In Vitro Assessment of Fluoropyrimidine-Metabolizing Enzymes: Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, and β-Ureidopropionase

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Abstract: Fluoropyrimidine drugs (FPs), including 5-fluorouracil, tegafur, capecitabine, and doxifluridine, are among the most widely used anticancer agents in the treatment of solid tumors. However, severe toxicity occurs in approximately 30% of patients following FP administration, emphasizing the importance of predicting the risk of acute toxicity before treatment. Three metabolic enzymes, dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase (DHP), and β-ureidopropionase (β-UP), degrade FPs; hence, deficiencies in these enzymes, arising from genetic polymorphisms, are involved in severe FP-related toxicity, although the effect of these polymorphisms on in vivo enzymatic activity has not been clarified. Furthermore, the clinical usefulness of current methods for predicting in vivo activity, such as pyrimidine concentrations in blood or urine, is unknown. In vitro tests have been established as advantageous for predicting the in vivo activity of enzyme variants. This is due to several studies that evaluated FP activities after enzyme metabolism using transient expression systems in Escherichia coli or mammalian cells; however, there are no comparative reports of these results. Thus, in this review, we summarized the results of in vitro analyses involving DPD, DHP, and β-UP in an attempt to encourage further comparative studies using these drug types and to aid in the elucidation of their underlying mechanisms.

Keywords: fluoropyrimidine; dihydropyrimidine dehydrogenase; dihydropyrimidinase; β-ureidopropionase; genetic polymorphism

1. Introduction

Fluoropyrimidine drugs (FPs), including 5-fluorouracil (5-FU) and its oral prodrugs tegafur, capecitabine, and doxifluridine, are widely used in the treatment of solid tumors in the gastrointestinal tract, breast, liver, lung, head, and neck [1–3]. FP-based treatments have a narrow therapeutic index, which has led to severe adverse effects in approximately 30% of cancer patients, including mucositis, diarrhea, neutropenia, thrombocytopenia, and hand–foot syndrome [4–8]. Additionally, severe treatment toxicities could lead to treatment interruption, which increases the subsequent risk of therapeutic failure as well as patient death [9].

Genetic polymorphisms of thymidylate synthase (TYMS), methylene tetrahydrofolate reductase (MTHFR), and miR-27a are associated with the development of severe toxicities as well as treatment...
resistance; however, FP-related toxicity is mainly dependent on FP catabolism. Over 80% of an administered dose of 5-FU is rapidly degraded by three consecutive enzymes belonging to the endogenous pyrimidine, uracil, and thymine catabolic pathways (Figure 1), the only known 5-FU in vivo degradation pathway. Initially, the rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2), mainly found in the liver, catalyzes the reduction of 5-FU to dihydro-5-fluorouracil (FUH2). Subsequently, dihydropyrimidinase (DHP, EC 3.5.2.2) catalyzes the hydrolytic ring opening of FUH2 to form fluoro-β-ureidopropionic acid (FUPA). Even though DPD and DHP catalysis is reversible, the positive reaction is dominant in vivo [10–15]. Lastly, β-ureidopropionase (β-UP, EC 3.5.1.6) catalyzes the hydrolysis of FUPA to fluoro-β-alanine. The three enzymes (DPD, DHP, and β-UP) are encoded by the *DPYD*, *DPYS*, and *UPB1* genes, respectively [16–18].

![Figure 1.](image)

**Figure 1.** Uracil and 5-fluorouracil degradation pathway. Uracil and 5-fluorouracil are catabolized successively by dihydropyrimidine dehydrogenase, dihydropyrimidinase, and β-ureidopropionase. β-Alanine and fluoro-β-alanine are the final metabolites in this pathway.

Decreased DPD and DHP enzymatic activities have been linked to genetic polymorphisms identified in patients with severe FP-related toxicities; for each causative polymorphism, the reduction in activity is caused mainly by the substitution or deletion of amino acids [19–21]. However, the relationship between β-UP activity and the development of FP-related toxicity is still unknown. To date, the specific effects of previously identified polymorphisms on enzymatic function are largely unknown. Only four *DPYD* variants (c.1905 + 1G > A (IVS14 + 1G > A, *DPYD*2A); c.1679T > G (*DPYD*13, p.I560S); c.1129 – 5923C > G /hapB3; and c.2846A > T (p.D949V)) have been characterized as predictive markers for FP-related toxicity in Caucasians [22]. However, significant racial and individual differences in polymorphism location and frequency make it challenging to safely extrapolate the clinical data and institute regional guidelines from one population to another. Thus, it is necessary to further clarify the effects of genetic polymorphisms in an attempt to establish their effect on in vivo enzymatic function. For example, before FP administration, PCR-Restriction Fragment Length Polymorphism (RFLP) analysis, Sanger sequencing, and next-generation sequencing analysis are often used for detecting genetic polymorphisms and establishing patient risk. Moreover, hepatic DPD activity, and thus DPD deficiency incidence, can be predicted by assessing peripheral blood mononuclear cell (PBMC) DPD activity. However, to date, there are no established methods to quantify DHP and β-UP activity clinically.

The most direct method to understand the effect of the genetic polymorphisms of these enzymes on FP pharmacokinetics is to measure metabolite concentrations in blood and urine from subjects with the respective genotypes after FP administration. However, in vivo testing is highly invasive due to
continuous blood sampling and poses a considerable risk of FP-related toxicity. Additionally, as the variants of interest are mainly low-frequency polymorphisms, the recruitment of an adequate subject pool to obtain statistically significant data is considerably difficult. While pyrimidine metabolites in blood and urine have been previously quantified to assess enzymatic activity in vivo, these have yielded contradictory results [23,24].

