The impact of IL-6 and IL-28B gene polymorphisms on treatment outcome of chronic hepatitis C infection among intravenous drug users in Croatia

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Short title: IL genotypes and hepatitis C outcome

ABSTRACT

Background. Several genes and their single nucleotide polymorphisms are associated with either spontaneous resolution of hepatitis C infection or better treatment induced viral clearance. We tested a cohort of intravenous drug users (IVDU) diagnosed with chronic hepatitis C (HCV) for treatment response and its association with single nucleotide polymorphisms in Interleukin-6 (rs1800795-IL6) and Interleukin-28B (rs12979860-IL28B) genes.

Methods. The study included 110 Croatian IVDU positive for anti-HCV antibody. Genotyping was performed by PCR based approach. Patients were treated by standard pegylated-interferon/ribavirin and followed throughout a period of four years, during which sustained virological response (SVR) was determined. All data were analysed with statistical package SPSS 19.0 (IBM Corp, Armonk, NY) and PLINK v1.07 software.

Results. Patients showed a significantly better response to treatment according to the number of copies of the C allele carried at rs1800795-IL6 (P=0.034). All but one of the patients with CC genotype achieved SVR (93%), whereas the response rate of patients with GG genotype was 64%. The association of rs1800795-IL6 with SVR status remained significant after further adjustment for patients' age, fibrosis staging, and viral genotype (OR 2.15, 95%CI 1.16-4.68, P=0.019). Distributions of allele frequencies at the locus rs12979860-IL28B among the study cohort and the underlying general population were suggestive of a protective effect of CC genotype in acquiring chronic hepatitis C in the Croatian IVDU population.

Discussion. The rs1800795-IL6 polymorphism is associated with positive response to treatment in IVDU patients positive for HCV infection. A protective role of rs12979860-IL28B CC genotype in acquiring chronic hepatitis C is suggested for Croatian IVDU population.

INTRODUCTION

It is estimated that chronic hepatitis C virus (HCV) infection affects nearly 170 million individuals worldwide. It poses as one of the most important and growing threats to public health. Being an intravenous drug user (IVDU) is one of the most important risk factors in acquiring this infection. Both, acute and chronic, hepatitis C infection is a great therapeutic challenge. Only a minority of patients are able to clear the virus, and so do not run the risk of developing HCV induced end liver damage. Among the different and diverse list of factors that influence the therapeutic response, the host's cytokines play a very important role. The cytokine levels are directly influenced by certain gene polymorphisms located within their coding or regulatory regions (Wilson et al. 1997).

Among others, IL-6 is reported to be elevated in chronic HCV infection compared to healthy controls (Malaguarnera et al. 1997). The low-producing Interleukin 6 genotype IL-6 CC (IL-6 rs1800795 G174C) was associated with spontaneous clearance of hepatitis C virus in patients infected by contaminated blood products (Barrett et al. 2001). On contrary, high producing Interleukin 6 genotypes of the rs1800795 174G/C polymorphism (i.e., GG or GC genotypes) were associated with a greater likelihood of SVR in patients coinfected with HCV and HIV (Nattermann et al. 2007).

Human Interleukin-28B gene encodes interferon lambda-3 (IFN-lambda-3). Interferon lambda has demonstrated antiviral activity against HCV genotype 1 *in vivo* (Pagliaccetti & Robek 2010) and *in vitro* (Muir et al. 2010). It has been shown that a single nucleotide polymorphism (SNP) of IL28B gene (IL-28B rs12979860 C/T) predicts hepatitis C treatment induced viral clearance (Ge et al. 2009) and is associated with spontaneous resolution of hepatitis C infection (Duggal et al. 2013).

[LH1] Comentário: Include hepatitis C therapeutic regimen, since
pegylated interferon-ribavirin until the new direct acting antivirals (DAAs)

[LH2] Comentário: Include reference. WHO for example.

