약학석사 학위청구논문

타목시펜 저항성 유방암에서 C-reactive protein(CRP)의 유도

C-reactive protein(CRP) induction in tamoxifen-resistant breast cancer

2014 년 2 월

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초록

타목시펜 저항성 유방암에서 C-reactive protein(CRP)의 유도

유방암은 폐경 여성에게서 흔히 발견되는 암 이다. 에스트로겐 수용체(estrogen receptor, ER)양성유방암의 대표적인 치료제는 타목시펜(tamoxifen) 이다. 하지만 타목시펜 반복 투여시에 가장 큰 문제점은 타목시펜 에 대해서 저항성을 가지게 되는 것이며, 현재도 명확한 치료법이 개발되지 않은 실정이다. 최근 문헌들에 의하면 많은 질병에서 염증인자로 알려져 있는 C-염증성 단백질(C-reactive protein, CRP)이 유방암과 관련을 가진다고 밝혀 졌다. 본 논문에서는 대조 유방암 세포(MCF-7) 에 비하여 타목시펜 저항성 유방암 세포(TAMR-MCF-7)에서 더 많은 양의 CRP 가 발현되는 것을 최초로 규명하였다. 이 결과는 CRP 가 유방암의 타목시펜 저항성에 어떠한 기여를 한다는 것을 의미 한다. 기질금속단백질 분해효소(Matrix Metalloproteinase, MMP)는 암의 진행(progression), 전이(metastasis), 이동(migration) 등에 관여하는 것으로 알려져 있다. 이 중 MMP-2 와 MMP-9 이 암의 이동성에 있어서 특히 중요한 담당을 한다. CRP 를 대조 MCF-7 세포에 처치 하였을 때 세포

이동능 평가(transwell migration assay)시험에서, 세포 이동능이 현저하게 증가하였으며, mRNA 정량 시험시 MMP-2 와 MMP-9 의 발현이 증가함을 알 수 있었다. 작은 헤어핀 RNA(small hairpin RNA, shRNA)를 이용해 타목시펜 저항성 유방암 세포에 들어있는 CRP 의 유전자를 결손시켰을때를 관찰한 결과, MMP-2 와 MMP-9 의 발현이 감소하는 것을 관찰 할 수 있었다. 결론적으로 타목시펜 저항성 세포에서 과다하게 발현되는 CRP 가 MMP-2 와 MMP-9 유도를 통하여 세포이동성을 증가시키는 것을 본 논문에서 규명하였다. Sphingosine-1-phosphate(S1P) 는 대표적인 염증인자로 알려져 있다. 그리고 이 S1P 는 유방암의 악성향 반응을 촉진한다고 알려져 있다. S1P 를 MCF-7 세포에 처치 후 CRP 단백질과 mRNA 량을 정량한 결과, S1P 에 의하여 CRP 의 단백질 그리고 mRNA 양 모두 증가되는 것을 관찰하였다. 그리고 S1P 를 합성할 때 필요한 효소인 Sphingosine kinase(SphK)-1 과 SphK-2 의 발현을 관찰한 결과 TAMR-MCF-7 에서 MCF-7 에 비하여 두 효소의 단백발현이 현저하게 증가된 것을 관찰하였다. S1P 의 여러가지 수용체 중 과연 어떤 수용체가 CRP 의 유도에 관여를 하는지 알아보기 위하여, S1P 수용체 저해제들을 사용하여 CRP 의 단백질 변화양을 측정한 결과, S1P 3 번 수용체가 CRP 의 유도에 관여하는 것을 규명하였다. 상기결과는 CRP 가 타목시펜 저항성 유방암을 치료하는 신규 표적이 될 수 있음을 보여준다.

주요어 : Tamoxifen-resistant breast cancer(TAMR-MCF-7), C-reactive protein, Sphingosine-1-phosphate, Matrix metalloproteinase.

학번 : 2012-21626 이름 : 최민창

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List of Abbreviations

C/ EBPß	Ccaat-enhancer-binding protein
CRP	C-reactive protein
EMT	Epithelial mesenchymal transition
IL	Interleukin
MMP	Matrix metalloproteinase
S1P	Sphingosine-1-phosphate
S1Pr3	Sphingosine-1-phosphate receptor 3
shRNA	Small hairpin RNA
SphK	Sphingosine Kinase
TAM	Tamoxifen
TAMR	Tamoxifen-resistant
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

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I. Introduction

Breast cancer is one of the most diagnosed cancer from women, especially when they are post-menopausal. One out of eight women in the United States will be likely to develop a breast cancer during their lifetime (Desentis et al., 2013). Most effective strategy to treat breast cancer is to target the estrogen receptor because most breast cancer is dependent on estrogen, which makes tamoxifen, known as selective estrogen receptor modulator (SERM), sufficient treatment. It has been known to increase quality of life and survival rate among breast cancer patient that has been used for more than a few decades (Suleiman et al., 2006). Despite of this sufficient treatment, major drawback of tamoxifen is that patient can develop a resistance. Statistically, every one out of three breast cancer patients who has been treated with tamoxifen for more than 5 years has a strong chance of developing a tamoxifen resistance (Chia et al., 2005). It is very difficult to treat breast cancer if they develop a resistance (Hoskins et al., 2009).

C-reactive protein (CRP) is an inflammatory marker and a plasma protein that appears drastically on a site of inflammation (Volanakis et al., 2001). CRP can be mainly induced by proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α in hepatocytes (Volanakis et al., 2001). Current clinical studies have discovered that elevated level of CRP is related to aggressiveness of breast cancer including its survival, recurrences, as well as metastasis and invasiveness (Erlinger et al., 2004; Pierce et al., 2009; Allin et al., 2011). However, exact

mechanism for the involvement of CRP in pathogenesis of breast cancer is still uncovered.

Recent findings suggest that matrix metalloproteinase(MMPs), which is known to degrade extracellular matrix, plays enormous part in microenviroment of tumor progression and they regulate physiological and signaling events of breast cancer (Kessenbrock et al., 2010). Relations between CRP and MMP have been researched in various fields. CRP induces MMP-10 in cardiomyocytes (Cui et al., 2012). CRP also induces MMP-1 and 10 in human endothelial cells (Montero et al., 2006).Biological process known as epithelial-mesenchymal transition (EMT) changes mesenchymal cell's migratory and invasive capability that usually relates with embryogenesis and organ development (Kalluri et al., 2009). It has been recently suggested that EMT process is related to growth of breast cancer by regulation of E-cadherin and N-cadherin, which is a crucial factor in EMT's invasive capacity (Bolós et al., 2013).

Bioactive lipid mediator sphingosine-1-phosphate (S1P) promotes breast cancer progression by activating angiogenesis via sphingosine kinase 1(SphK1) (Nagahashi et al., 2012). S1P enhances breast cancer progression through C/EBPß and CRP (Kim et al., 2013). S1P is a potent inflammatory mediator that acts through S1P receptor 3 and Gαq, induces MMP9, and therefore promotes breast cancer invasiveness (Kim et al., 2011).

In this study, we found for the first time that CRP is overexpressed in tamoxifen-resistant breast cancer cells (TAMR-MCF-7), and overexpression of CRP is related to

migration of breast cancer cells. We have also found out that S1P is related to CRP protein and mRNA expression, and MMP-2 and MMP-9, which is a known factor that is related to migration of cells, is regulated by CRP.

