UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

DOCTORAL THESIS

PHARMACOGENETICS AND THE APPLICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN RESPONSE TO PEGYLATED INTERFERON AND RIBAVIRIN THERAPY IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS INFECTION

- ABSTRACT –

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INTRODUCTION

More than 200 million people worldwide have been infected with hepatitis C virus. (1) The natural history of hepatitis C differs greatly; the reasons for this heterogeneity remain incompletely understood, but are closely related to viral, environmental and host factors. Chronic HCV infection can lead to cirrhosis and hepatocellular carcinoma (HCC). There are about 7 viral genotypes discovered so far, with Europe being the most prevalent for viral genotype 1b. Chronic Hepatitis C is the only chronic viral infection that can be cured by antiviral treatment. Importantly, successful antiviral treatment can prevent the short- and long-term complications of HCV infection in many patients. Although HCV infection leads to hepatic inflammation and steatosis, the major consequence of persistent HCV infection is the development hepatic fibrosis, which can degenerate into a hepatic cirrhosis with a risk or degenerating into hepatocellular carcinoma. Hepatic fibrosis is the net result of a complex dynamic process. Hepatic fibrosis occurs in response to all forms of hepatic injury, also in this case due to hepatitis C virus aggression on liver tissue.

Until 2011, the golden standard for the treatment of HCV infection was dual therapy with Peg-Interferon, administered once a week subcutaneously and Ribavirin administered orally, but by 2016 in the case of Romania. Several Phase 3 crucial studies on the treatment of chronic hepatitis C with Peg-IFN-α, ribavirin, and either boceprevir or telaprevir resulted in the approval of 2 protease inhibitors in 2011. Several types of DAA were tested in clinical trials Phase 2 and 3, in combination with Pegylated IFN and ribavirin. The main advantages of first generation protease inhibitors are better tolerability and their use in IFN free regimens. Combinations of new DAAs that target different stages of the HCV life cycle have led to viral clearance in vitro and in animal models.

MATERIAL AND METHODS

Creating the database of experimental groups

We included 267 patients diagnosed with chronic viral hepatitis C, from the Renașterea Medical Centre and Emergency County Hospital Craiova during 2013-2016. The control group consisted of healthy patients without evidence of hepatitis C

infection. The study involved evaluating the eligibility of patients, including them in the study after having signed the informed consent and monitoring them during therapy. Patients received PegInterferon and Ribavirin for 48 weeks, and were evaluated at 4,12,24 and 48 weeks during treatment, and 24 weeks after the completion of therapy.

METHODS

The included patients were biologically assessed at 1,3,6 months after the initiation of therapy and 6 months post-therapeutically. Patients received questionnaires during therapy for subjective reporting of adverse effects. Each patient was also provided with an evaluation sheet at each medical visit in which the data was recorded, the events that occurred since the last visit, and the changes observed during the visit.

Collection of samples and biological material

Biological material consisted in venous blood samples harvested on EDTA and maintained at 4 ° C until DNA extraction. For the patients in the control group, the biological material consisted in venous blood samples. Samples were coded with KAM letters and numbers assigned in order of harvest.

Diagnostic tests

It was performed by electrochemiluminescence detection (ECLIA) or serological tests for anti-HCV antibodies by the Enzyme Linked Immuno Sorbent Assay (ELISA)

HCV DNA Determination (viral load)

HCV DNA was determined at baseline, 4 weeks after initiation, 12 weeks after initiation, 24 weeks after initiation, at end of therapy and 6 months after completion.

Hematological tests

A complete blood count was performed at each medical visit of the subjects included in the study.

Evaluation of hepatic fibrosis

The evaluation of hepatic fibrosis was performed by the FibroTest® test, a non-invasive alternative to hepatic biopsy that was clinically validated in patients with

chronic hepatitis B and C, ethanol-induced hepatopathy and non-alcoholic liver steatosis. It is based on an algorithm combining the results obtained from the determination of serum biochemical markers in order to assess the degree of fibrosis and necro-inflammatory activity.

Genetic analysis

The Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) was used to isolate genomic DNA from EDTA-harvested blood samples and maintained at 4 ° C. As regards the working protocol for highlighting single-nucleotide polymorphisms (SNP), TaqMan® Universal Master Mix and TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA) were used for each of the studied polymorphisms.