[LH3] Comentário: Use abbreviation: HCV

[LH4] Comentário: There are more references to be included

[LH5] Comentário: There are more references to be included

Here, we studied whether interleukin-28B or interleukin-6 (IL-6) promoter ~~single nucleotide polymorphism~~SNP affects the response to the standard antiviral treatment in IVDU patients diagnosed with chronic hepatitis C. We determined IL-6 promoter and IL-28B gene polymorphisms in a cohort of 110 patients with chronic hepatitis C. All IVDU positive patients diagnosed with chronic hepatitis C were treated with a standard protocol of peg-interferon alpha-2a and ribavirin. Rates of SVR were compared between IL-6 and IL-28B wild type, heterozygous and homozygous genotypes.

SUBJECTS & METHODS

The study samples

The sample of 112 patients was recruited from the outpatient hepatology unit at the Split Medical Center, Croatia and was followed from September 2007 until November 2013. The sample was drawn from the total of 947 HCV positive patients but only 112 individuals were diagnosed, biopsied, genotyped, treated and followed for a period of four years. Two patients were excluded from further analysis due to an ambiguous SVR status, possible reuse of intravenous drugs and re-infection with a different viral strain, resulting in a cohort of 110 patients. All enrolled patients had an alcohol consumption of less than 14 units per week and other common forms of chronic liver disease were excluded in all cases. Patients were Caucasians with the median (IQR) age at diagnosis of 40 (35-45) years in the SVR group and 41.5 (39-47) in the non-responder (NR) group.

We also used the data from the 10,001 Dalmatians biobank as the source of population-based sample of the underlying, general Croatian population. All controls were apparently healthy subjects with no record of addiction, risky behaviors or detected HCV infection (Polasek 2013; Rudan et al. 2009).

Patients' data were collected as a part of standard clinical procedure and the informed consent was obtained prior to participating in the study in all cases. The Ethics Committee of the Clinical Hospital Split approved the study (No: 2181-147-06-01/01-M.J).

Diagnosis of HCV infection

A third generation enzyme immunoassay (ELISA; Abbott Diagnostics, Germany) was used to test all subjects for HCV specific antibodies. Reverse-transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor; Roche Diagnostic Systems, New Jersey, USA) was used to test for HCV RNA in all subjects, in order to determine SVR.

Include parameters to response definition: SVR and NR. What about relapsers (REL)?

Liver Histology

Percutaneous liver biopsy was performed at the time of initial diagnosis and at the beginning of the treatment, using the Trucut biopsy technique (Sterylab, Italy) following informed consent. Inflammation was graded using a histological activity index (HAI) (Knodell et al. 1981) and fibrosis (Ishak et al. 1995). Every fifth biopsy was independently validated by two pathologists. A minority of patients was not biopsied either due to secondary coagulopathy or refusal to sign the informed consent and to participate in the procedure.

DNA extraction

A salting out technique was used to extract DNA from whole blood or using the QLAmp DNA midi prep kit, (Qiagen Ltd., Crawley, UK). The obtained DNA was used for IL-6 promoter and IL-28B genotyping. During this process, all the RNA was removed by incubating the digested preparation with 1.5 ml ribonuclease A (Boehringer Mannheim UK Ltd, East Sussex, UK) per 400 ml of nuclear lysate.

[LH6] Comentário: Check: isn´t QIAmp?

IL-6 and IL28B genotyping

A 175 base pair (bp) fragment of the human IL-6 gene spanning the promoter IL-6 G-174C region was amplified with gene specific primers (Roche Diagnostics, Alameda, California, USA). The resulting PCR fragments were analyzed with hybridization probes labeled with LightCycler Red 640. The genotypes were identified by running a melting curve with specific melting points (Tm) (Tib Molbiol GmbH, Berlin, Germany).

