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Research Article

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The Effect of Genetic Variations of Methylene Tetrahydropholate Reductase Gene Polymorphisms on Ribavirin-Induced Anemia in Hepatitis C Patients

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Abstract

Aim: Ribavirin is known to inhibit the activity of S-adenosylhomocysteine hydrolase (AHCY) in erythrocytes. The aim of this study is to investigate the effect of AHCY gene variations and methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms on ribavirin-induced anemia.

Methods: Patients receiving interferon and ribavirin treatment with a diagnosis of chronic hepatitis C were included in the study. Patients with and without anemia were allocated into two groups; anemia being defined as a ≥ 2-gram reduction in hemoglobin level. C677T and A1298C polymorphisms for MTHFR, and 5'UTR-34 C→T, AHCY 112 C→T, AHCY Try143Cys A→G variations for AHCY were tested by PCR-RFLP method.

Results: The prevalence of anemia was 61.17% (52/85). There was no difference between the two groups in terms of gene polymorphisms or genetic variations. While genotype T/T of MTHFR C677T gene polymorphism was 6.029 times more prevalent in anemic patients; genotype C/C of MTHFR A1298C gene polymorphism was 3.586 times more prevalent. However, these differences were statistically insignificant (p=0.137 and p=0.124, respectively). AHCY genetic variations are not risk factors for ribavirin-induced anemia.

Conclusion: Genotype T/T for MTHFR C677T and genotype C/C for A1298C were more prevalent in patients with anemia. MTHFR gene polymorphism differences were not shown to be a contributing factor in ribavirin-induced anemia.

Keywords

Anemia; Hepatitis C; Methylenetetrahydrofolate reductase; Ribavirin; S- adenosylhomocysteine hydrolase

Introduction

Ribavirin is a purine nucleoside analogue [1]. Even though its mechanism of action is still controversial, it widely prevents the replication of DNA and RNA viruses through the inhibition of inosine monophosphate dehydrogenase, an essential enzyme in the synthesis of guanosine triphosphate [1]. The final step of this chain is the fatal mutagenesis of the RNA genome [1]. Currently, the treatment of patents with hepatitis C consists of interferon-α and ribavirin combination therapy [2,3]. Combination therapy, in comparison to single-agent interferon treatment, leads to an increase in side effects [2-9]. Dose reductions are common; essentially, dose reductions are required in 10% of patients under combination therapy who develop anemia [2-5]. The major toxicity related to ribavirin use is hemolytic anemia. This side effect has been associated with the accumulation of ribavirin triphosphate in red blood cells (RBCs), leading to the inhibition of erythrocyte functions [10,11].

Hemolytic anemia, a complication of ribavirin treatment, may cause the discontinuation of treatment [12]. The anemia is potentially related to the accumulation of ribavirin in erythrocytes. Ribavirin undergoes phosphorylation into its active form once inside the erythrocyte, and accumulates, such that its intracellular concentration exceeds its concentration in the plasma [13]. Although the exact mechanism is still unclear, ribavirin phosphate is assumed to delay ATP-dependent transport systems by competing with ATP on the cell membranes of RBCs, thus potentially interfering with the erythrocyte membrane stabilization.

Certain adenosine analogues such as ribavirin have been shown to inhibit the activity of S-adenosylhomocysteine hydroxilase (AHCY) in erythrocyte lysates [14]. Ribavirin (an inosinate dehydrogenase inhibitor), in addition to its recognized mechanisms of action, also has an inhibitory effect on the activity of AHCY, which may be an important component of its antiviral efficacy [14]. Homocysteine is a metabolic intermediate of demethylated methionine [15]. The first step in this reaction is the formation of S-adenosyl methionine (AdoMet), which is then demethylated into S-adenosylhomocysteine (AdoHcy) [15]. AdoHcy is hydrolyzed into adenosine and homocysteine (Hcy) by AHCY [15]. The thermodynamics of this reaction strongly induce AdoHcy synthesis. However, as both products are rapidly removed, the breakdown of AdoHcy is usually predominant in *in vivo* conditions [15].

AdoMet is an important methyl donor that participates in numerous transmethylation reactions [15]. AdoHcy, the metabolite of AdoMet, competes with the AdoMet binding sites, and is a strong inhibitor of methyltransferase enzymes, and a non-cytotoxic hypomethylation agent [16]. The concurrent reduction in AdoMet levels and increase in AdoHcy levels inhibit the methylation of various tissue components, including proteins, DNA, RNA, phospholipids and other small molecules [15]. Furthermore, these modifications in erythrocytes are not characteristic of patients with homocysteinemia and homocystinuria [17].

