CHAPTER IV
RESULTS

4.1 Cancer tissues

A total of 35 cancer tissues were obtained from cholangiocarcinoma patients who underwent surgical resection at the Department of Surgery, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. The demographic data of these specimens were shown in Table 2. The histological of CCA tissues and staging of patients were classified according to World Health Organization (WHO) classification.

Table 2 Demographic data of patients and cancer tissues

Characteristics	**Mumber**
Patient's gender	
- Male	26
- Female	9
Patient's age (yr)	
- Range	42-70
- Median	59
Histological type	
Well differentiate tubular adenocarcinoma	12
Moderately differentiated tubular adenocarcinoma	3
Mixed well and poorly differentiated adenocarcinoma	1
Invasive papillary adenocarcinoma	14
Adenocarcinoma squamousmetaplasia	1
Papillo-tubular adenocarcinoma	2
Colloid type	1
Intraductal papillary mucinous neoplasm	1

4.2 Response of CCA tissues to 5-FU determined by HDRA

Although 31 CCA tissues were used in order to determine the response of CCA tissue to 5-FU by HDRA, results were obtained from 28 specimens. Number of cancer cells obtained from 3 specimens after 4 day-incubation in culture medium were very low therefore these specimens were excluded from the study.

Figure 9A and 9B shown H&E-stained sections of CCA tissue before culture (day 0) and 4 day-incubation in the culture medium without 5-FU. Tumor cells with hyperchromatic nuclei/cytoplasm were considered as living cells (Figure 9A and 9B, arrow). In the presence of 5-FU at concentration of 100, 200, 400, 1,000 μg/ml. Some tumor cells with eosinophillic cytoplasm with shrinkage (condensed) nuclei (pyknotic nuclei), nuclei fragmentation (karyorrhexis) and/or disappearing nuclei (karyolysis) were considered as dead tumor cells (Figure 9C to 9E, arrow). The numbers of dead cell were increased depend on the concentration of 5-FU. At 5-FU concentration of 1,000 μg/ml, almost all of tumor cells were shrinkage and showed nuclear fragmentation.

The number of total tumor cells and living tumor cells were estimated in duplicate samples under microscope. The mean of percentage total living tumor cells of control at day 0 and day 4 of culturing were calculated. The mean of cell viability at day 4 was 83.3 $\pm$ 14.2 % of those observed at day 0 (Figure 10).