Genetic polymorphism in a SNP located near the IL-28B gene (rs12979860) was determined by enzymatic digestion of the PCR product. DNA fragments were amplified by using specific primers. Primer sequences were 5'-GCCTGTCGTGTACTGAACCA-3', and 5'-GCTCAGGGGTCAAATCACAGAAG-3', a PCR product of 143 bp was digested with HhaI enzyme and the resulting fragments of 27, 38, 65 and 78 bp were separated on acrylamide gel.

[LH7] Comentário: Include gel concentration. Include staining if important.

Statistical analysis

Data were analysed with the statistical package SPSS 19.0 (IBM Corp, Armonk, NY) and PLINK v1.07 software (Purcell et al. 2007). Absolute numbers and percentages were used to describe categorical data, whereas median and interquartile ranges (IQR) were used to describe quantitative data. One sample binomial test was used to assess distributions of sex, and response to the treatment. The association of response-to-treatment with sex or the viral genotype in infected patients was estimated with the Pearson's chi square test. Furthermore, nonparametric Mann-Whitney test was used to assess age differences, or differences in severity of fibrosis between the response groups.

Genetic association tests were mostly performed within PLINK software by using the case and control design. We defined cases as the group of patients that achieved SVR. Full

model association tests were run in PLINK for each SNP using either chi-square or, when appropriate, Fisher exact test; and the best-fit model was identified. Full model included basic allelic, Cochran-Armitage trend, genotypic, dominant gene action and the recessive gene action tests. Additionally, the difference in distribution of IL28B CC carriers vs CT+TT carriers across the response groups was estimated by the Pearson's chi square test using the SPSS v19 software (IBM, Armonk, NY) since the recessive model in PLINK tests only the minor allele and in both our samples (patients and the underlying, healthy general population) the allele C at the IL28B locus was the dominant allele. All significant p-values yielded by genetic tests were further controlled by empirical p-values which were generated by the permutation procedure. Cochran-Mantel-Haenszel statistics was used to test whether the predictive power of the SNP markers was independent of viral genotypes detected in infected patients. Associations of the SVR status with both SNPs were further evaluated by multivariate logistic regression while accounting for covariates/factors: age, viral genotype, stage of fibrosis.

RESULTS

A total of 110 IVDU patients with elevated liver function tests were diagnosed with a chronic hepatitis C, treated by standard interferon 2-alfa/ribavirin protocol and followed over at least one year for a SVR. The majority of treated patients (78 out of 110, or 71%) achieved SVR (one sample binomial test, $P<0.001$). Men were significantly more prevalent in our sample than women: 82 (75%) vs. 28 (25%) (one sample binomial test, $P<0.001$). Demographic and clinical characteristics of the sample are presented in Table 1. As shown in Table 1, non-responders were significantly older ~~that~~ than responders. We found no association of the SVR status with the sex, the initial Ishak score fibrosis stage, or the viral genotype.

Distributions of patients' genotypes across response-to-treatment groups are shown for both SNP loci, rs1800795- IL6 and rs12979860- IL28B, in Table 2.

With regard to genetic variation at the rs1800795-IL6 locus, we determined Hardy-Weinberg equilibrium in both response groups (exact test, p-values from 0.392 to 0.819). Overall, the genetic association tests indicated that the addition of allele 'C' has protective effect and increases the chance of achieving SVR. Specifically, several genetic association tests confirmed the association between rs1800795-IL6 polymorphism and SVR status, with the Cochran-Armitage trend test providing the best model fit (chi=4.477, df=1, p=0.034, empirical p=0.039). Patients with rs1800795-IL6 CC genotype had significantly better ~~sustained virological response~~SVR (14 out of 15, 93 %) compared to those with GC (37 out of 52, 71%) or GG (27 out of 43, 63%) genotypes (Figure 1).

