) play a critical role in innate immunity. TLRs are expressed not only in innate immune cells, but also in cancer cells. Stimulation of TLRs has been linked to prostate cancer development. TLRs may serve as a double-edged sword in prostate cancer tumorigenesis by promoting malignant transformation of epithelial cells such as tumor growth or inhibiting tumor progression. Recent reports showed that the stimulation of TLRs in cancer cells can inhibit or promote tumorigenesis dependently on receptors or tumor cell type. Also, the consequences might be dependent on complex signaling networks triggered by TLRs activation and tumor microenvironment.

Activation of TLRs in cancer cell induces production of variable molecules that has pro- or antitumor activity. TLR4 stimulation in lung cancer cells promoted immune escape by inducing secretion of immunosuppressive cytokines and resistance to TNF-related apoptosis (165) and high level of TLR3, TLR4, and TLR9 in breast cancer were associated with higher probability of metastasis (166). On the other hand, LPS treatment induced production of chemokines such as CCL2, CCL5, CXCL8, and CXCL10 in prostate cancer cells (167) and elicited T cell-mediated antitumor immune response resulting in tumor growth inhibition (8). Also, TLR3 or TLR5 agonist-treated prostate cells induced NF-κB-dependent upregulation of inflammatory molecules with recruitment of inflammatory cells (4).

In vitro and *in vivo* studies often lead to conflict results depending on experimental conditions. For instance, several *in vitro* studies revealed that TLR4

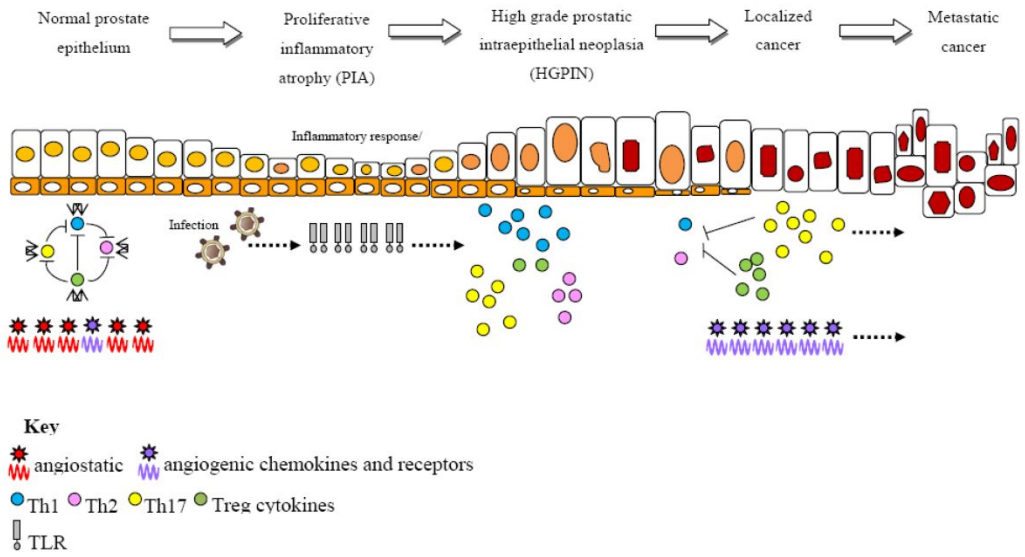

Figure IV. Multi-step process of prostate cancer development (168). Under normal conditions, the cytokines are maintained in a homeostatic state via self-regulating mechanisms and angiostatic chemokines are predominant. Regions of PIA are frequently associated with inflammation, possibly triggered by an infectious agent. The inflammatory response induces TLR-expressing inflammatory cells, which mediate cell proliferation or inhibition and increase cytokine and chemokine production.

signaling promotes tumor development and progression (6, 7, 98). In contrast, an *in vivo* study showed that TLR4 is associated with inhibition of tumor growth in a syngeneic rat model of prostate cancer (8). However, in accordance with first study (Figure 1-4 and 1-5), two independent studies showed that the percentage of epithelial cells expressing TLR4 is gradually reduced as pathologic grade increases in human prostate tissues (5) and that stimulation of prostate cancer cell MAT-LU with LPS *in vitro*, before inoculation, produces significant inhibition of tumor growth in Copenhagen rats (8). It seems that LPS stimulation induces chemokines production in prostate cancer cells, which result in alteration of phenotypic/functional pattern of tumor infiltrating lymphocytes *in vivo* (8). Although these discrepancies among studies could not be explained clearly, diversity of *in vivo* microenvironment including stroma-derived factors and immune cells might be an important factor to be explored. Therefore, *in vivo* study should be considered to determine the role of TLRs on tumorigenesis of prostate cancer.

