The Role of \textit{Dihydropyrimidine Dehydrogenase} and \textit{Thymidylate Synthase} Polymorphisms in Fluoropyrimidine-Based Cancer Chemotherapy in an Iranian Population

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\section*{Abstract}

\textbf{Background:} The fluoropyrimidine drug 5-Fluorouracil (5-FU) and the prodrug capecitabine have been extensively used for treatment of many types of cancer including colorectal, gastric, head and neck. Approximately, 10 to 25\% of patients suffer from severe fluoropyrimidine-induced toxicity. This may lead to dose reduction and treatment discontinuation. Pharmacogenetics research could be useful for the identification of predictive markers in chemotherapy treatment. The aim of the study was to investigate the role of five genetic polymorphisms within two genes (**DPYD**, **TYMS**) in toxicity and efficacy of fluoropyrimidine-based chemotherapy.

\textbf{Methods:} Total genomic DNA was extracted from 83 cancer patients treated with fluoropyrimidine-based chemotherapy. In this study, three polymorphisms were genotyped in \textit{dihydropyrimidine dehydrogenase} gene c.1905+1 G>A (**DPYD**\textsuperscript{2A}; rs3918290), c.1679 T>G (I5605; **DPYD**\textsuperscript{13}; rs55886062), and c.2846A>T (**D949V**; rs67376798) and two polymorphisms, besides the Variable Number of Tandem Repeat (VNTR) polymorphism and \textit{6-bp} insertion/deletion polymorphism in \textit{thymidylate synthase} gene. The analysis of polymorphisms for rs3918290, rs55886062, rs67376798 and \textit{6-bp} insertion/deletion in **TYMS** was done by Polymerase Chain Reaction-restriction Fragment Length Polymorphism (**PCR-RFLP**) **TYMS** VNTR analysis. 5-FU-related toxicities such as anemia, febrile neutropenia, neurotoxicity, vomiting, nausea, and mucositis were evaluated according to NCI-CTC criteria version 4.0. T-test and chi-square were used and \textit{p}-values less than 0.05 were considered statistically significant.

\textbf{Results:} **DPYD** gene polymorphisms were not observed in this study. The frequency of the **TYMS** \textit{6-bp} allele was 40.35\% and the \textit{-6-bp} allele was 59.65\% in this study. The frequency of VNTR **2R** allele was 48.75\% and **3R** allele was 51.15\%. Toxicity grade II diarrhea, mucositis, nausea, vomiting, and neurotoxicity was 2.2, 24.1, 15.7, 6, and 51.8\%, respectively. Thymidylate synthase ins/del polymorphisms were associated with increased grade III neurotoxicity (\textit{p}=0.02). Furthermore, anemia grade III was significantly associated with **2R/2R** genotype (0.009).

\textbf{Conclusion:} \textit{Thymidylate synthase} gene polymorphisms may play a key role in fluoropyrimidine-based chemotherapy. Although rare **DPYD** polymorphisms were not observed in our study, according to large population studies, **DPYD** gene polymorphisms could be used as a predictive biomarker for patient treatments.


\textbf{Keywords:} 5-fluorouracil, Dihydropyrimidine dehydrogenase, Fluoropyrimidines, Pharmacogenetics, Thymidylate synthase

\section*{Introduction}

The fluoropyrimidine drug 5-Fluorouracil (5-FU) and the prodrug capecitabine are used for treatment of a variety of solid cancers including gastrointestinal tract and breast \textsuperscript{1}.
5-FU/leucovorin combined with oxaliplatin (FOLFOX) is currently a standard chemotherapy regimen in treating colorectal and gastric cancers and has been shown clearly to improve response to treatment. However, the development of gastrointestinal, hematological and neurological toxicities is a major clinical problem following FOLFOX treatment. The clinical utility of DPYD polymorphisms has been demonstrated in a panel of markers to predict clinically actionable 5-FU toxicity. Other polymorphisms have been suggested as markers in TYMS, Enolase Superfamily Member 1 (ENOSF1), MethyleneTetraHydroFolate Reductase (MTHFR), ATP-binding cassette sub-family B member 1 (ABCB1), and Cytidine Deaminase (CD) 
Approximately, 85% of administered 5-FU dose is degraded via dihydropyrimidine dehydrogenase (DPD, encoded by the DPYD gene). Functional Single-Nucleotide Polymorphisms (SNPs) in the DPYD gene alter DPD activity which may lead to the development of severe 5-FU related toxicities. The clinically most relevant variant is DPYD*2A (c.1905+1G>A, previously named IVS14+1G>A or DPD*2A), or rs3918290. Second polymorphism is a 6-bp insertion/deletion in the 3′-untranslated region (3′-UTR) of TYMS. These two polymorphisms have been associated with altered TYMS expression, toxicity and an improved clinical response. The aim of this study was to determine whether the five genetic polymorphisms within two genes (DPYD, TYMS) are associated with severe toxicity in patients with cancer receiving fluoropyrimidine-based chemotherapy.

