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Gene expression evaluation of antioxidant enzymes in patients with hepatocellular carcinoma: RT-qPCR and bioinformatic analyses

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Abstract

Any condition leading to chronic liver disease is a potential oncogenic agent for hepatocellular carcinoma (HCC). Alterations in the expression of antioxidant enzymes could alter the redox balance. Our aim was to evaluate the expression of the genes *GPX1*, *GPX4*, *SEP15*, *SELENOP*, *SOD1*, *SOD2*, *GSR*, *CAT*, and *NFE2L2* in patients with HCC. Differential gene expression analysis was performed using RNA-Seq data from the TCGA and GTEx databases, and RT-qPCR data from HCC patient samples. Bioinformatic analysis revealed significant differential expression in most genes. *GPX4* expression was significantly increased ($p=0.02$), while *SOD2* expression was significantly decreased ($p=0.04$) in experimental data. In TCGA samples, alpha-fetoprotein levels (mg/dL) were negatively correlated with the expression of *SEP15* ($p<0.001$), *SELENOP* ($p<0.001$), *SOD1* ($p<0.001$), *SOD2* ($p<0.001$), *CAT* ($p<0.001$), and *NFE2L2* ($p=0.004$). Alpha-fetoprotein levels were positively correlated with the expression of *GPX4* ($p=0.02$) and *SELENOP* ($p=0.01$) in the experimental data. Low expression of *GPX1* ($p=0.006$), *GPX4* ($p=0.01$), *SELENOP* ($p=0.006$), *SOD1* ($p=0.007$), *CAT* ($p<0.001$), and *NFE2L2* ($p<0.001$), and higher levels of *GSR*, were associated with low overall survival at 12 months. These results suggest a significant role for these antioxidant enzymes in HCC pathogenesis and severity.

Keywords: Hepatocellular carcinoma, selenoproteins, antioxidant enzymes, oxidative stress, gene expression.

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Introduction

Hepatocellular carcinoma (HCC) has a high mortality rate, and ranks as the third leading cause of cancer deaths worldwide (Ozakyol, 2017; Forner *et al.*, 2018). Major risk factors for HCC include infection with hepatitis B and C viruses, alcohol intake, and fatty liver disease (Ozakyol, 2017; Yang *et al.*, 2019). Prognosis and treatment options vary according to tumor stage and liver function. The percentage of patients eligible for curative treatment varies between high and low-resource countries (Ozakyol, 2017; Yang *et al.*, 2019), but generally fluctuates between 20-30% of patients. The median survival of patients with untreated disease is nine months (Klungboonkrong *et al.*, 2017; Forner *et al.*, 2018). New markers or therapeutic targets are required for early diagnosis, and the development of novel treatment strategies for HCC (Klungboonkrong *et al.*, 2017).

Oxidative stress is associated with cancer, and has a dual role in disease development, due to the effects of reactive oxygen species (ROS) on cellular processes. Increased ROS levels are associated with oncogenic effects because of their ability to cause damage to biological macromolecules, such as DNA, lipids, and proteins (Reuter *et al.*, 2010; Moloney and Cotter, 2018). High concentrations of ROS have been

associated with activation of p53, oxidative lipid peroxidation, consumption of antioxidants, and can ultimately lead to cell death (Wang *et al.*, 2016b; Sajadimajd and Khazaei, 2017). Oxidative stress can play different roles: promoting carcinogenesis or cell apoptosis, or by providing sufficient components to promote cancer cell survival. The physiological functions of ROS include regulation of the expression and activity of several signaling regulators that are involved in key processes, such as proliferation and apoptosis (Gill *et al.*, 2016). Cancer cells, in turn, seem to maintain advantageously elevated levels of ROS to guarantee their survival, by adapting the content and regulation of their antioxidant machinery (Sajadimajd and Khazaei, 2017; Moloney and Cotter, 2018).

Cellular antioxidant defense systems include a series of antioxidant enzymes that maintain homeostasis by restricting ROS production or neutralizing ROS (Gill *et al.*, 2016). This group of enzymes includes major components such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GSR), and selenoproteins, including glutathione peroxidase 1 (GPX1), glutathione peroxidase 4 (GPX4), 15-kDa selenoprotein (SEP15), and selenoprotein P (SelP). All of these enzymes act in pathways of chain-breaking ROS molecules, or have other important functions, including detoxification of hydrogen peroxide (H_2O_2), inhibition of lipid peroxidation, quality control of protein folding, and transport of selenium to peripheral tissues (Gupta *et al.*, 2014; Labunskyy *et al.*, 2014; Zoidis *et al.*, 2018). Altered expression of these enzymes could be a useful resource for cancer cells. Hyperactivation of nuclear factor erythroid 2-related

factor 2 (Nrf2), a transcription factor that regulates the expression of several genes, including antioxidant enzymes, has been associated with a variety of cancers as well as with HCC (Cheng *et al.*, 2015; Menegon *et al.*, 2016; Ma-on *et al.*, 2017; Sajadimajd and Khazaei, 2017).

Previous studies have demonstrated an association between aberrant expression of antioxidant enzymes and cancer (Table 1). Abnormal expression of the *GPX1*, *GPX4*, *SEP15*, and *selenoprotein P (SELENOP)* genes has been detected in a variety of cancers, including gastric cancer (Lan *et al.*, 2017), colon carcinoma (Yagublu *et al.*, 2011), colorectal cancer (Hughes *et al.*, 2018), clear cell renal cell carcinomas (Rudenko *et al.*, 2015; Cheng *et al.*, 2019), laryngeal squamous cell carcinoma (Zhang Q *et al.*, 2018), breast cancer (Król *et al.*, 2018), MCF-7 adenocarcinoma cells (Rusolo *et al.*, 2017), lung cancer (Gresner *et al.*, 2009), and HepG2 liver cancer cells (Guariniello *et al.*, 2015; Zhao *et al.*, 2015). The *SOD1*, *SOD2*, *glutathione-disulfide reductase (GSR)*, *catalase (CAT)*, and *nuclear factor erythroid 2-related factor 2 (NFE2L2)* genes are deregulated in bladder cancer (Wieczorek *et al.*, 2017), oral squamous cell carcinoma (Pedro *et al.*, 2018), breast cancer (Wolf *et al.*, 2016), lung cancer (Zhang Y *et al.*, 2016), MCF-7 cells (Shi *et al.*, 2017), and HCC (Cheng *et al.*, 2015; Guerriero *et al.*, 2015; Wang *et al.*, 2016a).

