A Thesis for the Degree of Master of Science

Inhibition of Triple Negative Breast Cancer Metastasis by Methionine Restriction

메티오닌 제한이 나타내는 삼중음성 유방암 세포의 전이 억제 효능

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ABSTRACT

Nutrient deprivation strategies have been proposed as an adjuvant therapy for cancer cells due to their increased metabolic demand. Herein, I examined the specific inhibitory effects of amino acid deprivation on the metastatic phenotypes of the human TNBC lines MDA-MB231 and Hs 578T cells, as well as the orthotopic 4T1 mouse TNBC tumor model. Of the essential amino acids tested, methionine deprivation elicited the strongest inhibitory effects on the migration and invasion of these cancer cells. Methionine deprivation reduced the phosphorylation of focal adhesion kinase (FAK), as well as the activity and mRNA expression of matrix metalloproteinases MMP-2 and MMP-9, two major markers of metastasis, while increasing the mRNA expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) in MDA-MB-231 cells. Furthermore, methionine restriction downregulated the metastasis-related factor urokinase plasminogen activatior (uPA) and upregulated plasminogen activator inhibitor 1 (PAI-1) mRNA expression. For the *in vivo* lung metastasis model, female BALB/c mouse were injected into the tail vein with 4T1 mouse TNBC cells and fed on a

control diet, 0.1% methionine-containing diet, or a methionine-deprived diet. Animals on the methionine-deprived diet showed lower metastasis rates compared to mice on the control diet. Taken together, these results suggest that methionine deprivation has an inhibitory effect on the migration and invasion of TNBC cells, as well as lung metastasis in BALB/c mouse. Methionine restriction could therefore represent a potential nutritional strategy for more effective cancer therapy.

Keywords: Methionine; Met; Triple Negative Breast Cancer; TNBC; Migration; Invasion; Metastasis

Student ID: 2013-22560

CONTENTS

Ⅰ. INTRODUCTION

Breast cancer is the second most common form of cancer arising in women in Korea [1]. Recently, the mortality rate of breast cancer patients has been declining due to early detection methods and improvements in surgery, radiation therapy, chemotherapy, and hormone therapies [2]. However, metastasizing advanced stages of breast cancer remain very difficult to treat, and the ultimate cause of death in many patients is metastasis to a distant site, rather than due to the primary tumor itself [3,4]. Therefore, the control of metastasis is a key therapeutic goal for extending overall survival.

Triple-negative breast cancers (TNBC) account for 15-20% of all breast cancers. TNBC refers to an absence of the expression of three major hormone receptors; the estrogen receptor (ER), progesterone receptor (PR), and hormone epidermal growth factor receptor 2 (HER-2).

A number of potent receptor-targeting drugs such as tamoxifen and trastuzumab target these receptors, and are therefore ineffective for TNBC patients [5]. Consequently, patients with TNBC have lower survival rates and a greater risk of the disease relapsing within 5 years of diagnosis. TNBC patients also have a higher rate of metastatic recurrence (33.9%) compared to other breast cancer patients (20.4%) [6]. Continuous adjuvant therapy to prevent metastasis after surgery is typically required for the treatment of TNBC.

Cancer cells require larger quantities of nutrient resources such as glucose and amino acids than non-malignant cells, due to their rapid growth and aggressive characteristics. The increased rate of glycolysis exhibited by many tumors cells is referred to as the Warburg effect [7]. Due to the fact that glucose restriction in clinical settings can result in systemic toxicity, dietary amino acid restriction has emerged as a more viable nutritional therapeutic strategy [8]. Limited success has been

reported with nutrient deprivation strategies against various cancer cells that are amino acid-dependent. For example, L-asparaginase is a chemotherapeutic drug that has been used for the treatment of lymphoblastic leukemia [9,10]. Serine starvation also reduces cancer cell survival due to oxidative stress, particularly in cells lacking p53 [11], while tyrosine and phenylalanine restriction have been observed to inhibit B16-BL6 tumor growth and metastasis [12,13]. Taken together, these results demonstrate proof-of-concept for amino acid restriction as a viable adjuvant therapeutic strategy.

In the present study, I evaluated the effects of amino acid deprivation on the migration and invasion of TNBC cell lines. Of the 10 essential amino acids tested, methionine deprivation caused the strongest inhibitory effect on migration and invasion in these cells. Conditions of methionine deprivation suppressed metastasis-related biomarkers and increased levels of their inhibitors. I also analyzed the effects of

methionine restriction on lung metastasis in an orthotopic mouse metastasis model.

Ⅱ. MATERIALS AND METHODS

1. Reagents

Dulbecco's modified eagle medium (DMEM) and the amino acid-deprived DMEM formulations were purchased from Welgene (Daegu, Korea). Fetal bovine serum (FBS), mitomycin C, recombinant human epithelial growth factor and insulin solution were obtained from Sigma-Aldrich (St.Louis, MO). Dialyzed FBS (dFBS), HEPES, and anti-FAK (pY397) antibodies were purchased from Life Technologies (Grand Island, NY). The anti-FAK antibody was obtained from Millipore (Billerica, MA). Penicillin-Streptomycin (pen/strep) solution was purchased from Mediatech, Inc. (Manassas, VA). Matrigel was obtained from Corning (Corning, NY) and protein assay reagent kits were obtained from Bio-Rad Laboratories (Hercules, CA). A10021B and Modified Diets were obtained from Central Lab. Animal, Inc. (Seoul, Korea; details

in Table 1).

Table 1

Table 1. A10021B and Modified Diets. A10021B and Modified Diets were obtained from Central Lab Animal. A10021B is based on Hirakawa *et al* [14].

