TS, DHFR and GARFT Expression in Non-squamous Cell Carcinoma of NSCLC and Malignant Pleural Mesothelioma Patients Treated with Pemetrexed

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Abstract. Background: Recently, pemetrexed (PEM), a new generation antifolate, has been used for the treatment of patients with advanced non-squamous cell carcinoma (SQ) of non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM). However, no useful markers for selecting appropriate candidates exist at present. Materials and Methods: Tumor specimens were collected from 5 lung non-SQ and 8 MPM patients who underwent surgery and received PEM. Real-time PCR and immunohistochemical (IHC) staining of the primary tumor were used to analyze the mRNA and protein expressions of thymidylate synthase (TS)/dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), and to compare the expression status and clinical outcomes. Results: TS, DHFR, and GARFT mRNA levels had a median value of 2.39, 1.70, and 1.40 in non-SQ samples of NSCLC patients. The TS and DHFR protein levels had a mean total score of 2 and 4 in non-SQ of NSCLC patients. TS, DHFR, and GARFT mRNA levels had a median value of 5.55, 3.73, and 3.52 in MPM patients. TS and DHFR protein levels had a mean total expression score of 1 and 3 in MPM patients. No significant correlation was identified between the expression levels of TS/DPD/GARFT mRNA and clinical response for the non-SQ of NSCLC and MPM patients treated with PEM. Conclusion: TS, DHFR, and GARFT mRNA and protein expression may not be useful markers for predicting clinical response in Japanese patients with non-SQ of NSCLC and MPM. Further investigations are necessary in order to develop biomarkers to determine the clinical benefits of PEM treatment.

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Key Words: TS, DHFR, GARFT, NSCLC, malignant pleural mesothelioma, pemetrexed.

Lung cancer is the leading cause of cancer-related deaths in the majority of countries (1). Non-small cell lung carcinomas (NSCLCs) account for approximately 80% of all lung cancers, and the proportion of the adenocarcinoma is increasing (2). Conversely, MPM is a rare and a highly lethal tumor associated in the vast majority of cases with asbestos exposure (3). Furthermore, this fatal disease is largely unresponsive to conventional chemotherapy or radiotherapy, and surgical treatment has not shown a significant survival benefit in comparison to supportive treatments (4).

Recently, PEM has been developed as a new generation antifolate drug. A phase III study showed that PEM gave a significant improvement in response rate and overall patient survival when combined with cisplatin (CDDP), and PEM was more effective than conventional chemotherapy in both thoracic malignant tumors (3). PEM inhibits multiple enzymes in the folate metabolic pathway, and TS, DPD, and GARFT are the main targets (5). In regard to NSCLCs, the median *TS* gene expression is lower in adenocarcinomas than in squamous cell carcinomas (SQ) (6), and clinical trials have consistently reported a superior activity of PEM in patients with non-SQ of NSCLCs (7, 8). Moreover, high baseline *TS* gene expression levels conferred resistance to PEM *in vitro* (9). However, no useful markers that predict clinical response exist at present. Therefore, it is necessary to identify those patients who might benefit the most from PEM chemotherapy to not only precisely select those patients who require intensive treatment, but also to prevent the induction of adverse events in patients who do not require treatment.

Whether TS, DHFR, GARFT or/are useful predictive indicators of clinical response was examined here. The present study is the first to demonstrate the molecular analyses of TS, DHFR and GARFT expression and correlate these parameters with clinical responses in Asian patients with non-SQ of NSCLCs and MPMs.

Materials and Methods

Patients, clinical features, follow-up, and clinical response. The institutional review board approved this study and informed consent for the use of the tumor specimens was obtained from all the patients or from their legal guardians. Tumor samples were obtained from 291 patients with primary NSCLCs and 18 patients with MPMs who had undergone a surgical resection between 2005 and 2007 and 2004 and 2008 in our department, respectively. Eventually, 5 lung non-SQ of NSCLCs and 8 MPM patients were enrolled in this study under the following conditions: i: They received PEM treatment for recurrence after complete resection or advanced cases; ii: the tumor samples were appropriate for the evaluation of TS, DHFR and GARFT status; and 3: there were evaluable lesions for chemotherapy. The routine clinicopathological data included sex, age, histology, complete history and physical examination, thoracoscopic findings, and clinical response for chemotherapy. Patients received either cisplatin (75 mg/m^2) on day 1 plus PEM (500 mg/m^2) on day 1 (n=8) (7, 10), carboplatin (CBDCA) area under the curve (AUC) 5 on day 1 plus PEM (500 mg/m^2) on day 1 (n=1) (11, 12), or PEM as a single agent (500 mg/m^2) on day 1 (n=4) (13) (Tables I and II). Chemotherapy was repeated every 3 weeks for a maximum of six cycles until there was evidence of disease progression. All patients received a dexamethasone prophylaxis and the use of vitamin B12 and folic acid supplementation.

