Combined evaluation of dihydropyrimidine dehydrogenase and thymidine phosphorylate mRNA levels in tumor predicts the histopathological effect of 5-fluorouracil-based chemoradiotherapy

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Running title: TP and DPD mRNA levels predict effect of chemoradiotherapy in oral SCC.

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Abstract

Recent clinical studies have indicated that intra-tumoral gene expression levels of 5-fluorouracil (5-FU) metabolism-related enzymes may predict the clinical response of several cancers to 5-FU-based chemotherapy. However, few studies examining oral squamous cell carcinomas (OSCCs) have been reported. In this study, we determined the expression levels of 5-FU metabolism-related enzymes like thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylate (TP) and orotate phosphoribosyl transferase (OPRT) using reverse transcription-polymerase chain reaction (RT-PCR) combined with laser capture microdissection (LCM). We also evaluated the correlation between the mRNA expressions of these genes and clinico-pathological factors or the treatment effects of 5-FU-based chemotherapy combined with radiotherapy in 27 patients with OSCC. No significant correlation was observed between the mRNA expression levels of any of the examined genes and the T-stage, N-stage, differentiation grade or mode of tumor invasion. Although TS and OPRT mRNA were not correlated with the histopathological effects and the development of tumor recurrence, DPD and TP mRNA were significantly correlated with the histopathological effects and tumor recurrence. A significant positive correlation was also observed between the expression of TS and DPD mRNA, but no other correlations were observed among the other genes. Our results suggest that the combined evaluation of TP and DPD mRNA expression in tumor cells using LCM and RT-PCR may be

a useful predictor of the efficacy of 5-FU-based chemotherapy combined with radiotherapy in patients with OSCC.

1. **Introduction**

Squamous cell carcinoma (SCC) of the head and neck accounts for 6% of all cancers worldwide. The disease is potentially curable at an early stage, but most patients present with locally advanced disease. Various strategies to improve outcomes by coordinating chemotherapy with surgery and radiotherapy have been tried. Although the timing of chemotherapy has long been a matter of debate, radiotherapy plus concurrent chemotherapy (chemoradiotherapy) has become the standard of care for patients with unresectable SCC of the head and neck and for organ preservation [1,2].

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for the treatment of head and neck SCCs [3]. 5-FU itself is inactive and must be phosphorylated to exert an antitumor effect. Three different pathways for 5-FU phosphorylation exist. Thymidine phosphorylase (TP) [4,5], orotate phosphoribosyltransferase (OPRT) [6,7] and uridine phosphorylase (UP) [8,9] are the key enzymes in these pathways. 5-FU is phosphorylated to 5-fluoro-deoxyuridine (FdUR) by TP [4,5], which is converted to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) by thymidine kinase. FdUMP exerts its anticancer activity through the formation of a ternary complex with thymidylate synthase (TS) and 5, 10-methylenetetrahydrofolate (MTHF), resulting in the inhibition of TS and the blockade of the DNA synthetic process. 5-FU is also phosphorylated to 5-fluorouridine-monophosphate (FUMP) by OPRT [6,7] and to 5-fluorouridine (FUR), which

is subsequently converted to FUMP by UP [8,9]. FUMP is then phosphorylated to fluorouridine diphosphate, which can be either converted to FdUMP or phosphorylated to the active metabolite fluorouridine triphosphate, which is extensively incorporated into RNA (F-RNA), disrupting normal RNA processing and function. Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme for the catabolism of 5-FU. In summary, 5-FU is detoxified by DPD and activated by TP, OPRT and UP. Therefore, these enzyme may affect the efficacy of 5-FU [10,11].

Previous clinical studies have indicated that the intra-tumoral expression levels of TS, DPD, TP and OPRT genes may predict the clinical response of several cancers to 5-FU-based chemotherapy. The relative expression levels of these genes have also been reported to predict the survival of patients with advanced diseases. Thus, theoretically, a high amount of TS and DPD and a low amount of TP and OPRT in the tumor tissue is expected to be associated with a low probability of response to 5-FU and a poor prognosis. In line with this theory, retrospective clinical studies have shown that expression levels of 5-FU metabolism-related enzymes are associated with a response to 5-FU in several cancers [4-11]. Ichikawa et al. have reported that the response rate to a fluoropyrimidine-based protocol was 75% in colorectal tumors with low levels of DPD and TS mRNA, and the median survival time was longer in patients with these tumors than in patients with tumors with high DPD and TS mRNA levels [12]. A few studies examining this phenomenon in head and neck cancers have been reported

[5,13-16]. These studies have shown that a low level of DPD mRNA expression is associated with a high probability of response to 5-FU, but in each study some differences in the correlation between other enzymes and the response to 5-FU or the prognosis existed.