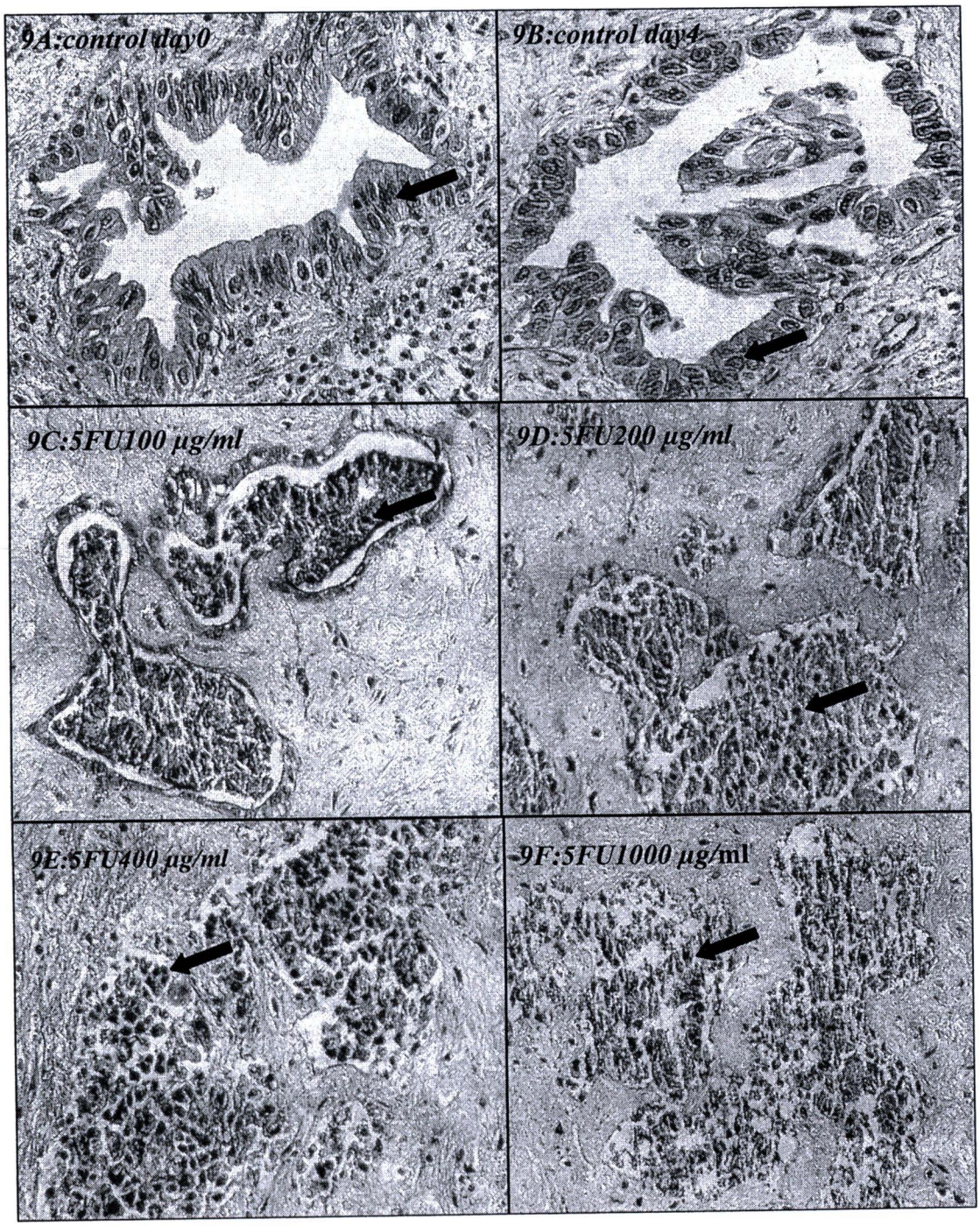

Figure 9 Representative H&E-stained sections of CCA tissues, original magnification is ×400 unless otherwise state. 9A and 9B were CCA tissues at day 0 before culture and at day4 cultured in the absence of 5-FU, respectively. 9C, 9D, 9E and 9F were CCA tissues at day 4 cultured in the medium containing 5-FU at 100, 200, 400 and 1000 μg/ml, respectively

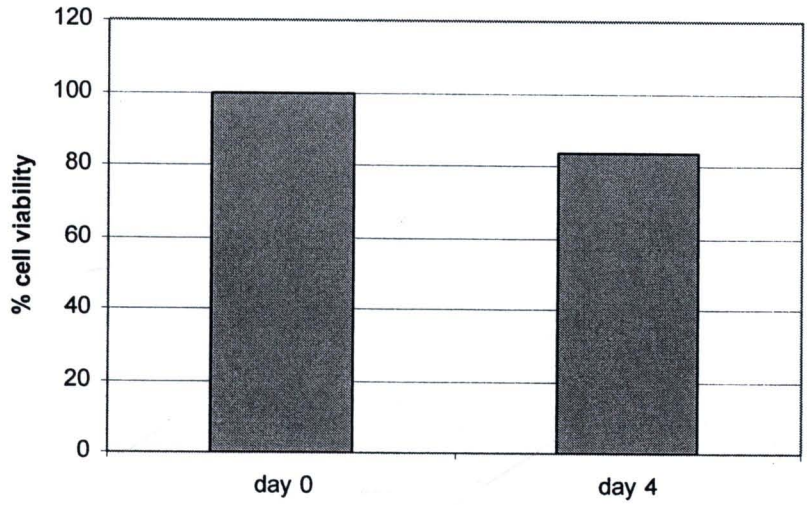

Figure 10 Distribution levels of % cell viability on day 0 and day 4 after culture

4.3 Evaluation of the 5-FU concentration as a cut-off point to differentiate the response of CCA tissues

The number of total tumor cells and living tumor cells in 5-FU treated tumor were observed under microscope and the percentage of inhibition index (%I.I.) was then calculated. Figure 11 showed relationship between the mean %I.I. value and concentration of 5-FU. In this study, we selected the concentration of 200 μg/ml and %I.I of 50% as an appropriate concentration for evaluating 5-FU sensitivity in CCA among the tested dosages. Poorly response CCA tissues were defined as the CCA tissues which exhibited %I.I. value at 200 μg/ml of 5-FU $< 50\%$ while well response CCA tissues were defined as the CCA tissues which exhibited %I.I. value at 200 μg/ml of 5-FU $\geq 50\%$.

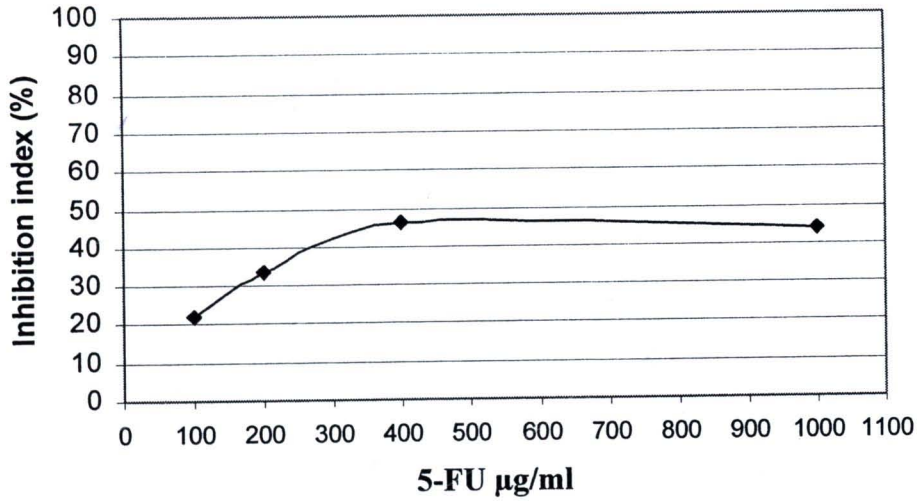

Figure 11 Relationship between the mean inhibition index values obtained from HDRA after 4 day incubation in the culture medium containing vary concentration of 5-FU

4.4 Expressions of target genes in cholangiocarcinoma tissues

Quantitative expression of genes encode for enzymes involved in 5-FU metabolic pathway included TS, DPD, TP and OPRT mRNA were successfully measured in all of 35 specimens by real time PCR using specific Taqman probes.