Statistical data processing

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) together with the XLSTAT suite for MS Excel (Addinsoft SARL, Paris, France) and IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY) , DOOR) were used.

RESULTS

Epidemiological study

The study included a total of 267 patients diagnosed with viral hepatitis C and admitted to the Medical Clinic II of the Emergency County Hospital Craiova or who presented themselves at a specialist consultation at the Renașterea Medical Centre. Our prospective study was conducted over a 4-year period, with an 18-month follow-up for each patient.

Of the initial group of 312 patients, only 267 met the inclusion criteria and did not show any exclusion criteria.

The highest share of chronic viral hepatitis C was recorded in the fifth decade of life (V), with a total of 80 patients (29.96%), followed by the fourth decade of age, representing 27.34% (73 patients) of the total included patients. The male / female ratio was 2: 1 without an additional risk factor for this population.

Study of adverse effects and response to treatment

We have investigated the incidence of adverse events during the antiviral treatment among patients. Of the total of patients enrolled in the study, 100 (37.45%) patients experienced weight loss, 78 (28.08%) patients had headache, 81 (30.33%) patients had myalgia ,100 (37.45%) of patients experienced fever or flu-like symptoms, 85 (31.83%) patients had arthralgia, 79 (29.58%) patients experienced local reactions at the injection site, 110 (41.19%) patients experienced nausea,126 (47.19%) of patients experienced loss of appetite, 29 patients (10.86%) experienced transit disorders,12 (4.49%) patients had post-impeditive pruritus, 16 (5.99%) patients had insomnia and irritability, 6 (2.24%) patients had depression, 4 (1.49%) patients experienced shortness of breath, 15 (5.62%) patients had rebellious cough and 3 (1.12%)patients developed hyperthyroidism, hypothyroidism and dermatitis.

Of the total patients included in the study, 139 (52.06%) of patients developed anemia, 123 (46.07%) patients had leucopenia, 95 (35.58%) patients had neutropenia and 119 (44, 56%) of patients experienced varying degrees of severity of thrombocytopenia.

Of the patients who responding to treatment, only 30.67% of the patients required a reduction in the dose of Ribavirin, which demonstrates that reducing the dose of Ribavirin negatively affects the response to treatment. The data demonstrate a strong statistical significance of the dose of Ribavirin in the development of anemia with a value of p calculated by the square Chi test of 0.001. The results of the study on the occurrence of anemia and the obtaining of sustained virological response were highly significant, demonstrating that patients with varying degrees of anemia responded better to antiviral therapy.

Results for the sustained virologic response study were as follows: 149 (55.81%) of patients achieved SVR at 24 weeks after the end of treatment and 118 (44.19) patients were non-responder. We could not identify a statistical link between the sex of patients in response to treatment. Neither women nor men responded better to Peg-Interferon and Ribavirin therapy.

We have studied the types of treatment response in our group. The results are as follows: 113 (42.32%) patients had EVR (early virologic response), 29 (10.86%) had brakethrough, 17 (6.37%) relapsers, 149 %) had RVS (sustained

virologic response), 167 (62.55%) had RVR (rapid virologic response) and 58 (21.72%) patients had DVR (delayed virological response). We demonstrated that RVS was positively influenced by obtaining rapid and early responses with a p <0.01.

We also demonstrated that patients with high viral loads(HVL) at initiation of therapy exhibited lower rates of sustained virologic response.

By studying the link between the viral load and the occurrence of a rapid or early virological response, we observed that 33 (12.36%) of patients who achieved RVR had a low viral load <400000UI / mL, and 83 (31.09%) patients did not obtain RVR. Of the total HVL patients, 134 (50.19%) patients achieved RVR and 17 (6.37%) patients did not get a rapid virological response. The results were unexpected, with a higher rate of rapid response for patients with HVL. The results of the study for high viral load and EVR have shown the following: 75 (28.09%) patients who achieved EVR had LVL and 38 (14.23%) had HVL; 76 (28.46%) of patients without EVR had HVL and 78 (29.21%) had LVL. The results were not statistically significant, but it can be seen that a high viral load is a negative predictive factor for obtaining an early virological response.