In contrast, in vitro testing using heterologous expression systems has yielded reproducible results using non-invasive methods to facilitate enzymatic activity assessment [25]. Amongst these, several in vitro FP analyses using Escherichia coli or mammalian cells have been reported. While other in vitro techniques have been used to evaluate genetic polymorphisms including gene expression profiling, in this review, we focus on the in vitro analysis of the FP-metabolizing enzymes: DPD, DHP, and β-UP, thus providing further information to aid in the application of genetic testing in a clinical setting in light of recent novel insights.

2. Dihydropyrimidine Dehydrogenase (DPD)

DPD, the rate-limiting enzyme of the pyrimidine degradation pathway, catalyzes the reduction of 5-FU and uracil to FUH₂ and dihydrouracil (UH₂). The DPD gene (DPYD) is expressed in most human tissues, but the expression level is highest in the liver and PBMCs [26]. Located on chromosome 1p21, human DPYD is comprised of 23 exons and features a 3078 bp open reading frame, encoding a polypeptide containing 1025 amino acid residues [27].

DPD deficiency is an autosomal recessive disorder first reported in a child with neurological symptoms by Bakkeren et al., which was characterized by the accumulation of uracil and thymine in urine, blood, and cerebrospinal fluid [28]. The clinical symptoms include convulsions, autism, microcephaly, growth impairment, and intellectual disability, although asymptomatic cases have also been reported [29–31]. The frequency of DPD-deficient patients varies greatly across world populations. While Caucasian frequencies range from 3–5% for partial deficiency and 0.2% for complete deficiency, it is estimated to be extremely rare in Asians [32,33]. In the case of asymptomatic DPD deficiency, there is a considerable risk of FP accumulation during treatment, including 5-FU, which could lead to severe toxicity in patients [34–36]. Therefore, it is imperative to diagnose DPD deficiency before chemotherapy administration, even in cases with no prior clinical evidence of this condition.

Of the three metabolic enzymes, DPYD is the most studied gene. More than 500 DPYD polymorphisms to date have been identified and have been linked to FP-related toxicity in cancer patients [22,37–45]. Several of these variants are known to alter amino acid sequence or mRNA splicing, resulting in decreased enzymatic activity. Within the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines, three variants (c.85T > C (DPYD*9A, p.C29R), c.1627A > G (DPYD*5, p.I543V), and c.2194G > A (DPYD*6, p.V732I)) are reported to have no effect on enzyme activity [46]. Four variants that cause exon 14 skipping or amino acid substitution (c.1905 + 1G > A (IVS14 + 1G > A, DPYD*2A), c.1679T > G (DPYD*13, p.I560S), c.1129 – 5923C > G/hapB3, and c.2846A > T (p.D949V)) are designated as having reduced enzymatic function and thus increase the risk of developing toxicity. Similarly, the Dutch Pharmacogenetics Working Group (DPWG) guidelines define these same four variants as risk factors for FP-related toxicity and recommend reducing treatment dosage when a patient possesses one of them [47]. Although DPYD*9A, *5, and *6 are common variants in many ethnic groups, these four risk variants have not yet been identified in Asians [48–50].

For most identified DPYD variants, except those mentioned above, the effect on DPD activity is unknown, and it is important to clarify the DPD phenotype [51]. The current standard to predict DPD activity measures its enzymatic activity in PBMCs, which correlates with hepatic DPD activity [52,53]. However, this method is not easily implemented in its current form in routine medical care, as it lacks solid evidence of clinical utility. Due to insufficient sensitivity, methods for quantifying pyrimidine metabolites in blood or urine might not identify patients with partial DPD deficiencies [23]. Moreover, additional studies on the clinical validity and utility of these tests are required before implementation can be justified.
In vitro testing is one of the methods used for estimating DPD phenotypes and for the functional analysis of identified non-synonymous variants [54–60]. Several studies of such tests using *E. coli* or mammalian cell expression systems have been reported (Table 1). Ogura et al. functionally analyzed two variants (G366A and T768K) identified from 150 healthy Japanese volunteers using an *E. coli* expression system [57]. Interestingly, while the G366A mutation produced a decreased intrinsic clearance (*CLint*) for 5-FU, reducing DPD activity by 50%, the T768K mutation did not. However, T768K-related activity decreased at a faster rate than that of wild-type DPD, suggesting protein instability. In a subsequent study, Offer et al. expressed 80 non-synonymous variants in HEK293T/c17 cells and measured their enzymatic activities using 5-FU as a substrate [58]. M166V, E828K, K861R, and P1023T exhibited significantly higher activity than wild-type DPD. In contrast, 31 variants, including D949V, exhibited significantly lower activity than wild-type DPD. Elraiyah et al. also analyzed 10 non-synonymous variants identified from 588 Somali and Kenyan individuals using HEK293T/c17 cells [59], in which P86L, P237L, A513V, T793I, V941A, and P1023S exhibited significantly reduced DPD activities. We have characterized 21 DPD allelic variants identified from 1070 Japanese individuals by transient expression in 293FT cells [60]. Among these, 10 (T298M, V313L, V335M, A380V, V434L, V515I, R592W, T768K, H807R, and V826M) showed significantly reduced *CLint* values relative to wild-type DPD, and the 5-FU metabolic activity of G926V was practically zero. These reports have yielded consistent results for DPYD*2A, which exhibited decreased activity, and for DPYD*5 (I543V) and *6 (V732I), which exhibited activities that were not considerably different from that of wild-type DPD. In contrast, there are variants such as DPYD*9A (C29R) and M166V, whose reported activities differ significantly among previous reports. Ogura et al. and our group found that M166V had a lower activity compared with that of wild-type DPD, while Offer et al. reported a reduction in activity for M166V. The differences in these activities are believed to be due to the differences in assay conditions and cell lines used. Notably, we and Ogura et al. reported DPD variants that were identified almost exclusively in Japanese individuals. Therefore, this raises awareness of the possibility of unidentified rare and relevant ethno-specific variants, which could lead to severe FP-related toxicity.