Time to progression (TTP) was defined as the time from the start of chemotherapy until the first evidence of disease progression, established in terms of objective clinical worsening or radiologic evidence. The objective response of the patients was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) (14). Clinical response was determined to be effective and non-effective by the evaluation of PR or SD with shrinking and SD with enlargement or PD, respectively. A follow-up examination was conducted in all patients. Routine clinical and laboratory assessments and chest X-rays were performed biweekly, and CT scans were performed 1 month after treatment and then every 3 months thereafter, and the imaging studies (bone scan and brain imaging) were performed every 3 months after the initiation of PEM treatment.

The characteristics of 5 NSCLC patients are listed in Table I. All of the patients were male. All of the tumors were pathologically confirmed to be adenocarcinoma except one pleomorphic carcinoma. The tumor stage was classified according to the International Union against Cancer tumor-node-metastasis classification of malignant tumors (15). All patients had recurrent disease after complete resection. According to the pathological staging, two patients were at stage at IIA, 1 at IIIA, and 2 at IIIB. Prior chemotherapy had been administered in 4 patients. Three patients and 2 had stable disease (SD) and progressive disease (PD), respectively. The characteristics of 8 MPM patients are also shown in Table II. All of the patients were male except 1 female. The histological types included 5 epithelial, 2 biphasic, and 1 sarcomatoid type. According to the International Mesothelioma Interest Groups' classification (16), two patients were at stage II, 3 at III, and 3 at IV. Four patients had recurrent disease after complete resection and 4 patients were advanced cases. Prior chemotherapy had been administrated in 2 patients. One case showed a partial response (PR). Five and two cases exhibited SD and PD, respectively.

Detection of TS/DHFR/GARFT mRNA. TS, DHFR, and GARFT expression was analyzed in all samples by a quantitative real-time PCR, performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) using a Fast SYBR Green Master Mix (Applied Biosystems, CA, USA). Gene expression was quantified by comparing the levels of the target gene to the levels of beta actin as an internal control. The quantification was based on a standard curve generated from human normal complementary DNA by previously described methods (17). The change in the copy number of the TS, DHFR and GARFT genes relative to actin and the calibrator DNA were determined by the following formula: (tumor-TS or DHFR or GARFT/tumor-beta actin)/(control-TS or DHFR or GARFT/control-beta actin). A PCR reaction was performed in triplicate for each primer set, and the means were reported. The conditions for the quantitative PCR reactions were as follows: one cycle of 95˚C for 20 s, and 40 cycles of 95˚C for 3 s and 60˚C for 30 s. At the end of the PCR reaction, the samples were subjected to a melting analysis to confirm the specificity of the amplicon. The primer sequences used in the present study for the TS gene were as follows: forward, 5'-TCGGTGTGCCTTTCA ACATC-3', and reverse, 5'-GATGTGCGCAATCATGTACGT-3' (59 bp). The primer sequences used in the present study for the DHFR gene were as follows: forward, 5'-TAAACTGCATCGTCGCTG TGT-3', and reverse, 5'-GGGCAGGTCCCCGTTCT-3' (59 bp). The primer sequences used in the present study for the GARFT gene were as follows: forward, 5'-GACAGTACTCGGGAACCAAATAG C-3', and reverse, 5'-ACTGCGGCTTTGTTGGAGAT-3' (65 bp).