Product #	**A10021B**		**A05080210**		**A05080226**		**A10033Y**	
	Control		**No Methionine**		**0.1 % Methionine**		**No Tryptophan**	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	17	18	16	17	16	17	17	17
Carbohydrate	69	71	69	71	69	71	69	21
Fat	5	12	5	12	5	12	5	12
Total kcal/gm		100		100		100		100

Table 1. continue

Product #	**A10021B**		**A05080210**		**A05080226**		**A10033Y**	
	Control		**No Methionine**		**0.1 % Methionine**		**No Tryptophan**	
Ingredient (gm)	gm	kcal	gm	kcal	gm	kcal	gm	kcal
L-Arginine	10	40	10	40	10	40	10	40
L-Histidine-HCl-H2O	6	24	6	24	6	24	6	24
L-Isoleucine	8	32	8	32	8	32	8	32
L-Leucine	12	48	12	48	12	48	12	48
L-Lysine-HCl	14	56	14	56	14	56	14	56
L-Methionine	6	24	0	0	1	4	6	24
L-Phenylalanine	8	32	8	32	8	32	8	32
L-Threonine	8	32	8	32	8	32	8	32
L-Tryptophan	2	8	2	8	2	8	0	0
L-Valine	8	32	8	32	8	32	8	32

Table 1. continue

Product #	**A10021B**		**A05080210**		**A05080226**		**A10033Y**	
	Control		**No Methionine**		**0.1 % Methionine**		**No Tryptophan**	
Ingredient (gm)	gm	kcal	gm	kcal	gm	kcal	gm	kcal
L-Alanine	10	40	10	40	10	40	10	40
L-Asparagine-H2O	5	20	5	20	5	20	5	20
L-Aspartate	10	40	10	40	10	40	10	40
L-Cystine	4	16	4	16	4	16	4	16
L-Glutamic Acid	30	120	30	120	30	120	30	120
L-Glutamine	5	20	5	20	5	20	5	20
Glycine	10	40	10	40	10	40	10	40
L-Proline	5	20	5	20	5	20	5	20
L-Serine	5	20	5	20	5	20	5	20
L-Tyrosine	4	16	4	16	4	16	4	16

Table 1. continue

Product #	**A10021B**		**A05080210**		**A05080226**		**A10033Y**	
	Control		**No Methionine**		**0.1 % Methionine**		**No Tryptophan**	
Ingredient (gm)	gm	kcal	gm	kcal	gm	kcal	gm	kcal
Corn Starch	550.5	2202	556.5	2226	556.5	2226	552.5	2210
Maltodextrin 10	125	500	125	500	125	500	125	500
Cellulose	50	0	50	0	50	0	50	0
Corn Oil	50	450	50	450	50	450	50	450
Mineral Mix S10001	35	0	35	0	35	0	35	0
Sodium Bicarbonate	7.5	0	7.5	0	7.5	0	7.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitrartrate	2	0	2	0	2	0	2	0
Yellow Dye, FD&C #5	0	0	0.025	0	0.05	0	0.05	0
Red Dye, FD&C #40	0	0	0	0	0	0	0	0
Blue Dye, FD&C #1	0.05	0	0.025	0	0.05	0	0	0
Total	1000.05	**3872**	1000.05	**3872**	1000.05	**3872**	1000.05	**3872**

2. Cell Culture

The human triple-negative breast cancer cell lines MDA-MB-231 and Hs 578T, and mouse triple negative breast cancer 4T1 cells were maintained in DMEM containing 10% (v/v) FBS and 1% (v/v) pen/strep at 37℃ and 5% CO_2. Human breast epithelial MCF 10A cells were maintained in DMEM with 10% (v/v) FBS, 1% (v/v) pen/strep, 15 mM hepes buffer, 10 μg/ml insulin and 20 ng/ml EGF. Each amino acid-deprived DMEM formulation was supplemented with 10% (v/v) dFBS and 1% (v/v) pen/strep.

3. Migration assay

In order to analyze the migratory capacity of the TNBC cells, Essen ImageLock 96-well plates (Essen Instruments, Hertfordshire, UK) were coated with BD Matrigel (100 μg/ml culture media, BD Biosciences, San Jose, CA) overnight. MDA-MB-231 (4 X 10^4

cells/well), Hs 578T (2 X 10^4 cells/well) and 4T1 (3 X 10^4 cells/well) were then seeded on the Essen ImageLock plates. The cells reached confluence after 24 hours, and mitomycin C (25 μg/ml serum free media) was treated 30 minutes prior to media change after the cells were washed with PBS. A single wound was made across each well automatically using a Wound Maker 96 Tool (Essen Instruments). The cell debris was washed with PBS and each amino acid-deprived media was applied as a treatment condition. Wound images were monitored with an IncuCyte Live-Cell Imaging System and software (Essen Instruments) and data were analyzed by custom algorithms in the IncuCyte software package.

4. Invasion assay

In order to determine the invasiveness of TNBC cells, Essen ImageLock 96-well plates (Essen Instruments) were coated with BD

Matrigel (100 µg/ml culture media, BD Biosciences, San Jose, CA) overnight. MDA-MB-231 (4 X 10^4 cells/well), Hs 578T (2 X 10^4 cells/well) and 4T1 (3 X 10^4 cells/well) were then seeded on the Essen ImageLock plates. After 24 hours, the cells reached confluence and a single wound was made across each well automatically using a Wound Maker 96 Tool (Essen Instruments). The cell debris was washed with PBS and each amino acid-deprived media containing BD Matrigel (500 µg/ml) was applied as a treatment condition. Wound images were monitored with the same IncuCyte Live-Cell Imaging System and software (Essen Instruments) and data were analyzed in terms of relative wound density (RWD) calculated by custom algorithms in the IncuCyte software package.

5. Cell viability assay

In order to assess cell viability, MDA-MB-231 (4 X 10^4

cells/well), Hs 578T (2 X 10^4 cells/well) and 4T1 (3 X 10^4 cells/well) and MCF 10A (2 X 10^4 cells/well) cells were seeded into 96-well plates. After 24 hours, the wells were treated with each amino acid-deprived media formulation and incubated for 24 hours. MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] solution (final concentration: 0.5 mg/ml) was added to each well, and the cells were incubated for 1 hour. The dark formazan crystals that were formed by the intact cells were dissolved in dimethyl sulfoxide, and the absorbance at 570 nm was measured with a microplate reader. The results are expressed as percent MTT reduction relative to the absorbance of the control cells.

6. Western blot analysis

After MDA-MB-231 cells were cultured in 6-cm dishes for 24 hours, the cells were incubated in either control media or methionine-deprived media for 2, 4, 8, or 12 hours. The cells were washed with cold

PBS and harvested, before the protein concentration was measured using a protein assay reagent kit as described by the manufacturer. The proteins were separated electrophoretically using a 10% SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (Merck Millipore, Billerica, MA). The membranes were blocked in 5% fat-free milk for 1 h, and then incubated with a specific primary antibody at 4℃ overnight. Protein bands were visualized using a chemiluminescence detection kit (GE Healthcare, London, UK) after hybridization with a HRP-conjugated secondary antibody (Life Technologies).