II. Material and Methods

1. Reagent and Antibodies

CRP, Sphk1 and Sphk2 antibodies were purchased from Santa Cruz biotechnology (CA, USA). Antibodies targeting E-cadherin and N-cadherin were purchased from BD bioscience (CA, USA). Horseradish peroxidase-conjugated donkey anti-rabbit, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were acquired from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin antibody, hematoxylin and eosin were obtained from Sigma (St. Louis, MO). W-146 (S1P receptor 1 antagonist), JTE-013 (S1P receptor 2 antagonist) and CAY-10444 (S1P receptor 3 antagonist) were all purchased from Cayman chemicals (MI, USA). C-reactive protein was purchased from Lee biosolution Inc (MO, USA). Sphingosine-1-phosphate was obtained from Avanti polar lipids (London, UK).

2. Cell Culture

MCF-7 and tamoxifen-resistant (TAMR) MCF-7 cell were used. MCF-7 cell were cultured in Dulbecco's modified eagle's medium (Thermo scientific, IL, USA) mixed with 10% FBS (Caisson, UT, USA) and 1% penicilin/streptomycin solution (Thermo scientific, IL, USA). TAMR-MCF-7 cells were established using previously reported methods (Phuong et al., 2011; Knowden et al., 2003; Choi et al.,

2007) and cultured in Dulbecco's modified eagle's medium mixed with 10% charcoal stripped FBS (Gemini bioproduct, CA, USA), 1% penicilin/streptomycin solution (Thermo scientific, IL, USA) and 3 μM of 4-hydroxytamoxifen (Sigma-aldrich, MO, USA)

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3. Immunoblot analysis

After washing the cultured cells with sterile PBS, cells were harvested and centrifuged at 3,000g for 5mins. After discarding the supernatant, cells were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerolphosphate, 2 mM sodium inorganic pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. Cells were then incubated in ice for 1hr, After incubation cells were centrifuged at 10,000g for 10 mins. Supernatants were then, used for protein measurement by Bradford analysis (Bio-Rad® protein assay kit, Bio-Rad, Hercules, CA). Samples were then transferred electrophoretically to sodium dodecylsulfate-polyacrylamide (SDS) gel with multiple gel caster (Hoefer, Inc., CA, USA). Protein samples were diluted with sterile water and 63mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% -mercaptoethanol, 0.0013% bromophenolblue. After electrophoresis, it was transferred to nitrocellulose paper with transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol, pH 8.3). After transfer, membranes were treated with specific 1st

and 2nd antibodies. Membrane pictures were taken with LAS-3000 (Fujifilm, OH, USA), using HRP-substrate luminol reagent and HRP-substrate peroxide solution (Millipore, MA, USA).

4. Reverse transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by using a Trizol and its isolation method (Invitrogen, CA, USA). The total RNA (1.0 μg) was reverse transcribed using an oligo(dT) 18-mer and Moloney murine leukemia virus reverse transcriptase (Bioneer, CA, USA). PCR was done using selective primers for human VEGF (sense primer, 5′-GCTACTGCCATCCAATCGAG-3′; antisense primer, 5′-TGCATTCACATTTGTTGTGC-3′), MMP-2 (Forward, AGTCTGAAGAGCGTGAAG; Reverse, CCAGGTAGGAGTGAGAATG), MMP-9 (Forward, TGACAGCGACAAGAAGTG; Reverse, CAGTGAAGCGGTACATAGG) and S16 ribosomal protein *(*S16r*)* genes (sense, 5′-TCCAAGGGTCCGCTGCAGTC-3′; antisense, 5′-CGTTCACCTTGATGAGCCCATT-3′). PCR was carried out for 40 cycles under the following conditions: denaturation at 95℃ for 10 s, annealing at 52℃ for 30 s, and elongation at 72℃ for 1 min. The band intensities of the amplified DNA were compared after visualization with an FLA-7000 (Fujifilm).

5. VEGF ELISA assay

VEGF ELISA Kit was purchased from Enzo life science (NY, USA). VEGF was measured as manufacturere's instruction with using culture medium. Concentration of VEGF was measured with Berthold Tristar LB 941 at 420nm and was normalized to total protein concentrations in each well.

6. Cell proliferation

Cells were cultured into 96 wells plate by 1 X 10^4 cells/ml each well. After 6hr incubation,

cells were treated with CRP for 72hrs. Then, MTT reagents were treated to each well for 2hrs. After MTT reagent treatment DMSO were treated each well and 570nm absorbance were measure with Berthold Tristar LB 941

7. Migration assay

In vitro migration assay was performed using a 24-well transwell unit with polycarbonate filters

(Corning, MA, USA). Lower part of filter was coated with type 1 collagen (Collaborative Research, KY, USA).Cells were cultured into a filter about 2 X 10^4 cell/ml each transwell with FBS-deprived media and outside of transwell were treated

with media that includes 10% FBS. CRP was treated alongside with cells in the inner part of filter. Cells were incubated for about 24hrs. Filters were fixed with formalin for 20mins and methanol for 1min. After that it was stained with hematoxylin for 10mins and 4mins with eosin. By using 400X magnification, lower side of the filter was used to analyze migrated cells. Each filters were counted with total of eight fields.

8. Short-hairpin RNA(shRNA) lentiviral transduction

Cells were first cultured in 12 well plate at 1 X 10^5 cells. After 24 hrs incubation, polybrene were added to each well with shRNA lentiviral infection solutions (Sigma-Aldrich, St. Louis, Missouri, USA) each targeting CRP and nontarget control shRNA. Next day, suction the supernatant and complete medium was added. Day after that, selection of puromycin was required to select the cells that has been successfully transduced. All of these following reagents were included in a kit purchased from Santa Cruz (TX, USA).

9. Reporter gene analysis

A dual-luciferase reporter gene assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were plated in 12-well plates and transiently transfected with 1 μg/ml reporter plasmids and phRL-SV plasmid (hRenilla luciferase expression for normalization) using Hillymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in culture

medium without serum for 18 h. Firefly and hRenilla luciferase activities in the cell lysates were measured using a Berthold Tristar LB 941. Relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase.

10. Statistical analysis

Scanning densitometry was performed using LAS-3000mini (Fujifilm, Tokyo, Japan). Student's t test was used to examine between group differences. Statistical significance was accepted at either $P < 0.05$ or $P < 0.01$..

III. Results

1. CRP increase cell migration but does not affect cell proliferation and VEGF (Vascular Endothelial Growth Factor) expression

Abnormal growth of cancer can be explained by uncontrolled proliferation, enhanced migration and VEGF production (Aktar et al., 2007). First, MCF-7 cells were treated with CRP for 72 hours, we then checked cell proliferation level by performing MTT assay. However, CRP did not change the cell proliferation level of MCF-7 cells (Fig. 1A). VEGF production, which is a key factor for angiogenesis, was checked on MCF-7 cells after treatment of CRP for 24 hours. VEGF mRNA level was also determined by RT-PCR analysis. Interestingly, CRP did not have any effect on mRNA and protein expression of VEGF (Fig 1B and C). We then checked the MCF-7 cell′s migration level by using transwell assay method. After incubation of MCF-7 cells with 10 or 30 μg/ml CRP for 24 hours, migration of MCF-7 cells was significantly increased. (Fig. 1D). These results suggest that CRP does not affect cell proliferation level and VEGF production. However, it increases migratory function of MCF-7.