In the present study, we produced new data on the gene expression levels of the antioxidant enzyme genes *GPX1*, *GPX4*, *SEP15*, *SELENOP*, *SOD1*, *SOD2*, *GSR*, *CAT*, and *NFE2L2* in human HCC tissues. Bioinformatic analyses using databases and RT-qPCR analysis of the original data were performed to investigate whether changes in the expression of these genes might be associated with severity and overall survival in HCC, and to explore possible relationships between the genes.

Material and Methods

Datasets and bioinformatic analyses of differentially expressed genes

Bioinformatic analyses were performed using two different experimental designs: transversal and case-control studies. For the transversal study, publicly available RNA-Seq data from the liver hepatocellular carcinoma (LIHC) project were downloaded directly from The Cancer Genome Atlas (TCGA) portal. These data included HTSeq-Counts of matched samples from 48 tumoral tissues and 48 normal solid tissues. The results published here are in whole or part based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga>. For the case-control study, RNA-Seq by Expectation-Maximization expected count data of 292 TCGA-LIHC tumoral samples (case) and 115 Genotype-Tissue Expression (GTEx) normal liver samples (control) were downloaded from “Figshare Data Record 1”, made available by Wang *et al.* (2018). Since TCGA and GTEx are studies from different sources, reprocessing of data and batch effect removal were necessary for adequate comparison. Therefore, in the present study, normalized datasets provided by Wang *et al.* (2018) were used. Publicly available clinical data were also collected from both datasets.

Differential expression analyses for both studies (tumoral × normal and case × control) were performed using the DESeq2 (Love *et al.*, 2014) package in the R language with $\text{padj} < 0.05$. The Edge R (Robinson *et al.*, 2009) package was also used for trimmed mean of M values (TMM) normalization and generation of logarithmic counts per million (logCPM) data for further statistical analyses.

Tissue samples and clinical data collection

Hepatic fresh tissue specimens (tumoral and adjacent peritumoral tissues) were collected from 14 cirrhotic patients with HCC who underwent liver transplantation between 2013 and 2015 at the Division of Gastroenterology of Irmandade Santa Casa de Misericórdia de Porto Alegre (ISCMPA), Brazil. Clinical data, such as age, sex, etiology, and metabolic panel, were collected from medical records. Informed consent was obtained from all patients. The study protocol was approved by the ISCMPA and Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) Ethics Committees (no. 2.400.119).

RNA extraction and quantitative real-time PCR analysis

Tissue samples from ISCMPA were collected from explanted liver and immediately dipped in RNAlater solution Ambion® (Thermo Fisher Scientific, USA). The stabilized tissue samples were frozen at -80°C until RNA isolation. Total RNA was extracted from the samples shortly after collection, using TRIzol™ reagent (Invitrogen, USA) according to the manufacturer’s specifications. RNA purity and concentration were evaluated by spectrometry using a Biospec-Nano device (Shimadzu, Japan). RNA integrity was evaluated by agarose gel electrophoresis of total RNA (Figure S1). Total RNA was reverse transcribed using the GoScript™ Reverse Transcription System (Promega, USA) according to the manufacturer’s instructions, in a PCR thermal cycler (Applied Biosystems, USA). Total RNA not used for RT-PCR was frozen at -80°C in case any additional experiments were necessary.

Quantitative polymerase chain reaction (qPCR) assays were performed on a StepOnePlus™ system (Applied Biosystems, USA), using SYBR™ Select Master Mix (Applied Biosystems, USA) and specific primers (Invitrogen, USA). The primer sequences are shown in Table S1. *Actin beta (ACTB)*, *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, and *18S* genes were tested for stability using the protocol described by Moura *et al.* (2014). The *ACTB* gene displayed higher stability, and was used as an endogenous control.

Gene expression was normalized to the *ACTB* housekeeping gene. The difference in gene expression between tumoral and peritumoral tissues (used as the calibrator) was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), where $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{tumoral}) - \Delta\text{Ct}(\text{peritumor})$ for tumoral tissue and $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{peritumor}) - \Delta\text{Ct}(\text{peritumor})$ for peritumoral tissue. Fold-change calculations were conducted as previously described (Schmittgen and Livak, 2008).

Table 1 – Comparison of gene expression in the present study and literature.

Type of cancer	GPXI	GPX4	SEP15	SELENOP	SOD1	SOD2	GSR	CAT	NFE2L2
Colon carcinoma (Yagublu <i>et al.</i> , 2011)	↑	↑	-	-	-	-	-	-	-
Colorectal cancer (Hughes <i>et al.</i> , 2018)	↑	NS	-	↓	-	↑	-	-	-
Bladder cancer (Wieczorek <i>et al.</i> , 2017)	↑	-	-	-	-	NS	-	NS	-
Laryngeal squamous cell carcinoma (Zhang <i>et al.</i> , 2018)	↑	-	-	-	-	-	-	-	-
Gastric Cancer (Lan <i>et al.</i> , 2017)	↓	↓	↓	-	-	-	-	-	-
Clear cell renal cell carcinomas (Rudenko <i>et al.</i> , 2015)	↓	↓	-	-	-	-	-	-	-
Clear cell renal cell carcinomas (Cheng <i>et al.</i> , 2019)	↑	NS	-	-	-	-	-	-	-
Breast Cancer (Król <i>et al.</i> , 2018)	↓	-	-	-	-	-	-	-	-
Breast Cancer (Wolf <i>et al.</i> , 2016)	-	-	-	-	-	-	-	-	↓
MCF 7 cells (Rusolo <i>et al.</i> , 2017)	↓	↓	-	-	-	-	-	-	-
MCF 7 cells (Shi <i>et al.</i> , 2017)	-	-	-	-	-	↑	-	-	-
Non-small cell lung cancer (Gresner <i>et al.</i> , 2009)	-	-	NS	↓	-	-	-	-	-
Lung squamous cell carcinoma (Zhang <i>et al.</i> , 2016)	-	-	-	-	-	-	-	-	↑
Oral squamous cell carcinoma (Pedro <i>et al.</i> , 2018)	-	-	-	-	↓	↑	↓	↓	-
Hepatocellular carcinoma (Wang <i>et al.</i> , 2016)	-	-	-	-	-	↓	-	-	-
Hepatocellular carcinoma (Guerrero <i>et al.</i> , 2015)	-	↑	-	-	-	-	-	-	-
Hepatocellular carcinoma (Cheng <i>et al.</i> , 2015)	-	-	-	-	-	-	-	-	↑
HepG2 cell line (Zhao <i>et al.</i> , 2015)	↑	↑	-	↑	-	-	-	-	-
HepG2 and Huh7 cell lines (Guariniello <i>et al.</i> , 2015)	-	↑	↑	-	-	-	-	-	-
TCGA (Tumor x normal adjacent tissue)	↑	NS	NS	↓	↓	↓	NS	↓	↓
TCGA x GTEX (Case x control)	↑	↑	↑	↑	NS	↓	↑	↓	↑
ISCMPA (Tumor x peritumor)	NS	↑	NS	NS	NS	↑	NS	NS	NS