7. Gelatin zymography

After MDA-MB-231 and Hs 578T cells were cultured in 6-cm dishes for 24 hours, the cells were incubated in either control media or methionine-deprived media for 24 hours. The media were harvested on ice, and then centrifuged at 18,620 g for 10 min. Protein concentration

was measured using a protein assay reagent kit as described by the manufacturer. The proteins were separated electrophoretically using a 12% polyacrylamide gel in the presence of gelatin (0.1% w/v) as a substrate for matrix metalloproteinases (MMPs). The protein samples were mixed with loading buffer [10% SDS, 25% glycerol, 0.25 M Tris (pH 6.8) and 0.1% bromophenol blue], and then run on a 12% SDS-PAGE gel without denaturation. The gel was then washed with renaturating buffer (Life Technologies) for 1 h at room temperature and incubated for 24 h at 37℃ in developing buffer (Life Technologies). After the enzyme reaction, the gel was stained with 0.5% Coomassie brilliant blue in 10% acetic acid.

8. Real-time quantitative PCR

MDA-MB-231 cells were incubated in either control media or methionine-deprived media for 24 hours and harvested using RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a

NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After reverse transcription with oligo-dT primers using a PrimeScript™ 1st strand complementary DNA (cDNA) synthesis kit (Takara Bio Inc.), real-time quantitative RT-PCR was conducted using IQ SYBR (Bio-Rad Laboratories) and 2 μl of cDNA in triplicate with 18s rRNA as an internal control. Prior to PCR amplification, the primers were denatured at 95℃ for 3 min. The amplification program consisted of 44 cycles at 95℃ for 10 sec, 60℃ for 30 sec, and 72℃ for 30. PCR was performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). cDNA was amplified using the following primers: MMP-2 forward (5'- TGG CAA GTA CGG CTT CTG TC -3'); MMP-2 reverse (5'- TTC TTG TCG CGG TCG TAG TC -3'); MMP-9 forward (5'- GCA CGA CGT CTT CCA GTA CC -3'); MMP-9 reverse (5'- CAG GAT GTC ATA GGT CAC GTA GC -3'); TIMP-1 forward (5'- TGC GGA TAC TTC CAC AGG TC -3'); TIMP-1 reverse

(5’- GCA TTC CTC ACA GCC AAC AG -3’); TIMP-2 forward (5’- AAG AGC CTG AAC CAC AGG TA -3’); TIMP-2 reverse (5’- GAG CCG TCA CTT CTC TTG AT -3’); u-PA forward (5’- AGC CCT GCC CTG AAG TCG TTA -3’); u-PA reverse (5’- CAG GGC ATC TCC TGT GCA TG -3’); PAI-1 forward (5’- CTC CTG GTT CTG CCC AAG TT -3’); pai-1 reverse (5’- GAG AGG CTC TTG GTC TGA AAG -3’) ; 18s rRNA forward (5’- GTA ACC CGT TGA ACC CCA TT -3’); 18s rRNA reverse (5’- CCA TCC AAT CGG TAG TAG CG -3’).

9. Animals

Eight-week-old female BALB/c mice were purchased from Orient Bio Inc. (Seongnam, Korea). Animals were acclimated for 5 days prior to the study and had free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee (Case Number: SNU-141006-4) of Seoul National University,

Seoul, Korea. The animals were housed in climate-controlled quarters (24℃ at 50% humidity) with a 12 h light/12 h dark cycle.

10. *In vivo* lung metastasis experiments

4T1 cells were harvested and diluted in sterile PBS. The cells (2 x 10^5 cells/mouse) were injected into the tail veins of nine-week-old female BALB/c mice. On the day of the injection, the mice were divided randomly into 3 treatment groups and diets were modified accordingly with the following: 1) control diet, 2) 0.1% methionine containing diet, 3) methionine-deprived diet. The food and water were supplied ad libitum. Ten days after the injection, all mice were sacrificed and the lungs and livers were isolated and weighed. The lungs were fixed in Bouin's solution (Sigma-Aldrich, St.Louis, MO) to quantify tumor nodules.

11. Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Duncan's Multiple Range Test, and p values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Methionine deprivation inhibits cell migration and invasion of MDA-MB-231 and Hs 578T cells.

To investigate the effect of deprivation of each amino acid in regards to triple negative breast cancer (TNBC) cell migration, MDA-MB-231 and Hs 578T cells were incubated with each amino acid-deprived media formulation for 24 hours. Wound widths at each time point were expressed as the percentage relative to the wound width at 0 hours in each well (Fig. 1A(a) and B(a)). The relative wound widths of the methionine-deprived group were 174% for MDA-MB-231 (Fig. 1A(b) and 131.1% for Hs 578T cells (Fig. 1B(b)). The cells of the control group migrated to a greater extent than the cells of the methionine-deprived group (Fig. 1A(c) and B(c)).

To examine the effect of deprivation of each amino acid on

TNBC cell invasiveness, MDA-MB-231 and Hs 578T cells were incubated with each amino acid-deprived media formulation containing BD Matrigel (500 μg/ml) for 24 hours. The relative wound density (RWD) at each time point was expressed as the percentage relative to the wound density at 0 hours in each well (Fig. 1C(a) and D(a)). The RWD of the methionine-deprived group was 73.5% for MDA-MB-231 (Fig. 1C(b)) and 70.2% for Hs 578T cells (Fig. 3D(b)). The cells of the control group were more invasive than the cells of the methionine-deprived group (Fig. 1C(c) and D(c)). Overall, these results indicate that methionine deprivation elicits the strongest inhibitory effect on cell migration and invasion in these cell lines.