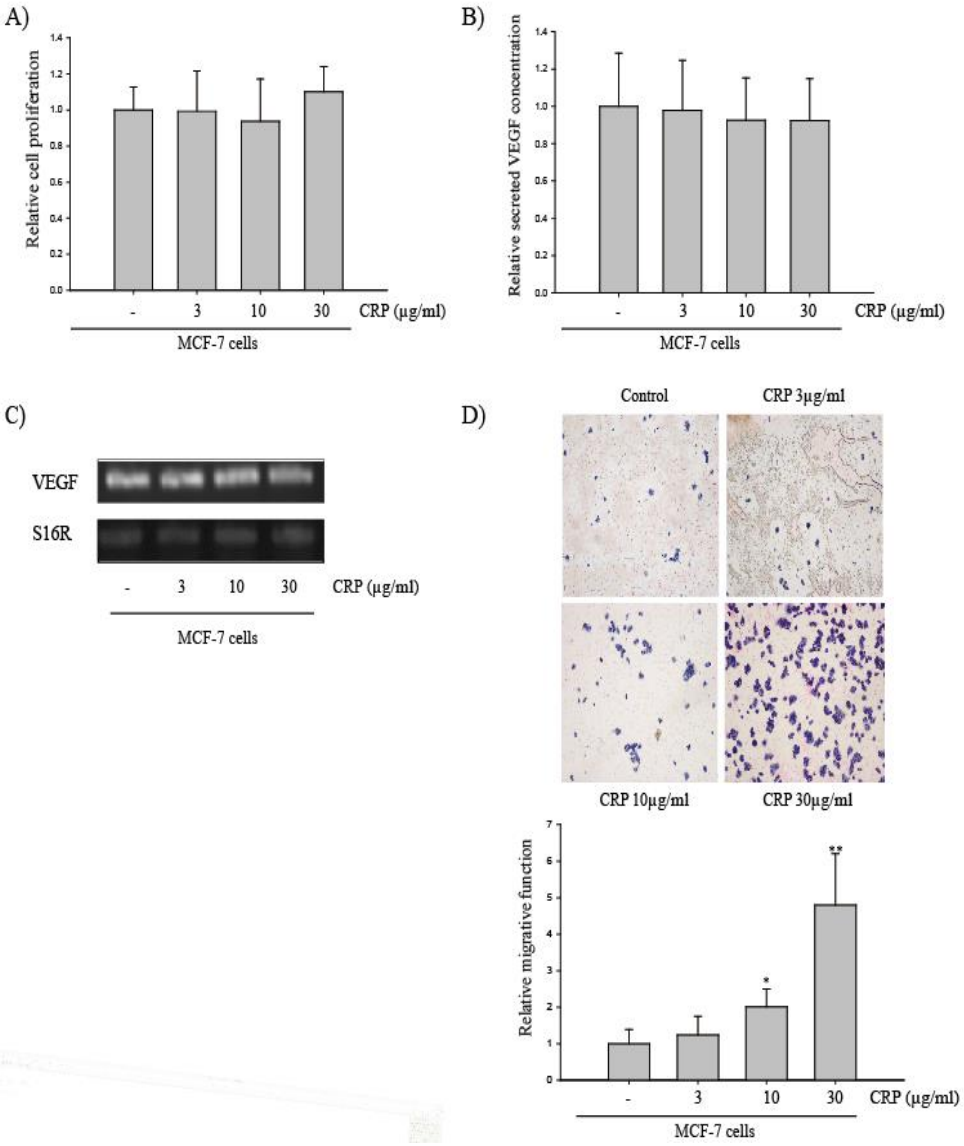
A)
Relative cell proliferation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-
3
10
30
CRP (μg/ml)
MCF-7 cells
B)
Relative secreted VEGF concentration
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-
3
10
30
CRP (μg/ml)
MCF-7 cells
C)
VEGF
S16R
-
3
10
30
CRP (μg/ml)
MCF-7 cells
D)
Control
CRP 3μg/ml
CRP 10μg/ml
CRP 30μg/ml
Relative migrative function
7
6
5
4
3
2
1
0
*
**
-
3
10
30
CRP (μg/ml)
MCF-7 cells

Figure 1. CRP increase cell migration but does not affect cell proliferation and VEGF (Vascular Endothelial Growth Factor) production

(A) Effect of CRP on proliferation of MCF-7 cells. Cell proliferation rate was measured by MTT assay. MCF-7 cells were treated with CRP (3, 10, 30 μg/ml) for 72hours. Data represent mean±SD with 8 different samples.

(B) VEGF ELISA in MCF-7 cells. MCF-7 cells were treated with CRP (3, 10, 30 μg/ml) for 24 hours and culture media were applied for ELISA. Data represent mean±SD with 5 different samples.

(C) RT-PCR analysis of VEGF mRNA level on MCF-7 cells. MCF-7 cells were treated with CRP (3, 10, 30 μg/ml) for 24 hours.

(D) Representative migratory function of MCF-7 cells. MCF-7 cells were incubated in transwell filter in the absence or presence of CRP (3, 10, 30 μg/ml). Cells were stained with hematoxylin and eosin. The relative cell numbers of control was assigned as 1. Data represent mean±SD with 3 different samples (significant versus non-treated MCF-7 cells, *P<0.05, **P<0.01).

2. CRP overexpression in TAMR-MCF-7

In our previous study, it has been found that TAMR-MCF-7 cells show the typical phenotype of epithelial mesenchymal transition (Kim et al., 2009). When we compared CRP protein expression between MCF-7 and TAMR-MCF-7 cells, TAMR-MCF-7 cells had more than 3 times of CRP protein expression compared to MCF-7 cells (Fig. 2A). RT-PCR experiments showed that mRNA level of CRP was increased in TAMR-MCF-7 cells (Fig. 2B). CRP reporter gene analysis showed an increased level of CRP promoter activity (Fig. 2C). All of these results are congruent with the fact that CRP level is overexpressed in TAMR-MCF-7 cells.

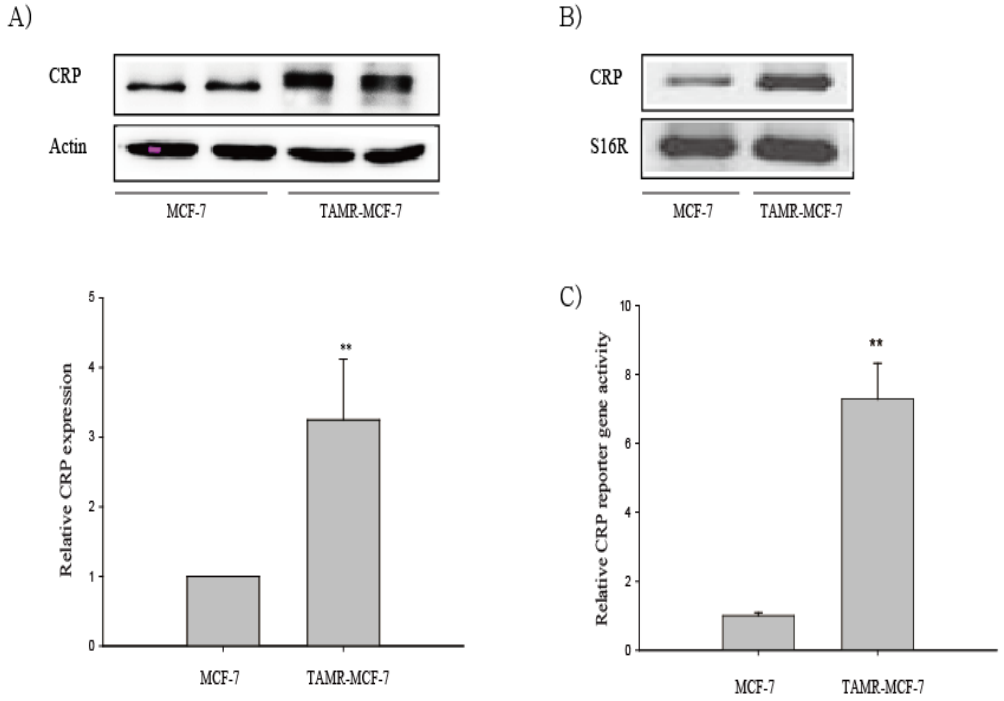
A)
CRP
Actin
MCF-7
TAMR-MCF-7
B)
CRP
S16R
MCF-7
TAMR-MCF-7
Relative CRP expression
0
1
2
3
4
5
**
MCF-7
TAMR-MCF-7
C)
Relative CRP reporter gene activity
0
2
4
6
8
10
**
MCF-7
TAMR-MCF-7

