RESEARCH ARTICLE

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Blood-Based vs. Tissue-Based mRNA Expression of Thymidylate Synthase, Dihydropyrimidine Dehydrogenase, and Methylenetetrahydrofolate Reductase: A Prospective Study to Predict the Neoadjuvant CAPEOX Response in Advanced Colorectal Cancer

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Abstract

Background: Fluoropyrimidine-based chemotherapy is a fundamental treatment for colorectal cancer (CRC), yet its therapeutic efficacy is often limited by significant inter-individual variability. Enzymes involved in 5-fluorouracil (5-FU) metabolism thymidylate synthase (TS), dihydropyrimidine dehydrogenase (*DPD*), and methylenetetrahydrofolate reductase (*MTHFR*) are critical determinants of drug response. This study aimed to evaluate the molecular correlation between *TS*, *DPD*, and *MTHFR* mRNA expression in paired tumor tissue and peripheral blood samples, while simultaneously developing and validating a non-invasive predictive nomogram to forecast therapeutic response to neoadjuvant CAPEOX (capecitabine–oxaliplatin) chemotherapy in advanced CRC. **Methods:** A prospective cohort study was conducted on 36 patients with stage III–IV CRC receiving CAPEOX. mRNA expression of *TS*, *DPD*, and *MTHFR* was quantified using qRT-PCR from paired tumor and peripheral blood samples. Chemotherapy response was evaluated using RECIST 1.1 criteria. Statistical analyses included Spearman correlation, the Mann–Whitney U test, and multivariate logistic regression. A nomogram was constructed based on significant predictors. **Results:** *DPD* and *MTHFR* expression levels were significantly lower in responders than in non-responders ($p < 0.001$), while *TS* expression showed no significant difference. Gene expression in tissue and blood was strongly correlated ($r = 0.820$ for *TS*, $r = 0.658$ for *DPD*, and $r = 0.623$ for *MTHFR*; all $p < 0.001$). Multivariate analysis identified blood-based *DPD* and *MTHFR* expression as independent predictors of response. The predictive nomogram demonstrated excellent discrimination (AUC = 0.932). **Conclusion:** Lower *DPD* and *MTHFR* expression predicts a favorable response to neoadjuvant CAPEOX in advanced CRC. These biomarkers offer a promising, non-invasive approach to personalizing treatment strategies potentially enhancing therapeutic efficacy while minimizing unnecessary toxicity.

Keywords: Colorectal cancer- dihydropyrimidine dehydrogenase- methylenetetrahydrofolate reductase

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related death globally, characterized by a high incidence of metastasis [1]. Despite advances in treatment, tumor recurrence and chemotherapy resistance remain formidable challenges [2]. Chemotherapy regimens based on 5-fluorouracil (5-FU), such as Capecitabine, are a cornerstone of CRC treatment. However, patient response to 5-FU is highly variable, necessitating the identification of predictive biomarkers to tailor therapeutic strategies effectively [3, 4].

The therapeutic efficacy and toxicity profiles of 5-fluorouracil are primarily dictated by the metabolic interplay of three key enzymes: Thymidylate Synthase

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(*TS*), Dihydropyrimidine Dehydrogenase (*DPD*), and Methylenetetrahydrofolate Reductase (*MTHFR*) [5]. As the primary target of 5-FU, *TS* catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a critical step in DNA synthesis [6–9]. In contrast, *DPD* serves as the rate-limiting enzyme in 5-FU catabolism, where its activity determines the bioavailability of active metabolites [6, 7]. Furthermore, *MTHFR* regulates the folate pool, specifically the availability of 5,10-methylenetetrahydrofolate, which is essential for the stabilization of the inhibitory ternary complex between 5-FU and *TS* [10].

The exploration of *TS*, *DPD*, and *MTHFR* as biomarkers is not entirely without precedent. Extensive research has investigated Single Nucleotide Polymorphisms (SNPs) of these genes, such as the *TS* 5'-UTR tandem repeat or the *MTHFR* C677T variant [11, 12]. However, findings from these genetic studies remain highly inconsistent; for instance, while some meta-analyses suggest a link between *TS* polymorphisms and 5-FU sensitivity, others report no clinical significance [5, 13, 14]. These discrepancies are likely due to the fact that DNA-based markers do not account for the epigenetic and post-transcriptional regulations that ultimately determine enzymatic activity. In contrast to static genetic markers, mRNA expression profiling provides a more accurate representation of the current metabolic state of the tumor.