Abbreviations: ↑=higher expression in tumoral compared to normal/peritumoral tissue (or non-tumoral cell line); ↓= lower expression in tumoral compared to normal/peritumoral tissue (or non-tumoral cell line); NS = not significant; - = not analyzed; TCGA= The Cancer Genome Atlas; GTEX = Genotype-Tissue Expression; ISCMPA = Irmandade Santa Casa de Misericórdia de Porto Alegre. Each line corresponds to one study for better understanding. MCF 7 cells constitute a type of breast cancer cell line, and HepG2 and Huh7 cells constitute types of HCC and liver cancer cell line.

Protein-protein interaction (PPI) network analysis

PPI network visualization and analysis were performed using Cytoscape 3.8 software (Shannon, 2003). PPI network construction was carried out using the Search Tool for the Retrieval of Interacting Genes (STRING) database v.11 (Szklarczyk *et al.*, 2019), using STRINGapp from Cytoscape 3.8. A confidence score of 0.4 was set as the cut-off criterion.

Gene ontology (GO) and pathway enrichment analyses

To analyze the genes and biological characteristics, bioinformatic enrichment analysis of GO and pathways was performed using the STRINGapp plugin from Cytoscape 3.8. This plugin has a feature that performs enrichment retrieval from databases. GO (Ashburner *et al.*, 2000; Carbon *et al.*, 2019), the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (Kanehisa, 2000; Kanehisa *et al.*, 2019) and REACTOME Pathway (Jassal *et al.*, 2020) were the databases selected for retrieval by the STRINGapp plugin. Two groups of genes were examined separately: upregulated and downregulated. All genes were analyzed with “*Homo sapiens*” as background species. The statistical criterion was a corrected p-value <0.05.

Statistical analyses

Continuous data are shown as mean \pm standard deviation or median (interquartile range). Categorical values are shown as absolute frequency (relative frequency). Shapiro-Wilks or Kolmogorov-Smirnov tests were used to test the normality of variables. Comparison of mRNA levels between tumoral and peritumoral tissues was performed using Wilcoxon signed-rank tests. Correlations were made using Spearman’s tests, and are presented as p-values and r coefficients. Survival analysis was performed using Kaplan–Meier log-rank tests and Cox regression. Evaluate Cutpoints (Ogłuszka *et al.*, 2019) software was used to choose the optimal cut-off point for the dichotomization of continuous variables. SPSS 20.0 (SPSS Inc., USA) and R software version 4.0.0 were used for statistical analysis. The significance level was set at p<0.05.

Results

Differential gene expression of antioxidant enzymes in HCC

Comparison of matched tumoral and adjacent normal tissues from TCGA patients revealed significantly differential expression of six genes (Figure 1A). One gene (*GPX1*) was