Figure 1

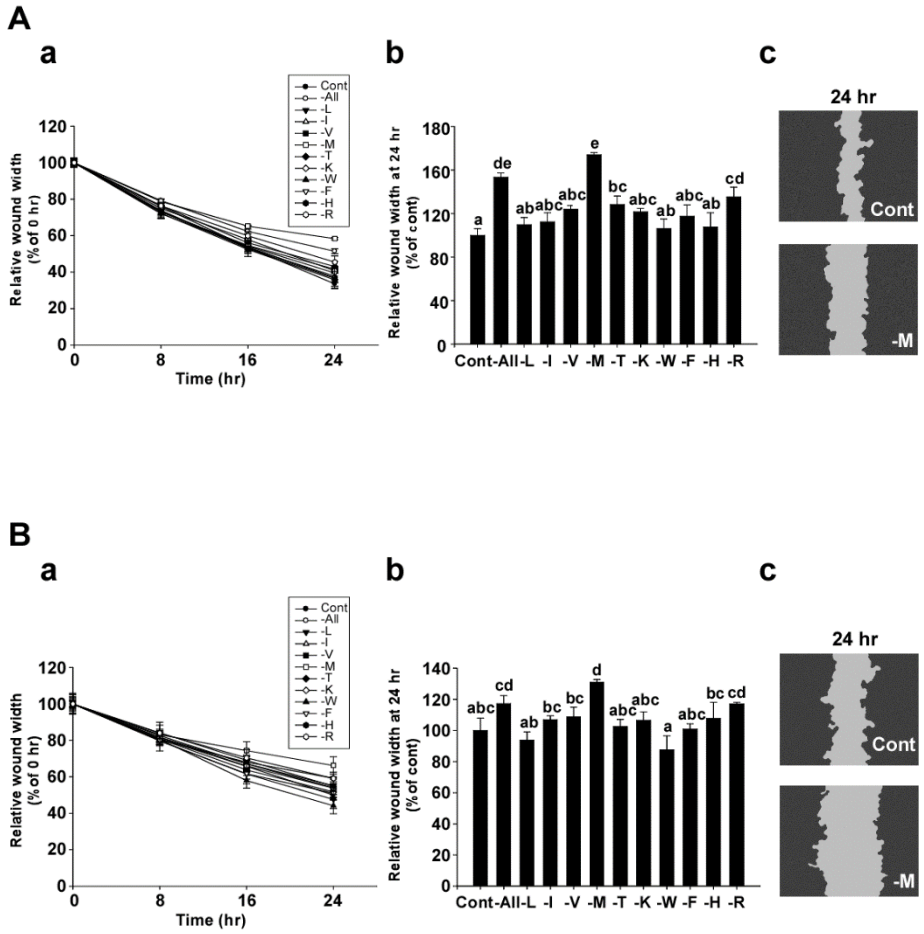
A
a
b
c
Relative wound width (% of 0 hr)
Time (hr)
Cont
-All
-L
-I
-V
-M
-T
-K
-W
-F
-H
-R
Relative wound width at 24 hr (% of cont)
Cont -All -L -I -V -M -T -K -W -F -H -R
24 hr
Cont
-M
B
a
b
c
Relative wound width (% of 0 hr)
Time (hr)
Relative wound width at 24 hr (% of cont)
Cont -All -L -I -V -M -T -K -W -F -H -R
24 hr
Cont
-M

Figure 1. continue

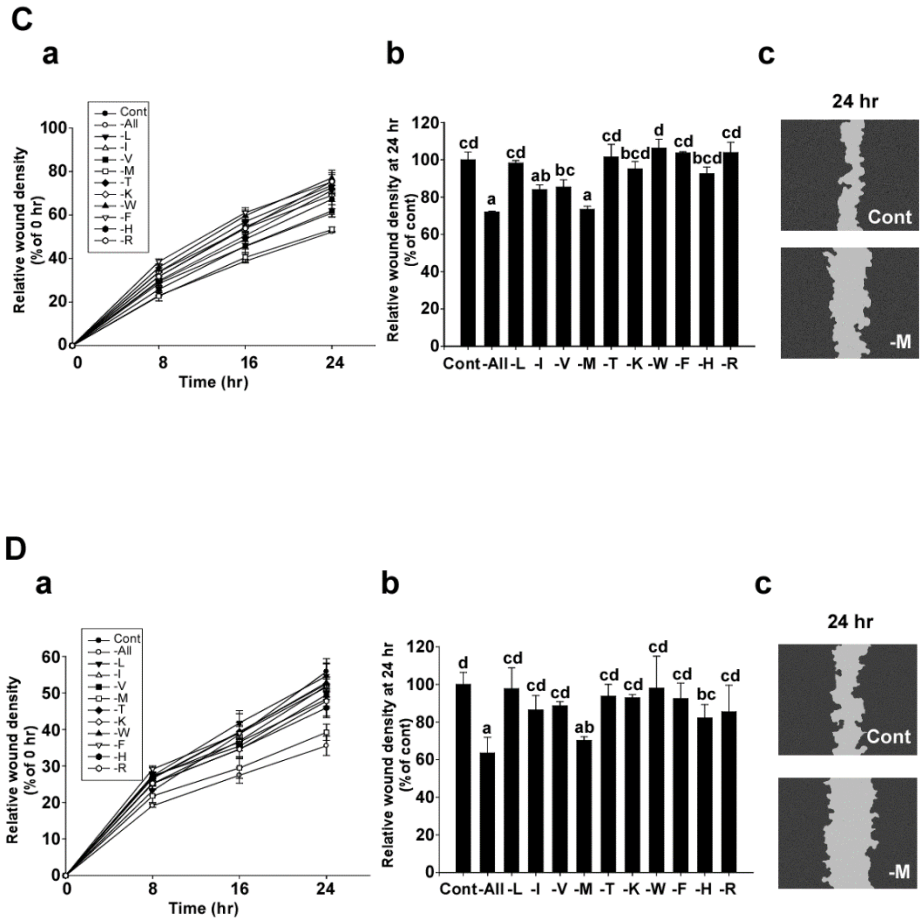

Figure 1. Effect of methionine deprivation on cell migration in MDA-MB-231 and Hs 578T cells. *A*. Migration assay results using MDA-MB-231 cells. The relative wound widths of each amino acid-deprived group were compared with the control group over time (a). Comparison of relative wound widths between treatment groups after 24 hours (b). Representative image of a scratch wound mask after 24 hours (c). *B*. Migration assays using Hs578T cells. The relative wound widths of each amino acid-deprived group were compared with the control group over time (a). Comparison of the relative wound widths of each amino acid-deprived and control group after 24 hours (b). Representative image of a scratch wound mask after 24 hours (c). The wound widths were automatically measured using IncuCyte™ software, with the following equation: Wound width (μm) = $\frac{1}{N}\sum w_i$ (*N*: vertical line of the scratch wound image, *w*: distance between the edges of the scratch wound mask, *i*: number of 924 horizontal lines of resolution in a scratch wound image).