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism [18,19]. MTHFR causes the irreversible conversion of 5,10 methylenetetrahydrofolate (5,10-methylenee THF), into 5-methyl tetrahydrofolate (5-methyl THF) [19-21]. 5-methyl THF provides the methyl group for DNA methylation and methionine synthesis [20-22]. 5,10-methylenee THF, on the other hand, is utilized in the conversion of deoxyuridilate into timidilate, meanwhile being oxidated into 10-formyl THF for purine synthesis [20,21]. A polymorphism in the MTHFR gene (most commonly,

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C677T polymorphism) diminishes enzyme activity [22,23]. The reduced MTHFR activity leads to a decrease in 5-methyl THF levels, and increases in 5,10-methylene THF levels and plasma homocysteine levels [18,20-23]. The prevalence of A1298C and C677T polymorphism greatly varies in different populations, and according to age [24]. When A1298 and C667T polymorphisms are heterozygous, the activity of the MTHFR enzyme is only 50-60% of the expected enzyme activity [24]. This activity is lower than the activity in subjects who are heterozygous for C677T polymorphism [24].

MTHFR is a key enzyme in single-carbon metabolism. Methyltetrahydrofolate, a product of the reduction of methylenetetrahydrofolate by MTHFR, provides methyl groups for various biological methylation reactions, and single-carbon units for timidine and purine synthesis [15-17]. It is also used as the methyltetrahydrofolate methyl donor in the catalysis of Hcy into methionine, by methionine synthetase [15,17]. The reduction in MTHFR activity results in the augmentation of Hcy levels. Intracellular Hcy is derived from AdoHcy by AHCY [15]. This is a dynamic, balanced reversible reaction that tends more strongly towards AdoHcy synthesis, rather than hydrolysis [15]. Therefore, the efficient metabolic removal of Hcy is necessary for the prevention of AdoHcy accumulation [15]. The toxicity of intracellular AdoHcy accumulation is the result of the inhibition of numerous AdoMet-dependent methyltransferases [15]. The action of ribavirin is also dependent on AHCY [14]. Ribavirin may block all transmethylation processes through the increase in AdoHcy, that results from the interruption of the demethylated conversion of AdoMet into AdoHcy by inhibiting AHCY in the homocysteine metabolism. Thus, genetic modifications in MTHFR and AHCY may have a role in ribavirin-induced anemia [25-29]. The aim of this study is to investigate the effect of AHCY gene variations and MTHFR genetic polymorphisms on ribavirin-induced anemia.

Materials and Methods

Patients

Patients who presented to our gastroenterology outpatient clinic were diagnosed with chronic C hepatitis, and received treatment with interferon (conventional or pegylated) and ribavirin (weight-adjusted dose; 800 mg/day for <65 kg, 1000 mg/day for 65-85 kg, and 1200 mg/day for ≥ 85 kg) were included in this study. Patient data obtained from their medical records by retrospective screening. The ribavirin dose was reduced by 200 mg when hemoglobin levels were below 10 g/dl, and ribavirin treatment was discontinued when hemoglobin levels were below 8.5 g/dl. A ≥ 2-gram decline in hemoglobin levels was significant in terms of treatment-related anemia. Patients were thus allocated into two groups: those with or without anemia. The date and treatment week of the lowest hemoglobin level was recorded. The delta hemoglobin level was calculated by the subtraction of the lowest hemoglobin value from baseline hemoglobin levels. Besides baseline hemoglobin, platelet count, AST, ALT, GGT, LDH levels and body mass index [Weight (kilograms)/Height (meters)^2], concomitant therapies with ribavirin (conventional interferon or pegylated interferon), were recorded for all patients. Following approval by the Mersin University School of Medicine's Ethics Committee (06.03.2006-8), informed written and verbal consent was obtained from all participants.

DNA extraction and analysis

A blood sample was drawn from each individual. Venous blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). DNA was extracted from whole blood by salting out procedure [30].

