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Thymidine phosphorylase gene variant, platelet counts and survival in gastrointestinal cancer patients treated by fluoropyrimidines

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The predictive value of thymidine phosphorylase gene variants (TP, also called platelet-derived endothelial cell growth factor) and thrombocytosis were controversial and worthy of further study in gastrointestinal cancer (GIC) patients. We screened all of the common missense single nucleotide polymorphisms (MAF ≥ 0.1) in fluoropyrimidines (FU) pathway genes (including *TP*, *TS*, *ENOSF1* and *DPD*). Three of them were selected and genotyped using Sequenom MassARRAY in 141 GIC patients. TP expression was assessed by immunohistochemistry. Our aim was to evaluate the prognostic significance of studied genes and platelet counts in GIC patients. Multivariate analyses indicated in rs11479-T allele carriers, platelet counts negatively correlated to overall survival. In addition, T allele of *TP*: rs11479 was associated with higher TP expression in cancer tissues. We suggest *TP*: rs11479 variant combined with platelet counts may be useful prognostic makers in GIC patients receiving first-line FU chemotherapy and thrombopoietin factor should be used with caution in the rs11479 T allele bearing patients.

A s we all know that fluoropyrimidines (FU, including 5-Fluorouracil (5-FU) and capecitabine) continue to be the backbone of gastrointestinal cancer (GIC) treatment. Thymidine phosphorylase (TP) is the enzyme responsible for conversion of FU to its active metabolite fluorodeoxyuridine monophosphate. TP was also proved to be an angiogenesis-promoting factor with a similar structure to that of platelet-derived endothelial cell growth factor (PD-ECGF)[1] and played an important role in angiogenesis and extracellular matrix remodeling and then can stimulate tumor growth and metastasis[2]. So, TP may play a dual role in cancer development and responding to FU chemotherapy. On the one hand, high level of TP in cancer patients resulted in more aggressive cancer growth and metastasis; on the other, since TP plays a positive role in FU activation, higher TP level patients may benefit more from FU treatment theoretically. In most cases, TP expression of cancer tissues appeared to be associated with poor prognosis[2], but the predictive value of *TP* gene variants were unclear.

Some studies indicated that preoperational thrombocytosis was an independent indicator of poor survival in operable colorectal cancer patients[3–5], but the others reported conflict results[6,7]. Platelets are one of the richest sources of TP. It may by speculated that platelet counts (PC) may interacts with TP in predicting survival of GIC patients.

Till now, the relationships among PC, TP gene variants and FU related survival were unknown and worthy of further study. Our aim was to find out how TP gene variants and PC influence the treatment outcomes of FU in GIC patients.

The present study took all of the common missense single nucleotide polymorphisms (mSNPs) in *TP*, thymidylate synthase (*TS*), Dihydropyrimidine dehydrogenase (*DPD*) and the Enolase superfamily member 1 (*ENOSF1*) genes into account because (1) The positive predictive value of rare genomic polymorphisms (minor allele frequency [MAF]<0.10) was considered to have limited clinical utility in individualized therapy because more than 90% patients may not benefit from the maker detection and thus lead to an increased medical costs;

Table 1 | Patient and disease characteristics

Characteristic	No. of Patients (%)
Age, years	
<60 years	105(74.5)
≥60 years	36(25.5)
Median (range)	53(18–79)
Sex	
Male	89(63.1)
Female	52(36.9)
KPS	
60%	16(11.3)
70%	52(36.9)
≥80%	73(51.8)
Smoker	
never	94(66.7)
ever	47(33.3)
Chemotherapy regimen	
Fu + irinotecan	72(51.1)
Fu + platinum	65(46.1)
Capecitabine	4(2.8)
Primary tumor	
Colon	63(44.7)
Rectum	46(32.6)
Gastric	32(22.7)

KPS, Karnofsky's index of performance status; FU, fluoropyrimidines.

