약학석사 학위논문

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**2017 년 8 월**

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**2017 년 7 월**

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# ABSTRACT

K-Ras is the most famous oncogene that is frequently mutated in human cancers, and it influences on poor prognosis with low survival rate of cancer patients. Although K-Ras is a strong target of cancer therapeutics, the mechanism underlying pathological increase of K-Ras is unclear. Here we report that AIMP2-DX2, oncogenic splicing variant of AIMP2-F, is critical determinant of K-Ras stabilization. Through transcriptome analysis in AIMP2-DX2 high- and low-expressing lung cancer cells, we identified AIMP2-DX2 is positively correlated with Ras signaling. In AIMP2-DX2-inducible *in vitro* and *in vivo* model, we monitored the stabilization of K-Ras by induction of AIMP2-DX2. AIMP2-DX2 GST-N domain binds to the positively charged hypervariable region of K-Ras and inhibits the β-TrCP-, E3 ligase of K-Ras, mediated ubiquitination. Therefore, this work unveiled that the stabilization of K-Ras can be determined by interaction with AIMP2-DX2 and suggested that the interaction of two oncogenic proteins leads to pathologically synergic cancer-promoting property.

# INTRODUCTION

Ras protein is a critical protein of signal cascade in proliferative condition. Ras proteins are regulated by guanine nucleotide exchange factors (GEF) that promote GDP dissociation and GTP binding, and GTPase-activating proteins (GAP) that stimulate the GTPase activity of Ras (1). In human cancer, Ras genes are frequently mutated and function as oncogenes. Aberrant Ras function is associated with a single mutation typically at codon 12, 13, or 61 in cancer (2). Ras with the single mutation leads to constitutive activation of Ras by blocking GAP binding. When Ras is the active form, Ras-GTP can bind to numerous effectors. In the active GTP-bound state, Ras proteins interact with downstream effectors, such as Raf, Ral-GEF and PI3K (phosphoinositide 3-kinase), to transmit signals from the cell membrane to intracellular signaling pathways that ultimately influence gene transcription and therefore regulate cellular function related on proliferation, survival, and angiogenesis (3). Also, there is evidence to suggest that Ras activity is regulated by other mechanisms, such as its ubiquitination-dependent stability (4).

Three RAS genes encode four proteins: K-Ras4A, K-Ras4B, N-Ras, and H-Ras (5). All Ras isoforms share sequences in all of the regions that are related with GDP/GTP binding, GTPase activity, and effector interactions (6). Nevertheless, Ras proteins have isoform-specific functions. These functional differences are most likely associated with the unique C-terminal hypervariable region (HVR) in each isoform, which is thought to modulate the Ras–membrane interaction to specify distinctive localizations (7). Also, although N-ras and H-ras, after palmitoylation, traffic through the classical secretory pathway through the Golgi to the plasma membrane, K-ras traffics to the plasma membrane through a poorly characterized pathway that bypasses the Golgi (8).

MSC (multi-synthetase complex) is composed of nine ARSs (aminoacyl tRNA synthetase), enzyme which ligates the specific amino acid and its cognate tRNA, and AIMP1, 2, and 3, three auxiliary factors (9). Three AIMPs facilitate the assembly of MSC through the interactions with each other as well as with their specific target enzymes (10). In addition to stabilization of MSC, these factors also play the regulatory roles in signaling pathways (11, 12). Especially, upon DNA damage, AIMP2 is dissociated

from the complex and works as a potent tumor suppressor by promoting apoptosis through the protective interaction with p53 (13, 14).

However, AIMP2 produces a variant lacking a part of its structure in cancer, and it was designated as AIMP2-DX2. The splicing variant of AIMP2 lacking exon 2 (AIMP2-DX2) blocks the tumor suppressive activity of AIMP2 full length (AIMP2-F) by competitive binding to p53 (15). Moreover, many cancer cell lines and patients shows the higher expression of AIMP2-DX2 than normal. Additionally, the patients with higher expression of AIMP2-DX2 showed lower survival and poor prognosis. Suppression of AIMP2-DX2 inhibited tumor growth, suggesting it as a new therapeutic target (15).

In this work, we found the functional connection of two oncogenic proteins, AIMP2-DX2 and Ras. Positive correlation between AIMP2-DX2 and Ras was identified by transcriptomic analysis. Through the interaction screening of all the Ras isoforms, the specific interaction of AIMP2-DX2 to K-Ras was confirmed and that binding enhances the stability of K-Ras proteins. Also, in *in vitro* and *in vivo* AIMP2-DX2-inducible system, AIMP2-DX2 induction leads to stabilization of K-Ras. Therefore, this study

indicates a potential mechanism for tumor promotion by synergistic effect of two oncogenic proteins, K-Ras and AIMP2-DX2.

# Results

## Positive connection between AIMP2-DX2 and Ras signaling

To identify the insight how AIMP2-DX2 affects cancer progression, we analyzed the genetic background in AIMP2-DX2 high- or low-expressing lung cancer cell lines. Through transcriptome analysis to check the differentially expressed genes in those cells, we found some significant differences on the level of genes which are related with AIMP2-DX2 levels (Figure 1A). Interestingly, we identified that the proliferative and apoptotic genes are upregulated and downregulated in AIMP2-DX2 high-expressing cells, respectively (Figure 1B). And also the signals of Ras-upstream and -downstream gene expression are specifically increased in cell lines with high expression of AIMP2-DX2 (Figure 1C).

## AIMP2-DX2 specifically increases not N- and H-Ras but K-Ras of protein level regardless of mutation

To study the meaning of positive correlation of AIMP2-DX2 and Ras, we checked whether AIMP2-DX2 enhances endogenous Ras protein in H460

cells. And then, we found that AIMP2-DX2 increases endogenous Ras on protein level (Figure 2A). Also, to further study, we cloned all the Ras isoforms and each frequent mutant in major cancers (Figure 2B). All GFP-Ras isoform wild types (WT) and each cancer-associated mutant were expressed in HEK 293T cells in presence or absence of strep-AIMP2-DX2, and the amounts of GFP were determined by immunoblotting. Specifically, AIMP2-DX2 increased the expression of K-Ras only, neither N-Ras nor H-Ras. Moreover, AIMP2-DX2-mediated increases of K-Ras seem to be totally irrelevant to mutation. Also, we checked whether AIMP2-DX2-mediated increase of K-Ras happen on transcription level. But, AIMP2-DX2 does not affect the level of Ras mRNA. We concluded that AIMP2-DX2 increases K-Ras on protein level (Figure 2C).