Materials and Methods

Patients and study design

Eighty three cancer patients received 5-FU-based chemotherapy at Hazrat-e Rasool hospital and Masoud clinic, Tehran, Iran between February 2014 and June 2016. The cancer types among the patients were distributed as follows: 28 colon (33.7%), 37 rectum and rectosigmoid (44.5%), and 18 stomach (21.6%). Patients were questioned about nausea and vomiting, mucositis, diarrhea, hand-foot syndrome, and neutropenia at every cycle. Eligible patients were treated with FOLFOX4 (Oxaliplatin 85 mg/m² (2 hr infusion on day 1), leucovorin (100 mg/m² as 2 hr infusion on day 1), 5-fluorouracil bolus (400 mg/m²) and 22 hr infusion (600 mg/m²) on days 1 and 2 every 2 weeks for 6 months (12 cycles). Toxicity was evaluated according to Common Terminology Criteria for Adverse Events (CTCAE), version 4. Neurotoxicity was evaluated according to the oxaliplatin-specific scale. The study was approved by the ethics committees (NIGEB) (940101-IV-503). All patients signed a written informed consent before entering the study.

Genotyping

Total genomic DNA was extracted from 200 µl whole blood using the GeneAll kit (South Korea) and stored at -20°C until genotyping. The TYMS VNTR polymorphism was amplified by polymerase chain reaction with Taq DNA Polymerase 2×Master Mix RED (Ambicon, Denmark). Other polymorphisms were performed using a PCR–RFLP technique. RFLP analysis was performed with fast digest enzymes (Thermo Fisher Scientific, the USA). After restriction enzyme analysis, PCR fragments were visualized in a 2.5%-3% agarose gel. The information of studied genetic variants is shown in table 1.

Statistical analysis

Statistical analysis was done by SPSS software version 21. The quantitative analysis was performed by descriptive variables. T-test was used for comparison of different groups and chi-square was used for qualitative analysis. p-value less than 0.05 was considered statistically significant.

Results

Genotyping

Eighty three cancer patients (58 males, 25 females, and mean age of 57.17 years old, range of 23-86 years) were evaluated in this study. Three variants in DPYD gene were not polymorphic in this study (Table 2, Figure 1). The distribution of the rs45445694 genotype was 27.7% for 2R/2R, 30.1% for 2R/3R and 42% for 3R/3R. The distribution of the rs16430 was 38.5% for del6/del6, 42.1% for del6/ins6 and 19% for ins6/ins6 (Table 3).

Toxicity

Toxicity data included hematological toxicities (anemia and neutropenia) in 50 patients and nonhematological toxicities (nausea, vomiting, diarrhea, mucositis, neurotoxicity) in 83 patients. In this study, 20% (17/83) developed non-hematological grade III-IV toxicity and 29% (13/44) developed hematological grade III-IV toxicity. Anemia (18%) was the most common severe hematological toxicity, whereas mucositis (3%) and neurotoxicity (2%) were the most frequent non-hematological severe toxicities. Also, 68% of patients experienced neurotoxicity as the most severe toxicity of any grade developed during the treatment. Thymidylate synthase ins/del polymorphisms were significantly associated with increased grade III neurotoxicity (p=0.02). Anemia grade III was significantly associated with 2R/2R genotype (0.009) (Table 4).

Discussion

Colorectal cancer is the second most common cancer in women and the third most common cancer in
men worldwide. Colorectal cancer incidence is increasing in Iran, China and South Korea. Fluorouracil in combination with leucovorin, and oxaliplatin (FOLFOX) or with irinotecan (FOLFIRI) is the backbone of chemotherapy treatment for colorectal and gastric cancers. Gene polymorphisms are associated with cancer susceptibility and response to treatment in different types of cancer.