From a biochemical perspective, human DPD is a flavoprotein containing a single flavin mononucleotide (FMN), a single flavin adenine dinucleotide (FAD), and four iron-sulfur (FeS) clusters. Human DPD consists of five major domains [61–64]. Domain I (residues 27–172) and domain V (residues 1–26, 848–1025) each contain two FeS clusters. FAD- and nicotinamide adenine dinucleotide phosphate (NADPH)-binding sites are located in domain II (residues 173–286, 442–524) and domain III (residues 287–441), respectively. FMN and the substrate both bind to domain IV (residues 525–847). Domains II and IV are essential for DPD activity in the structural analysis of variants. Amino acid substitutions that have been observed to affect protein conformation adjacent to the FeS clusters have also caused a significant decrease in enzyme activity.

Henricks et al. described a prediction method using an activity score system and divided DPYD alleles into three categories, consisting of fully functional alleles (wild-type; value of 1), reduced activity alleles (c.2846A > T and HapB3; value of 0.5), and nonfunctional alleles (DPYD*2A and *13; value of 0) [66]. Allele values are totaled for a given patient, leading to an individual gene activity score that represents the DPD phenotype of the patient. Moreover, Shrestha et al. developed a DPYD-specific variant classifier (DPYD-Varifier) using machine learning of in vitro functional data from 156 variants [67]. This model exhibited an accuracy of 85% and outperformed other in silico prediction tools, including PROVEAN, SIFT, and Polyphen-2. In the future, it may be possible to easily predict in vivo DPD activity using machine learning by creating compound databases by gathering detailed information from in vitro analyses. Recently, a list of DPYD variants has been added to the Pharmacogene Variation Consortium website (https://www.pharmvar.org/gene/DPYD). It is expected that evidence-based decisions on FP therapeutic regimens and patient-specific dose guidelines could be applied on the basis of an activity score formula, as has been recommended and implemented with other clinically relevant metabolic enzymes.
| dbSNP rsID   | PharmVar ID | Location | Nucleotide Change | Amino Acid Substitution | Domain | Expression System | Substrates | Effect | References |
|------------|-------------|----------|------------------|-------------------------|--------|------------------|-----------|--------|------------|
| rs150036960 | PV00901     | Exon 2   | 46C > G          | L16V                    | V      | HEK293T/c17      | 5-FU      | Normal function | [58]       |
| rs72549310  | PV01042     | Exon 2   | 61C > T          | R21X                    | I      | HEK293T/c17      | 5-FU      | No function      | [58]       |
| rs80081766  | PV01307     | Exon 2   | 62G > A          | R21Q                    | I      | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs1801265   | PV00910     | Exon 2   | 85T > C          | (DPYD*9A)               | I      | HEK293T/c17      | 5-FU      | Increased function | [54]       |
| rs371587702 | PV00962     | Exon 3   | 194C > T         | T65M                    | I      | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs143986398 | PV00887     | Exon 4   | 257C > T         | P86L                    | I      | HEK293T/c17      | 5-FU      | No function      | [59]       |
| rs150385342 | PV00911     | Exon 4   | 295delTCAT       | F100fs                  | I      | HEK293T/c17      | 5-FU      | No function      | [58]       |
| rs141462178 | PV00878     | Exon 5   | 2T > G           | A105T                   | I      | HEK293T/c17      | 5-FU      | 79% of Clint ratio | [60]       |
| rs200562975 | PV00927     | Exon 5   | 451A > G         | N151D                   | I      | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs2297595   | PV0943      | Exon 6   | 496A > G         | M166V                   | I      | HEK293T/c17      | 5-FU      | Increased function | [58]       |
| rs139834141 | PV00871     | Exon 6   | 498G > A         | M166I                   | I      | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs371792178 | –           | Exon 6   | 524T > G         | S175L                   | II     | 293FT            | 5-FU      | 131% of Clint ratio | [60]       |
| rs115232998 | PV00862     | Exon 6   | 557A > G         | Y109N                   | I      | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs72549308  | PV01040     | Exon 6   | 601A > C         | S201R                   | II     | HEK293T/c17      | 5-FU      | No function      | [58]       |
| rs72549307  | PV01039     | Exon 6   | 632A > G         | Y211C                   | II     | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs1801266   | PV00911     | Exon 7   | 703C > T         | R235W                   | II     | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs790025995 | PV01299     | Exon 7   | 710C > T         | P237L                   | II     | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs45589337  | PV00984     | Exon 8   | 775A > G         | K259E                   | II     | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs777220476 | PV01275     | Exon 9   | 851G > T         | G284V                   | II     | HEK293 Flp-In    | Thymine   | No function      | [56]       |
| rs146356975 | PV00895     | Exon 9   | 868A > G         | K290E                   | III    | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs143878757 | PV00886     | Exon 9   | 893C > T         | T298M                   | III    | 293FT            | 5-FU      | 50% of Clint ratio | [60]       |
| rs183105782 | PV00914     | Exon 9   | 910T > C         | Y304H                   | III    | HEK293T/c17      | 5-FU      | Decreased function | [58]       |
| rs150437414 | PV00904     | Exon 9   | 929T > C         | L310S                   | III    | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| rs145112791 | PV00891     | Exon 9   | 934C > T         | L312F                   | III    | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
| –           | –           | Exon 9   | 937G > T         | V313L                   | III    | 293FT            | 5-FU      | 30% of Clint ratio | [60]       |
| rs201018345 | PV00933     | Exon 10  | 967G > A         | A323T                   | III    | HEK293T/c17      | 5-FU      | Normal function  | [58]       |
Table 1. Cont.