The expressions of 5-FU metabolism-related enzymes have been analyzed using immunohistochemistry and enzyme-linked immunosorbent assays (ELISAs) for proteins or quantitative reverse transcription-polymerase chain reaction (RT-PCR) for mRNAs in solid tumors. Recently, a laser-captured microdissection (LCM) technique combined with RT-PCR was developed and combined with RNA extraction to examine the mRNA levels of 5-FU-related metabolic enzymes [17,18]. This technique enables the separate analysis of gene expression in cancer cells and cancerous stroma. However, few analyses of these enzymes using LCM with RT-PCR have been performed, especially in oral SCCs (OSCCs). In this study, we evaluated the mRNA expressions of TS, DPD, TP and OPRT using TaqMan PCR after using LCM to extract RNA from tumor cells in paraffin-embedded specimens; the relations between the expression levels and clinicopathological factors and histopathological effects of chemoradiotherapy in OSCCs were then examined.

2. Material and methods

2.1. Patients

Twenty-seven patients with OSCC were enrolled in this study. This study was

approved by the Institutional Ethical Committee of Kochi Medical School, Kochi University, and written informed consent was obtained from all patients. Eligible criteria included the followings; (i) primary OSCC in the tongue or floor of the mouth; (ii) patients in whom the performance status was evaluated as 0–2 according to the classification described by the Eastern Cooperative Oncology Group; (iii) bone marrow function was maintained (leukocyte count: 3000/mm^2 or more, platelet count: 100 000/mm^2 or more); (iv) patients without liver, kidney, heart or lung dysfunction; (v) patients without active double cancer at the start of treatment who had not undergone radiotherapy in the head and neck region. Eligible patients were between the ages of 39 and 90 years (mean, 62.9 years) (Table 1). The primary site of the tumor was the tongue in 16 patients and the floor of the mouth in 11 patients. TNM staging categories were determined according to the criteria established by the American Joint Committee on Cancer and the International Union against Cancer (UICC) [19]. The patients were concurrently treated with 5 mg of peplomycin (PEP) 3 times/week intramuscularly, 250 mg of 5-FU 3 times/week intravenously and 2 Gy of x-rays 5 times/week in the Kochi Medical School Hospital. This combined treatment was given to all patients for 3 to 4 weeks depending on the grade of stomatitis. Mean doses were 3254 ± 1659 mg, 39.1 ± 10.8 mg and 39.7 ± 12.6 Gy in 5-FU, PEP and x-rays, respectively.

After the treatment course, 14 patients exhibited clinically complete response although 6 patients developed local recurrence. Notably, 3 of 13 patients who underwent

surgery exhibited pathologically complete response, and 5 of 10 patients who exhibited pathologically non-lethal effect in the resected specimens had local recurrence.

2.2. Histopathologic evaluation of biopsied specimens

The biopsied specimens before the treatment and surgically resected materials after the concurrent chemoradiotherapy were fixed in 4% buffered formalin and embedded in paraffin. Thin sections (4 to 5 μm thick) were prepared using the paraffin embedded samples, and the sections were stained with hematoxylin-eosin. A histopathologic examination was carried out by evaluating the mode of tumor cell invasion according to Willen's classification [i.e., well-defined border (grade 1), cords and less marked border (grade 2), groups of cells and no distinct border (grade 3), and diffuse invasion (grade 4)] [20] and the grade of differentiation according to the criteria of the World Health Organization [21].

2.3. Grade of histological degeneration

The degeneration of the tumor cells was microscopically evaluated on semi-serial sections of the surgically extirpated materials. Degeneration was graded according to the previously reported Ohboshi-Shimosato's classification [22]. Briefly, the 4 grades were as follows: Grade I, the tumor cells showed no degenerative changes; grade IIA, less than 70% of the tumor cells had died; grade IIB, > 70% of the tumor cells showed lethal degenerative damage; grade III, only markedly degenerated non-surviving cells were visible; and grade IV,

only tumor cell fragments remained. When a tumor of grade III or IV recurred, the degree of degeneration was corrected to grade IIB.