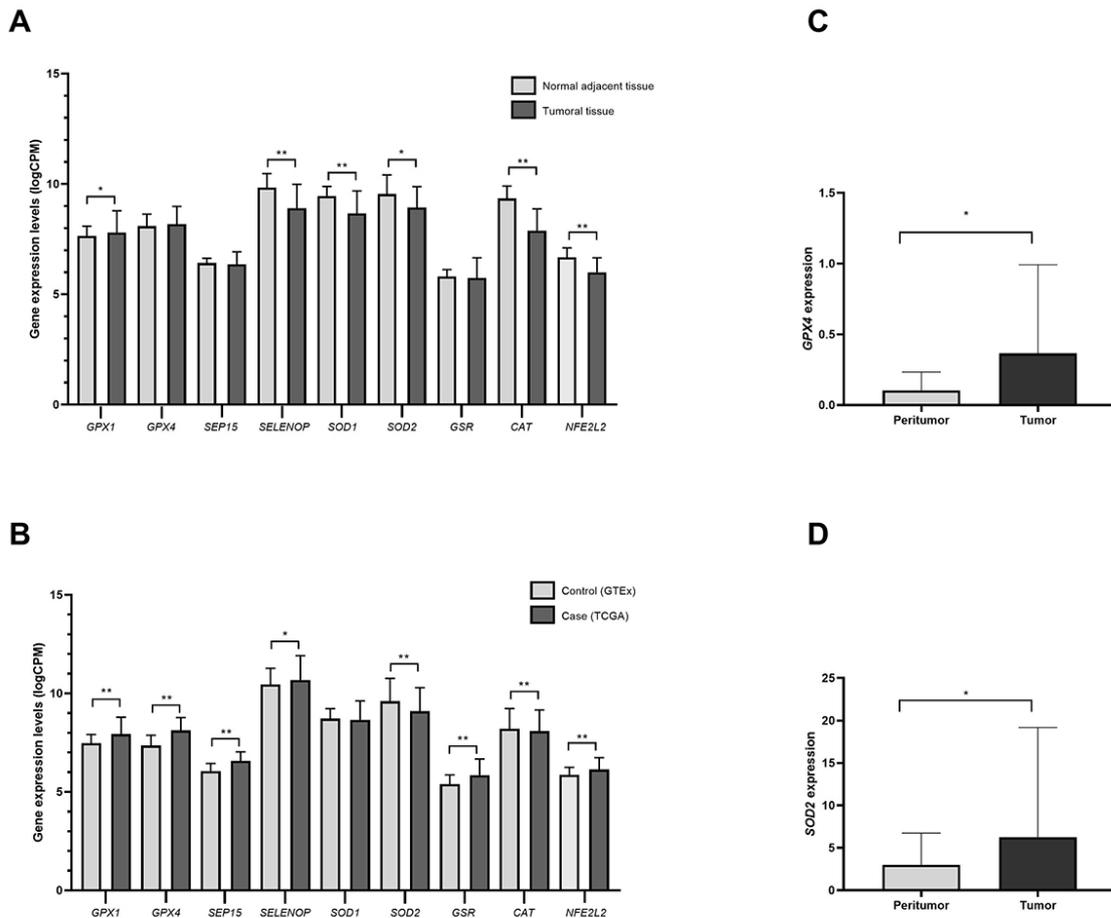


Figure 1 – Gene expression profiles. (A) Gene expression levels in the TCGA-LIHC dataset for tumoral and normal adjacent tissues. (B) Gene expression levels in case (TCGA) versus control (GTEx) analysis. (C) and (D) Significant differentially expressed genes in ISCMPA’s sample (tumoral versus peritumoral tissues). *p<0.05, **p<0.001.

upregulated and five (*SELENOP*, *SOD1*, *SOD2*, *CAT*, and *NFE2L2*) were downregulated. All genes except *SOD1* showed significant differential expression in TCGA (case) and GTEX (control) comparisons (Figure 1B). Six genes (*GPX1*, *GPX4*, *SEP15*, *SELENOP*, *GSR*, and *NFE2L2*) were upregulated, and two genes (*SOD2* and *CAT*) were downregulated. Detailed data from these analyses are provided in Table S2.

In RT-qPCR analysis of 28 fresh frozen matched samples of HCC patients from ISCMPA, two genes displayed significantly higher expression in tumoral tissue than in peritumoral tissue (Figure 1C, D). *GPX4* displayed a 2.70-fold

increase ($p=0.02$), and *SOD2* a 2.59-fold increase ($p=0.04$). In contrast, the *GPX1*, *SEP15*, *SELENOP*, *SOD1*, *GSR*, *CAT*, and *NFE2L2* genes were not significantly differentially expressed when comparing tumoral and peritumoral tissues (data not shown).

Correlation analysis

Correlations between gene expression levels of TCGA data and alpha-fetoprotein levels were also examined (Figure 2A). Alpha-fetoprotein levels (mg/dL) were negatively correlated with the expression of *SEP15* ($p<0.001$), *SELENOP* ($p<0.001$),

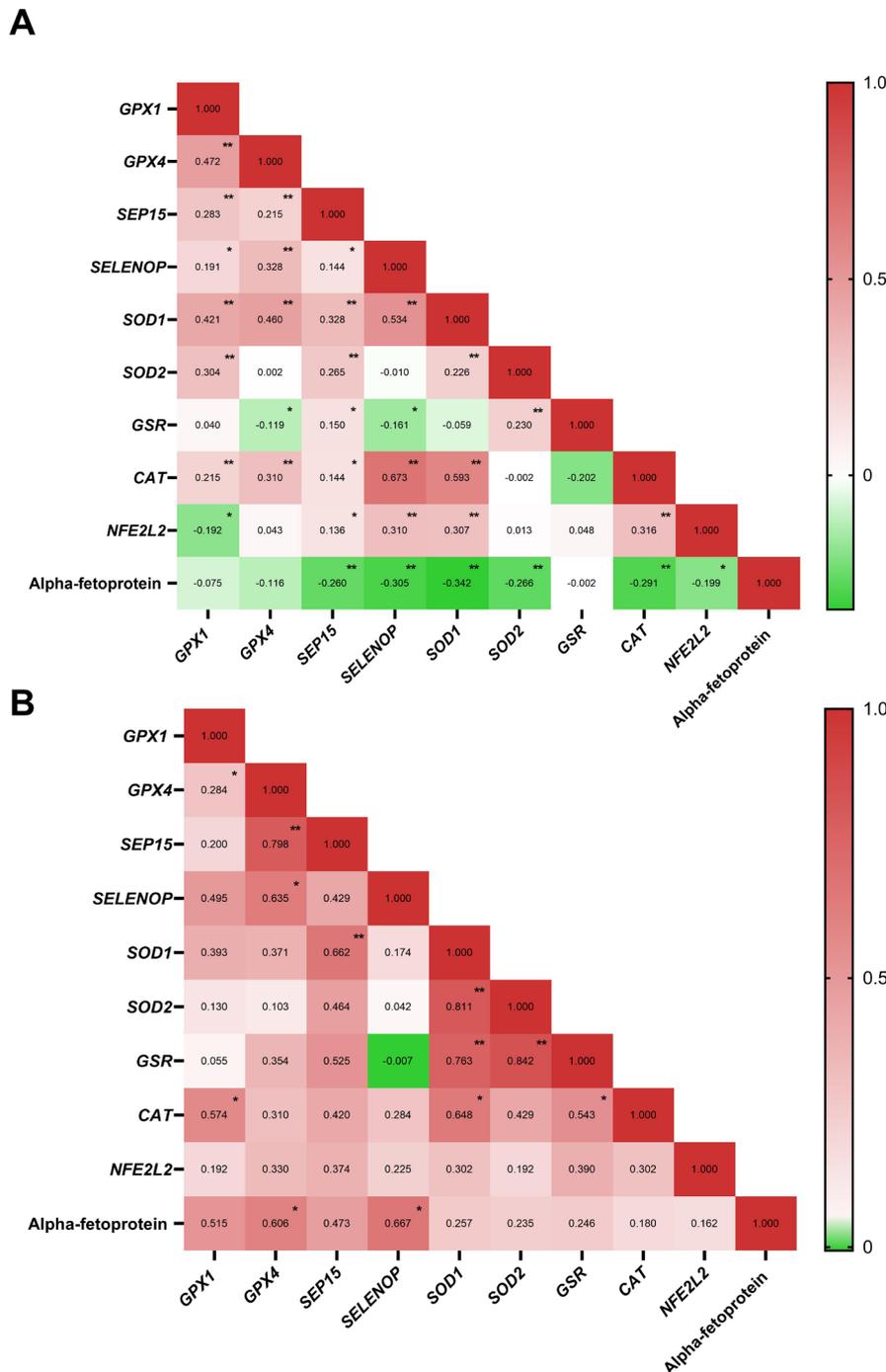


Figure 2 – Spearman correlation analysis. (A) Spearman correlation coefficients (r) for gene expression levels in TCGA tumoral samples and clinical data. (B) Spearman correlation coefficients (r) for gene expression levels in ISCMPA tumoral samples. * $p<0.05$, ** $p<0.001$.