While individual studies have examined mRNA levels in tumor tissue, the results have been hampered by the inherent limitations of invasive sampling, tumor heterogeneity, and the lack of standardization across different tumor sites [5, 11, 12, 15, 16]. Furthermore, although the concept of liquid biopsy is gaining traction, the majority of CRC research has focused on circulating tumor DNA (ctDNA) for mutation detection, rather than quantifying metabolic enzyme mRNA in peripheral blood [17, 18]. Our study advances the current knowledge by not only comparing paired tissue-blood expression to resolve sampling inconsistencies but also by transitioning from single-marker analysis to a multi-marker predictive model specifically optimized for the CAPEOX regimen.

Despite these advancements, there is a paucity of prospective studies that simultaneously evaluate the molecular concordance between paired tumor tissue and peripheral blood samples within the same patient cohort, particularly under the neoadjuvant CAPEOX protocol. Furthermore, most existing research focuses on single-gene associations, failing to provide an integrated multi-marker framework that is clinically actionable. Therefore, this study was designed to address these gaps through a dual-objective approach. The primary objective was to elucidate the correlation between the mRNA expression levels of *TS*, *DPD*, and *MTHFR* in paired tumor tissue and peripheral blood samples, thereby evaluating the validity of systemic markers as non-invasive surrogates for intratumoral molecular activity. The secondary objective was to develop and validate a clinically applicable predictive nomogram based on these systemic biomarkers to forecast therapeutic response to neoadjuvant CAPEOX in patients with advanced CRC. Through this approach, we aim to provide a robust tool for patient stratification and contribute to the advancement of precision oncology in the management of CRC.

Materials and Methods

Study Design

This study was designed as a prospective cohort, observational study conducted between December 2024 and June 2025. The clinical phase of the research was carried out at Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia, and its network hospitals. The molecular and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were performed at the Clinical Microbiology Laboratory, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia.

Participants

Eligible participants were adult patients (≥18 years) with histologically confirmed stage III or IV colorectal adenocarcinoma who were planned to receive standard neoadjuvant chemotherapy using the CAPEOX regimen. Inclusion criteria were patients diagnosed with stage III or IV colorectal adenocarcinoma confirmed by histopathological examination, patients eligible and clinically fit for neoadjuvant CAPEOX chemotherapy, and willingness to provide written informed consent. Exclusion criteria were damaged or inadequate tumor tissue samples, patients who failed to complete at least three chemotherapy cycles or were lost to follow-up, and histopathological variants such as signet ring cell carcinoma or mucinous adenocarcinoma.

Sample Size

Sample size determination was performed using the formula for comparing two independent means, assuming a 95% confidence level and 80% power. The calculation was informed by local prevalence data from the Indonesian National Cancer Registry (Global Cancer Observatory/ GLOBOCAN Indonesia) [19, 20] and institutional records from Dr. Wahidin Sudirohusodo General Hospital, which rank colorectal cancer among the top four most frequent malignancies in South Sulawesi [19, 21]. Based on these data and an anticipated effect size derived from previous biomarker studies, a minimum of 30 participants was considered sufficient to detect significant differences in gene expression. Ultimately, 36 patients were enrolled to provide a buffer against potential attrition and ensure the stability of the multivariate analysis.

Ethical Considerations

The study protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Hasanuddin, and Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia (Approval No. UH25040303). Written informed consent was obtained from all participants prior to inclusion. All procedures complied with the Declaration of Helsinki and Indonesian national ethical guidelines.

Clinical Assessment and Response Classification

Therapeutic response to neoadjuvant CAPEOX was

evaluated following the final treatment cycle, prior to surgical intervention. Assessment was performed in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Baseline tumor measurements obtained via contrast-enhanced computed tomography scan or magnetic resonance imaging were compared with post-treatment imaging.

Patients were categorized into two groups: Responders, which included those achieving a Complete Response (CR) or a Partial Response (PR; ≥30% decrease in the sum of diameters of target lesions); and Non-responders, which included those with Stable Disease (SD) atau Progressive Disease (PD; ≥20% increase in target lesion diameters or appearance of new lesions).

Chemotherapy Protocol

All participants received standard neoadjuvant CAPEOX chemotherapy, consisting of capecitabine (1,000 mg/m² orally twice daily, days 1–14) and oxaliplatin (130 mg/m² intravenously on day 1 of each 21-day cycle). Four cycles were administered prior to surgery.

Sample Collection

Prior to the initiation of neoadjuvant chemotherapy, paired specimens were obtained from each participant. Tumor tissue samples were collected via endoscopic biopsy, while peripheral blood samples (5 mL) were drawn intravenously using vacuum tubes. To ensure the stability of the transcriptome, both specimen types were immediately placed in sterile containers containing L6 lysis buffer and transported at 4 °C to the Clinical Microbiology Laboratory at the Faculty of Medicine, Universitas Hasanuddin, for immediate molecular processing.