Molecular analysis of MTHFR C677T and A1298C polymorphisms

Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) assays were used to determine MTHFR C677T and A1298C polymorphisms. The primer pairs used were forward 5'-TGAAGGAGAAGGTGTCTGCGGGA-3', reverse 5'-AGGACGGTGCGGTGAGAGTG-3' for MTHFR C677T polymorphism [23,24], and forward 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' reverse 5'-AGGACGGTGCGGTGAGAGTG -3' for MTHFR A1298C polymorphism [22]. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µM dNTPs, 20 pmol of each primer, 1,5 mM $MgCl_2$, 1×PCR buffer with $(NH_4)_2SO_4$ (Fermentas), and 1 U Taq DNA polymerase (Fermentas). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 minutes for initial denaturation at 95°C; 35 cycles at 95°C for 45 seconds for denaturation, 1 minute at 60°C for annealing (30 seconds at 64°C for MTHFR A1298C polymorphism), and 1.5 minutes at 72°C for extension, followed by 7 minutes at 72°C for final extension. PCR products were digested with specific restriction enzymes. Digestion of the PCR product was carried out using 10 U *Hinf*I (Fermentas), for MTHFR C677T polymorphism, and 10 U *Sat*I (Fermentas) for MTHFR A1298C polymorphism, overnight at 37°C. The *Hinf*I restricted products of MTHFR C677T; genotypes CC, CT and TT had band sizes of 198 bp, 198 bp/175 bp/23 bp and 175 bp/23 bp, respectively. The *Sat*I restricted products of MTHFR A1298C; genotypes AA, AC and CC had band sizes of 138 bp, 138 bp/119 bp/19 bp and 119 bp/19 bp, respectively. The digest products were resolved at 120 V for 30-40 minutes on a 3.5% agarose gel containing 0.5 µg/ml ethidium bromide.

Molecular analysis of AHCY 5' UTR-34 C→T, AHCY Exon 2 Arg38Trp 112 C→T and AHCY Exon 4 Try143Cys A→G variations

PCR-RFLP assays were used to determine AHCY-34 C→T, AHCY 112 C→T and AHCY Try143Cys A→G variations. The primer pairs used were forward 5'-CGCCACGCGCATATCCCTG-3', reverse 5'-CCCCCGCCACGAACAAGC-3' for AHCY -34 C→T variations [31], and forward 5'-GTGACCGCCCCTCTTGGTTGG-3' reverse 5'-CCACCCTGGCACAGTCGTCTTC-3' for AHCY 112C→T variations [32], and forward 5'-GTTGGGAAGGAGGTAGTTTTGGC-3', reverse 5'-GCTGCTTGAGGTGATGGGAGTC-3' for AHCY Try 143Cys A→G variations [32]. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µM dNTPs, 20 pmol of each primer, 1,5 mM $MgCl_2$, 1×PCR buffer with $(NH_4)_2SO_4$ (Fermentas), and 1 U Taq DNA polymerase (Fermentas). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 minutes for initial denaturation at 95°C; 35 cycles at 95°C for 45 seconds for denaturation, 1 minute at 58°C for annealing and 1.5 minutes at 72°C for extension, followed by 7 minutes at 72°C for final extension. PCR products were digested with specific restriction enzymes. Digestion of the PCR product was carried out using 10 U *Mnl*I (Fermentas) for AHCY-34C→T variations, and 10 U *Mbi*I (Fermentas) for AHCY 112C→T variations, and 10 U *Sdu*I (Fermen-

tas) overnight at 37°C. The *Mnl*I restricted products of AHCY-34C→T variations; genotypes CC, CT and TT had band sizes of 148 bp/47 bp, 148 bp/121 bp/47 bp/27 bp and 121 bp/47 bp/27 bp, respectively. The *Mbi*I restricted products of AHCY 112C→T variations; genotypes CC, CT and TT had band sizes of 189 bp/149 bp, 338 bp/189 bp/149 bp and 338 bp, respectively. The *Sdu*I restricted products of AHCY Try 143Cys A→G variations; genotypes AA, AG and GG had band sizes of 179 bp/171 bp, 179 bp/171 bp/120 bp/59 bp, and 171 bp/120 bp /59 bp, respectively. The digest products were resolved at 120 V for 30-40 minutes on a 3.5% agarose gel containing 0.5 µg/ml ethidium bromide.

A GeneRuler 100 bp DNA ladder marker (Fermantas), was used as a size standard for each gel lane. The gel was visualized under UV light, using a gel electrophoresis visualizing system (Vilber Lourmat Marne La Vallée, France). Genotyping was based upon independent scoring of the results by two reviewers, who were unaware of case/control status.

Statistical analysis

The correlation between anemia and considered risk factors was determined by the multiple binary logistic regression models. The balance of genotypes, according to the Hardy-Weinberg principle, was evaluated by the chi-square test. The one-way variance analysis was used for delta values, in the comparison of carrier and normal genotypes, and comparison of the three genotypes. The comparisons for maximum hemoglobin reduction, delta Hb, ribavirin dose reduction, and ribavirin discontinuation and between genders were performed by the chi-square test. The statistical significance level was set at P<0.05. The SPSS (version 11.5) program was used for computerized data analyses.

