High Frequency of Low Expression Genotypes in Mexican Population for the TYMS Gene Predicted From the TSER and G>C Variants

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Abstract

Background. The *TYMS* gene contains one of the 68 very important polymorphisms because affect the expression level of the thymidylate synthetase (TS), such as TSER that is involved in the toxicity and therapeutic response to 5'-Fluoracil (5'-FU), the drug of choice for different cancers. However, the inclusion of additional *TYMS* variants have improved the association with the expression levels. Although the TSER variants have been studied in the Mexican population, genotypes including the G>C SNP in the *TYMS* gene are completely unknown in this country.

Methods and results. A Mexican population sample (n= 156) was genotyped for the TSER and G>C polymorphisms by PCR and PCR-RFLPs followed by PAGE and silver staining, respectively. Allele and genotype frequencies were estimated, and Hardy-Weinberg equilibrium was demonstrated for both *TYMS* variants in the studied population sample. For TSER, the most frequent allele was 2R (52.56%), as well as the genotype 2R/3R (42.3%). Comparison with Latin American, European, and American (USA) populations suggest a heterogeneous worldwide distribution (*FST-value*=0.01564; *P-value*=0.0000). When the G>C variant was included, a high frequency of the genotypes 2RG/2RG, 2RG/3RC, and 3RC/3RC was observed (84.6%). This finding allows predicting a high frequency of low expression for the thymidylate synthase (TS) in the Mexican population, which can predispose to peculiar treatment responses of 5´-FU, methotrexate, and pemetrexed.

Conclusion. This study justifies obtaining the pharmacogenetic profile of *TYMS* variants in candidate Ca patients for 5´-FU treatment, given the high frequency of low TS expression in the Mexican population.

Introduction

Chemotherapy is among the most common cancer treatments, being 5-fluoracil (5´-FU) a drug of choice for gastric, colorectal carcinoma, and bladder cancers, mainly. This antineoplastic drug has been shown to have high variability, both in efficacy and adverse reactions (e.g., toxicity), which can be largely explained by genetic factors [1]. Among the enzymes involved in the toxicity and therapeutic response to 5´-FU, thymidylate synthetase (TS) is inhibited for the 5´-FU and methotrexate [2]. The TS is encoded by the *TYMS* gene that is located on the short arm of chromosome 18, which is constituted by seven exons encoding a 313 amino acids protein [3]. The *TYMS* contains one of the 68 very important polymorphisms (https://www.pharmgkb.org/), with large pharmacogenetic potential in cancer because *TYMS* expression alterations allow predicting the response to specific treatments, mainly with 5'-FU [1].

The main polymorphism of clinical relevance as modulator of the *TYMS* expression can be detected in the 5'-UTR region of the *TYMS* mRNA as a Variable number of tandem repeat (VNTR) (rs45445694), which involves a 28 base pair repeat in the promoter region (TSER) ranging from two (TSER*2) to nine (TSER*9) duplicated copies [3]; the most common alleles of this polymorphism are TSER*2 (2R) and TSER*3 (3R). The presence of the 3R allele is associated with increased mRNA expression and protein production. Some studies have predicted the association between the toxicity and effectiveness of some drugs, such as 5´-FU, methotrexate, and pemetrexed [1, 4]. Another important *TYMS* variant is a Single nucleotide polymorphism (SNP) located in the *TSER*3R* allele involving a G > C transversion (rs2853542), which decreases the expression level of the TS enzyme because avoids the corresponding upstream stimulatory factor (USF-1) binding site [1, 4, 5]. Among the expected *TYMS* haplotypes formed by TSER and the SNP (2RG, 3RG, and 3RC), 3RG presented 3–4 times greater translation efficiency in the functional analysis [4]. However, the "genotypes" from these haplotypes support the following classification based on the *TYMS* expression level: i) low expression is 2R/2RG, 2R/3RC, and 3RC/3RC; ii) medium expression is 2R/3RG and 3RC/3RG; and iii) high expression is 3RG/3RG [4, 5].