The expression levels of DPD and TP mRNA among individual samples were markedly different (169-fold; range 0.04-6.76 and 100-fold; range 0.03-3.01, respectively). Whereas, only moderately different in the expression levels of TS and OPRT mRNA were observed (31-fold; range 0.04-1.22 and 34-fold; range 0.03-1.02, respectively) (Figure 12).

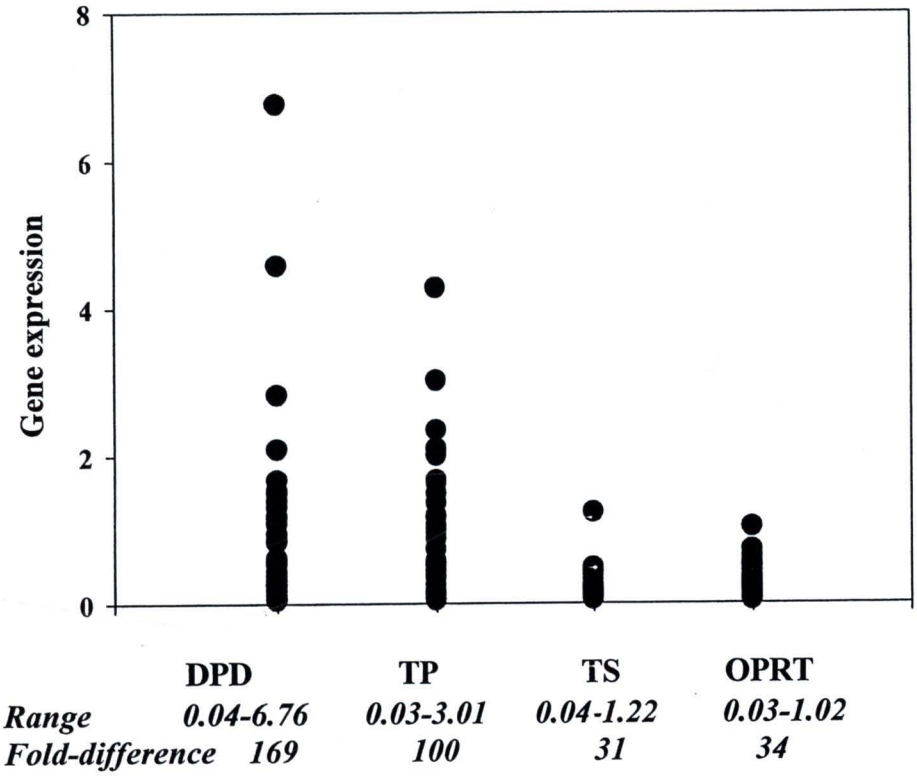

Figure 12 Expression levels of target mRNA among 35 CCA tissues

4.5 Relationships in the expression level of target mRNAs

In the 35 specimens for which OPRT, TS, TP and DPD mRNA expression findings were available. OPRT and TS are the key enzymes for DNA synthesis in the *de novo* pathway (Danenberg, 1977; Navalgund *et al.*, 1980), we therefore examined the relationship of these mRNAs in CCA tissues. As shown in Figure 13, there was a positive correlation between the levels of OPRT and TS mRNA expression in CCA ($R = 0.602, p = 0.0007$).

Similarly, a positive correlation between the levels of OPRT and DPD mRNA expression in CCA was observed ($R = 0.621, p = 0.0004$) (Figure 14).

Moreover, there was a positive correlation between the levels of TP and DPD mRNA expression in CCA ($R = 0.596, p = 0.0008$) (Figure 15).

Apart from the above results, no correlation was observed between any other pairs of the four 5-FU-related enzymes.