**AIMP2-DX2 enhances the stability of K-Ras**

As we found that AIMP2-DX2 increases K-Ras protein expression, we investigated whether AIMP2-DX2 enhances the stability of Ras and contributes to its ubiquitination. To check up the stability of Ras protein, we treated the cycloheximide time-dependently, and we monitored that the

cellular stability of K-Ras, not N- and H-Ras, is only enhanced in condition of AIMP2-DX2 overexpression (Figure 3A, 3B). Moreover, as Ras protein is degraded by ubiquitin-dependent proteasomal degradation (16), we checked the ubiquitination of Ras isoforms in HEK 293T cells in presence or absence of strep-AIMP2-DX2. Likewise, in AIMP2-DX2-overexpressing cells we observed the decreased amount of K-Ras ubiquitination, not N- and H-Ras (Figure 3C).

To confirm the effect of AIMP2-DX2 on endogenous K-Ras in cancer cells, we established strep-AIMP2-DX2 inducible stable cell line with H460, lung cancer cell line. In order to induce AIMP2-DX2, we treated doxycycline time-dependently and monitored the expression of the induced AIMP2-DX2 and endogenous K-Ras. We found that endogenous K-Ras protein level followed the increase of AIMP2-DX2 (Figure 4A). Also this result was confirmed *in vivo*. We manufactured the AIMP2-DX2-inducible mouse and we checked up the expression of AIMP2-DX2 and endogenous K-Ras in spleen of a transgenic mouse. As above, the similar effect of inducible AIMP2-DX2 on the endogenous K-Ras was also observed in the mouse spleen (Figure 4B).

**Specific interaction between AIMP2-DX2 and K-Ras**

As we found that AIMP2-DX2 stabilizes K-Ras, we investigated whether AIMP2-DX2 binds to K-Ras. Through binding screening using immunoprecipitation with all the Ras isoform wild types and mutants, we found that only K-Ras binds with AIMP2-DX2 regardless of mutation, not N- and H-Ras (Figure 5A). Next, we also checked up the imaging assay using confocal microscopy. Through the immunofluorescence experiment, we observed that all the tested GFP-K-Ras wild types and mutant types were co-localized with RFP-AIMP2-DX2, and this result was as same as immunoprecipitation (Figure 5B). Altogether, AIMP2-DX2 specifically interacts with K-Ras.

**Positive patch of K-Ras HVR is critical for interaction with AIMP2-DX2**

To investigate which peptide region of AIMP2-DX2 is critical for its interaction with K-Ras, we performed domain mapping using various AIMP2-DX2 deletion mutants by *in vitro* pull down assay. We purified

GST-proteins containing AIMP2-DX2 1-87, 88-151, 152-251, 1-151 and 88-251 amino acids (Figure 6A), and each purified protein was mixed with the lysate expressing GFP-K-Ras4B wild type. Through the result of *in vitro* pull down assay, we observed GFP-K-Ras4B wild type binds to peptide region containing AIMP2-DX2 88-151 amino acids (Figure 6B). This result shows that 88-151 region of AIMP2-DX2, GST N-terminal domain, is critical for binding with K-Ras. To find the information of this binding region, we checked the structure of K-Ras and GST domain of AIMP2-DX2. Specifically, although the structures of the G domain of K-, N- and H-Ras, are almost identical, K-Ras only has positively charged patch in hypervariable region (HVR) (Figure 6C). Moreover, through the structural analysis of AIMP2-DX2 GST domain, we found that GST domain of AIMP2-DX2 has negatively charged patch (Figure 6D). Hence, we suggest that the each opposite charge peptide region of AIMP2-DX2 and K-Ras is critical for the interaction, but the validation is needed.

**AIMP2-DX2 inhibits the β-TrCP-mediated ubiquitination of K-Ras**

AIMP2-DX2 enhances the protein stability of K-Ras, and protein stability is

usually controlled by proteasome-dependent degradation. Because β-TrCP was reported as an E3 ligase of Ras protein in previous study (16), we tested whether AIMP2-DX2 affects β-TrCP-mediated degradation. First, we checked whether AIMP2-DX2 suppress the β-TrCP-dependent turnover of K-Ras. We observed that β-TrCP-mediated degradation of K-Ras is recovered by overexpression of AIMP2-DX2 (Figure 7A). Next, we checked the ubiquitination of K-Ras. Likewise, the amount of ubiquitinated K-Ras by β-TrCP is declined when AIMP2-DX2 was exogenously introduced (Figure 7B). Lastly, we investigated whether AIMP2-DX2 inhibits interaction between K-Ras and β-TrCP, substrate and E3 ligase, respectively. In the immunoprecipitation, when AIMP2-DX2 was overexpressed, binding of β-TrCP to K-Ras was decreased (Figure 7C). Altogether, AIMP2-DX2 inhibits the interaction between K-Ras and β-TrCP, resulting in suppression of ubiquitination of K-Ras.

All of these results suggest that AIMP2-DX2 enhances the stability of K-Ras via interaction with K-Ras. In AIMP2-DX2 overexpression condition, like cancer, the binding of AIMP2-DX2 to K-Ras blocks the approach of β-TrCP, E3 ligase, and then, it leads to stabilization of K-Ras.

That results in synergic oncogenic property through connecting with two oncogenic proteins, AIMP2-DX2 and K-Ras (Figure 8).

# DISCUSSION

This study proposes AIMP2-DX2, oncogenic protein, as a key determinant required in the K-Ras-stabilizing effect. Through transcriptome analysis, we found that AIMP2-DX2 is positively associated with Ras signaling. And, it was also found that AIMP2-DX2 specifically binds to K-Ras and increases K-Ras, not N- and H-Ras, of protein level, regardless of mutation. AIMP2-DX2 is a positive regulator contributed to the stabilization of K-Ras by inhibiting the recruitment of β-TrCP, E3 ligase, with K-Ras. The mechanism implies AIMP2-DX2 stabilizing K-Ras and amplifying the signal transductions of oncogenic K-Ras. To further study on synergistic oncogenecity of two proteins via Ras stabilization, *in vitro* and *in vivo* phenotype analysis, and patient analysis need to be performed. Also, as K-Ras has oncogenic properties through its downstream signal transduction related on proliferation and survival, influences of AIMP2-DX2 on downstream effectors of K-Ras need to be involved in further study.