The most common alleles for these *TYMS* variants are related to increased levels of the TS enzyme, which produces resistance to drugs, such as 5´-FU and oral pro-drugs (capecitabine, methotrexate, among others). Different worldwide studies have demonstrated the clinical impact of these *TYMS* polymorphisms [2–4, 6–9]. However, the haplotype distribution based on the TSER-SNP variants has been poorly analyzed in worldwide populations, and only one study in Mexican breast Ca patients and volunteers has reported the TSER allele and genotype frequencies [10]. Therefore, in this study, we aimed to analyze two *TYMS* variants in one Mexican population sample to estimate the potential prevalence of resistance to some drugs, and to compare results with available populations.

Subjects And Methods

Population sample and DNA extraction method

We included 156 volunteers from two Mexican geographic regions, including 103 western individuals from the Jalisco state, and 53 northern volunteers from the Chihuahua state. Because they have been described with similar admixture components [11], and they displayed similar genetic frequencies in this study ($p > 0.05$), they were reported as one Mexican population sample. The genomic DNA was extracted from peripheral blood samples by the standard phenol-chloroform method. DNA was quantified into a Nanodrop 2000™ instrument (Thermo Scientific, USA), and it was diluted to 25–30 ng/μL for working samples. The TSER 2R/3R polymorphisms were amplified by PCR using the primers *TSER-F* (GTG GCT CCT GCG TTT CCC CC) y *TSER-R* (GCT CCG AGC CGG CCA CAG GCA TGG CGC GG). The PCR was carried out in a total volume of 20 μL including 4 μL of template DNA. The reaction included 10μL of the múltiplex PCR master mix (QIAGEN), 2μL of oligonucleotides at 4μM, 0.75 μL of formamide, and 3.25μL of HPLC water. The amplification conditions were: initial denaturation to 95°C for 15 min, denaturation to 94°C for 30s, alignment to 57°C for 90s, extension to 72°C for 90s, and a final extension to 72°C for 10 min. Using the above described amplified products for TSER genotyping, the G > C variation was established by the technique of Restriction fragment length polymorphisms (RFLPs). We took 9.75μL of the TSER 2R/3R amplified product, which was mixed with 0.25μL of restriction enzyme Hae III and incubated 1h at 37°C. Enzime inactivation was done by thermoblock incubation at 80°C for 30m. In both cases, the amplification products were submitted to polyacrylamide gel electrophoresis (PAGE 6%; 29:1) to 250 V for 2.5h followed by silver staining. Genotypes were established according to the respective amplified and RFLP patterns as described by Kawakami & Watanabe (2003) and Marcuello et al. (2004) [3, 4].

Data analysis

Descriptive statistics was accomplished by estimation of the allele and genotype frequencies by the gene counting method. Fisher exact tests were performed to confirm that the genotype distribution agreed with the Hardy-Weinberg equilibrium (HWE). Significance levels of exact tests were empirically determined in 5000 simulations. Similarly, pairwise F_{st} distances and F_{st} *p*-values were done to establish differences regarding previous worldwide studies of the TSER polymorphism. For these purposes, we used the software Arlequin 3.5 [12].

Results

Allele, Genotype, and Haplotype frequencies

The allele and genotype frequencies were estimated in the Mexican population sample for TSER and their internal G > C SNP in the *TYMS* gene, whose results were compared to previously reported Mexican and worldwide populations (Table 1). Comparison with Latin American, European, and American (USA) populations suggest a heterogeneous

worldwide distribution (*FST-value* = 0.01564; *p-value* = 0.0000) (Online Resource 1). The modal allele in the TSER polymorphism was 2R (52.6%), whereas the heterozygous 2R/3R was the most frequent genotype (42.3%). Similarly, the 2R allele was the most frequent in previously studied Mexican cancer control (MxCaCo = 58.6%), in Florida (FlorUSA = 54.7%), and Italian control (ItaC = 48.9%) population samples (Table 1). However, in most worldwide populations the modal allele was 3R. On the other hand, for the SNP G > C, the wild type allele G (63.5%) and their homozygous genotype G/G (44.87%) were the most common. In our Mexican population sample, and most of the cited populations, the genotype distribution agreed with HWE expectations ($p > 0.05$) (Table 1).

Table 1
Allele and genotypes frequencies for the TSER2R/3R variants in worldwide populations used for comparison purposes.