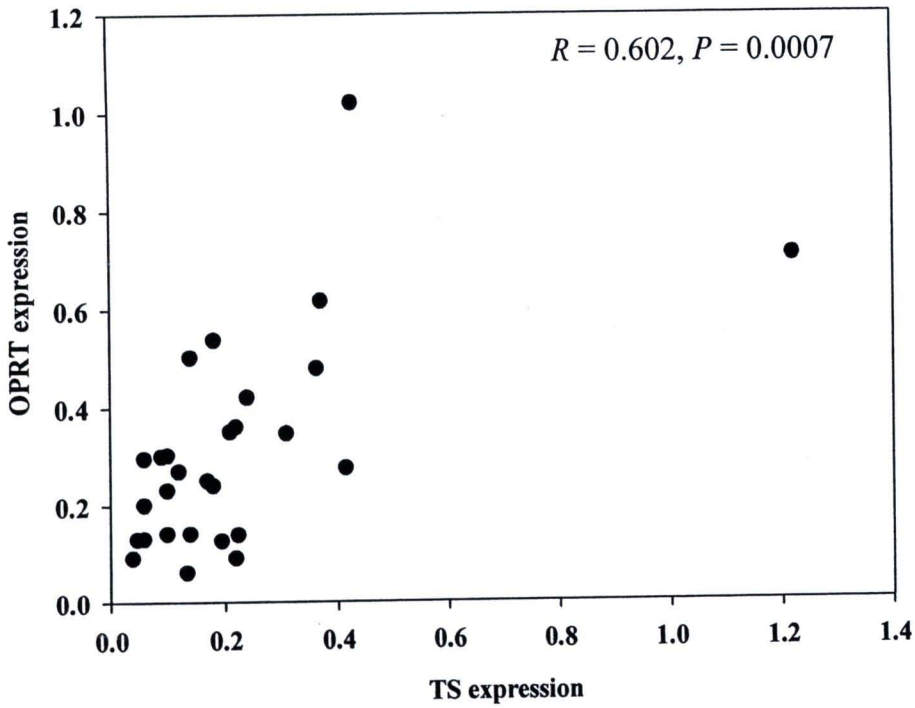

Figure 13 Relationship between OPRT and TS mRNA levels

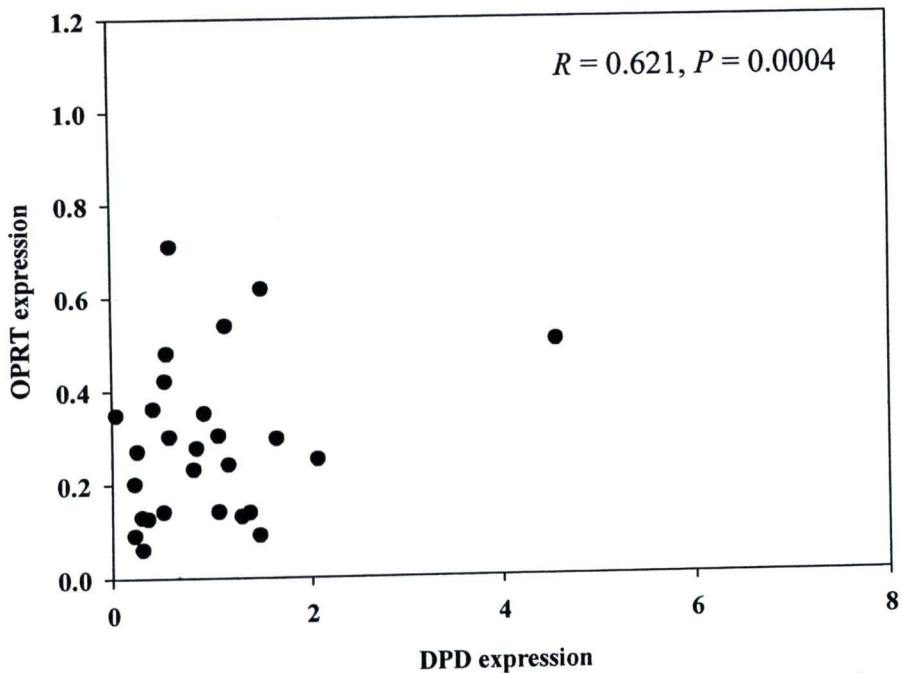

Figure 14 Relationship between OPRT and DPD mRNA levels

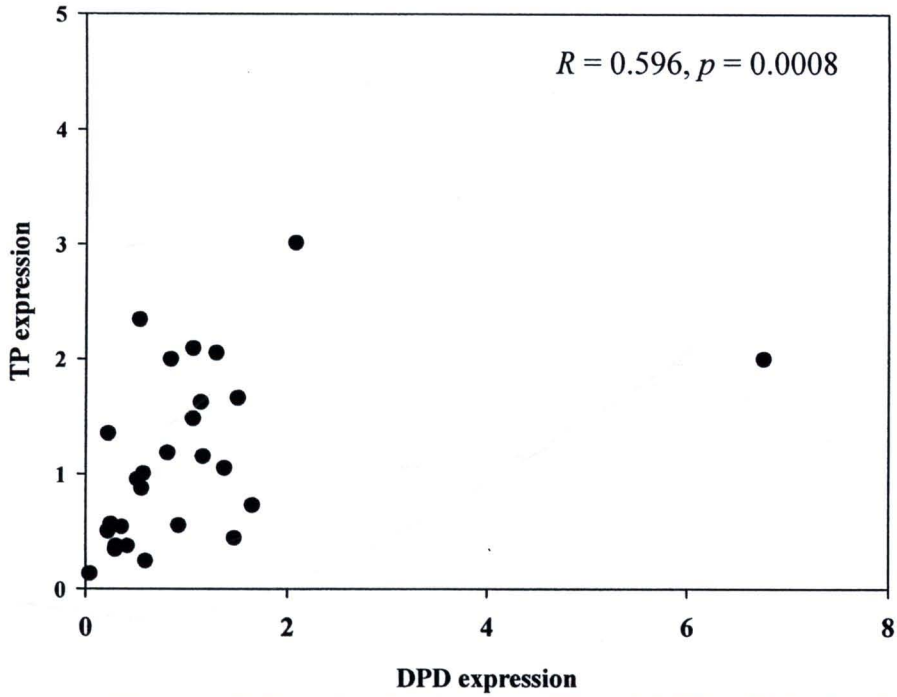

Figure 15 Relationship between TP and DPD mRNA levels

4.6 Relationship between gene expression levels and response to 5-FU

To determine the role of TS, DPD, TP and OPRT expression in the response of CCA tissues to 5-FU, we examined the correlation of TS, DPD, TP and OPRT expression and the sensitivity to 5-FU *in vitro* by using HDRA. Using %I.I. value of 5-FU at 200 μg/ml, we defined CCA tissues which exhibited % I.I. $\geq$ 50 as well response to 5-FU whereas those with % I.I.< 50 as poorly response.

The mean expression level of TP was appeared to be higher in CCA tissues with poorly response to 5-FU compared to those which well response to 5-FU, however there was not statistically significance (1.38 vs 0.93, $p = 0.178$; Figure 16). Whereas, the mean expression level of OPRT was appeared to be higher in CCA tissues with well response to 5-FU compared to those which poorly response to 5-FU, although there was not statistically significance (0.27 vs 0.42, $p = 0.162$; Figure 17).