C. Invasion assay using MDA-MB-231 cells. The relative wound density of each amino acid-deprived group was compared with the control group over time (a). Relative wound densities for each amino acid-deprived group compared with the control group after 24 hours (b). Representative image of a scratch wound mask after 24 hours (c). *D*. Invasion assay using Hs578T cells. The relative wound density of each amino acid-deprived group was compared with the control group over time (a). Comparison of relative wound densities between treatment groups after 24 hours (b). Representative image of a scratch wound mask after 24 hours (c). The relative wound density (RWD) is a measure of (%) density of the wound region relative to the density of the cell region, which was automatically measured using IncuCyte™ software, with the equation as follows: *% RWD (t)* = $100 \times \{w(t) - w(0)\}/\{c(t) - w(0)\}$

(*w(t)* = Density of wound region at time, t; *c(t)* = Density of cell region at time, t). Data represent the mean values ± S.D. Mean values with letters

(a-e) within a graph are significantly different from each other at $p < 0.05$.

2. Methionine deprivation has no significant effect on cell viability of MDA-MB-231, Hs 578T, or MCF 10A cells.

Methionine deprivation had no significant effect on cell viability for 24 hours in MDA-MB-231 (Fig. 2A) and Hs 578T cells (Fig. 2B) when assessed using MTT assay. This suggests that the migration and invasion results previously obtained (Fig. 1) were a direct result of the inhibition of migration and invasion factors, rather than an indirect effect of proliferative inhibition. In addition, there was no significant difference on cell viability between the methionine-deprived group and the control group for 24 hours when assessed using the normal breast cell line MCF 10A (Fig. 2C).

Figure 2

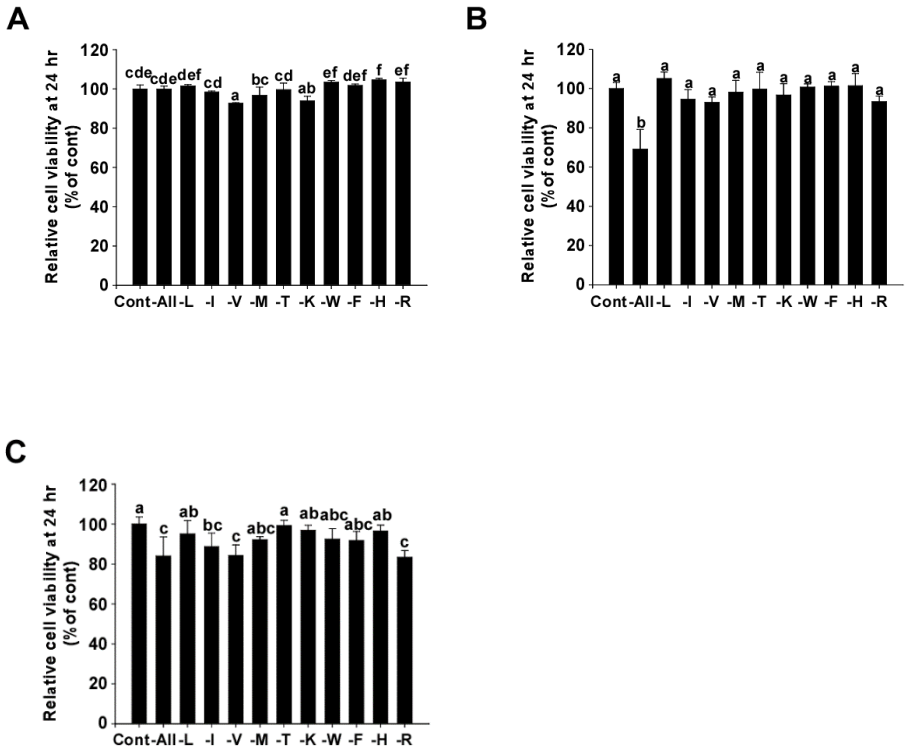
A
Relative cell viability at 24 hr (% of cont)
120
100
80
60
40
20
0
cde cde def cd a bc cd ab ef def f ef
Cont -All -L -I -V -M -T -K -W -F -H -R
B
Relative cell viability at 24 hr (% of cont)
a b a a a a a a a a a
Cont -All -L -I -V -M -T -K -W -F -H -R
C
Relative cell viability at 24 hr (% of cont)
a c ab bc c abc a ab abc abc ab c
Cont -All -L -I -V -M -T -K -W -F -H -R

Figure 2. Effect of methionine deprivation on cell viability of MDA-MB-231, Hs 578T cells and the non-invasive breast cell line MCF 10A. *A.* Relative cell viability of MDA-MB-231. *B.* Relative cell viability of Hs 578T. *C.* Relative cell viability of MCF 10A. Viability was measured using MTT assay. Data (n = 3) represent the mean values ± S.D. Mean values with letters (a-f) within a graph are significantly different from each other at $p < 0.05$.

3. Methionine deprivation suppresses FAK phosphorylation and activity, in addition to mRNA expression of MMP-2 and MMP-9 in MDA-MB-231 cells.

I subsequently investigated how methionine deprivation suppresses cell migration and invasion. I first analyzed levels of focal adhesion kinase (FAK), an important protein in cell adhesion, motility and metastasis [15]. Methionine deprivation effectively inhibited phosphorylation of focal adhesion kinase (FAK) after 8 hours (Fig. 3A), while also causing a reduction of activity and mRNA expression of MMP-2 and MMP-9, two extracellular matrix-degrading enzymes (Fig. 3B and 3C). Tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA levels were observed to increase in conditions of methionine deprivation (Fig. 3D). Furthermore, mRNA levels of urokinase plasminogen activator (uPA) decreased (Fig. 3E), while plasminogen activator inhibitor-1 (PAI-1) increased (Fig. 3F).