Figure 2. CRP overexpression in TAMR-MCF-7

(A) CRP overexpression in TAMR-MCF-7 cells. Western blot analysis of CRP in MCF-7 and TAMR-MCF-7 cells. Relative changes in the CRP protein expression were assessed by scanning densitometry. Data represent mean±SD with 5 different samples (significant versus MCF-7 cells, *P<0.05, **P<0.01).

(B) mRNA expression of CRP in TAMR-MCF-7 cells. RT-PCR analysis of CRP in MCF-7 and TAMR-MCF-7 cells

(C) CRP promoter activities in MCF-7 and TAMR-MCF-7 cells. Each cell type was transiently co-transfected with CRP-Luc reporter plasmid containing -2 kb human CRP promoter (1 μg/ml) and phRL-SV (hRenilla)(1 ng/ml). Dual luciferase reporter assays were performed on the lysed cells 18 h after transfection. Reporter gene activity was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent mean±SD with 4 different samples (significant versus MCF-7 cells, **P<0.01).

3. Role of CRP in cell migration of TAMR-MCF-7 cells

In order to determine whether CRP induction in TAMR-MCF-7 cells is crucial for the enhanced cell migration, CRP-knockdown TAMR-MCF-7 cells were established after performing lentiviral shRNA transduction. Colony number 3 TAMR-MCF-7 CRP-knockdown cells showed a definite decrease in protein and mRNA level of CRP (Fig 3 A and B). After establishment of cell, we first checked the cell proliferation and VEGF production to see if it was any different from previous findings in CRP treatment of MCF-7 cells (Fig 1A, B and C). Cell proliferation rate in CRP-knockdown TAMR-MCF-7 cells was not different from control shRNA-tranduced cells (Fig. 3C). Surprisingly, in comparison to control cells, VEGF production level significantly increased in CRP-knockdown TAMR-MCF-7 cells (Fig. 3D). The reason why CRP-knockdown cells showed an increase level of VEGF production is still enigmatic. Lastly, we then checked if CRP's effect on migratory function. In TAMR-MCF-7 cell which has a higher expression of CRP than MCF-7 cell, showed an increased migratory cell number by more than 3 times (Fig. 3E). CRP-knockdown cells showed about 60% decreased level of migration level than control shRNA cells (Fig. 3F). These results suggest that overexpression of CRP in TAMR-MCF-7 is related to cell migration.

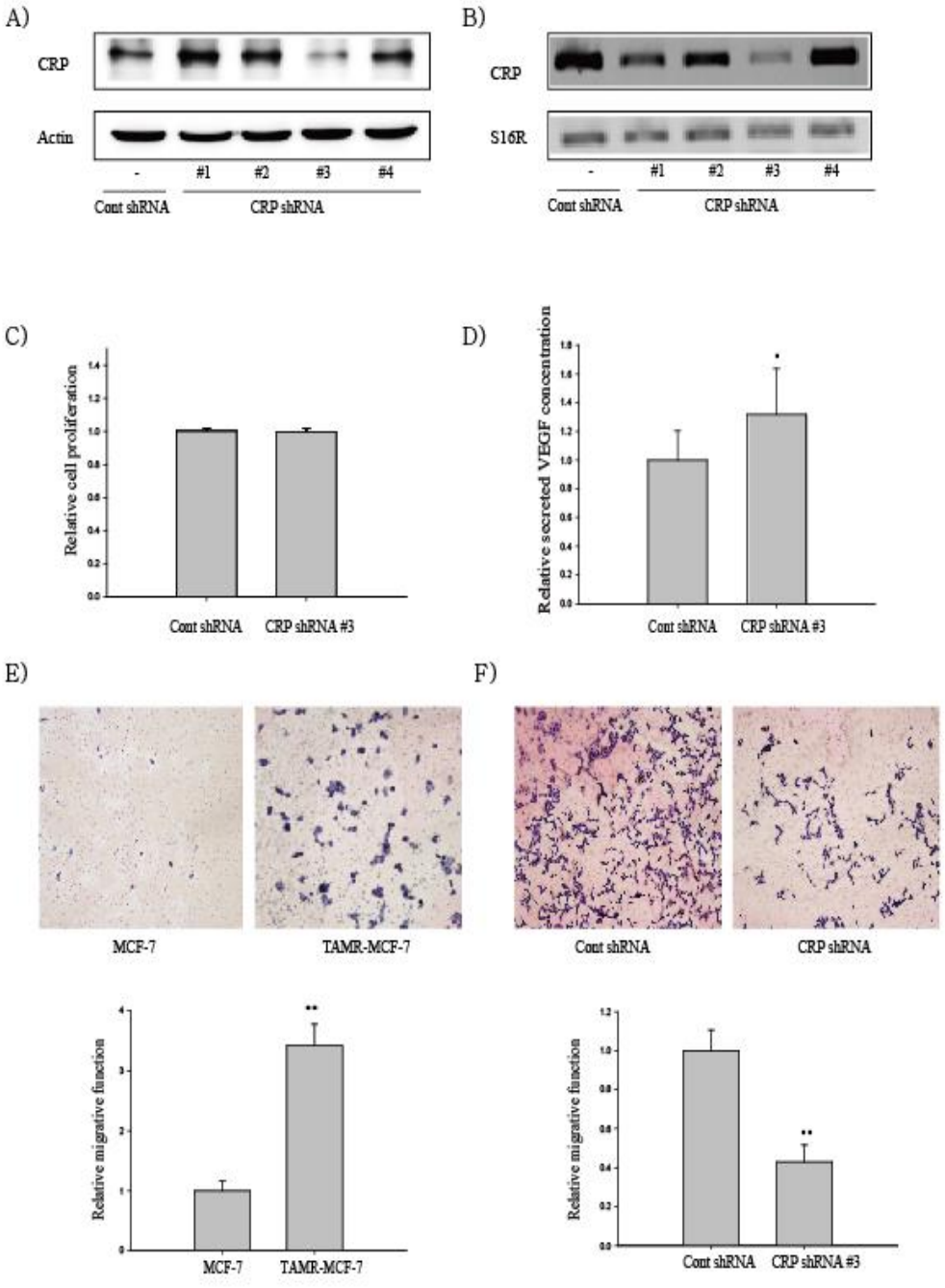
A)
CRP
Actin
-
#1
#2
#3
#4
Cont shRNA
CRP shRNA
B)
CRP
S16R
-
#1
#2
#3
#4
Cont shRNA
CRP shRNA
C)
Relative cell proliferation
Cont shRNA
CRP shRNA #3
D)
Relative secreted VEGF concentration
Cont shRNA
CRP shRNA #3
E)
MCF-7
TAMR-MCF-7
Relative migrative function
MCF-7
TAMR-MCF-7
F)
Cont shRNA
CRP shRNA
Relative migrative function
Cont shRNA
CRP shRNA #3

Figure 3. CRP knockdown TAMR-MCF-7 decreases cell's migration but does not affect cell proliferation nor VEGF production

(A) TAMR-MCF-7 cells were transducted with a lentiviral shRNA targeted against CRP or with a nontargeting shRNA as a control. After transduction, western blot analysis was performed to confirm the CRP knockdown.

