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BRIEF ARTICLE

Intrahepatic expression of genes related to metabotropic receptors in chronic hepatitis

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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, concomitant 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, phosphoinositol-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 PDPK1, 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

limitations as a validation tool for oligonucleotide microarray studies.

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

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

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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 metabotropic 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 metabotropic 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 (*GNAS*), 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 (*EGR1*) 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*, *EGR1*, *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 I (*IL1R1*), IL1 type II (*IL1R2*), *CALCR*, *EDG1*, *CCL4*, *AGT* and adhesion molecules vascular cell adhesion molecule 1 (*VCAM1*)[7-14].

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 ℃ 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 (ASPAT/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 RNeasy 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

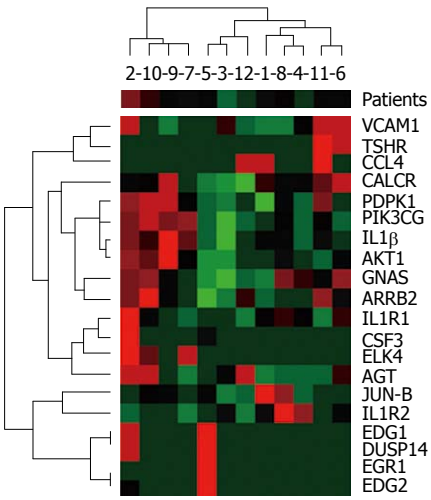

Figure 1 Agglomerative hierarchical clustering methods of genes expression. The dendrogram assigned to the numerical value on the horizontal axis describes the patients' correlation; the dendrogram assigned to individual genes on the vertical axis describes the genes' correlation. Figures on the x-axis denote patient order. *VCAM1*: Vascular cell adhesion molecule 1; *TSHR*: Thyroid stimulating hormone receptor; *CCL4*: Chemokine (C-C motif) ligand 4; *CALCR*: Calcitonin receptor; *PDPK1*: 3-phosphoinositide-dependent protein kinase-1; *PIK3CG*: Phosphoinositide 3-kinase; *IL1β*: Interleukin 1β; *AKT1*: Protein kinase A; *GNAS*: α subunit of G-coupled protein; *ARRB2*: Arrestin β; *IL1R1*: Interleukin 1 receptor, type Ⅰ; *CSF3*: Colony stimulating factor-3; *ELK4*: ETS-domain protein; *AGT*: Angiotensinogen; *JUN-B*: Transcription factor jun-B; *IL1R2*: Interleukin 1 receptor, type Ⅱ; *EDG1*: Sphingosine-1-phosphate receptor 1; *DUSP14*: Dual-specificity protein phosphatases 14; *EGR1*: Early growth response protein 1; *EDG2*: Lysophosphatidic acid receptor.

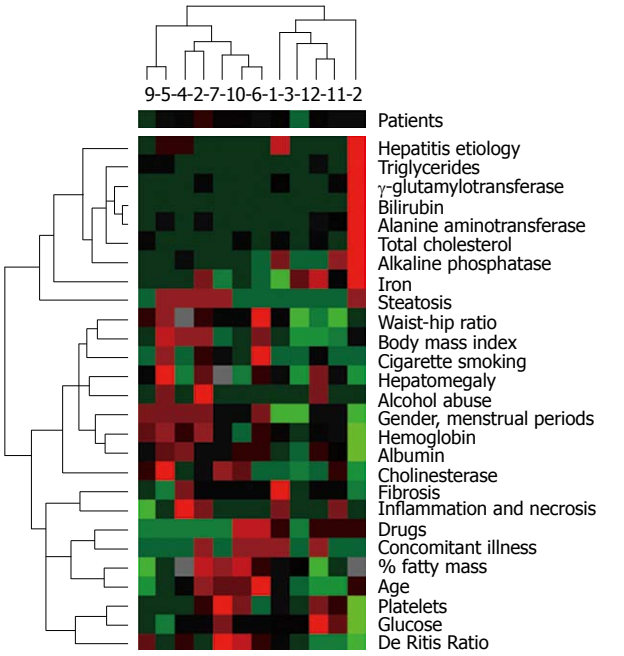

Figure 2 Agglomerative hierarchical clustering methods of clinical features expression. The dendrogram assigned to a numerical value on the horizontal axis describes the patients' correlation; the dendrogram assigned to individual clinical features on the vertical axis describes the clinical features' correlation. Figures on the x-axis denote patient order.

measured by a capillary electrophoresis system (Experion, Bio-Rad, Hercules, United States). For further analysis only samples without evidence of RNA degradation were qualified (RQI > 8.5 Rna Quality Index). Subsequently, based on the obtained RNA, probes were synthesized according to the manufacturer's instructions (SABiosciences, Frederick, United States), and then hybridized to a microarray. We used the Oligo GEArray Human GPCR Signaling Pathway Finder Microarray (OHS-071) supplied by SABioscences. Detection of the array probes is achieved based on chemiluminescence, using the FujiLAS System (FujiFilm, Tokyo, Japan). The resulting images (the signal density) were quantified using the OligoAnalyser (SABiosciences). 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 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, 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*, *EDG2*, *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 sequences of genes are indicated by microarray. They are commercially available at OriGene (Rockville, United States), but were additionally checked for specificity with National Center for Biotechnology Information (NCBI) The Basic Local Alignment Search Tool (BLAST). The optimum annealing temperature for each primer set was determined prior to the analysis of experimental samples. Following amplification, dissociation curves were

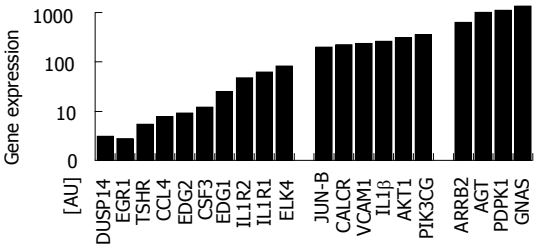

Figure 3 Arithmetic mean of gene activity. There was a statistically significant difference in the Wilcoxon signed-rank test between the genes with the highest expression, *GNAS*, *PDPK1*, *AGT*, and *ARRB2*, and moderate expression, *PIK3CG*, *AKT1*, *IL1β*, *VCAM1*, *CALCR*, and *JUN-B*, and between the genes with moderate expression and the remaining genes with low expression.

analyzed for each reaction. 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 ℃ for 10 min, 95 ℃ for 40 s, gene specific annealing temperature (58-62 ℃) for 60 s for 40 cycles, followed by a gradual increase in temperature from 55 ℃ to 95 ℃ 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, *IL1B*, *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[9,15]. 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, *GNAS*, *PDPK1* and *ARRB2*. Their high transcriptional activity is related to their essential role in the metabotropic receptor system. *GNAS* 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 *GNAS*, *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*, *EGR*, *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 β 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 governing the course of chronic hepatitis and form the basis for future investigations.

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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