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The Role of Thymidylate Synthase in Pemetrexed-Resistant Malignant Pleural Mesothelioma Cells

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ABSTRACT

We established new pemetrexed-resistant cells originating from malignant pleural mesothelioma MSTO-211H cells to clarify the mechanism involved in pemetrexed resistance in malignant pleural mesothelioma. In the pemetrexed-resistant cells, only thymidylate synthase (*TYMS*) mRNA was overexpressed among other well-known molecular targets and chemosensitivity determinants of pemetrexed, and the role of the *TYMS* gene was ascertained by artificial regulation induced by specific siRNA. Silencing the *TYMS* expression partially restored the cytotoxicity of pemetrexed. The resistant cells did not display other gene alterations related to folate metabolism. We conclude that the primary mechanism imparting resistance to these cells is specific up-regulation of *TYMS* function. Further, the *TYMS* gene may serve as a useful biomarker for the prediction of pemetrexed chemosensitivity in patients with malignant pleural mesothelioma. We also investigated the efficacy of 1-(3-*C*-ethynyl-*ß*-D-ribo-pentofuranosyl)cytosine (ECyd) in overcoming pemetrexed resistance; this compound is presently undergoing clinical trials in the USA as TAS-106. ECyd had a similar antitumor effect on the resistant cells as that on the parental cells. In the clinical treatment of malignant pleural mesothelioma, ECyd promises to emerge as a novel drug.

Keywords: Malignant Pleural Mesothelioma; Thymidylate Synthase; Pemetrexed; ECyd

1. Introduction

Malignant pleural mesothelioma is considered to be caused by previous exposure to asbestos fibers [1-3]. Regardless of the stage at diagnosis, it is generally viewed as a treatment-resistant tumor having poor prognosis [4] owing to serious difficulties, including the availability of effective drugs. Until recently, although no chemotherapeutic agent was effective against malignant pleural mesothelioma, in 2004, the Food and Drug Administration (FDA) approved administration of pemetrexed in combination with cisplatin for the treatment of patients whose condition is unresectable or who are otherwise not candidates for curative surgery [5]. Pemetrexed is an antifolate drug that targets some folate enzymes [5,6]; this drug is initially transported into the cytoplasm by reduced folate carrier (*RFC*) and other transporters and then metabolized to a polyglutamated form by folypoly-gamma-glutamate synthetase (*FPGS*). Polyglutamation increases cellular retention and confers an affinity for some enzymes involved in folate metabolism. Pemetrexed and its polyglutamated derivatives inhibit thymidylate synthase (*TYMS*), dihydrofolate reductase (*DHFR*), and glycinamide ribonucleotide transformylase (*GARFT*), all of which are involved in the *de novo* biosynthesis of thymidine and purine nucleotides. Antimetabolite agents, including pemetrexed, induce an imbalance in the cellular nucleotide pool and inhibit nucleic acid biosynthesis that results in arresting the proliferation of tumor cells and inducing cell death [5-7].

In pemetrexed-resistant cells, *TYMS* overexpression is one of the major factors leading to resistance [8] and the regulation of *DHFR*, *RFC*, and *FPGS* expression is associated with acquired resistance to pemetrexed [8,9]. These resistance mechanisms have been investigated in the colon [10], breast [11], gastric [12], small cell lung cancer [13], non-small cell lung cancer [14], and leukemia cell lines [15]. These tumor types had not been recognized by FDA. In this study, we have newly established peme-

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trexed-resistant malignant pleural mesothelioma cells from MSTO-211H cells and studied its resistant mechanisms against pemetrexed. Further, we also examined the efficacy of 1-(3-*C*-ethynyl-*ß*-D-ribo-pentofuranosyl)cytosine (ECyd) against malignant pleural mesothelioma in overcoming the established resistance.

2. Materials and Methods

2.1. Drugs and Tumor Cells

1-(3-*C*-Ethynyl-*ß*-D-ribo-pentofuranosyl)cytosine (ECyd, TAS-106) was provided by TAIHO Pharmaceutical (Tokyo, Japan), while pemetrexed was purchased from Toronto Research Chemicals (North York, Canada). Human mesothelioma MSTO-211H cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The resistant cell line (H/pemetrexed) was established by a stepwise drug increase method. Both parental and resistant cell lines were maintained at 37˚C and 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA).