After controlling for viral genotypes, the association of rs1800795-IL6 polymorphism with SVR status remained significant (Mantel-Haenszel 2x2 chi^2=4.483, p=0.034, with odds ratio of 1.99, 95% CI 1.05-3.78). The association was further evaluated by multivariate logistic regression analysis while accounting for covariates: age, viral genotype, and fibrosis (F≥3). The result of regression analysis confirmed that the addition of allele 'C' increased the chance of achieving SVR (OR 2.45, 95% CI 1.13-5.30, P=0.023). Besides the rs1800795-IL6 ~~polymorphysm~~polymorphism, age was the only covariate that significantly affected SVR status, with each additional year slightly decreasing the chance of SVR response (significance at the level of 0.1, OR 0.95, 95%CI 0.89-1.01, P=0.096).

The genotype frequencies at the rs12979860-IL28B locus met the Hardy–Weinberg expectations in nonresponders (exact test, p=0.473), and deviated from the Hardy-Weinberg equilibrium in SVR cases (i.e. SVR responders, p=0.004). When we analysed the genotype frequencies at the rs12979860 locus in the IVDU cohort, and in the underlying, apparently healthy, general-population sample (n=531 individuals; HWE, p=0.999), we observed that the

frequencies of CC, CT and TT genotypes in our cohort were: 27%, 63%, and 10%; whereas the corresponding frequencies in the population-based sample were 49%, 42%, and 9% (Figure 2). The finding demonstrated a large and significant reduction of CC genotype (z-score test for proportions, $p<0.001$), and a significant increase in frequency of heterozygous TC genotype ($p<0.001$) in the IVDU cohort of chronic HCV patients compared to the underlying, apparently healthy population. The frequencies of TT genotype were comparable between the groups ($p=0.741$).

With regard to the SVR responder and non-responder groups, we did not, however, observe a significant association of SVR response with the allele-carrier groups: CC and CT+TT (chi square=0.118, df=1, p=0.732); although the frequency of CC genotype was in fact somewhat higher in responders (by 3%). The result persisted after we controlled for viral genotypes (Mantel-Haenszel 2x2 chi2=0.630, p=0.427) and after multivariate logistic regression using age, viral genotype and fibrosis stage as additional predictors (data not shown).

DISCUSSION

This study investigated the role of IL-6 and IL-28B gene polymorphisms on SVR in IVDU patients diagnosed with chronic hepatitis C infection. The treatment was conventional and included Peg IFN combined with ribavirin for either 48 weeks (genotype 1) or 24 weeks (genotype 3). We defined sustained virological response as an absence of ~~detectible~~ detectable virus at the end of follow up evaluation and or disease relapses according to the standard definitions (Ghany et al. 2009).

Interleukin-6 was originally discovered as a protein that caused the final differentiation of B cells into immunoglobulin secreting cells (Muraguchi et al. 1988).

Additional work showed that IL-6 and its receptor - sIL-6Rα suppress neutrophil recruitment at site of acute inflammation, making way for the influx of monocytes as the inflammatory response is sustained (Kopf et al. 1994). IL-6 is well known pro-inflammatory cytokine with pro-tumorigenic potential (Grivennikov et al. 2009) and is emerging as a key regulatory signal in the development of the newly described pro-inflammatory effectors T-cell subset, so called Th17 cells (Harrington et al. 2005). The IL-6 rs1800795 G allele has been also associated with higher degrees of liver necroinflammation (Falleti et al. 2010) and fibrosis (Cussigh et al. 2011).

In our study, allele C at rs1800795-IL6 - a ~~single nucleotide polymorphism~~SNP in the IL-6 gene promoter, was associated with sustained virological response (OR 2.45, 95%CI 1.13-5.30, P=0.023). The genotype that confers the highest degree of protection in terms of achieving SVR, rs1800795-IL6 CC, demonstrated an overwhelming lower relapse rate in HCV treated patients (1 out of 15 patients, 7%). Similar results were reported for Italian non-IVDU HCV infected patients thus corroborating the importance of our findings (Cussigh et al. 2011). According to prior studies, the CC genotype appears to be associated with significantly lower level of plasma IL-6, whereas the GG and GC genotypes appear to have higher levels of plasma IL-6 (Fishman et al. 1998; Lapinski 2001). This implies a possible connection of IL-6 status with the therapy outcome. The putative low producing IL-6 phenotype may play a protective role against chronic hepatitis C infection by helping to clear the viral particles during standard therapy. Chronic hepatitis C patients with rs1800795-IL6 CC genotype and lower IL-6 serum level may have an attenuated adoptive immune response, directed away from damaging, pro-inflammatory and autoimmune to predominately suppressive and anti-viral inflammatory response.