(2)TS and DPD are the other two key enzymes in FU metabolism pathway (The mechanism of 5-FU toxicity is primarily through inhibition of TS, the rate-limiting enzyme in the pyrimidine nucleotide synthesis[8]. Up to 80% of administered 5-FU is broken down by DPD in the liver[9]) and *ENOSF1* was originally identified as a naturally occurring antisense transcript to the human *TS* gene[10]. They had putative influence on fluorouracil activity and could be the covariates when study TP and FU treatment outcomes; (3) The previous results suggested controversial predictive role of *TS*, *ENOSF1*, *DPD* and *TP* gene variants[11–15] and whether common mSNPs (MAF ≥ 0.10) in those genes predict outcomes of FU chemotherapy was still unclear.

Results

Patient characteristics and treatment outcome. Characteristics of the 141 patients are presented in Table 1. The median age was 53 years (range, 18 to 79 years). Response evaluation was available for 131 patients. Ten patients could not be evaluated for response because of: early cessation of chemotherapy (fewer than three cycles) due to insufferable toxicity ($N = 2$) or to non-medical reasons ($N = 4$); other anti-cancer therapy interfering with the efficacy assessment ($N = 4$). Among the 131 patients, 2 (1.5%) achieved a complete response (CR), 44 (33.6%) experienced a partial response (PR), 58 (44.3%) and 27 patients (20.6%) had stable and progressive disease (PD) respectively.

Blood tests were done regularly during and after chemotherapy in 136 patients, of whom, 21 patients (15.4%) experienced thrombocytopenia, 43 (31.6%) experienced thrombocytosis, 27 (19.8%) experienced hypoalbuminemia.

Information and genotypic frequencies of SNPs. Three mSNPs (*DPD* rs1801159; *TP* rs11479 and *ENOSF1* rs2612086) were selected and genotyped. For *TS* gene, no mSNP met our selection criteria. The MAF of each SNP was similar to reported data in the NCBI database. Call rates were 100% ($N = 141$) for rs11479 and rs2612086; for rs1801159, it was 99.3% ($N = 140$). All genotype frequencies were in Hardy-Weinberg equilibrium (HWE) (Table 2.).

Correlation between polymorphisms, PC and response. The overall objective response rate (ORR), calculated as CR plus PR

was 35.1%. Gastric cancer patients had higher ORR (17 out of 30,56.7%) compared to colorectal cancer patients (29 out of 101, 28.7%), $p = 0.005$. No significant link of ORR was noted to studied genotypes or PC in all of the patients (Table 3) or in patients according to different primary tumor sites (see Supplementary Table S1 online). No association was found between TP expression of cancer tissues and ORR (Logistic regression, $p = 0.945$). There was no significant associations between *TP*:rs11479 and thrombocytopenia or thrombocytosis (Chi-Square tests, $p = 0.146$ and 0.321 respectively).

Correlation between mSNPs, PC and survival. At the median follow-up of 328 days (range 36–1956 days), 85 patients had died and the median OS was 408 days. Thirty seven patients without PD chose to give up the anti-cancer therapy due to socioeconomic reasons, 14 non-PD patients accepted surgery or transcatheter arterial chemoembolization therapy and 10 patients suffered intolerable grade ≥ 3 toxicities and stopped chemotherapy before PD. To rule out that the differences in progression-free survival (PFS) and time to treatment failure (TTF) were due to non-chemotherapy factors, the PFS and TTF evaluation excluded those patients with no medical reasons for withdrawal ($N = 37$) and non-PD patients who accepted an anti-cancer therapy other than chemotherapy ($N = 14$). Thus, 80 and 90 patients were analyzable for PFS and TTF, respectively.

PFS, TTF and OS did not show any differences between the gastric and colorectal cancer patients (see Supplementary Fig. S1 online). So we did not carry out subgroup analysis according to different primary tumor sites.