2.2. Drug Sensitivity Test

The growth-inhibitory effects of the drugs on human tumor cells were examined using a colorimetric assay involving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, 190-µl aliquots of an exponentially growing cell suspension (1000 cells/190 µl/well) were incubated with 10 µl of varying concentrations of drugs. After exposure to the drugs for 48 - 72 h, 20 µl of MTT solution (3 mg/ml) was added to each well and the cell cultures were incubated at 37˚C for 4 h. After removal of the medium, the formed formazan was dissolved in 200 µl of dimethyl sulfoxide. The absorbance of each well was measured at 570 nm with an immunoreader (MTP-800AFC, CORONA Electric, Hitachinaka, Japan), and the inhibition ratio (IR) was calculated using the following formula: IR (%) = (1 − T/C) × 100, where C is the mean of optical densities of the control group and T of the treatment group. The IC_{50} value was defined as the concentration of the drug needed to effect a 50% reduction in growth relative to the control. The IC_{50} value was determined by a graphical correlation of the dose-response curve with at least three drug concentration points.

2.3. siRNA Transfection

All small-interfering RNAs (siRNAs) were purchased from Stealth RNAi (Life Technologies). The sequences of three siRNAs (TYMS-1 to -3) targeting *TYMS* gene (NM_001071) were
5’-GCTGTGGTTTATCAAGGGATCCACA-3’ (403 - 427),
5’-GGGAGATGCACATATTTACCTGAAT-3’ (895 - 919), and
5’-CAGAGATATGGAATCAGATTATTCA-3’ (577 - 919), respectively. Stealth RNAi glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) Positive Control (Life Technologies) and Stealth RNAi Negative Control (Low GC Duplex #2; Life Technologies) were served as a control. Upon preincubation at 37˚C for 24 h, cells in 35-mm dishes were transfected with 250 pmol siRNA using lipofectamine 2000 (Life Technologies) following the manufacturer’s protocol. Cells were treated with the transfection agent/siRNA complex for 24 h and subjected to further analysis.

2.4. Quantization of mRNA Expression

The cells were washed with PBS (−) and then total RNA was extracted using ISOGEN (Nippon Gene, Osaka, Japan) according to the manufacturer’s instructions. The concentration of the total extracted RNA was determined by measuring the OD at 260 nm, and the RNA was diluted to 200 µg/ml. First strand cDNA synthesis was carried out using 2 µg of total RNA, 5 pmol oligo (dT) 12 - 18 (GE Healthcare, Buckinghamshire, UK) and ReveTraAce (TOYOBO, Osaka, Japan) at 42˚C for 90 min. cDNA prepared by the reverse transcription reaction was subjected to PCR amplification in a Thermal Cycler Dice Real-Time System (Takara Bio, Shiga) with SYBR Green PCR Master Mix (Takara Bio, Japan) using specific primers (**Table 1**). The expression of the target genes was standardized using the regular housekeeping genes [ribosomal protein large P2 (*RPLP*2), ribosomal protein S18 (*RPS*18), phosphoglycerate kinase 1 (*PGK1*), and beta-actin (*ACTB*)], and the relative expression levels were quantified by using the $2^{-\Delta\Delta CT}$ method.

2.5. Establishment of TYMS-Overexpressing Tumor Cell Line

Cells were transfected with the *TYMS* gene to clarify and understand the effect of the drugs on *TYMS* function. Human *TYMS* was overexpressed by PCR amplification of the full coding sequence of human *TYMS* cDNA using the sense primer 5’-ATGCCTGTGGCCGGCTCGGA-3’ and the antisense primer 5’-ATATCCTTCGAGCTCCTTTG-3’. The *TYMS* cDNA was cloned into the pEF6/V5-His TOPO vector (Life Technologies) to construct pEF6/*TYMS*. This plasmid was transfected into the MSTO-211H cells by using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. The transfectants were grown in a cultured medium containing 500 µg/ml blasticidin S. Blasticidin-

Table 1. Primers used in real-time PCR.

