Effect of 5-FU and MTX on the Expression of Drug-resistance Related Cancer Stem Cell Markers in Non-small Cell Lung Cancer Cells

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Cancer stem cells (CSCs) are often characterized by the elevated expression of drug-resistance related stem-cell surface markers, such as CD133 and ABCG2. Recently, we reported that CSCs have a high level of expression of the IL-6 receptor (IL-6R). The purpose of this study was to investigate the effect of anticancer drugs on the expression of the drug resistance-related cancer stem cell markers, ABCG2, IL-6R, and CD133 in non-small cell lung cancer (NSCLC) cell lines. A549, H460, and H23 NSCLC cell lines were treated with the anticancer drugs 5-fluorouracil (5-FU; 25 μg/ml) and methotrexate (MTX; 50 μg/ml), and the expression of putative CSC markers was analyzed by fluorescent activated cell sorter (FACS) and the gene expression level of *abcg2, il-6r and cd133* by reverse transcriptase-polymerase chain reaction (RT-PCR). We found that the fraction of ABCG2-positive(+) cells was significantly increased by treatment with both 5-FU and MTX in NSCLC cells, and the elevation of *abcg2, il-6r and cd133* expressions in response to these drugs was also confirmed using RT-PCR. Also, the number of IL-6R(+) cells was increased by MTX in the 3 cell lines mentioned and increased by 5-FU in the H460 cell line. The number of CD133(+) cells was also significantly increased by both 5-FU and MTX treatment in all of the cell lines tested. These results indicate that 5-FU and MTX considerably enhance the expression of drug-resistance related CSC markers in NSCLC cell lines. Thus, we suggest that antimetabolite cancer drugs, such as 5-FU and MTX, can lead to the propagation of CSCs through altering the expression of CSC markers.

Key Words: Cancer stem cell, 5-FU/MTX, ABCG2/IL-6R/CD133, Drug resistance

INTRODUCTION

The cancer stem cell (CSC) theory suggests that tumors contain rare subpopulations of undifferentiated cells that possess stem cell properties, such as self-renewal, heterogeneity, and resistance to anticancer drugs [1,2]. CSCs show resistance to chemotherapy because of their low proliferation rate and resistance mechanisms, such as the expression of multidrug transporters of the ATP-binding cassette (ABC) superfamily. The transporter ABCG2 is known to promote resistance to multiple drugs and has been observed in various types of CSCs [3-5]. Due to the drug resistant properties of CSCs, many anticancer drugs show transient effects and often do not improve patient prog-

nosis. Even therapies that cause complete tumor regression may spare CSCs, allowing for regeneration of the tumor. The pentaspan protein, CD133, is a universal marker of organ-specific stem cells and tumor-initiating cells. CD133 was originally identified as a marker for CD34(+) hematopoietic stem and progenitor cells [6] and has been used to identify CSCs in several types of cancer [7-11]. Lung cancer is the leading cause of cancer death in many countries [12]. Poor prognosis of lung cancer patients is due mainly to poor response, early relapse, and metastasis after treatment with chemotherapy and radiotherapy. Several studies have demonstrated that CD133(+) lung cancer cells possess features of stemness and display a higher tumorigenic potential than CD133-negative(−) cells [13-15]. CD133 expression has also been linked to a drug-resistant phenotype in lung cancer patients [16].

In our previous study, lung CSCs, isolated with Hoechst 33342, were found to express significantly higher levels of the IL-6 receptor (IL-6R) and *abcg2* [17]. IL-6/IL-6R signaling has been well documented to play a role in the growth and malignancy of cancer. The aberrant production and increased secretion of IL-6 in cancer patients is linked to tu-

ABBREVIATIONS: CSC, cancer stem cell; IL-6R, interleukin 6 receptor; NSCLC, non-small cell lung cancer; 5-FU, 5-fluorouracil; MTX, methotrexate; ABCG2, ATP-binding cassette superfamily G2; FACS, fluorescence activated cell sorter.

mor progression and poor prognosis in many cancer types, including lung cancer [18-20]. IL-6 is also known to promote multi-drug resistance to chemotherapy [21]. Recently, it was reported that the expression of IL-6 and IL-6R in CSCs was much greater than in non-CSCs [11,22]. These findings suggest that IL-6R is a potential CSC marker related to the drug resistance phenotype of some tumors. However, the expression of CSC markers and mechanisms by which CSCs acquire resistance to many anticancer drugs remains unknown. Therefore, we carried out this study to assess the effect of anticancer drugs on the expression of the drug resistance-related cancer stem cell markers, ABCG2, IL-6R, and CD133 in non-small cell lung cancer (NSCLC) cell lines.