2.4. LCM and real-time RT-PCR

Ten-micrometer thick sections obtained from identified areas of formalin-fixed paraffin-embedded biopsy specimens with the highest tumor cell concentration were mounted on uncoated glass slides. Before microdissection, the sections were stained with nuclear fast red (American MasterTech Scientific, Lodi, CA). The sections of interest were selectively isolated using laser-captured microdissection (PALM Microsystem; Leica, Wetzler, Germany) according to standard procedures. The dissected particles of tissues were transferred to a reaction tube containing 400 μL of RNA lysis buffer.

The samples were homogenized and heated at 92°C for 30 min. Fifty microliters of 2 M sodium acetate, pH 4.0, was added followed by 600 μL of freshly prepared phenol/chloroform/isoamylalcohol (250:50:1). The tubes were placed on ice for 15 min and then centrifuged at 13000 rpm for 8 min in a chilled centrifuge. The upper aqueous phase was then carefully removed. Glycogen (10 μL) and 300 μL of isopropanol were added. The tubes were chilled at –20°C for 30 min to precipitate the RNA. The samples were washed in 500 μL of 75% ethanol and air-dried for 15 min. The pellet was resuspended in 50 μL of 5 mM Tris buffer. Finally, cDNA was prepared as described by Lord and colleagues [23].

Quantification of the four genes of interest and an internal reference gene (β-actin)

was performed using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System TaqMan®; Perkin-Elmer Applied Biosystems, Foster City, CA) using the standard curve method. The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dNTP, 3.5 mM of $MgCl_2$ and 1 × TaqMan buffer A containing a reference dye. The final volume of the reaction mixture was 20 μL. The cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 46 cycles of 95°C for 15 sec and 60°C for 1 min. The gene expression values (relative mRNA levels) were expressed as ratios (differences between C_t values) between the gene of interest and an internal reference gene (β-actin). Real-time RT-PCR was performed in a single run.

2.5. Statistical analysis

In Table 2, results are expressed as the mean ± SEM. To evaluate correlations between the expression of these genes and the response to chemotherapy, the Student *t*-test was applied. A Spearman correlation analysis was used to evaluate correlations among the expressions of the four genes. P value of less than 0.05 was considered statistically significant. Cut-off values were determined through analysis of the receiver operating characteristic curve.

3. Results

3.1. Levels of TS, DPD, TP and OPRT mRNA are not correlated with T stage, N stage, differentiation grade or mode of invasion

The expression levels of TS, DPD, TP and OPRT mRNA in each group classified according to T stage, N stage, differentiation grade and mode of invasion are summarized in Table 2. No correlations between the mRNA expression levels of 5-FU metabolism-related enzyme genes and clinico-pathologic factors like the T and N stage classifications, the differentiation grade, and the mode of invasion were observed.

3.2. Histopathological effect of chemoradiotherapy is correlated with DPD and TP mRNA levels, but not with TS and OPRT mRNA levels

First, we assessed the relation between histopathological effects and tumor sites and doses of chemotherapeutic drugs and radiation. However, histopathological effects were not correlated with tumor sites and doses of chemotherapeutic drugs and radiation (data not shown). Next, we evaluated the relation between histopathological effects and mRNA levels of 5-FU metabolism-related enzymes. The mean level of DPD mRNA in OSCCs with lethal histopathological effects (≥ grade III) was significantly lower than that in OSCCs with nonlethal histopathological effects (≤ grade IIB) (Fig. 1, p=0.0053, cut-off value=1.3). In contrast, the mean level of TP mRNA in OSCCs with lethal histopathological effects was significantly higher than that in OSCCs with nonlethal histopathological effects (p=0.0218, cut-off value=4.3). No correlations between the histopathological effects and the levels of TS

or OPRT mRNA were observed. A lethal histopathological effect was obtained in all cases with a DPD mRNA level of less than 1.8 and a TP mRNA level of more than 3.0.

3.3. Local recurrence is correlated with mRNA levels of DPD and TP, but not with mRNA levels of TS and OPRT

As shown in Fig. 2, significant correlations were observed between local recurrence and the levels of DPD (p=0.0488, cut-off value=2.0) and TP (p=0.0465, cut off value=3.7). The mean level of DPD mRNA in cases with local recurrence was significantly higher than that in cases without local recurrence. In contrast, the mean level of TS mRNA in cases with local recurrence was significantly lower than that in cases without local recurrence. No differences in the mean levels of TS and OPRT mRNA were noted between cases with and those without local recurrence. Almost all the cases without local recurrence had low levels of DPD mRNA and high levels of TP mRNA.