Total RNA Extraction (Boom Method)

Total RNA was isolated using the Boom method [22, 23], which utilizes the silica-adsorption principle in the presence of chaotropic agents. Briefly, 100 µL of the sample was homogenized with 900 µL of L6 buffer (120 g guanidinium thiocyanate in 100 mL of 0.1 M Tris-HCl, pH 6.4; 22 mL of 0.2 M EDTA, pH 8.0; and 2.6 g Triton X-100). Following the addition of 20 µL of diatom suspension, the mixture was vortexed and centrifuged at 13,000 rpm for 15 s. The resulting pellet was washed sequentially with L2 buffer, 70% ethanol, and acetone. After drying at 56 °C, the RNA was eluted in 60 µL of Tris-EDTA (TE) buffer and stored at −80 °C until further analysis.

RNA Quality Control and cDNA Synthesis

The concentration and purity of the extracted RNA were assessed using spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, USA). Samples with an A260/A280 ratio of 1.8–2.0 were selected for downstream applications. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The reaction was performed in a 20 µL volume under the following thermal conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was conducted using the Applied Biosystems Power SYBR Green® PCR system. Targeted gene expression was normalized against two internal controls to ensure robust data interpretation. The specific primer sequences for the target genes (*TS*, *DPD*, *MTHFR*) and housekeeping genes (β-actin and GAPDH) used for normalization are detailed in Table 1.

Each assay was run in triplicate, and relative expression levels were calculated using the ΔΔCt method [24]. To validate the use of the $2^{-\Delta\Delta Ct}$ method, the amplification efficiency (E) for each target and reference gene was determined prior to experimental analysis. A five-point standard curve was constructed using a 10-fold serial dilution of pooled cDNA samples. The efficiency was calculated using the following formula:

$$E=(10-1/slope-1)\times 100\%$$

All primer sets demonstrated a linear relationship between the log of the cDNA concentration and the Ct values, with correlation coefficients (R^2) exceeding 0.99. The calculated efficiencies ranged from 92% to 98%, falling within the acceptable range of 90–100%. This high level of efficiency ensures that the doubling of the target sequence occurs at a near-theoretical rate during each cycle, thereby permitting the accurate comparative quantification of mRNA levels.

Data Analysis

All statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) and R Studio (v4.2.3) for nomogram construction. Descriptive statistics were applied to summarize patient demographics and biomarker distributions. Associations between gene expression and chemotherapy response were evaluated using non-parametric tests, including the Mann–Whitney U test for two-group comparisons and the Kruskal–Wallis

Table 1. Primer Sequences Used for qRT-PCR Analysis

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
TS	GCCAGAATCTGTTCGCTTCAAC	AGGAAACTGAGTGCCGGCTT
DPD	CTTTGGGTGCGACTTGACG	GTCGACCCCGCTCCTTTT
MTHFR	CATTTCCGACTCCAGCATCG	GGGATGCCTGTAAAGGGTGT
β-actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

test for multiple-group comparisons, while correlations between tissue and blood expression levels were analyzed using Spearman's rank correlation coefficient.

To identify independent predictors of chemotherapy response, variables demonstrating a p-value <0.25 in the bivariate analysis were entered into a multivariate logistic regression model. This screening threshold was chosen based on the Hosmer–Lemeshow purposeful selection strategy to prevent the premature exclusion of clinically relevant predictors that might only reach significance upon adjustment for other covariates [Hosmer et al., 2013]. Prior to model construction, multicollinearity among candidate predictors particularly blood-based *DPD* and *MTHFR* expression levels was assessed using the Variance Inflation Factor (VIF). Multicollinearity was considered significant if the VIF exceeded 5.0; however, all included variables demonstrated a VIF <2.0, confirming the stability of the regression coefficients.

The final model was refined using a backward stepwise elimination procedure. Model fit was assessed via the Hosmer–Lemeshow goodness-of-fit test, and predictive performance was quantified using the Area Under the Curve (AUC) from Receiver Operating Characteristic (ROC) analysis. A nomogram was subsequently developed based on the regression coefficients of significant predictors (*DPD* and *MTHFR* expression in blood), with model calibration performed both graphically through calibration plots and statistically. A p-value <0.05 was considered statistically significant.