Of the patients who received a sustained virologic response, 81 (30.34%) patients had F1 grade fibrosis versus 23 (8.61) patients who did not respond, 48 (17.98%) of patients had had F2 grade fibrosis versus 44 (16.48%) of unresponsive patients and 20 (7.49%) patients had grade F3 versus 51 (19.10%) who did not respond. The results were statistically high, with a calculated p value of <0.001.

The result of the study of IL28B gene polymorphisms

Depending on the response to treatment, the results were as follows: 69 (70.41%) patients with CC genotype responded to treatment in 29 (29.59%) did not respond, 76 (56.72%) patients with CT genotype recorded SVR while 58 (43.28%) patients were nonresponsive, and 4 (11.43%) patients with TT genotype responded to treatment while 31 (88.57%) patients were non- of patients had detectable viremia at the end of treatment. The results of the study were highly significant with a p value of <0.001, demonstrating that patients with CC genotype are more likely to respond to PegInterferon and Ribavirin versus those with CT or TT genotype.

We also conducted a descriptive analysis of the rate of viral load decrease during treatment according to genetic polymorphism. The fastest decrease was recorded for genetic polymorphism CC but also patients with this genotype were those with the highest viral load.

We further investigated whether the genetic polymorphism rs12979860 influenced the rate of early and rapid virological response. The results were not statistically significant, demonstrating that the IL28B gene polymorphisms lack predictive power for rapid or early virological response during dual therapy.

The result of the gene polymorphism study rs8099917

We studied the relationship between response to treatment and the genetic polymorphism rs8099917. The results were as follows: 2 (0.75%) patients with CC genotype recorded SVR while 16 (5.99%) patients were nonresponsive, 54 (20.22%) patients with GT genotype responded to treatment while 47 (17.60%) of patients did not respond, and in the TT genotype, 93 (34.83%) patients responded to treatment compared with 55 (20.60%) patients who had detectable viremia at the end of treatment. The calculated p-value of <0.001 demonstrates that the study results were highly significant, demonstrating that patients with TT genotype are more likely to respond to treatment with PegInterferon and Ribavirin.

We continued with the analysis of the genetic polymorphism rs8099917 on the types of virological response.

Of the patients who had a rapid virological response, 9 (3.37%) patients had GG genotype, 62 (23.22%) patients had GT genotype and 96 (35.96%) patients with TT genotype while 9 (3.37%) patients with GG genotype, 39 (14.61%) of GT genotype patients and 52 (19.48%) patients with TT genotype, did not achieve rapid virological response. The results were statistically significant with a calculated p <0.001 which demonstrates that the TT genotype influences the rate of RVR occurrence.

As regards the early virological response, 1 (0.37%) GG genotype patient, 48 (17.98%) GT genotype patients and 64 (23.97%) TT genotype patients achieved EVR while 17 (6.37%) patients with GG genotype, 53 (19.85%) GT genotype patients and 84 (31.46%) patients with TT genotype did not achieve a

decrease of 2-log of viral load at 12 weeks of treatment. We obtained statistically significant results with a p value of 0.04 in terms of TT genotype and early virological response.

For the control group study, we noticed that in the case of genetic polymorphism rs 8099917, most patients presented the CC genotype, and in the case of the rs12979860 polymorphism, the proportions were relatively equal for CC and TT genotypes.

The result of the study of the ITPA gene polymorphisms

One of the most important side effects in dual therapy was indisputably, anemia. We tried to investigate whether the polymorphism of the ITPA gene correlated with the occurrence of anemia in PegInterferon and Ribavirin treated patients. The results obtained were statistically significant, demonstrating that patients carrying the minor A allele are protected against anemia.

We also investigated whether the single nucleotide polymorphism rs1127354 influenced to some extent the decrease in Hemoglobin over 2.5g / dl at week 4.