SOD1 ($p < 0.001$), *SOD2* ($p < 0.001$), *CAT* ($p < 0.001$), and *NFE2L2* ($p = 0.004$). In contrast, analysis of relative mRNA expression ($2^{-\Delta\Delta Ct}$) in tumoral tissue revealed that patients from ISCMPA, alpha-fetoprotein (mg/dL) levels were positively correlated with *GPX4* ($p = 0.02$) and *SELENOP* ($p = 0.01$) expression levels (Figure 2B).

Survival analysis

In TCGA samples, patients were divided into two groups according to gene expression (high or low), using the optimal cut-off point generated by the Evaluate Cutpoints (Ogłuszka et al., 2019) software. Kaplan–Meier and Cox multivariate regression were used to generate overall survival data (Figure 3 and Table 2). The final models used for multivariate analysis are presented in Table S3. Low overall survival at 12 months was correlated with low expression of *GPX1* ($p = 0.006$), *GPX4* ($p = 0.01$), *SELENOP* ($p = 0.006$), *SOD1* ($p = 0.007$), *CAT* ($p < 0.001$), and *NFE2L2* ($p < 0.001$). Higher levels of *GSR* ($p < 0.001$) were associated with low overall survival in the same period. Complete sample data from patients from ISCMPA were not available for survival analysis.

PPI network analysis

Since correlation analyses revealed several significant correlations (Figure 2) between gene expression in both TCGA and ISCMPA samples, a PPI network analysis was performed. The PPI network contained nine nodes and 25 edges (Figure 4A), with an average node degree of 5.55 and PPI enrichment p -value $< 1.0e-16$. *GPX1* and *GPX4* had the highest degree and betweenness centrality values, of 8 and 0.166, respectively. Proteins *SELP* and *SEP15* had the lowest degree value, interacting with only three other proteins in this analysis: *GPX1*, *GPX4*, and each other.

GO and pathway enrichment analyses

Gene enrichment analysis using the enrichment tool STRINGapp returned a series of sets. GO terms are divided into three groups: biological process (BP), molecular function (MF), and cellular component (CC). The most significant results are presented in Table 3.

Upregulated genes were mainly enriched in the biological processes of cellular detoxification, response to oxidative stress, and cellular response to toxic substances. With respect to GO molecular function, antioxidant activity and glutathione peroxidase activity were implicated. According to GO cellular component, genes were mainly enriched in the intracellular organelle lumen. Downregulated genes were mainly enriched in biological processes of response to ROS, protein homotetramerization, and aging. GO molecular functions returned antioxidant activity and oxidoreductase activity as the main enriched terms.

KEGG pathways and REACTOME Pathways analyses revealed that genes were enriched in several pathways. The most significant results are presented in Table 4. Upregulated genes were mainly enriched in pathways involving glutathione metabolism, synthesis of eicosatetraenoic acids, detoxification of ROS, and diseases that included amyotrophic lateral sclerosis and Huntington's disease. Downregulated genes were also enriched in pathways of detoxification of ROS, as well as pathways involving peroxisomes, longevity regulation, FoxO signaling, and the immune system.

Discussion

We performed bioinformatic and experimental analyses to evaluate the expression patterns of eight antioxidant enzymes, including four selenoproteins, and one important transcription factor, to assess their association with HCC pathogenesis.

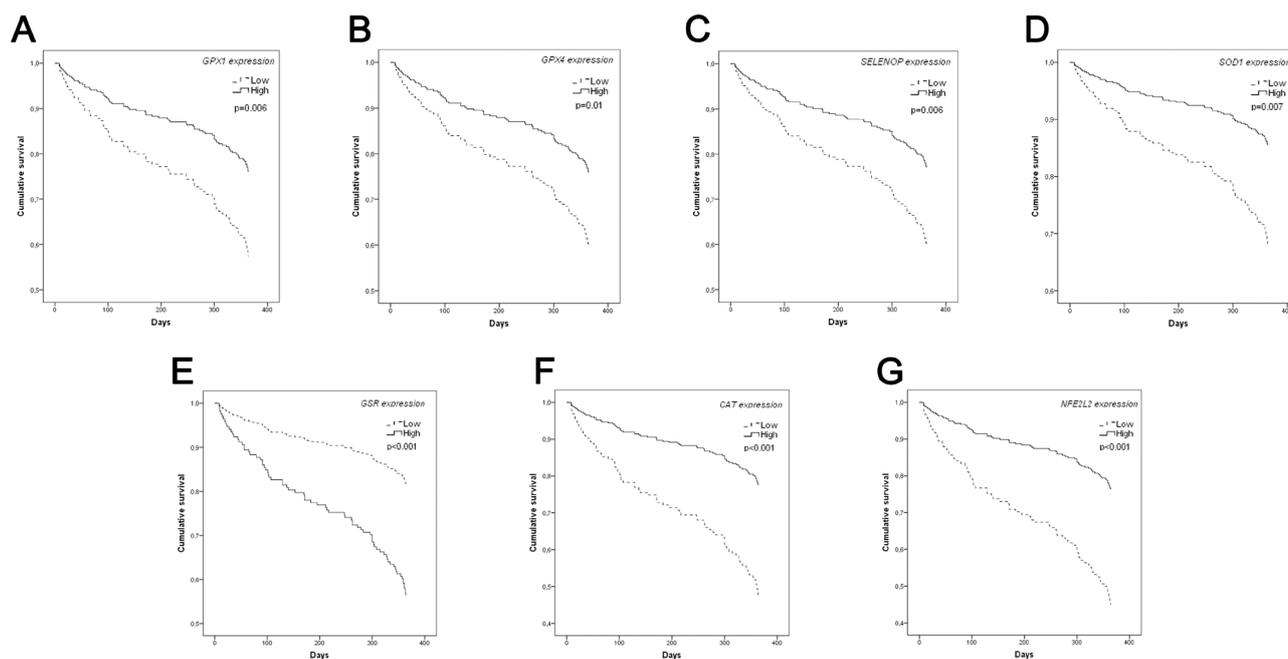


Figure 3 – Survival analysis. Cumulative survival plot of dichotomized gene expression levels of TCGA tumoral tissues.