Symbol	Gene name		GenBankacc No.
	Forward primer	Reverse primer	
TYMS	thymidylate synthetase		**NM_001071.2**
	ATCATCATGTGCGCTTGGAATC	TGTTCACCACATAGAACTGGCAGAG	
DHFR	dihydrofolate reductase		**NM_000791.3**
	AGGGTTGGTTAGGCAATCATTC	AGGATTTAGCTCTTACACCATCACA	
GGH	gamma-glutamyl hydrolase		**NM_003878.2**
	AACCTCTGACTGCCAATTTCCATAA	TCTCTGGATGCCACTGGACAC	
*GART*_v1	phosphoribosylglycinamideformyltransferase transcript variant 1		**NM_000819.3**
	CATAGCAATGGATTTAGCCTTGTGA	GCTGTAGATTCTGGTAGGCGTGAG	
*GART*_v2	phosphoribosylglycinamideformyltransferase transcript variant 2		**NM_175085.2**
	ATGGACTGCTCTGCACATCTCTG	AGTGCCTGCATGGAACACCTC	
*FPGS*_v1	folylpolyglutamate synthase transcript variant 1		**NM_004957.4**
	TCTGCCCTAACCTGACAGAGGTG	TCGTCCAGGTGGTTCCAGTG	
*FPGS*_v2	folylpolyglutamate synthase transcript variant 2		**NM_001018078.1**
	TCTGCCCTAACCTGACAGAGGTG	TCGTCCAGGTGGTTCCAGTG	
*SLC*19*A*1	solute carrier family 19 (folate transporter) member 1 transcript variant 1		**NM_194255.1**
	ACTTTCATTGTCTCGGACGTG	GTAGATGATGGACAGGATCAGGA	
*SLC*46*A*1	solute carrier family 46 member 1 (folate transporter)		**NM_080669.3**
	CCGCAGCTTAAAGCAGTCACAA	CAGCACCTGCCTGGCTACAA	
*RRM*1	ribonucleotide reductase M1		**NM_001033.3**
	GAGCAGGGCCCATACGAAAC	CCCAGGATCTGAGCAGTGGAA	
*RRM*2	ribonucleotide reductase M2, transcript variant		**NM_001034.1**
	TGATGTTCAAACACCTGGTACACAA	AACCCAGTTCCAGCATAAGTCTGTC	
RRM2B	ribonucleotide reductase M2B (TP53 inducible) transcript variant 1		**NM_015713.3**
	AATGATAAAGCTGCAGATGGGCTAA	AATTCTGTGCCATTCATCCAGATTC	

resistant cells were isolated and designated as MSTO/TYMS. The empty vector pEF6 was also transfected into the MSTO-211H cells to generate control cells, which were designated as MSTO/Mock.

2.6. Western Blot Analysis for Protein Expression of Human TYMS

Cell lysates were prepared in CelLytic-M reagent (Sigma-Aldrich, St. Louis, MO, USA) containing 10% Protease Inhibitor Cocktail (Sigma-Aldrich). Protein samples were mixed with a loading buffer [50 mMTris-HCl (pH 6.5), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 40 mM dithiothrei tol] and electrophoresed on a 10% - 20% gradient SDS-poly-acrylamide gel (mini-quick gel, Anatech, Tokyo, Japan) after which the proteins were transferred to a polyvinylidene difluoride membrane filter (Immobilon; Millipore, Bedford, MA, USA). The membrane was blocked in TBS-Tween containing 5% blocking agent (GE Healthcare) for 1 h and then probed with the mouse monoclonal antibody to *TYMS* (ab3145, Abcam, Cambridge, UK) or with rabbit polyclonal antibody to ß-actin (ab8227, Abcam) for 2 h at room temperature. Horseradish peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (GE Healthcare) was used for the detection with enhanced chemiluminescence detection reagent (GE Healthcare). Chemiluminescence was detected by LAS-3000 (Fuji Film, Tokyo, Japan).

3. Results

3.1. Establishment of Pemetrexed-Resistant Cells Originating from Malignant Pleural Mesothelioma

The MSTO-211H cells were initially cultured with 1 nM pemetrexed. Incremental increase in the concentration of

pemetrexed during logarithmic cell growth allowed the determination of maximum growth rate, and eventually, the cells were grown in the culture medium containing 0.1 μM pemetrexed. The dose-response curves for pemetrexed exposed to parental MSTO-211H cells and resistant cells (H/pemetrexed) are presented in **Figure 1**. The IC_{50} value of the H/pemetrexed cells was estimated to be over 100 times that of the parental cells.