Immunohistochemical (IHC) staining in paraffin-embedded tumor samples. IHC staining was conducted using serial sections from the same paraffin-embedded blocks as previously described (18, 19). Briefly, all tissue specimens were formalin-fixed and processed similarly, according to the standard histology practices. Several 3-μm-thick formalin-fixed paraffin-embedded tissue sections were prepared from each specimen. All specimens were stained with hematoxylin-eosin for the histological diagnosis. The sections were briefly immersed in citrate buffer [0.01 mol/l citric acid (pH 6.0)] and were incubated for two 10-minute periods at 121˚C in a high-pressure sterilization oven for antigen retrieval. They were then incubated with anti-TS (TS106, Santa Cruz Biothechnology, CA) diluted at 1:25, or anti-DHFR (ab82171, Abcam, Cambridge, MA) diluted at 1:50, in phosphate-buffered saline for 60 minutes at room temperature. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer's instructions (18, 19). The positive and negative controls were analyzed using HeLa cells and exclusion of the primary antibody, respectively.

Evaluation of the stained specimens. Following the IHC detection of protein expression in each specimen, the percentage of immunoreactive tumor cells in five randomly-selected ×400 fields from one slide was recorded, and then the final value of positive tumor cells was determined as the average of the positive number of immunostained cells. To evaluate any correlations with clinicopathological characteristics, the stained specimens for cytoplasm of cancer cells were then categorized into eight degrees according to a previous report (20). Initially, 6 degrees of the proportional score (PS) for the positive staining cells were assigned according to the frequency of positive tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3). Thereafter, 4 degrees for the intensity score (IS) were assigned according to the intensity of the staining (0, none; 1, weak; 2,

Table I. *Characteristics of the non-SQ patients treated with PEM.*

Case	Age (years)	Sex	Histology	Surgical procedure[b]	Stage	Line	Combined drug	Clinical response
1	78	M	AD	L	IIIA	3rd	-	PD
2	76	M	Pleo	L	IIA	1st	-	SD*
3	64	M	AD	L	IIIB	4th	-	SD*
4	70	M	AD	L	IIIB	2nd	-	PD
5	27	M	AD	L	IIA	3rd	CBDCA	SD*

AD: Adenocarcinoma, Pleo: pleomorphic carcinoma, [b]L: lobectomy with systematic nodal dissection, *shrinkage.

Table II. *Characteristics of the MPM patients treated with PEM.*

Case	Age (years)	Sex	Histology	Surgical procedure[b]	Stage	Line	Combined drug	Clinical response
1	68	M	B	EPP	III	1st	CDDP	SD*
2	69	M	E	biopsy	IV	1st	CDDP	SD**
3	68	F	E	EPP	II	1st	CDDP	SD*
4	68	M	B	EPP	III	1st	CDDP	PD
5	58	M	S	biopsy	IV	1st	CDDP	PD
6	67	M	E	biopsy	III	1st	CDDP	PR
7	57	M	E	EPP	IV	2nd	CDDP	SD*
8	60	M	E	biopsy	II	2nd	CDDP	SD*

E: Epithelial, B: biphasic, S: sarcomatoid type, [b]EPP: extrapleural pneumonectomy, **enlargement.

Table III. *TS, DHFR and GARFT status in non-SQ of NSCLC patients.*

Case	*TS* mRNA	*DHFR* mRNA	*GARFT* mRNA	TS IHC	DHFR IHC
1	2.48	1.30	0.75	0	0
2	2.39	0.85	1.16	4	5
3	6.08	3.63	3.47	4	5
4	1.40	1.70	1.40	0	7
5	2.16	1.94	2.36	2	4

Table IV. *TS, DHFR and GARFT status in MPM patients.*

Case	*TS* mRNA	*DHFR* mRNA	*GARFT* mRNA	TS IHC	DHFR IHC
1	2.97	1.23	1.26	0	5
2	13.20	0.26	3.47	3	5
3	15.20	8.93	5.61	2	1
4	2.41	1.28	1.64	0	4
5	3.87	1.78	3.29	0	0
6	5.39	5.76	3.57	0	3
7	85.10	208.20	139.20	0	2
8	8.75	5.67	6.41	0	0