3.4. Level of TS mRNA is correlated with level of DPD mRNA but not with levels of TP and OPRT mRNA

Using a Spearman correlation analysis, a significant correlation was observed between the TS and DPD mRNA levels (r=0.627, p=0.0005) but not with the TP and OPRT mRNA levels (Fig. 3). No correlations among the mRNA levels of DPD, TP and OPRT were observed.

4. Discussion

5-FU metabolism-related enzymes like TS, DPD, TP and OPRTP are reported to be predictive markers for 5-FU sensitivity in several cancers [4-11,13-16]. In some cancers, including oral cancers, however, it is still controversial whether these enzymes predict the clinical response to 5-FU-based chemotherapy. One possible reason for the difficulty in finding predicting factors is the histological variety in biopsied specimens, which sometimes contain large amounts of cancerous stroma. The activity of 5-FU metabolism-related enzymes in cancer cells and cancerous stroma is considered to be different, and TS and TP mRNA expressions have been reported to be higher in cancer cells than in cancerous stroma, although DPD gene expression was lower in cancer cells than in cancerous stroma [24]. Almost all previous reports concerning these enzymes have discussed results that were obtained using ELISA, immunohistochemistry and RT-PCR without LCM. The surrounding normal tissues and cancerous stroma can become contaminated in methods such as ELISA and RT-PCR without LCM. In immunohistochemistry, furthermore, the preservation method used to prepare the tissue samples and the sensitivity of the antibody being used affect the results. Recently, LCM has been developed as a sophisticated method to quantify the mRNA levels of these enzymes in cancer cells [17,18,24], which are selectively harvested from target cells in paraffin-embedded specimens. In this study, we quantified the mRNA expression levels of TS, DPD, TP and OPRT in paraffin-embedded specimens of OSCCs using a method consisting of

LCM and RT-PCR and then evaluated the correlations between the mRNA expressions of these enzymes and clinicopathological factors or treatment effects.

The relationships between the mRNA expression levels of the above-mentioned enzymes and clinicopathological factors, such as the T-stage, the N-stage, lymph node metastasis, the differentiation degree and the invasion mode of the tumor, have been previously reported [25,26]. In gastric cancers, TS gene expression was reportedly higher in differentiated cases than in undifferentiated cases, although DPD gene expression in the undifferentiated cases was higher than that in the differentiated-type cases [27]. Because the labeling index of [^{3}H]-thymine in differentiated gastric cancer cells is higher than that in undifferentiated cancer cells [28], a synthesis enzyme (e.g., TS) is thought to be facilitated, whereas a degradation enzyme (e.g., DPD) is inhibited in differentiated-type cases. However, DPD expression in well-differentiated OSCCs is reportedly significantly higher than that in moderate and poorly differentiated OSCCs, and no correlation exists between the Ki-67-labeling index and DPD expression [29]. In this study, the expression of the enzymes was not correlated with T-stage, N-stage, the differentiated grade or the mode of invasion. Because the T-stage, N-stage and mode of invasion closely depend on the interaction between tumor cells and mesenchymal cells, the obtained results seem reasonable. Further studies are needed to determine whether cell proliferation is related to the mRNA expressions of these enzymes.

In OSCCs, TS expression, but not TS, DPD or OPRT expression, was shown to predict the response to S-1 [30]. Kobayashi et al. reported that DPD expression may provide a clue to predicting the sensitivity to 5-FU in patients with OSCC [29]. In the present study, the DPD mRNA levels in patients with histopathological effects of grade III or IV were significantly lower than those in patients with histopathological effects less than grade IIB. On the other hand, the TP mRNA levels in patients with histopathological effects more than grade III were significantly higher than those in the patients with histopathological effects less than grade IIB. Further, a lethal histopathological effect was obtained in all of the cases with a DPD mRNA level of less than 1.8 and a TP mRNA level of more than 3.0. Furthermore, DPD mRNA expression in the recurrence group was significantly higher than that in the no recurrence group, whereas TP mRNA expression in the recurrence group was significantly lower than that in the no recurrence group. However, correlations between the histopathological effects and the TS or OPRT mRNA levels were not detected. These results suggest that the combined evaluation of the DPD and TP mRNA levels in tumor cells may predict the clinical effect of 5-FU-based chemotherapy combined with radiotherapy in OSCCs.

