RESEARCH ARTICLE

Editorial Process: Submission:10/09/2017 Acceptance:12/15/2017

Immunohistochemical and Biochemical Expression Patterns of TTF-1, RAGE, GLUT-1 and SOX2 in HCV-Associated Hepatocellular Carcinomas

Tarek Aboushousha[1]*, Samah Mamdouh[2], Hussam Hamdy[3], Noha Helal[1], Fatma Khorshed[2], Gehan Safwat[4], Mohamed Seleem[5]

Abstract

Objective: To investigate the expression of TTF-1, RAGE, GLUT1 and SOX2 in HCV-associated HCCs and in surrounding non-tumorous liver tissue. **Material and Methods:** Tissue material from partial hepatectomy cases for HCC along with corresponding serum samples and 30 control serum samples from healthy volunteers were studied. Biopsies were classified into: non-tumor hepatic tissue (36 sections); HCC (33 sections) and liver cell dysplasia (LCD) (15 sections). All cases were positive for HCV. Immunohistochemistry (IHC), gene extraction and quantitative real-time reverse-transcription assays (qRT-PCR) were applied. **Results:** By IHC, LCD and HCC showed significantly high percentages of positive cases with all markers. SOX2 showed significant increase with higher HCC grades, while RAGE demonstrated an inverse relation and GLUT-1 and TTF-1 lacked any correlation. In nontumorous-HCV tissue, we found significantly high TTF-1, low RAGE and negative SOX2 expression. RAGE, GLUT-1 and SOX2 show non-significant elevation positivity in high grade HCV compared to low grade lesions. TTF-1, RAGE and SOX2 exhibited low expression in cirrhosis compared to fibrosis. Biochemical studies on serum and tissue extracts revealed significant down-regulation of RAGE, GLUT-1 and SOX2 genes, as well as significant up-regulation of the TTF-1 gene in HCC cases compared to controls. All studied genes show significant correlation with HCC grade. In non-tumor tissue, only TTF-1 gene expression had a significant correlation with the fibrosis score. **Conclusion:** Higher expression of TTF-1, RAGE, GLUT-1 and SOX2 in HCC and dysplasia compared to non-tumor tissues indicates up-regulation of these markers as early events during the development of HCV-associated HCC.

Keywords: IHC- TTF1- RAGE- GLUT1- SOX2- HCV- HCC

Asian Pac J Cancer Prev, **19 (1)**, 219-227

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in the world and the third most common cause of cancer-related deaths (Siegel et al., 2012). The incidence of HCC is inconstant worldwide with the highest rates in Southeast Asia and Sub-Saharan Africa (Blechacz and Mishra, 2013). It is estimated that 70%–90% of HCC develop within an established background of chronic liver disease (Sherman, 2010). Carcinogenesis of HCC is a complex process that is associated with miscellaneous risk factors, including but not limited exposure to aflatoxin B, chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive consumption of alcohol and tobacco, iron overload, and diabetes (Forner et al., 2012).

Thyroid transcription factor-1 (TTF-1) has been commonly used in the diagnosis and prediction for prognosis of thyroid and pulmonary tumors (Folpe et al., 1999; Agoff et al., 2000). TTF-1 staining was well known to be nuclear, with only occasional reports of cytoplasmic staining. However, increasing attention has been drawn to the significance of cytoplasmic staining of TTF-1 in both HCC and non-neoplastic hepatic tissue recently (Wieczorek et al., 2002; Gokden and Shinde, 2005; Lei et al., 2006) who suggested that TTF-1 may serve as a potential valuable marker to confirm the diagnosis of HCC in uncertain cases.

Receptor for advanced glycation end products (RAGE) belongs to an immunoglobulin superfamily of cell surface molecules that could bind to a number of ligands such as advanced glycation end products, high-mobility group protein box-1, S-100 calcium-binding protein, and amyloid-β-protein, inducing a series of signal transduction cascades and being involved in a variety of cellular function, including inflammation, proliferation, apoptosis, angiogenesis, migration, and fibrosis. RAGE is expressed in hepatic stellate cells, hepatocytes and hepatoma cells.

*[1]Pathology Department, [2]Biochemistry Department, [3]Surgical Department, Theodor Bilharz Research Institute, [4]Faculty of Biotechnology, October University of Modern Sciences and Arts, Giza, [5]National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt. *For Correspondence: t.aboushousha@tbri.gov.com*

There is accumulating evidence that engagement of RAGE with various ligands elicits oxidative stress generation and subsequently activates the RAGE downstream pathway in the liver, thereby contributing to the development and progression of numerous types of hepatic disorders (Yamagishi and Matsui, 2015). These observations suggest that inhibition of the RAGE signaling pathway could be a novel therapeutic target for liver diseases.