3.2. Comparison of the Expression of Related Genes during Folate Metabolism

Expression of several typical genes related to pemetrexed chemosensitivity was measured by real-time PCR, and the *TYMS* mRNA expression (32.9 ± 9.7 times) was found to be significantly increased in H/pemetrexed cells (**Figure 2(a)**). The expression of DHFR, gamma-glutamyl hydrolase (*GGH*), ribonucleotide reductase M1 (*RRM*1), and ribonucleotide reductase M2B (*RRM2B*) genes also increased slightly in the H/pemetrexed cells (3.2 to 4.6-fold, statistically insignificant). On the other hand, the expression of solute carrier family 19 member (*SLC*19*A*) mRNA, which is one of the folate transporters, was significantly reduced compared to the parental cells (0.53 ± 0.05 times). Overexpression of the *TYMS* protein was also confirmed by western blot analysis (**Figure 2(b)**).

3.3. Drug Sensitivity to Assess the Effect of Transfection with siRNA Targeted to the TYMS Gene

Intracellular mRNA expression can be downregulated by specific siRNA. All three *TYMS* siRNAs, which targeted independent sequences within the *TYMS* coding region, effectively induced downregulation of the intracellular *TYMS* mRNA expression by over 80% in H/pemetrexed and MSTO-211H cells (**Figure 3**). Control siRNA, targeted towards the *GAPDH* gene, also specifically downregulated the expression of *GAPDH* mRNA. Each siRNA had an effect exclusively on the expression of its specific target gene in both the cells. The cells treated with siRNA were exposed to varying concentrations of pemetrexed for 48 h after which their chemosensitivity was evaluated. Pemetrexed was applied at two varying concentrations, as each cell line possessed considerably

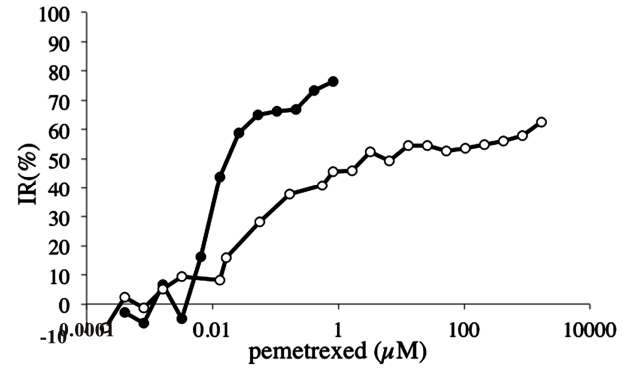


Figure 1. Chemosensitivity in pemetrexed-resistant cells originating from malignant pleural mesothelioma. Dose-response curves for pemetrexed in MSTO-211H cells (closed circles) and in H/pemetrexed cells (open circles). Continuous exposure to variable concentrations of pemetrexed applied for 72 h. Each point was plotted as an average of easily three independent experiments.

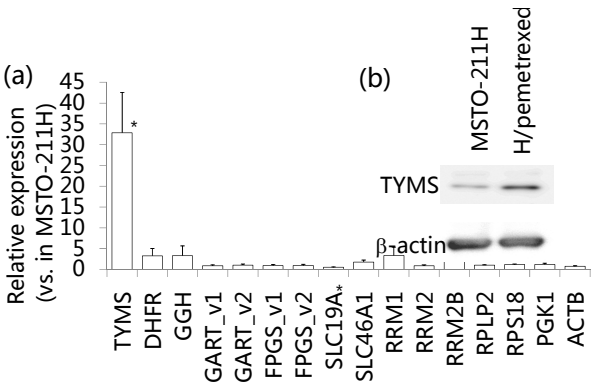


Figure 2. Comparison of anti-folate metabolism genes between parental and resistant cells. (a) mRNA expression was evaluated by real-time RT-PCR using the ΔΔCT method normalized by housekeeping genes. * shows significant difference from parental MSTO-211H cells by the t-test ($p < 0.01$); (b) The level of TYMS protein in MSTO-211H cells and H/pemetrexed cells as detected by the western blot analysis.