Figure 18 and 19 showed the differences in TS and DPD expression according to the response of cancer tissue to 5-FU. No significant differences in the mean expression levels of TS and DPD mRNAs were observed in CCA tissues which

poorly response to 5-FU compared to those with well response to 5-FU (0.17 vs 0.34, $p = 0.461$ and 1.08 vs 1.40, $p = 0.611$, respectively).

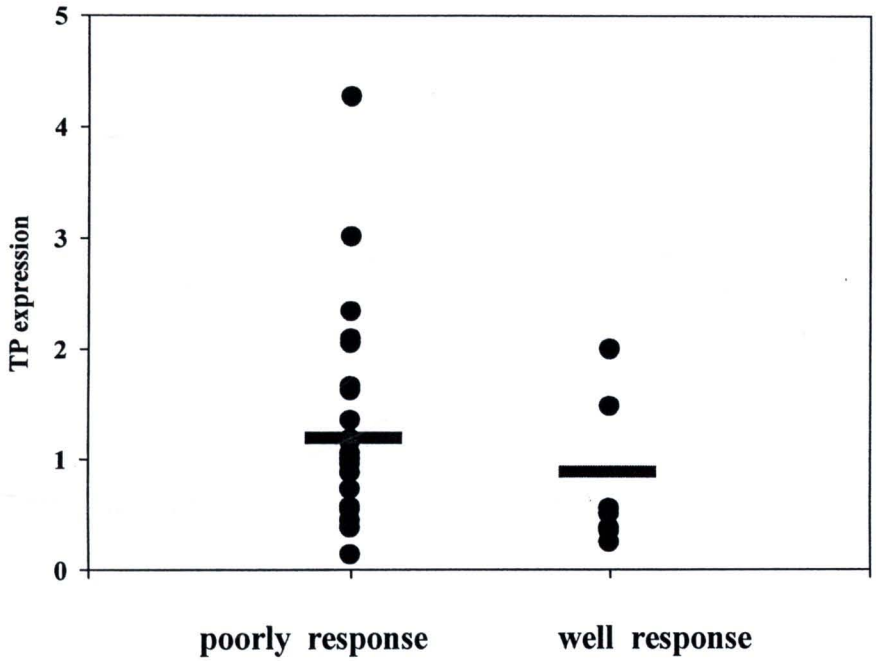

Figure 16 Distribution of TP mRNA levels and the response of CCA tissues to 5- FU (1.37 vs 0.93, $p = 0.178$)

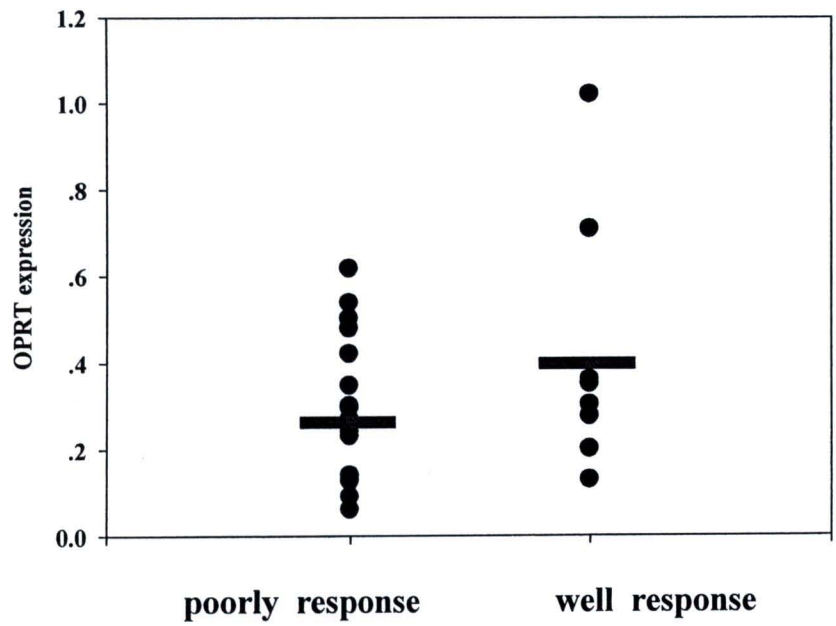

Figure 17 Distribution of OPRT mRNA levels and the response of CCA tissues to 5- FU (0.27 vs 0.42, $p = 0.162$)