Results

85 patients who had been diagnosed with chronic C hepatitis and received treatment with interferon (conventional or pegylated), and ribavirin combination therapy, were included in the study to determine MTHFR C677T, MTHFR A1298C gene polymorphisms and AHCY 5' UTR-34 C→T, AHCY Exon 2 Arg38Trp 112 C→T, AHCY Exon 4 Try143Cys A→G variations. The allele and genotypes of MTHFR C677T, MTHFR A1298C gene polymorphisms and AHCY -34 C→T, AHCY 112 C→T, AHCY4 Try143Cys A→G gene variations were verified by PCR and RFLP methods. The prevalence of anemia was 61.17% (52/85). The ribavirin dose had been reduced in 31 patients (36.47%), and discontinued in 9 (10.58%) patients. There was no difference between the two groups in terms of gender, body mass index, hemoglobin level, platelet count, ALT, AST, GGT, LDH, type of interferon, and gene polymorphisms and genetic variations (Table 1). In the comparison of risk factors between the two groups, patients' age showed the only marked relationship. The risk of anemia increased 1.112-fold (OR) with age, and this result was statistically significant (p=0.009). The mean age of the 33 subjects in the group who did not develop anemia was 48.51 ± 8.15 years, while the mean age of the 52 subjects in the group who had anemia was 53.01 ± 8.21 years (p=0.016).

Of the 85 individuals with HCV that were included in the study, 55 were female and 30 male. Mean age was 52.74 ± 8.55 years in female patients, and 48.56 ± 7.64 years in male patients (p=0.028). There was no correlation between gender and development of anemia (p=0.737). Ribavirin dose reductions were required in 28 patients (25 female, 3 male) in the group with anemia (55.76%), and 3 patients (3 female) in the group without anemia (9.09%). The three dose reductions in the group without anemia were due to complaints of severe fatigue and weakness, despite Hb levels above 10 g/dl, and the difference between the two groups in terms of treatment discontinuation was significant (p=0.001). All 9 patients who discontinued treatment were female, and were in the group who developed anemia (p=0.019). In the comparison of genders in terms of genotype and allele distribution of gene polymorphisms and variations; there was no significant difference for MTHFR C677T, MTHFR A1298C, AHCY-34 C→T and AHCY 112 C→T (p=0.175, p=0.582, p=0.458 and p=0.458, respectively). The distribution of the AHCY Try143Cys A→G variation was constant between genders, and thus, a statistical analysis was not carried out.

Table 1: Patient characteristics.

Criterion	Group with Anemia (n=52)	Group without Anemia (n=33)	p
Age (years, mean ± SD)	53.01 ± 8.21	48.51 ± 8.15	0.016
Gender (F/M)	33/19	22/11	0.737
Body Mass Index	27.03 ± 4.08	26.13 ± 3.37	0.316
Hemoglobin (g/dl)	13.70 ± 1.36	13.18 ± 1.82	0.135
Platelet count (/mm^3)	192115 ± 62223	195303 ± 79336	0.837
ALT (IU)	82.30 ± 93.57	76,48 ± 72.61	0.762
AST (IU)	66.15 ± 71.78	62.66 ± 46.25	0.805
GGT (IU)	60.68 ± 68.11	48.69 ± 42.75	0.362
LDH (IU)	309.59 ± 80.57	319.45 ± 81.75	0.586
Time of hemoglobin reduction (weeks)	11.84 ± 9.13	6.42 ± 3.25	0.002
Minimum hemoglobin level (g/dl)	10.31 ± 1.59	12.06 ± 1.36	0.0001
Delta hemoglobin	3.40 ± 1.03	1.12 ± 0.80	0.0001
Dose reduction (n (%))	28 (55.76)	3 (9.09)	0.001
Discontinuation of drug (n (%))	9 (17.30)	0 (0)	0.019

43 patients had received conventional interferon and ribavirin, while 42 patients had received pegylated interferon and ribavirin. 27 of the 43 patients on conventional interferon and ribavirin (51.9%), and 25 of the 42 patients on pegylated interferon and ribavirin (48.1%) had developed anemia. There was no significant difference between treatment modalities in terms of the prevalence of anemia (p=0.543).