| dbSNP rsID | PharmVar ID | Location | Nucleotide Change | Amino Acid Substitution | Domain | Expression System | Substrates | Effect | References |
|------------|-------------|----------|-------------------|-------------------------|--------|-------------------|------------|--------|------------|
| rs72549306 | PV01038     | Exon 10  | 1003G > A         | V335M                   | III    | 293FT             | 5-FU       | 47% of CLint ratio | [60]       |
| rs72549306 | PV01037     | Exon 10  | 1003G > T         | V335L                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs183385770| PV00915     | Exon 10  | 1024G > A         | D342N                   | III    | HEK293T/c17       | 5-FU       | Decreased function | [58]       |
| rs190577302| PV00919     | Exon 10  | 1054C > G         | L352V                   | III    | HEK293T/c17       | 5-FU       | Decreased function | [58]       |
| rs143154602| PV00882     | Exon 10  | 1057C > T         | R353C                   | III    | HEK293T/c17       | 5-FU       | No function        | [58]       |
|            |             |          |                   |                         |        | 293FT             | 5-FU       | 71% of CLint ratio | [60]       |
| rs72549305 | PV01036     | Exon 10  | 1097G > C         | G366A                   | III    | Escherichia coli   | 5-FU       | 47% of CLint ratio | [57]       |
| rs61622928 | PV01302     | Exon 11  | 1156G > T         | E386X                   | III    | HEK293T/c17       | 5-FU       | No function        | [58]       |
| rs140602333| PV00874     | Exon 11  | 1180C > T         | R394W                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs143815742| PV00883     | Exon 11  | 1181G > T         | R394L                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [59]       |
| rs143815742| PV00884     | Exon 11  | 1181G > A         | R394Q                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [59]       |
| rs61622928 | PV01018     | Exon 11  | 1218G > A         | M406I                   | III    | HEK293 Flp-In     | 5-FU       | Thymine            | [55]       |
| rs200064537| PV00925     | Exon 11  | 1260T > A         | N420K                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs764666241| PV0183      | Exon 11  | 1278G > T         | M426I                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs200693895| PV00931     | Exon 11  | 1280T > C         | V427A                   | III    | HEK293 Flp-In     | 5-FU       | Thymine            | [56]       |
| rs142512579| PV00880     | Exon 11  | 1294G > A         | D432N                   | III    | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
|            |             |          |                   |                         |        | 293FT             | 5-FU       | 44% of CLint ratio | [60]       |
| rs186169810| PV00916     | Exon 11  | 1314T > G         | P438L                   | III    | HEK293T/c17       | 5-FU       | Decreased function | [58]       |
| rs27957510 | PV01043     | Exon 12  | 1349C > T         | A450V                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs144395748| PV00888     | Exon 12  | 1358C > G         | P453R                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs199549923| PV00921     | Exon 12  | 1403C > A         | T468N                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs72549304 | PV01035     | Exon 12  | 1475C > T         | S492L                   | II     | HEK293T/c17       | 5-FU       | Decreased function | [58]       |
| rs111858276| PV00857     | Exon 12  | 1484A > G         | D495G                   | II     | HEK293T/c17       | 5-FU       | Decreased function | [58]       |
| rs138391988| PV00867     | Exon 12  | 1519G > A         | V507I                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs760663364| PV01150     | Exon 13  | 1538C > T         | A513V                   | II     | HEK293T/c17       | 5-FU       | Decreased function | [59]       |
| rs148994843| PV00900     | Exon 13  | 1543G > A         | V515I                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
|            |             |          | 1567C > T         | L523F                   | II     | HEK293T/c17       | 5-FU       | Normal function    | [59]       |
| dbSNP rsID      | PharmVar ID | Location | Nucleotide Change | Amino Acid Substitution | Domain | Expression System | Substrates | Effect                          | References |
|----------------|-------------|----------|------------------|------------------------|--------|------------------|------------|--------------------------------|------------|
| rs190951787    | PV00920     | Exon 13  | 1577C > G        | T526S                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs1180771326   | PV00864     | Exon 13  | 1582A > G        | IS28V                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [59]       |
| rs1801158      | PV00907     | Exon 13  | 1601G > A (DPYD*4) | S534N                  | IV     | HEK293T/c17      | 5-FU       | Increased function             | [54]       |
| rs142619737    | –           | Exon 13  | 1615G > C        | G539R                  | IV     | HEK293 FLp-In    | Thymine    | Decreased function             | [55]       |
| rs1801159      | PV00908     | Exon 13  | 1627A > G (DPYD*5) | I543V                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [54]       |
| rs142619737    | –           | Exon 13  | 1615G > C        | G539R                  | IV     | HEK293 FLp-In    | Thymine    | Decreased function             | [55]       |
| rs55886062     | PV01000     | Exon 13  | 1679T > G (DPYD*13) | I560S                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs201615754    | PV00937     | Exon 13  | 1682G > T        | R561L                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs59086055     | PV01015     | Exon 14  | 1774C > T        | R592W                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [60]       |
| rs138616379    | PV00869     | Exon 14  | 1775G > A        | R592Q                  | IV     | HEK293T/c17      | 5-FU       | Decreased function             | [58]       |
| rs145773863    | PV00894     | Exon 14  | 1777G > A        | G593R                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [58]       |
| rs147601618    | PV00898     | Exon 14  | 1796T > C        | M599T                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| Rs72549304     | PV01034     | Exon 4   | 1898delC (DPYD*3) | P633fs                 | IV     | HEK293T/c17      | 5-FU       | No function                     | [58]       |
| rs3918289      | PV00982     | Exon 14  | 1905C > T/G (DPYD*2A) | N635K                 | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs3918290      | PV00983     | Intron 14 | 1905 + 1G > A    | I636L                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [54]       |
| rs55971861     | PV01003     | Exon 15  | 1906A > C        | I636L                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs138545885    | PV00868     | Exon 16  | 1990G > T        | A664S                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs137999090    | PV00866     | Exon 16  | 2021G > A        | G674D                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [58]       |
| rs145548112    | PV00893     | Exon 17  | 2096G > C        | R699T                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs146529561    | PV00896     | Exon 18  | 2186C > T        | A729V                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs1801160      | PV00909     | Exon 18  | 2194G > A        | V732I                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [54]       |
| rs60511679     | PV01017     | Exon 18  | 2195T > G        | V732G                  | IV     | HEK293T/c17      | 5-FU       | Normal function                | [58]       |
| rs112766203    | PV00858     | Exon 18  | 2279C > T        | T760I                  | IV     | HEK293T/c17      | 5-FU       | Decreased function             | [58]       |
| rs56005131     | PV01004     | Exon 19  | 2303C > A        | T768K                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [58]       |
| rs199634007    | PV00922     | Exon 19  | 2336C > A        | T779N                  | IV     | HEK293T/c17      | 5-FU       | No function                     | [58]       |
| dbSNP rsID   | PharmVar ID | Location | Nucleotide Change | Amino Acid Substitution | Domain | Expression System | Substrates | Effect          | References |
|-------------|-------------|----------|-------------------|-------------------------|--------|-------------------|------------|-----------------|------------|
| rs547099198 | PV00994     | Exon 19  | 2378C > T         | T793I                   | IV     | HEK293T/c17       | 5-FU       | Decreased function | [59]       |
| rs200687447 | PV00930     | Exon 20  | 2420A > G         | H807R                   | IV     | HEK293T/c17       | 5-FU       | 50% of CLint ratio | [60]       |
| rs60139309  | PV01016     | Exon 20  | 2582A > G         | K861R                   | V      | HEK293T/c17       | 5-FU       | Increased function  | [58]       |
| rs201035051 | PV00934     | Exon 21  | 2623A > C         | K875Q                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs55674432  | PV00996     | Exon 21  | 2639G > T         | G880V                   | V      | HEK293T/c17       | 5-FU       | No function        | [58]       |
| rs147545709 | PV00897     | Exon 21  | 2656C > T         | R886C                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs1801267   | PV00912     | Exon 21  | 2657G > A (DPYD*9B) | R886H                  | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs188052243 | PV00918     | Exon 21  | 2678A > G         | N893S                   | V      | HEK293T/c17       | 5-FU       | 61% of CLint ratio | [60]       |
| rs67376798  | PV01031     | Exon 22  | 2846A > T         | D949V                   | V      | HEK293 Flp-In     | Thymine    | Decreased function  | [55]       |
| rs141044036 | PV00876     | Exon 22  | 2872A > G         | K958E                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs145529148 | PV00892     | Exon 23  | 2915A > G         | Q972R                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs72547602  | PV01033     | Exon 23  | 2921A > G         | D974V                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs72547601  | PV01032     | Exon 23  | 2933A > G         | H978R                   | V      | HEK293T/c17       | 5-FU       | No function        | [58]       |
| rs61757362  | PV01019     | Exon 23  | 2948C > T         | T983I                   | V      | HEK293T/c17       | 5-FU       | Decreased function  | [58]       |
| rs202144771 | PV00941     | Exon 23  | 2977C > T         | L993F                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs139459586 | PV00870     | Exon 23  | 2978T > G         | L993R                   | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs1801268   | PV00913     | Exon 23  | 2983G > T (DPYD*10) | V995F                  | V      | HEK293T/c17       | 5-FU       | No function        | [58]       |
| rs140114515 | PV00873     | Exon 23  | 3049G > A         | V1017I                  | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs148799944 | PV00899     | Exon 23  | 3061C > A         | V1021L                  | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs114096998 | PV00860     | Exon 23  | 3067C > A         | P1023T                  | V      | HEK293T/c17       | 5-FU       | Normal function    | [58]       |
| rs114096998 | PV00861     | Exon 23  | 3067C > T         | P1023S                  | V      | HEK293T/c17       | 5-FU       | Decreased function  | [59]       |
3. Dihydropyrimidinase (DHP)