Accelerated glycolysis is one of the biochemical characteristics of cancer cells. The glucose transporter isoform 1 (GLUT-1) is a key rate-limiting factor in the transport and metabolism of glucose in cancer cells. GLUT-1 expression is primarily undetectable in normal epithelial tissues and benign epithelial tumors; however, it is overexpressed in a significant proportion of human carcinomas (Medina and Owen, 2002; Airley and Mobasheri, 2007). However, GLUT-1 expression level and functional significance in HCC are still disputed. Therefore, we aimed to analyze the expression of the GLUT-1 gene in cases of HCC.

SOX2, one of the key members of the SOX family gene, plays critical roles in embryonic pluripotent stem cells and is a key factor to reprogram differentiated cells into induced pluripotent stem cells (Yu et al., 2007). Many publications showed that SOX2 participated in oncogenesis and tumor progression of human colorectal (Han et al., 2012), pancreatic (Sanada et al., 2006), gastric (Li et al., 2004) and breast (Rodriguez-Pinilla et al., 2007) cancers, osteosarcomas (Basu-Roy et al., 2012) and glioma (Fang et al., 2011). SOX2 is a novel predictor of poor prognosis for HCC patients after hepatectomy (Huang et al., 2011). Nevertheless, there is still no report describing the molecular role of SOX2 in HCC.

Materials and Methods

Groups of patients

Our study included 84 biopsy materials (tumor and non-tumor) from cases of partial hepatectomy done for patients suffering of hepatocellular carcinoma. The study included also their corresponding serum samples in addition to serum samples from 30 healthy volunteers served as controls. Tissue specimens were processed into paraffin blocks at Pathology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The specimens were histopathologically classified into: non-tumor hepatic tissue (36 sections); HCC (33 sections) and liver cell dysplasia (15 sections). All cases were proved serologically to be of hepatitis virus C etiology. This study was carried out in full accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Ethics Committee of Theodor Bilharz Research Institute and by National Hepatology and Tropical Medicine Research Institute (NHTNRI). A written informed consent was obtained from each participant, in accordance with the institutional guidelines.

Histological Study

Paraffin blocks were made and 4 microns thick sections were stained with hematoxylin and eosin stain (H and E stain) for routine histopathological examination, grading and staging of hepatitis activity and tumor grade. Sections were also stained by Masson's trichrome stain for assessment of fibrosis stage using METAVIR scoring system (Poynard et al., 2000).

Grade of hepatitis activity; based on amount of inflammation

A1:- mild activity, A2:- moderate activity, A3:- severe activity.

Stage of fibrosis; representing amount of fibrosis or scarring:

F1:-portal fibrosis without septa, F2: portal fibrosis with few septa, F3: numerous septa without cirrhosis, F4: cirrhosis. In our study, for simplicity, we have grouped A1 and A2 as low hepatitis activity and a separate A3 as high hepatitis activity. We have also grouped F1, F2 and F3 as fibrosis and F4 as cirrhosis.

HCC grade was done according to the WHO classification of tumors of the liver and intrahepatic bile ducts (Bosman et al., 2010) into:

Grade 1: (well differentiated).
Grade 2: (Moderately differentiated).
Grade 3: (Poorly differentiated).

Immunohistochemical technique

Formalin-fixed paraffin sections (5µm in thickness) were cut. Sections underwent deparaffinization and rehydration. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide. Antigen retrieval was performed by microwaving the sections in citrate buffer, pH 6.0. The slides were allowed to cool at room temperature and washed 3 times by immersing them in TBST (Tris Buffered Saline having 0.05% Tween 20). Sections were incubated with the primary antibodies overnight at 4oC in humid chamber:

1) TTF-1 monoclonal antibody (Thermo Fisher Scientific, Cheshire, UK) at an optimal dilution of 1:50 in PBS (phosphate buffered saline).

2) RAGE Antibody (A11) (Santa Cruz Biotechnology, sc- 80652, USA) at an optimal dilution of 1:100 in PBS.

3) GLUT-1 Antibody (Abcam 41525, Cambridge, USA) at an optimal dilution of 1:100 in PBS.

4) SOX2 Antibody (NBP2-29623) (Novus Biologicals, USA) at an optimal dilution of 1:100 in PBS.

Next day, the slides were washed 4 times, 5 minutes each with TBST. Sections were then incubated for 30 minutes with the secondary biotinylated antibody followed by avidin peroxidase complex for another 30 minutes according to the manufacturer's instructions (Universal Detection Kit, Dako, Denmark). The antigen was localized by the addition of DAB (Diaminobenzidine) substrate chromogen solution. Finally, slides were counterstained with hematoxylin, dehydrated in alcohol, and mounted.