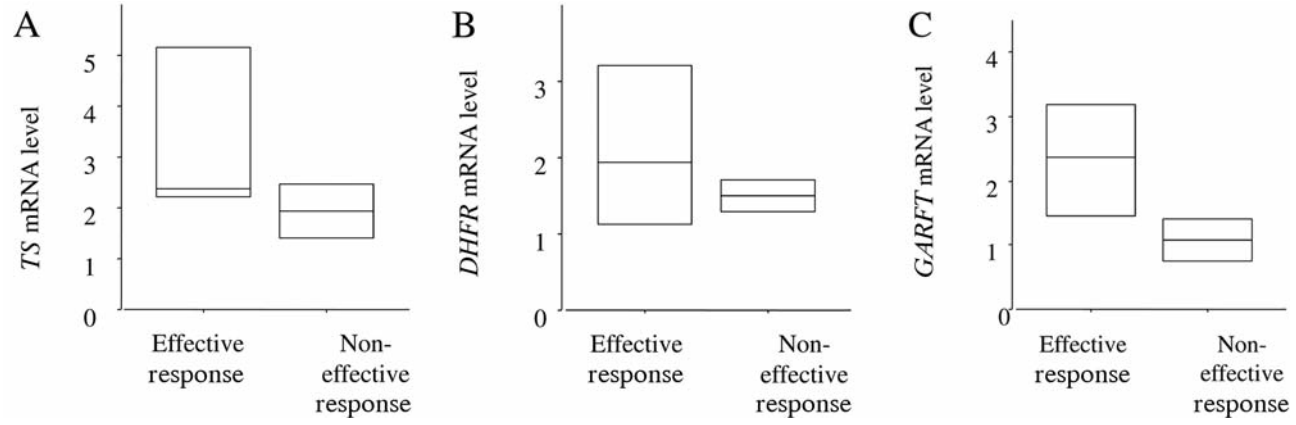

Figure 1. *The mean and standard deviation of the expression levels of TS, DHFR, and GARFT mRNA for non-SQ of NSCLC patients with effective responses.*

intermediate; and 3, strong). The PS and the IS were added to obtain the total score, which ranged from 0-8. The slides were independently examined by two of the investigators (HS and TO) who were blinded to the clinicopathological data. When a discrepancy was found between the two investigators, a consensus was reached via their simultaneous examination using a double-headed microscope.

Statistical analyses. The statistical significance was evaluated using the *t*-test. The Kaplan-Meier method was used to estimate the probability of patient survival, and the survival differences were analyzed by the log-rank test. Differences were considered to be statistically significant for *p*-values of less than 0.05. The data were analyzed using Stat View software (Abacus Concepts, Inc, Berkeley, California, USA).

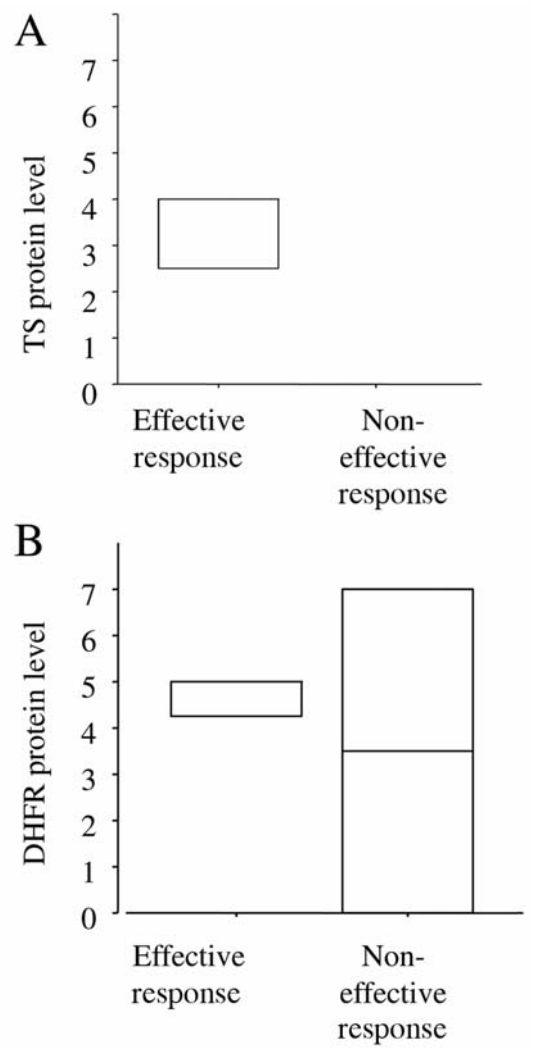

Figure 2. *The mean and standard deviation of expression levels of the TS and DHFR protein for non-SQ of NSCLC patients with effective response.*