Aberrant Ras function is associated in various cancers. As the mechanisms underlying increase of pathological level of K-Ras are unclear,

many clinical trials targeting K-Ras yielded disappointing results. However, this study shows AIMP2-DX2 suppresses the degradation of K-Ras, regardless of mutation, and it means AIMP2-DX2-targeting will be covered both wild type and mutant of K-Ras degradation. Therefore, this study is a highly significant discovery that unveils the mechanism of pathological K-Ras stabilization.

Our results are meaningful in the aspect that a gain-of-function of AIMP2-DX2 was unveiled for the first time. Oncogenic roles of AIMP2-DX2 have been limited to blocker of AIMP2-F since it was found that AIMP2-DX2 is an alternative splicing form of AIMP2-F. The role of AIMP2-DX2 related to K-Ras stabilization is distinguishable from that of AIMP2-F. These results will be used for the basis on the study of oncogenic AIMP2-DX2. And, the connection between AIMP2-DX2 and K-Ras implies the synergistic oncogenecity of two proteins.

Based on the results from this study, AIMP2-DX2 could be a novel target in various cancers that K-Ras is crucial such as pancreatic cancer, colorectal cancer and lung cancer. Additionally, interaction between AIMP2-DX2 and K-Ras supposes new approaches toward drug development on

targeting K-Ras. Downregulation of AIMP2-DX2 via targeting it has two merits: 1) Decrease of AIMP2-DX2 leads to decline of oncogenic protein K-Ras since the result of this study, AIMP2-DX2-mediated stabilization mechanism of K-Ras. 2) Since AIMP2-DX2 blocks the tumor suppressor activity of AIMP2-F in various signals, decline of AIMP2-DX2 leads to the enhanced tumor suppressor activity of AIMP2-F, resulting in regression of cancer. This study gives a solution, or suggests a potential candidate that could be an efficacious therapeutic medicine for suffering patients from the absence of proper drugs. Therefore, we propose possibility of new therapy related with K-Ras by targeting AIMP2-DX2. Targeting AIMP2-DX2 will be significant in treatments for major cancers without good drugs.

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**Figure 7. AIMP2-DX2 inhibits the β-TrCP-mediated ubiquitination of K-Ras**

**Figure 8. Schematic model**

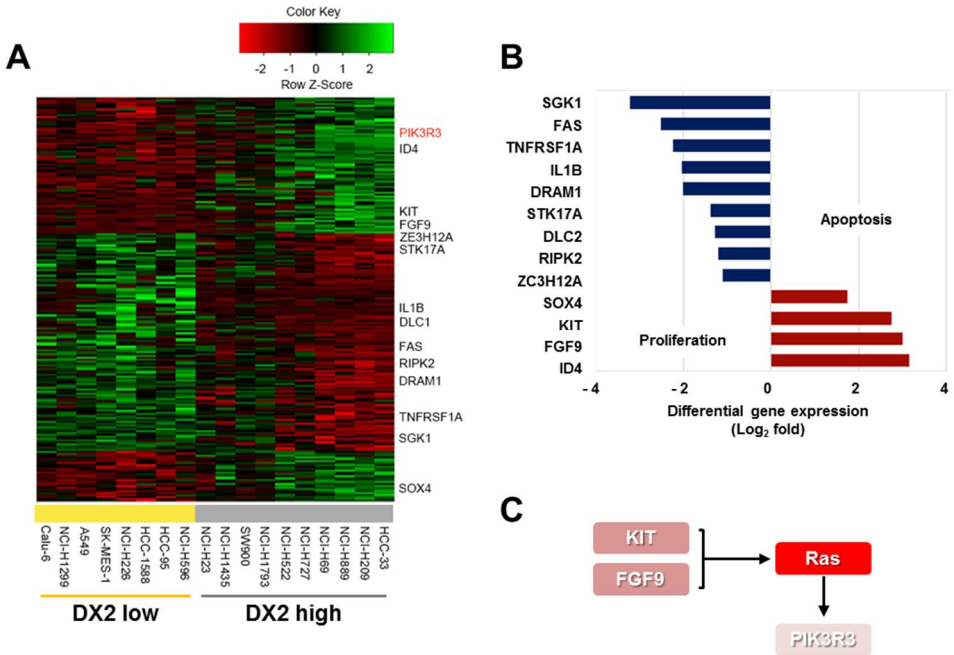
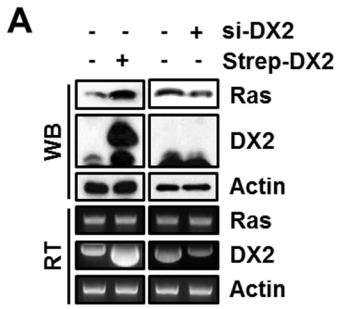



**Figure 1. AIMP2-DX2 is positively associated with Ras signaling**

**(A) Gene signature by transcriptome analysis to find DEG (differentially expressed genes) in AIMP2-DX2 low or AIMP2-DX2 high lung cancer cell lines. The expression of AIMP2-DX2 in 18 lung cancer cell lines was analyzed by western blotting. Green and red signature means the increased and decreased gene expression, respectively.**

**(B) Differentially expressed proliferative or apoptotic genes in AIMP2-DX2 high or low cell lines are classified and displayed.**

**(C) Signals of Ras-upstream and -downstream gene expression are specifically increased.**




B

| Ras isoform | Mutation | Disease |
|---|---|---|
| K 4A / 4B | G12C | Lung |
| | G12D | Pancreas, colorectal |
| N | Q61R | Haematopoietic /lymphoid, skin |
| H | G12V | Bladder |