The effect of TLR5 stimulation on prostate tumorigenesis has not been well studied. Galli *et al*. showed that TLR5 stimulation on human prostate cancer cell triggered the production of inflammatory cytokines and chemokines, which mediate recruitment of inflammatory cells (4) and Konig *et al.* showed the decreased level of TLR5 mRNA in human prostate cancer tissue compared with that of benign prostate hyperplasia (104). However, the expression of TLR5 was not defined in prostate. My IHC data showed that normal prostate, low-grad PIN, high-grad PIN and poorly differentiated adenocarcinoma tissue expressed TLR4 and 5 in TRMAP mice. Expression of TLR4 and TLR5 in TRAMP prostate was decreased as pathologic grade became more aggressive,

which could be due to loss of differentiation during tumor progression and may provide tumor cells with a strategy to escape host immune surveillance.

Previous studies have reported the expression of TLRs in prostate epithelial cells and tissues. Specifically, Galli *et al*. screened the gene expression of various TLRs in three most well-studied human prostate cancer cell lines (4). Interestingly, TLR7 expression was not identified in any of the three prostate cancer cell lines. The main finding of my study is that TLR7 is highly expressed in a non-tumor prostate epithelial cell line. TLR7 protein expression is relatively high in normal prostate epithelia or low grade of PIN and decreased in prostate adenocarcinomas from TRAMP mice (Figure 2-2). Taken together, it seems likely that TLR7 expression gradually reduces during prostatic tumor development, with the expression level dependent on prostate cancer malignancy. Moreover, TLR7 stimulation was shown to induce the production of proinflammatory cytokines/chemokines and IFN-β gene expression in prostate cancer cell lines. Inflammation induced by microorganisms such as bacteria and viruses is considered a risk factor for prostate cancer, with evidence now showing that chronic infection with uropathogenic *E. coli* induces prostatic intraepithelial neoplasia or high-grade dysplasia in TLR4-defective C3H/HeOuJ mice (128, 129). Regarding virus-associated disease, the most common that infect human prostate tissue are polyomavirus, human papilloma virus (HPV), and cytomegalovirus. Viral nucleic acids are detected by endosomal TLRs – including TLR3, TLR7, and TLR9 – and have been associated with prostate cancer pathogenesis in several previous studies. One of TLR7 ligand, loxoribine inhibited the growth and colony formation of TRAMP-C2 cells dependent of TLR7. *In vitro* and *in*

vivo studies often lead to conflict results depending on experimental conditions. Several *in vitro* studies revealed that TLR4 signaling promotes tumor development and progression (6, 7, 96). In contrast, an *in vivo* study showed that TLR4 is associated with inhibition of tumor growth in a syngeneic rat model of prostate cancer (8) and my 'Chapter I' study. Therefore, *in vivo* study should be considered to determine the role of TLR7 on tumorigenesis of prostate cancer. These findings suggest that TLR7 may suppress prostate cancer cell proliferation.

In addition, I examined *in vitro* and *in vivo* antitumor effect of the small molecule imiquimod against prostate cancer. Although imiquimod is known as a TLR7 agonist, it can also induce various cellular signaling TLR7-independently. Schön *et al*. suggest that imiquimod triggers inflammatory response via TLR7/8 or adenosine receptor signaling, whereas it induces direct apoptosis independently of these signalings (155). I investigated that an endosomal TLRs inhibitor chloroquine inhibited imiquimod-induced production of IL-6 in TRAMP-C2 cells, whereas it did not affect cell growth inhibition by imiquimod. These results suggest that imiquimod may primarily induce growth inhibition in prostate cancer cells through a TLR7-independent mechanism. Treatment with imiquimod inhibited the growth of mouse (TRAMP-C2) and human (PC-3) prostate cancer cells through inducing cell cycle arrest at the G2/M phase in TRMPA-C2 cells, confirmed by the changes of G_2/M checkpoint regulators such as reduction of cyclin B1 expression and increase of phospho-CDC2 and p21 in TRAMP-C2 cells treated with imiquimod. Also, flow cytometry and Western blot analysis revealed that imiquimod induced direct apoptosis in TRAMP-C2 cells via a mitochondrial-dependent pathway.