Results

Participant Characteristics

A total of 36 CRC patients undergoing neoadjuvant chemotherapy were included in the study, with 18 patients classified as responders and 18 as non-responders. The majority of subjects were male (66.7%). The mean age was similar between the non-responder (46.11 ± 8.78 years) and responder (45.17 ± 7.51 years) groups. The most common tumor location was the rectum (66.7%), and most tumors were well-differentiated (63.9%). There were no significant differences in sex, age, tumor location, or histological grade between the responder and non-responder groups (p>0.05 for all, Table 2).

mRNA Expression and Tissue-Blood Correlation

The relative mRNA expression levels of the three metabolic enzymes were successfully quantified in all 36 paired samples. As shown in Table 3, significant differences in expression levels between responders and non-responders were observed for *DPD* and *MTHFR*. In peripheral blood, the median expression of *DPD* was significantly lower in responders (4.42, IQR: 3.12–6.85) compared to non-responders (12.54, IQR: 9.15–18.42; p<0.001). Similarly, *MTHFR* blood expression was lower in the responder group (median:5.12 vs. 10.28; p<0.001). In contrast, *TS* expression levels in both tissue and blood showed no statistically significant difference between the two groups (p=0.412 and p=0.385, respectively).

Spearman's rank correlation analysis revealed a strong and significant positive correlation between intratumoral and systemic mRNA expression for all three genes. The strongest concordance was observed for *TS* (r=0.820,p<0.001), followed by *DPD* (r=0.658,p<0.001) and *MTHFR* (r=0.623,p<0.001). These findings confirm that peripheral blood mRNA levels accurately reflect the molecular landscape of the primary tumor.

Correlation of Gene Expression in Tissue and Blood

Spearman correlation analysis revealed a very strong and significant positive correlation between the expression of *TS* in tissue and blood (r=0.820; p<0.001). Similarly, strong and significant correlations were found for *DPD* (r=0.658; p<0.001) and *MTHFR* (r=0.623; p<0.001), as shown in Table 4. All three genes (*TS*, *DPD*, and *MTHFR*) demonstrated strong and statistically significant positive correlations between tumor tissue and blood, indicating that peripheral expression levels reliably reflect intratumoral expression patterns.

Gene Expression and Chemotherapy Response

The relationship between gene expression levels and chemotherapy response is summarized in Table 5. There was no significant difference in the median expression of *TS* in either tissue or blood between the responder and non-responder groups (p=0.584 and p=0.339, respectively).

In contrast, the median expression levels of *DPD* and *MTHFR* were significantly lower in the responder group

Table 2. Participant Characteristics

Variable	Non-responders (n = 18)	Responders (n = 18)	p-value
Age (years, mean ± SD)	46.11 ± 8.78	45.17 ± 7.51	0.673
Sex			0.362
Male	11(61.1%)	13(72.2%)	
Female	7(38.9%)	5(27.8%)	
Tumor location			1
Right colon	4 (22.2%)	4 (22.2%)	
Left colon	2 (11.1%)	2 (11.1%)	
Rectum	12 (66.7%)	12 (66.7%)	
Histological grade			0.567
Well differentiated	13 (72.2%)	10 (555.6%)	
Moderately differentiated	4 (22.2%)	6 (33.3%)	
Poorly differentiated	1 (5.6%)	2 (11.1%)	
Clinical Stage (AJCC 8th)			0.742
Stage III	12 (66.7%)	13 (72.2%)	
Stage IV	6 (33.3%)	5 (27.8%)	
T-Primary Tumor			0.815
T3	14 (77.8%)	15 (83.3%)	
T4	4 (22.2%)	3 (16.7%)	
N-Regional Nodes			0.65
N0	3 (16.7%)	2 (11.1%)	
N1–N2	15 (83.3%)	16 (88.9%)	
M–Metastasis			0.592
M0	12 (66.7%)	13 (72.2%)	
M1	6 (33.3%)	5 (27.8%)	

Table 3. Relative mRNA Expression Levels of *TS, DPD,* and *MTHFR* in Tissue and Blood Samples

Gene Marker	Sample Type	Non-responders (n=18)	Responders (n=18)	p-value*
TS	Tissue	8.45 (5.12–12.30)	7.12 (4.85–10.54)	0.412
	Blood	6.90 (4.25–9.80)	6.15 (3.90–8.42)	0.385
DPD	Tissue	15.20 (11.45–21.10)	6.12 (4.15–8.90)	< 0.001
	Blood	12.54 (9.15–18.42)	4.42 (3.12–6.85)	< 0.001
MTHFR	Tissue	11.85 (8.90–15.42)	5.80 (4.25–8.15)	< 0.001
	Blood	10.28 (7.50–13.90)	5.12 (3.65–7.25)	< 0.001

