NFE2L2 is associated with *NQO1* expression and low stage of hepatic fibrosis in patients with chronic hepatitis C

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Abstract

Background. Oxidative stress is extremely important in the pathogenesis of chronic hepatitis C virus (HCV). In response to oxidative stress, adaptive antioxidant defenses are upregulated in the liver. The balance between antioxidant response and oxidative stress plays a key role in hepatic injury in HCV infection.

Objectives. The objective of this study was to assess the hepatic expression of the antioxidant genes *GFER* (growth factor erythroid 1-like) and *NQO1* (NAD(P)H:quinone oxidoreductase-1) and the regulatory gene *NFE2L2* (nuclear factor erythroid 2-related factor-2) in liver biopsy specimens obtained from chronic HCV patients with regard to selected clinical parameters and histology, and to determine whether *GFER* and *NQO1* expression is dependent on *NFE2L2*.

Material and methods. The study group consisted of 42 patients with chronic HCV. Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of antioxidant and regulatory genes in liver biopsy samples.

Results. Positive correlation was observed between the hepatic expression of *NFE2L2* and *NQO1* in the chronic HCV patients ($p < 0.0001$). The hepatic expression of *NFE2L2* was significantly lower in patients with advanced liver fibrosis ($p = 0.05$). However, there was no significant difference in the hepatic expression of *GFER* and *NQO1* in relation to the progression of liver steatosis, inflammation and fibrosis.

Conclusions. The hepatic expression of *NFE2L2* is associated with *NQO1* and low stage of hepatic fibrosis in patients infected with HCV.

Key words: hepatitis C, liver fibrosis, antioxidant genes, regulatory gene

Cite as

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Introduction

Despite increasing access to effective antiviral therapies, HCV infection remains an important epidemiological issue worldwide, with about 3 to 4 million cases reported each year.¹ Approximately 75–85% of HCV-infected patients develop chronic HCV infection, 20% of whom progress to cirrhosis.²

It is well known that oxidative stress plays a significant role in HCV pathogenesis.^{3,4} Hepatitis C virus core proteins induce the mitochondrial production of reactive oxygen species (ROS), leading to the peroxidation of membrane lipids. In response to this oxidative stress, adaptive antioxidant defenses such as antioxidant enzymes are upregulated in the liver. The main regulator of this self-protective antioxidant mechanism is nuclear factor erythroid 2-related factor-2 (Nrf2), encoded by the *NFE2L2* gene, which activates the expression of antioxidant proteins such as glutathione peroxidase, NAD(P)H-quinone oxidoreductase-1, superoxide dismutase, catalase, glutathione S-transferases, glutamate-cysteine ligase, heme oxygenase-1, thioredoxin reductase-1, and augments liver regeneration.⁵ NQO1 (NAD(P)H:quinone oxidoreductase-1) is a cytosolic flavoprotein encoded by the gene *NQO1* that inhibits oxidative stress by the detoxification of superoxide anions and the regeneration of reduced forms of protective endogenous antioxidant compounds.⁶

Augmenter of liver regeneration (ALR) is encoded by the growth factor *erv1*-like (*GFER*) gene and is responsible for liver regeneration through the upregulation of the mitogen-activated protein kinase pathway, as well as interleukin-6 (IL-6) and tumor necrosis factor- α .⁷

The aim of the present study is to assess the hepatic expression of the antioxidant genes *GFER* and *NQO1* and the regulatory gene *NFE2L2* in liver biopsy specimens obtained from patients with chronic HCV in relation to selected clinical parameters and histological factors, and to determine whether *GFER* and *NQO1* expression is dependent on *NFE2L2*.

Material and methods

The study included 42 adult patients with chronic HCV. Inclusion criteria were as follows: 1. The presence of positive serum anti-HCV antibodies and detectable HCV viremia lasting more than 6 months; 2. HCV genotype 1. Exclusion criteria:

1. other systemic or inflammatory diseases;
2. other causes of liver disease;
3. cirrhosis (revealed by liver biopsy);
4. previous anti-HCV treatment;
5. treatment with other medications including antioxidant vitamin and diet supplements;
6. HIV/HBV co-infection;
7. pregnancy.