The lowest level of hemoglobin, 10.99 ± 1.72 g/dl, was reached in 9.74 ± 7.86 weeks, with an average of 2.51 ± 1.46 g/dl reduction. The lowest level of hemoglobin occurred in 11.84 ± 9.13 weeks in the group who developed anemia, and 6.42 ± 3.25 weeks in the group without anemia (p=0.002). The maximum drop in Hb levels was 12.06 ± 1.36 g/dl in the group without anemia, and 10.31 ± 1.59 g/dl in the group who developed anemia (p=0.0001). Delta Hb was 1.12 ± 0.80 g/dl anemia in the group without anemia, and 3.40 ± 1.03 g/dl in the group who developed anemia; this difference was statistically significant, i.e. delta Hb was higher in patients who developed anemia (p=0.0001).

Distribution of genotypes and alleles in MTHFR C677T gene polymorphism

Upon comparison of the two groups in terms of MTHFR C677T

polymorphism, 52 of the 85 individuals carried genotype C/C (61.2%), 24 genotype C/T (28.2%), and 9 had genotype T/T (10.6%). Distribution according to gender was found to be 34 genotype C/C (61.8%), 13 genotype C/T (23.6%), 8 genotype T/T (14.5%) in female and 18 genotype C/C (60.0%), 11 genotype C/T (36.7%), 1 genotype T/T (3.3%), in 30 male patients. There was no difference in distribution across genders (p=0.175) (Figure 1).

In the evaluation of the two groups in terms of MTHFR C677T polymorphism, 33 of the 52 anemic subjects were genotype C/C (63.5%), 12 genotype C/T (23.1%), and 7 genotype T/T (13.5%), while in the group without anemia (n=33), distribution of genotypes were 19 (57.6%), 12 (36.4%) and 2 (6.1%), respectively (Figure 2).

In the evaluation of the mean reduction in hemoglobin levels, according to MTHFR C677T polymorphism, there was no difference between genotypes (p=0.623).

Genotype T/T of MTHFR C677T gene polymorphism was 6.029-fold more prevalent in anemic patients, compared with genotype C/C. However, this result was statistically insignificant (p=0.137).

We investigated the balance of genotypes of MTHFR C677T gene polymorphism, according to the Hardy-Weinberg principle, in groups with and without anemia. While results were balanced in the group without anemia (chi-square=0.20, SD=2), results in the group that developed anemia was aberrant, according to the Hardy-Weinberg principle (chi-square=7.5, SD=2, P=0.024). Our results show that genotypes C/C and T/T are more prevalent than would be expected in the group with anemia, while genotype C/T is less common than would be anticipated.

Distribution of genotypes and alleles in MTHFR A1298C gene polymorphism

Upon comparison of the two groups in terms of MTHFR A1298C polymorphism, we were not able to obtain any results in 3 individuals. 30 of the remaining 82 subjects had genotype A/A (36.6%), 27 genotype A/C (32.9%), and 25 genotype C/C (30.5%). Distribution according to gender was found to be 20 genotype A/A (38.5%), 15 genotype A/C (28.8%), 17 genotype C/C (32.7%) among the 52 female patients, and 10 genotype A/A (33.3%), 12 genotype A/C (40%), 8 genotype C/C (26.7%), among the 30 male patients. There was no difference in distribution across genders (p=0.582) (Figure 3).

Upon comparison of the two groups in terms of MTHFR A1298C polymorphism, of the 50 patients who developed anemia, 18 were genotype A/A (36%), 14 genotype A/C (28%), 18 genotype C/C (36%), while the corresponding numbers in the group without anemia (n=32) was 12 (37.5%), 13 (40.6%) and 7 (21.9%), respectively (Figure 4).

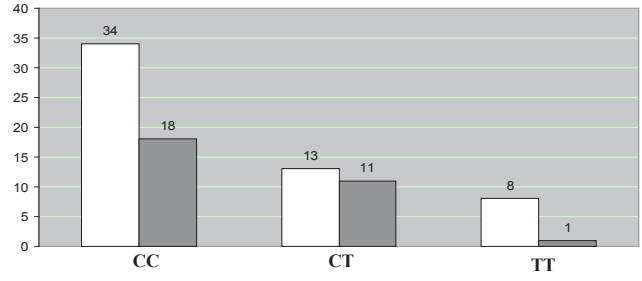

Figure 1: Distribution of MTHFR 677 C/T genotypes according to gender (White columns indicate female, and gray columns male patients).

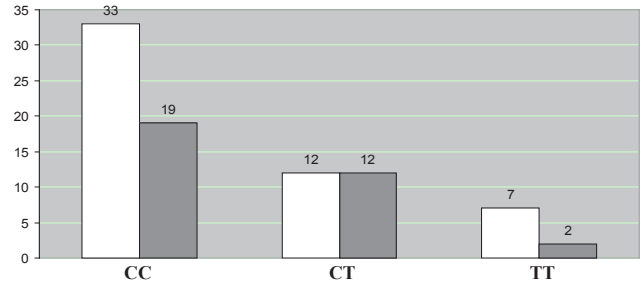

Figure 2: Distribution of MTHFR 677 C/T genotypes by patient group (White columns indicate the group who developed anemia, and gray columns the group without anemia).