DHP, as previously mentioned, catalyzes the hydrolytic ring opening of FUH$_2$ and UH$_2$ and is expressed mainly in the liver and kidneys [15,68]. The human DHP gene (DPYS) consists of 10 exons mapped to chromosome 8q22, and features a 1560 bp open reading frame, corresponding to a 519 amino acid protein [17].

DHP deficiency is an autosomal recessive disease characterized by the accumulation of UH$_2$ and dihydrothymine (TH$_2$) in blood, urine, and cerebrospinal fluid [69]. The clinical phenotype of DHP-deficient patients is highly variable, ranging from asymptomatic to exhibiting symptomatology similar to that of DPD deficiency, including seizures, intellectual disability, growth impairment, and dysmorphic facial features [70–72]. To date, 35 genetically confirmed patients with DHP deficiency have been reported [33,73–77]. However, potential asymptomatic deficiencies might be present in a population with a low frequency of DPD deficiencies. In screening 21,200 healthy Japanese infants, Sumi et al. estimated the deficiency frequency to be approximately 1/10,000 [73]. Akai et al. analyzed the DPYS coding regions from 183 Japanese individuals, in which the c.349T $>$ C (p.W117R) and c.1001A $>$ G (p.Q334R) variants were identified with an allelic frequency of 0.27% and 1.09%, respectively [78].

To date, multiple studies have reported on the relationship between DPD deficiency and the risk of developing FP-related toxicity. However, there is an increasing awareness that patients with DHP deficiencies are also prone to the development of severe FP-associated toxicity. One such study identified severe FP-related toxicity in a female breast cancer patient with the DPYS heterozygous mutation c.833G $>$ A (p.G278D) [21]. We previously reported about a patient with severe capecitabine-associated toxicity and DHP deficiency caused by a compound DPYS heterozygous mutation, c.1001A $>$ G (p.Q334R) and c.1393C $>$ T (p.R465X), including a genetic analysis of the patient’s family [79]. Urinary pyrimidine analysis of the patient’s family revealed that the UH$_2$/uracil ratio of heterozygous individuals was similar to that of wild-type individuals. Although heterozygous patients are predominantly asymptomatic, severe toxicity might occur during chemotherapy containing FPs, rendering the need for genetic testing before FP administration [80].