The study was approved by the ethical committee of the Faculty of Medicine, Medical University of Lodz, Poland, and informed written consent was obtained from every patient. Liver biopsies were performed as part of the routine standard of care for the subjects studied. The grade of inflammation and the state of fibrosis were determined according to the Batts and Ludwig scale. Steatosis was assessed with Bruntet and Kleiner's histological scoring. Hepatitis C virus RNA was measured using reverse transcription PCR (RT-PCR) with COBAS AMPLICOR HCV v. 2.0 (Roche Diagnostic Inc., Basel, Switzerland). Hepatitis C virus genotype was assessed using a hybridization method (InnoLipa HCV; Innogenetics, Ghent, Belgium).

Statistical analysis and polymerase chain reaction

Total RNA isolation

Total RNA was isolated using the mirVana™ miRNA isolation kit (Ambion® Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol.

mRNA expression

Homo sapiens-specific TaqManGene Expression Assay (Applied Biosystems Inc., Foster City, USA) for *GFER*, *NQO1* and *NFE2L2* mRNA was used for gene expression assays. cDNA generation was performed using 250 ng of total RNA with High Capacity cDNA Reverse Transcription Kits according to the manufacturer's protocols (Applied Biosystems). Expression levels of mRNA were determined using beta-actin (ACTB) as an endogenous control.

Real-time polymerase chain reaction analysis

TaqMan PCR assays were determined using Sequence Detection System v. 2.0 software (Thermo Fisher Scientific). Fold induction values were measured according to $2^{\Delta\Delta Ct}$, where ΔCt defines the differences in cycle threshold numbers between the target gene and endogenous control, and $\Delta\Delta Ct$ defines the relative change in these differences between study and control groups.

Statistical analysis

The Mann–Whitney test was used to determine differences between the examined groups. Correlations between the hepatic expression of antioxidant genes *GFER* and *NQO1* and the regulatory gene *NFE2L2* were assessed with Spearman's rank correlation coefficient. Values of $p \leq 0.05$ were considered to be statistically significant.

Results

Study group

The study group consisted of 42 patients with chronic HCV infection: 11 women and 31 men. The median age of patients was 32 years (LQ 21 years–UQ 49 years). The median plasma HCV viral load was 4,145,000 IU/mL (LQ 388,000 IU/mL–UQ 6,890,000 IU/mL). The characteristics of the patients are presented in Table 1.

Table 1. Characteristics of the study group

	HCV infection (n = 42)		
	median	LQ–UQ	min–max
Age [years]	32	21–49	18–67
ALT U/L	54	39–83	23–336
HCV-RNA IU/mL	4,145,000	388,000–6,890,000	118,000–118,000,000
<i>GFER</i>	1.46	0.98–2.05	0.06–3.98
<i>NQO1</i>	1.46	0.98–1.69	0.01–17.47
<i>NFE2L2</i>	2.30	1.50–3.83	0.21–12.33

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotransferase.

Hepatic expression of *GFER*, *NQO1*, *NFE2L2*, and HCV viral load

The study group was divided into 2 subgroups: one with HCV-RNA > 600,000 IU/mL and the other with

HCV-RNA < 600,000 IU/mL. No significant difference in the hepatic expression of *GFER*, *NQO1* and *NFE2L2* was observed between the 2 groups (Table 2).

Hepatic expression of *GFER*, *NQO1* and *NFE2L2* in the context of inflammation, steatosis and fibrosis

Advanced fibrosis (S ≥ 2) was detected in 34 patients. No significant difference in the hepatic expression of *GFER* or *NQO1* was observed in relation to the progression of liver fibrosis. However, the hepatic expression of *NFE2L2* was significantly lower in patients with advanced liver fibrosis (p = 0.05) (Table 3). No relationships were found between the hepatic expression of *GFER*, *NQO1* or *NFE2L2* and the grade of inflammatory activity (Table 4). Again, the hepatic expression of *GFER*, *NQO1* or *NFE2L2* was not associated with the presence of liver steatosis (Table 5). However, older age was reported in patients with the higher grade of liver inflammation (p < 0.0001) and HCV-RNA > 600,000 IU/mL (p = 0.03) (Tables 2,4).