Table 2 – Multivariate Cox proportional hazards regression analysis to assess the independent effect of gene expression on survival in 12 months

	Adjusted HR	95% CI	p
<i>GPX1</i>	2.023	1.222-3.347	0.006
<i>GPX4</i>	1.859	1.147-3.015	0.012
<i>SEP15</i>	0.75	0.315-1.783	0.514
<i>SELENOP</i>	1.968	1.217-3.183	0.006
<i>SOD1</i>	2.447	1.273-4.702	0.007
<i>SOD2</i>	1.461	0.824-2.590	0.194
<i>GSR</i>	0.354	0.224-0.559	<0.001
<i>CAT</i>	2.924	1.774-4.820	<0.001
<i>NFE2L2</i>	2.955	1.779-4.908	<0.001

HR: hazard ratio; CI: confidence interval

A number of differentially expressed genes were identified in tumoral samples from TCGA patients (Figure 1A, B), along with replication of two genes in the experimental data (Figure 1C, D). Previous studies have demonstrated variable expression patterns of antioxidant genes, depending on the type of cancer analyzed (Table 1). The present results add new information about the expression of these genes in HCC.

Some studies evaluated the expression of the genes for these antioxidant enzymes separately in HCC tumoral samples, HepG2 and Huh7 cell lines (Table 1). Results for *GPX1*, *GPX4*, *SEP15*, *SELENOP*, *SOD2*, and *NFE2L2* seem to be corroborated by our analysis (Cheng *et al.*, 2015; Guariniello *et al.*, 2015; Guerriero *et al.*, 2015; Wang *et al.*, 2016a; Zhao *et al.*, 2015). We observed that the pattern of gene expression of these enzymes seemed to vary when analysis was performed in TCGA tumoral versus normal adjacent tissues, and TCGA versus GTEx databases (Figure 1A, B and Table 1). The tumor microenvironment seems to play a role in HCC progression, influencing progression by modulation of liver fibrosis, initiation of the epithelial-to-mesenchymal transition, invasion, alterations of oxidative stress status, and other processes (Novikova *et al.*, 2017). Differences in antioxidant enzyme levels between normal, tumoral, and peritumoral tissues may represent different stages of adaptation of this system against oxidative stress, or the use of ROS as signaling molecules (Moloney and Cotter, 2018).

Differences in the expression of two genes, *GPX4* and *SOD2*, were statistically significant in TCGA and experimental data. Increased expression of *GPX4* was present in TCGA versus GTEx analysis and experimental data (Figure 1B, C). However, this difference was not significant in TCGA tumoral × non-tumoral tissues (Figure 1A). In agreement with our findings, studies with grade III HCC tissues (Guerriero *et al.*, 2015), HepG2 cell lines (Guariniello *et al.*, 2015; Zhao *et al.*, 2015), and Huh7 cells (Guariniello *et al.*, 2015) also revealed higher expression of *GPX4* (Table 1). Lower expression was found in gastric cancer (Lan *et al.*, 2017), clear cell renal cell carcinoma (Rudenko *et al.*, 2015), and human breast cancer cell lines (Rusolo *et al.*, 2017) (Table 1).

GPX4 has the same detoxification function as *GPX1* in the cell, including the ability to reduce lipid peroxides (Figure 4B) (Labunskyy *et al.*, 2014). Even though mRNA expression was analyzed, and gene transcription is not always directly related to protein synthesis, it is possible that overexpression

of *GPX4* could affect the cell environment. Increased *GPX4* expression could enhance the levels of antioxidant components in cells and protect against oxidative stress (Davis *et al.*, 2012; Rohr-Udilova *et al.*, 2018). However, overexpression of *GPX4* could also benefit cancer cells. *GPX4* plays an important role in preventing oxidative stress-induced apoptosis by decreasing lipid peroxidation. In so doing, *GPX4* blocks posterior signaling, leading to cell death (Figure 4B). Therefore, overexpression of this enzyme could be an advantageous mechanism used by tumoral cells to sustain growth and avoid apoptosis (Labunskyy *et al.*, 2014; Rohr-Udilova *et al.*, 2018). A previous study showed that overexpression of *GPX4* in HCC *in vitro* protected the cells from oxidative stress and reduced the intracellular free radical level (Rohr-Udilova *et al.*, 2018).

SOD2 had different expression patterns between TCGA analyses and experimental data (Figure 1A, B, D and Table 1). Reduced expression of *SOD2* has been previously reported (Wang *et al.*, 2016a), in a study into HBV-positive HCC tumors in a cohort. Higher gene expression of *SOD2* has been reported in oral squamous cell carcinoma (Pedro *et al.*, 2018) and colorectal adenoma and cancer (Hughes *et al.*, 2018). Most of the patients from TCGA and Wang *et al.* (2016a) presented with hepatitis B as the main etiology, while patients from our experimental data were mostly HCV-positive. No statistically significant differences were found in gene expression levels between risk factor types (data not shown). A diversity of tumor types can develop in HCC, in terms of staging and its molecular subclasses, which could explain, in part, the variety of findings in gene expression and deregulated pathways (Hoshida *et al.*, 2013).

SOD2 is located in the mitochondrial matrix (Figure 4B) and acts to catalyze dismutation of the superoxide anion ($O_2^{\cdot-}$) to H_2O_2 , playing a crucial role in alleviating oxidative stress (Kim *et al.*, 2017). Loss of this antioxidant component could impair the oxidative balance in cells. However, its overexpression could favor the cancer cell environment (Kim *et al.*, 2017). Intensification of *SOD2* expression in tumoral cells seems to ensure H_2O_2 flow from mitochondria, which is a crucial step for the occurrence of the Warburg effect (Che *et al.*, 2016), a strategy used by cancer cells to increase the generation of additional metabolites. Upregulation of *SOD2* could favor H_2O_2 accumulation (Figure 4B), which is involved in a variety of signaling pathways related to proliferation, migration, and invasion in cancer cells (Glorieux *et al.*, 2015).