The anti-proliferative effects of imidazoquinolines family are mediated by cell cycle arrest and/or apoptosis in several cancer cells (158, 159). Finally, I showed that imiquimod effectively inhibited tumor growth in mice s.c. implanted with TRAMP-C2 cells. These results suggest that imiquimod can be an effective therapeutic against locally generated prostate cancer. Although imiquimod induce growth inhibition in prostate cancer cells through a TLR7-independent mechanism, imiquimod can be an alternative therapeutic for locally generated prostate cancer.

In my study, the expression of TLR4, 5 and 7 in TRAMP prostate was decreased as pathologic grade became more aggressive, which could be due to loss of differentiation during tumor progression and may provide tumor cells with a strategy to escape host immune surveillance suggesting that TLR4, 5, and 7 may contribute to antitumor immune response.

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국문 초록

TRAMP 모델의 전립선종양의 진행에서

Toll-Like receptors 4, 5, 7 의 영향

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전립선암은 세계에서 두 번째로 많이 발생하는 암이다. 이 질환은 90%가 선진국에서 발생하고 있으며, 개발도상국에서도 발생률과 사망률이 급증하고 있다. 폐암에 의한 남성 사망률을 능가하여 남성사망률 1위를 차지할 것으로 예견된다. 최근 전립선의 만성염증이 전립선암의 발생과 밀접하게 연관되어 있으며, 전염성 물질, 물리적 외상, 호르몬, 면역 관용의

휴식 (break of immune tolerance) 등, 전립선 모든 항원이 전립선 염증의 근본적인 원인이 되는 것으로 알려져 왔다. Toll-Like Receptor (TLR) 은 미생물 유래 분자 패턴을 인식하는 수용체 (pattern recognition receptor, PRR) 로서 미생물 침입 등과 같은 외부 자극을 인식하여 선천성 면역반응 (innate immune response) 을 활성화시킬 뿐만 아니라 획득면역반응 (adaptive immune response) 을 조절하는 역할을 수행하여 염증반응을 활성화 시키는데 중요한 역할을 수행한다고 알려져 있다. 최근 TLRs는 면역세포뿐만 아니라 암세포 활성에도 직접 영향을 미친다는 것으로 보고되고 있다. 하지만, 종양 세포에서의 세포면역기전의 중요한 분자의 신호전달 경로는 잘 알려지지 않았다. 최근 종양세포의 종류에 따라 TLRs을 자극시켰을 때 TLRs이 종양의 진행 (tumor progression) 이나 억제 (inhibition) 에 영향을 미친다는 연구결과가 발표되고 있다. TLRs은 세포 또는 조직 특이적인 발현 양상에 따라 리간드 (ligand) 에 의한 활성화 정도 및 세포반응성은 달라지며, 활성화된 TLRs는 종양의 증식과 진행하는데 양면성을 지니고 있다. 게다가, 연구조건에 따라 *in vitro* 와 *in vivo* 연구는 종종 대립되는 연구결과가 나오기도 한다. 비록 TLRs와 전립선암의 염증반응 및 종양형성과 관련된 연구가 많이 진행되고 있지만 아직까지 전립선암 발달에 있어 TLR5와 TLR7의 역할이 보고되지 않았다. 또한, 전립선암 진행에 있어 TLR4 발현에 대한 상반된 연구보고들이 있다. 이는 임상샘플의 유전적 혹은 환경적 요인에서 기인했을 수 있다. 따라서, TRAMP

마우스와 전립선암 세포를 이용하여 동일한 유전적 배경과 환경에서 전립선암의 진행에 따라 TLR4, TLR5, TLR7이 전립선 암세포의 활성 및 기능에 미치는 영향과 그 작용기전에 대한 연구를 진행 하였다.