The aims of this study were to investigate the association of DPYD rare risk variants and TYMS polymorphisms and toxicity that may help predict the response to fluorouracil-based chemotherapy. These polymorphisms were evaluated in 83 colorectal and gastric cancer patients treated with FOLFOX regimen. Dihydropyrimidine Dehydrogenase (DPD) is responsible for conversion of 5-FU to 5-fluorodihydropyrimidinuracil (5-FHU2) and plays a key role in the catabolism of 5-FU. Deficiency in DPD activity leads to severe toxicity which may lead to treatment discontinuation or even death. Three DPYD variants of rs3918290, rs55886062 and rs67376798 were associated with decreased DPD enzyme activity and a high risk of severe 5-FU toxicity. The development of severe toxicity results in dose reduction, discontinuation of treatment or even death in cancer patients.

Regarding the low minor allele frequency of the DPYD rare risk variants, three DPYD variants of rs3918290, rs55886062 and rs67376798 were not identified in the study group. This finding is consistent with some small studies. Similar to our study, rs3918290 polymorphism was not found in Korean, Taiwanese, Turkish, Caucasian, and African populations. In some large studies, heterozygous patients for the rs3918290 experienced severe toxicity. Some reported toxicity in just 46% of patients with rs3918290 polymorphism. For the rs67376798 variant, previous studies reported that 75% of patients with a heterozygous genotype experienced severe toxicity in the first two cycles of therapy. Other studies showed 60% or 62% of heterozygous patients experiencing toxicity within the first two cycles. The single patient with a heterozygous rs55886062 genotype experienced late death even death in cancer patients. Three polymorphisms were evaluated in 83 colorectal and gastric cancers.

Table 1. Characteristics of studied DPYD and TYMS polymorphisms

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP in dbSNP</th>
<th>Location</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Primer</th>
<th>Annealing temperature</th>
<th>Restriction enzyme</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPYD</strong></td>
<td></td>
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<tr>
<td>rs3918290</td>
<td>Intron 4</td>
<td>Exon 14 skipping</td>
<td>1905p1G 4A</td>
<td>F:ACTCAATATCTTTACTCTTTCA TCAGGAC R:ACATTCAACACTTTGACCA TICT</td>
<td>60</td>
<td>HpyCH4</td>
<td>N: 190+54 M: 244+190+54</td>
<td></td>
</tr>
<tr>
<td>rs55886062</td>
<td>Exon 13</td>
<td>I560S</td>
<td>1679 T4G</td>
<td>F:TCACCAATACAAATGTTAC ACTGAGA R:TTAAACCGATGCTTGTGA AGTG</td>
<td>61</td>
<td>Bsp119I</td>
<td>N: 469,265 M: 724,469,265</td>
<td></td>
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<tr>
<td><strong>TYMS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>rs45445694</td>
<td>131 (1)</td>
<td>28 bp deletion</td>
<td>F:CTAAGACTTCTGCTGTG GCC C</td>
<td>64</td>
<td>-</td>
<td>2R: 276 3R:304</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs16430</td>
<td>6 bp deletion</td>
<td>Insertion or deletion of TTAAG</td>
<td>F:CCACAGGCATGGCCGC GCC C:CAAATCGGAGGGAGCTGAGT</td>
<td>60</td>
<td>DraI</td>
<td>Del/del: 123 Ins/ins: 60,63 Ins/del: 1,63,60</td>
<td></td>
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</tr>
</tbody>
</table>