Table 3 – Top Most significant enriched gene ontology (GO) terms.

Category	Id	Term	Count	Genes	P-value ^a
Upregulated					
BP	GO.0098754	Detoxification	5	GSR SOD1 GPX4 NFE2L2 GPXI	5.92E-08
BP	GO.0006979	Response to oxidative stress	6	GSR SOD1 GPX4 NFE2L2 GPXI SELENOP	1.50E-07
BP	GO.0097237	Cellular response to toxic substance	5	GSR SOD1 GPX4 NFE2L2 GPXI	6.26E-07
BP	GO.0098869	Cellular oxidant detoxification	4	GSR SOD1 GPX4 GPXI	3.10E-06
BP	GO.1902175	Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	3	SOD1 NFE2L2 GPXI	2.18E-05
BP	GO.0034599	Cellular response to oxidative stress	4	GSR SOD1 NFE2L2 GPXI	6.32E-05
BP	GO.0006749	Glutathione metabolic process	3	GSR SOD1 GPXI	6.95E-05
BP	GO.0045454	Cell redox homeostasis	3	GSR NFE2L2 GPXI	1.20E-04
BP	GO.0042542	Response to hydrogen peroxide	3	SOD1 NFE2L2 GPXI	4.20E-04
BP	GO.0019372	Lipoxygenase pathway	2	GPX4 GPXI	7.50E-04
MF	GO.0016209	Antioxidant activity	4	GSR SOD1 GPX4 GPXI	7.40E-07
MF	GO.0004602	Glutathione peroxidase activity	2	GPX4 GPXI	8.10E-04
MF	GO.0016491	Oxidoreductase activity	4	GSR SOD1 GPX4 GPXI	0.0014
CC	GO.0070013	Intracellular organelle lumen	7	GSR SOD1 SEPI5 GPX4 NFE2L2 GPXI SEPP1	0.0067
CC	GO.0005759	Mitochondrial matrix	3	GSR SOD1 GPXI	0.0082
CC	GO.0005739	Mitochondrion	4	GSR SOD1 GPX4 GPXI	0.0163
Downregulated					
BP	GO.0000302	Response to reactive oxygen species	2	CAT SOD2	0.0057
BP	GO.0034599	Cellular response to oxidative stress	2	CAT SOD2	0.0057
BP	GO.0051289	Protein homotrimerization	2	CAT SOD2	0.0057
BP	GO.0072593	Reactive oxygen species metabolic process	2	CAT SOD2	0.0057
BP	GO.0098869	Cellular oxidant detoxification	2	CAT SOD2	0.0057
BP	GO.0007568	Ageing	2	CAT SOD2	0.006
BP	GO.0010035	Response to inorganic substance	2	CAT SOD2	0.0157
BP	GO.0043066	Negative regulation of apoptotic process	2	CAT SOD2	0.0419
BP	GO.0055114	Oxidation-reduction process	2	CAT SOD2	0.043
MF	GO.0016209	Antioxidant activity	2	CAT SOD2	5.8E-4
MF	GO.0016491	Oxidoreductase activity	2	CAT SOD2	0.0229

GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component; ^a Corrected p-value

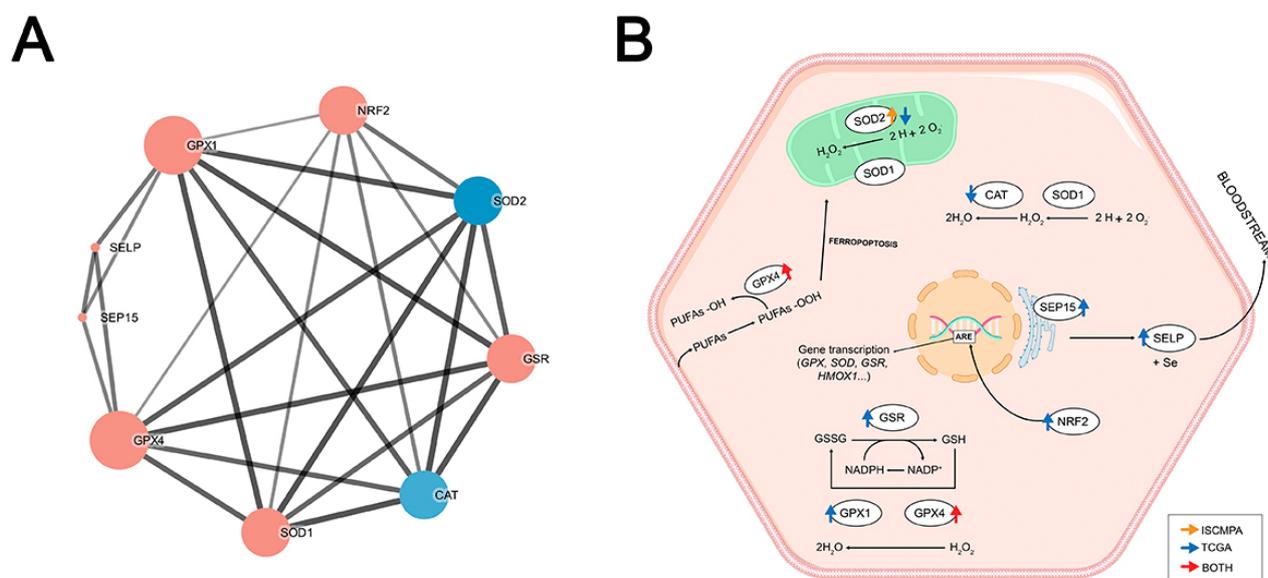


Figure 4 – Enriched annotation pathways and interaction network analysis of antioxidant proteins investigated in the present study. (A) Protein-protein interaction network of the analyzed genes. Lines represent interaction associations between nodes and line thickness indicates the strength of data support (score). Size of nodes indicates the degree of associations. Blue nodes represent downregulated genes and red nodes represent upregulated genes according to data from case (TCGA) vs control (GTEx) analyses. (B) Illustrative representation of antioxidant enzymes functions in hepatocytes. Up- and down-arrows denote significant upregulated or downregulated genes, respectively. Red arrows: both analysis; Blue arrows: only in TCGA analysis; Orange arrows: only in experimental analysis. Abbreviations: GPX1, glutathione peroxidase 1; GPX4, glutathione peroxidase 4; GSR, glutathione reductase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; SEP15, 15 kDa selenoprotein; SelP, selenoprotein P; GSSG, oxidized glutathione; GSH, reduced glutathione; NRF2; nuclear factor erythroid 2-related factor 2; HMOX1; heme oxygenase-1 gene; ARE; antioxidant response element; Se, selenium; and PUFAs; Polyunsaturated fatty acids.

