Intrahepatic expression of genes related to metabotropic receptors in chronic hepatitis

Andrzej Cieśla, Maciej Kuśmider, Agata Faron-Górecka, Marta Dziedzicka-Wasylewska, Monika Bociąga-Jasik, Danuta Owczarek, Irena Ciečko-Michalska, Dorota Cibor, Tomasz Mach

Andrzej Cieśla, Monika Bociąga-Jasik, Danuta Owczarek, Irena Ciečko-Michalska, Dorota Cibor, Tomasz Mach, Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, 31-521 Kraków, Poland
Maciej Kuśmider, Agata Faron-Górecka, Marta Dziedzicka-Wasylewska, Institute of Pharmacology, Polish Academy of Sciences, 31-343 Kraków, Poland
Author contributions: Cieśla A, Kuśmider M, Faron-Górecka A, Dziedzicka-Wasylewska M, Mach T designed the research; Cieśla A, Kuśmider M, Faron-Górecka A, Dziedzicka-Wasylewska M performed the study; Kuśmider M, Faron-Górecka A, Dziedzicka-Wasylewska M performed the analytical part of study; Cieśla A, Kuśmider M, Faron-Górecka A, Dziedzicka-Wasylewska M, Owczarek D, Ciečko-Michalska I, Cibor D performed the study; Cieśla A, Kuśmider M, Faron-Górecka A, Dziedzicka-Wasylewska M, Ciećko-Michalska I, Owczarek D, Ciečko-Michalska I, Cibor D, Mach T wrote the paper.

Correspondence to: Andrzej Cieśla, MD, Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, 31-521 Kraków, Poland. acielas@us.krakow.pl
Telephone: +48-12-4247340 Fax: +48-12-42473480
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Abstract

AIM: To screen for genes related to metabotropic receptors that might be involved in the development of chronic hepatitis.

METHODS: Assessment of 20 genes associated with metabotropic receptors was performed in liver specimens obtained by punch biopsy from 12 patients with autoimmune and chronic hepatitis type B and C. For this purpose, a microarray with low integrity grade and with oligonucleotide DNA probes complementary to target transcripts was used. Evaluation of gene expression was performed in relation to transcript level, correlation between samples and grouping of clinical parameters used in chronic hepatitis assessment. Clinical markers of chronic hepatitis included alanine and aspartate aminotransferase, γ-glutamyltranspeptidase, alkaline phosphatase and cholinesterase activity, levels of iron ions, total cholesterol, triglycerides, albumin, glucose, hemoglobin, platelets, histological analysis of inflammatory and necrotic status, fibrosis according to METAVIR score, steatosis, as well as anthropometric body mass index, waist/hip index, percentage of adipose tissue and liver size in ultrasound examination. Gender, age, comitant diseases and drugs were also taken into account. Validation of oligonucleotide microarray gene expression results was done with the use of quantitative real-time polymerase chain reaction (qRT-PCR).

RESULTS: The highest (0.002 < P < 0.046) expression among genes encoding main components of metabotropic receptor pathways, such as the α subunit of G-coupled protein, phosphoinositid-dependent protein kinase or arrestin was comparable to that of angiotensinogen synthesized in the liver. Carcinogenesis suppressor genes, such as chemokine ligand 4, transcription factor early growth response protein 1 and lysophosphatidic acid receptor, were characterized by the lowest expression (0.002 < P < 0.046), while the factor potentially triggering hepatic cancer, transcription factor JUN-B, had a 20-fold higher expression. The correlation between expression of genes of protein kinases PDK1, phosphoinositide 3-kinase and protein kinase A (Spearman’s coefficient range: 0.762-0.769) confirmed a functional link between these enzymes. Gender (P = 0.0046) and inflammation severity, measured by alanine aminotransferase activity (P = 0.035), were characterized by diverse metabotropic receptor gene expression patterns. The Pearson’s coefficient ranging from -0.35 to 0.99 from the results of qRT-PCR and microarray indicated that qRT-PCR had certain
INTRODUCTION
The natural course of chronic viral hepatitis is affected by progression of fibrosis and the risk of hepatocellular carcinoma development[1]. Current data indicate that intracellular signaling disturbances have an impact on progression of inflammation and fibrosis, as well as carcinogenesis, in the course of chronic hepatitis.

G-protein-coupled receptors (GPCRs) are a family of cell surface receptors which receive, integrate and enhance the majority of extracellular signals. After stimulation with different signals, GPCRs activate amplifying enzymatic cascades, regulatory proteins and ion channels. This activation regulates cellular responses, including growth, proliferation, and cell survival.