METHODS

Cell culture

NSCLC cell lines, A549, H460, and H23, were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). Cells were grown in RPMI-1640 medium (Gibco, CA, USA), with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Gibco, CA, USA) in a humidified atmosphere at 37°C with 5% CO_2 as recommended by the KLCB.

Anticancer drug treatment

The anticancer drugs, 5-FU and MTX, were purchased from Sigma Aldrich Inc. (MO, USA) and dissolved in phosphate buffered saline (pH 7.4). NSCLC cells were seeded in a 6-well cell culture plate (Corning Inc., NY, USA) and incubated overnight in a humidified atmosphere at 37°C with 5% CO_2. Cells were then incubated with 5-FU (25 μg/ml) or MTX (50 μg/ml) in media for 6, 12, and 48 hrs prior to RT-PCR and for 24 hrs prior to FACS analysis.

FACS analysis

A549, H460, and H23 NSCLC cells were harvested and suspended at a density of 1×10^6 cells/ml in FACS buffer containing PBS (pH 7.4) with 2% fetal bovine serum (FBS). The cells were incubated with PE-anti-ABCG2 (Abcam, MA, USA), FITC-anti-IL-6R (Abcam, MA, USA), and PE-anti-CD133 (Miltentyi Biotec, Bergisch Gladbach, Germany) antibodies for 30 min. After washing the cells three times with FACS buffer, the expression of ABCG2, IL-6R, and CD133 were analyzed by fluorescent activated cell sorter (FACScalibur; BD, NJ, USA).

RNA extraction and semi-quantitative RT-PCR

We performed semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to assess the effect of

the anticancer drugs on mRNA expressions of *abcg2*, *il-6r*, and *cd133*. Total RNA of the cells was extracted using Trizol Reagent (Invitrogen, CA, USA) following manufacturer's directions. Briefly, cells were washed with phosphate buffered saline three times and mixed with 1 ml of Trizol reagent. The mixture was extracted with 200 μl of chloroform. After centrifugation at 10,000×g for 30 minutes, the RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol. The precipitated RNA was collected by centrifugation at 10,000×g for 20 minutes, washed by 70% ethanol, dissolved in 20 μl of RNase-free water. The amount of RNA was quantified by its absorption at 260 nm and stored at -80°C before use. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), was used as an internal control, and the mRNA expression of different molecular markers was analyzed by a semiquantitative RT-PCR assay. Briefly, first-strand cDNAs were synthesized from 5 μg of total RNA using superscript III reverse transcriptase (Invitrogen, CA, USA). PCR amplification was performed with specific primer pairs designed from published human gene sequences (Table 1) using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

RESULTS

FACS analysis for the expression of ABCG2, IL-6R, and CD133 on the cell surface

The effect of the anticancer drugs 5-FU and MTX on the expression of CSCs markers was studied in A549, H460, and H23 NSCLC cell lines using FACS analysis. The fractions of ABCG2(+), IL-6R(+), and CD133(+) cells were significantly increased by treatment with 5-FU or MTX in these NSCLC cell lines.

In vehicle-treated A549 cells, approximately 17% of the cells were ABCG2(+). Following 5-FU and MTX treatment, the ABCG2(+) cell-fraction was significantly increased by 2.3- and 4.6-fold, respectively (Fig. 1). H460 cells also showed a 3.5- and 3.9-fold increase in the number of ABCG2(+) cells in response to 5-FU and MTX, respectively. Also, H23 cells displayed a 10~20% increase in the ABCG2(+) fraction by treatment with 5-FU and MTX compared to control treated cells.

As shown in Fig. 2, the population of IL-6R(+) cells in A549, H460, and H23 were 1.4, 0.04, and 0.74%, respectively. IL-6R(+) fractions were significantly increased by MTX treatment in all 3 cell lines. Alternatively, the IL-6R (+) fraction was not increased by 5-FU in A549 and H23 cells, but H460 cells displayed a large increase in the IL-6R (+) cell population. In the 3 NSCLC cell lines tested, the CD133(+) population varied from 0.17% to 0.82% (Fig. 3). Specifically, in A549 cells, the CD133(+) fraction was slightly increased by 5-FU or MTX treatment, while the