The results of univariate Cox regression analysis of PFS, TTF and OS are shown in Supplementary Table S2. No significant association was found between PFS, TTF or OS and studied genotypes. Age, smoking status and PC correlates to OS. In Kaplan-Meier analysis, as shown in Fig. 1, patients aged above 60 years had longer OS ($N = 141$, median survival time was 625 vs. 380 days, log rank $p = 0.036$, Fig. 1-A). Thrombocytopenia was associated with longer OS while thrombocytosis was linked to shorter OS ($N = 136$, median survival time was 640 vs. 364 days and 353 vs. 538 days, log rank $p = 0.017$ and 0.030 respectively, Fig. 1 B and C). Ever smokers had worse OS ($N = 141$, median 312 vs. 528 days, log rank $p = 0.010$, Fig. 1 D).

The Multivariate Cox regression analysis (Table 4) revealed that age and thrombocytopenia were independent prognostic factors of OS (Hazard ratios [HR] = 0.448 and 0.436, 95% confidence interval [CI] = 0.236–0.853 and 0.209–0.911, $p = 0.014$ and 0.027 respectively) in GIC patients. Ever smokers had a trend toward worse OS ($p = 0.050$). None of the studied SNPs was a significant predictor of survival endpoints.

Prognostic value of PC in patients with different genotypes of rs11479. We further analyze the prognostic value of thrombocytopenia and thrombocytosis to OS by different genotypes of rs11479. Survival curves were shown in Fig. 1 (E to H). In CT/TT genotype of TP: rs11479 group, thrombocytopenia was associated with longer OS ($p = 0.036$) and thrombocytosis predicted worse OS ($p = 0.004$). In patients with CC genotype of rs11479, PC did not predict OS. Multivariate Cox regression analysis (Enter) got similar results (Supplementary Table S3), showing that PC linked to OS in the T allele bearing GIC patients.

Correlation between T allele of rs11479 and cancer TP expression. Formalin-fixed, paraffin-embedded specimens of cancer tissues from 26 patients were immunostained for TP expression. Tumor sections stained for TP were semiquantified based on percentage of positive cells (see Fig. 2), 14 of them were recognized as TP = 0, 5 as TP = 1 and 7 as TP = 2. T allele of rs11479 was associated with higher TP expression in cancer tissues ($N = 26$, Odds ratios [OR] = 3.951, 95% CI = 1.216–12.838, $p = 0.022$, Supplementary Table S4).

Table 2 | SNPs information and genotypic frequencies

SNPs	Gene	Allelic change	AA change	Call rate, N(%)	HWE, (p)	MAF	Genotype frequency, n(%)		
							wt/wt	wt/var	var/var
rs1801159	DPYD	A > G	I543V	140(99.3)	0.832	0.261	77(55.0)	53(37.9)	10(7.1)
rs11479	TYMP	C > T	S471L	141(100.0)	0.991	0.188	93(66.0)	43(30.5)	5(3.5)
rs2612086	ENOSF1	T > C	M145T	141(100.0)	0.157	0.124	110(78.0)	27(19.1)	4(2.8)

SNPs, Single-nucleotide polymorphisms; AA, amino acid; N, No. of Patients; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; wt, wild type; var, variant.

Discussion

We found that PC negatively correlated to OS in GIC patients carrying T allele of *TP*: rs11479. To the best of our knowledge, this is the first study to evaluate the effect of PC on survival of GIC patients receiving FU chemotherapy based on different *TP*: rs11479 genotypes. Neither thrombocytosis nor rs11479 was associated with FU treatment outcomes separately in our study.

Previous studies showed controversial results about FU treatment outcomes and PC. Some studies supported that OS was significantly worse in patients who had elevated platelet count[5,16,17] but the others showed negative results[6,18,19]. Our results supported that elevated PC was not associated with OS in GIC patients receiving FU treatment independently. However, PC correlated to FU-related OS in rs11479 T allele bearing GIC. It might partly explain the controversial prognostic value of PC in those previous studies.