It is noteworthy that a sizable number of DHP-deficient patients have been identified in East Asian populations. Hamajima et al. identified a single frameshift mutation and five DPYS missense variants in six Japanese patients with dihydropyrimidinuria [17]. Nakajima et al. reported two Chinese pediatric patients with DHP deficiency caused by the compound DPYS heterozygous mutation c.1443 + 5G $>$ A (exon 8 skipping) [81]. Moreover, Nakajima et al. identified eight variants, including four novel missense mutations and one novel deletion in four DHP-deficient patients [77]. Thus, DPYS polymorphisms could emerge as novel pharmacogenomic markers associated with severe FP-related toxicity in diverse global populations.

Recently, in vitro functional characterization of DHP variants using heterologous expression systems, including E. coli and mammalian cells, has been reported (Table 2). Van Kuilenburg et al. reported that in the case of 14 variants (L7V, M70T, D81G, G278D, R302Q, L337P, T343A, W360R, V364M, S379R, R412M, R465X, R475X, and R490C) expressed in E. coli, the hydrolytic ring opening of radiolabeled UH$_2$ was markedly altered [71,76]. Hamajima et al. and Thomas et al. reported that six variants (L7V, T68R, Q334R, W360R, G435R, and R490C) showed lower activities than wild-type DHP in COS-7 and RKO cells expression systems [17,82]. We have characterized 21 DHP variants and wild-type DHP expressed in 293FT cells using UH$_2$ and FUH$_2$ as substrates [83]. Among these, 13 variants (N16K, T68R, M70T, D81G, G278D, R302Q, L337P, W360R, S379R, G435R, R465X, R475X, and R490C) demonstrated no enzymatic activity, and five variants (W117R, Q334R, T343A, V364M, and R412M) showed significantly lower $CL_{int}$ values than wild-type DHP. Except for L7V, the results of this study corroborated those of other in vitro studies, suggesting that the specific experimental conditions reflected the in vivo activities of the assayed variants. The divergence observed for L7V might be due to differences in assay conditions, substrate concentrations, or expression systems used.
Table 2. DPYS variants reported in in vitro analysis.

| dbSNP rsID   | Location | Nucleotide Change | Amino Acid Substitution | Expression System | Substrates          | Effect                     | References |
|-------------|----------|-------------------|-------------------------|-------------------|---------------------|----------------------------|------------|
| rs199618701 | Exon 1   | 17G > A           | R6Q                     | 293FT             | FUH₂               | 120% of CL<sub>int</sub> ratio | [83]       |
| rs57732538  | Exon 1   | 19C > G           | L7V                     | RKO E. coli       | FUH₂, UH₂          | 65% of wild-type DHP       | [82]       |
| rs572241599 | Exon 1   | 48C > G           | N16K                    | 293FT             | FUH₂               | No function               | [83]       |
|             | Exon 1   | 203C > G          | T68R                    | COS-7             | FUH₂, 5-bromo-UH₂  | 1.5% of wild-type DHP     | [17]       |
| rs370718225 | Exon 1   | 209T > C          | M70T                    | 293FT E. coli     | FUH₂, UH₂          | No function               | [83]       |
|             | Exon 1   | 242A > G          | D81G                    | 293FT E. coli     | FUH₂, UH₂          | No function               | [83]       |
|             | Exon 2   | 349T > C          | W117R                   | 293FT             | FUH₂               | 44% of CL<sub>int</sub> ratio | [83]       |
| rs36027551  | Exon 3   | 541C > T          | R181W                   | RKO               | FUH₂, UH₂          | 99% of wild-type DHP      | [82]       |
| rs751371011 | Exon 4   | 750G > A          | M250L                   | HEK293            | UH₂                | 2% of wild-type DHP       | [77]       |
|             | Exon 5   | 833G > A          | G278D                   | HEK293            | FUH₂, UH₂          | No function               | [83]       |
|             | Exon 5   | 884A > G          | H295R                   | HEK293            | UH₂                | 9.8% of wild-type DHP     | [77]       |
| rs200913682 | Exon 5   | 905G > A          | R302Q                   | 293FT E. coli     | FUH₂, UH₂          | No function               | [83]       |
| rs121964923 | Exon 6   | 1001A > G         | Q334R                   | HEK293 COS-7      | FUH₂, UH₂, 5-bromo-UH₂ | 9.7% of wild-type DHP | [77]       |
| rs530911437 | Exon 6   | 1010T > C         | L337P                   | 293FT E. coli     | FUH₂, UH₂          | 2.5% of wild-type DHP     | [17]       |
| rs201457190 | Exon 6   | 1027A > G         | T343A                   | 293FT E. coli     | FUH₂, UH₂          | No function               | [83]       |
|             | Exon 6   | 1078T > C         | W360R                   | 293FT E. coli     | FUH₂, UH₂, 5-bromo-UH₂ | 43% of CL<sub>int</sub> ratio | [83]       |
| rs121964924 | Exon 6   | 1090G > A         | V364M                   | 293FT E. coli     | FUH₂, UH₂          | 1.2% of wild-type DHP     | [17]       |
| rs138282507 | Exon 6   | 1090G > A         | V364M                   | 293FT E. coli     | FUH₂, UH₂          | 8% of CL<sub>int</sub> ratio | [83]       |
Table 2. Cont.