An IL-28B gene single nucleotide polymorphism is located 8 kb upstream of the start codon of IL-28B gene that encodes interferon lambda (IFN-λ) a member of type III IFN

family. IFN-λ interacts with a transmembrane receptor to induce a potent antiviral response (Donnelly & Kotenko 2010; Fox et al. 2009; Li & Huang 2007). The antiviral activity is mediated through the activation of the either JAK-STAT (IFN α, λ and γ) or MAPK (IFN α and λ) pathways (Arslani et al. 2013). There is a strong association of genetic variations in IL-28B gene with response to therapy (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009), and with spontaneous HCV clearance (Duggal et al. 2013). In our cohort of IVDU patients diagnosed with chronic hepatitis C no association between the therapy outcome and the single nucleotide polymorphism in the IL-28B gene, rs12979860-IL28B was identified. However, our data do support involvement of the rs12979860-IL28B CC genotype in both of these patho-physiological/immunological processes: spontaneous HCV clearance and the response to therapy.

Firstly, when we compared our cohort of IVDU patients suffering from chronic HCV with the population-based sample taken from the apparently healthy, underlying population; the frequency of the favorable rs12979860-IL28B CC genotype which has been also associated with the spontaneous clearance of HCV, was largely decreased in the patient group. Conversely, the frequencies of genotypes considered to have a neutral effect on acquiring HCV infection/response to therapy were either increased in patients (TC) or showed no difference between the samples (TT). In other words, the protective genotype rs12979860-IL28B CC was found at much lower frequency in infected IVDU individuals with chronic HCV (27%) than in the underlying, healthy population (49%), thus pointing towards the selective loss of CC homozygotes in the patient population.

In line with our finding, Gelinas et al. have observed significantly higher prevalence of the responder genotype rs12979860 CC in a group of IVDU who were spontaneous resolvers from a HCV infection than in a baseline population of IVDU users (Gelinas et al. 2013); suggesting a dilution of CC genotype in chronic IVDU HCV patients. Additionally, also

supporting our findings, Ezzikouri et al. found that patients who had cleared HCV spontaneously were from 2.7 to 4.7 times more likely to carry CC genotype than the TC, or the TT genotype, respectively (Ezzikouri et al. 2013) while Montes-Cano et al. observed the CC genotype in 73% of individuals with spontaneous resolution of HCV infection versus only 46% in individuals with the persistent infection (Montes-Cano et al. 2010).

Secondly, our results still suggest the positive effect of rs12979860-IL28B CC genotype in acquiring SVR. In particular, the distribution of rs12979860-IL28B genotypes in the cohort of IVDU chronic patients significantly deviated from HWE-P only in the group of responders, whereas in non-responders, and in healthy controls the rs12979860-IL28B genotypes followed the HWE. Since the case (SVR responder) genotypes will only be in HWE under the multiplicative genetic model (Lewis & Knight 2012), the departure from this equilibrium, if found exclusively in cases, can be expected in relatively small samples of patients over a range of genetic models and is indicative of the actual association to the trait under study (Wittke-Thompson et al. 2005). In addition, we have observed somewhat higher percentage of CC genotype in SVR cases, although this percentage did not reach a statistical significance.