Blood platelets are one of the richest sources of TP[20]. Chemotherapy induced thrombocytopenia may reduce the amount of TP and then weak the role of TP. We found that PC negatively predicted FU related OS only in rs11479 T allele carriers and the latter was associated with higher TP protein expression in cancer tissues, intimating that (1) Platelets control may be important in patients carried rs11479 T allele or with high TP expression in cancer tissues, chemotherapy related thrombocytopenia illustrate the inhibition of TP related tumorigenesis and angiogenesis and then led to survival benefits; (2)TP may play a more important role in promoting tumor growth and metastasis in the patients originally with high TP expression or born with rs11479 T allele comparing to its role in activating FU; (3)TP might be a potential target to control tumor progression in rs11479 T allele carriers or patients with high TP expression in cancer tissues.

A previous study considered T allele of rs11479 as a novel finding associated with early FU related adverse events, consistent with our results that the activity of TP might be different in patients with different rs11479 genotypes[21]. Further studies can be carried out to investigate the associations between the plasma concentrations of FU metabolites and rs11479 genotypes.

TP induces angiogenesis, increases the metastatic potential of cancer cells and protects cancer cells against apoptosis, therefore, in most cases, high tumor tissue TP expression appeared to be associated with poor prognosis[2]. However, rs11479 T allele failed to predict worse OS independently in our study even though we found it correlated to higher TP protein expression in cancer tissues. This may due to (1) FU treatment in high TP expression patients may partly compensate the adverse influences of TP; (2) relatively small sample size may bring bias into our study.

Because of the small amount of cancer tissues from inoperable cancer patients, we could only successfully detect TP expression in 26 patients. The sample size is too small to analyze associations between TP expression and ORR or survival endpoints in the present study.

The present study made small steps forward to find out that *TP*: rs11479 T allele carriers may have higher TP expression in cancer tissues. The underline mechanisam of why the T-allele would result in more protein in tumor tissue and the associations between genomic polymorphisms and the relevant proteins' expression in cancer tissues are worth pursuing in additional studies with lager sample size since it may benefit those patients who failed to get cancer tissues to analyze.

We just took the mSNPs of relative genes into our study due to(1) mSNPs may change the activities of relevant proteins; (2) To date, whether mSNPs in *TS*, *ENOSF1*, *DPD* and *TP* genes predict outcomes of FU chemotherapy was unclear. In the past years, several studies have been conducted to investigate the predictive value of *TS* gene polymorphisms (28-bp tandem repeat polymorphism in the 5′-UTR, a guanine instead of a cytosine (G/C) in 3R and 3′-UTR 6-bp deletion polymorphisms), and TS, DPD and TP expressions (most of them analyzed mRNA, rare detect protein) in cancer tissues, however, no proof-of-principle results can be drawn at this point[22–33]. Our study demonstrated that none of the studied mSNPs can predict ORR or survival of GIC patients receiving first-line FU treatment independently and in addition we found that in *TP*: rs11479 T allele carriers (who may have higher TP level in tumor tissues), PC

Table 3 | Univariate and multivariate analysis (logistic regression) of ORR

Variate	Univariate			Multivariate		
	N	OR(95%CI)	P	N	OR(95%CI)	P
Age	131	1.412(0.606–3.290)	0.424	125	1.659(0.656–4.194)	0.285
Sex	131	0.801(0.376–1.708)	0.566	125	0.723(0.225–2.322)	0.586
PS	131	0.991(0.947–1.036)	0.683	125	0.962(0.910–1.016)	0.159
SA	131	2.316(0.232–23.154)	0.474	125	4.517(0.147–138.580)	0.388
SS	131	0.802(0.372–1.732)	0.574	125	0.673(0.263–1.725)	0.410
Thrombocytopenia	126	1.296(0.486–3.457)	0.604	125	1.117(0.383–3.255)	0.839
Thrombocytosis	126	0.685(0.307–1.530)	0.357	125	0.555(0.224–1.379)	0.205
Hypoalbuminemia	126	0.929(0.429–2.014)	0.853	125	1.056(0.427–2.609)	0.906
rs11479 (CT + TT vs. CC)	131	1.039(0.483–2.239)	0.921	125	0.965(0.413–2.255)	0.935
rs1801159 (AG + GG vs. AA)	130	0.989(0.478–2.047)	0.977	125	1.049(0.490–2.246)	0.903
rs2612086 (TC + CC vs. TT)	131	1.257(0.531–2.974)	0.602	125	1.230(0.509–2.973)	0.646