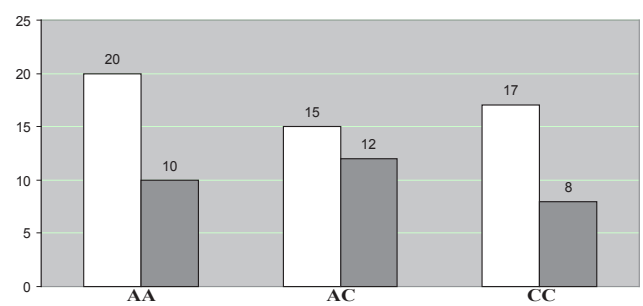

Figure 3: Distribution of MTHFR 1298 A/C genotypes according to gender (White columns indicate female, and gray columns male patients).

In the evaluation of the mean reduction in hemoglobin levels according to MTHFR A1298C polymorphism, there was no significant difference between genotypes (p=0.159).

Although genotype C/C of the MTHFR A1298C gene polymorphism was 3.586-fold more prevalent in anemic patients compared with genotype A/A, this difference was statistically insignificant (p=0.124). We investigated the balance of genotypes of MTHFR A1298C gene polymorphism, according to the Hardy-Weinberg principle, in groups with and without anemia, and found that while results were balanced in the group without anemia (chi-square=0.91, SD=2), results in the group that developed anemia were aberrant according to the Hardy-Weinberg principle (chi-square=7.68, SD=2, P=0.021). Our results show that genotypes A/A and C/C are more prevalent than would be expected in the group with anemia, while genotype A/C is less common than would be anticipated.

Distribution of genotypes and alleles in the AHCY 5'UTR-34 C→T variation

Upon evaluation of the two groups in terms of AHCY-34 C→T variations, 84 of the total 85 subjects were genotype C/C (98.8%), and 1 genotype C/T (1.2%). We did not encounter any patients with genotype T/T. Upon comparison of the two groups in terms of AHCY-34 C→T, all 52 patients who developed anemia were genotype C/C (100%), while patients without anemia (n=33) were 32 genotype C/C (97%) and 1 genotype C/T (3%). Mean reduction in hemoglobin levels, according to AHCY variations, could not be evaluated due to the low prevalence in our study population.

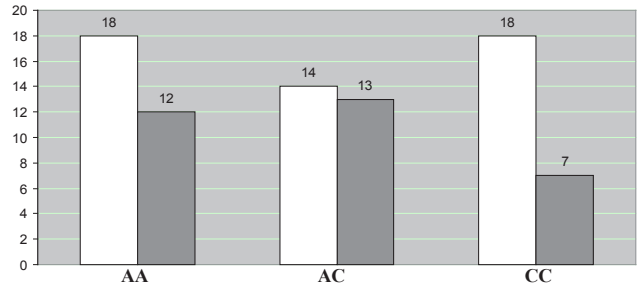

Figure 4: Distribution of MTHFR 1298 A/C genotypes by patient group (White columns indicate the group who developed anemia, and gray columns the group without anemia).

Distribution of genotypes and alleles in the AHCY Exon 2 Arg38Trp 112 C→T variation

In the evaluation of the two groups in terms of AHCY 112 C→T variations, 84 of the total 85 subjects were genotype C/C (98.8%) and 1 was genotype C/T (1.2%); there were no genotype T/T subjects. Upon comparison of the two groups in terms of AHCY 112 C→T, all 52 patients who developed anemia (%100) were genotype C/C, while patients without anemia (n=33) were 32 (97%) genotype C/C, and 1 (3%) genotype C/T.

Distribution of genotypes and alleles in the 4.5. AHCY Exon 4 Try143Cys A→G variation

In the evaluation of the two groups in terms of AHCY Try143Cys A→G conversion, all 84 patients were genotype A/A (100%). We did not encounter any patients with genotype A/G or G/G. A statistical assessment was therefore not possible.

Discussion

Our study results indicate that C677T and A1298C polymorphisms of MTHFR and AHCY-34 C→T, AHCY 112 C→T, AHCY Try143Cys A→G variations of AHCY do not constitute a risk factor for ribavirin-induced anemia. We determined that genotype T/T of the MTHFR C677T gene polymorphism is 6.029-fold more prevalent than genotype C/C in patients who develop anemia, but this difference was statistically insignificant (p=0.137). Meanwhile, genotype C/C of the MTHFR A1298C gene polymorphism was 3.586-fold more prevalent than genotype A/A in patients who develop anemia, but again, this difference was statistically insignificant (p=0.124).