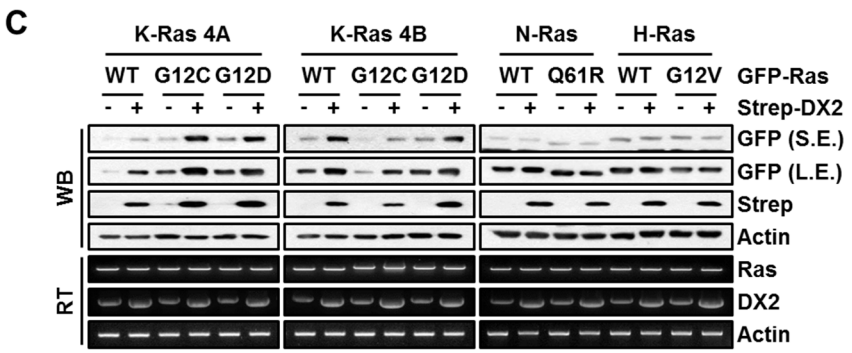



**Figure 2. AIMP2-DX2 specifically increases K-Ras not N- and H-Ras of protein level regardless of mutation**

**(A) AIMP2-DX2 increases endogenous Ras protein level in H460 cells.**

**(B) Ras isoform-specific point mutation and disease.**

**(C) GFP-Ras isoforms wild type (WT) and each mutant were expressed in HEK 293T cells in presence or absence of strep-AIMP2-DX2, and the amounts of GFP were determined by immunoblotting. Actin was used as a loading control. S.E. and L.E. means short expose and long expose, reslectively. Also, Same cells as above were subjected to RT-PCR for checking the mRNA level of Ras. Actin was used as a loading control of RT-PCR.**

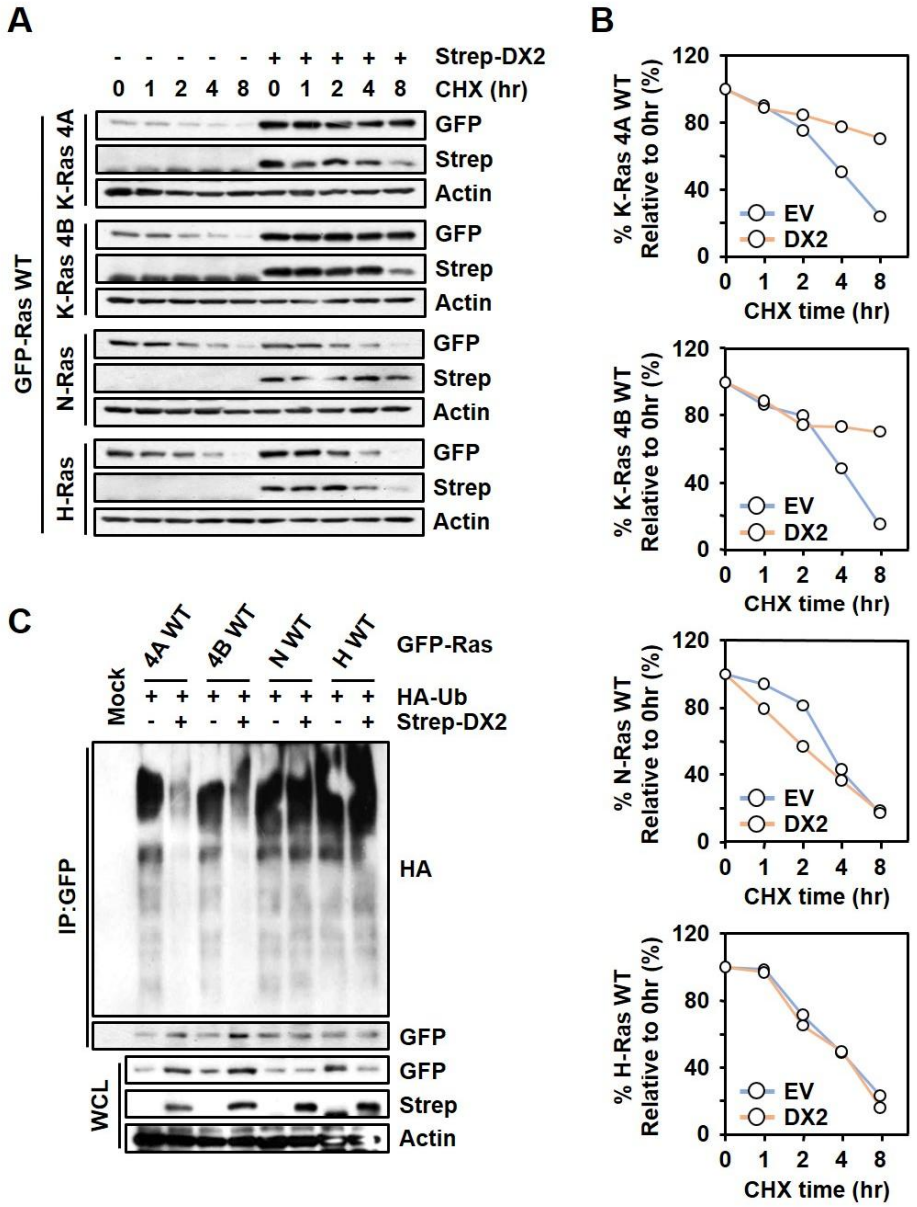



**Figure 3. AIMP2-DX2 enhances the stability of K-Ras**

**(A and B) The cellular stability of GFP-Ras isoforms wild type (WT) was determined by treatment of cycloheximide (CHX) time-dependently as indicated above. The amounts of Ras were determined**

**by immunoblotting using antibody against GFP (A). Results of immunoblotting were quantified and displayed as a graph (B). Actin was used as a loading control.**

**(C) The effect of AIMP2-DX2 on ubiquitination of Ras was confirmed. The same cells as above were treated with MG-132 for 4 hours and subjected to ubiquitination assay.**