Table 1. Human primer sequences used for semi-quantitative RT-PCR

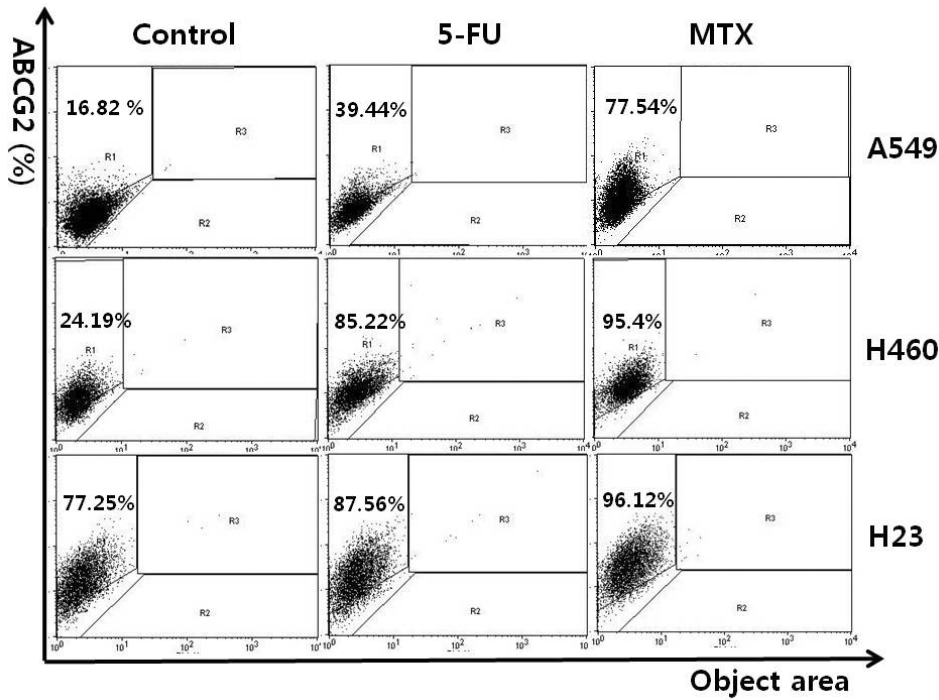| Gene	Sense primer	Antisense primer	Product size
abcg2	5'-ACGCCTTGGACAGAATCCAG-3'	5'-TGGCTCGAGGTATTGTCAGA-3'	379 bp
il-6r	5'-ACGCCTTGGACAGAATCCAG-3'	5'-TGGCTCGAGGTATTGTCAGA-3'	398 bp
cd133	5'-GCATGCAAAAGCCATCATAG-3'	5'-GGGAATGCCTACATCTGGAA-3'	446 bp
gapdh	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'	498 bp

Fig. 1. Analysis of ABCG2 expression after 5-FU or MTX treatment. A549, H460, and H23 non-small cell lung cancer cell lines were treated with 5-FU (25 μg/ml) or MTX (50 μg/ml) for 24 hours and incubated with a PE-anti-ABCG2 antibody. The number of fluorescent-conjugated cells in 10^4 cells was analyzed by fluorescence activated cell sorter (FACS).
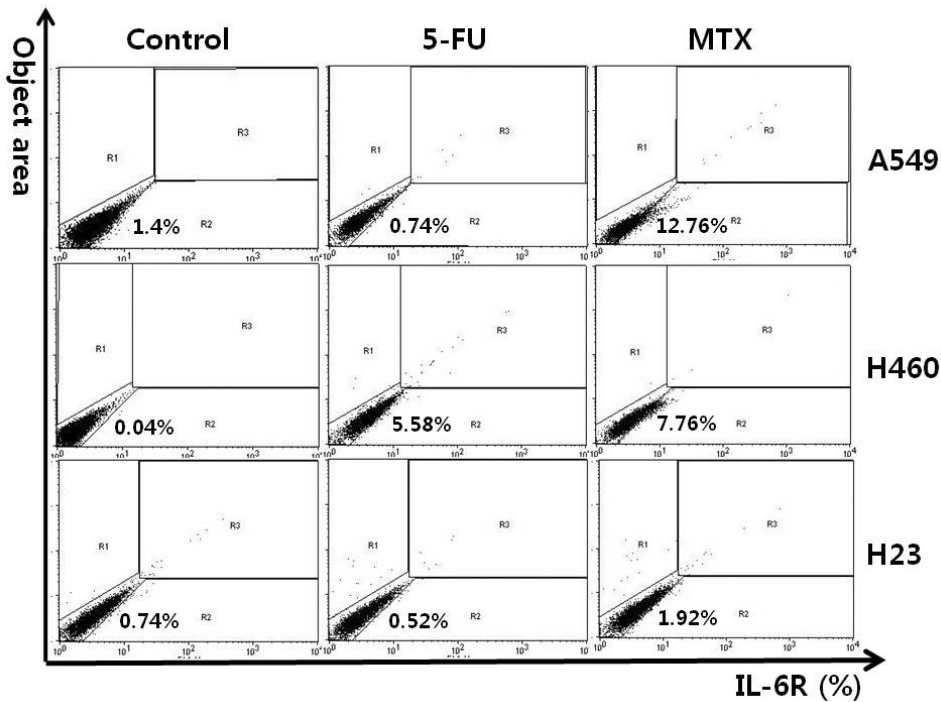