Results

Relationship between TS, DHFR and GARFT status and clinical response. The *TS*, *DHFR*, and *GARFT* mRNA levels had a median value of 2.39 (range, 1.40 to 6.08), 1.70, (range, 0.85 to 3.63), and 1.40 (range, 0.75 to 3.7) in lung non-SQ patients. The TS and DHFR protein levels had a mean total score of 2 (range, 0 to 4) and 4 (range, 0 to 7) in non-SQ of NSCLC patients (Table III). The *TS*, *DHFR*, and *GARFT* mRNA levels had a median value of 5.55 (range, 2.41 to 85.1), 3.73, (range, 0.26 to 208.20), and 3.52 (range, 1.26 to 139.20) in MPM patients. TS and DHFR protein levels had a mean total score of 1 (range, 0 to 3) and 3 (range, 0 to 5) in MPM patients (Table IV). No significant correlation was identified between the expression levels of *TS*, *DHFR* and *GARFT* mRNA and the clinical response for the non-SQ of NSCLCs (Figure 1). No significant correlation was identified between the expression levels of TS and DHFR by the total IHC score and the clinical response for the patients with lung non-SQ (Figure 2). The relationship between TS, DHFR and GARFT status and clinical response produced the same results for the patients with MPM (Figure 3 and 4). To examine the patient survival, two groups were divided according to the median mRNA value. There was no significant difference in TTP compared to the group with high or low mRNA levels of *TS*, *DHFR* and *GARFT* mRNA in either thoracic tumor.

Discussion

Molecular-targeted drugs have two-sided clinical features, including beneficial effects and unexpected adverse events. Therefore, novel strategies for the prediction of clinical response are urgently required for individualized therapy. PEM is a promising drug for the treatment of patients with advanced NSCLCs and MPMs (3, 21). A treatment-by-histology interaction for PEM is significant (21). Previous clinical studies have also demonstrated that high TS expression of AD is lower than in SQ samples, which suggests the therapeutic difference in histological samples in patients following PEM treatment (6), but this is not entirely supported by the presented data. Recently, low TS protein levels were reported to be a predictive marker for improved TTP in MPM patients treated with PEM (22). This report was not consistent with the current results. These divergent results might be due to the differences in race and statistical power related to the number of patients examined (23). On the other hand, Smit *et al*. did not find any correlation between high and low TS expression genotype by a pharmacogenetic study of PEM-treated patients with NSCLC (11). Recently, the selection of patients by gene markers has enabled molecular-targeted drug therapy to be proposed, yielding extraordinary results (24). The rationale behind this effect is the oncogenic addiction of the enriched subgroup (25, 26). Targeted molecules depend on protein overexpression or an addicted pathway (25). Therefore, using low expression levels as a predictive biomarker might appear to be adverse logic, in comparison to clinically successful studies to date in various types of cancer (27-29).

The present study had three limitations. Firstly, the number of patients was small due to certain conditions such as sufficient sample materials and reliable assaying. Moreover, PEM was only recently approved by the national health and welfare minister in Japan for the treatment of patients with MPM in 2007 and for the treatment of patients with NSCLC in 2009. The current study also included those