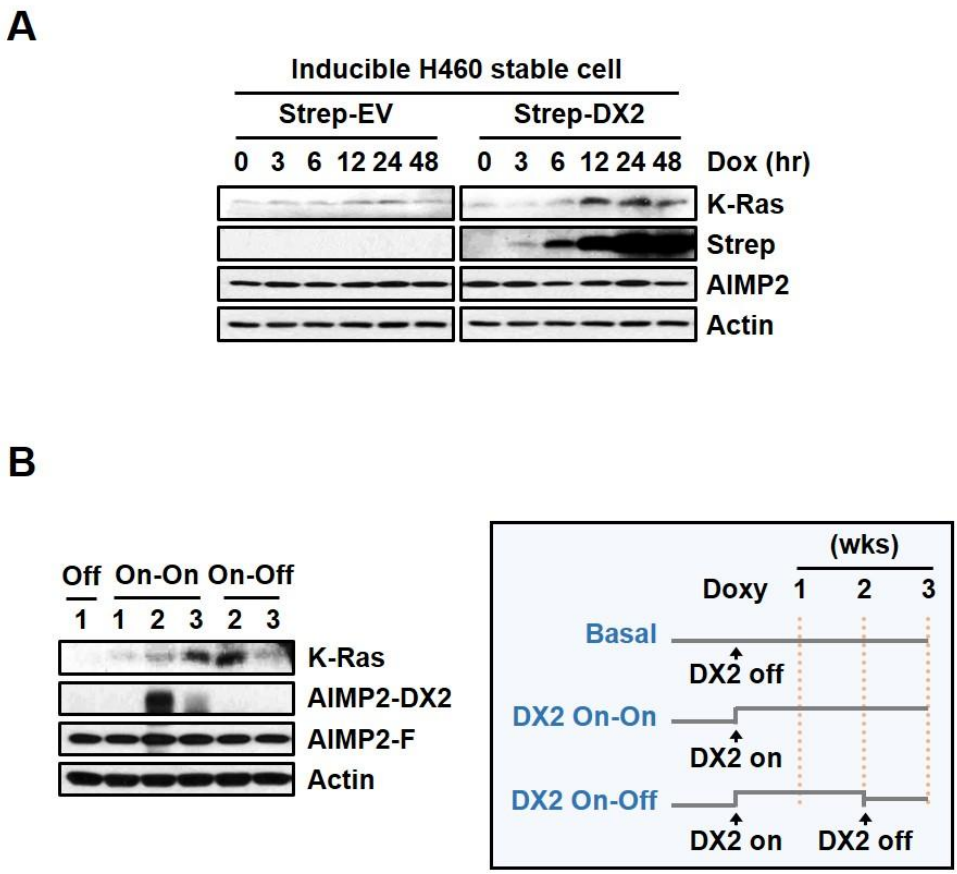



**Figure 4. AIMP2-DX2 enhances the stabilization of K-Ras**

**(A) AIMP2-DX2-inducible H460 cells were treated with doxycycline (Dox) time-dependently for induction of AIMP2-DX2. The expression of K-Ras and AIMP2-DX2 were checked by western blotting using their specific antibodies. EV means empty vector for using negative control to AIMP2-DX2. Actin was used as a loading control.**

**(B) AIMP2-DX2-inducible mouse was treated with doxycycline (Doxy) time-dependently or not, and spleen of sacrificed mouse was analyzed by immunoblotting. Experimental schedule of AIMP2-DX2 turn on or off was shown in right table.**

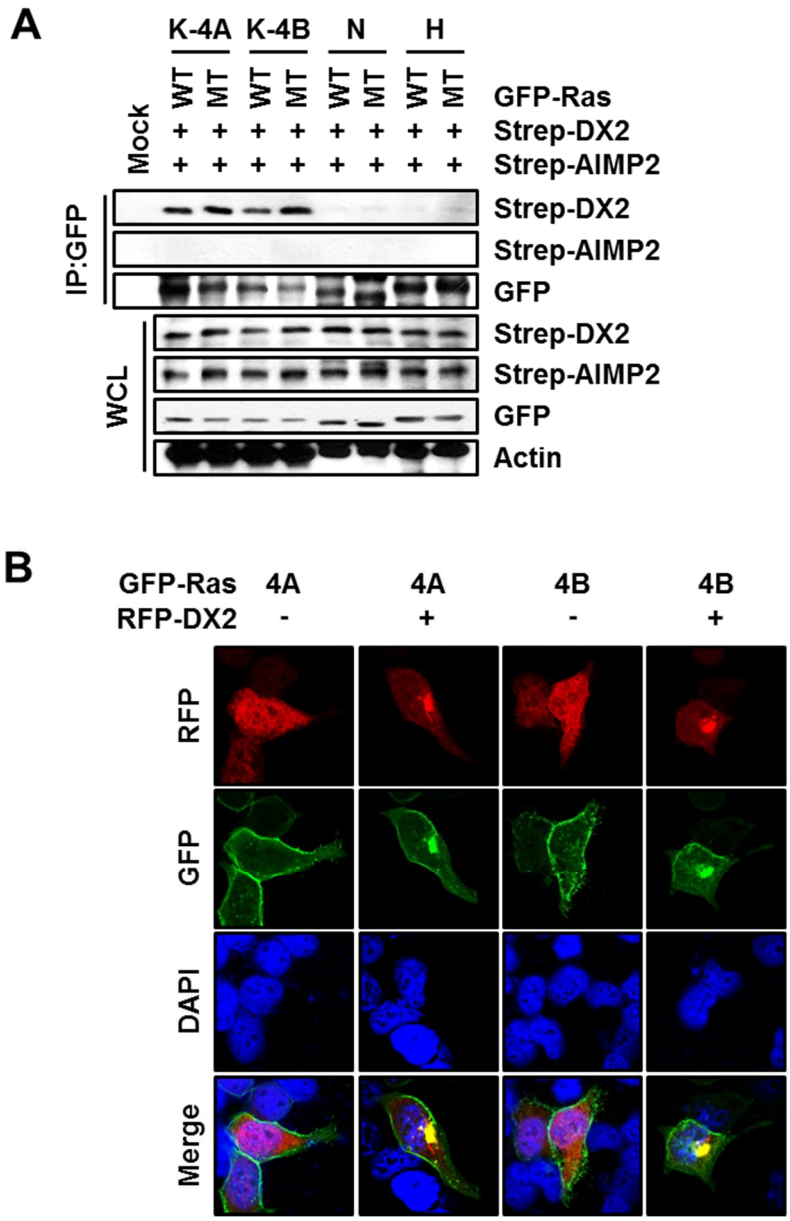



**Figure 5. AIMP2-DX2 binds to K-Ras**

**(A) Interaction of AIMP2-DX2 with GFP-Ras isoforms wild type (WT) and each mutant was confirmed by co-immunoprecipitation. GFP-Ras-expressing HEK 293T cells were introduced by strep-AIMP2-DX2 and**

**the cells were subjected to immunoprecipitation with GFP-tag column. Co-precipitated AIMP2-DX2 with Ras was analyzed by western blotting using antibody against Strep.**

**(B) RFP-AIMP2-DX2 and GFP-Ras isoforms wild type or mutant type were expressed in HEK 293T cells. Colocalization of two proteins was monitored with GFP and RFP by using confocal microscopy. DAPI staining means the nucleus.**