Table 4. Correlation of Gene Expression Levels in Tissue and Blood

Gene (Tissue vs. Blood)	Spearman's r	p-value
TS	0.82	< 0.001*
DPD	0.658	< 0.001*
MTHFR	0.623	< 0.001*

Note: *, Significant correlation at p < 0.05.

compared to the non-responder group. For *DPD*, this difference was highly significant in both tissue (p<0.001) and blood (p<0.001). For *MTHFR*, the difference was also significant in both tissue (p=0.010) and blood (p<0.001). In contrast, *TS* expression showed no significant association with treatment response. These findings suggest that *DPD* and *MTHFR* may serve as reliable predictive biomarkers for CAPEOX efficacy in advanced colorectal carcinoma.

Development of a Predictive Model for Chemotherapy Response

A multivariate logistic regression model was developed using blood-based *DPD* and *MTHFR* gene expression levels to predict the probability of response to CAPEOX. The Hosmer-Lemeshow test indicated good model fit (p=0.357). The Nagelkerke R^2 was 0.65, indicating that the model explains 65% of the variance in chemotherapy response. Both *DPD* and *MTHFR* expression levels in blood remained significant predictors in the final model (p=0.016 and p=0.027, respectively) (Table 6). The Receiver Operating Characteristic (ROC) curve analysis for this model yielded an Area Under the Curve (AUC) of 0.932, demonstrating excellent discriminatory power (Figure 1).

Regression coefficients were used to construct a predictive nomogram (Figure 2). Calibration plot analysis

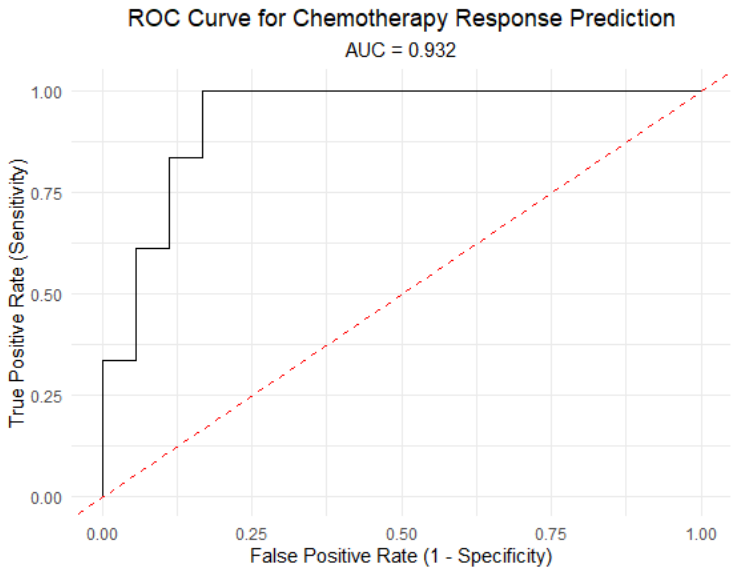

Figure 1. ROC Curve of the Multi-Marker Predictive Model. The AUC of 0.932 (95% CI: 0.854–1.000) indicates high accuracy in distinguishing responders from non-responders.

Table 5. Gene Expression Levels in Tissue and Blood by Chemotherapy Response

Gene Expression	Non-responders (Median, IQR)	Responders (Median, IQR)	Total (Median, IQR)	p-value
TS (Tissue)	8.949 (0.495)	9.002 (0.634)	8.985 (0.563)	0.584
TS (Blood)	10.010 (0.824)	10.037 (0.757)	10.010 (0.853)	0.339
DPD (Tissue)	8.288 (0.963)	7.637 (0.693)	7.879 (0.711)	< 0.001*
DPD (Blood)	9.270 (0.590)	8.835 (0.362)	9.009 (0.555)	< 0.001*
MTHFR (Tissue)	9.699 (0.871)	9.112 (2.433)	9.408 (0.954)	0.010*
MTHFR (Blood)	8.469 (1.075)	7.675 (0.765)	8.057 (0.844)	< 0.001*