The superior efficacy of interferon (IFN)/ribavirin combination therapy, as opposed to single-agent treatment in patients with HCV infection has been demonstrated, and hemolytic anemia is an important side effect of treatment with ribavirin. Anemia is possibly the most important hematological defect related to IFN/ribavirin therapy, and besides the decline in health-related quality of life, it may be the major influential factor of fatigue [31]. In a meta-analysis of three large studies comparing conventional interferon and pegylated interferon (Peginterferon), deterioration of the fatigue score was established as the main indicator of anemia, resulting in the discontinuation of treatment [32]. Untimely dose reduction or cessation of antiviral therapy undermines the efficacy of antiviral treatment. In multi-center clinical studies of IFN/ribavirin combination therapy for HCV infection, dose reduction due to anemia was required in 23% of all patients [33-35]. Discontinuation of therapy was rare in these studies. Conversely, the ratio of discontinuation was found to be higher in real-life settings (outside of clinical studies). Gaeta et al. [32] demonstrated that anemia was the leading cause of premature cessation of therapy in IFN/ribavirin combination treatment, responsible for 36% of all discontinuations, and that untimely cessation of therapy due to anemia occurred in 8.8% of all patients. Significant anemia (hemoglobin <10 g/dL) is observed in 9-13% of patients receiving IFN/ribavirin treatment (99), moderate anemia (hemoglobin <11 g/dL) in 30% [36]. The average maximum reduction in hemoglobin levels was 3.1 g/dL with ribavirin plus conventional interferon, and 3.7 g/dL with ribavirin plus pegylated interferon [36-38]. Ribavirin causes various degrees of erythrocyte hemolysis in nearly all patients, and necessitates a dose reduction in 7-9% of patients under combination therapy [2,3,33-35].

The prevalence of anemia in our study was 61.17% (52/85). The higher frequency compared to that reported in the literature may be attributable to our definition of anemia as a ≥2 g/dl drop in hemoglobin levels. Ribavirin dosage was decreased in 31 patients (36.47%), and ribavirin treatment was discontinued in 9 (10.58%); these ratios are consistent with those reported in the literature [37].

Ribavirin concentrations reach steady state within 2-4 weeks with continuous dosing [38]. Hematocrit levels generally reach the lowest values within 2-8 weeks after initiation of therapy; this is the phase when ribavirin reaches its highest concentration. After this, hematocrit values are stable, and return to baseline values following cessation of therapy [39,40]. The patients in our study group showed a mean reduction of 2.5 g/dl in hemoglobin levels within 10 weeks after initiation of therapy, and minimum values of around 11 g/dl were reached within the following week.

Little is known about the mechanism of anemia developing during IFN/ribavirin combination therapy for HCV infection. Ribavirin causes dose-dependent and reversible hemolytic anemia. Once inside the red blood cells, ribavirin undergoes phosphorylation to its active form, that causes depletion of adenosine triphosphate and ribavirin triphosphate, which interrupts cellular functions, accumulates inside the erythrocytes [41,42]. The accumulation of ribavirin triphosphate inside the RBCs disrupts antioxidant mechanisms on the erythrocyte membrane and causes oxidative damage, which leads to the removal of the damaged erythrocytes by the reticuloendothelial system [42]. It has been demonstrated that interferon interrupts the compensatory reticulocyte response against ribavirin-induced hemolytic anemia, and that the suppression of bone marrow by interferon contributes to the anemia related to combination therapy [3,43].

When erythrocytes are exposed to *in-vitro* ribavirin, the osmotic fragility and deformability of red blood cells is maintained [44]. Ribavirin in high doses inhibits the release of erythrocytes from the bone marrow; ribavirin shortens the life cycle of erythrocytes (30-69 days), and also diminishes erythrocyte mass [44,45].

Little is known about the variables that influence the development of hemolytic anemia in patients receiving treatment for HCV infection. A study on 244 patients with chronic HCV has reported that three factors have an effect on ribavirin-related hemolysis: pre-treatment platelet count, the amount of interferon alfa administered, and haptoglobin phenotype [46]. A possible explanation that has been contended for haptoglobin phenotype, the third factor that affects anemia, is that haptoglobin phenotypes differ in the extent

of intracellular migration of ribavirin or competitive intracellular migration of ribavirin [42].

