약학석사학위논문

Studies on methionyl-tRNA synthetase -dependent stabilization of cyclin-dependent kinase 4 in lung cancer

폐암에서의 Methionyl-tRNA synthetase (MRS) 에 의한 Cyclin-dependent kinase 4 (CDK4) 안정화 기전 연구

2017년 2월

서울대학교 융합과학기술대학원
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이 논문을 약학석사학위논문으로 제출함

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ABSTRACT

Cyclin-dependent kinase 4 (CDK4) is an important regulator of cell cycle progression which is related to cell proliferation. Interestingly, the level of CDK4 protein is remarkably elevated compared to the gene amplification rates in lung cancer. It suggests the existence of underlying mechanism that can further increase the CDK4 level after gene expression as well as the potential dependence on the CDK4 level in lung cancer.

Our previous study reveals that methionyl-tRNA synthetase (MRS) specifically stabilizes CDK4 protein, while other CDKs are not affected in lung cancer. However, the mechanism of MRS-mediated stabilization of CDK4 is not well understood yet.

Here, this study demonstrates that MRS enhances the complex formation among CDK4, heat shock protein 90 (HSP90) and cell division cycle 37 (CDC37), leading to increase in the CDK4 level. The increased CDK4 level by MRS overexpression was reduced by HSP90 inhibitor. MRS directly interacted with HSP90 and formed the ternary complex with CDK4 and HSP90. It implies that the function of MRS on stabilizing CDK4 requires the chaperone function of HSP90. To find out which functions of HSP90 MRS modulates for the stabilization of CDK4, the effects of MRS on the ATPase activity of HSP90 and the interaction between HSP90 and CDK4 were investigated. Whereas the ATPase activity of HSP90 was not affected by MRS addition, the association between CDK4 and HSP90 was clearly increased in a dose-dependent manner. It suggests that MRS plays a significant role in the recruitment of CDK4 into HSP90. Depletion of MRS

using small interference RNA dissociated CDK4 from HSP90. The association was synergistically reduced by addition of a compound, the MRS-CDK4 interaction inhibitor. In particular, other HSP90 clients, such as CDK7 and p70S6K, known to be stabilized by HSP90 were not affected in this condition. It suggests that MRS exclusively works on the CDK4 stability, but not other HSP90 clients. Furthermore, MRS level was positively critical for the association between CDC37 and HSP90, as well.

Taken together, this research reveals that MRS specifically stabilizes CDK4 by recruiting CDK4 and CDC37 into HSP90.

Key words : CDK4, MRS, HSP90, CDC37, stabilization, interaction, complex formation

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INTRODUCTION

Uncontrolled cell proliferation is one of the key hallmarks of cancer and is caused by dysregulation of cell division (1, 2). Thus, blocking the uncontrolled cell division has been considered as an effective cancer targeting strategy. One of the known mechanisms causing the uncontrolled cell division is aberrantly upregulated function of cyclin-dependent kinases (CDKs).

During cell division, cell must pass through a predetermined series of stages, termed cell cycle which is conserved in mammalian cells (2). Each stage is strictly controlled by different subtypes of CDKs which are associated with and activated by Cyclins. Among the CDK and Cyclins complexes, CDK4 and Cyclin D1 are in charge of G1 phase to early S phase transition. In response to mitogen stimuli, cells synthesize cyclin D1, which associates with CDK4. The CDK4-Cyclin D1 complex phosphorylates retinoblastoma protein (pRb), followed by release of E2F family transcription factors from pRb. It results in the expression of essential genes for the next phase of cell cycle.

Noticeably, CDK4 inhibition has been regarded as a selective targeting strategy on cancer with less toxicity on normal cells (3). Recent genetic studies suggest that CDK4 is only required for the proliferation of specific cell types, not essential for the cell cycle in all cell types. Whereas systematic knockout of CDK4 causes little effects on mouse germ line development or proliferation, CDK4 is critical for the development of cancers initiated by specific oncogenes such as Erbb2, Hras or Myc depending on cellular context. It seems that the function of CDK4 can be compensated by other CDKs under normal condition, but not be substituted by other factors in cancer.

It is note worth that the level of CDK4 protein is detected at much higher frequency such as 30-50% in lung cancer, whereas the gene amplification rates are observed at 1.7-4.3% (9-13). It suggests the existence of underlying mechanism that can further increase the CDK4 level after gene expression as well as the importance of CDK4 pathway in lung cancer.

Methionyl-tRNA synthetase (MRS) is known as one of aminoacyl-tRNA synthetases (ARSs) and plays an essential role in protein synthesis, especially in translation initiation by transferring methionine to initiator tRNA (14). Considering the importance of MRS in translation initiation, it is not surprising that MRS regulates global translation in response to diverse cellular conditions, such as mitogenic signals, UV irradiation and oxidative stress (15-17). These data also support that MRS can sense environmental signals and has the ability to cope with the stress or stimuli. Our previous study demonstrates that MRS specifically stabilizes CDK4 protein without affecting other CDKs in lung cancer. Depletion of MRS reduced the CDK4 protein level, not the CDK4 transcript, while other CDKs were not affected. Based on the MRS-CDK4 interaction, we tested several methionine analogues and found FSMO [Fmoc-Sec(Mob)-OH], which is a specific MRS-CDK4 interaction inhibitor. FSMO inhibits the interaction between MRS and CDK4 at relatively low concentration (25-50 μM), without affecting MRS catalytic activity although it affects both the interaction as well as MRS catalytic activity at relatively high concentration (> 100 μM) (18). FSMO treatment decreased the CDK4 level, resulting in G1 cell cycle arrest and suppressed cell proliferation, mimicking MRS depletion.