For each setting, negative controls were carried out in which the primary antibody was omitted and replaced by PBS. Positive controls were squamous cell carcinoma of the lung for SOX2, red blood cells and perineurium of nerves (internal positive control) for GLUT-1, thyroid tissue for TTF-1 and lung tissue for RAGE.

Interpretation of Immunostaining

All immunostained slides were assessed and scored. The sections were examined by using light microscope (Scope A1, Axio, Zeiss, Germay). Photomicrographs were taken using a microscope-camera (AxioCam, MRc5, Zeiss, Germany).

TTF-1, GLUT-1 and SOX2: The expression level of these antibodies was judged according to the percentage positive cells in each tumor tissue. Specifically, a percentage of ≤10 % was judged negative and >10 % was positive (Cao et al., 2011; Sun et al., 2013; Mano et al., 2014).

RAGE

The expression of RAGE was semi quantitatively estimated as the total membrano-cytoplasmic immunostaining scores, which were calculated as the product of a proportion score and an intensity score. The proportion and intensity of staining were evaluated independently. The proportion score reflected the fraction of positive staining cells (score 0: <5%, score 1: 5%-10%, score 2: 10%-50%, score 3: 50%-75%, score 4: >75%) and the intensity score represented the staining intensity (score 0: no staining, score 1: weak positive, score 2: moderate positive, score 3: strong positive). Finally, a total expression score was given ranging from 0 to 12. Based on the analysis in advance, RAGE was regarded as negative expression in gastric cancer tissues if the score <2, and positive expression if the score ≥2 (Xu et al., 2013).

Biochemical Investigations

Laboratory tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea (B. Urea), albumin (ALB) and alpha-fetoprotein (ALP) were performed for all subjects in a duplicate as routine tests.

Genes extraction

Total RNAs were extracted from 200 µl serum according to the manufacturer's instructions of Abbott mSample preparation system kit (Abbott Molecular, Inc., Des Plaines, IL) and from 100 mg tumor and non-tumor liver tissues according to the manufacturer's instructions of Promega kit (Promega Corporation, Madison, WI 53711 USA) and the extracted RNA was aliquoted and stored at -80°C until used.

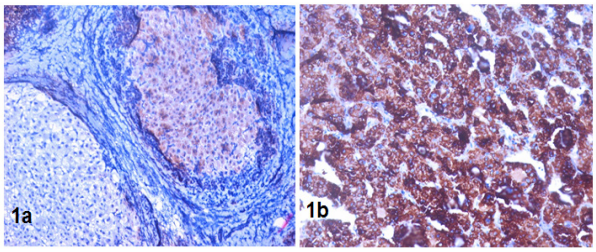

Figure 1. a, Section in Viral Hepatitis C Showing Liver Cirrhosis, with Mild Cytoplasmic Brownish Positivity for TTF1in the Smaller Regenerating Nodule. (IHC for TTF1 with DAB detection, X100). b, Section in Viral Hepatitis C Showing HCC, with Strong Cytoplasmic Brownish Positivity for TTF1in Malignant Hepatocytes. (IHC for TTF1 with DAB detection, X200).

Quantitative Real-Time Reverse-Transcription Assay (qRT-PCR)

TTF-1, RAGE, GLUT-1 and SOX-2 expression quantification were performed through two step PCR amplification. Firstly, the total RNA was reversely transcripted using poly-dT primers into cDNA according to the manufacturer's instructions of (High-capacity cDNA kit, AB Applied Biosystems, Foster City, CA, USA). Secondly 5 µl of the cDNA was used for the real time PCR amplification step using TTF-1, RAGE, GLUT-1 and SOX-2 specific primers and syber green master mix (Maxima SYBR Green/ROX qPCR Master Mix 2X,Thermofisher, UK) using StepOne™ Real-Time PCR System (AB Applied Biosystems, Foster City, CA, USA). All reactions were run in duplicate. The ΔΔCT method was used for the relative quantification in all samples (Yilmaz et al., 2012).

Statistical analysis

SPSS for Windows, version 20 was used for statistical analysis (IBM corporation, Armonk, new York, USA). The comparisons of quantitative variables were performed between two groups using Mann Whitney U-test. The receiver operating characteristic (ROC) curve analysis determined sensitivity, specificity and the accuracy of TTF-1, RAGE, GLUT-1 and Sox-2 genes. Associations between genes expressions, tumor grade and fibrosis stage were evaluated by chi square test and Fisher's exact test. The P value < 0.05 was considered statistically significant.