ORR, objective response rate; N, No. of assessable patients; OR, odds ratio; CI, confidence interval; SA, surface area; PS, performance status; SS, smoking status.
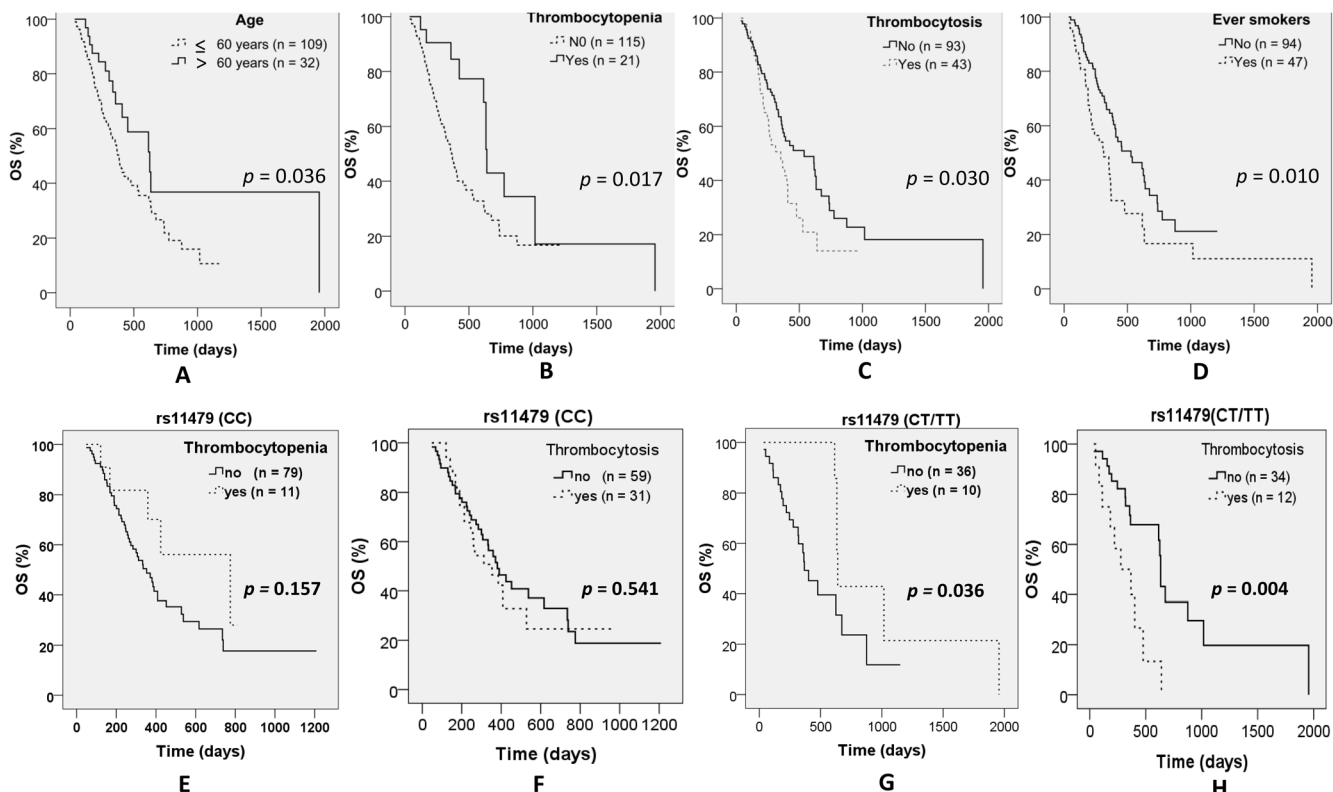
Figure 1 | (A; B; C; D): OS in patients according to Age, Thrombocytopenia, Thrombocytosis, and Smoke status; (E; F; G; H): Prognostic value of platelet counts in patients with different rs11479 genotypes (log-rank test).

negatively correlated to OS of metastasis GIC patients, revealing the complexity among cancer progression, drug metabolism, tumor response to chemotherapy and the survival benefits.

