Abschlussbericht Teilprojekt 10.1.2

Projekttitel: Systematic High Through-Put Association Mapping in Chronic Hepatitis C

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Berichtszeitraum: 01.02.2005 – 31.01.2007

1. Summary

The genetic makeup of the hosts may play a role in the likelihood to develop chronic hepatitis and influence disease progression after infection with the hepatitis C virus. Polymorphisms resulting in a genotype associated with high secretion levels of TGF-ß1 or angiotensinogen II (1), respectively, as well as missense mutation in the HFE gene (2) are associated with a faster progression to cirrhosis. Polymorphisms in the TNF promotor (3) have been implicated in the recurrence of chronic hepatitis C after liver transplantation. Chemokine receptor 2 and 5 (CCR2 and CCR5) polymorphisms have been shown to influence the natural course of the disease.

2. Aims

We systematically investigated pathophysiological candidate genes and their promoters as well as dense map of SNPs on several chromosomes for association with a particularly aggressive phenotype. An integrated model of epigenetic and (multiple) genetic factors has been formed, which will cluster genetic variations with phenotype characteristics (including viral elimination kinetics)

3. Previous work

Until December 2005 more then the planned 1000 DNA samples of hepatitis C patients have been recruited in this project. DNA of all blood samples has been isolated. A platform for TaqMan based high-throughput SNP typing (5000 SNP genotypes per day) as well as an integrated genotype-phenotype database has been created. A capacity of 1000-2000 SNP genotypes per day and high troughput mutation detection capacity (ABI3700) was available for this project. Although final determination of SNP´s has been started as the calculated sample size of 1000 is reached, initial analyses in a subgroup of 465 patients were performed. Six single nucleotide polymorphisms and a 32 bp deletion in the genes coding for CCR3, CCR2, and CCR5 (which are all located in a cluster on chromosome 3) were investigated in 465 consecutively recruited patients infected with HCV and 370 matched controls during the first funding period. The G190A polymorphism (variant allele Ile64) in the first transmembrane domain of CCR2 was underrepresented in the 29 patients who had spontaneously cleared the hepatitis C virus (P=0.018). None of the other variants in the CCR gene cluster showed association with the natural course of the infection, progression of fibrosis or response to therapy. The manuscript has been accepted by Clinical and Experimental Immunology.

4. Current Work

The large cohort (> 1000 individuals) with different phenotypes (HCV infection with spontaneous recovery, chronic HCV infection, cirrhosis) has been completed. Phenotypic characterisation defined severity as a quantitative trait (e.g. stage of cirrhosis as a function of time) and included potential confounders (e.g. age, sex, genotype, alcohol consumption, iron load, viral load). The clinical database has been actualised every 3 months, if new clinical aspects are available (i.e. outcome of therapy). This has not stopped at the end of the funding period. It will be done in future as well. Although I left the institution on December 31th 2006 the medical faculty named Dr. Susanne Ross to follow up our initial work n the future.

SNPs in pathophysiological candidate molecules have been obtained from public databases and new polymorphisms have been generated by direct sequencing on an ABI 3700. In addition, 400 anonymous SNPs which are in pairwise linkage disequilibrium have been already established from public databases and by own sequencing in a region of about 20 cM around the HLA complex on chromosome 6p. This region includes a host of inflammation relevant genes (e.g. TNF, LTA, NFkB). The search for detection of associated haplotypes is still ongoing, although funding of the project ended in early 2006 and genes if detected will be identified (if not already annoted in the public sequence by exon trapping, gene prediction) and functionally characterized (expression patterns, immunohistology,

promotor studies). An intranet interface has be maintained to give other scientists (e.g. researchers wanting to look for pharmacogenetic predictors or who want to relate functional findings to certain viral antigenic structures or viral kinetics) access to individual genotyping data and the bioinformatics tools necessary for analysis. The high throughput sequenzing and genotyping platform and cohort has also been available for the establishment of new polymorphisms and the typing of gene variations of pathophysiological candidate genes identified in other projects of the HepNet or other networks like the competence network CED supported by the BMBF.

5. Original aims of the project

To investigate systematically pathophysiological candidate genes and their promoters as well as dense map of SNPs on chromosome 6 for association with a particularly aggressive phenotype. An integrated model of epigenetic and (multiple) genetic factors will be formed, which will cluster genetic variations with phenotype characteristics (including viral elimination kinetics) in 1000 patients with hepatitis C virus infection.

6. Scientific results

1100 consecutively recruited patients infected with HCV, mainly of German origin (more than 96% Caucasian origin from local, north-German referral areas), attending the outpatient clinic of the 1st Department of Medicine, University of Schleswig-Holstein, Campus Kiel and the 1st Department of Medicine, University Hospital Eppendorf (Hamburg), were included during the funding period (table 1). 370 age and sex matched blood donors, for whom infection with HCV and HIV could be excluded were used as controls.