Although the clear effect of MRS on CDK4 stabilization was observed via MRS depletion as well as chemical inhibition, the mode of action of the MRS-

mediated stabilization of CDK4 is not well understood yet. Thus, this study was designed to clarify how MRS stabilizes CDK4.

MATERIALS AND METHODS

Cell cultures

H460 cells and CHO cells were purchased from the American type culture collection bank and were cultured using RPMI supplemented with 10% FBS and 1% penicillin. Cells were maintained at 37^{o}C, 5% CO2 in a humidified incubator.

Plasmids were transfected using X-treme transfection reagents (Roche, Cat.6366546001) and si-RNAs were transfected using Lipofectamine 2000 (Invitrogen, Cat. 11668-019), according to the manufacturer's instructions.

Table of si-RNA sequences

si-RNA	**Sequences**
si-MRS	CAG AGC AAG UGG ACC UGU AUC AGU U
si-CDC37	CCC ACC AGA CAA UCG UCA U
3' UTR si-CDC37	CAG CAA UGA UCU UCC AAU A

Immunoblotting

Cells were lysed in cold lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, supplemented with protease inhibitor (Calbiochem, Cat. 535140) and phosphatase inhibitor (Thermo scientific, Cat. 78427)] for 30 min at 4^{o}C. Then, each lysate was collected into Eppendorf tubes and centrifuged at 13,200 rpm for 20 min at 4^{o}C (Eppendorf, DE/5415R).

The supernatant proteins were quantified by Bradford assay (Biorad, Cat. 500-0006). Sample buffer and lysis buffer were added to quantified proteins to make the final samples.

After boiling samples for 7 min at 100^{o}C, they were loaded on SDS page gels and separated by electrophoresis. Gel which obtained the protein was transferred to polyvinylidenefluoride membranes (Milipore, Cat. IPVH 00010) at 55 mA, 6 V for 1 hr 30 min (BioRad, Cat. Powerpac 3000). The membranes were incubated with 5% skim milk solution based 0.5% TBS-T for 1 hr to prevent non-specific bindings.

After removing the skim milk solution, primary antibodies were added to the each membrane during overnight at 4^{o}C. Next day, the membranes were washed with 0.5% TPBS-T buffer for 5 min, 3 times repeatedly. Secondary antibodies were added for 1 hr. The membranes were washed equally to the previous step, and ECL solution (Santacruz biotechnology, Cat. sc-2048, GE healthcare life sciences, Cat. RPN2232) was applied to the membrane.

Immunoprecipitation

Cells were lysed in cold lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, supplemented with protease inhibitor (Calbiochem, Cat. 535140) and phosphatase inhibitor (Thermo Scientific, Cat. 78427)] for 30 min at 4^{o}C.

Then, the cell lysates prepared as described above. Equal amounts of protein (700-1000 μg) were incubated with primary antibody for 2 hr at 4^{o}C, and then added to protein G beads (Invitrogen, Cat. 15920-010) and incubated for 2 hr at 4^{o}C. The samples were washed for 7 min, 3 times repeatedly.

Sample buffer was added to the beads and heated for 7 min at 100^{o}C to elute the immunoprecipitated proteins. The samples were analyzed by immunoblotting.

Table of antibodies for immunoblotting

Protein	**Product no.**	**Source**
Strep	2-1509-001	iba
CDK4	sc-260	Santacruz biotechnology
CDK4	sc-23896	Santacruz biotechnology
MRS	NMS-01-0003	Neomics
MRS	ab50793	Abcam
β-Actin	A1978	Sigma
Cyclin D1	04-221	Millipore
Cyclin D1	#2922	Cell signalingtechnology
HSP 90 α/β	sc-7947	Santacruz biotechnology
Flag	F3165	Sigma
HA	sc-7392	Santacruz biotechnology
HA	sc-805	Santacruz biotechnology
CDC37	sc-5617	Santacruz biotechnology
c-Myc	A190-205A	Bethyl
c-Myc	M192-3	MBL
Phospho-Rb (Ser780)	3590S-CST	Cell signaling technology
CDK7	2916P	Cell signaling technology

In vitro pull down assay

Glutathione-S transferase (GST)- and maltose-binding protein (MBP)-fusion proteins were expressed in Rosetta competent cell and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 hr at 18^{o}C. Harvested cells were lysed by sonication (Qsonica, Q125) and lysates were incubated with glutathione Sepharose 4B (GE healthcare life sciences, Cat. 17-5279-01) or amylose resin (New england biolabs, Cat. E8021S) in the lysis buffer (PBS containing 0.5% Triton X-100 and protease inhibitor) at 4^{o}C for 12 hr.