Fig. 2. Analysis of IL-6R expression after 5-FU or MTX treatment. A549, H460, and H23 non-small cell lung cancer cell lines were treated by 5-FU (25 μg/ml) or MTX (50 μg/ml) for 24 hours and incubated with a FITC-anti-IL-6R antibody. The number of fluorescent-conjugated cells in 10^4 cells was analyzed by fluorescence activating cell sorter (FACS).

CD133(+) fractions were markedly increased by 2- to 7-fold by 5-FU or MTX treatment in H460 and H23.

RT-PCR gene expression analysis of abcg2, il-6r and cd133

The expression of ABCG2, IL-6R, and CD133 was confirmed at the mRNA level in the A549, H460, and H23 NSCLC cell lines using semi-quantitative RT-PCR (Figs. 4~6). The expression levels of *abcg2 and il-6r* were increased in the 3 cell lines following treatment with 5-FU or MTX. As shown in Fig. 6, the expression level of *cd133* was increased in A549 cell line following treatment with 5-FU or MTX.

DISCUSSION

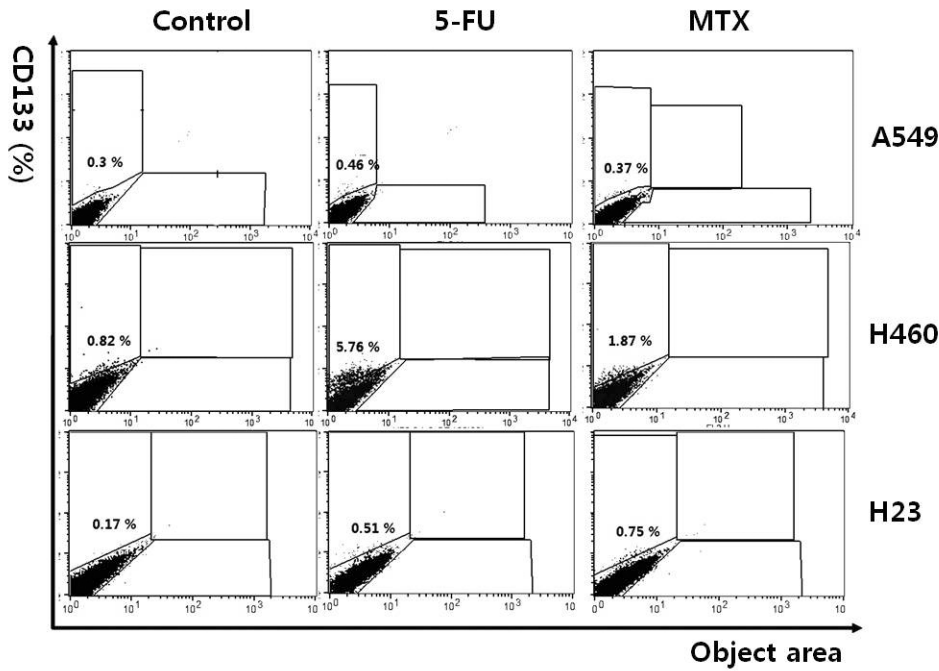Various studies have revealed that CSCs develop resist-