In the present study, using microarray DNA analysis, we attempt to define genes related to metabolotropic receptors associated with progression of chronic hepatitis.

DNA technology with genomic profiling and cluster analysis allows determination of the role of genes in the pathogenesis of liver injury[2]. We assessed the activity of 20 genes encoding metabolotropic receptors, some of which have been documented to have probable significance in the progression of chronic hepatitis.

The assessment of expression of genes of the main component of GPCR, such as the G-protein α subunit (GN-A), 3-phosphoinositide-dependent protein kinase-1 (PDPK1), phosphoinositide 3-kinase (PIK3CG), protein kinase A (AKT1) and arrestin β (ARRB2) was performed. We determined transcription factor JUN-B, ETS-domain protein (ELK4), early growth response protein 1 (EGFR) activated as the result of GPCR stimulation, angiotensinogen (AGT), which is a GPCR-ligand, dual-specificity protein phosphatases 14 (DUSP14), which is responsible for dephosphorylation of kinase products, calcitonin receptor proteins (CALCR), thyrotropin receptor (TSHR), colony stimulating factor-3 (CSF3), sphingosine-1-phosphate receptor 1 (EDG1), and lysophosphatidic acid receptor (EDG2) associated with G-protein. The selection of EDG2, EDG1, JUN-B, chemokine (C-C motif) ligand 4 (CCL4), ELK4 genes was based on their association with cellular proliferation, differentiation and apoptosis[3-6]. Additionally, the selected group represented genes involved in regulation of inflammatory response and liver fibrosis, which included genes for interleukin 1β (IL-1β) and its receptors IL1 type 1 (IL1R1), IL1 type II (IL1R2), CALCR, EDG1, CCL4, AGT and adhesion molecules vascular cell adhesion molecule 1 (V-CAM1)[7-9].

MATERIALS AND METHODS
In the group of 12 patients (7 men, 5 women; age 36 ± 10.8 years) with chronic hepatitis type B (2 patients) and C (8 patients) and autoimmune hepatitis (2 patients), according to clinical indications, a liver biopsy was performed by the Menghini technique. From the obtained liver sections, a sample of 2-3 mm in length was frozen at -75 °C until the analysis of mRNA of 20 selected genes was performed (gene list Figure 1). During the histopathological investigation of the biopsies, the degree of inflammation and fibrosis was assessed by the METAVIR score and steatosis by the steatosis scoring system.

On the day of liver biopsy, the activity of serum alanine aminotransferase (ALT/GPT Cobas, Roche Diagnostics, Mannheim, Germany), aspartate aminotransferase (ASAP/GOT Cobas), γ-glutamyltransferase (GGT Cobas), alkaline phosphatase (ALP Cobas), cholinesterase (CHE Cobas), the level of iron (Fe Cobas), total cholesterol (CHOD-PAP Cobas), triglycerides (TG Cobas), albumin (ALB plus Cobas), glucose (GLU Cobas), hemoglobin and platelet (Sysmex XE-2100, Sysmex Europe GMBH, Norderstedt, Germany) were assessed. Anthropometric measurements, including body mass index, waist/hip index and the percentage of fatty mass was assessed by skin fold thickness. The size of the liver was measured during ultrasound examination of the abdomen. Patient characteristics included the presence of concomitant illnesses, drug history, as well as the use of such substances as alcohol and cigarettes (Figure 2). In women, the menstrual cycle phase and menopause were taken into account. Biochemical, histological and anthropometric parameters are presented in Figure 2. The gene expression studies were performed using microarray (with low integration level), with DNA oligonucleotides complementary to the investigated transcripts. Total RNA was isolated from liver samples using the TRI Reagent (Sigma-Aldrich, St. Louis, United States), and then purified using the RNaseasy MiniElute spin columns with a DNA eliminator (Qiagen, Hilden, Germany). The quantity of isolated RNA was assessed by a spectrophotometer (NanoDrop, Wilmington, United States), and subsequently its degradation level was limitations as a validation tool for oligonucleotide microarray studies.

CONCLUSION: A microarray-based analysis of hepatocyte metabolotropic G-protein-related gene expression can reveal the molecular basis of chronic hepatitis.