Our neoadjuvant therapy consists of radiotherapy and chemotherapy with 5-FU and PEP. Kocakova et al. reported that responders to preoperative radiotherapy and concomitant capecitabine treatment exhibited a significantly higher expression of TP mRNA than

non-responders among patients with rectal carcinoma [31]. Liersch et al. also showed that all of their patients who developed cancer recurrence after 5-FU-based chemoradiotherapy had a significantly higher TS gene expression level than those patients without recurrences among patients with rectal cancer [32]. Thus, the activity of 5-FU metabolism-related enzymes seems to affect the efficacy of 5-FU-based chemoradiotherapy.

In the present study, a significant positive correlation was observed between TS and DPD mRNA expressions, but not among other genes. A correlation between TP and DPD mRNA expression, as well as between OPRT and TS mRNA expression, was reportedly observed in gastric cancer [33], while a correlation between TP and TS mRNA expression was reportedly observed in oropharyngeal cancer [5]. Although these results were also obtained using LCM combined with RT-PCR, our results differed substantially from the results of these previous reports. TS is an enzyme for the de novo synthesis of deoxythymidine monophosphate, and DPD is the rate-limiting enzyme in the degradation of pyrimidine bases as well as being the key enzyme in the degradation of 5-FU. Our results suggest that cancer cells with high TS activity also possess high DPD activity to maintain the homeostasis of nucleotide metabolism. The precise mechanism responsible for the correlation between TS and DPD is presently unknown, and further studies on this topic are needed.

The addition of chemotherapy to radiotherapy has recently become the standard of care for organ preservation. To improve the clinical benefit of combined therapies at an

acceptable level of toxicity, useful predictors for the clinical response of the combined therapy must be identified. Our results suggest that the combined evaluation of TP and DPD mRNA expression levels in tumor cells using LCM and RT-PCR may be a useful predictor of the efficacy of 5-FU-based chemotherapy combined with radiotherapy in patients with OSCC.

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Table 1. Relation of expression levels of TS, DPD, TP, and OPRT with each stage of carcinomas, differentiation grade and mode of invasion.

Site	No. of cases	T stage				N stage	
		T1	T2	T3	T4	N0	≥N1
Tongue	16	0	9	5	2	12	4
Floor of the mouth	11	1	5	1	4	6	5
Total	27	1	14	6	6	18	9

Age: 62.9 ± 13.8 (39 – 90), Radiation: 39.7 ± 12.6 Gy, 5-FU: 3254 ± 1659mg, PEP: 39.1 ± 10.8mg

Table 2. mRNA levels of four enzymes stratified according to the T-stage, N-stage, differentiation grade and mode of invasion.

		TS	DPD	TP	OPRT
T stage	T1 (n=1)	0.79	2.08	4.96	0.84
	T2 (n=14)	2.56 ± 2.00	2.22 ± 2.38	4.01 ± 2.23	1.46 ± 0.74
	T3 (n=6)	2.67 ± 2.18	1.36 ± 0.66	4.79 ± 2.45	2.70 ± 1.78
	T4 (n=6)	2.08 ± 1.21	1.89 ± 0.94	3.54 ± 2.04	2.44 ± 1.83
N stage	N0 (n=18)	1.99 ± 1.32	1.68 ± 0.94	4.57 ± 1.92	1.82 ± 1.50
	≥N1 (n=9)	3.24 ± 2.46	2.49 ± 2.84	3.20 ± 2.42	2.16 ± 1.15
Differentiation grade	Low (n=1)	7.61	9.87	1.61	1.19
	Moderate (n=9)	2.35 ± 1.62	1.70 ± 1.00	3.42 ± 2.60	2.28 ± 1.64
	High (n=17)	2.13 ± 1.54	1.62 ± 0.81	4.63 ± 1.80	1.79 ± 1.27
Mode of invasion	Grade 2 (n=1)	0.79	2.08	4.96	0.84
	Grade 3 (n=13)	2.50 ± 1.42	1.89 ± 1.03	4.93 ± 2.12	2.10 ± 1.21
	Grade 4 (n=13)	2.45 ± 2.24	2.01 ± 2.43	3.24 ± 2.00	1.85 ± 1.58

Figure legends

Figure 1. The mRNA levels of DPD and TP but not TS and OPRT mRNA levels are significantly associated with histopathological effects. The mRNA levels of the four enzymes are stratified according to the histopathological effects. The mRNA expression levels of TP and DPD are plotted according to the histopathological effects.