| dbSNP rsID     | Location | Nucleotide Change | Amino Acid Substitution | Expression System | Substrates         | Effect                          | References |
|---------------|----------|-------------------|-------------------------|-------------------|-------------------|--------------------------------|------------|
| rs201258823   | Exon 7   | 1137C > A         | S379R                   | 293FT             | FUH₂UH₂           | No function                    | [83]       |
|               |          |                   |                         | E. coli           |                   | 0.20–9% of wild-type DHP       | [76]       |
| rs267606774   | Exon 7   | 1235G > T         | R412M                   | 293FT             | FUH₂UH₂           | 36% of \( CL_{int} \) ratio    | [83]       |
| –             | Exon 8   | 1253C > T         | T418I                   | HEK293            | UH₂               | 64% of wild-type DHP           | [77]       |
| rs267606773   | Exon 8   | 1303G > A         | G435R                   | 293FT             | FUH₂5-bromo-UH₂   | 5.1% of wild-type DHP          | [17]       |
| rs201280871   | Exon 8   | 1393C > T         | R465X                   | 293FT             | FUH₂UH₂           | No function                    | [83]       |
| rs61758444    | Exon 8   | 1423C > T         | R475X                   | 293FT             | FUH₂UH₂           | 0.2–0.9% of wild-type DHP      | [76]       |
| rs142574766   | Exon 9   | 1468C > T         | R490C                   | 293FT             | FUH₂UH₂5-bromo-UH₂| 0.2–0.9% of wild-type DHP     | [76]       |
| Rs189448963   | Exon 9   | 1469G > A         | R490H                   | HEK293            | UH₂               | 1.7% of wild-type DHP          | [17]       |

References:
- [83]
- [76]
- [77]
Hsieh et al. reported that dimer formation is essential for DHP activity [84]. Within the cell, DHP is known to form a tetramer composed of subunits containing two zinc ions each [85–87]. Each DHP subunit consists of two domains, a large (β/α)8-barrel domain that binds the catalytic dimetal center and a small β-sandwich domain [88]. Each subunit also has two dynamic loops, which act as a lid for the substrate-binding pocket. DHP activity is exerted by the interaction of the C-terminus with the dynamic loop of the neighboring subunit [89–91]. We have performed immunoblotting assays of native proteins following blue native polyacrylamide gel electrophoresis and showed that oligomer formation is very important for DHP activity [83]. In the reduced or null-activity variants, the ability of DHP to form oligomers was reduced. The five variants G435R, R465X, R475X, R490C, and R490H introduce mutations in the C-terminus or lead to truncation of the C-terminus, thus affecting oligomer formation and resulting in loss of enzymatic activity. In contrast, the substitutions T68R, M70T, D81G, W117R, M250I, G278D, R302Q, Q334R, L337P, T343A, and R412M exist near the active site of the two dynamic loops, which result in conformational changes in the active site that reduce or eliminate activity. Thus, it has been clarified that changes in DHP activity are associated with amino acid substitutions, as well as changes in oligomer formation and the resulting three-dimensional structure. DHP deficiencies are rarely reported in Caucasians but are highly prevalent in Asians. Thus, we consider that these variants could serve as novel pharmacogenomic markers for the prevention of FP-related toxicity, especially in populations that have a low frequency of symptomatic DPD-deficiency cases.

4. β-Ureidopropionase (β-UP)

β-UP catalyzes the irreversible last step, converting FUPA and β-ureidopropionic acid (bUPA) to fluoro-β-alanine and β-alanine, respectively. The human β-UP gene, UPB1, is located on chromosome 22q11, contains 10 exons, and features an 1155 bp open reading frame; the gene encodes a polypeptide containing 384 amino acids [18]. Human β-UP activity has been detected predominantly in the liver and kidney [26,92].

β-UP deficiency is an autosomal recessive disease characterized by the accumulation of bUPA and N-carbamoyl-β-aminoisobutyric acid (NCBA) in urine, blood, and cerebrospinal fluid [93,94]. To date, 33 genetically confirmed patients with β-UP deficiency have been reported [94–100]. The clinical phenotype of these patients is highly variable but tends to center around neurological problems. Similar to DHP deficiency, β-UP deficiency is often reported in East Asian populations, including Japan and China. Although it has been reported that severe FP-related toxicity is caused by DPD and DHP deficiencies, little is known about the relationship between β-UP deficiency and FP-related toxicity.

There have been several reports of the in vitro analysis of 13 UPB1 variants with amino acid substitutions identified in β-UP-deficient patients (Table 3). Van Kuilenburg et al. and Thomas et al. reported that variant A85E expressed in E. coli and RKO cells was inactive [93,101]. In a separate study, van Kuilenburg et al., using an E. coli expression system, analyzed six β-UP variants (L13S, G235R, R236W, S264R, R326Q, and T359M) that had been previously identified in 16 β-UP-deficient patients, showing a significant reduction or loss of activity in all of them [95]. Nakajima et al. reported that the G31S, E271K, and R326Q variants expressed in HEK293 cells showed profound reductions in activity [97]. Moreover, Nakajima et al. performed native polyacrylamide gel electrophoresis of β-UP expressed in HEK293 cells and showed that octamer formation is necessary for β-UP activity as well as DHP activity. The majority of variants showed a significant reduction in enzymatic activity. However, whether these variants contribute to the development of FP-related toxicity remains unclear.
Table 3. UBP1 variants identified in β-UP deficient patients.