(B) RT-PCR analysis of CRP mRNA level on CRP knockdown cells to confirm the CRP knockdown.

(C) Effect of CRP knockdown in proliferation of CRP-knockdown cells. As seen from results above, CRP level in colony number 3 has been successfully knocked down. Using colony number 3, MTT assay was perform to see a change in proliferation between control shRNA and CRP shRNA-transduced cells. Data represent mean±SD with 8 different samples.

(D) VEGF ELISA analysis of VEGF secretion in CRP-knockdown cells. Data represent mean±SD 5 different samples.

(E) Representative migratory function of MCF-7 cells and TAMR-MCF-7. Cells were stained with hematoxylin and eosin. The relative cell numbers of control was assigned as 1. Data represent mean±SD with 3 different samples (significant versus MCF-7 cells, **P<0.01).

4. S1P/S1PR3-mediated CRP induction in TAMR-MCF-7 cells

It has been very recently reported that sphingosine-1-phosphate (S1P), which is an important inflammatory marker, induces CRP in mammary epithelial cells (Moon et al., 2013). From this finding, we then checked if S1P induces CRP in an estrogen receptor-positive breast cancer cell. After treatment of MCF-7 cells with S1P for 24 hours, we were able to see an increase of protein and mRNA expression of CRP in MCF-7 cells (Fig. 4 A and B). S1P production is absolutely catalyzed by Sphingosine kinase (Sphk) 1 or Sphk2 (Price et al., 2008). Western blot analysis showed that, protein expression of Sphk1 and Sphk2 was up-regulated in TAMR-MCF-7 cells. (Fig. 4 C and D). There are total of 5 S1P receptors and each of them has different functions. We wanted to know which S1P receptor(s) is involved in S1P-mediated CRP induction in TAMR-MCF-7 cells. After treatment of different S1P receptor antagonist for 24 hours, S1P receptor 3 antagonist (CAY-10444) selectively inhibited protein expression of CRP in TAMR-MCF-7 (Fig. 4E). The data demonstrate that S1P binding toS1P receptor 3 plays a key role in CRP induction in TAM-resistant breast cancer.

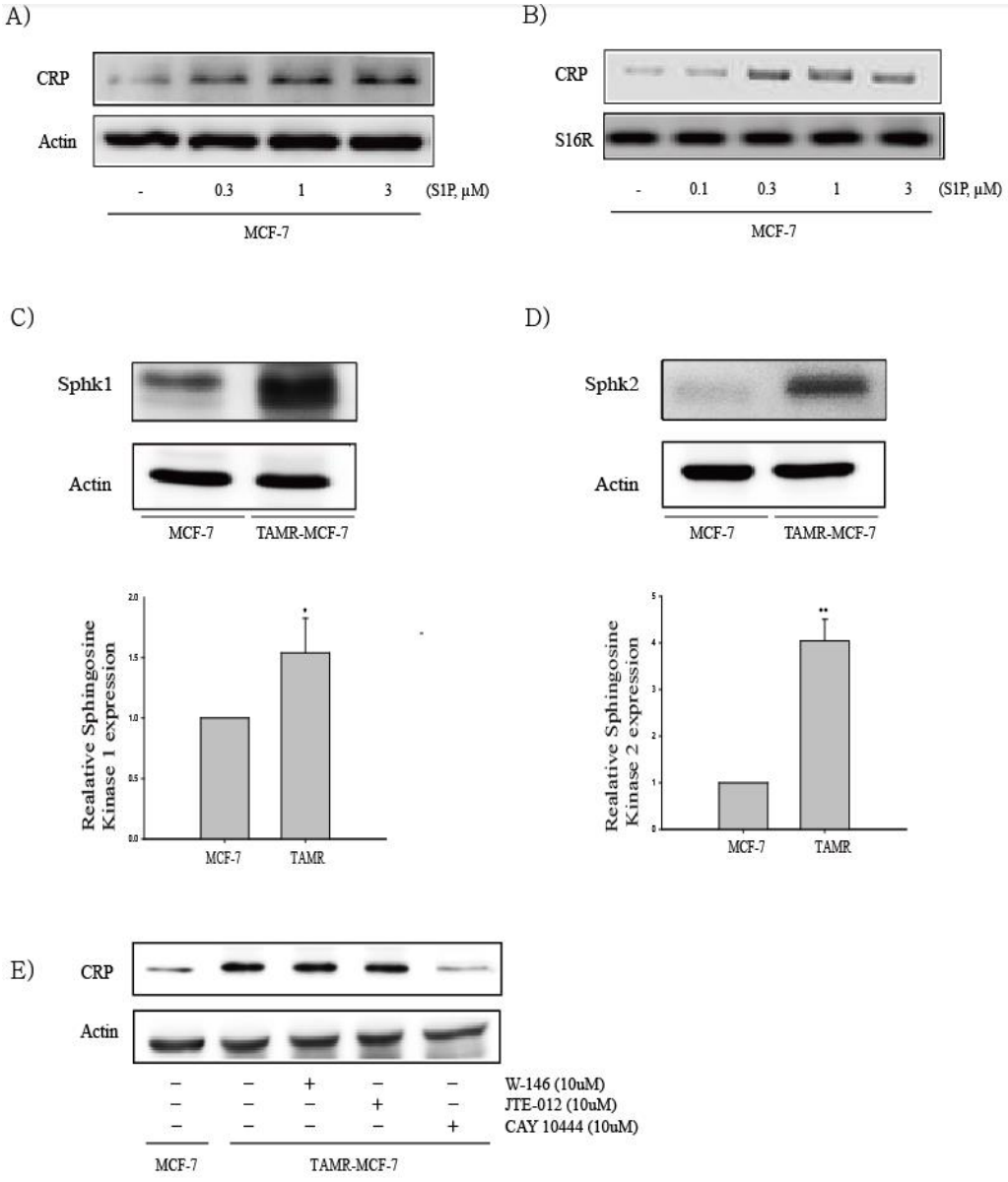
A)
CRP
Actin
- 0.3 1 3 (S1P, μM)
MCF-7
B)
CRP
S16R
- 0.1 0.3 1 3 (S1P, μM)
MCF-7
C)
Sphk1
Actin
MCF-7 TAMR-MCF-7
Realative Sphingosine Kinase 1 expression
MCF-7 TAMR
D)
Sphk2
Actin
MCF-7 TAMR-MCF-7
Realative Sphingosine Kinase 2 expression
MCF-7 TAMR
E)
CRP
Actin
W-146 (10uM)
JTE-012 (10uM)
CAY 10444 (10uM)
MCF-7 TAMR-MCF-7

Figure 4. S1P/S1PR3-mediated CRP induction in TAMR-MCF-7 cells

(A) S1P-mediated CRP protein up-regulation in MCF-7 cells. MCF-7 cells were serum deprived for 18hours. After serum deprivation, S1P (0.3, 1 and 3μm) was treated for 24 hours. Cell lysates were subjected to western blot analysis.