In conclusion, the results of this study suggest that in *TP*: rs11479 T allele carriers, platelet could lead to bad survival. It can be speculated that thrombopoietin factor should be used with caution in those patients. The underline mechanistic studies about the role of platelet and TP palying in GIC patients are worth pursuing in the future.

Methods

Patient eligibility and study design. This study was approved by the Ethics Committee of Huazhong University of Science and Technology. Written informed consent was obtained from each patient before blood samples were collected. Methods were carried out in accordance with the approved guidelines. From 2007 to 2013, 141 metastatic GIC patients treated with FU based first-line chemotherapy at the department of oncology, Tongji Hospital who also had blood samples available for

analysis were selected in this study. We choose type I error $\alpha = 0.05$, $1 - \beta = 0.8$, two-sided test, provided the target SNP allele frequency in the population was about 12% to 40%, treatment efficacy was about 30%, OR ≥ 3.0, the maximum calculated samples size was 140 by Quanto (Version 1.2.4). Eligibility criteria were shown in supplementary file.

The primary objectives were to assess the correlation between genetic variants and the ORR. Response was defined as at least a 30% decrease in the sum of the longest diameter of target lesions during first-line chemotherapy. Secondary objectives included the relationship between interested variants and PFS, time to TTF, and overall survival (OS). PFS was defined as the time elapsed between the first day of FU treatment and disease progression (PD) or death from any cause, whichever occurred first. TTF was calculated from the start of FU treatment to its discontinuation for reasons including PD, intolerable toxicity, or death. OS was calculated as the time from treatment initiation until death from any cause or the date of last follow-up[34]. Thrombocytopenia, thrombocytosis and hypoalbuminemia were defined as a blood platelet count below 150,000 per microliter induced by chemotherapy, over 300,000 per microliter and the albumin level in blood serum below 30 gram per liter of blood respectively before chemotherapy.

Table 4	Multivariate Cox regressionanalysis (Enter) of PFS, TTF and OS					
	PFS ($N = 77$)		TTF($N = 87$)		OS($N = 135$)	
Variate	HR(95%CI)	P	HR(95%CI)	P	HR(95%CI)	P
Age	0.543(0.254–1.163)	0.116	0.706(0.367–1.359)	0.298	0.448(0.236–0.853)	**0.014**
Sex	1.187(0.523–2.692)	0.682	1.165(0.568–2.389)	0.677	0.680(0.325–1.420)	0.304
PS	1.010(0.968–1.053)	0.652	1.007(0.970–1.047)	0.707	0.984(0.953–1.016)	0.330
SA	0.812(0.062–10.604)	0.874	0.664(0.064–6.942)	0.733	0.275(0.034–2.252)	0.229
SS	1.614(0.743–3.509)	0.227	1.472(0.734–2.952)	0.276	1.754(1.000–3.078)	**0.050**
Thrombocytopenia	1.231(0.592–2.561)	0.578	1.117(0.584–2.137)	0.738	0.436(0.209–0.911)	**0.027**
Thrombocytosis	1.043(0.563–1.931)	0.893	1.017(0.572–1.807)	0.955	1.407(0.838–2.362)	0.197
Hypoalbuminemia	1.324(0.728–2.408)	0.358	1.230(0.701–2.158)	0.470	1.174(0.677–2.034)	0.568
rs11479 (CT + TT vs. CC)	0.536(0.284–1.011)	0.054	0.557(0.308–1.005)	0.052	0.611(0.363–1.030)	0.064
rs1801159 (AG + GG vs. AA)	0.733(0.406–1.321)	0.301	0.836(0.485–1.441)	0.519	0.667(0.422–1.055)	0.083
rs2612086 (TC + CC vs. TT)	1.134(0.652–1.974)	0.655	1.109(0.659–1.865)	0.698	1.239(0.729–2.106)	0.428