Figure 3

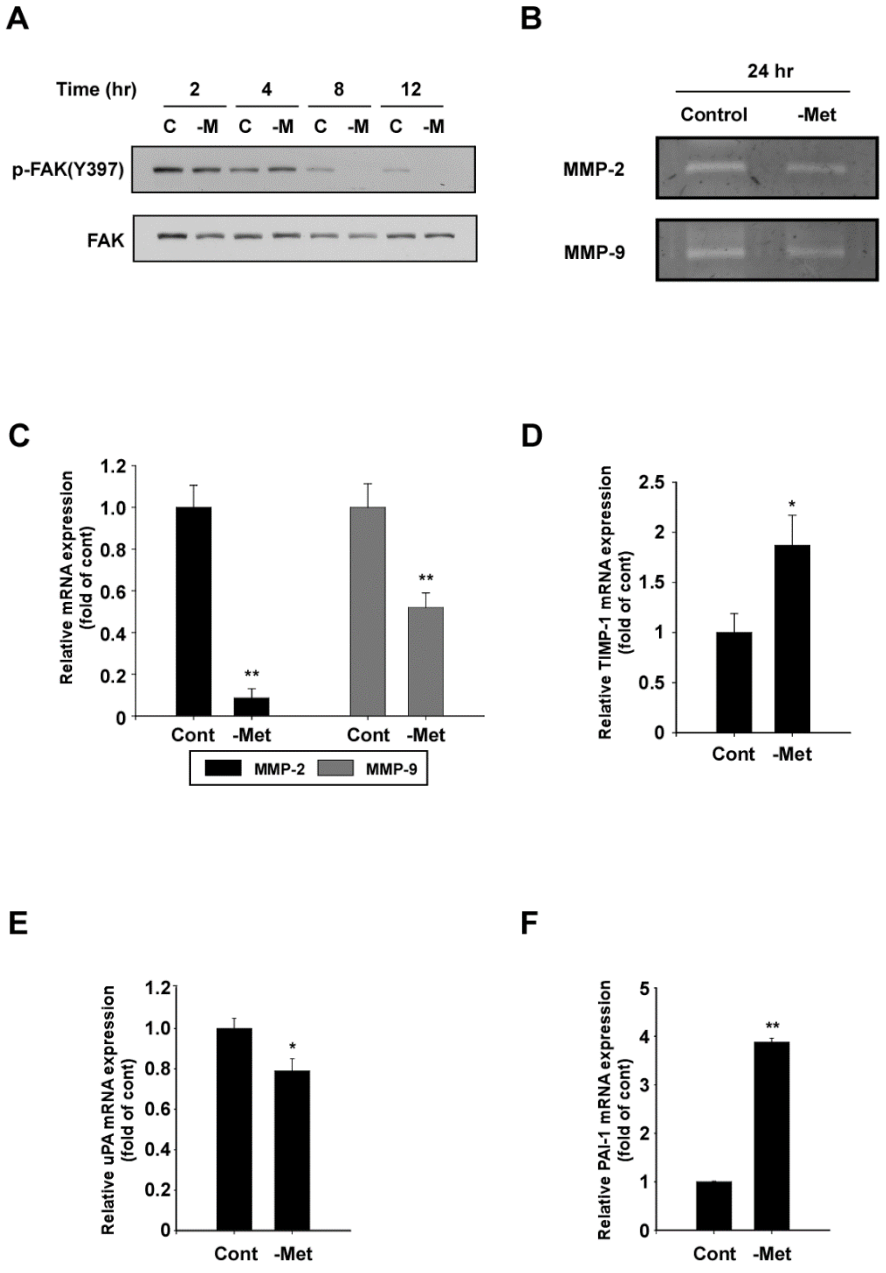
A
Time (hr)
2
4
8
12
C
-M
C
-M
C
-M
C
-M
p-FAK(Y397)
FAK
B
24 hr
Control
-Met
MMP-2
MMP-9
C
Relative mRNA expression (fold of cont)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
Cont
-Met
Cont
-Met
MMP-2
MMP-9
D
Relative TIMP-1 mRNA expression (fold of cont)
2.5
2
1.5
1
0.5
0
*
Cont
-Met
E
Relative uPA mRNA expression (fold of cont)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Cont
-Met
F
Relative PAI-1 mRNA expression (fold of cont)
5
4
3
2
1
0
**
Cont
-Met

Figure 3. Effect of methionine deprivation on FAK, MMP-2, MMP-9, TIMP-1, uPA, and PAI-1 expression in MDA-MB-231 cells. *A.* Methionine deprivation inhibits phosphorylation of FAK at Tyr 397, a major autophosphorylation site, as determined by Western blot. Total FAK was used as a loading control. Cells were incubated in control and methionine-deprived media for the indicated durations and harvested simultaneously. *B.* MMP-2 and MMP-9 activity was determined by gelatin zymography as described in the Materials and Methods. *C*. mRNA levels of MMP-2 and MMP-9 were analyzed by real-time quantitative PCR. Cells were incubated with control and methionine-deprived media for 24 hours and RNA was harvested. *D*. mRNA expression of TIMP-1. *E*. mRNA expression of uPA. *F*. mRNA expression of PAI-1. Data represent the mean values ± S.D. The asterisks (* or **) indicate a significant difference ($p < 0.05$ or $p < 0.01$) between the control group and methionine deprivation group.

4. Methionine deprivation inhibits cell migration and invasion in 4T1 cells.

I next examined the inhibitory effect of methionine deprivation on the migration and invasion of 4T1 mouse triple negative breast cancer cells. The wound width at each time point was expressed as the percentage relative to the wound width at 0 hours in each well (Fig. 4A). The relative wound width of the methionine-deprived group at 12 hours was 100.9% compared to a 60% relative wound width for the control group (Fig. 4B). The RWD at each time point was expressed as the percentage relative to the wound density of 0 hours in each well (Fig. 4C), and the RWD of the methionine-deprived group was 16.4% compared to a 49.1% RWD for the control group (Fig. 4D). These inhibitory effects of methionine deprivation were evident within the same time duration that cytotoxicity was absent (Fig. 4E).