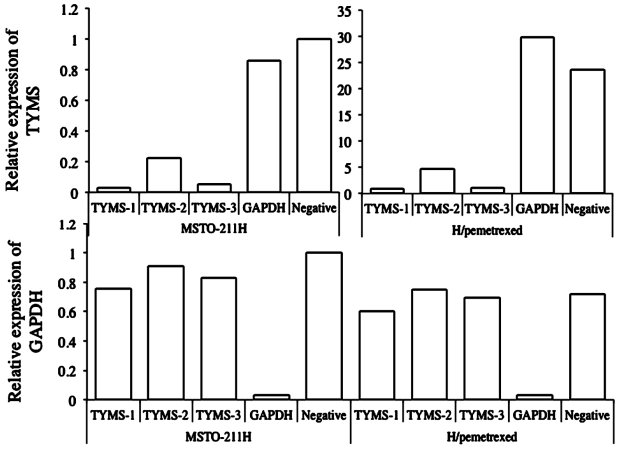


Figure 3. Quantization of mRNA expression in siRNA treated MSTO-211H and H/pemetrexed cells. Three TYMS siRNAs were targeted for different TYMS gene sequences and transfected into MSTO-211H and H/pemetrexed cells. GAPDH and negative siRNA were used as controls. mRNA expression was evaluated by quantitative real-time PCR. Upper and lower graphs represent the expression of TYMS and GAPDH mRNAs, respectively. Each expression fold was calculated from the ratio of MSTO-211H cells treated with negative siRNA.

different chemosensitivity to pemetrexed. Treating the parental cells with two siRNAs targeting *TYMS* (TYMS −2 and −3) enhanced the antitumor activity (27 and 15%) at 0.007 μM pemetrexed (**Figure 4**). Moreover, the effect of *TYMS* siRNA was confirmed even in H/pemetrexed cells that had overexpressed *TYMS* mRNA. In both cell lines, siRNAs targeted at *TYMS* increased the cytotoxicity of pemetrexed.

3.4. Reduction of Drug Sensitivity in Transfected Cells with TYMS Expression Vector

In order to determine whether *TYMS* gene expression was affected by pemetrexed sensitivity, the parental cells were transfected with a *TYMS* expression vector. The *TYMS* expression vector (pEF6/TYMS) was constructed from pEF6/V5-His TOPO vector according to the given protocol, and the constructed vector was transfected into the MSTO-211H cells to establish stable *TYMS* overexpressed cells (MSTO/TYMS). The expression of *TYMS* mRNA in these cells was confirmed by real-time PCR (**Figure 5**) and the expression levels in the MSTO/TYMS cells against parental MSTO-211H cells (21.9 ± 3.9 fold) was similar to that of H/pemetrexed cells. Pemetrexed chemosensitivity in the established MSTO/TYMS cells was investigated (**Figure 6**) and found to be reduced, having IC_{50} values higher than those of the parental or mock cells. Furthermore, a cross-resistance to methotrexate (MTX) was also observed.

3.5. Antitumor Activity of ECyd against Pemetrexed-Resistant Cells

The cytotoxicity of other antimetabolic drugs was inves tigated for overcoming the high pemetrexed-resistance in H/pemetrexed cells (**Table 2**). ECyd showed similar antitumor effect against both parental and resistant cells.

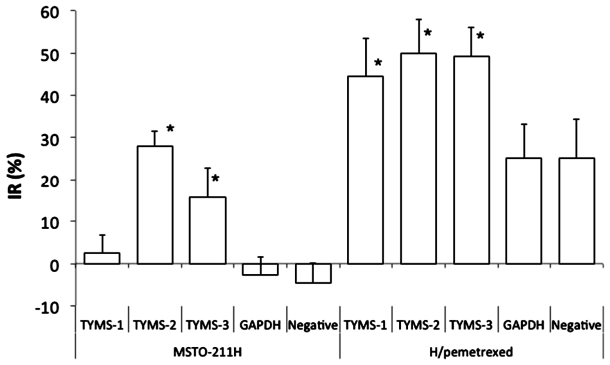


Figure 4. Effect of TYMS siRNA on cytotoxicity of pemetrexed in MSTO-211H and H/pemetrexed cells. MSTO-211H and H/pemetrexed cells were exposed to 0.007 and 840 μM of pemetrexed for 48 h after transfection with specific siRNA. *shows significant difference from negative siRNA treated cells by the t-test ($p < 0.01$).