Results

Patient's clinical parameters

Our study included 84 biopsy materials from cases of partial hepatectomy done for patients suffering of hepatocellular carcinoma and their corresponding serum samples in addition to 30 control serum samples from healthy volunteers (50 of these patients were males and 34 were females). Data presented in Table 1 shows the clinical analysis performed for the samples. The mean age of the studied cases of male was significantly higher than that of female patients (p<0.01) (Table 1). There was

Table 1. Biochemical and Histopathological Analysis of HCC Patients and Controls

parameters	HCC Patients	Healthy Volunteers	P Value
Age			
(Mean±S.D. in years)	51.89± 8.84	41.66± 8.02	0.0374*
Males	54.79±7.59		
Females	48.57±9.08		
ALB (Mean±S.D. unit)	2.84± 0.17	4.2 ± 0.26	0.0001***
ALT (Mean±S.D. unit)	48.16± 3.50	26.37± 1.18	0.0238*
AST (Mean±S.D. unit)	42.85± 3.39	23.56± 2.14	0.0286*
Tumor grade (number of cases) I/II/III	10/15/8	NO BIOPSY TAKEN	
Fibrosis score (number of cases) (F)/(C)	12/30		

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; S.D, Standard Deviation; ***, significant at p<0.001; *, significant at p<0.05; (F), Fibrosis; (C), Cirrhosis

Table 2. Difference in Tissue Expression Scores of Studied Markers in Examined Lesions

Lesions		TTF-1		RAGE		GLUT-1		SOX2	
		Negative	Positive	Low	High	Negative	Positive	Negative	Positive
Non-tumor (36)	N (%)	9_a (25.0%)	27_b (75.0%)	32_a (87.5%)	4_b (12.5%)	18_a (50.0%)	18_a (50.0%)	30_a (83.33%)	6_b (16.67%)
Dysplasia (15)	N (%)	0_a (0.0%)	15_b (100%)	0_a (0.0%)	15_b (100%)	3_a (25.0%)	12_b (75.0%)	0_a (0.0%)	15_b (100%)
HCC (33)	N (%)	0_a (0.0%)	33_b (100%)	8_a (24.42%)	25_b (75.76%)	4_a (12.12%)	29_b (87.88%)	6_a (18.18%)	27_b (81.82%)

Each subscript letter denotes a subset of marker's score categories whose column proportions do not differ significantly from each other at the .05 level.

Table 3. Difference in Markers Expression Scores in Different Ggrades of Hepatitis Activity and Stages of Fibrosis

			TTF-1		RAGE		GLUT-1		SOX2	
HCV Hepatitis			Negative	Positive	Low	High	Negative	Positive	Negative	Positive
Hepatitis Grade	Low (31)	N (%)	5 (16.10%)	26 (83.90%)	17 (54.80%)	14 (45.20%)	8 (25.80%)	23 (74.20%)	29 (83.50%)	2 (6.50%)
	High (11)	N (%)	3 (27.30%)	8 (72.70%)	4 (36.40%)	7 (63.60%)	2 (18.20%)	9 (81.80%)	8 (72.70%)	3 (27.30%)
Z-score			0.8086		1.05		0.5101		1.831	
p value			0.41		0.29		0.61		0.067	
Fibrosis Stage	Cirrhosis (30)	N (%)	9 (30.00%)	21 (70.00%)	18 (60.00%)	12 (40.00%)	15 (50.00%)	15 (50.00%)	26 (86.70%)	4 (13.30%)
	Fibrosis (12)	N (%)	2 (16.70%)	10 (83.30%)	5 (41.70%)	7 (58.30%)	6 (50.00%)	6 (50.00%)	8 (66.70%)	4 (33.30%)
Z-score			0.89		1.078		0		1.49	
p value			0.37		0.28		1		0.136	

significant difference in terms of age with P=0.0374, ALT with P= 0.0238 and AST with P=0.0286. Biochemical analysis has showed that ALB was highly significant in HCC than control group (P =0.0001).