| db SNP rsID   | Location | Nucleotide Change | Amino Acid Substitution | Expression System | Substrates | Effect                  | References |
|--------------|----------|-------------------|-------------------------|-------------------|------------|-------------------------|------------|
| –            | Exon 1   | c.38T > C         | p.L13S                  | E. coli           | bUPA       | 6% of wild-type β-UP    | [95]       |
| rs200145797  | Exon 1   | c.91G > A         | p.G31S                  | HEK293            | bUPA       | 52% of wild-type β-UP   | [97]       |
| rs121908066  | Exon 2   | c.209G > C        | p.R70P                  |                   |            | No reports of in vitro study | [98]       |
| rs34035085   | Exon 2   | c.254C > A        | p.A85E                  | E. coli           | bUPA       | No function             | [93]       |
| –            | Exon 6   | c.703G > A        | p.G235R                 | E. coli           | bUPA       | 2.7% of wild-type β-UP  | [101]      |
| rs144135211  | Exon 6   | c.706C > T        | p.R236W                 | E. coli           | bUPA       | No function             | [95]       |
| rs145766755  | Exon 7   | c.792C > A        | p.S264R                 | E. coli           | bUPA       | No function             | [95]       |
| –            | Exon 7   | c.811G > A        | p.E271K                 | HEK293            | bUPA       | 20% of wild-type β-UP   | [95]       |
| –            | Exon 7   | c.851G > T        | p.C284F                 |                   |            | 0.7% of wild-type β-UP  | [97]       |
| rs1375840064 | Exon 7   | c.853G > A        | p.A285T                 |                   |            | No reports of in vitro study | [99]       |
| –            | Exon 7   | c.857T > C        | p.I286T                 | HEK293            | bUPA       | 70% of wild-type β-UP   | [97]       |
| rs118163237  | Exon 9   | c.977G > A        | p.R326Q                 | E. coli           | bUPA       | No function             | [95]       |
| rs369879221  | Exon 10  | c.1076C > T       | p.T359M                 | E. coli           | bUPA       | No function             | [95]       |
Fidlerova et al. performed an analysis of the entire UPB1 coding sequence from 113 Czech cancer patients treated using FP-based chemotherapy \[102\]. Nine UPB1 variants were detected in a subpopulation of patients exhibiting severe toxicity, including a novel mutation affecting the coding sequence. An analysis of the effect of UPB1 variants on FP-related toxicity in the population of all analyzed patients revealed an association between the c.−80C > G (rs2070474) variant and gastrointestinal toxicity. In addition, a strong positive correlation was found between carriers of the homozygous c.−80G variant and the development of severe mucositis. Thomas et al. deduced that the c.−80G variants might alter the potential binding sites of transcription factors, resulting in a statistically non-significant decrease in UPB1 gene expression in patients who are homozygous for the c.−80G allele. This indicates the possibility that UPB1 variants have an additive and relatively minor effect on the development of FP-related toxicity compared with that of the DPYD and DPYS variants.

5. Other Considerations

Genetic variations in TYMS, MTHFR, and miR-27a have also been associated with FP-related toxicity. Clinical and preclinical studies have shown the importance of intracellular levels of TYMS, a target for 5-FU involved in DNA repair and synthesis \[103\], as a determinant of sensitivity to 5-FU treatment. Its overexpression stemming from polymorphic TYMS variations lead to differing response rates to 5-FU therapy \[104\]. The three most studied TYMS genetic polymorphisms are the variable numbers of tandem repeat (VNTR) polymorphisms comprising 28 bp sequence repeats (rs34743033), rs2853542C > G, and the 3’-untranslated region polymorphism 1494delTTAAAG (rs34489327). These polymorphisms alter gene expression, mRNA stability, or TYMS expression levels, resulting in the development of treatment resistance and toxicity \[105–107\]. MTHFR plays a role in the metabolism of folate and forms the reduced folate cofactor essential for TYMS inhibition by 5-FU. Two non-synonymous variants, c.677C > T (p.A222V , rs1801133) and c.1298A > C (p.E429A, rs1801131), alter intracellular folate distribution and decrease enzymatic activity \[105,107\]. The micro RNA miR-27a polymorphism (rs895819A > G) has been associated with FP-related toxicity, more so in DPD-deficient patients, as increased miR-27a expression leads to decreased DPD mRNA expression \[108–110\]. To date, however, studies involving these genetic polymorphisms have yielded inconsistent results, and further assessment is needed to assess their clinical utility and potential use as biomarkers.

6. Conclusions

FPs are degraded by three metabolic enzymes (DPD, DHP, and β-UP), and a reduction or elimination of their activities leads to severe FP-related toxicity. Therefore, predicting enzymatic activity is critical before the administration of FPs, in which in vitro testing has proven to be a useful complementary method to in vivo testing. This review summarized the findings on the functional characterization of DPD, DHP, and β-UP using in vitro analysis. To date, a large number of DPD variants have been analyzed, giving rise to a significant body of evidence regarding the four most commonly identified risk variants in Caucasians (DPYD*2A, DPYD*13, c.1129− 5923C > G/hapB3, and c.2846A > T) that are associated with an increased risk of 5-FU-related toxicity. Additionally, a system for predicting in vivo DPD activity has been developed on the basis of in vitro analysis results. This has provided further evidence that rare DHP variants might be useful predictive biomarkers of FP-related toxicity in populations with low frequencies of DPD deficiency, as is the case for Asians. Notably, β-UP is not known to be associated with FP-related toxicity, although variants with reduced function have been identified. Currently, studies comprising in vivo and in vitro correlation of frequent DPYD polymorphisms are advancing applicability as well as underlying the importance of including infrequent DPYD, DPYS, and UPB1 variants, as their collective data is insufficient to establish their clinical consequences fully. Additional in vitro and large-scale in vivo studies using standardized methodologies are needed to generate clear evidence for rare variants and verify existing associative studies.
Recently, the underlying mechanisms by which amino acid substitutions alter enzymatic activities by influencing three-dimensional structures have been elucidated; these findings have significant implications toward the interpretation of previously acquired data and how they could be further used to aid clinical decision making for optimal treatments and forewarning the need for alternative chemotherapy regimens. We expect that this report and others related to genetic FP-metabolizing enzyme variants will be useful in the development and further validation of pharmacogenetic testing with the future inclusion of additional biomarkers. In this way, these developments could lead to optimal personalized medicine grounded on genetic polymorphisms.

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