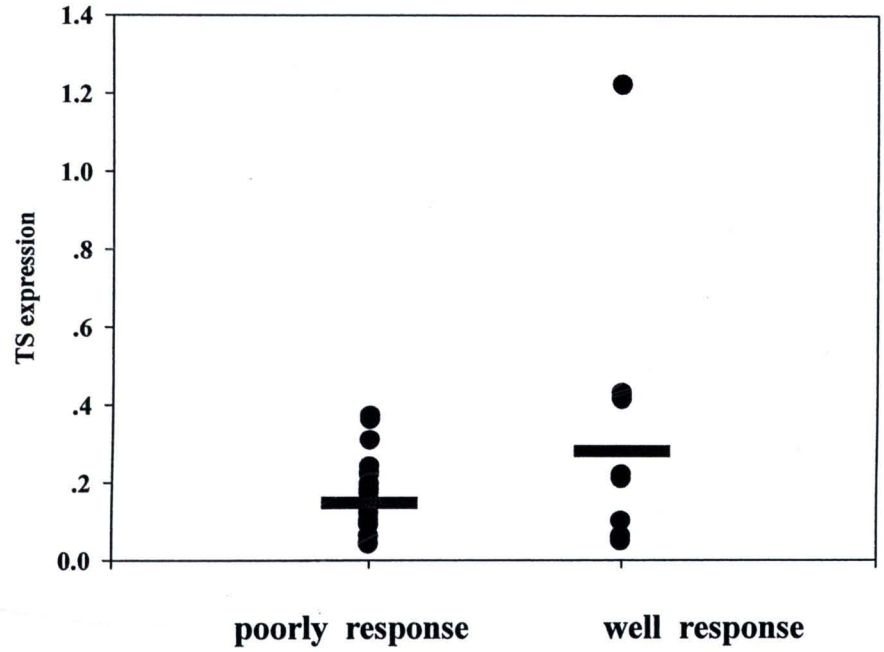

Figure 18 Distribution of TS mRNA levels and the response of CCA tissues to 5-FU (0.17 vs 0.34, $p = 0.461$)

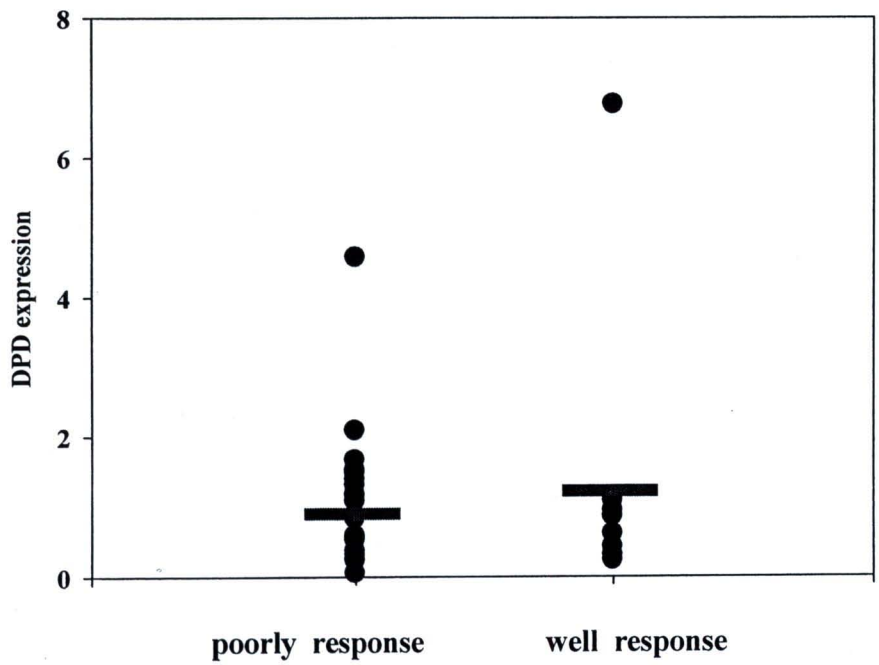

Figure 19 Distribution of DPD mRNA levels and the response of CCA tissues to 5- FU (1.08 vs 1.40, $p = 0.611$)

When the ratio between the expression levels of target genes were calculated, the data revealed that the ratio of OPRT/TP was apparently higher in the well response group compared to the poorly response group (0.77 vs 0.35, $p = 0.025$; Figure 20) with statistically significance, but other ratios including DPD/TS, DPD/OPRT, TS/OPRT, TP/TS and TP/DPD was not statistically significance (Figure 21-25).

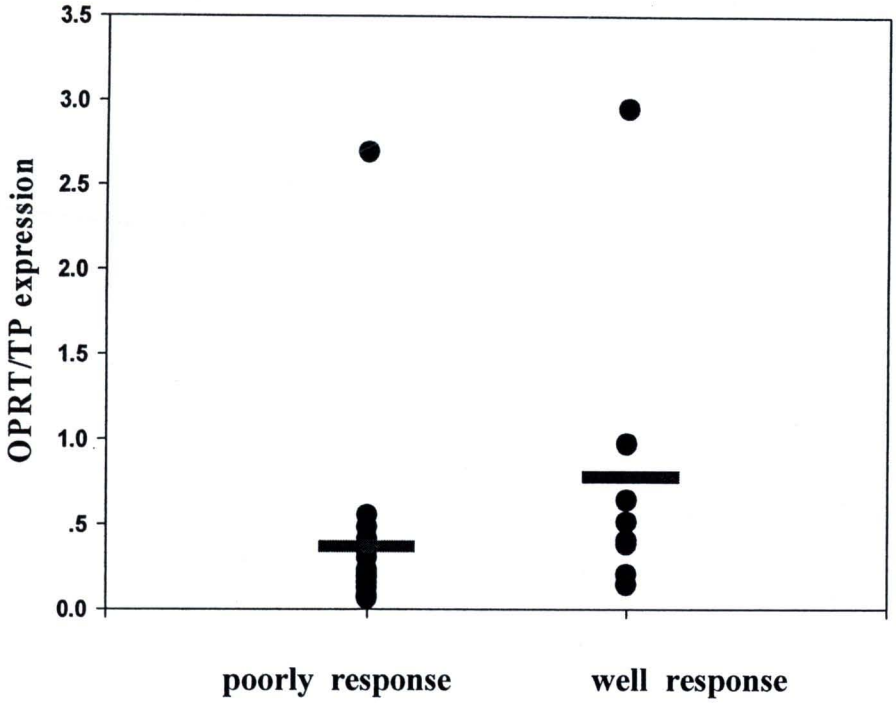

Figure 20 Distribution of ratio of OPRT/TP expression and the response of CCA tissues to 5- FU (0.35 vs 0.77, $p = 0.025$)