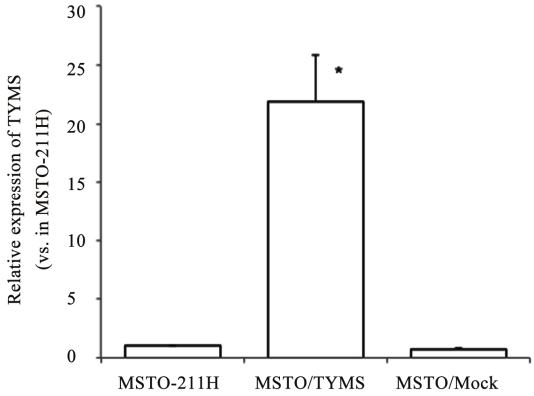
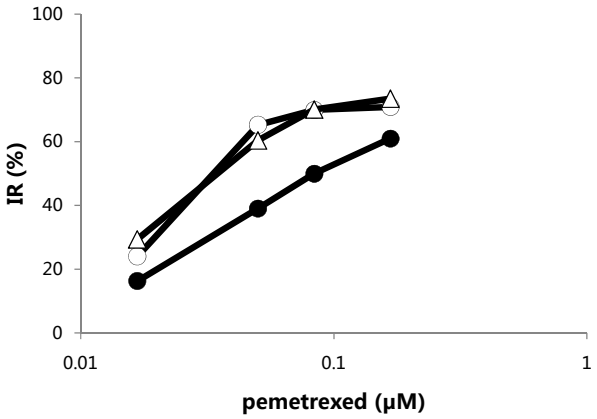
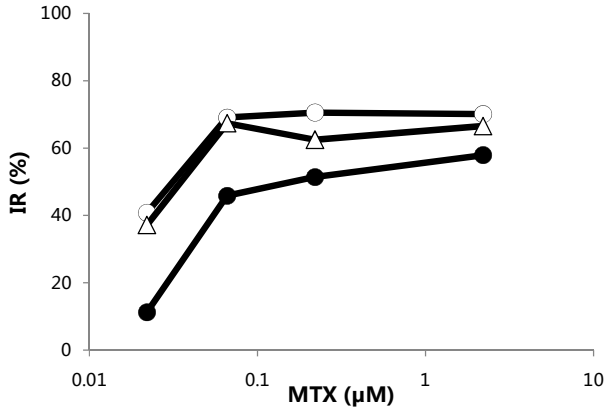


Figure 5. mRNA expression in MSTO-211H cells transfected with TYMS expression vector. MSTO-211H cells transfected with TYMS expression vector were established by a selection agent over a few weeks. The mRNA expression was evaluated by quantitative real-time PCR. Each expression fold was calculated by the ratio against MSTO-211H cells. *shows significant difference from parental MSTO-211H cells by the t-test ($p < 0.01$).



(a)



(b)

Figure 6. Inhibition of antitumor effect by transfection with TYMS expression vector. The chemosensitivity of pemetrexed and MTX is shown in MSTO-211H cells (open circles), MSTO/TYMS cells (closed circles) and MSTO/Mock cells (open triangles).

Table 2. Cytotoxicity profiles of antimetabolic drugs in H/pemetrexed cells.

Drug	IC_{50} (μM)		Resistant index*
	MSTO-211H	H/pemetrexed	
Pemetrexed	0.067	8.70	130
MTX	0.012	0.066	5.67
5FU	7.46	13.7	1.84
ECyd	0.029	0.012	0.43

*Resistance index was calculated as a ratio of IC_{50} for resistant cells to IC_{50} for parental cells.

Since MTX had a similar mechanism as pemetrexed, partial cross-resistance was observed in H/pemetrexed cells. Additionally, 5FU had an equivalent antitumor effect, which was revealed even in H/pemetrexed cells.

4. Discussion

Although several pemetrexed-resistant cells originating from colon, breast, gastric and non-small cell lung cancers and especially leukemia cell lines have already been reported, there is no report of pemetrexed-resistance cells from malignant pleural mesothelioma cell lines. Recently, pemetrexed has been clinically approved against malignant pleural mesothelioma, but continuous and repeated treatment can result in resistance against permetrexed in the future. We attempted to establish pemetrexed-resistant cells originating from some malignant pleural mesothelioma cell lines in order to clarify the mechanism underlying pemetrexed resistance in mesothelioma. Among the cell lines tested, only the MSTO-211H cells could acquire resistance against pemetrexed. Treatment of MSTO-211H cells with pemetrexed induced higher expression of *TYMS* mRNA compared to other mesothelioma cell lines, such as NCI-H2452 and ACC-MESO-1. This characteristic nature of MSTO-211H cells may contribute to the acquisition of high resistance against pemetrexed.