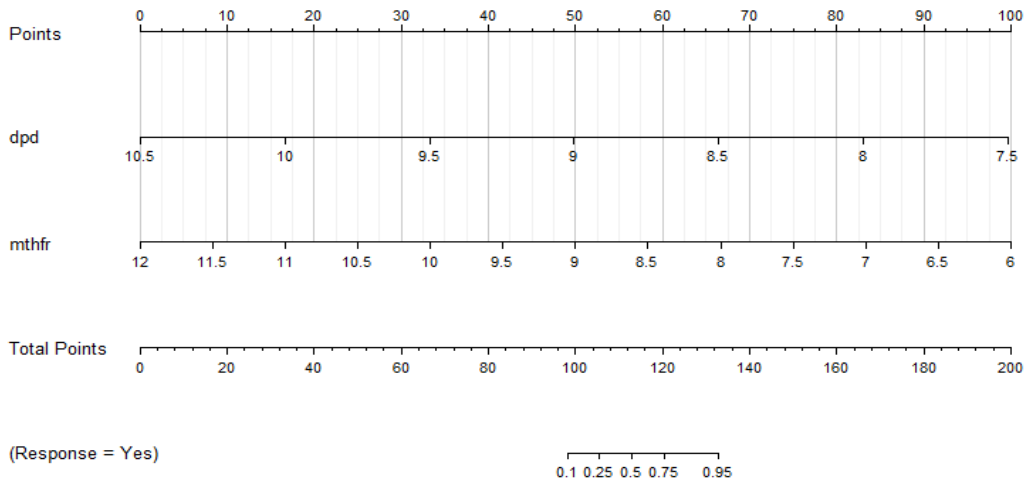

Figure 2. Predictive Nomogram for Forecasting Chemotherapy Response. Points are assigned based on DPD and *MTHFR* blood levels; a higher total point score corresponds to a higher probability of response.

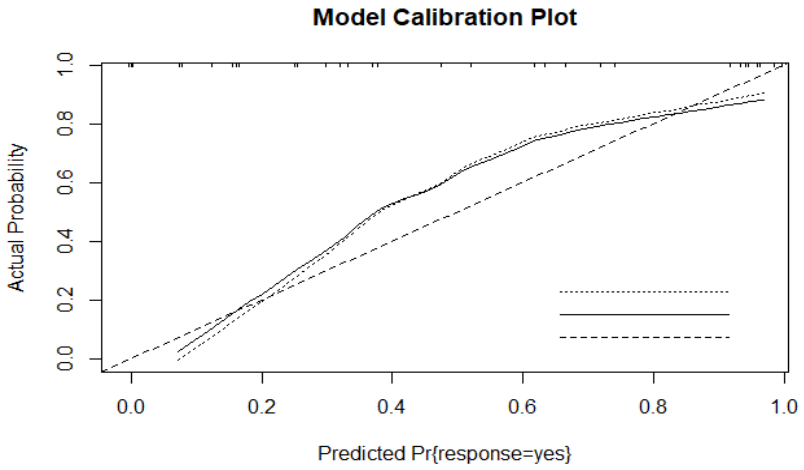

Figure 3. Calibration Plot of the Nomogram Model. The x-axis represents the predicted probability of response, and the y-axis represents the observed response rate. The diagonal 45-degree dashed line represents perfect prediction. The solid line indicates the model's performance, with close proximity to the diagonal line demonstrating excellent calibration and minimal bias.

confirmed excellent agreement between predicted and observed probabilities (Figure 3).

Discussion

This study demonstrated that patient demographic and clinicopathological characteristics including age, sex, tumor location, and histologic grade did not significantly influence the response to neoadjuvant CAPEOX chemotherapy in advanced CRC. These findings align with previous studies indicating that traditional clinical parameters have limited predictive value for chemotherapy outcomes [25, 26]. Although tumor biology is often influenced by histologic differentiation and anatomical site, therapeutic response to fluoropyrimidine-based regimens is primarily governed by molecular determinants rather than demographic or morphological factors [27]. The lack of association between these variables and treatment response underscores the importance of identifying reliable molecular biomarkers that can better predict sensitivity or resistance to chemotherapy, ultimately guiding individualized treatment decisions.

Table 6. Multivariate Logistic Regression Model for Predicting Chemotherapy Response

Variable	B	SE	p-value	Exp(B)	95% CI
DPD expression (blood)	-4.916	2.039	0.016*	0.007	0 – 0.399
MTHFR expression (blood)	-2.465	1.111	0.027*	0.085	0.1 – 0.750
Constant	64.486	23.539	0.006*	—	—

Note: *Significant at $p < 0.05$.