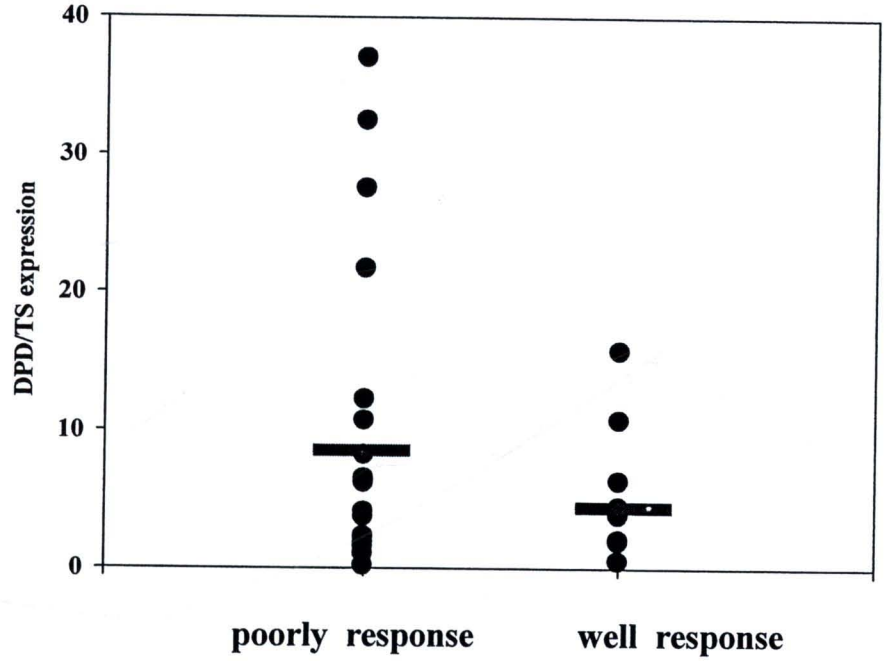

Figure 21 Distribution of ratio of DPD/TS expression and the response of CCA tissues to 5- FU (9.72 vs 5.68, $P = 0.525$)

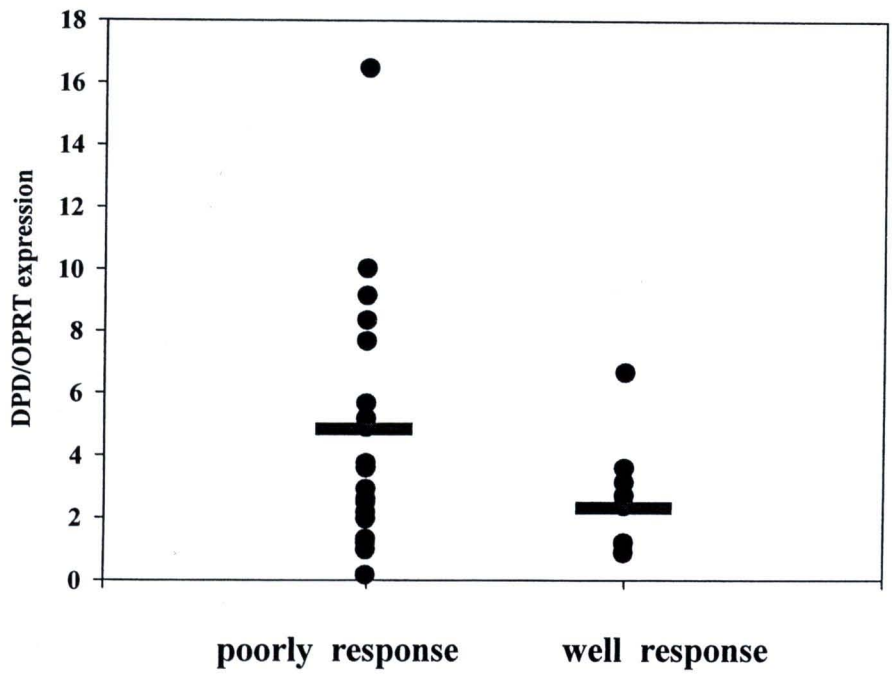

Figure 22 Distribution of ratio of DPD/OPRT expression and the response of CCA tissues to 5- FU (5.00 vs 2.68, $P = 0.147$)