(B) S1P-mediated CRP mRNA up-regulation in MCF-7 cells.. MCF-7 cells were serum deprived for 18hours. After serum deprivation, S1P (0.1 0.3, 1 and 3μm) was treated for 24 hours.

(C) Sphk 1 protein expression in MCF-7 and TAMR-MCF-7 cells. Relative changes in the Sphk 1 protein expression were assessed by scanning densitometry. Data represent mean±SD with 3 separate samples (significant versus MCF-7 cells, *$P<0.05$).

(D) Sphk 2 protein expression in MCF-7 and TAMR-MCF-7 cells. Relative changes in the Sphk 2 protein expression were assessed by scanning densitometry. Data represent mean±SD with 3 separate samples (significant versus MCF-7 cells, **$P<0.01$).

(E) Effect of different S1P receptor inhibitors on CRP expression in TAMR-MCF-7 cells. (W-146 : S1P receptor 1 inhibitor, JTE-013 : S1P receptor 2 inhibitor, CAY-10444 : S1P receptor 3 inhibitor.)

5. No involvement of CRP in EMT of TAMR-MCF-7 cells

Previous findings suggest that migratory function of a breast cancer is related to EMT pathway and both down-regulation of E-cadherin and up-regulation N-cadherin is considered a typical marker for EMT progression (Bolós et al., 2013). Earlier findings discovered that, N-cadherin is up-regulated while E-cadherin is down-regulated in TAMR-MCF-7 cells, which may be related with higher migratory phenotype function of TAMR-MCF-7 cells (Kim et al., 2009). We also confirmed the results by performing western blot analysis of MCF-7 and TAMR-MCF-7 (Fig. 5A). Stable CRP knockdown TAMR-MCF-7 cells, however did not affect the expression of E-cadherin and N-cadherin (Fig. 5B). Moreover, S1P receptor 3 inhibition which causes a decrease in CRP also did not alter the protein levels of E-cadherin and N-cadherin (Fig. 5C). The data imply that CRP does not have any effect on EMT phenotype of TAMR-MCF-7 cells.

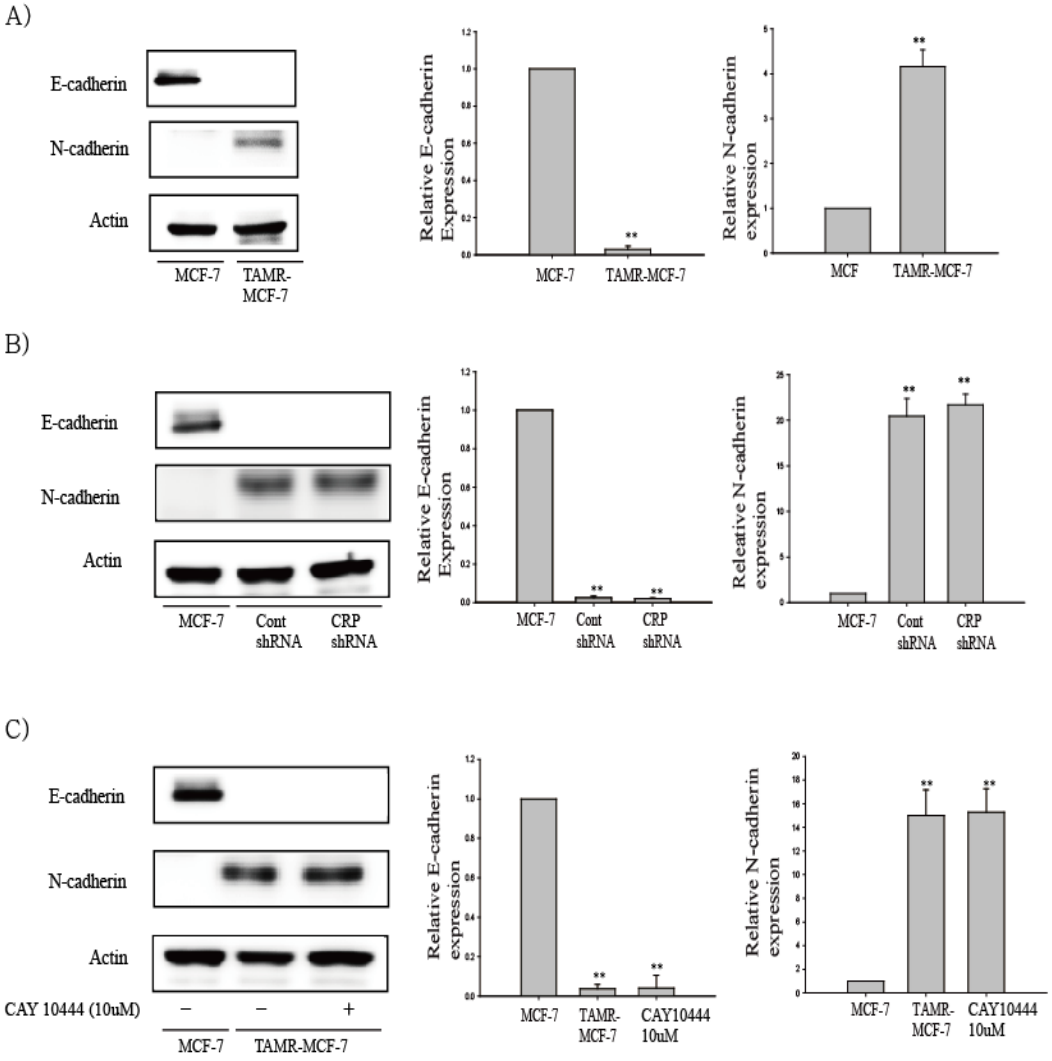
A)
E-cadherin
N-cadherin
Actin
MCF-7
TAMR-MCF-7
Relative E-cadherin Expression
MCF-7
TAMR-MCF-7
Relative N-cadherin expression
MCF
TAMR-MCF-7
B)
E-cadherin
N-cadherin
Actin
MCF-7
Cont shRNA
CRP shRNA
Relative E-cadherin Expression
MCF-7
Cont shRNA
CRP shRNA
Releative N-cadherin expression
MCF-7
Cont shRNA
CRP shRNA
C)
E-cadherin
N-cadherin
Actin
CAY 10444 (10uM)
– – +
MCF-7
TAMR-MCF-7
Relative E-cadherin expression
MCF-7
TAMR-MCF-7
CAY10444 10uM
Relative N-cadherin expression
MCF-7
TAMR-MCF-7
CAY10444 10uM

Figure 5. No involvement of CRP in EMT of TAMR-MCF-7 cells

(A) E-cadherin and N-cadherin expression in MCF-7 and TAMR-MCF-7 cells. Relative changes in the E-cadherin and N-cadherin expression were assessed by scanning densitometry. Data represent mean±SD with 3 separate samples (significant versus MCF-7 cells, *P<0.05).

(B) Effect of CRP knockdown in E-cadherin and N-cadherin expression. Relative changes in the E-cadherin and N-cadherin expression were assessed by scanning densitometry. Data represent mean±SD with 3 separate samples (significant versus MCF-7 cells, *P<0.05). However, no significant change was observed between control shRNA- and CRP-shRNA -transduced cells.