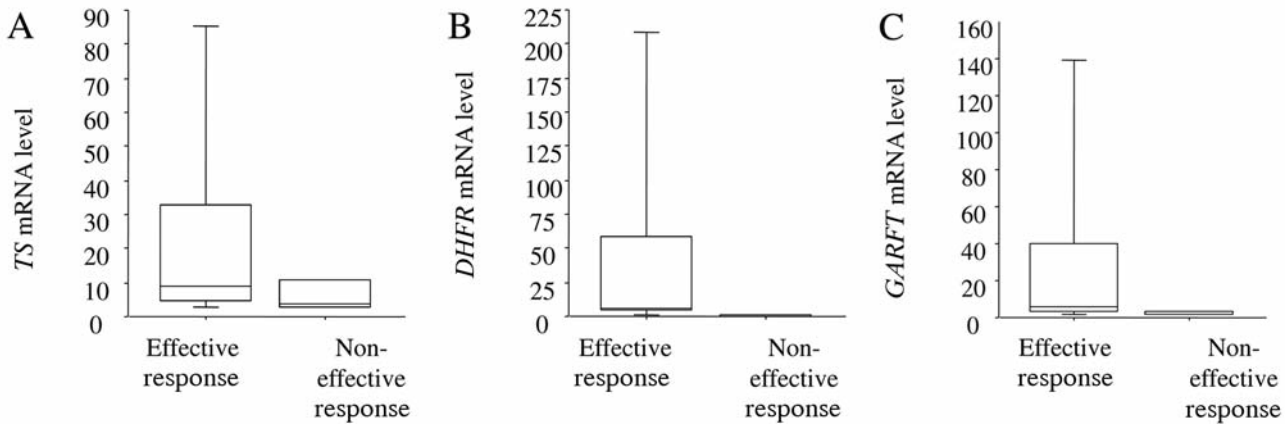

Figure 3. *The mean and standard deviation of the expression levels of TS, DHFR and GARFT mRNA for MPM patients with effective response.*

cases who were treated with PEM as a second-line or later therapy. In general, the response rate of front-line treatment is higher than the rate of second-line therapies (13). Therefore, the clinical response might be altered or improved when PEM is used as a first-line therapy. However, PEM is also an active drug as a second-line therapy (13, 30). We compared the TTP between patients with high and low expression of *TS*, *DHFR* and *GARFT* mRNA to exclude the bias of chemotherapeutic timing as much as possible. We were unable to determine any relationships with statistical significance. Finally, the present study was a retrospective study, and a patient selection bias exists related to the treatment policy at a single institution.

A firm conclusion cannot be drawn from the result of this study because of the small number of patients analyzed an retrospective nature of this study; nonetheless, it is proposed that the current results indicate that *TS*, *DHFR* and *GARFT* status was not a significant predictive factor for patients with non-SQ of NSCLCs and MPMs. Further investigation is necessary to develop biomarkers that determine the clinical benefit of PEM in a larger cohort of patients and in prospective studies.

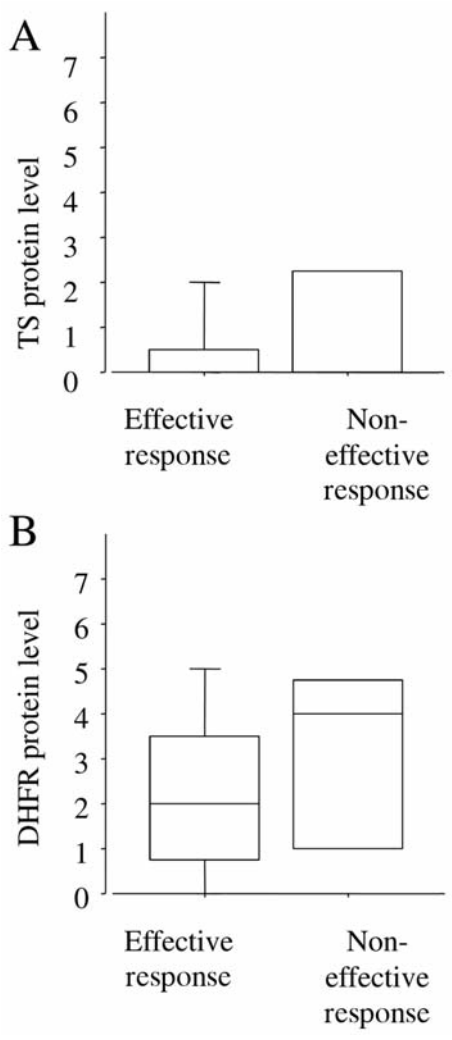

Figure 4. *The mean and standard deviation of the expression levels of TS and DHFR for MPM patients with effective response.*

Acknowledgements

We thank Misako Fukumoto and Kanako Sasaki for their valuable technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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Received May 21, 2010
Revised June 16, 2010
Accepted June 25, 2010