Radiolabeled MRS was synthesized by in vitro translation with the TNT-coupled translation kit (Promega, Cat. L1170) and incubated with immobilized GST-fusion protein at 4^{o}C for 4 h in binding buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5mM EDTA, 1 % NP-40, supplemented with protease inhibitor (Calbiochem, Cat. 535140). The beads were washed three times with binding buffer. Eluted proteins were separated by SDS/PAGE and detected by autoradiography.

In vitro malachite green assay

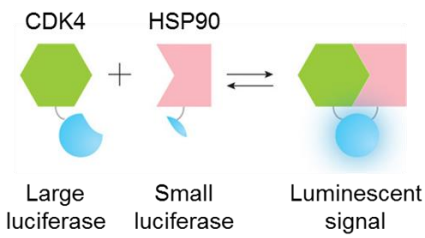
Purified His tagged human HSP90 α protein was purchased from Sigma (Cat. SRP5191). The ATPase activity of HSP90 was monitored by malachite green assay kit (Sigma, Cat. MAK113). The malachite green reagent forms a stable dark green color with free phosphate liberated by the HSP90 resulting in a colorimetric product, measured at 620 nm, proportional to the HSP90 activity present.

We optimized the HSP90 concentrations and incubation times with large amounts of ATP (data not shown). We incubated 0.5 μM Hsp90 and 1 mM ATP with the indicated amounts of MRS for 2 hr at 37^{o}C.

NanoBiT (nanoluc binary technology) assay

NanoBiT assay is a structural reporter that uses two small complementary subunits as fusion partners with proteins of interest. When those proteins interact, the two subunits of NanoBiT are brought together to form an active enzyme.

CHO-K1 cells were transfected with 0.5 μg of each pBiT1.1-C [TK/LargeBiT] Vector cloned with CDK4 and pBiT2.1-C [TK/SmallBiT] vector inserted with HSP90, respectively, and with different concentrations of pEXPR-IBA105-MRS plasmid (from 0.13 to 2 μg), using Turbofect transfection reagents (Fermentas, Cat. R0531). After 48 hr incubation, cells were incubated with Nano-Glo live cell substrate (Promega) and buffer (Promega) and the luminescence was read with Glomax 96 microplate luminometer (Promega).



Gel filtration

H460 cells were treated with 50 μM methionine analog [Fmoc-Sec(Mob)-OH] for 8 hr and then harvested. For size exclusive chromatography, cells were prepared in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 5 mM β-mercaptoethanol) containing protease inhibitor cocktail (Calbiochem, Cat. 535140). After centrifugation, cell lysates were filtered through a 0.22 μm syringe filter. A total of 4 mg protein was loaded onto gel filtration column (GE healthcare life sciences, Superdex 200 10/300 GL) in AKTA FPLC system at a flow rate of 0.4 ml/min. Proteins of each fraction were separated by SDS-PAGE and analyzed by immunoblotting.

RESULTS

MRS stabilizes CDK4 in HSP90-dependent manner

Since it has been known that heat shock protein 90 (HSP90), a chaperone, allows the newly synthesized CDK4 to be properly folded and matured, we investigated whether the function of MRS on stabilizing CDK4 requires the function of HSP90 (19). We treated Geldanamycin (Tocris), HSP90 activity inhibitor, in H460 cells, after transfection of MRS, and found that the increased CDK4 by MRS overexpression was abolished by the HSP90 inhibitor (Fig. 1a). It implies that MRS-mediated CDK4 stabilization is affected by the chaperone function of HSP90.

Next, we examined the direct interaction between MRS and HSP90 via *in vitro* glutathione-S transferase (GST)-pull down assay, to identify the possibility of their interplay, and observed that MRS co-precipitated with GST-tagged HSP90 (Fig. 1b). In addition, the formation of ternary complex among endogenous MRS, CDK4 and HSP90 was observed by co-immunoprecipitation (Fig. 1c).

Taken together, it suggests that MRS requires the chaperone function of HSP90 for the stabilization of CDK4. Thus, we further investigated which functions of HSP90 would be modulated by MRS.

MRS does not modulate the ATPase activity of HSP90

The ATPase activity of HSP90 is essential for the maturation of proteins (known as clients) (19). Recent work is beginning to identify the role of ATP hydrolysis in large and highly dynamic conformational shifts of HSP90 which termed the HSP90 chaperone cycle. The weak intrinsic ATPase activity of HSP90 is modulated by its interaction with certain co-chaperones. Thus, we examined the dose-dependent effect of MRS on the ATPase activity of HSP90 with *in vitro* malachite green assay (20). However, the ATPase activity of HSP90 was neither increased nor decreased by the addition of MRS (Fig. 2a).

MRS enhances the interaction between CDK4 and HSP90

The function of HSP90, also, can be regulated by enhancing the recruitment of client proteins, which are stabilized by HSP90 (21). Even though it has been reported that cell division cycle 37 (CDC37), known as a co-chaperone, aids HSP90 to recognize CDK4, it has numerous clients, especially protein kinases. It suggests that CDK4 may require a specific factor in order to be recruited into HSP90 and MRS would be in charge of it.