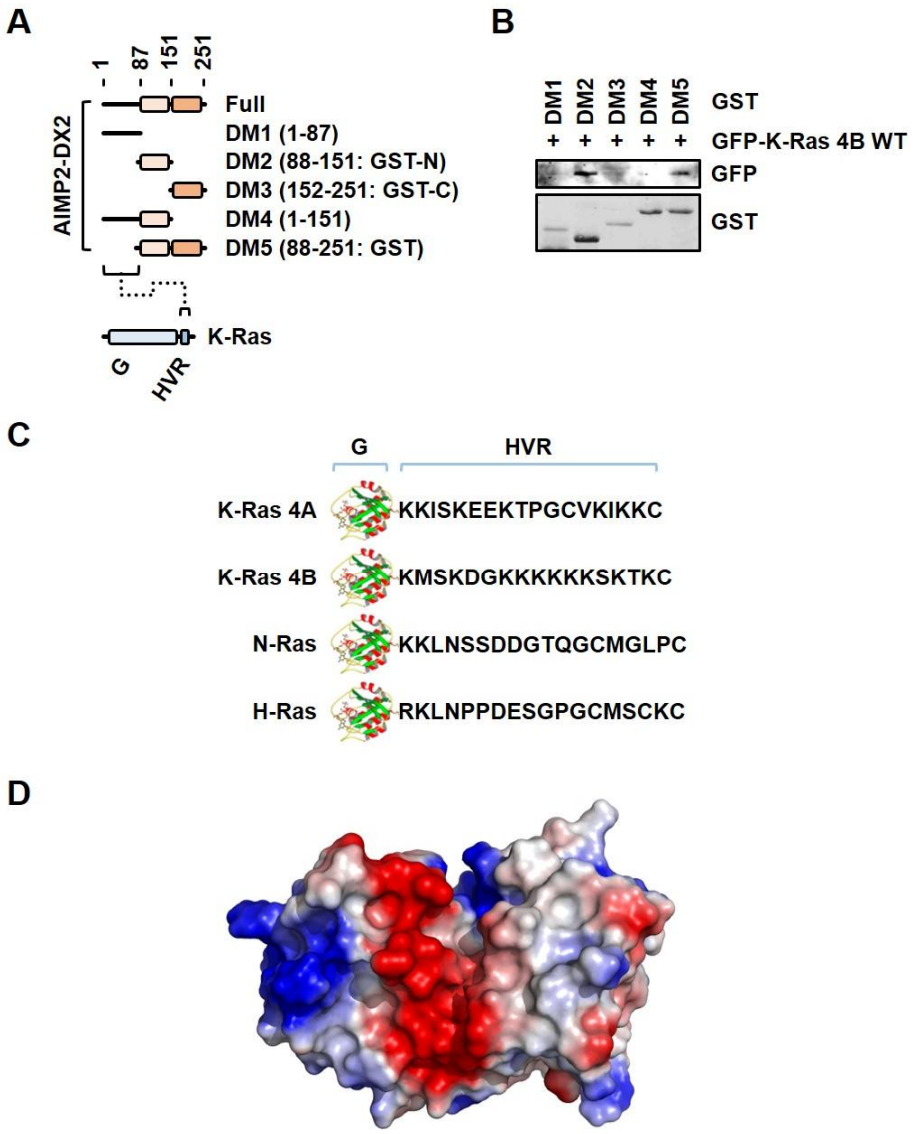



**Figure 6. Negative patch of AIMP2-DX2 interacts with positive patch of K-Ras hyper variable region (HVR)**

**(A) Domain arrangement of AIMP2-DX2 and K-Ras protein. AIMP2-DX2 is composed of three peptide region as indicated.**

**(B) GST proteins containing 1-87, 88-151, 152-251, 1-151 and 88-251 amino acids region of AIMP2-DX2 were purified. Each purified GST protein was incubated with extracts from HEK 293T cells expressing GFP-K-Ras4B wild type (WT) and pulled down with Glutathione Sepharose beads. The precipitated Ras was detected by western blotting using anti-GFP antibody. The amounts of each purified GST proteins were determined by coomassie staining.**

**(C) Domain arrangement of Ras isoforms wild type (WT). The structures of the G domain of K-, N- and H-Ras have been solved and are virtually identical, but the structure of processed hypervariable regions (HVR) has not been solved and is depicted as a linear sequence. K-Ras only has positively charged patch.**

**(D) The structure of GST domain of AIMP2-F. Negatively and positively charged patch was shown as red and blue colors, respectively.**

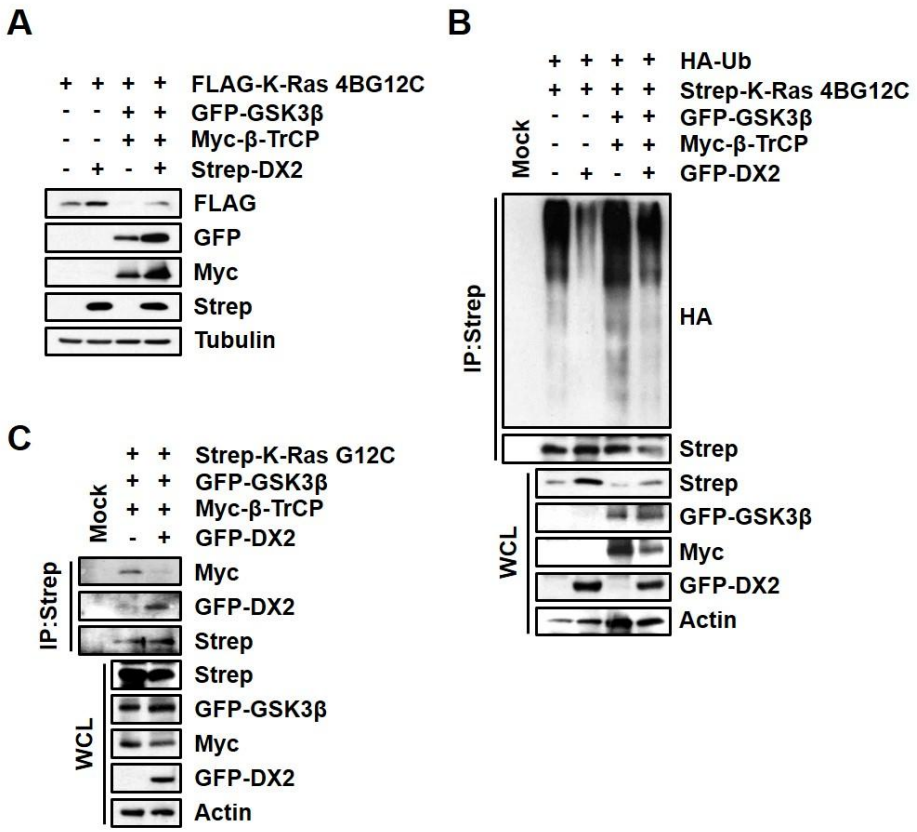



**Figure 7. AIMP2-DX2 inhibits the β-TrCP-mediated ubiquitination of K-Ras**

**(A) The effect of AIMP2-DX2 on β-TrCP-mediated degradation of K-Ras. Each plasmid as indicated was expressed in HEK 293T cells and the cells were subjected to SDS-PAGE. The expression of each protein was checked by immunoblotting with its specific antibody. Tubulin was used as a loading control.**