Fig. 3. Analysis of CD133 expression after 5-FU or MTX treatment. A549, H460, and H23 non-small cell lung cancer cell lines were treated with 5-FU (25 μg/ml) or MTX (50 μg/ml) for 24 hours and incubated with a PE-anti-CD133 antibody. The number of fluorescent-conjugated cells in 10^4 cells was analyzed by fluorescence activating cell sorter (FACS).
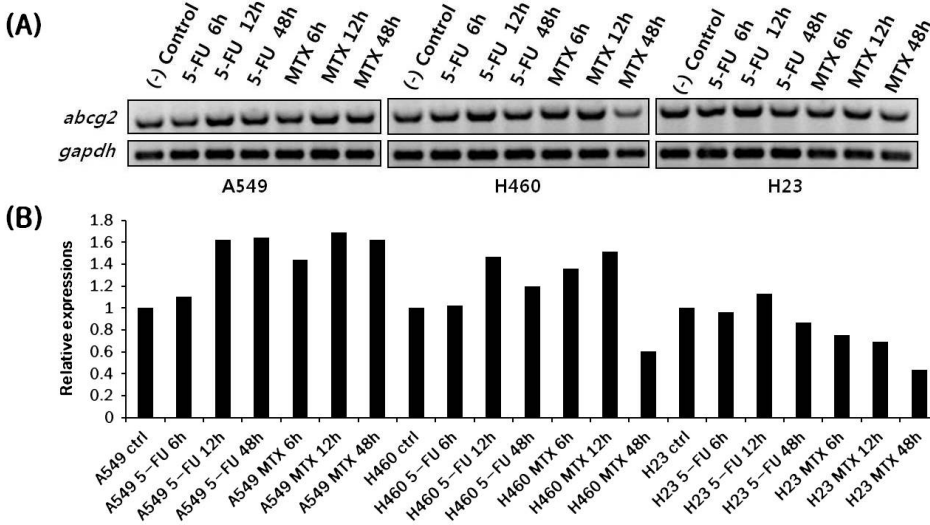

Fig. 4. Gene expression of *abcg2* after 5-FU or MTX treatment. (A) A549, H460, and H23 non-small cell lung cancer cell lines were treated with 5-FU (25 μg/ml) or MTX (50 μg/ml) for 6, 12, and 48 hr. After the incubation period, total RNA was extracted, and mRNA levels were analyzed by RT-PCR. PCR amplification was performed using specific primer pairs designed from published human gene sequences. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), was selected as an internal control. (B) The densities of the DNA bands of *abcg2* were analyzed by Image J Software (NIH, http://rsb.info.nih.gov/nih_image/index.html).

ance to anticancer drugs [3,11,23], but the mechanisms responsible for this trait have not been well established. In our study, 5-FU and MTX treatment increased ABCG2(+) cell populations to a similar extent in 3 NSCLC cell lines. Gene expression of *abcg2* was also increased by 5-FU and MTX treatment. 5-FU has been used to select quiescent or slow-dividing stem/progenitor cells based on the fact that only rapidly dividing cells are sensitive to 5-FU [24-26]. There is growing evidence that CSCs are more resistant to 5-FU than non-CSCs [27,28]. This explains why 5-FU treatment can spare CSCs, while inducing cell death in cancer cells. MTX is a typical substrate of the membranous efflux transporter, ABCG2. Therefore, overexpression of ABCG2 in cancer cells results in an extensive reduction in the amount of MTX absorption, leading to drug resistance

[29]. Previously, we have shown that lung CSCs have higher expression of ABCG2 and display greater resistance to MTX than non-CSCs [17,30]. This indicates that treating cancer cells with MTX can allow for CSC survival. Although we have no direct evidence for the increase of ABCG2(+) cell populations due to 5-FU and MTX, it is speculated that these anticancer drugs can induce the expression of the ABCG2 transporter, thus sparing drug-resistant CSCs.

Our results indicate that MTX increased the population of IL-6R(+) cells in A549, H460, and H23 cells, while 5-FU increased the IL-6R(+) population in only H460 cells. Although the responses of IL-6R expression to 5-FU treatment were not the same among the 3 NSCLC cell lines tested, the up-regulation of IL-6R(+) cells by MTX was similar in the 3 NSCLC cell lines. Conze et al. demonstrated that
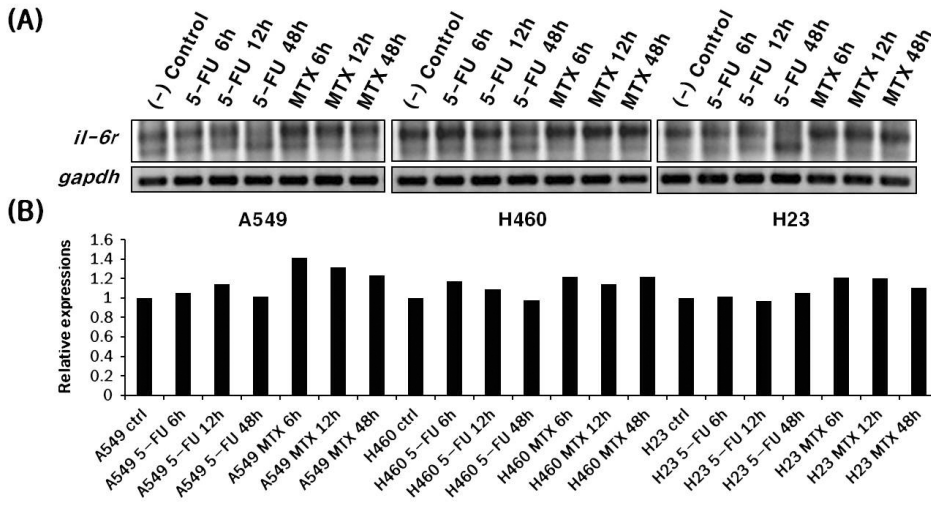
(A)