Chapter I 연구에서, TLR4와 TLR5의 발현을 전립선 암모델 마우스 (Transgenic adenocarcinoma of mouse prostate, TRMAP mouse)를 이용하여 종양발달 정도에따라 관찰하였다. TRAMP 마우스의 정상 전립선의 상피세포에서 TLR4와 TLR5가 강하게 발현되었으며 low-grade prostate intraepithelial neoplasia (PIN), high-grade PIN 그리고 고분화 선암 (poorly differentiated adenocarcinoma) 조직으로 진행될수록 TLR4, TLR5의 발현은 감소하였다.

Non-small cell lung cancer (NSCLC), esophageal squamous cell carcinoma (SCC), pancreatic cancer에서 TLR7의 역할에 대한 보고는 있지만, 전립선암 발달에 있어 TLR7의 역할이 보고되지 않았다. Chapter II 연구에는 전립선암에서의 TLR7의 발현 및 역할에 관한 연구를 진행하였다. 정상 전립선 상피세포인 RWPE-1세포에서 TLR7의 mRNA가 강하게 발현되었지만, 전립선암 상피세포인 PC3와 DU145에서는 거의 발현되지 않았다. 면역염색으로 전립선종양모델인 TRAMP 마우스의 정상 전립선 상피세포에서 암 (adenocarcinoma) 조직의 병리학적 등급이 더 공격적 (aggressive) 으로 진행될수록 TLR7의 발현이 줄어들었다. 특히, TLR7 아고니스트 (agonists) 인 loxoribine은 전립선암 상피세포주 (TRAMP-C2) 에서 TLR7의 유전자와

단백질의 발현이 증가하였고 염증관련 사이토카인과 케모카인 (pro-inflammatory cytokines /chemokines) 및 IFN-β 의 생성을 유도하여 TLR7 기전에 따라 전립선암 상피세포를 억제 하였다. 이와 같은 결과는 TLR7이 전립선암 상피세포에서 종양 억제에 관여를 하고 있다는 것을 추측할 수 있다.

Chapter III 연구는 또 다른 TLR7의 아고니스트 (agonists) 로 알려진 imiquimod는 세포 주기억제 (G_2/M arrest) 및 mitochondrial dependent한 세포사멸 (apoptosis) 유도에 의한 항암활성을 보유함을 확인하였다. 마우스에 TRAMP-C2세포를 피하로 주사하여 암을 유발한 모델을 이용하여 imiquimod를 intratumoral injection하여 전립선 암의 크기가 줄어드는 것을 확인하였고, apoptotic 세포가 증가하는 것을 확인하였다. 하지만, TLR7 inhibitor 인 chloroquine와 imiquimod를 같이 처치했을 때 염증관련 사이토카인과 케모카인의 생성은 억제하였으나 전립선암세포의 증식억제에는 관여하지 않았다. 이것은 TLR7의 아고니스트의 종류에 따라 전립선암세포에서 종양억제에 관여하는 것을 추측 할 수 있었다.

이 연구에서 TRAMP모델을 이용하여 TLRs 4, 5, 7가 전립선의 정상 상피세포에서 강하게 발현되며 암세포로 진행되면서 TLRs 4, 5, 7의 발현이 줄어드는 것을 알 수 있었다. *In vivo* 시스템에서 TLRs을 매개로한 염증성 사이토카인과 케모카인은 염증성 미세환경을 조장하고 종양세포에 직접적으로 작용하여 염증이 종양의 발생 (carcinogenesis)이나 진행 (tumor progression) 등에

중요한 역할을 하여 암 억제에 관여 할 수 있다는 것을 추측 할 수 있다. 게다가, TLR7 아고니스트인 loxoribine과 imiquimod를 사용하여 전립선 상피세포에서 TLR7 경로가 기능적으로 존재하며, 그 결과로 염증성 사이토카인과 케모카인을 생성하였다. 특히, loxoribin은 TLR7 경로를 통하여 전립선암 억제에 관여 할 수 있다는 것을 알 수 있었다. 이상의 결과로 전립선암에서 TLR7 신호전달 의한 사이토카인과 케모카인은 염증성 미세환경을 조절하여 항암작용을 나타낼 수 있음을 시사하며, 따라서 TLR7 아고니스트는 전립선암의 치료에 유용하게 사용될 수 있으리라 생각된다.

주요어: 전립선암, 선천면역수용체, TLR, 면역조절, 종양형성

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