PFS, progression-free survival; TTF, time to treatment failure; OS, overall survival; N, No. of assessable patients; HR, hazard ratio; CI, confidence interval; SA, surface area; PS, performance status; SS, smoking status.
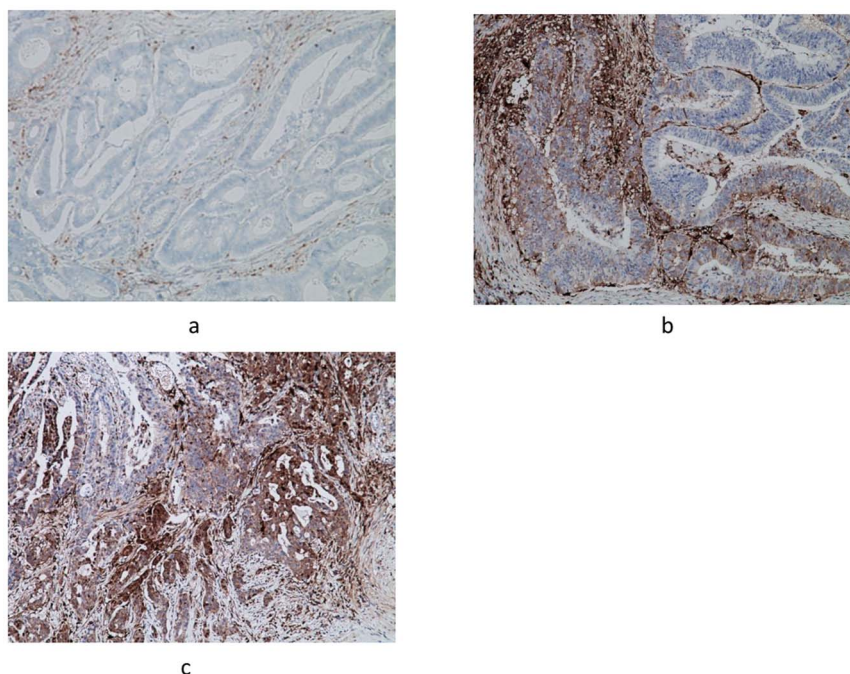
Figure 2 | Immunohistochemical staining for thymidine phosphorylase (TP)in cancer tissues (original magnification × 100). TP = 0, Stromal cells TP-positive but TP-negative tumor cells (a);TP = 1, more than 5% but less than 50% of the cancer cells were TP-positive (b); TP = 2, more than 50% of the cancer cells were TP-positive (c).

Efficacy assessment. Efficacy was evaluated with a consistent imaging technique (magnetic resonance imaging or computed tomography scan) every 6 weeks by the RECIST 1.1. At least 6 weeks of chemotherapy would be needed in the response assessment analysis. Earlier response evaluation was only allowed when patients had severe symptoms indicating progression. Blood counts and biochemistry tests were performed within 72 hours of the beginning of each cycle. Evaluations were performed blinded to the genetic results and were assessed independently by two doctors. A third doctor resolved inconsistencies.

Treatment. As recommended by the guidelines of the National Comprehensive Cancer Network, patients were allocated to FU-based treatment. **FOLFIRI**, irinotecan (Camptosar; Pfizer) 180 mg/m² intravenous (IV) infusion over 30–90 minutes, day 1; leucovorin 400 mg/m² IV infusion to match the duration of irinotecan infusion, day 1; 5-FU 400 mg/m² IV bolus, day 1; then 1200 mg/m²/day × 2 days (total 2400 mg/m² over 46–48 hours) continuous infusion; repeat every 14 days or **mCapeIRI**[35] regimen (irinotecan 125 mg/m², days 1 and 8; capecitabine (Xeloda; Roche)825–1000 mg/m², twice daily on days 1–14; repeat every 21 days) or **FOLFOX** (oxaliplatin 80 mg/m² IV infusion over 2 hours, day 1; leucovorin 400 mg/m² IV over 2 hours, day 1; 5-FU 400 mg/m² IV bolus, day 1; then 1200 mg/m²/day × 2 days continuous infusion; repeat every 14 days) or **CapeOX** (Oxaliplatin 130 mg/m² day 1, **Capecitabine** 825–1000 mg/m², twice daily on days 1–14; repeat every 21 days) or single-agent chemotherapy of capecitabine (1250 mg/m² twice daily on days 1–14; repeat every 21 days). All patients accepted 5-hydroxytryptamine receptor antagonist (5 mg once a day) 30–60 minutes before irinotecan or oxaliplatin. The chemotherapy continued until disease progressed or intolerable toxicities came out or patients asked to withdrew due to any reason.