Effect of S1P receptor 3 antagonist, CAY 10444 in E-cadherin and N-cadherin expression. CAY 10444 was treated to TAMR-MCF-7 cell for 24 hours. Relative changes in the E-cadherin and N-cadherin expression were assessed by scanning densitometry. Data represent mean±SD with 3 separate samples (significant versus MCF-7 cells, *P<0.05). However, no significant change was observed between TAMR-MCF-7 and CAY-10444-treated TAMR-MCF-7.

6. CRP-mediated MMP-2 and MMP-9 induction in TAMR-MCF-7 cells

Matrix metalloproteinase (MMP) is extracellular matrix degrading enzyme which is known to be the most important proteinase family for cancer metastasis (Deryugina et al., 2006). RT-PCR analysis using control shRNA or CRP shRNA-infected TAMR-MCF-7 cells depicted that mRNA levels of MMP-2 and MMP-9 were diminished by CRP knockdown (Fig. 6A) Moreoever, S1P receptor 3 inhibitor, CAY-10444 also reduced mRNA expression of MMP-2 and MMP-9 (Fig. 6B).

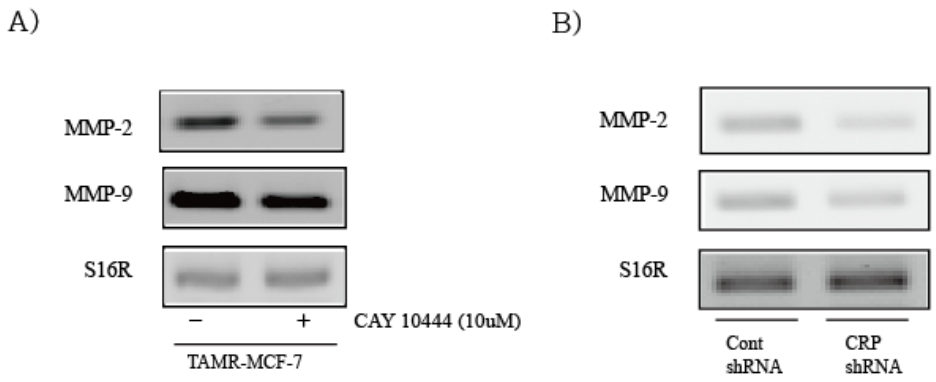
A)
MMP-2
MMP-9
S16R
−
+
CAY 10444 (10uM)
TAMR-MCF-7
B)
MMP-2
MMP-9
S16R
Cont shRNA
CRP shRNA

Figure 6. CRP-mediated MMP-2 and MMP-9 induction in TAMR-MCF-7 cells

(A) Down-regulation of MMP-2 and MMP-9 expression by CAY 10444 on TAMR-MCF-7 cells. CAY-10444 was treated to TAMR-MCF-7 cells for 24 hours. After treatment, RT-PCR analysis was performed to check mRNA expression of MMP-2 and MMP-9.

(B) Down-regulation of MMP-2 and MMP-9 expression by CPR knockdown TAMR-MCF-7 cells. RT-PCR analysis was performed to check mRNA expression of MMP-2 and MMP-9.

IV. Discussion

Most of breast cancer patients have estrogen receptor(ER) positive breast cancer. In this particular breast cancer, it is important to block the binding of estrogen and ER. Tamoxifen as a selective estrogen receptor modulator (SERM) is a primary treatment or prevention option for ER positive breast cancer (Taneja et al., 2010). Problems occur when ER positive breast cancer patients acquire resistance to tamoxifen. Every one out of three ER-positive breast cancer patients who has been given a treatment of tamoxifen for five years develop a resistance (Schiff et al., 2003). Therefore, it should be no surprise that figuring out the treatment for this type of breast cancer is very important. Recent studies have suggested that CRP may be related to breast cancer progression (Brandon et al., 2009). In this study, we first found that migratory function of MCF-7 cells (ER-positive human breast cancer) was enhanced by CRP. Previous studies have already found the information of CRP inducing proliferation of endothelial cell and migration of vascular smooth muscle cell (Cirillo et al., 2005; Wang et al., 2003). However, direct effect of CRP on the proliferation and migration of cancer cells has not been fully covered. In myeloma cells, CRP enhanced cell proliferation rate under stressed conditions, and suppressed anti-cancer agent-induced apoptosis via blocking caspase cascade (Yang et al., 2007). In this study, we surprisingly found that cell proliferation and VEGF production were not altered by CRP exposure to MCF-7 cells. We are still unsure why cell proliferation and VEGF production, which is also an

important factor that relate to cancer progression, were not affected by CRP. Hence, CRP may be related to the increased migration and metastasis potential of breast cancer.

CRP has been widely used as a biomarker marker for inflammation and malignancy of certain tumors including bladder, pancreatic, renal and many other tumors (Wang et al., 2009). However, there is not much information about relationship between CRP and breast cancer, especially TAM-resistant breast cancer. Here, we further showed overexpression of CRP in TAMR-MCF-7 cells. Moreover, we have also discovered that TAMR-MCF-7 cells exhibit higher migratory function than MCF-7 cells. To make sure CRP is related to breast cancer cell migration, we established CRP-knockdown cells by using lentiviral shRNA system. After CRP-knockdown cells were established, we checked the migratory function of CRP-knockdown cells. As a result, CRP-knockdown cells exhibit a lower migration level than control shRNA cells. From all of these results we can conclude that CRP is definitely related to breast cancer cell's migration.

Sphingosine-1-phosphate (S1P) is known to be related to breast cancer progression, metastasis and invasion (Kim et al., 2011). Recent studies have discovered that S1P induces CRP in mammary epithelial cells (Kim et al., 2013). In the present study we also confirmed that S1P increasesd the expression of CRP in MCF-7, human breast cancer cells and SphK 1 and SphK 2, which are the key factor in developing S1P, expression was increased in TAMR-MCF-7 cells. However, the exact amount of S1P made in TAMR-MCF-7 cells compared to MCF-7 cells is currently being researched. But from these results we can probably speculate that TAMR-MCF-7 has more amount

of S1P than MCF-7 cells. There are total of 5 different S1P receptors. Not much is known about S1P receptor 4 and 5, S1P receptor 1 is known to be related to tumor cell intravasation and adhesion. S1P receptor 2 negatively regulates tumor growth and angiogenesis. Lastly, S1P receptor 3 is related to brain tumor progression (Feng et al., 2010, Watter et al., 2011). From different S1P receptor inhibitor study, we were able to conclude that S1P receptor 3 is related to induction of CRP in TAMR-MCF-7 cells. Recently, Dr. Moon group also revealed that S1P receptor 3 is crucial for S1P-stimulated CRP induction in mammary epithelial cells (Kim et al., 2013). Hence, cellular signaling pathway(s) required for CRP expression in chemo-resistant breast cancer cells and mammary epithelial cells seems to be conserved.

During our research, we have found from previous study that E-cadherin, N-cadherin and MMP-2 and MMP-9 are deeply related to cancer metastasis (Kessenbrock et al., 2010, Kalluri et al., 2009). However, no significant relation between E-cadherin, N-cadherin and CRP was observed in the present study. MMP-2 and MMP-9 expression in TAMR-MCF-7 cells was significantly decreased by either CRP-shRNA transduction or S1P receptor 3 antagonist treatment. However, CRP treatment to MCF-7 cells does not change the expression of MMP-2 and MMP-9 (results not included). From this result, we cannot completely conclude that MMP-2 and MMP-9 is related to CRP-stimulated migration of breast cancer cells. However, other studies such as enzyme activity, MMP-2, MMP-9 reporter gene analysis and xenograft could be required for the final conclusion.