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Key words: Metabotropic receptors; Gene expression; DNA oligonucleotides; Quantitative real-time polymerase chain reaction; Chronic hepatitis

Peer reviewer: Dr. Shashi Bala, Department of Medicine, Umass Medical School, 346 Plantation Street, LRB2701, Worcester, MA 01605, United States

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Cieśla A et al. Metabotropic receptors in chronic hepatitis
Diversification in gene expression was assessed by agglomerative hierarchical clustering methods. Using the distinction was analyzed using Fisher’s exact test, to determine the difference between clustering of patients achieved on the basis of a dendrogram of clinical signs and a dendrogram of gene expression (Figures 1 and 2). Differentiation of clinical parameters between groups of patients with the biggest difference in gene expression was performed by the Mann-Whitney U test and Fisher’s exact test. In the present study, these were patients number 2, 10, 9, and 7 vs the rest (Figure 1). The Wilcoxon signed-rank test was used to assess differential expression between the selected genes, to determine the three groups of genes with the highest, moderate, and lowest activity (Figure 3).

The results of the microarray experiment were verified by means of quantitative real-time polymerase chain reaction (qRT-PCR) for IL1B, VCAM1, PIK3CG, AGT, PDPK1, GNAS, JUN-B, EDG1, CCL4, EGR1, IL1R1, IL1R2, CALCR, AKT1 and ARRB2. Total RNA acquired from the tissues of interest was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, United States) for the 2-step qRT-PCR assays. The qRT-PCR was performed on a Chromo 4 (Bio-Rad), using the ABI SYBR Green master mix (Applied Biosystems). Primer sets against the selected genes were commercially available at OriGene (Rockville, United States), but were additionally checked for specificity with the Basic Local Alignment Search Tool (BLAST). The obtained results describing the relative levels of gene expression (with respect to the reference gene) were further examined.

Diversification in gene expression was assessed by agglomerative hierarchical clustering methods. Using the Spearman’s rank correlation coefficient for activity of the investigated genes, we searched for pairs of objects and then for clusters with the smallest distance (Figure 1). An analogous classification was carried out using biochemical, histological and anthropometric parameters (Figure 2). Determination of a direct correlation between gene expression and clinical features was done based on agglomerative hierarchical clustering of both the investigated indicators of chronic hepatitis. The genotype-phenotype distinction was analyzed using Fisher’s exact test, to determine the difference between clustering of patients with the biggest difference in gene expression was performed by the Mann-Whitney U test and Fisher’s exact test.

Determination of a direct correlation between gene expression and clinical features was done based on agglomerative hierarchical clustering of both the investigated indicators of chronic hepatitis. The genotype-phenotype distinction was analyzed using Fisher’s exact test, to determine the difference between clustering of patients achieved on the basis of a dendrogram of clinical signs and a dendrogram of gene expression (Figures 1 and 2). Differentiation of clinical parameters between groups of patients with the biggest difference in gene expression was performed by the Mann-Whitney U test and Fisher’s exact test. In the present study, these were patients number 2, 10, 9, and 7 vs the rest (Figure 1). The Wilcoxon signed-rank test was used to assess differential expression between the selected genes, to determine the three groups of genes with the highest, moderate, and lowest activity (Figure 3).
There was a statistically significant difference in the Wilcoxon signed-rank test between the genes with the highest expression, GNAS, PDPK1, AGT, and ARRB2, and moderate expression, PIK3CG, AKT1, IL1β, VCAM1, CALCR, and JUN-B, and between the genes with moderate expression and the remaining genes with low expression. A sample volume of 20 μL was used for all assays which contained a 1X final concentration of SYBR green PCR master mix, 100 nmol gene specific primers, and 1 μL of template. The assays were run using the following protocol: 95 °C for 10 min, 95 °C for 40 s, gene specific annealing temperature (58-62 °C) for 60 s for 40 cycles, followed by a gradual increase in temperature from 55 °C to 95 °C during the dissociation stage.

 Following amplification, the instrument software was used to set the baseline and threshold for each reaction, as well as to determine the reaction efficiency. A cycle threshold (Ct) was assigned at the beginning of the logarithmic phase of PCR amplification and the difference in the Ct values [corrected for reaction efficiency: Ct = Ct × log(efficiency)/log(2)] of the housekeeping genes [mean Ct of glucuronidase (GUS) and beta-actin (BAKT)] and the gene of interest were used to determine the relative expression of the gene in each sample. Relative expression levels were then calculated as fold changes to the housekeeping genes, where each PCR cycle represented a 2-fold change.

 The data of PCR and microarray experiments were then correlated using Pearson’s coefficient.