Detailed pemetrexed-resistant mechanisms in H/pemetrexed cells were investigated by analyzing mRNA expression, which were closely related to folate metabolism. Remarkably, *TYMS* mRNA was expressed in H/pemetrexed cells, and the expression of some genes, including *DHFR*, *GGH*, and *RR*, increased slightly. The main reason for pemetrexed-resistance in H/pemetrexed cells was considered to be an up-regulation of the *TYMS* function. However, contribution of the up-regulation of *DHFR*, *GGH*, and *RR* genes in the elevated pemetrexed-resistance could not be completely ruled out. The expression of *SLC*19*A*, a folate-transporter gene, decreased significantly and there appeared to be a weak relationship with pemetrexed resistance. Additionally, pemetrexed may be specifically transported intracellularly via *SLC* 19*A*1 and not *SLC*46*A*1.

Single nucleotide polymorphism (SNP) in the 5'-UTR of the *TYMS* gene is well known, and the tandem repeat is related to the chemosensitivity of 5FU [16-19]. The overexpression of *TYMS* mRNA could not be attributed to SNP in the 5'-UTR, since the genomic polymorphisms (3R type) among the parental and resistant cells were not altered. Moreover, since the *E*2*F* of a transcription factor resided within the promoter region of the *TYMS* gene, the mRNA expression of the *E*2*F* family was investigated. We observed that the expression of *E*2*F* family mRNA was not upregulated in the H/pemetrexed cells. Therefore, even though the activation mechanism of the *TYMS* gene in H/pemetrexed cells has not yet been identified, the *TYMS* gene is considered to be an important factor in the acquisition of pemetrexed-resistance.

The *TYMS* gene was artificially regulated to clarify its involvement in pemetrexed resistance. *TYMS* down-regulation by specific siRNA partially restored the chemosensitivity for pemetrexed in the parental and H/pemetrexed cells. However, the effect of TYMS-1 siRNA on pemetrexed chemosensitivity did not appear unexpectedly. Although TYMS-1 siRNA knocked down *TYMS* mRNA, the chemosensitivity for pemetrexed in only the parental MSTO-211H cells were similar to that treated with control siRNA. Since *TYMS* mRNA expression in MSTO-211H cells were steady at a lower level than that in H/pemetrexed cells, the enhanced cytotoxic effect on being treated with specific siRNA in MSTO- 211H cells are plausible. On the other hand, MSTO/ TYMS cells, which stably over-expressed *TYMS* mRNA, tended to be resistant against pemetrexed and MTX. Even though the expression level of *TYMS* mRNA in MSTO/TYMS cells were over 20 times higher than that in MSTO-211H cells, its resistance were weak relative to H/pemetrexed cells, which similarly over-expressed *TYMS* mRNA. In H/pemetrexed cells, the expression of other genes, including DHFR and GGH, increased slightly but not in MSTO/ TYMS cells. It was considered that the functions of *DHFR* and *GGH* were partially related to acquisition of pemetrexed resistance in the MSTO-211H cells.

High resistance to an antitumor drug is a serious problem in chemotherapy. We have examined the cytotoxicity of some antimetabolic drugs in H/pemetrexed cells (**Table 2**) and found that these cells showed cross-resistance to MTX, which had cellular metabolism and targets similar to pemetrexed. Although 5FU targets the same cellular factors as MTX, the chemosensitivity for 5FU in H/pemetrexed cells were retained, suggesting that the main target of 5FU in this cell line is not *TYMS*. It appeared that the pyrimidine salvage pathway was more prominent than in other cell lines. ECyd, having an antitumor mechanism different from pemetrexed, was an

effective drug even in pemetrexed-resistant cells. However, the mechanism of ECyd involves inhibition of RNA biosynthesis, and ECyd has been considered a superior antitumor nucleoside whose clinical trials are in progress as TAS-106 in USA [20,21]. ECyd and pemetrexed belong to the same category as anti-metabolic drugs. However, their metabolic and activated pathways differ through the pyrimidine and folate metabolic pathways, respectively, bestowing ECyd with excellent antitumor activity against many solid tumors [22-28]. Even in H/pemetrexed cells, ECyd showed the same antitumor effect as in MSTO-211H cells. Therefore, in the clinical treatment of malignant pleural mesothelioma patients, ECyd may be extremely useful as a promising second line drug. The *TYMS* gene may be considered as a useful biomarker for predicting pemetrexed chemosensitivity in malignant pleural mesothelioma patients.

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