Population			*Allele frequencies*		*Genotype frequencies*				
	Abbrev	**n**	**2R**	**3R**	**2R/2R**	**2R/3R**	**3R/3R**	**HWE†**	**Reference**
Mexican	Mx	156	0.5256	0.4744	0.3141	0.4230	0.2628	0.05800	*This study*[¥]
Mexican Control	MxC	145	0.3965	0.6034	0.1586	0.4759	0.3655	0.00000	[10]
Mexican BCa (Breast Ca)	MxBCa	230	0.3826	0.6174	0.1304	0.5043	0.3652	0.33202	
Mexican PrM (pre-menopausal)	MxPrM	53	0.3490	0.6510	0.0943	0.5094	0.3962	0.54511	
Mexican PM (post-menopausal)	MxPM	143	0.3741	0.6260	0.1329	0.4825	0.3846	0.85850	
Caucasian Americans	CauAm	96	0.4600	0.5400	0.1900	0.5400	0.2700	0.41870	[2]
Florida USA	FlorUSA	74	0.5473	0.4459	0.3510	0.3920	0.2430	0.15551	[9]
Chilean	Chi	368	0.4483	0.5516	0.2119	0.4728	0.3152	0.39925	[6]
Portuguese	Port	130	0.4423	0.5577	0.2000	0.4846	0.3153	0.85875	[5]
Spanish Control	SpC	347	0.4294	0.5706	0.1960	0.4668	0.3372	0.38654	[7]
Spanish AML (Acute Myeloblastic Leukemia)	SpAML	169	0.3195	0.6805	0.1952	0.2485	0.5562	0.00000	
Spanish ALL (acute lymphoblastic leukemia)	SpALL	108	0.412	0.588	0.2130	0.3981	0.3888	0.07316	
Spanish CoCa (Colon Ca)	SpCaCo	89	0.3989	0.6011	0.1910	0.4157	0.3932	0.27040	[4]
Italian Control	ItaC	139	0.4892	0.4892	0.2230	0.5324	0.2230	0.38837	[8]
Italian SCa (Stomach Ca)	ItaSCa	134	0.4179	0.5672	0.1343	0.5672	0.2836	0.75580	

†Hardy Weinberg equilibrium (HWE) estimated by exact tests.

Table 1, TABLE S1

When the G > C SNP was included in the TSER polymorphism, three haplotypes were observed: 2R, 3RG, and 3RC. As could be expected, the 2RC absence is explained by the association (linkage disequilibrium) between the 3R and C

alleles. The 2RG haplotype was the most frequent in the studied Mexican population sample (52.56%), followed by the 3RC (36.54%), and 3RG (10.9%), respectively. Similarly, 2R was the most frequent in Spain (39.9%) [4], and Portugal (44.2%) [5], followed by the haplotypes 3RC (range: 34.27 to 35%) and 3RG (range: 20.77 to 25.8%), respectively. In addition, we were able to estimate the frequency of six “genotypes” based on these three haplotypes and to compare results indicating the *TYMS* expression level [4, 5] (Fig. 1). The genotype distribution pattern in the Spanish and Portuguese populations was similar to each other but different from the studied Mexican population sample ($p < 0.0001$). However, some similarities were observed, such as the same modal genotype, the heterozygote 2RG/3RC, followed by the homozygous 2RG/2RG, among others.

FIGURE 1

Population pairwise comparisons

Based on the TSER polymorphism, Fst genetic distances and Fst *p*-values from pairwise comparisons were accomplished among all the worldwide populations enlisted in Table 1, and the results are presented in Online Resource 1. We detected two main worldwide population clusters including different Mexican samples, which is appreciated in the MDS plot that shows their genetic relationships (Fig. 2). The first population cluster included our sample and another Mexican control sample, *plus* one Italian and American (Florida, USA) population. The second population cluster included Mexican Breast Cancer, pre-menopausal, and post-menopausal patients, plus Caucasian Americans and most of the Spanish population samples (excepting AML patients).