Tab. 1: Clinical characterization of the cohort of HCV infected patients (n=1100).

Trait	Category	%
Gender	Men	56%
	Women	44%
Viral genotype	1	78%
	2	3%
	3	16%
	4	3%
Infection status	Chronic disease	93%
	Spontaneous viral clearance	7%
Grading of fibrosis	Mild (stages 0, 1, 2)*	78%
	Severe (stages 3, 4)	22%
IFN based therapy if performed	Sustained response	43%
	Lack of sustained response	57%

*

Considering only patients infected since more than 10 years

CCR-polymorphisms (first funding period)

The CCR cluster has been extensively sequenced in Caucasians and Asians in previous studies. A *de novo* mutation detection was therefore not necessary. A series of 16 SNPs, which have been reported in the literature, was preliminarily investigated in 96 individuals (HCV infected patients and healthy controls). The following SNPs were monomorphic in the population studied (Table 1): +971 in CCR3 (previously identified in a Japanese population but not in a British one of 142 individuals) [44], and +811, +714, +684, +630, +626, +612 in CCR5 (counting from the start of exon 1) representing the rare promoter haplotypes P5, P6, P7, P8, P9, P10 [29]. SNPs at nucleotide positions +240, +824 and +1052 in CCR3 were observed at a low frequency (<1%) as previously reported [27] and not genotyped in the full sample. Therefore, the following variants were analysed in our sample: G190A (Val64Ile) and T780C (Asn260Asn) in CCR2, T51C (Tyr17Tre) in CCR3, +208, +627, +676 in the CCR5 promoter and □32 in CCR5. SNPs +208, +627 and +676 in CCR5 occurred in the

known haplotypes P1 (G, C, A), P2 (G, T, A), P3 (T, T, A) and P4 (T, T, G) [26 29 45]. CCR2-64Ile and CCR5-Δ32 never occurred together but were always found on a CCR5-P1 bearing haplotype. The complete CCR2-CCR5 haplotypes were therefore: +.P1.+, 64Ile.P1.+, +.P1.Δ32, +.P2.+, +.P3.+ and +.P4.+.
Genotype distributions approximated Hardy-Weinberg equilibrium for the HCV infected patients as well as the healthy controls. Allele and genotype frequencies for each SNP, as well as haplotype frequencies, did not show any statistically significant difference between the HCV infected patients and the healthy controls ($p>0.75$).
Protection against chronic HCV Infection:
The CCR2-64Ile variant was under-represented in the 29 patients with spontaneous viral elimination compared with the 406 patients suffering from chronic HCV infection (Table 3). Comparison of genotype frequencies resulted in a P value of 0.018. Assumption of a dominant effect and therefore analysis of carrier status (combination of Ile64Ile and Ile64Val) resulted in a similar statistical significance for the association (P=0.02). The haplotype 64Ile.P1.+ showed a reduced frequency of 0.02 in patients, who eliminated the hepatitis C virus in comparison with a frequency of 0.10 in both patients with chronic infection and in healthy controls. The corresponding wildtype haplotype +.P1.+ was more frequent in the patients characterised by spontaneous viral elimination (0.44) than in the chronically infected (0.35) and healthy controls (0.35). None of the other variants in the CCR gene cluster showed a statistically significant association with spontaneous viral elimination.

Polymorphisms in the IL1 cluster, IL4, IL4R, IL6 and IL10

The following 12 SNPs were genotyped by Allelic Discrimination, TaqMan technology (ABI 7700, Applied Biosystems, Foster City, CA): IL1a –889 (T/C) (from the start of the primary transcript, corresponding to –1612 from the ATG), IL-1b -1903 (T/C) (from the start of the primary transcript, corresponding to –587 from the ATG, AluI restriction site), IL1b +5810 (A/G) in intron 4 (numbering from the start of the primary transcript, corresponding to +484 from the end of exon 4, BsoFI restriction site), IL1RA +389 (C/T, Ser130Ser, MspA1I restriction site), IKKE +201 (C/T, Ile67Ile, rs1539243, IL4 –589 (C/T), IL4R +224 (G/A, Val50Ile of mature peptide), +1726 (A/G) (Gln576Arg of mature peptide) and +3044 (A/G) in the 3'UTR, IL6 –174 (G/C) and –572 (G/C) in the 5'UTR, IL-10 –592 (C/A) and -819 (C/T) in the promoter region (counting from the transcription starting site)

These 12 polymorphisms in the genes coding for IL1a, IL1b, IL1RA, IL4, IL4R, IL6, IL10 and IRAK1 were investigated in 370 consecutive HCV patients (73% infected with viral genotype 1). None of the mutations tested was found associated with spontaneous viral elimination against chronic disease, cirrhosis against mild fibrosis (patients infected for more than 10 years) or response to therapy (IFN and IFN+R) (manuscript in preparation).