We tested the effect of MRS overexpression on the interaction between CDK4 and HSP90 via immunoprecipitation. MRS overexpression clearly increased the association of CDK4 with HSP90, and also enhanced the total level of CDK4 (Fig. 3a). And it was further confirmed by *in vitro* maltose binding protein (MBP)-pull down assay. CDK4 and MRS were co-precipitated with MBP-tagged HSP90 (Fig. 3b).

Furthermore, to investigate the dose-dependent effect of MRS on the intracellular association between CDK4 and HSP90, we used nanoluc binary technology (NanoBiT) assay which can quantitate the association with nanoluciferase luminescence (22). As a result, MRS addition apparently increased the association between CDK4 and HSP90 in a dose-dependent manner (Fig. 3c). It implies that MRS plays a significant role in the recruitment of CDK4 to HSP90, leading to increase in CDK4 stability.

MRS suppression dissociates CDK4 from HSP90

We further investigated the effects of MRS depletion using small interference RNA (si-RNA) on the CDK4-HSP90 interaction with or without MRS-CDK4 interaction inhibitor, FSMO. Consequently, MRS knockdown and inhibitor treatment synergistically dissociated CDK4 from HSP90 and reduced the total level of CDK4, further illustrating the importance of MRS for the recruitment of CDK4 to HSP90 (Fig. 4a). Moreover, it demonstrates that FSMO also facilitates the dissociation of CDK4 from HSP90. It is notable that other clients of HSP90, such as CDK7 and p70S6K, known to be stabilized by HSP90 were not affected, suggesting that the function of MRS on stabilizing CDK4 is specific to CDK4.

We further performed gel filtration assay to fractionize the whole protein of H460 cells treated with FSMO and investigated the effects on the CDK4-HSP90 complex formation. FSMO treatment diminished the number of fractions which contained CDK4 and HSP90 together and the total level of CDK4 (Fig. 4b). It shows that the CDK4-HSP90 complex is disrupted by restricting the direct

interaction between MRS and CDK4, implying that MRS is critical for the CDK4-HSP90 complex formation.

Taken together, the recruitment of CDK4 to HSP90 is regulated by MRS and requires the interaction between MRS and CDK4.

MRS enhances the CDK4-CDC37-HSP90 complex formation

It has been reported that interplay among co-chaperones contributes to various modulations on the HSP90 chaperone function (19, 21). For example, the stabilization of progesterone receptor is mediated by the cooperation among HSP90, HSP70 and co-chaperones. Thus, we examined whether MRS interplays with CDC37 to stabilize CDK4 via modulating the chaperone function of HSP90, since CDC37 is known as a co-factor to help HSP90 to stabilize CDK4 (23-25).

We investigated the effects of MRS overexpression and MRS knockdown on the association among CDK4, HSP90 and CDC37. As a result, MRS overexpression increased the association among CDK4, HSP90 and CDC37 (Fig. 5a). In the same context, MRS knockdown caused the dissociation of CDK4 and CDC37 from HSP90 (Fig. 5b).

In conclusion, MRS specifically stabilizes CDK4 by recruiting CDK4 and CDC37 into HSP90 (Fig. 6), and leads to cell cycle progression and cell proliferation as previously reported (18).

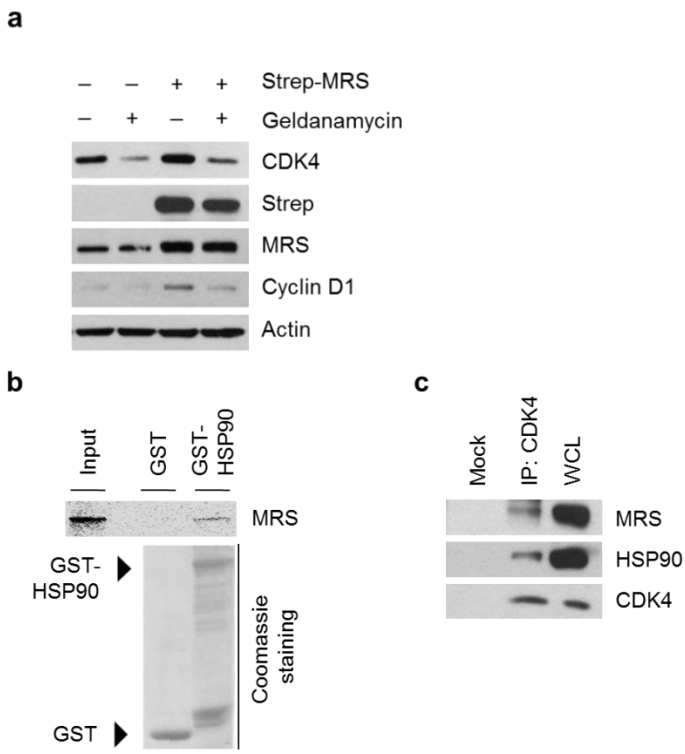


Figure 1. MRS stabilizes CDK4 in HSP90-dependent manner.