Figure 4

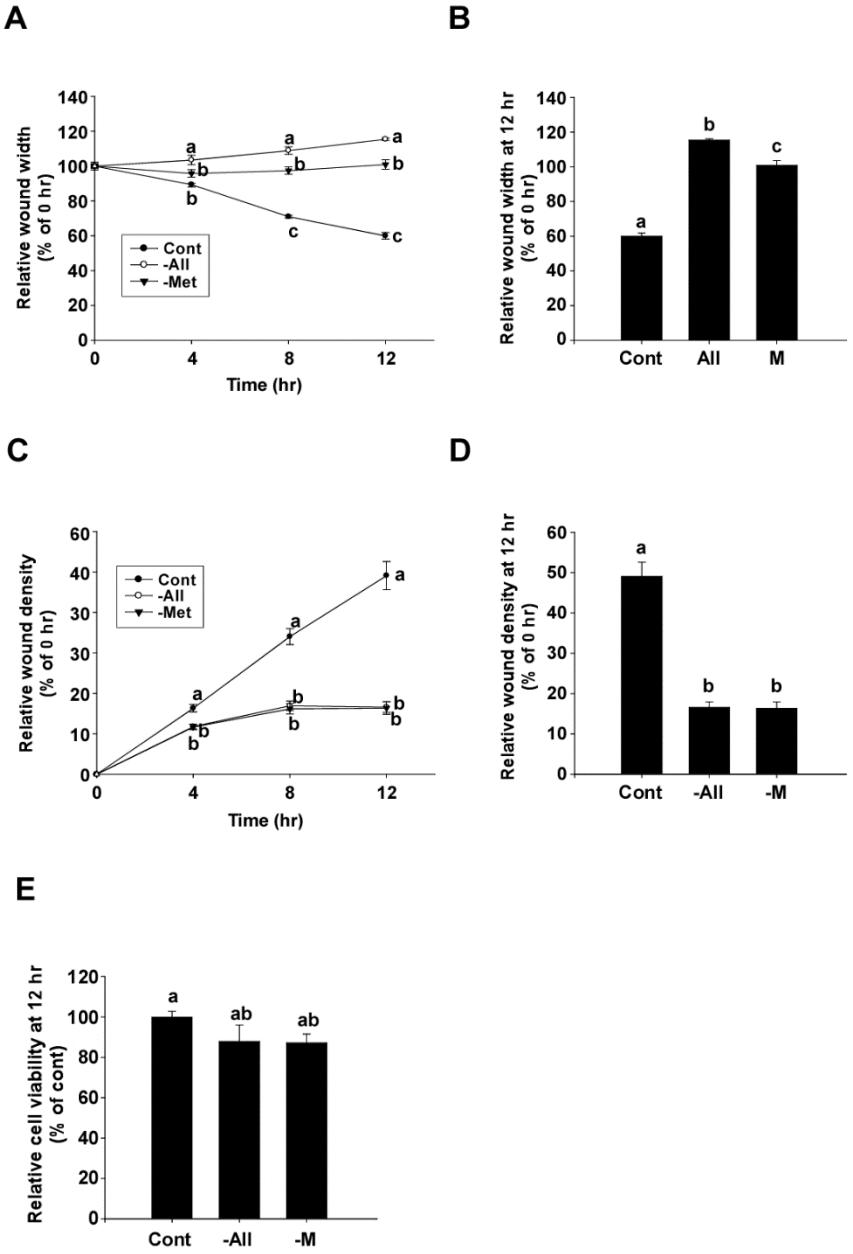
A
Relative wound width (% of 0 hr)
Time (hr)
Cont
-All
-Met
B
Relative wound width at 12 hr (% of 0 hr)
Cont
All
M
C
Relative wound density (% of 0 hr)
Time (hr)
Cont
-All
-Met
D
Relative wound density at 12 hr (% of 0 hr)
Cont
-All
-M
E
Relative cell viability at 12 hr (% of cont)
Cont
-All
-M

Figure 4. The inhibitory effect of methionine deprivation on migration and invasion of 4T1 cells. *A and B*. Relative wound widths of 4T1 cells were evaluated by migration assay. *C* and *D*. The relative wound density in 4T1 cells was determined by invasion assay. *E*. Relative cell viability of 4T1 cells. Data represent the mean values ± S.D. Mean values with letters (a-c) within a graph are significantly different from each other at $p < 0.05$.

5. Methionine deprivation reduces lung metastasis of 4T1 cells in BALB/c mice.

Tail vein injections of 4T1 cells (2 X 10^5 cells/mouse) caused the development of tumor nodules in the lungs. An intake of 0.1% methionine in the diet was observed to have no effect, but the intake of a methionine-deprivation diet for 10 days significantly reduced the number of tumor nodules in the lungs (Fig. 5A and 5B). The diets were provided ad libitum, and the intake of the methionine deprived-diet (1.69 g/day/mouse) was found to be lower than that of the control diet (2.27 g/day/mouse) (Fig. 5C). The body weights of the mice between the control and methionine-deprived group were significantly different after 6 days of injection (Fig. 5D).

Figure 5

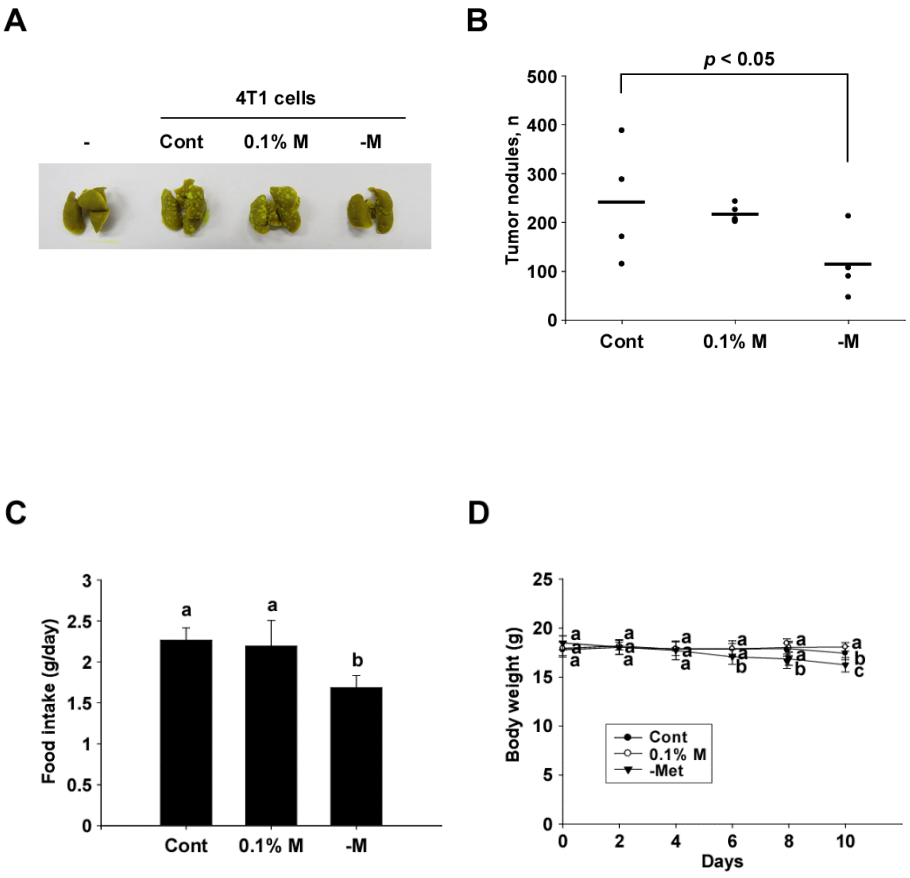
A
4T1 cells
-
Cont
0.1% M
-M
B
p < 0.05
Tumor nodules, n
500
400
300
200
100
0
Cont
0.1% M
-M
C
Food intake (g/day)
3
2.5
2
1.5
1
0.5
0
a
a
b
Cont
0.1% M
-M
D
Body weight (g)
25
20
15
10
5
0
Cont
0.1% M
-Met
0
2
4
6
8
10
Days

Figure 5. Inhibitory effect of a methionine deprived-diet on lung metastasis in BALB/c mice. *A and B.* Numbers of tumor nodules detected. *C*. Food intake details for each group. *D*. Mouse body weights. Data (n = 4-5) represent the mean values ± SEM. Mean values with letters (a-c) within a graph are significantly different from each other at $p < 0.05$.