Similar to our results, Searberg et al. also did not found the association between rs12979860-IL28B genotypes and the spontaneous clearance of HCV in men who have sex with men (Seaberg et al. 2015). There ~~is~~ are obviously a large number of factors: demographic, viral, and human genetic factors; which influence HCV viremia and the results on distribution of particular SNPs should be interpreted in a larger context. The fact that the frequency of rs12979860 CC genotype varies in different ethnic groups or geographical areas adds to this complexity. In particular, several studies have estimated that East Asians had a high percentage of CC genotype, whereas the frequency of this genotype was intermediate in Europeans and minor frequency in African cohorts (Ge et al. 2009; Thomas et al. 2009).

Concerning the geographic variability of rs12979860 CC genotypes, previous studies on Caucasian patients who were infected with the HCV viral genotype 1 estimated the prevalence of CC genotypes to be between 35% and 39% (Cavalcante et al. 2012; Montes-Cano et al. 2010; Nattermann et al. 2011). The percentage of CC genotypes observed in our patients infected with the same viral genotype was 27% (95% CI 19-36%) which is somewhat lower, suggesting that the impact of IL-28B polymorphism on acquiring of infection or spontaneous clearance of HCV might be more prominent in the Croatian population. Having in mind that the population differences in rates of spontaneous clearance have also been proposed for IVDU patients (Fischer et al. 2004; Miedzinski & Taylor 2008), the impact of IL-28B polymorphism on the spontaneous clearance of HCV in Croatian population should be investigated in more details in order to increase our knowledge on the therapeutic effectiveness of pegylated-interferon/ribavirin on rs12979860 genotypes in different populations, particularly in high-HCV-risk IVDU population.

CONCLUSIONS

In conclusion, we have identified that the rs1800795-IL6 CC genotype is associated with significantly better sustained virological response to the standard Peg IFN and ribavirin treatment in IVDU/HCV patients. Also, findings point towards strong protective role of rs12979860-IL28B CC genotype in acquiring chronic hepatitis C infection in Croatian IVDU population. Finally, among all covariates, age is the most important, where every additional year slightly decreases the chance of SVR response. Further prospective and large scale clinical studies are necessary to confirm our results before we can prospectively identify IVDU and HCV patients for whom therapy is likely to be successful.

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Table 1. Demographic and clinical characteristics of IVDU patients by the response-to-treatment status

	SVR, N=78	NR, N=32	Statistics
Sex, N (%)			
men	56 (72%)	26 (81%)	Chi square test, p=0.301
women	22 (28%)	6 (19%)	
Age, median (IQR)	40 (35-45)	41.5 (39-47)	Mann-Whitney, p=0.041
Fibrosis stage, N (%)			
Mean Ishak score †	3 (2.0-3.0)	2 (1.0-3.0)	Mann-Whitney, p=0.077
Mean Knodell score ‡	7 (5.0-9.0)	6 (5.0-9.0)	Mann-Whitney, p=0.803
Viral genotype, N (%)			
1	51 (73%)	19 (27%)	Chi square test P=0.706
3	27 (68%)	13 (33%)	

SVR-sustained viral response; NR-non responders

† Liver fibrosis was scored by Ishak fibrosis score (0-6) as previously described. (Ishak et al. 1995)10

‡ Histology activity index by Knodell score (0-18) in chronic active hepatitis. (Knodell et al. 1981)9

485 **Table 2. Distributions of rs1800795- IL6 and rs12979860- IL28B genotypes, by the**
486 **response-to-treatment group**
487

SNP	Genotype	SVR, N=78	NR, N=32	Total
IL-28B, N (%)	CC	22 (28%)	8 (25%)	30 (27%)
	CT	50 (64%)	19 (59%)	69 (63%)
	TT	6 (8%)	5 (16%)	11 (10%)
IL-6, N (%)	GG	27 (35%)	16 (50%)	43 (39%)
	GC	37 (47%)	15 (47%)	52 (47%)
	CC	14 (18%)	1 (3%)	15 (14%)