(a) H460 cells were treated with 1 μM Geldanamycin for 6 hr as a HSP90 inhibitor, after transfection of Strep-MRS. CDK4 were determined by immunoblotting with anti-CDK4 antibody.

(b) Purified GST or GST-HSP90 protein was incubated with [^{35}S] Met-labeled MRS, which was synthesized by *in vitro* transcription and translation (see materials and methods). The co-precipitated MRS was determined by autoradiography. Inputs are the amount of 1% MRS used.

(c) H460 cell lysates were immunoprecipitated with anti-CDK4 antibody. Co-precipitated MRS and HSP90 were determined by immunoblotting with anti-MRS and anti-HSP90 antibodies.

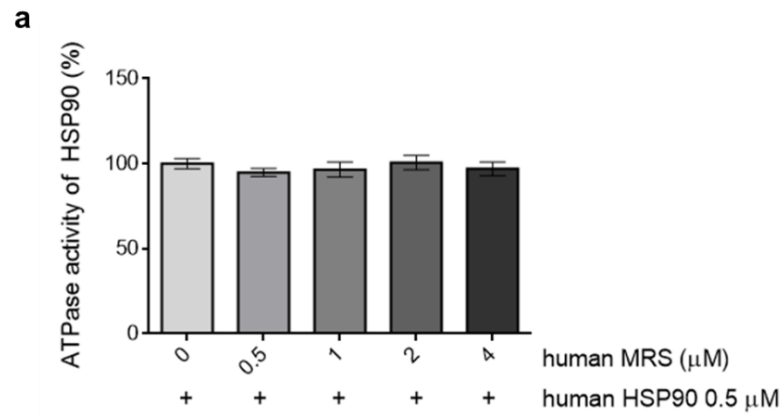


Figure 2. MRS does not modulate the ATPase activity of HSP90

(a) We analyzed the ATPase activity of HSP90 by *in vitro* malachite green assay. We incubated 0.5 μM HSP90 and 1 mM ATP with the indicated amounts of MRS for 2 hr at 37^{o}C. Generation of free phosphate was detected and measured using malachite green. ATPase activity is expressed as a percentage of HSP90 alone (Mean ± SD, duplicates).

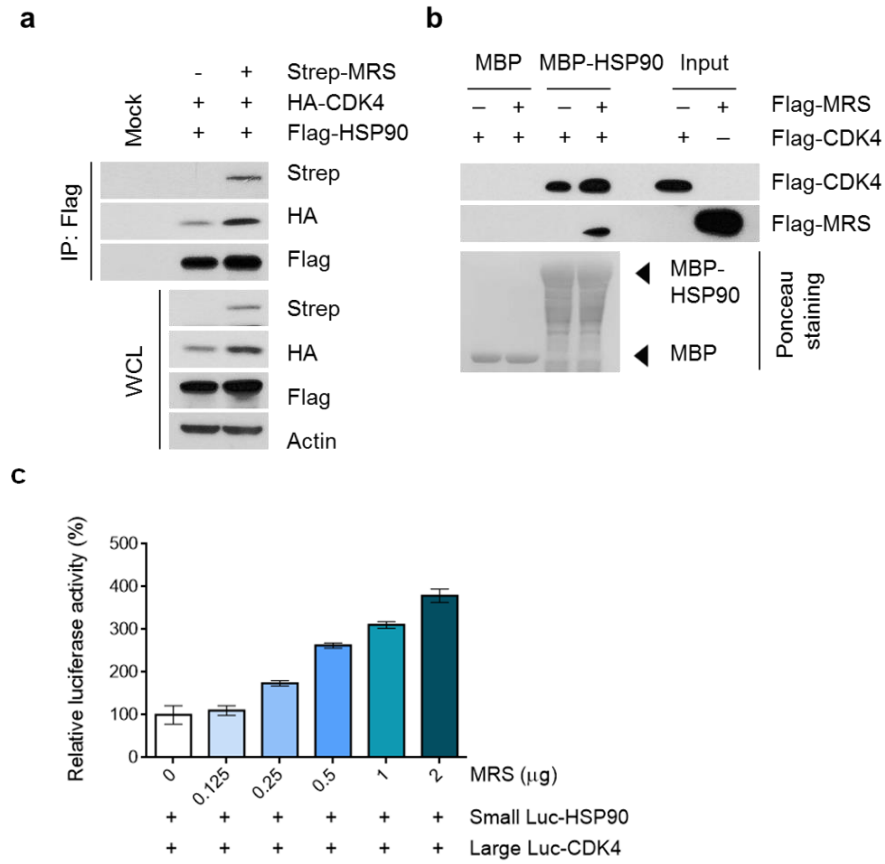


Figure 3. MRS enhances the interaction between CDK4 and HSP90

(a) After cotransfection of Flag-HSP90 with Strep-MRS, cell lysates were immunoprecipitated with anti-Flag antibody. And the co-precipitated MRS and CDK4 were determined by immunoblotting with anti-Strep and anti-CDK4 antibodies.