Fig. 5. Gene expression of *IL-6R* after 5-FU or MTX treatment. (A) A549, H460, and H23 non-small cell lung cancer cell lines were treated with 5-FU (25 μg/ml) or MTX (50 μg/ml) for 6, 12, and 48 hr. After the incubation period, total RNA was extracted, and mRNA levels were analyzed by RT-PCR. PCR amplification was performed using specific primer pairs designed from published human gene sequences. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), was selected as an internal control. (B) The densities of the DNA bands of *il-6r* were analyzed by the Image J Software.
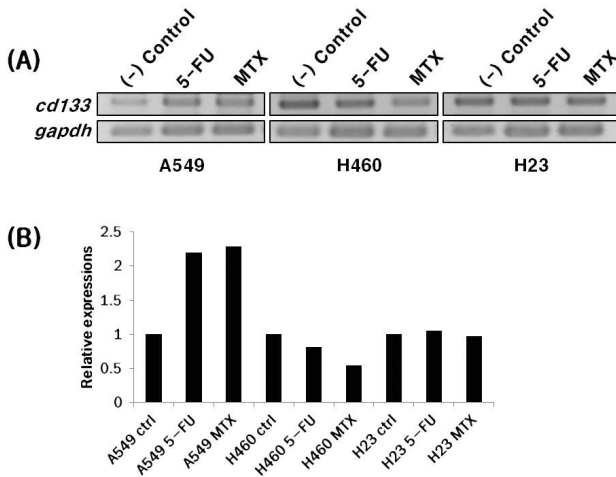

Fig. 6. Gene expression of *CD133* after 5-FU or MTX treatment. (A) A549, H460, and H23 non-small cell lung cancer cell lines were treated with 5-FU (25 μg/ml) or MTX (50 μg/ml) for 12 hr. After the incubation period, total RNA was extracted, and mRNA levels were analyzed by RT-PCR. PCR amplification was performed using specific primer pairs designed from published human gene sequences. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), was selected as an internal control. (B) The densities of the DNA bands of *cd133* were analyzed by the Image J Software.

IL-6 release could promote resistance to chemotherapy [21] through the induction of multi-drug resistance 1 (MDR1) gene expression [21]. Also, Levina et al. reported that drug resistant-CSCs, isolated from H460 NSCLC cell line, have significantly elevated levels of IL-6 compared to non-CSCs. These reports indicate that IL-6/IL-6R signaling and drug resistance are closely related in both cancer cells and cancer stem cells. Our results demonstrate that the anticancer drugs 5-FU and MTX markedly increased the CD133(+) cell population in 3 NSCLC cell lines. Consistent with our results, Sozzi et al. demonstrated that CD133(+) cells display stem cell-like features and are resistant to anticancer drugs [3]. In addition, Herr et al. showed that CD133(+) cells had a resistance phenotype in lung cancer patients

[16]. Therefore, these findings suggest that the elevation of CSC marker-positive cells by 5-FU or MTX treatment results in CSC resistance in NSCLC cell lines.

In conclusion, 5-FU and MTX considerably alter the expression of drug-resistance related CSC populations in the NSCLC cell lines A549, H460, and H23. Thus, we suggest that antimetabolite cancer drugs, such as 5-FU and MTX, can lead to propagation of CSCs by enhancing the expression of several CSC markers. These findings provide a novel mechanism for the highly drug resistant phenotype of lung CSCs and their ability to survive chemotherapy.

ACKNOWLEDGEMENTS

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