Figure 2. The mRNA levels of DPD and TP but not TS and OPRT mRNA levels are significantly associated with local recurrence. The mRNA levels of the four enzymes are stratified according to the local recurrence. The mRNA expression levels of TP and DPD are plotted according to local recurrence.

Figure 3. The level of TS mRNA is significantly correlated with the level of DPD mRNA but not with levels of TP and OPRT mRNA. Diagram of the mRNA expression levels of TS, DPD, TP and OPRT is scattered.

Figure 1

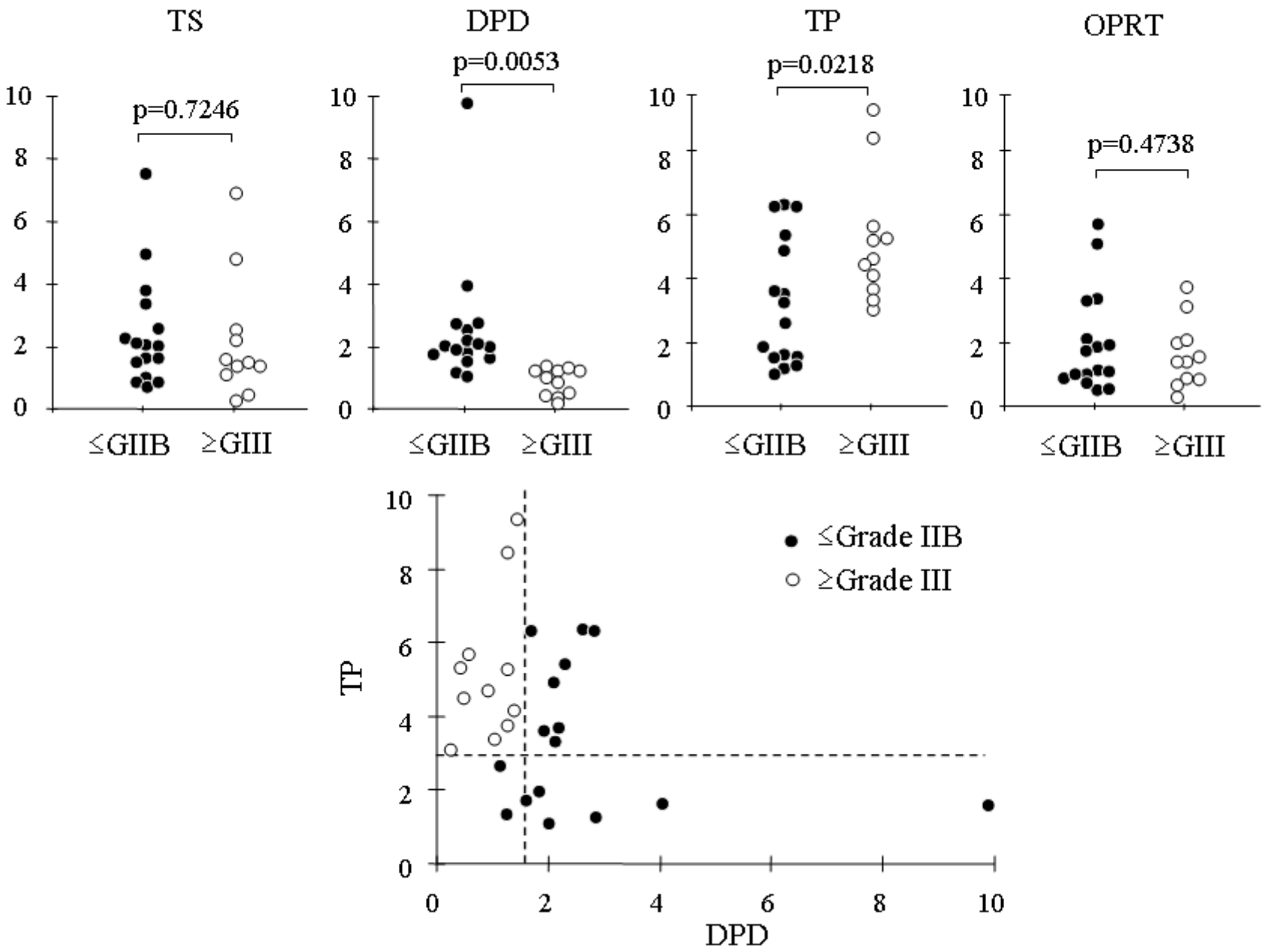

TS
p=0.7246
DPD
p=0.0053
TP
p=0.0218
OPRT
p=0.4738
10
8
6
4
2
0
≤GIIB
≥GIII
TP
DPD
≤Grade IIB
≥Grade III