From this study, we have discovered for the first time that CRP is overexpressed in TAMR-MCF-7 cells and the enhanced CRP production may be closely related to the higher migration phenotype of TAM-resistant breast cancer. We have also revealed that S1P/S1P receptor 3 is responsible for induction of CRP. CRP may be new therapeutic target against treatment of TAM-resistant breast cancer.

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Abstract

CRP induction in tamoxifen-resistant breast cancer

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Breast cancer is one of the most commonly found cancer among post-menopausal women. Tamoxifen has been known as a most effective treatment for breast cancer. One of the main issues of breast cancer is acquisition of tamoxifen resistance, and if it does, the way to treat tamoxifen-resistant breast cancer has not been discovered. It has been recently suggested that c-reactive protein (CRP), inflammatory marker found in many diseases may be related to breast cancer progression. Here, we have discovered that compared to normal breast cancer (MCF-7) cells, tamoxifen-resistant breast cancer (TAMR-MCF-7) cells expresses much higher protein and mRNA level of CRP, which suggests that CRP induction could be related to tamoxifen-resistant function of breast cancer cell. Matrix metalloproteinases (MMPs) has been known to have a critical role in

metastasis, progression and migration of cancer. Especially MMP-2 and -9 have a major role in migration of cancer cells. Through transwell migration assay, we have discovered that, treatment of CRP to MCF-7 cells, increases the expression levels of MMP-2 and MMP-9 and migration potential. Moreover, CRP knockdown by shRNA lentivirus significantly reduced the mRNA expression of MMP-2 and -9. Taken these findings together, tamoxifen-resistant breast cancer expresses high level of CRP and it induces migration through the activation of MMP-2 and -9 induction. Sphingosine-1-phosphate (S1P), which is a biomarker for inflammation, has been suggested by previous findings that it may be related to progression of breast cancer. We have found that both protein and mRNA level of CRP increased in MCF-7 after S1P treatment. Moreover, expression of Sphingosine kinase (SphK) 1 and 2, which is a key factor in making S1P, increases in TAMR-MCF-7 cells. We have also found that S1P receptor 3 is related to induction of CRP by S1P receptor 3 inhibitor studies. CRP may be an important therapeutic target for the treatment of tamoxifen-resistant breast cancer.

Key words: Tamoxifen-resistant breast cancer(TAMR-MCF-7), C-reactive protein, Sphingosine-1-phosphate, Matrix metalloproteinase

Student number: 2012-21626

감사의 글

대학교를 갓 졸업하고 대학원 생활을 시작한 것이 엊그제 같은데 벌써 2 년 이라는 시간이 지나가 버렸습니다. 대학을 처음 들어갔을 때 설렜던 그 마음가짐으로 대학원에 입학을 하여 좋은 사람들과 훌륭한 교수님 밑에서 공부를 하다 보니 시간이 가는 줄 몰랐던 것 같습니다. 대학과정을 마치고 열심히 공부하여 약사고시를 합격 하였을 때도 기분이 홀 가분 하였는데, 석사 졸업논문을 마무리 하게 되니 저의 인생을 살아가는데 있어서 거쳐야 되는 과정을 마친 것 같아 더욱 더 홀 가분 해진 것 같습니다.

우선 저를 이렇게 까지 성장 할 수 있게 끝까지 저를 믿고 도움과 희망을 아낌없이 준 우리 가족들에게 이 감사의 글을 전하고 싶습니다. 아버지는 제가 어렸을 때부터 때론 듣기 싫은 잔소리와 꾸중을 하셨지만, 그것이 아버지가 저를 더욱 더 성장하고 성공하기를 바라기 때문에 그러는 것이라는걸 아주 나중에 알게 되었습니다. 어머니는 아버지가 잔소리와 꾸중을 하시고 나면 상처받은 저를 위해 아낌없이 위로를 해주셨고 1 주일에 한두 번씩은 꼭 안부전화를 하실 정도로 저를 굉장히 아끼고 사랑해주셔서 감사합니다. 비록 두분 다 미국이라는 먼 나라에 계셔 자주 뵙지는 못하지만, 저의 마음 한구석에는 항상 저의 부모님이 있고, 부모님께 사랑하고 못나고

부족한 자식 믿어주시고 도와주셔서 정말로 감사 드린다고 전해 드리고 싶습니다.

부모님이 미국에서 계셔서 부모님께서 해주시는 집안일을 대신 도맡아 해주는 이모께도 이 감사의 글을 전하고 싶습니다. 처음에 이모께서 해주신다 하셨을 때 정말로 불편한 마음을 가졌었지만, 이모께서는 그런 마음 가지지 말고 엄마가 해준다고 생각하라고 말씀하셨을 때 정말로 고마웠습니다. 지금까지도 인천이라는 먼 거리 에서 오셔서 집안일을 해주셔서 정말 고맙고 사랑합니다. 안부전화를 드렸을 때도 항상 저의 팬이라고 하며 저를 아껴주시고 사랑해 주시고 걱정해 주셔서 정말로 감사합니다.

제가 명절 때 인사 드리러 내려가면 역까지 친히 마중까지 나오셔서 반겨주실 정도로 저를 사랑하는 할아버지와 할머니에게 역시 이 감사의 말씀을 전하고 싶습니다. 부모님과 멀리 떨어져 살아서 밥은 챙겨 먹고 다니는지 안부전화도 자주 해주시고, 반찬이나 무엇이든지 한가지라도 더 많이 챙겨 주시려고 해주시는 할아버지 할머니께 감사의 말씀을 전해드리고 싶습니다.

저와 한집에서 같이 산다고 고생한 친 누나에게 또 감사의 말씀을 전하고 싶습니다. 누나도 저랑 사느라고 불편한 것이 이만 저만이 아니었을 텐데, 가끔씩 저에게 맛있는 것도 사주면서 불평 한마디 안 해주고 잘 참아주어서

저에게 많은 도움이 되었습니다. 이 같이 좋은 누나를 둔 저 자신이 행복하고 행운이 많은 것 같아 정말 고맙습니다.

제가 모르는 부분이 있을 때 많이 도와주신 실험실 선배, 후배와 동기들에게 이 감사의 말씀을 전하고 싶으며, 저에게 실험적인 것 외에 여러 가지 다른 일들에 대해서도 아낌없이 조언해주고 도와주신 양진원 선배님께 이 감사의 말씀을 전하고 싶습니다. 그리고 저에게 여러 가지 도움을 준 저의 동기인 김지원 님, 김효선 님 그리고 고기훈 님, 김나연 님, 임지혜 선배님, 정성백 님 그리고 Quyen 에게 감사의 말씀을 전합니다. 실험적인 면에 있어서 저에게 정말 많은 도움을 준 Phuong 에게 역시 이 감사의 말씀을 전합니다.

마지막으로 제가 여기까지 올 수 있게 너무나도 큰 도움을 주신 강건욱 교수님, 김상건 교수님 그리고 이승희 교수님께 이 감사의 말씀을 전해 드립니다. 학 부생 때 부족했던 저의 모습에 대한 의심보다는 저에게 끝까지 믿음을 주시고, 포기하지 않게 도와주셔서 정말로 감사 드립니다. 교수님들께서 계셔서 이 자리에 올 수 있었다는 것을 절대 잊지 않을 것이며, 앞으로 더 나아가 박사과정을 시작하게 되었을 때 지금까지 저를 도와주셨던 많은 분들을 잊지 않고 감사하는 삶을 사는 사람이 되겠습니다.