488 SVR-sustained viral response; NR-non responders

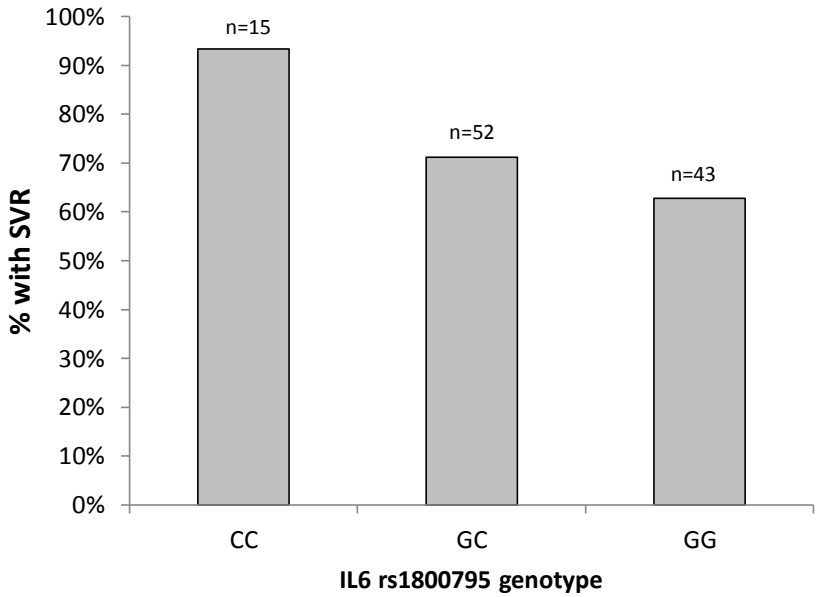
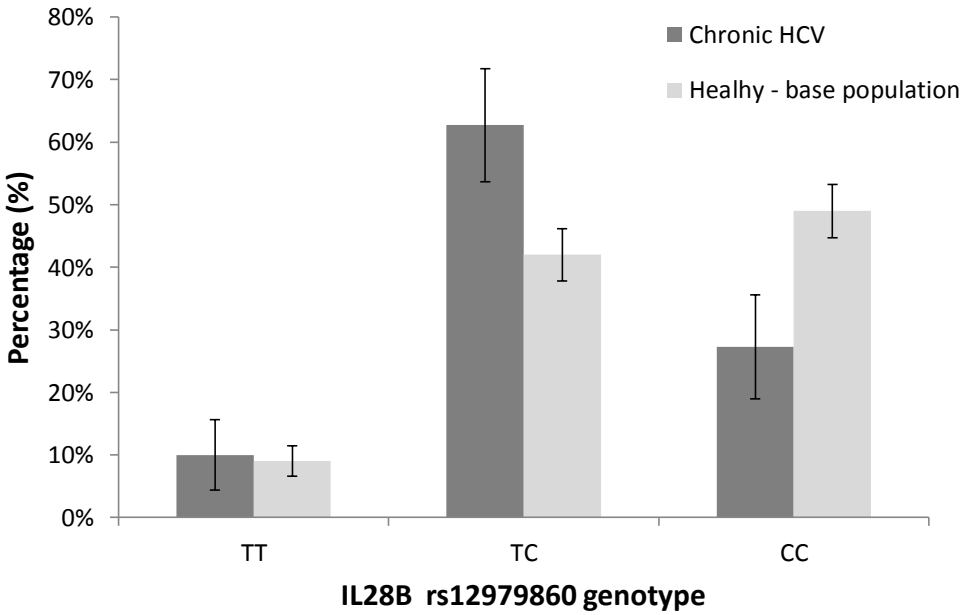


Figure 1. The percentage of patients with SVR by the rs1800795-IL6 genotypes

n = total number of patients with each genotype.



496

497 **Figure 1.** The distribution of rs12979860-IL28B genotypes among: IVDU with chronic HCV
498 (in black bars), and the underlying (base), apparently healthy population (in gray bars).
499 Shown are percentages with accompanying 95% CI.
500 Show statistically significances using * above the bars.

501