Correlations between hepatic expression of *GFER*, *NQO1* and *NFE2L2*

A significant positive correlation was found between the hepatic expression of *NFE2L2* and *NQO1* in chronic HCV patients (p < 0.0001). However, the hepatic expression of *NFE2L2* was not associated with *GFER* (Table 6).

Table 2. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to HCV viral load

	HCV-RNA < 600,000 IU/mL (n = 15)			HCV-RNA > 600,000 IU/mL (n = 27)			p-value
	median	min–max	LQ–UQ	median	min–max	LQ–UQ	
Age [years]	26	18–59	21–48	37	20–67	29–56	0.03
ALT U/L	55	24–336	37–91	52	23–201	39–77	>0.01
<i>NFE2L2</i>	2.42	0.21–12.32	1.72–4.40	2.01	0.22–6.03	1.00–3.50	>0.01
<i>GFER</i>	1.45	0.09–17.47	0.97–2.57	1.46	0.68–2.01	0.05–2.55	>0.01
<i>NQO1</i>	0.89	0.02–17.47	0.47–2.03	0.58	0.38–1.62	0.01–2.36	>0.01

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotransferase.

Table 3. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to liver fibrosis

	Staging <2 (n = 8)			Staging ≥2 (n = 34)			p-value
	median	min–max	LQ–UQ	median	min–max	LQ–UQ	
Age [years]	26	18–61	20–33	35	18–67	26–50	>0.01
ALT U/L	54.5	24–336	42–83	41.5	23–95	25–79.5	>0.01
HCV viral load	580,000	392,000–11,800,000	2671,000–7,535,000	3,040,000	118,000–9,780,000	381,000–6,390,000	>0.01
<i>NFE2L2</i>	3.96	0.21–12.32	2.44–6.16	2.05	0.22–7.76	1.42–3.13	=0.05
<i>GFER</i>	1.14	0.09–3.76	0.35–1.82	1.48	0.05–3.98	1.04–2.14	>0.01
<i>NQO1</i>	0.79	0.24–9.32	0.02–17.47	0.77	0.01–5.48	0.44–1.68	>0.01

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotransferase.

Table 4. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to liver inflammation

	Grading <2 (n = 30)			Grading ≥2 (n = 12)			p-value
	median	min-max	LQ-UQ	median	min-max	LQ-UQ	
Age [years]	26	18–51	20–33	53	27–201	48.5–2.68	<0.0001
ALT U/L	51	23–336	35–73	76	24–67	53–108	>0.01
HCV viral load	4,760,000	118,000–11,800,000	452,000–7,200,000	809,000	221,000–9,780,000	4,522,000–4,895,000	>0.01
<i>GFER</i>	1.50	0.06–3.76	0.97–2.14	1.26	0.07–3.98	0.89–1.96	>0.01
<i>NQO1</i>	0.79	0.01–17.24	0.39–1.80	0.76	0.05–3.66	0.52–1.34	>0.01
<i>NFE2L2</i>	2.51	0.21–12.32	1.72–4.40	1.84	0.22–6.03	1.13–2.68	>0.01

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotransferase.

Table 5. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to the presence of liver steatosis

	Without steatosis (n = 30)			With steatosis (n = 12)			p-value
	median	min-max	LQ-UQ	median	min-max	LQ-UQ	
Age [years]	27	18–67	21–48	38.5	18–59	30–52.5	>0.01
ALT U/L	52	23–201	32–73	76	39–336	52–96	0.03
HCV viral load	4,370,000	118,000–11,800,000	392,000–6,390,000	2,930,000	221,000–8,750,000	383,499.5–7,085,000	>0.01
<i>GFER</i>	1.45	0.06–3.98	0.90–1.96	1.46	0.68–3.32	1.15–2.30	>0.01
<i>NQO1</i>	0.76	0.03–17.47	0.41–1.68	0.87	0.01–3.17	0.52–1.85	>0.01
<i>NFE2L2</i>	2.26	0.21–12.32	1.75–4.16	2.34	0.80–6.03	1.46–3.21	>0.01

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotransferase.