Table 4 – Top Most significant enriched pathways.

Category	Id	Term	Count	Genes	P-value ^a
Upregulated					
K	hsa00480	Glutathione metabolism	3	GSR GPX4 GPX1	8.46E-06
R	HSA-2142770	Synthesis of 15-eicosatetraenoic acid derivatives	2	GPX4 GPX1	7.05E-05
R	HSA-2142712	Synthesis of 12-eicosatetraenoic acid derivatives	2	GPX4 GPX1	7.05E-05
R	HSA-2142688	Synthesis of 5-eicosatetraenoic acids	2	GPX4 GPX1	7.05E-05
R	HSA-3299685	Detoxification of Reactive Oxygen Species	2	SOD1 GPX1	4.20E-04
K	hsa05014	Amyotrophic lateral sclerosis (ALS)	2	SOD1 GPX1	9.40E-04
K	hsa04918	Thyroid hormone synthesis	2	GSR GPX1	0.0013
R	HSA-114608	Platelet degranulation	2	SOD1 SEPP1	0.0033
K	hsa05016	Huntington's disease	2	SOD1 GPX1	0.0065
Downregulated					
R	HSA-3299685	Detoxification of Reactive Oxygen Species	2	CAT SOD2	6.61E-5
K	hsa04146	Peroxisome	2	CAT SOD2	9.18E-5
K	hsa04211	Longevity regulating pathway	2	CAT SOD2	9.18E-5
K	hsa04068	FoxO signaling pathway	2	CAT SOD2	1.0E-4
R	HSA-2262752	Cellular responses to stress	2	CAT SOD2	0.003
R	HSA-8953897	Cellular responses to external stimuli	2	CAT SOD2	0.003
R	HSA-168256	Immune System	2	CAT SOD2	0.046

K, KEGG Pathways; R, REACTOME Pathways; a Corrected p-value

Survival analysis of patients from TCGA revealed an interesting aspect of gene expression patterns in HCC. Although higher expression of antioxidant enzymes was present in HCC patients, patients with lower gene expression also displayed lower overall survival, except for *GSR* expression (Figure 3). As previously discussed, ROS act as a two-edged sword in cancer, with beneficial and detrimental roles in cells, and are tightly regulated by cancer cells (Moloney and Cotter, 2018). On the one hand, higher expression of antioxidant enzymes could not only control ROS accumulation, preventing cell death being triggered by them, but could also perpetuate tumoral cells. On the other hand, lower expression could cause apoptosis and kill tumoral cells. However, regarding the level of expression, tumors could also be favored due to the accumulation of ROS used as signaling factors (Sajadimajd and Khazaei, 2017; Moloney and Cotter, 2018). This mechanism and the extent to which the levels of these antioxidant enzymes vary in each stage of hepatocarcinogenesis should be explored in depth in further studies.

Correlation (Figure 2), PPI network (Figure 4A), and GO (Tables 3 and 4) analyses highlighted possible relationships between the antioxidant enzymes investigated and their interactions in different pathways (Table 4). *NFR2*, which is encoded by *NFE2L2*, seems to be important because of its interaction with Antioxidant Responsive Element (ARE) (Figure 4B), an interaction that increases the expression of several genes, including genes that encode glutathione peroxidases and SODs (Raghunath *et al.*, 2018). Further analysis at the protein level should be performed to clarify the knowledge about this protein network, especially in the context of carcinogenesis.

None of the other genes in the experimental data had significantly altered expression, even though there was a tendency towards underexpression or overexpression of some genes. The limitations of our study, such as the difficulty in acquiring fresh liver tissue for mRNA analysis, resulted in a small sample size, which could be one of the explanations for the lack of statistical significance. To improve our analysis, we also investigated data from larger databases, such as TCGA and GTEx, which provided us with a better understanding of the results. In addition, peritumoral tissue, due to its proximity to the tumor, could already contain alterations, and could complicate the examination of gene expression differences. However, the availability of fresh, healthy liver tissue was also limited. Peritumoral tissue was collected with a safety margin and, in this case, allowed us to perform a paired analysis. These data permitted an evaluation of differences in gene expression in the tumoral and adjacent peritumoral tissues of the same patient. To verify the presence of differences between tissue types, we performed bioinformatic analyses between matched samples, as well as between case and control samples.

We present preliminary findings of the gene expression patterns of antioxidant enzymes in HCC. The findings highlight the importance of further evaluation of these components in the pathology of cancer studies with larger sample sizes. We were able to replicate data from TCGA analysis for at least two genes. The present study is one of few investigations to investigate a diversity of antioxidant enzyme genes in the context of cancer. We were also able to examine clinical

parameters and survival data for different gene expression levels in TCGA patients. Our study highlights the need for further studies to better understand the role of these enzymes in HCC.

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Conflict of interest

The authors have no conflict of interest to disclose.

Authors Contributions

AFA conceived and designed the study, conducted all the analysis and wrote the manuscript; ACM conducted laboratory experiments; HFA and ABGV collected samples and clinical data from patients; MG, MF and SA revised the manuscript, helped in statistical analysis, provided financial support and administered the project. All authors read and approved the final version.

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Supplementary material

The following online material is available for this article:

Table S1 – Sequences of primer pairs.

Table S2 – Detailed results of gene expression analysis using RNA-Seq data.

Table S3 – Final models of multivariate Cox regression analysis.

Figure S1 – Sample of agarose gel electrophoresis of total RNA.

Internet Resources

The Cancer Genome Atlas – Liver Hepatocellular Carcinoma (TCGA-LIHC), <https://portal.gdc.cancer.gov/projects/TCGA-LIHC> (accessed 01 June 2020).

Genotype-Tissue Expression database (GTEx), <https://gtexportal.org/home/eqtls/tissue?tissueName=Liver> (accessed 05 June 2020).

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