Figure 2

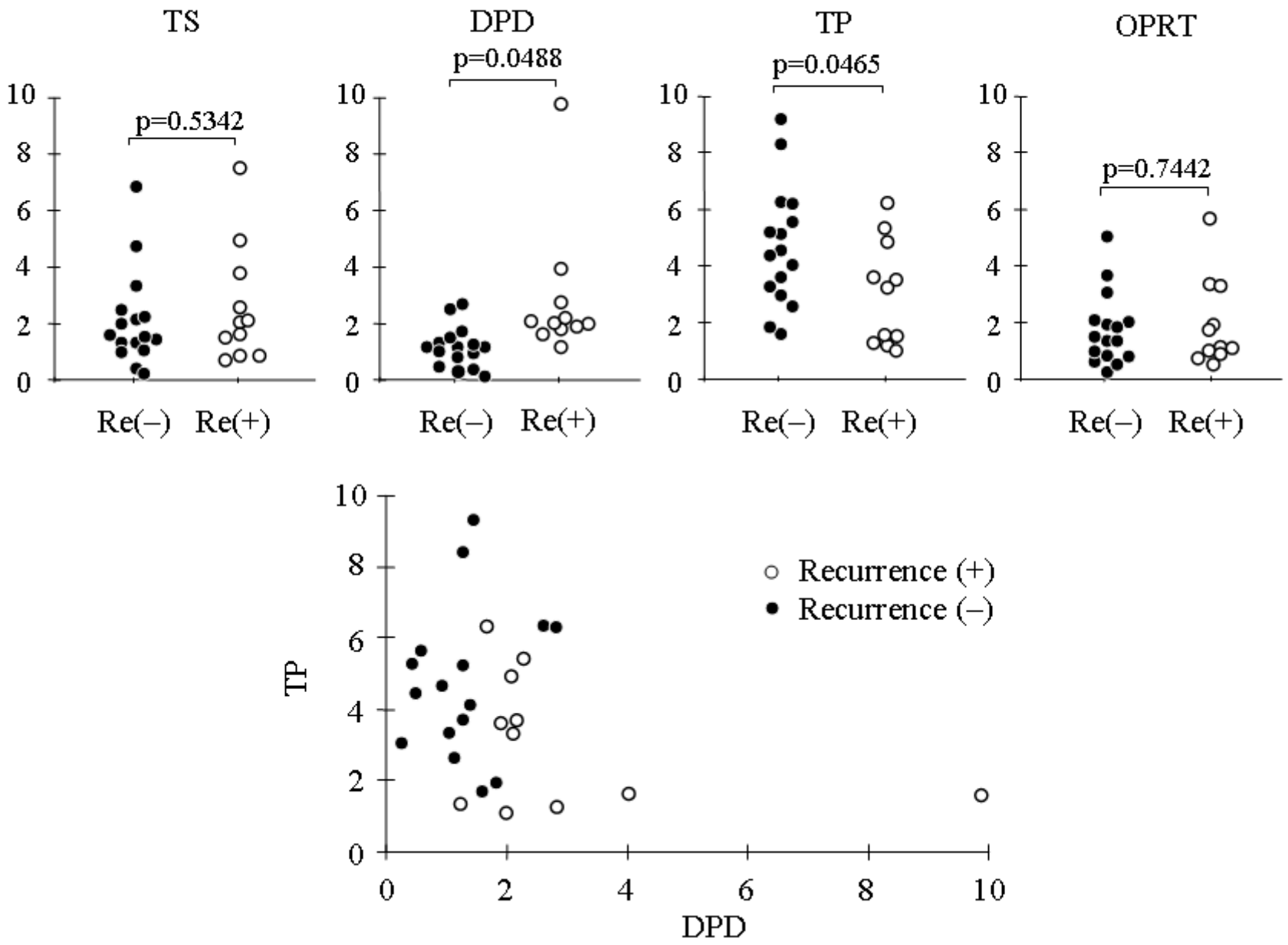
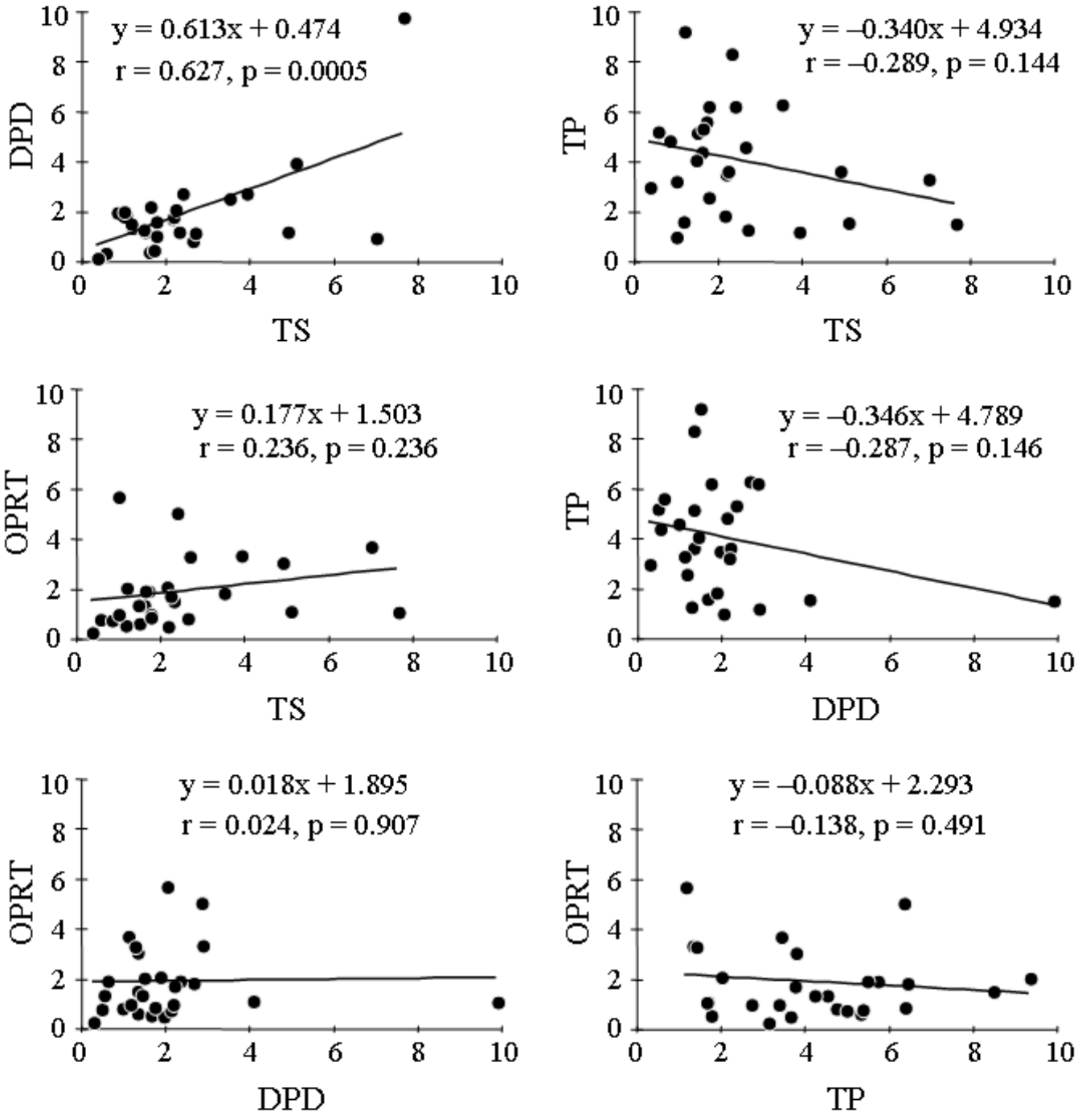

TS
p=0.5342
DPD
p=0.0488
TP
p=0.0465
OPRT
p=0.7442
10
8
6
4
2
0
Re(–)
Re(+)
TP
DPD
Recurrence (+)
Recurrence (–)

Figure 3


y = 0.613x + 0.474
r = 0.627, p = 0.0005
DPD
TS
y = –0.340x + 4.934
r = –0.289, p = 0.144
TP
TS
y = 0.177x + 1.503
r = 0.236, p = 0.236
OPRT
TS
y = –0.346x + 4.789
r = –0.287, p = 0.146
TP
DPD
y = 0.018x + 1.895
r = 0.024, p = 0.907
OPRT
DPD
y = –0.088x + 2.293
r = –0.138, p = 0.491
OPRT
TP