Genotyping. We searched the National Center for Biotechnology Information (NCBI) SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/snp/) and related literature to identify mSNPs from the *DPD, TP, TS* and *ENOSF1* genes. The criteria for SNP selection were as follows: (1) With a MAF of more than 0.10 in Asian population, (2) Genotypes call rate ≥ 95%, (3) Missense SNP. Three SNPs (*DPD* rs1801159; *TP* rs11479 and *ENOSF1* rs2612086) were selected for genotyping. None SNP in *TS* gene met the inclusion criteria. Genomic DNA was extracted from peripheral blood using a FUJI whole blood DNA kit (Fujifilm Corporation, Tokyo, Japan). Primers were designed by Genotyping Tools and MassARRAY Assay Design software (version 3.0, Sequenom Inc., San Diego, California). SNPs were genotyped using the Sequenom MassARRAY iPLEX platform. Data were processed and analyzed by Sequenom MassArray TYPER 4.0 software. Details of PCR reactions are in supplementary file. Primer sequences are presented in Supplementary Table S5. Five percent of the samples were randomly selected and genotyped by direct sequencing, with a resulting concordance rate of 100%. Call rate threshold was set at least 95% for each SNP. HWE was tested through χ^2 test and $p < 0.05$ indicated deviation from the equilibrium.

TP expression. Immunohistochemical analysis was performed on formalin fixed, paraffin embedded tissue sections (4 μm) using a labeled streptavidin biotin method (LSAB kit; Zhongshanjinqiao Beijin). The concentration of the mouse anti-TP antibody was 100 μg/mL, working dilution was 1 : 50. The second antibody was biotinylated rabbitanti-rat immunoglobulin (Zhongshanjinqiao Beijin). Tumor sections stained for TP were scored based on percentage of positive cells. The scale was 0: positive cells < 5%, 1: positive cells 5%–50%, 2: positive cells > 50%. TP expression was evaluated by two researchers who had no knowledge of the patient's clinicopathological factors.

Statistical analysis. The univariable and multivariable logistic regression model was performed to evaluate the associations between variants and ORR while the cox regression model was used to evaluate the associations of variants and PFS, TTF, and OS. Covariates included dichotomous variables (age, sex, smoking status, thrombocytopenia, thrombocytosis, hypoalbuminemia and genotypes of rs2612086, rs11479 and rs1801159) and continuous variables (surface area, and performance status). Smoking was significantly associated with shorter disease-free survival and time to recurrence in colon cancer patients[36], therefore, we accounted for smoking status in the multivariate analysis. OR, HR and their 95% CI were calculated as estimates of the correlations. Kaplan-Meier analysis and log-rank test were performed to estimate the distribution of PFS, TTF, and OS and to compare differences between survival curves. A value of $p < 0.05$ was considered statistically significant in a two-tailed test. Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL).

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Study design: L.H. and X.L.Y.; data collected and assembly: X.M.Y., F.J.C., Y.Y.C., G.Y.H., P.Z. and S.L.F.; performed the experiments: X.L., T.F.C., S.P.X. and Q.Q.Y.; data analysis: S.W.G. and L.H.; drafted the manuscript: L.H. and X.L.Y. All authors read and approved the final manuscript.

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