(b) Purified MBP or MBP-HSP90 protein was incubated with Flag-CDK4 and Flag-MRS, which were synthesized by *in vitro* transcription and translation (see materials and methods). The co-precipitated CDK4 and MRS were determined by immunoblotting with anti-Flag antibody.

(c) The intracellular association between CDK4 and HSP90 was measured by NanoBiT assay, which can quantitate the association with nanoluciferase luminescence (see materials and methods). The indicated amounts of MRS plasmid (from 0.13 to 2 μg) were cotransfected with 0.5 μg SmallBiT-HSP90 and 0.5 μg LargeBit-CDK4 in CHO-K1 cells. After 48 hr incubation, cells were incubated with Nano-Glo live cell substrate and buffer. And the luminescence was read with Glomax 96 microplate luminometer.

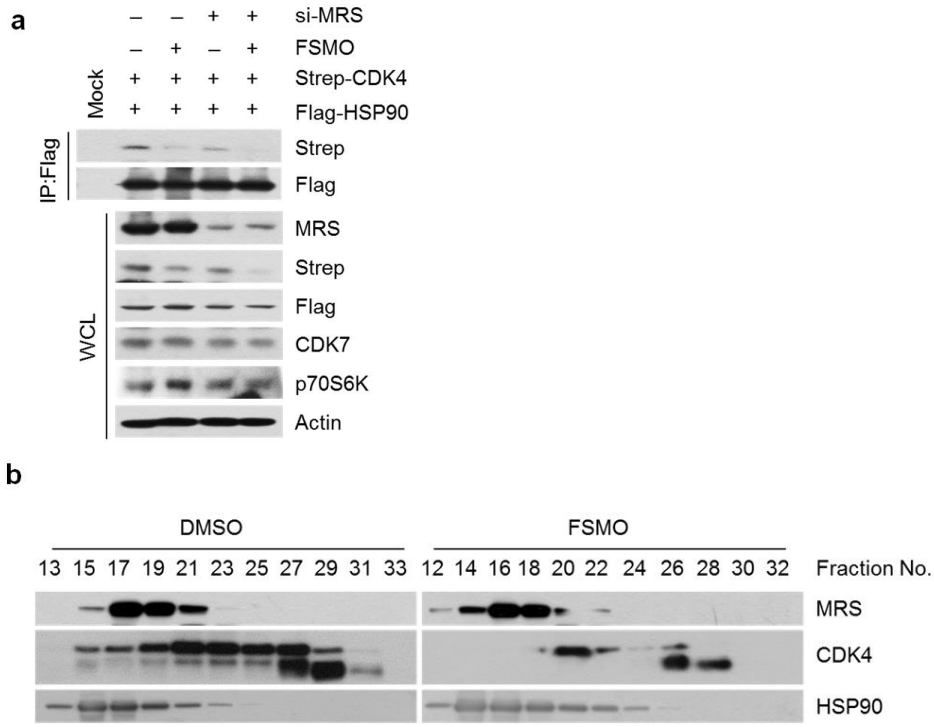


Figure 4. MRS suppression dissociates CDK4 from HSP90

(a) H460 cells were transfected with 100 nM control or si-RNA specific to MRS (si-MRS) for 72 hr and treated with 50 μM FSMO**.** After cotransfection of Flag-HSP90 with Strep-CDK4, cell lysates were immunoprecipitated with anti-Flag antibody, and the co-precipitated CDK4 was determined by immunoblotting with anti-Strep antibodies**.** CDK7 and p70S6K, known as HSP90 clients, were used as negative control to show the specific effect of MRS suppression on CDK4 level.

(b) H460 cells were treated with 50 μM FSMO and the protein extracts (4 mg) were subjected to size-exclusion chromatography. Fractions were selected based on the whole protein peaks to represent the same fraction between two different samples preparation and the distribution of the proteins was investigated by immunoblotting with ani-MRS, ani-CDK4 and ani-HSP90 antibodies.