Taking into account the results presented above, we wanted to investigate whether a correlation with the need for Ribavirin dose reduction can be made during treatment. CC homozygous patients proved to be the least susceptible to this measure, with a share of 62.55% of patients (p <0.001) not requiring a change in the dose of Ribavirin. The study continued with the investigation of the same parameters in relation to the occurrence of leucopenia and neutropenia. The results were not conclusive, so it was not possible to demonstrate a link between the occurrence of these hematologic adverse effects and the ITPA gene rs 1127354 polymorphism. As far as thrombocytopenia was concerned, we investigated the relationship with the ITPA gene polymorphisms. We have conducted correlation studies between mild, moderate, severe thrombocytopenia and its development as a whole. We have not been able to demonstrate a statistical link between the parameters studied.

We also investigated whether the occurrence of adverse effects is influenced by the genotype rs12979860, the genotype rs8099917 and the genotype rs6051702. We have found that although there can be no prediction relationship, some polymorphisms have been shown to protect the occurrence of some of the adverse

events investigated. As with the other polymorphisms, we investigated whether genetic variants of ITPA rs 1127354 may be correlated with the occurrence of adverse effects in our study. It has been demonstrated that a very large proportion of patients with CC genotype are protected against the development of depression during treatment; no other correlation could be established.

The results of the study on the relationship between treatment response and genetic polymorphism were statistically significant with a p value of <0.001, demonstrating that patients with AA genotype were most likely to respond to Peginterferon and Ribavirin treatment.

We investigated whether anemia was influenced by any of the genetic polymorphisms investigated. Data were statistically significant, demonstrating that A allele predisposes to the development of anemia during treatment with PegInterferon and Ribavirin (p <0.001).

The result of the C20orf194-rs6051702 gene polymorphism study

We further investigated whether the single nucleotide polymorphisms of the C20orf194-rs6051702 gene were associated with a decrease in Hemoglobin value at week 4 of over 2.5g / dl. There was no association between AA, AC, CC and Hemoglobin at week 4 with a p calculated by the Chi square test of 0.1, but the AA genotype was found to be a protective factor. As far as neutropenia was concerned, we found the allele A to be a protective factor.

Of the total patients who experienced varying degrees of thrombocytopenia during treatment, 77 (%) of patients had AA genotype, 46 (%) of patients had AC genotype and 2 patients, CC genotype. The statistical study demonstrated that homozygous allele A patients are protected against the development of thrombocytopenia during antiviral treatment.

We investigated the relationship between the single nucleotide polymorphism rs6051702 and the Hemoglobin decrease over 2.5 g / dl at week 4. The results obtained were statistically significant in that the minor allele A had a protective role for the anemia developed at week 4 of treatment. However, we could not demonstrate that allele A retains its protective role throughout the treatment.

Regarding the control group study, we observed that in the case of the genetic polymorphism rs 1127354, the majority of patients presented the genotype CC and the genotype AA was not identified in any patient, and in the case of polymorphism rs6051720, most patients presented the genotype AA.

GENERAL CONCLUSIONS

The study group comprised 267 patients diagnosed with chronic hepatitis C virus infection, during 2013-2016.

The largest proportion of patients belonged to decades 4-6 of age, with the majority of them being between 49 and 59 years of age.

As regards the distribution of the batch studied by gender, 68.54% of the patients included in the study were female. The women: men ratio was 2: 1.

Most of the patients had an urban background, with 69.29% of the population, compared with 30.71% of the rural patients.

Adverse events during the study were varied, with predominance of gastrointestinal and pseudogripal side effects such as fever, myalgia and headache. Also, an important percentage of patients experienced arthralgia and local reactions at the injection site.

In terms of haematological adverse effects, 139 (52.06%) of patients had secondary anemia, 123 (46.07%) patients had leucopenia, 95 (35.58%) patients had neutropenia and 119 44.56%) of patients experienced varying degrees of thrombocytopenia.

Reduction of doses of Ribavirin negatively influenced sustained virological response. Thus, of the 149 (55.81%) patients responding to treatment, only 30.67% of the patients required a dose reduction of Ribavirin, while 69.33% of the patients who did not have a favourable treatment response, have required reduced doses of Ribavirin.

The dose of Ribavirin influenced the occurrence of anemia, the results being statistically significant. Due to the large number of patients receiving the 1000 mg

dose, proportions have remained relatively uneven, our study demonstrating that the 1000 mg dose affects the most the occurrence of anemia.

Anemia had a higher frequency among women than men; 40 men developed anemia during treatment, and in 99 women there were low Hb values during the study. However, women have better tolerated anemia, with lower doses of Ribavirin reductions.