**(B) The effect of AIMP2-DX2 on β-TrCP-mediated ubiquitination of K-Ras. The same cells as above expressed exogenous HA-Ub and were treated with MG-132 for 4 hours. The cells were subjected to**

**ubiquitination assay. The amounts of ubiquitinated K-Ras were determined by immunoblotting with the antibody against HA. Actin was used as a loading control.**

**(C) The effect of AIMP2-DX2 on the interaction between β-TrCP and K-Ras. Indicated plasmids were expressed into HEK 293T cells and K-Ras was precipitated with strep-tag column. Co-precipitated β-TrCP and AIMP2-DX2 with K-Ras were detected by western blotting.**

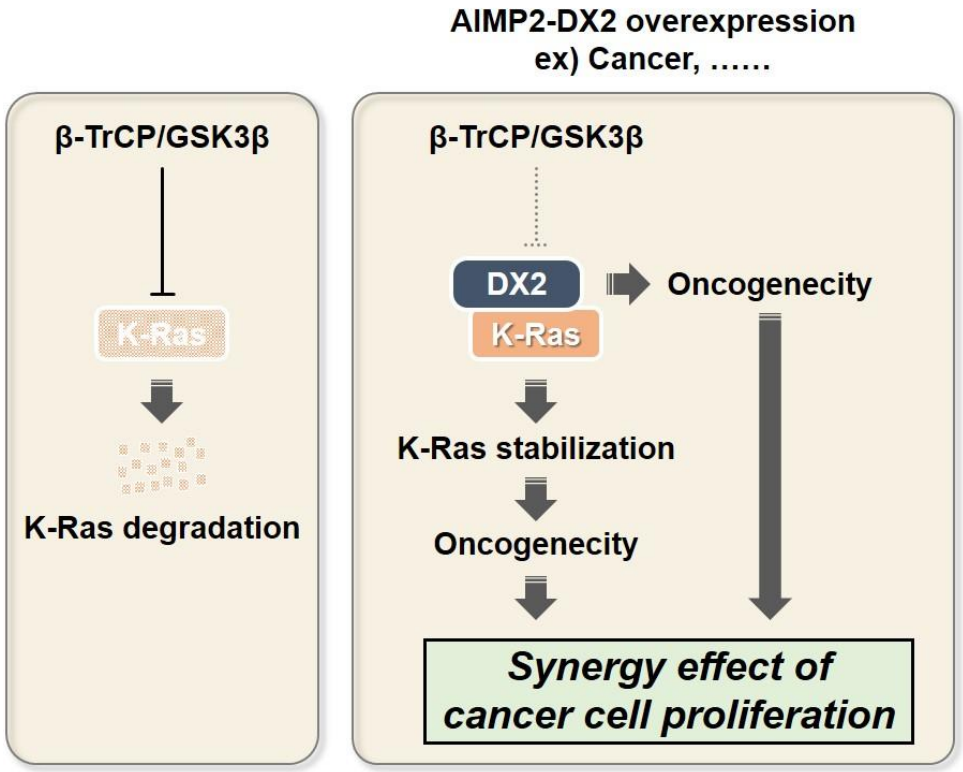



**Figure 8. Schematic model**

**In normal condition, β-TrCP/GSK3β, K-Ras degradation complex, leads to the proteasome-dependent degradation of K-Ras (left). Binding overexpressed AIMP2-DX2, oncogeninc protein, to K-Ras blocks the approach of β-TrCP/GSK3β complex for protection the ubiquitination, resulting in stabilization of K-Ras. Interaction of two oncogenic proteins, K-Ras and AIMP2-DX2, makes the synergetic oncogenecity, leading to enhancement of cancer cell proliferation.**

# Material and methods

## Cell culture and material

Mammalian cells such as H460 in RPMI with HEPES (hyclone), HEK 293T in DMEM (hyclone) were cultured at 37˚C with $CO_2$ 5%. Strep-DX2 or strep-EV inducible H460 stable cell was cultured in RPMI with HEPES. Strep-tagged human AIMP2-DX2 was lab material. GFP-tagged human K-Ras4A, K-Ras4B, N-Ras and H-Ras were gifted by Mark Philips (NY Univ.). HA-tagged Ub, Flag-tagged human K-Ras, and RFP-tagged DX2 were lab materials. GFP-tagged GSK3β and Myc-tagged β-TrCP were gifted by Kang-Yell Choi (Yonsei Univ.) GFP-Ras isoforms containing point mutation of G12 or Q61 were cloned using the QuickChange®Ⅱ (Agilent), following the manufacturer's instruction. , Protease Inhibitor and MG132 were purchased from Calbiochem. Cycloheximide was purchased from Sigma-Aldrich. Antibody against Strep was purchased from IBA. The other primary antibodies using in this study were purchased from Santa Cruz Biotechnology. Secondary antibodies, Mouse (Thermo scientific Immunopure Ab, Host Goat-Anti, Antigen Mouse IgG [H+L], Cat 31430),

and Rabbit (Thermo scientific Immunopure Ab, Host Goat-Anti, Antigen Mouse IgG [H+L], Cat 31430) were used. Streptavidine Sepharose™ High Performance and Glutathione Sepharose were purchased from GE Healthcare.