| Table 2. Genotype frequencies of DPYD rs3918290, rs67376798, rs55886062 polymorphism |
|-----------------|-----------------|-----------------|-----------------|
| **Reference** | **Sample type** | ds3918290 n (%) | ds67376798 n (%) | rs55886062 n (%) |
| | | GG | GA | AA | TT | TA | AA | AA | AC | CC | GG | GA | AA | TT | TA | AA | AA | AC | CC |
| (25) | Colorectal and gastric cancers | 83 (100) | 0 (0.0) | 0 (0.0) | 83 (100) | 0 (0.0) | 83 (100) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| (23) | Colorectal cancer | 99 (99) | 1 (1) | 0 (0.0) | 100 (100) | 0 (0.0) | 0 (0.0) | 99 (99) | 1 (1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| (10) | Colorectal and oesophageal, anal and hepatobiliary cancers | 427 (99) | 3 (0.006) | 0 (0.0) | 426 (99) | 4 (0.9) | 0 (0.0) | 429 (99) | 1 (0.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| (13) | Colorectal and oesophageal, anal and hepatobiliary cancers | 2859 (99) | 27 (0.94) | 0 (0.0) | 2882 (99.8) | 4 (0.14) | 0 (0.0) | 2854 (99.9) | 32 (1.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| (26) | Rectal cancer | 131 (100) | 0 (0.0) | 0 (0.0) | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

a. Underlined capital letter labeled nucleotides show mismatch to complementary DNA.
b. F:forward; R:reverse; N:normal; M:mutant.
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Loganayagam et al reported, in the largest study to date, the incidence of grade ≥3 5FU-toxicities in rs3918290, rs55886062, and rs67376798 carriers as 88.0, 50.0, and 81.5%, respectively, whereas incidence of grade ≥3 overall toxicities was 88.0, 75.0, and 88.9%, respectively. Carrier of rs3918290, rs55886062 and rs67376798 had at least one grade IV toxicity of 64.0, 25.0 and 66.7%, respectively.

The Clinical Pharmacogenetics Implementation Consortium (CPIC) recommended reduction of fluoropyrimidine dosage in patients who are heterozygous for rs3918290, rs55886062 and rs67376798 variants which may prevent severe and possibly life-threatening toxicities. The use of 5-fluorouracil or capecitabine is not recommended in patients who are homozygous for rs3918290, rs55886062 and rs67376798.

Thymidylate synthase is a main intracellular target of the active 5-FU metabolite, FdUMP, which forms a ternary complex with TS and 5, 10-MTHF. rs3474303 as a variable number of tandem repeats (VNTR) is present in the 5'-untranslated region (5'-UTR) of the TS gene (TYMS). The three 28-bp repeat (3R) is associated with 3-4-fold translational efficiency, compared to two 28-bp repeat. In our study, the two 28-bp repeat and the 6-bp deletion allele frequencies of the TYMS gene were 48.75 and 59.65%, respectively. Significant associations were found between anemia grade III and 2R/2R TYMS genotype (0.009). In our study, association between polymorphism rs3474303 with toxicity is the same as other studies. 2R/3R or 3R/3R genotypes were significantly associated with a lower risk of toxicity. Although some studies have shown association between rs3474303 and toxicity, this association is likely to be small and clinical test would not be useful.

The 6-bp deletion in the 3'-UTR region of the TYMS gene affects TYMS mRNA expression. In our study,
the del/del genotype in rs16430 was significantly associated with increased grade III neurotoxicity (p=0.02). Other studies have shown del/del genotype in rs16430 associated with diarrhea, neutropenia and mucositis (p=0.0123). However, certain other studies have failed to show an association between a homozygous del/del genotype and severe toxicity. 53,54.

TS mRNA level predicts fluoropyrimidine and raltitrexed sensitivity in gastric cancer patients 55. A meta-analysis has shown that response rates for fluoropyrimidine-based chemotherapy were significantly lower in gastric cancer patients with high TS expression 56. In Meulendijks et al’s study, TYMS VNTR 3R/3R genotype was formally associated with Objective Response Rate (ORR) 57.

Homozygous genotype rs1801159A/A was associated with response to fluorouracil-based adjuvant chemotherapy in gastric cancer patients 58. Another study revealed that gene polymorphisms of DPYD could be considered as a biomarker for prediction of gastric cancer patients survival treated with 5-fluorouracil-based adjuvant chemotherapy 59.

**Conclusion**

In conclusion, an association was found between VNTR and TYMS 1494 ins/del polymorphism in TYMS gene and anemia and neurotoxicity, respectively. Due to the relatively limited sample size, DPYD rare risk variants of rs3918290, rs55886062 and rs67376798 were not identified in our study. Further investigations on larger sample size are needed to demonstrate the role of rare risk variants of DPYD gene. In addition, to achieve better biomarkers for FOLFOX chemotherapy regimen, additional variation in genes involved in metabolism of other drugs like oxaliplatin should be investigated.

**References**