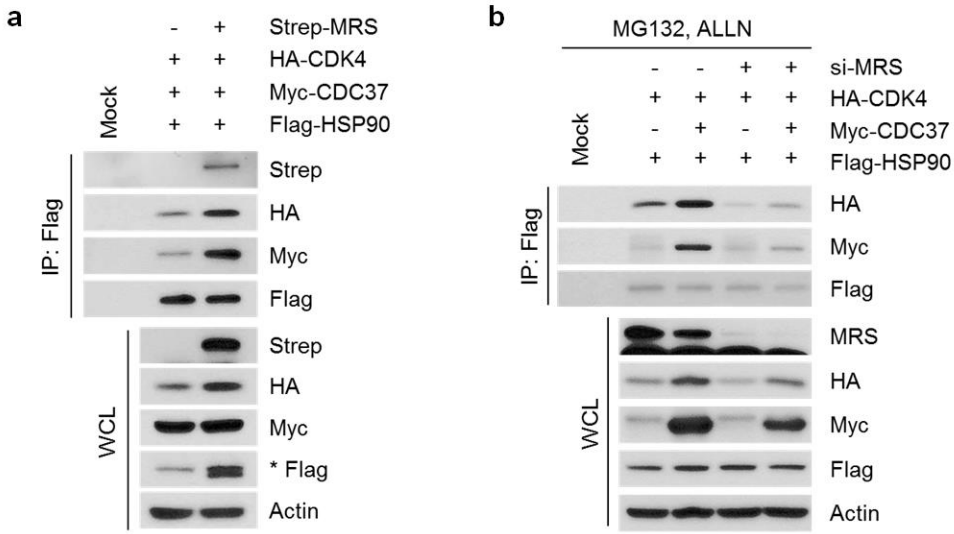


Figure 5. MRS enhances the CDK4-CDC37-HSP90 complex formation

(a) After cotransfection of Flag-HSP90, Strep-MRS, HA-CDK4 and Myc-CDC37, H460 cell lysates were immunoprecipitated with anti-Flag antibody, and the co-precipitated MRS, CDK4 and CDC37 were determined by immunoblotting with anti-Strep, anti-HA and anti-Myc antibodies. * The lower band is non-specific.

(b) H460 cells were transfected with 100 nM control or si-MRS for 72 hr and treated with 50 μM MG132 and 20 μM ALLN for 7 hr. Cell lysates were immunoprecipitated with anti-Flag antibody, and the co-precipitated CDK4 and CDC37 were determined by immunoblotting with anti-HA and anti-Myc antibodies.

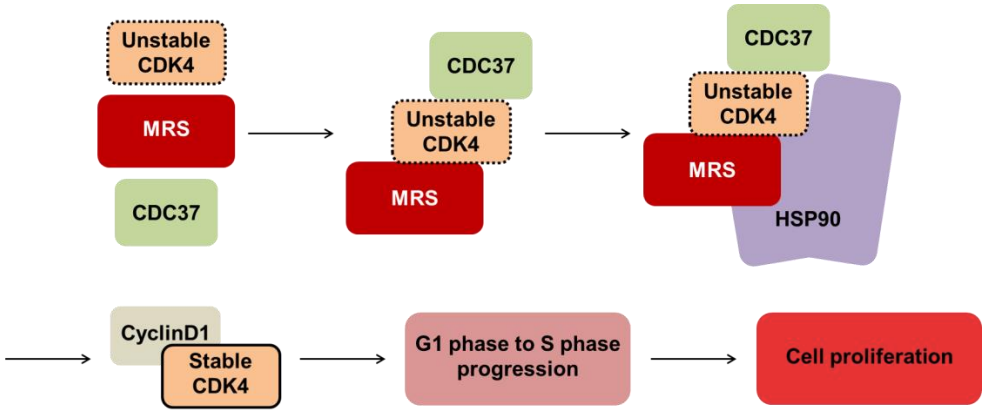


Figure 6. Schematic model

Schematic model represents the MRS-mediated CDK4 stabilization. MRS specifically stabilizes CDK4 by recruiting CDK4 and CDC37 into HSP90. Consequently, CDK4 stability is enhanced, leading to cell cycle progression and cell proliferation.

DISCUSSION

Over the past decades, it has been known that the newly synthesized CDK4 is stabilized by HSP90 and CDC37 (23-25). CDC37 is regarded to help the chaperone function of HSP90 by loading CDK4 onto HSP90, which induces the proper folding of CDK4. However, in fact, HSP90 and CDC37 are involved in the stabilization of a wide range of its clients which have no consensus sequences (17). It has remained unclear how HSP90 and CDC37 recognize CDK4 at the right time, suggesting the existence of another factor to help the CDK4-specific stabilization. Based on our previous study, we already knew that MRS affects the CDK4 stability. In this study, we illustrate the MRS-mediated CDK4 stabilization through investigation of the interaction among related chaperons. We propose here that MRS specifically helps the recruitment of CDK4 to HSP90 by enhancing the complex formation among CDK4, CDC37 and HSP90

It has long been considered that the function of CDC37 on the recruitment of CDK4 into HSP90 is indispensable to stabilize CDK4 (23-25). However, a recent mutant study from the Workman group shows that the loss of function of CDC37 on the recruitment does not destabilize CDK4, suggesting that there would be other mediators to recruit CDK4 into HSP90, instead of CDC37 (26). In this context, our study supports that MRS may cooperate with CDC37 to recruit CDK4. But, it still remains unclear whether the function of MRS requires CDC37 to stabilize CDK4, and it should be further investigated.

Interestingly, our study shows the connection between translation and cell cycle. MRS and CDK4 are inevitable enzymes in protein synthesis and cell cycle, respectively (2, 14). It is well accepted that protein synthesis and cell cycle are

closely related, since protein synthesis is a fundamental measure to synthesize necessary proteins for cell growth and cell division (27, 28). MRS and CDK4 are especially in charge of translational initiation and G1 cell cycle progression, respectively. It has been previously reported that impairing translation initiation specifically impairs G1 transit (27, 29). However, currently, very little is known about the interactions between translational machinery and cell cycle regulators (30). Taken together, our findings suggest that a novel evidence to connect between translation initiation and G1 cell cycle progression.