 The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

RESULTS

Using the agglomerative hierarchical clustering methods we found statistically significant correlations between the EDG1, DUSP14, EGR1, and EDG2 genes and PDPK1, PIK3CG, AKT1, as well as clustering, though not statistically significant, for GNAS and ARRB2 (Figure 1). Spearman’s rank correlation coefficients were 0.762 for PDPK1 and AKT1, 0.769 (P < 0.05) for PDPK1 and PIK3CG, 0.769 (P < 0.05) for AKT1 and PIK3CG, and 0.699 (P < 0.05) for GNAS and ARRB2. In case of EDG1, DUSP14, EGR1 and EDG2, in 10 out of 12 patients there was no transcriptional activity. Except for the transcriptional factor JUN-B and the stage of fibrosis r = 0.667 (P < 0.05), we did not observe any correlation between the activity of the selected genes and the investigated clinical factors. Assessing patient distribution based on dendrograms of the investigated genotype and phenotype, a difference on the border of statistical significance was found in Fisher’s exact test (P = 0.08). The patients with the highest diversity in gene expression (Figure 1) showed a statistically significant difference in gender and ALT activity (P = 0.0046 and P = 0.035, respectively). Based on expression activity, the genes were divided into a group with high activity, which included GNAS, PDPK1, AGT and ARRB2 and statistically differed (0.002 < P < 0.046) from the genes with moderate and low expression. The group of genes with moderate activity of AKT1, PIK3CG, IL1β, VCAM1, JUN-B and CALCR also showed a statistically significant difference (0.002 < P < 0.041) compared with genes which were classified as belonging to the group with low expression (Figure 3).

For the following genes: JUN-B, EDG2, CCL4 and EGR1, estimated with the use of microarrays and qRT-PCR, there was a strong positive correlation, with Pearson’s coefficient within the range of 0.61-0.99. For other genes, IL1β, VCAM1, PIK3CG, AGT, PDPK1, and GNAS, the correlation was weakly positive, with Pearson’s coefficient ranging from 0.07 to 0.43; and for IL1R1, IL1R2, CALCR, AKT1, and ARRB2 the correlation of expression estimated with the two methods was weakly negative (Pearson’s coefficient ranging from -0.1 to -0.35).

DISCUSSION

With the exception of the correlation between JUN-B and fibrosis stage, we did not reveal any direct relationship between expression of genes related to metabotropic receptors in hepatocytes and anthropometric, histological and biochemical parameters that are commonly used for monitoring progression of chronic hepatitis. In assessing the connection between gene expression and the investigated clinical features, we found an impact of gender and concentration of ALT in the serum on changes in gene expression. The effect of these two factors was detected as the result of the analysis of not any single, but rather all the investigated genes. In the probability test, it was gender which better determined changes in gene activity rather than ALT. In chronic hepatitis C and B, gender is the factor which defines the course and prognosis of the disease[1]. Changes in gene expression resulting from gender differences can influence the progression of liver disease.

In the present study, among different markers of inflammation and fibrosis in the liver parenchyma, only the ALT level differentiated the gene activity. Among the investigated genes, there were important mediators of inflammation, including IL-1β, with their receptors, as well as chemokines and adhesion factors. Also protein products of such genes as EDG1, EDG2, CALCR are involved in the induction of the inflammatory response[13]. In contrast to ALT, a small histological difference used to assess the inflammatory process in the
liver, classified in the majority of cases as score 1 or 2 according to METAVIR, were not useful in assessing gene expression.

The reported values of genes expression and their statistical diversity allow distinction between three groups of genes with high, moderate and low activity. Because there is no direct correlation between gene activity and clinical markers of chronic hepatitis, it is difficult to determine if the observed gene expression is induced or constitutional.

In the group of genes with the highest activity there were genes of the main metabotropic receptor proteins, GN-A1, PDPK1 and ARRB2. Their high transcriptional activity is related to their essential role in the metabotropic receptor system. GN-A1 expression, despite individual differences, showed the highest correlation with ARRB2 among the investigated genes. Proportional to the level of stimulation of GPCR, ARRB2 triggers the mechanism of receptor internalization, which is an adaptation to overstimulation\(^{16}\). Also mRNA encoding AGT was characterized by high expression. This observation remains in accordance with current literature and is associated with the liver being the main site of AGT synthesis\(^{17}\). Because AGT is incorporated only indirectly by the renin-angiotensin system in the progression of liver fibrosis\(^{18,19}\), its gene activity did not correlate with the histological assessment of this process.

In the group of genes with moderate activity, there were genes which encode products involved in the pathogenesis of liver injury. Disturbances of PIK3CG described in chronic hepatitis as caused by NS5 protein of hepatitis C virus and HBx protein of hepatitis B virus destabilize and damage hepatocytes\(^{20,21}\). However, in the present study, expression of PIK3CG did not correlate with the markers of liver injury.

As there were no cases with advanced fibrosis among the investigated patients, we did not observe any increased expression of VCAM1, which is characteristic only for liver cirrhosis\(^{22}\).