Interferon alfa, acknowledged to have a suppressive effect on the bone marrow, contributes to anemia in these patients, when given in high doses [47,48]. Nomura et al. [47] established a significant correlation between the female gender, advanced age (>60 years), and weight-adjusted ribavirin dosage (>12 mg/kg), and anemia. The only factor that posed a risk for the development of anemia in our study was age. The risk of anemia increased 1.112-fold with age. Of the 85 subjects with HCV that were included, 55 were female and 30 male. The mean age of female patients was statistically significantly higher than male patients. In both groups, the majority of individuals that required a dose reduction were female. All 9 patients who discontinued treatment were female. Despite these findings, we could not establish a correlation between gender and the development of anemia.

The decrease in hemoglobin levels during IFN/ribavirin combination therapy may be due the level of ribavirin in erythrocytes. Two studies that investigated the correlation between erythrocyte ribavirin concentrations and alterations in hematological criteria in patients receiving IFN/ribavirin therapy for the eradication of HCV, reported that the overload of ribavirin, and its phosphorylated metabolite inside the erythrocyte is related to the decrease in hemoglobin levels, that causes interferon/ribavirin-induced anemia [49,50].

Furthermore, *in-vitro* studies have shown that RBCs of patients who develop hemolytic anemia during HCV treatment are more susceptible to oxidative stress [50]. Marked differences have been ascertained in the oxidative stress markers and membrane proteins of patients, with or without a history of ribavirin-induced anemia [50]. These findings suggest that there exist risk factors related to erythrocytes for ribavirin-induced anemia. It has been suggested that the augmented susceptibility of erythrocytes to oxidative stress, and modifications in the erythrocyte membrane content may be a result of alterations in the AdoMet/AdoHcy ratio inside erythrocytes that is caused by ribavirin; however; this has not been investigated [51]. It has been shown that total cholesterol, total phospholipids and the cholesterol/phospholipid ratio distinctly increase in the erythrocyte membrane lipid contents in patients receiving IFN/ribavirin treatment [52]. While phosphatidylcholine and the ratio of phosphatidylcholine/sphingomyelin ratio is diminished, other phospholipid fractions is increased [52]. These findings indicate that there are changes in the serum lipid and erythrocyte membrane lipid profile of patients with chronic HCV; who are treated with interferon and ribavirin [52]. It is particularly contended that these changes in the erythrocyte membrane lipids lead to a decrease in erythrocyte resilience and membrane viscosity, and may result in the hemolytic anemia related to IFN/ribavirin treatment [52].

The frequency of MTHFR A1298C lower than that of C677T, and the incidence of A1298C homozygosis is 9% [29,36]. We found a proportion of 36.6% genotype A/A, 32.9% genotype A/C, and 30.5% genotype C/C for MTHFR A1298C. The incidence of A1298C homozygosis in our study is considerably higher than reported in the literature. We are unable to explain this higher ratio at this time. The reported incidence of MTHFR C677T homozygosis is 1-20% [53]. In the 85 patients in our study, we found a proportion of 61.2% genotype C/C, 28.2% genotype C/T, and 10.6% genotype T/T for MTHFR C677T.

Gellekink et al. [25] investigated AHCY-34 C→T and AHCY 112 C→T gene variations in 172 patients with recurrent venous thrombosis. They found a ratio of 96.5% genotype C/C, 3.5% genotype C/T, and no genotype T/T in AHCY-34 C→T [25]; 94.2% genotype C/C, 5.8% genotype C/T, and no genotype T/T in AHCY 112 C→T [25]. They concluded that this gene variation was a minor risk factor in recurring venous thrombosis [25]. In our study, we observed 98.8% genotype C/C, 1.2% genotype 2 C/T, and no genotype T/T for both AHCY-34 C→T and AHCY 112 C→T gene variations. We did not encounter any AHCY Try143Cys A→G alleles.

In conclusion, although both MTHFR and AHCY play an important role in methionine metabolism and hypomethylation, we could not establish any correlation in this first study, investigating the impact of genetic defects that may cause alterations in the activity of these enzymes on IFN/ribavirin-induced anemia. However, even if it does not pose a risk for the development of anemia, genotype T/T of the MTHFR C677T gene polymorphism was approximately 6-fold, and genotype C/C of the MTHFR A1298C gene polymorphism around 3.5-fold more prevalent in patients who developed anemia. Further studies are needed to conclusively verify the lack of any effect of these gene polymorphisms on IFN/ribavirin-induced anemia. Since the risk of ribavirin-induced anemia increases with age, elderly patients should be closely monitored for anemia during treatment.

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