However, it still remains unsolved which oncogenic upstream promotes MRS to stabilize CDK4 in lung cancer and how the function of MRS on stabilizing CDK4 is modified in normal cells. To address these issues, it should be taken into consideration that cancer needs to accelerate proliferation and shows aberrant dysregulation on the CDK4-Cyclin D1-pRb axis (2). For example, the loss of function of $p16^{INK4a}$, known as an endogenous inhibitor of CDK4, is commonly observed in diverse cancer types and causes the uncontrolled activity of CDK4 (2, 31, 32). According to our previous study, interestingly, the effects of the depletion of MRS by si-RNA and the inhibition of interaction between MRS and CDK4 with FSMO treatment on the CDK4 stability are dramatically sensitized in $p16^{INK4a}$-negative cancer. The function of MRS on stabilizing CDK4 seems dependent on $p16^{INK4a}$ status. Thus, it may be one of clues to address the different regulation and effect of MRS on cancer.

Lastly, our study supports that the interaction between MRS and CDK4 can be targeted therapeutically to prevent the uncontrolled lung cancer cell proliferation. As shown in this study, blocking the interaction significantly dissociated the CDK4-HSP90 complex and reduced the level of CDK4 (Fig. 4b). In

addition, high expression of MRS in cancer has been well reported (33-35). And according to our previous study, it is highly related to poor prognosis of lung cancer patient and MRS level is positively correlated with CDK4 level (ref). Therefore, it may enable us to target the interaction in more appropriate populations, which shows dependence on the MRS-mediated stabilization of CDK4.

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요약(국문초록)

폐암에서의 Methionyl-tRNA synthetase (MRS) 에 의한 Cyclin-dependent kinase 4 (CDK4) 안정화 기전 연구

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오성은

사이클린 의존성 인산화 효소 4 (cyclin-dependent kinase 4, CDK4)는 세포 증식과 관련된 세포 주기 진행에 중요한 조절인자이다. 흥미롭게도, 폐암에서 CDK4 단백질 양은 유전자 증폭 비율보다 현저하게 증가해 있다. 이것은 유전자 발현 이후 CDK4 단백질 양을 더 증가시키는 기전의 존재와 폐암에서의 CDK4 양에 대한 잠재적 의존성을 시사한다.

이전 연구에서는 폐암에서 메티오닐 운반 리보핵산 합성효소 (methionyl-tRNA synthetase, MRS) 가 CDK4 만을 특이적으로 안정화시키며, 다른 CDKs 는 영향을 받지 않는 것을 밝혔다. 하지만, MRS 가 어떠한 기전으로 CDK4 를 안정화시키는지에 대한 후속 연구가 필요하였기에 본 연구가 진행되었다.

본 연구는 MRS 가 CDK4, 열 충격 단백질 90 (heat shock protein

90, HSP90) 그리고 세포 분열 주기 37 단백질 (cell division cycle 37, CDC37) 의 복합체 형성을 증가시키며, 그에 따라 CDK4 양을 증가시키는 것을 밝혔다. MRS 의 과발현에 의한 CDK4 양 증가는 HSP90 억제제에 의하여 억제되었다. 또한, MRS 는 HSP90 와 직접적으로 결합을 하며, HSP90 그리고 CDK4 와 함께 3 차 복합체를 형성하였다. 이는 CDK4 를 안정화 시키는 MRS 의 기능이 HSP90 를 필요로 하는 것을 알 수 있다. CDK4 의 안정화를 위하여 MRS 가 HSP90 의 어떤 기능을 조절하는지 밝히고자, HSP90 의 아데노신 삼인산 분해 활성 (ATPase activity) 과 HSP90 와 CDK4 의 결합에 MRS 가 미치는 영향을 확인하였다. ATPase activity 는 MRS 의 증가에도 영향을 받지 않았지만, HSP90 와 CDK4 의 결합은 MRS 양에 의존성을 보이며 명확하게 증가하였다. 이는, CDK4 가 HSP90 와 결합할 때의 MRS 의 중요한 역할을 시사한다. 짧은 간섭 리보핵산 (si-RNA) 을 이용한 MRS 의 양 감소는 CDK4 를 HSP90 로부터 와해시켰으며, 이 결합은 MRS 와 CDK4 의 결합 저해제에 의하여 상승 작용으로 더욱 감소되었다. 특별히, HSP90 에 의하여 안정화가 된다고 알려진 CDK7 과 p70S6K 의 경우는 전혀 영향을 받지 않았다. 즉, MRS 는 오직 CDK4 의 안정화에 관여하며, HSP90 에 의하여 안정화가 된다고 알려진 다른 단백질들의 안정화에는 관여하지 않는 것을 알 수 있다. 더 나아가, MRS 의 양은 CDC37 과 HSP90 의 결합에도 양의 방향으로 중요하였다.

결론적으로, 본 연구는 MRS 가 CDK4 와 CDC37 를 HSP90 에 결합시켜줌으로써 CDK4 만을 특이적으로 안정화시키는 것을 밝혔다.

주요어 : 사이클린 의존성 인산화 효소 4, 메티오닐 운반 리보핵산 합성효소, 열 충격 단백질 90 , 세포 분열 주기 37 단백질, 안정화, 결합, 복합체 형성

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