Actual genotyping

The following genes which are still under investigation including members of the TNF-alfa / NF-kB (TNF-alfa, TNF-beta, TNFR1, TNFR2, TRAF1, TRAF2, NFKBIA, NFKBIB) and apoptotic (Fas, FasL, FADD) pathways, as well as genes directly involved in viral recognition (RANTES, SDF, SDF receptor, Mx1, SCYA4). A manuscript concerning the role of the interleukins has been submitted recently
(Hinrichsen H et al. Investigation of polymorphisms in the IL1 cluster, IL4, IL4R, IL6 and IL10 in relation to Hepatitis C infection outcome). Furthermore in cooperation with the other groups in this BMBF competence net a publication concerning the role of complement factor 5 has been submitted by Juliane Halangk to the Journal of Hepatology recently (Evaluation of complement factor 5 variants as genetic risk factors for the development of advanced fibrosis in chronic hepatitis C infection). In cooperation with the other genetic working groups in the HepNet a publication concerning the role of gender in CTL4-polymorphisms and spontaneous recovery from hepatitis C by E. Schott has been accepted by the Journal of Hepatology recently (Gender-dependent association of CTLA4 polymorphisms with resolution of hepatitis C virus infection).

7. Publications

- **H. Hinrichsen,** S. Mascheretti, S. Ross, P. Buggisch, J. Hampe, U.R. Foelsch and S. Schreiber. Genetic variants in the CCR gene cluster and spontaneous viral elimination in Hepatitis C infected patients. Clin Exp. Immunol 2004
  This publication was possible by the funding of the competence network Hepatitis and the competence network CED, as the infrastructure for the genetic testing was provided by the local group of the competence network CED (Prof. Schreiber).
- E. Schott, H. Witt, **H. Hinrichsen**, K. Neumann, V. Weich, A. Bergk, J. Halangk, T. Müller, S. Tinjala, G. Puhl, P. Neuhaus, B. Wiedenmann and T. Berg Gender-dependent association of CTLA4 polymorphisms with resolution of hepatitis C virus infection. J Hepatol 2007
- J. Halangk, C. Sarrazin, K. Neumann, G. Puhl, T. Mueller, G.Teuber, H. Klinker, **H. Hinrichsen**, P. Buggisch, O. Landt, V. Weich, A. Bergk, B. Wiedenmann, P. Neuhaus, T. Berg and H. Witt. Evaluation of complement factor 5 variants as genetic risk factors for the development of advanced fibrosis in chronic hepatitis C infection. J Hepatol 2007 submitted
- **H. Hinrichsen**, S. Ross, S. Mascheretti, C.Rosati, J. Hampe and P. Buggisch. Investigation of polymorphisms in the IL1 cluster, IL4, IL4R, IL6 and IL10 in relation to Hepatitis C infection outcome. Eur J Gastroenterol Hepatol 2007 submitted

Networking

The cooperating partners from the inital part of the funding period (PD. Dr. Berg, Berlin, Prof. Schmidt, Bochum and Dr. Buggisch, Hamburg) could be enlarged by Prof. Schott from the RKI in Berlin and Dr. Witt from the Universitätsmedizin in Berlin. An IT platform for the clinical database has been designed. There has been no double testing of SNP`s in these groups. Furthermore DNA of patients has been already shared within the genetic projects of the HepNet (PD Dr. Berg, Prof. Schott, Dr. Buggisch, Dr. Witt)). Furthermore first plans for a common clinical database of the cohorts of Berlin and Kiel have been undertaken to optimise research activities in the two largest cohorts in Germany. The establishment of the big cohorts and their combining database will be in the near future the working platform for the majority of genetic tests in hepatitis C. Only this two databases are able the study special aspects in hepatitis C i.e. spontaneous cure, as all other cohorts only compromise patients with treatment. In comparison with the general DNA bank in Essen this cohorts are still under examination for further clinical details (prospective updating of the clinical database, allowing to answer several questions in the future. The input in the clinical database does allow to study several questions which might be unresolved using other cohorts without informations about stage of disease, co-factors like alcohol abuse, genotype, iron load, duration of infection, chronicity or spontaneous cure.

The genetic testing of polymorphisms in a large cohorts of hepatitis C patients will contribute to the natural course of the disease and possible to the effect of antiviral therapy (pharmacogenomics). This genetic testing is only reliable in large cohorts of patients, which will only be reached in cooperations of different centers, ideally in the competence network HepNet. Furthermore due to the local activities of the competence network CED there was /is still a cooperation between boths networks, as several equipments of the CED network can be used for the genetic testing in HCV and furthermore the knowledge of genetic testing in chronic inflammatory bowel disease will provide new candidate genes for chronic infection which will be of interest in hepatitis C as well. Thus cooperations are not limited to other partners of the competence network HepNet but are still established with the competence network CED.