Molecular Concordance: Blood as a Surrogate for Tissue

A major finding of this study is the strong positive correlation between *TS*, *DPD*, and *MTHFR* mRNA expression in tumor tissue and peripheral blood. These results suggest that peripheral blood analysis can serve as a valid and less invasive surrogate for tumor gene expression profiling. This finding supports the growing role of liquid biopsy in oncology, where circulating tumor-derived nucleic acids reflect intratumoral molecular activity [16, 24]. The high correlation observed for *TS* (r = 0.820), *DPD* (r = 0.658), and *MTHFR* (r = 0.623) in this study indicates systemic regulation of these metabolic genes involved in 5-fluorouracil (5-FU) metabolism. This systemic consistency reinforces the potential for blood-based gene expression assays to replace invasive tumor biopsies for predictive assessment, particularly in patients with unresectable disease or those unfit for repeat tissue sampling [17].

Predictive Value of DPD and MTHFR

In this cohort, *DPD* and *MTHFR* expression levels both in tissue and blood were significantly associated with chemotherapy response, whereas *TS* expression showed no such relationship. These findings are consistent with prior research showing that *DPD* overexpression confers resistance to fluoropyrimidine-based chemotherapy due to increased catabolism of 5-FU, resulting in reduced intracellular concentrations of active metabolites [28, 29]. Patients with lower *DPD* expression demonstrated better responses to CAPEOX, reflecting higher effective drug exposure.

Similarly, reduced *MTHFR* expression was associated with improved therapeutic outcomes. The enzyme *MTHFR* regulates folate availability for thymidylate synthesis, influencing DNA replication and repair. Decreased *MTHFR* activity may enhance cytotoxic effects by augmenting the stability of the 5-FU–*TS* inhibitory complex [30–32]. Our results suggest that in the context of multi-agent neoadjuvant therapy, rate-limiting catabolic factors (*DPD*) and cofactor availability (*MTHFR*) provide more stable predictive signals than the target enzyme itself. The absence of correlation between *TS* expression and therapeutic response, despite its known role as a 5-FU target, may reflect the multifactorial nature of resistance mechanisms including alternative nucleotide salvage pathways, tumor heterogeneity, and oxaliplatin-induced DNA damage mechanisms independent of *TS*.

The TS Paradox in CAPEOX Therapy

A notable finding in our study was the lack of a significant association between pre-treatment *TS* expression and clinical response, contrasting with the traditional paradigm identifying *TS* as the primary determinant of fluoropyrimidine sensitivity. Several mechanisms may explain this observation. Primarily, the synergistic effect of oxaliplatin in the CAPEOX regimen may diminish the relative impact of *TS* levels, as oxaliplatin induces cytotoxicity through the formation of DNA adducts and inter-strand crosslinks mechanisms that bypass the thymidylate synthesis pathway targeted by 5−FU [33, 33]. Consequently, high *TS* expression might be 'overcome' by the DNA-damaging potency of Oxaliplatin, neutralizing its independent predictive value.

Second, the presence of compensatory nucleotide salvage pathways may decouple *TS* inhibition from cell death. Tumor cells often upregulate enzymes like thymidine kinase (TK), which enables the utilization of exogenous thymidine for DNA synthesis, effectively bypassing the 5-FU-mediated block on the de novo *TS* pathway [34, 35]. Furthermore, the lack of significance may reflect intratumoral heterogeneity; a single pre-treatment biopsy may not capture the varying *TS* expression profiles throughout the tumor mass, especially in advanced-stage CRC where clonal evolution is prominent. Lastly, recent evidence suggests that *TS* mRNA levels are highly dynamic and may undergo rapid post-treatment 'rebound' overexpression, rendering baseline levels less predictive of the sustained response required to achieve clinical remission [insert citation]. This reinforces our conclusion that in the context of multi-agent neoadjuvant therapy, rate-limiting catabolic factors (*DPD*) and cofactor availability (*MTHFR*) may provide more stable predictive signals than the target enzyme itself.

The robust correlation between intratumoral and systemic mRNA levels of *TS*, *DPD*, and *MTHFR* establishes a strong biological foundation for transitioning toward blood-based pharmacogenomic testing. From a clinical perspective, utilizing peripheral blood as a surrogate for tumor tissue offers several transformative advantages. Unlike endoscopic biopsies, which are inherently invasive and capture only a localized snapshot of the tumor, blood-based assays reflect the systemic metabolic capacity of the host and the cumulative molecular profile of the disease, potentially mitigating the confounding effects of tumor heterogeneity. This 'liquid biopsy' approach is particularly feasible for patients with anatomical obstructions or those whose clinical status precludes repeated invasive procedures, allowing for a more dynamic assessment of chemosensitivity prior to each treatment cycle.