**Western Blot**

HEK 293T cell line was used. Lysis buffer (1% Triton X-100, 0.1% SDS, and protease inhibitor in PBS) was applied to the cells and it was incubated for 45 min at 4℃. The supernatant was taken after centrifuge, which was quantified with Bradford assay (BioRad, Cat. 500-0006). Final sample was maded by adding 5X laemmli sample buffer. Samples were boiled for 7min before gel running. After that, samples were loaded on 12% SDS-PAGE gel and separated by electrophoresis. Proteins at the gel were transferred to PVDF membranes from Merck Millipore for 100mins, 55mA per gel. Blocking with 5% skim milk based 0.5% TBS-T for 1hr was performed, followed by 1$\mu g/\mu \ell$ of primary antibody (1% skim milk) binding for 2hr at RT. Incubate with secondary antibody in 1:20000 was for 1hr at RT. The film was exposed normally 2 to 15min.

**RT-PCR**

Transcript level of AIMP2-DX2 and Ras was measured by RT-PCR. RNA was gathered by H460, which was transfected with plasmid DNA, using RNeasy Mini kit from Qiagen as manufacturer's instruction. 2$\mu g$ of RNA was reverse-transcribed with M-MLV Reverse Transcriptase (Invitrogen), Random hexamer (Qiagen), dNTP (Takara), forward primer, reverse primer and reaction buffer.

| Gene | Primer Sequence (5'-3') |
|---|---|
| K-Ras | F_CAGTGCAATGAGGGACCAGT<br>R_ AGTCCTGAGCCTGTTTTGTGT |
| N-Ras | F_ACCAATACATGAGGACAGGCG<br>R_ AACTCTTGGCCAGTTCGTGG |
| H-ras | F_AGCAGGTGGTCATTGATGGG<br>R_ AGGCATCCTCCACTCCCTG |
| AIMP2 | F_CCGGAATTCATGCCGATGTACCAGGTAAAG<br>R_CCGCTCGAGTTAAAAAGGAGCCAGGTTTTC |

After synthesizing cDNA from the extracted RNA, transcript level of AIMP2-DX2 and Ras was measured using PCR Premix from Bioneer through PCR.

**Immunoprecipitation**

HEK 293T cell line was lysed with 50mM Tris-HCl (pH7.4) buffer containing 100mM NaCl, 0.5% Triton X-100, 0.05% SDS, 10% glycerol,

1mM EDTA and protease inhibitor. To remove the nonspecific IgG bound proteins, protein extracts were incubated with normal IgG and protein agarose for 2hr and then centrifuged. The supernatants were mixed with specific antibody, incubated the mixture for 4hr at 4℃ with gentle agitation, added protein agarose. After agitation, precipitates were washed with the cold lysis buffer three times, and precipitates were dissolved in the SDS sample buffer and subjected to SDS-PAGE.

**Immunofluorescence staining**

HEK 293T cell line was used. Cells were transfected with both GFP-Ras isoforms wild type or mutant type and mcherry-DX2. Cells were washed briefly with cold PBS. After incubation with the blocking buffer, 1% CAS, for 30min and mounted with mounting solution containing DAPI (ImmunoBioScience Corp.). The mounted samples were observed by confocal laser-scanning microscopy.

**GST fusion protein purification and pull down assay**

GST-DX2 domain fragment proteins were purified as follows. BL21

competent cells were transformed with each DNA and cultured in LB media containing ampicillin. The competent cells were induced by 1mM IPTG and cultured overnight at 18℃. The competent cells were centrifuged at 3,000 rpm for 30min at 4℃. The pellet was resuspended by PBS containing 0.5% Triton X-100 and protease inhibitor and lysed by sonication on ice. The lysate was centrifuged at 13,000 rpm for 30min at 4℃. The supernatants were incubated with Glutathione Sepharose (GE Healthcare) overnight at 4℃ under rotary agitation. The purified proteins were quantified by InstantBlue.

Purified GST-tagged proteins containing beads were incubated with lysates, which were transfected HEK 293T cell lysates, overnight at 4℃ under rotary agitation. After agitation, precipitates were washed with the cold lysis buffer three times, and precipitates were dissolved in the SDS sample buffer and subjected to SDS-PAGE.

**Ubiquitination assay**

The cells were pre-incubated with MG132 for 4hr (50μM), and were lysed with lysis buffer used in Western Blot. The interesting proteins were immunoprecipitated with specific antibody and subjected SDS-PAGE for immunoblotting to determine the amounts of ubiquitinated protein.

**Lenti-X Tet-One Inducible Expression System**

The following are the steps required to create a doxycycline-inducible expression system using lentivirus. Strep-EV and Strep-DX2 were cloned into the pLVX-TetOne Vector using In-Fusion HD. Lentiviral supernatants were produced using the Lenti-X HTX Packaging System. H460 cells were transduced with TetOne virus.

# 국문초록

K-Ras 는 암 세포에서 가장 빈번하게 변형된 종양 형성 유전자로 잘 알려져 있다. 뿐만 아니라 암 환자의 생존률 감소에 영향을 미치며, 초기 진단을 통한 처방에도 어려움을 주는 요소이다. K-Ras 가 암 치료에 중요한 타겟임에도, 암세포 내의 K-Ras 단백질 증가와 관련한 기전 연구가 잘 밝혀져 있지 않다. 우리는 본 연구를 통해, AIMP2-F 의 변성체로 알려진 종양 형성 유발인자인 AIMP2-DX2 가 K-Ras 의 단백질 안정화에 기여하는 데에 중요한 역할을 한다고 보고한다. 다양한 AIMP2-DX2 의 발현을 보이는 폐암 세포의 전사체 분석을 통하여, AIMP2-DX2 가 Ras 신호체계와 관련이 있음을 확인하였다. 또한 AIMP2-DX2 를 유도하는 *in vitro* 와 *in vivo* 모델을 통하여, AIMP2-DX2 를 유도함으로써 K-Ras 안정화가 이루어지는 것을 확인하였다. AIMP2-DX2 GST-N 범위가 양전하를 띠는 K-Ras 의 hypervariable region 과 결합하는 것을 확인하였고, AIMP2-DX2 와의 결합을 통해 K-Ras 의 E3 ligase 로 알려진 β-TrCP 에 의한 ubiquitination 이 감소하는 것을 확인 할 수 있었다. 본 연구는 K-Ras 의 안정화가 AIMP2-DX2 와의 결합을 통하여 이루어질 수 있음을 제시하며, 이는 두 병리적인 종양형성 유전자 간의 상호 연결 관계를 밝힘으로써 암세포 성장을 촉진하는 데에 상승적인 역할을 함을 밝힌다.

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