Ⅳ. DISCUSSION

Although there have been many advances in the treatment of breast cancer with the emergence of receptor-targeted therapies, TNBC remains largely insensitive to such approaches as tumors of this subtype do not express three major hormone receptors. Because TNBC has highly metastatic and aggressive features, new therapeutic strategies are urgently needed.

In this study, I employed two human TNBC cell lines, MDA-MB-231 and Hs578T, which are commonly studied human cell lines for treatment of triple negative breast cancer [17]. I also used the 4T1 orthotopic model of TNBC with female BALB/c mice. The tail vein metastasis model with 4T1 cells has established itself as a relevant model for lung metastasis in breast cancer [18]. I screened 10 essential amino acids and observed that methionine restriction conferred the highest

inhibitory effect on migration and invasion of human TNBC cells. These inhibitory effects occurred within a short duration after treatment initiated, during which the effect of deprivation was not observed to be cytotoxic, as evidenced by MTT assay for the human TNBC cells and MCF 10A normal breast epithelial cells. Furthermore, the effects of methionine deprivation on migration, invasion and cell viability were confirmed in our 4T1 mouse breast cancer model, and a methionine-deprived diet suppressed metastasis to the lungs of 4T1 cells in BALB/c mice. Although the body weights of methionine-restricted mice were slightly decreased, this is unlikely to be the sole factor responsible for the decrease in metastasis observed. A previous study reported that a methionine-restricted diet negatively impacts the growth of animals, but extends the life span of various rat strains. The study showed that the life span extension effects of low methionine was not due to reduced energy intake, which was also observed to a slight degree [19]. Therefore, the slight

weight loss I observed may be a reasonable side effect of a methionine restricted diet.

The Warburg effect refers to the significantly increased need for nutrients and stronger metabolic processes exhibited by cancer cells [7]. In addition to calorie restriction, specific amino acid restriction has been used in the past to treat cancer. Methionine, one of the essential amino acids, is a major therapeutic target for the treatment of cancer. Methionine dependence of malignant cells were first observed in Walker-256 carcinosarcoma-transplanted rodents in 1959 [20]. An absolute methionine dependence of human, mouse, and rat malignant cells was identified by growth in methionine-depleted and in homocysteine (the immediate precursor of methionine)-supplemented media (Met^- Hcy^+). In contrast, normal cells were not affected by the Met^- Hcy^+ media [21]. Several other studies have suggested that various cancer cells are methionine dependent, and simple dietary depletion of methionine has

been shown to reduce the proliferation of numerous cancer cell lines. For example, methionine starvation has been observed to inhibit tumor growth of PC-3 cells, and a methionine analogue, ethionine, was shown to potentiate these effects [22]. A methionine-restricted diet also suppressed colon cancer development in F344 rats [23]. In addition to the growth inhibitory effects of methionine depletion, it has also been shown to enhance the efficacy of chemotherapeutic agents in refractory cancers [24]. Furthermore, methionine restriction is known to extend the life-span of various rat strains, indicating that basic health is not threatened by methionine restriction [19,25]. Taken together, these observations suggest that methionine restriction should be regarded as a possible adjuvant therapeutic strategy for cancer treatment.

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Ⅵ. 국문 초록

유방암은 우리나라 여성암 중 발병률 2위를 차지하며 치료 후 전이성 재발이 잘 일어나는 대표적인 암종이다. 조기 진단의 증가와 의료기술의 발달로 국소암의 경우는 치료 후 5년 생존율이 약 92%로 높은 편이지만, 다른 장기로 원격 전이된 경우 생존율이 급감하여 전이의 예방 및 치료에 대한 관심이 높아지고 있다. 특히, 유방암의 약 20%를 차지하며 치사율과 전이율이 높은 삼중음성유방암은 에스트로겐, 프로게스테론, HER2 수용체를 발현하지 않아서 타목시펜, 허셉틴 등의 호르몬 치료제로는 억제가 불가능 하며, 따라서 삼중음성유방암의 전이를 막기 위한 보조 치료법에 대한 연구가 필요하다. 암세포는 여러 영양소에 대한 의존도가 증가해있으며, 이러한 영양소를 제한함으로써 암세포를 제어하려는 연구들이 있어왔다. 따라서 본 연구는 삼중음성유방암의 전이와 관련된 특정 아미노산 의존성을 밝히고자 하였다. 10 종류의 필수 아미노산 중

메티오닌 결핍이 삼중음성유방암의 세포 이동 및 침투를 가장 효과적으로 저해하였다. 또한 메티오닌 결핍이 세포 부착 및 이동에 관여하는 Focal adhesion kinase (FAK)의 인산화를 저해하였으며, 세포 외 기질 분해 효소 (Matrix metalloproteinase, MMP-2 and MMP-9)의 전사 및 활성을 저해하였다. 또한, MMP의 저해인자인 Tissue inhibitor of metalloproteinase (TIMP-1)의 전사 정도를 증가시켰으며, 또 다른 세포 이동 촉진인자인 Urokinase plasminogen activator (uPA)의 전사 정도는 감소시키고 그 저해인자인 Plasminogen activator inhibitor (PAI-1)의 전사 정도는 증가시켰다. 또한, 쥐의 꼬리 미정맥으로 주입된 삼중음성유방암 세포의 폐로의 전이를 메티오닌 제한 식이가 억제함을 확인하였다. 본 연구 결과, 메티오닌 제한이 삼중음성유방암의 전이를 억제함을 밝혔으며, 삼중음성유방암의 전이를 조절하는 보조적 치료법으로써 메티오닌 제한 식이의 가능성을 제시하였다.