Table 6. Correlations between hepatic expression of *GFER*, *NQO1* and the *NFE2L2* in patients with chronic HCV

	<i>GFER</i>		<i>NQO1</i>	
	Spearman's rank correlation ρ	p-value	Spearman's rank correlation ρ	p-value
<i>NFE2L2</i>	0.22	NS	0.59	<0.0001

NS – not significant.

Discussion

Polymerase chain reaction proteins induce the production of more ROS in the liver than other hepatitis viruses.^{8–10} As excessive production of ROS can result in hepatocyte damage, an adequate antioxidant response is extremely important. The transcription factor Nrf2 plays a central role in stimulating the expression of various antioxidant-associated genes. Numerous reports describe that the antioxidant response is disturbed in subjects with HCV infection.^{11,12}

It is possible that HCV can directly affect regulatory genes responsible for the antioxidant response, such as *NFE2L2*. Carvajal-Yepes et al. indicate that HCV prevents the entry of Nrf2 in the nucleus by the delocalization of sMaf proteins, thereby inhibiting the induction of Nrf2/ARE-regulated genes.¹³ Reduced levels of glutathione and other antioxidants have been reported in liver biopsies in chronic HCV.^{14,15} Interestingly, these results correspond to those of an in vitro study conducted by Wen et al., which indicates the decreased induction of HMOX-1 in hepatocyte

cell lines expressing the HCV core protein in response to oxidative stress.¹² Conversely, Inanov et al. demonstrated that HCV proteins activate the Nrf2/ARE pathway in HCVcc infected Huh7.5 cells and induce the transcription of antioxidant genes.⁹ Our results confirm the involvement of Nrf2 in the regulation of the antioxidant defense system, in that a positive correlation was observed between *NFE2L2* and *NQO1* expression.

Interestingly, the literature presents several contradicting opinions on the role of antioxidant enzymes in HCV replication. Zhu et al. demonstrated that heme oxygenase-1 overexpression is associated with decreased HCV replication in vitro.¹⁶ In contrast, Ghaziani et al. found the heme oxygenase-1 gene to be upregulated in Huh-7 cells expressing HCV proteins.¹⁷ It is still not clear whether antioxidant enzymes directly affect HCV replication in vivo; however, our present findings indicate that the hepatic expression of *GFER*, *NQO1* and *NFE2L2* was not related to HCV viremia.

A number of studies have examined the role of the antioxidant response in hepatocyte injury.^{18,19} In a study

conducted by Kumar et al., the decreased hepatic expression of ALR was found in alcohol-induced fibrosis in mice model.¹⁹ Conversely, Cheng et al. reported the expression of *NQO1* to be increased in alcoholic cirrhosis.²⁰

Our present findings did not indicate any association between hepatic expression of *GFER* and *NQO1* with the progression of liver inflammation and fibrosis in chronic HCV. However, higher hepatic expression of *NFE2L2* was observed in patients with a lower stage of fibrosis.

Interestingly, Nrf2 activation directly inhibits the functioning of TGF- β 1 (transforming growth factor- β 1) and subsequently suppresses the collagen-producing hepatic stellate cells in a liver fibrosis mouse model.²¹ These findings are in accordance with Jiang et al., who reported stronger Nrf2 staining in HCV infected Huh 7.5.1 hepatocytes and in liver biopsy specimens obtained from chronic HCV patients with a low grade of inflammation and fibrosis.²²

Moreover, experimental studies show that the decreased expression of ALR and Nrf2 can result in liver steatosis by lowering the activity of several enzymes important for fatty acid β -oxidation and the promoting factors that regulate adipocyte differentiation and lipogenesis.^{19,23,24}

However, our findings do not confirm the influence of *NFE2L2* and *GFER* on the presence of liver steatosis in HCV infection in vivo. The pathogenesis of liver steatosis in HCV infection is complex. Not only the host but also viral mechanisms likely contribute to the development of hepatic steatosis in HCV infection.^{25,26}

The limitation of this study is the lack of the assessment of oxidative stress markers in the liver. Therefore, further studies are needed to precisely clarify the simultaneous role of the oxidative stress and antioxidant response in pathogenesis of chronic HCV. In conclusion, the hepatic expression of *NFE2L2* is associated with *NQO1* and low stage of fibrosis in patients infected with HCV. However, the study subgroup with mild fibrosis was limited to 8 patients.

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