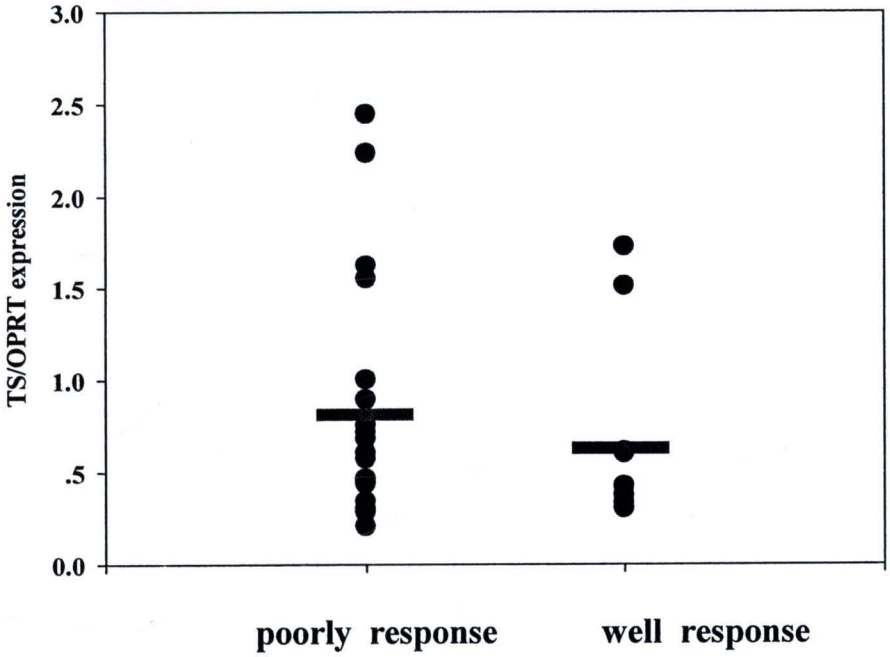

Figure 23 Distribution of ratio of TS/OPRT expression and the response of CCA tissues to 5- FU (0.84 vs 0.73, $P = 0.508$)

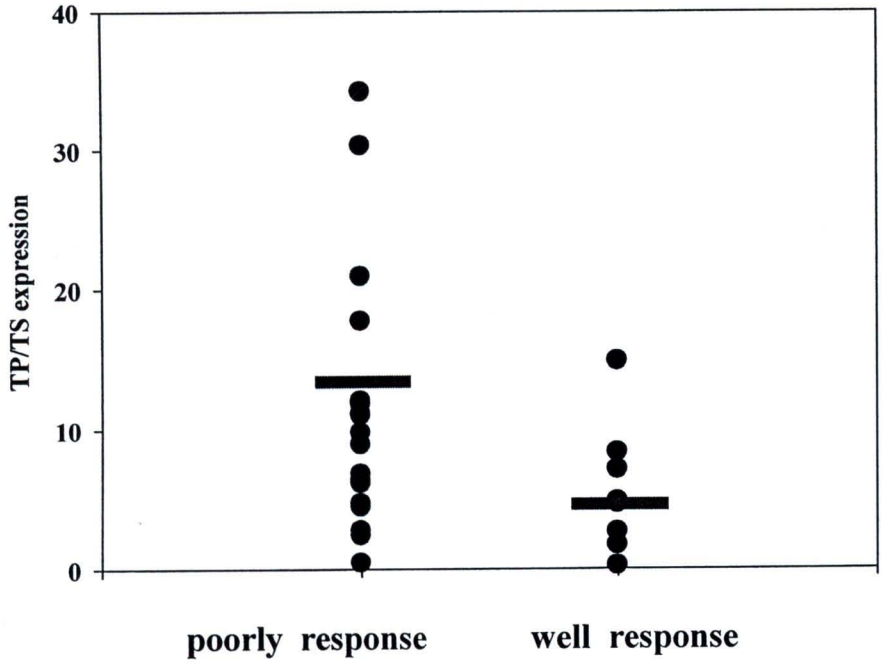

Figure 24 Distribution of ratio of TP/TS expression and the response of CCA tissues to 5- FU (10.46 vs 5.52, $P = 0.147$)

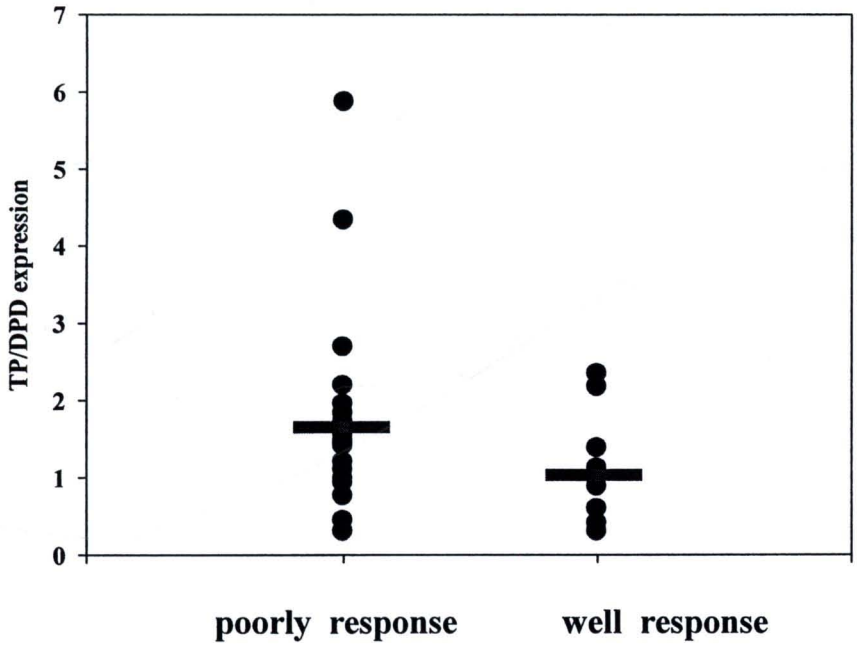

Figure 25 Distribution of ratio of TP/DPD expression and the response of CCA tissues to 5- FU (1.76 vs 1.15, $P = 0.162$)