From the studied group, 149 (55.81%) patients achieved SVR at 24 weeks after treatment and 118 (44.19) patients were non-responders.

Obtaining early and early virological response is a positive predictive factor for obtaining SVR. Of 113 patients who achieved EVR, 75 of them achieved SVR, and of 167 patients who achieved RVR, 133 received SVR.

Obtained sustained virological response was strongly influenced by anemia. 80 (57.55%) of patients had anemia and responded favourably to treatment, while 59 (42.45%) of those with anemia did not respond favourably.

Patients with low viral load at initiation of therapy are more likely to respond to dual therapy with PegInterferon and Ribavirin.

The degree of hepatic fibrosis influences the response to antiviral therapy. Of the patients who received a sustained virologic response, 81 (30.34%) patients had F1 grade fibrosis versus 23 (8.61) patients who did not respond, 48 (17.98%) of patients had had F2 grade fibrosis versus 44 (16.48%) unresponsive patients and 20 (7.49%) patients had grade F3 versus 51 (19.10%) who did not respond. Patients with F1 grade liver fibrosis responded best to treatment.

Patients with the IL28B gene CC genotype are more likely to respond to treatment. Of the 149 patients who achieved RVS, 69 (70.41%) of patients were homozygous for C allele.

No statistical link could be demonstrated between the IL28B gene polymorphism and the occurrence of anemia or rapid and early virological responses.

We have demonstrated that the CC genotype of the rs12979860 gene is protective for the occurrence of myalgia, arthralgia and severe thrombocytopenia.

Further in-depth studies are needed, on larger numbers of patients, to demonstrate this correlation.

As far as the polymorphism of the rs8099917 gene is concerned, the results of our study have shown that the TT genotype is a positive predictive factor for obtaining RVS.

We also demonstrated that the TT genotype of the rs809917 gene has a positive influence on the occurrence of rapid and early virologic responses with 96 TT genotype patients, 62 GT genotype patients, and 9 patients with genotype GG who achieved RVR and 64 patients with genotype TT, 48 GT genotype patients and 1 GG genotype patient who achieved EVR.

No statistical link could be demonstrated between the polymorphism of the rs8099917 gene and the occurrence of anemia.

We have shown that the TT genotype of the rs8099917 gene is protective for headache, arthralgia, transit disorders, moderate irritability and thrombocytopenia.

We tried to investigate whether the polymorphism of the ITPA gene correlated with the occurrence of anemia in PegInterferon and Ribavirin treated patients. The results obtained were statistically significant, demonstrating that patients carrying the minor A allele are protected against anemia.

In our study, it was demonstrated that the AC genotype was the strongest protector against the occurrence of anemia. We could not determine whether the genetic variants of the polymorphisms rs 1127354 and rs6051702 may have predictive value for antiviral therapy in patients with chronic viral hepatitis C in the South West of Romania.

Patients with the genotype AC of the rs1127354 gene are the most protected against a Hemoglobin drop of over 2.5g / dl at week 4 of treatment. Allele A of gene 1127354 has been shown to be a protective factor for a drop in Hemoglobin at week 4 of treatment.

It has been demonstrated that a very large proportion of patients with CC genotype are protected against the development of depression during treatment; no other correlation could be established.

As regards the single nucleotide polymorphisms of the C20orf194 gene, the data demonstrated that patients carrying AC / CC genotypes were protected against the development of anemia. Genotype AA has been associated with higher rates of anemia. Instead, no correlations could be established between these genotypes and a weekly decrease in Hemoglobin of over 2.5 g / dl. The data obtained corresponded to our previous findings in a smaller group of patients.

The CC genotype of the ITPA gene has been shown to have a negative predictive power to obtain early and rapid virological responses, and no correlation could be demonstrated in what concerns the obtaining of SVR.

A surprising finding was to association between the AA genotype of the c20orf194 gene with the sustained virological response.

We have found that although there can be no prediction relationship, some polymorphisms have been shown to protect against the appearance of some of the adverse effects investigated. Homozygous patients for the A allele, seem to be protected against the occurrence of moderate and severe thrombocytopenia and nausea secondary to antiviral therapy.