The correlation of expression of phosphatidylinositol kinases observed in the study confirms the functional association of the investigated enzymes. A significantly higher expression of PDPK1 compared with PIK3CG is due to the amplification of PDPK1 during phosphorylation catalyzed by PIK3CG. PDPK1 in turn activates AKT1 correspondingly; therefore, the 3-fold higher activity of PDPK1 is noteworthy.

Among the investigated phosphatases that dephosphorylate active kinases, DUSP14 was measured. However, this phosphatase, which is not functionally associated with the presently studied kinases, showed low expression values and was not correlated with the expression of kinases. Interestingly, a statistically significant correlation was determined between the stage of fibrosis and expression of transcriptional factor JUN-B. This factor, in the malfunctioning of the liver, is responsible for reprogramming of hepatocyte genes to the phase of cell proliferation\(^{23,24}\). Its increase is frequently observed in liver injury and hepatic carcinogenesis\(^{25}\).

The higher expression of this factor linked to carcinogenesis correlated with a low activity of the genes CCL4, EGR1, EDG2 involved in the induction of apoptosis and suppression of neoplasia\(^{26}\).

The results obtained in the present study indicate that qRT-PCR has certain limitations as a validation tool for oligonucleotide microarray studies. Only four genes have shown a similar expression pattern between results obtained with the use of both techniques. A weak positive and negative correlation observed for other genes might result from potential pitfalls inherent in both approaches, and might be a source of errors encountered while employing each method.

However, the range of differences in the correlation coefficient observed in the present study remains within the range described in the literature, from -0.48 to 0.94\(^{27,28}\). The results obtained in this study reflect the debate over which methods produce the most accurate measurements of gene expression.

In conclusion, gender and inflammation activity, as determined by ALT level, were associated with a more diverse pattern of metabotropic receptor gene expression. The highest gene expression was observed for mRNA of the main components of the metabotropic receptor pathway, such as GN-A1, PDPK1, ARRB2, and correlated with mRNA of angiotensinogen synthesized in the liver. The correlation of expression of protein kinases PDPK1, PIK3CG and AKT1 points to a functional association of these enzymes. The genes suppressing carcinogenesis, CCL4, EGR1, EDG2, were characterized by the lowest expression levels among the investigated genes. On the other hand, JUN-B, a factor potentially involved in the development of hepatocellular cancer, was characterized by a 20-fold higher level of expression.

**COMMENTS**

**Background**
Metabotropic G protein-coupled receptors activate various signaling pathways, which trigger multiple sub-cellular reactions. Microarray-based analysis of expression of hepatocyte genes related to metabotropic receptors can reveal the molecular basis of liver diseases.

**Research frontiers**
The natural course of chronic viral hepatitis is associated with progression of fibrosis and the risk of hepatocellular carcinoma development. Current data indicate that intracellular signaling disturbances have an impact on progression of inflammation and fibrosis as well as carcinogenesis in the course of chronic hepatitis.

**Innovations and breakthroughs**
The highest gene expression was in the mRNAs of the main components of the metabotropic receptor pathways, such as the α subunit of G-coupled protein, phosphoinositol-dependent protein kinase (PDPK1) and arrestin γ and correlated with the mRNA for angiotensinogen synthesized in liver. Carcinogenesis suppressor genes such as chemokine CCL4, transcription factor EGR1 and lysophosphatidic acid receptor were characterized by the lowest expression, while the factor potentially triggering hepatic cancer, JUN-B, had 20-fold higher expression. Comparable expression of genes encoding protein kinases PDPK1, phosphoinositide 3-kinase and protein kinase A confirms a functional link between these enzymes. Gender and inflammation severity, measured by alanine aminotransferase activity, were characterized by different expression patterns.
of genes related to metabotropic receptors.

**Applications**

Results of the presented work enables better delineation of mechanisms gov-
erning the course of chronic hepatitis and form the basis for future investiga-
tions.

**Terminology**

G-protein-coupled receptors are a family of the cell surface receptors, which
receive, integrate and enhance most of the extracellular signals implicated in
cell growth, proliferation, and survival. Microarray DNA technology with genomic
profiling and cluster analysis allows determination of the role of genes in the pathogenesis of liver injury.

**Peer review**

This is a good descriptive study in which authors screen for genes related to
metabotropic receptors family that might be involved in the development of
chronic hepatitis. The results are interesting and suggest that a microarray-
based analysis of hepatocyte metabotropic G-protein-related gene expression
can reveal the molecular basis of chronic hepatitis.

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