FIGURE 2

Discussion

Different studies have reported allele and genotype frequencies for the TSER polymorphism in worldwide populations, mainly in patients with different types of cancer [2, 4–10]. However, for the first time, we report the TSER-G > C haplotype distribution in a Mexican population sample, including individuals from the north region of this country. In addition, we compared our findings with related European, Latin American, and American (USA) populations.

The 3R variant of TSER associated with high production of the enzyme TS [2] (Marsh et al, 2005), was lower than the previous report in Mexico including control, Breast cancer, premenopausal, and postmenopausal patients ($p < 0.0001$), which could be explained by the illness presence or due to the small population sample previously analyzed [10] (Quintero-Ramos et al., 2014). However, after the Bonferroni correction ($p > 0.003125$) the allele distribution of TSER in Mexico was similar to the majority of the compared worldwide populations (Online Resource 1).

Many studies have demonstrated the clinical importance of the presence of TSER-G > C on response to treatment, toxicity with chemotherapy agents such as 5´-FU, and the relationship with the prognosis and survival of Ca patients. Initially, only the 2R and 3R alleles were associated with low or high expression of the *TYMS*, respectively; with their corresponding genotypes and possible clinical consequences [2–4, 6–9]. However, in posterior studies, the complexity of these associations was increased. For instance, although initial studies showed a poor response to 5´-FU with the high expression genotype 3R/3R [2, 3, 13–15], eventually the 3RG haplotypes were associated with high expression and better response [5, 16]. In addition, some studies to elucidate the influence of *TYMS* polymorphisms concerning treatment response, survival, and disease progression remain contradictory. Some possible explanations for these opposing findings could be: 1) the type of tissue or samples, such as those from patients with tumor that have shown greater heterozygosity, unlike those derived from control people [16]; 2) the drug-administration route used between studies, because intravenous bolus administration shows a greater effect on mRNA synthesis, whereas continuous

infusion administered from 3 to 15 min shows an effect on the TS [16]; 3) the effect of the *TYMS* polymorphisms, either associated with the mRNA expression [17, 18] and synthetized protein mostly associated with 5´-FU sensitivity in patients with higher amount of enzyme that correspond to high expression genotypes [19, 20]; and 4) the presence of additional polymorphisms, such as the TYMS3'UTR or 1494del6, which modulates the *TYMS* expression and are associated with poor response to treatment [21, 22].

In this study, we observed an important frequency of low expression genotypes for the *TYMS* gene in a Mexican population sample (84.62%) (Fig. 1), which imply a high probability of not responding to treatment and toxicity to 5'-FU [3, 5]. In addition, these findings support clinical decisions on the administration of methotrexate, considering that the 2R/2R genotype has a greater chance of responding to this treatment [2]. Interestingly, the 2R/2R genotype had a higher frequency (31.4%) than the homozygous 3R/3R (26.3%). On the other hand, the studied Mexican populations would have elevated survival and free progression of the disease regarding the pemetrexed administration, due to the association with low expression genotypes [23].

This work allows predicting the combined impact of two *TYMS* polymorphisms in Mexican populations. Due to the elevated frequency for low expression *TYMS* genotypes, and to avoid predictable adverse drug effects and better treatment response, a previous genetic analysis is recommended in candidate Mexican patients for 5´-FU, methotrexate, or pemetrexed treatment [4, 5, 23]. Although the health status of the studied sample is unknown, results suggest a high proportion of Mexican patients at risk of unfavorable response to the 5´-FU treatment, linked to the survival rate and prognosis value of some types of cancer, as previously described [10]. In brief, our results justify obtaining the pharmacogenetic profile for the *TYMS* gene in Mexican candidate patients for 5´-FU treatment from oncology clinics.

Conclusions

We described a high frequency of genotypes associated with low expression of the *TYMS* gene in the Mexican population (84.62%), based on the following TSER and G > C haplotypes: 2R/2RG, 2R/3RC, and 3RC/3RC. This result predicts a problematic response in a large proportion of Mexican cancer patients to 5´-FU, methotrexate, and pemetrexed treatment. Consequently, this study justifies conducting the routine implementation of pharmacogenetic profiles of these TYMS polymorphisms in Mexican candidate patients for 5´-FU treatment.

Declarations

Authors declare no conflict of interest.