However, the clinical implementation of circulating mRNA markers requires careful consideration of both pre-analytical and biological confounders. mRNA is inherently labile; thus, variability in sample handling such as the time elapsed between venipuncture and RNA stabilization in L6 buffer can significantly alter expression profiles. Furthermore, systemic factors such as the chronic inflammatory state often observed in advanced CRC may influence leukocyte-derived mRNA levels, potentially overlapping with the signals derived from circulating tumor-associated nucleic acids. These factors emphasize the necessity of standardized blood collection protocols and the potential inclusion of inflammatory markers (e.g., C-reactive protein) in future iterations of the predictive model. Acknowledging these constraints does not diminish the nomogram's value but rather provides a rigorous framework for its refinement as a precision medicine tool in oncology.

Model Performance and Clinical Implementation

Following this biological validation, a logistic regression model incorporating *DPD* and *MTHFR* blood

expression was developed, which accurately predicted chemotherapy response with an AUC of 0.932. The model's good calibration and Nagelkerke R2 value of 0.65 suggest that 65% of the variance in treatment response was explained by these two genes. The resulting nomogram provides a clinically practical tool for estimating individualized probability of response to neoadjuvant CAPEOX. For instance, patients with low *DPD* (≤8.0) and low *MTHFR* (≤7.5) expression exhibited >90% predicted probability of favorable response. Such visual and quantitative models are invaluable for clinical decision-making, particularly in resource-limited settings, as they simplify complex molecular interactions into actionable predictions.

This approach reflects the shift toward precision oncology, where biomarker-guided algorithms help tailor chemotherapy regimens to maximize efficacy and minimize unnecessary toxicity. Importantly, the use of blood biomarkers rather than tissue samples makes this model more feasible for real-world clinical implementation, offering a non-invasive, cost-effective, and repeatable assessment method.

The novelty of this study lies in its integrated evaluation of *TS, DPD*, and *MTHFR* expression across both tissue and blood samples, and in demonstrating that blood-based mRNA profiles can serve as accurate surrogates for predicting therapeutic response. While earlier studies investigated these genes individually or within tumor tissue alone, this research uniquely develops and validates a nomogram model based on blood *DPD* and *MTHFR* expression to predict CAPEOX efficacy. This dual biological and statistical integration represents a step forward in non-invasive precision treatment planning for CRC. The findings highlight the potential of molecular stratification prior to chemotherapy, allowing clinicians to identify patients unlikely to respond to CAPEOX and consider alternative regimens early in treatment. Furthermore, by confirming strong correlation between tissue and peripheral gene expression, the study provides foundational evidence supporting the future use of liquid biopsy–based pharmacogenomic assays in treatment monitoring and adaptive therapy planning.

Strengths and Limitations

This study has several strengths, including its prospective design, the direct comparison of tissue and blood samples, and the development of a clinically applicable multi-marker predictive model. Despite the significant predictive performance of our model, several limitations warrant careful consideration. First, the modest sample size (n=36) inherently limits the statistical power of the multivariate analysis and increases the risk of model overfitting. In predictive modeling, a common rule of thumb is to maintain at least 10 'events' per candidate predictor variable (EPV) to ensure model stability. With 18 responders in our cohort and two independent predictors (*DPD* and *MTHFR* expression in blood), our model achieves an EPV of 9.0. While this ratio is close to the recommended threshold, it suggests that the resulting nomogram should be interpreted with caution until it undergoes more rigorous validation.

Second, as a single-center study conducted in a specific regional population in Indonesia, the external validity of our findings may be constrained. Genetic and environmental factors specific to this cohort could influence baseline gene expression levels, potentially affecting the nomogram's calibration in different ethnic or geographic populations. Finally, the high AUC (0.932) observed in our study might be an optimistic estimate due to the lack of an independent external validation set. Future research utilizing larger, multicenter cohorts and prospective external validation is essential to refine the nomogram's coefficients and confirm its generalizability across the broader clinical spectrum of CRC.

In conclusion, this study demonstrated that lower *DPD* and *MTHFR* gene expression in both tumor tissue and peripheral blood is significantly associated with a better response to neoadjuvant CAPEOX chemotherapy in advanced CRC, while *TS* expression showed no predictive value. The strong correlation between tissue and blood expression suggests that peripheral blood can serve as a reliable, non-invasive biomarker source for predicting chemotherapy response.

Multivariate and nomogram analyses confirmed that blood *DPD* and *MTHFR* expression independently predict treatment outcomes with high accuracy (AUC = 0.932). These findings propose a practical and clinically applicable framework for personalized chemotherapy, enabling optimization of CAPEOX therapy and reduction of unnecessary toxicity. Further large-scale validation is recommended to confirm these results and support clinical integration.

Author Contribution Statement

All authors contributed equally in this study.

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