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Author contributions

Conceived and designed the experiments: Favela-M, Rangel-V. Performed the experiments: Godinez-A, Favela-M., Chávez-M. Analyzed the data: Aguilar-V, Favela-M. Contributed reagents/materials/analysis tools: Favela-M, Martinez-C, Rangel-V. Wrote the paper: Favela-M, Rangel-V.

Funding & competing interest disclosure

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Ethical approval and informed consent

All individuals signed a written informed consent according to the ethical guidelines of the Helsinki Declaration. For this purpose, ethical approval was obtained from the Committee of Ethics and Research of the Centro Universitario de la Ciénega of the Universidad de Guadalajara (CUCI-UdeG, Mexico). The anonymity of the recruited individuals was always preserved.

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Figures

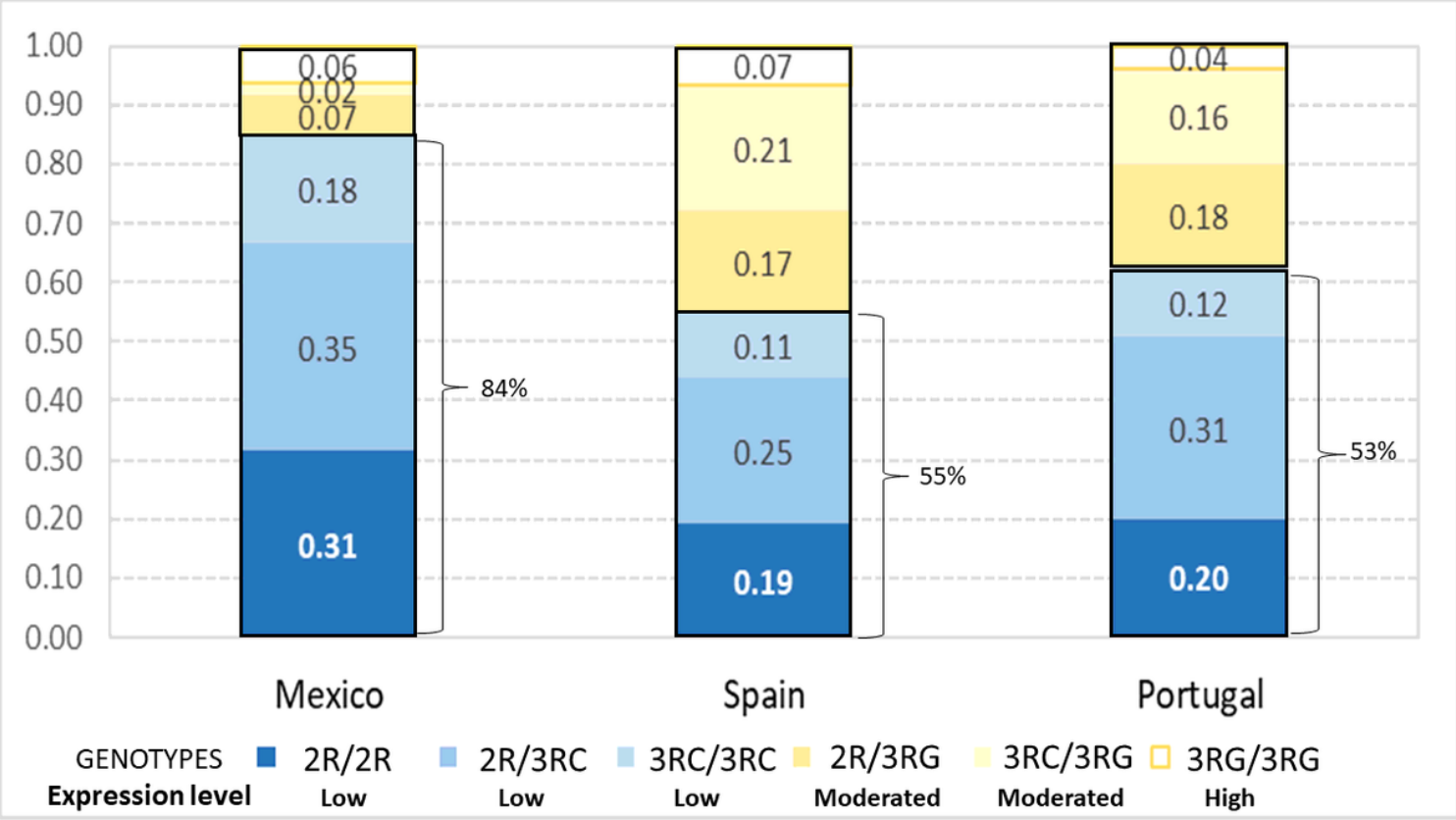

Figure 1

Genotype frequency and expression level based on haplotypes from TSER and G>C *TYMS* gene polymorphisms in the studied Mexican sample and two European populations.

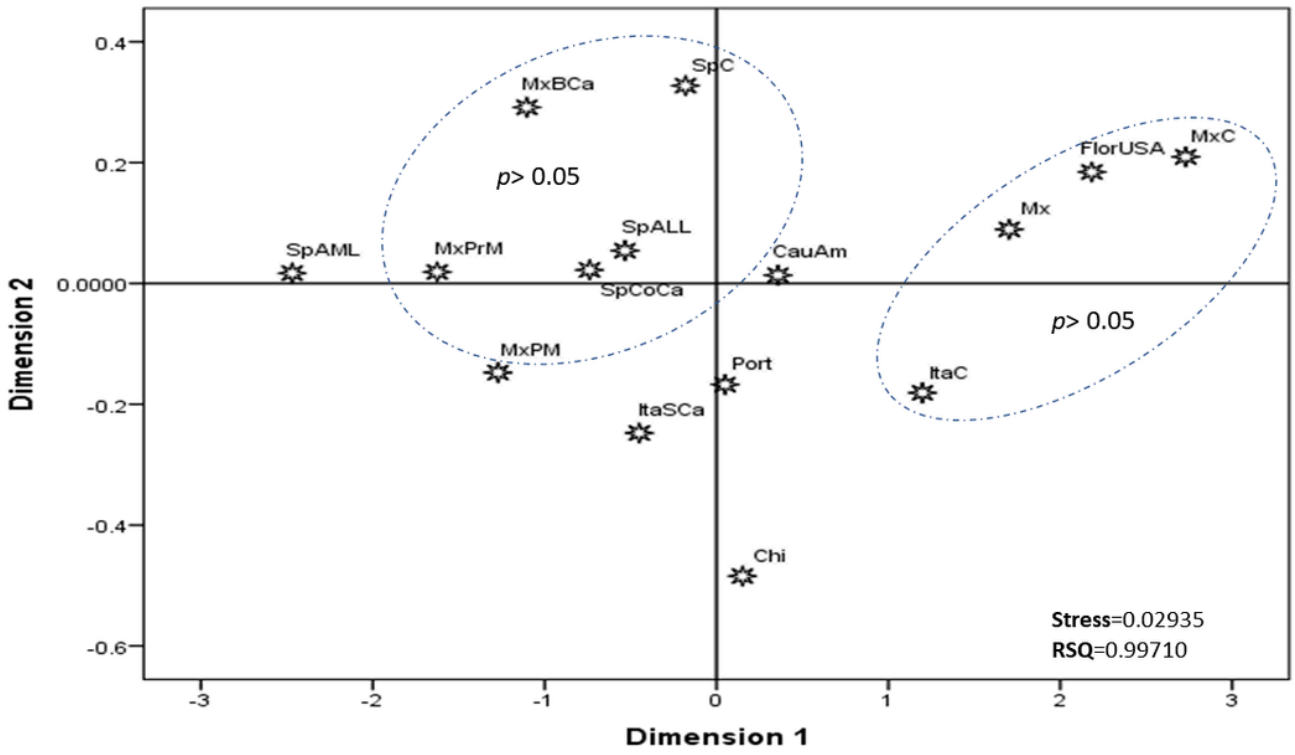

Figure 2

MDS plot representing Fst genetic distances among worldwide populations for the *TSER* polymorphism. For discussion purposes, two population clusters are indicated.

Supplementary Files

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- OnlineResource1.Pairwisecomparison.xlsx