Immunohistochemical Results

Non-tumor tissue sections showed significantly low expression of RAGE ($p<0.05$) and negative expression of SOX2, while they showed non-significant difference between negative and positive GLUT-1 expression ($p>0.05$) and significantly higher percentage of TTF-1 positive expression ($p<0.05$). On the other hand, liver cell

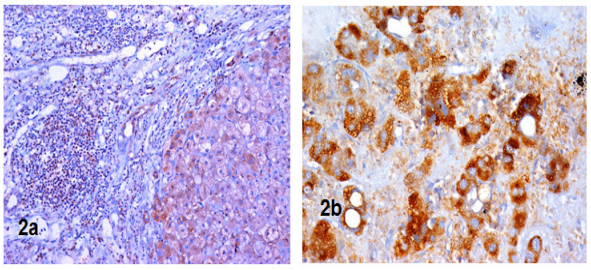

Figure 2. a, Section in Viral Hepatitis C Showing Liver Cirrhosis, with Mild Cytoplasmic Brownish Positivity for RAGE in the Regenerating Nodule. (IHC for RAGE with DAB detection, X100). b, Section in Viral Hepatitis C Showing HCC, with Strong Cytoplasmic Brownish Positivity for RAGE in Malignant Hepatocytes. (IHC for RAGE with DAB detection, X200).

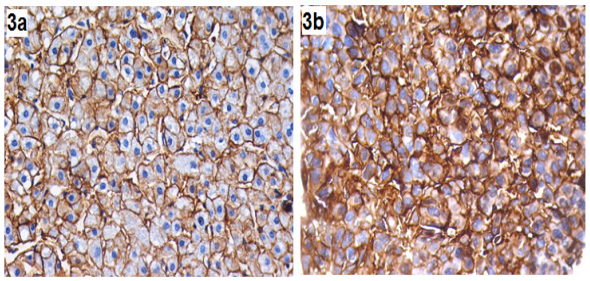

Figure 3. a, Section in Viral Hepatitis C Showing Liver Cirrhosis, with Moderate Membranous Brownish Positivity for GLUT1 in Hepatocytes within a Regenerating Nodule. (IHC for GLUT1 with DAB detection, X200). b, Section in Viral Hepatitis C Showing HCC, with Strong Membrano-Cytoplasmic Brownish Positivity for GLUT1 in Malignant Hepatocytes. (IHC for GLUT1 with DAB detection, X200).

Table 4. Relation between the Expression of Tissue Markers and the Grades of HCC

Grade of HCC and Dysplasia		TTF-1		RAGE		GLUT-1		SOX2	
		Negative	Positive	low	High	Negative	Positive	Negative	Positive
G1(10)	N(%)	1_a (10.0%)	9_b (90.0%)	1_a (10.0%)	9_b (90.0%)	0_a (0.0%)	10_b (100%)	2_a (20.0%)	8_a (80.0%)
G2(15)	N(%)	3_a (25.0%)	12_b (75.0%)	3_a (25.0%)	12_b (75.0%)	6_a (40.0%)	9_b (60.0%)	2_a (13.33%)	13_b (86.67%)
G3(8)	N(%)	2_a (25.0%)	6_a (75.0%)	4_a (50.0%)	4_a (50.0%)	0_a (0.0%)	8_b (100%)	1_a (12.5%)	7_b (87.5%)
LCD(15)	N(%)	0_a (0.0%)	15_b (100%)	0_a (0.0%)	15_b (100%)	3_a (25.0%)	12_b (75.0%)	0_a (0.0%)	15_b (100%)

Each subscript letter denotes a subset of marker expression score categories whose column proportions do not differ significantly from each other at the .05 level; LCD, Liver cell dysplasia

Table 5. The Correlation between Serum TTF-1, RAGE, GLUT-1 and SOX-2 Genes Expression in Relation to Tumor Grade and Stage of Liver Fibrosis

	cut off value	Tumor grade			P[a] value	Stage of Fibrosis		P[b] value
		GI	GII	GIII		(F=12)	(C=30)	
SOX-2	>4.460	2	18	19	<0.0001***	5	21	0.058 NS
	<4.460	10	4	1		7	9	
GLUT-1	>3.560	4	16	15	0.0346*	6	21	0.292 NS
	<3.560	8	6	5		6	9	
RAGE	>3.560	5	17	16	0.0040**	4	20	0.083NS
	<3.560	7	5	4		8	10	
TTF-1	>3.285	10	2	2	<0.0001***	8	7	0.013*
	<3.285	2	20	18		4	23	

[a], Fisher exact test between miRNAs expressions and fibrosis score; [b], chi-square test between miRNAs expressions and tumor grade; P value considered significant at P<0.05; NS, non-significant;*, significant; **, highly significant; ***, very highly significant.

Table 6. The Validation of SOX-2, GLUT-1, RAGE and TTF-1 Genes as a Diagnostic Biomarker in the Serum

	Cut off value	Specificity	Sensitivity	AUC
TTF-1	<3.285	84.22%	82.44%	0.88
RAGE	3.56	82.31%	83.33%	0.866
GLUT-1	4.475	78.23%	76.45%	0.763
SOX-2	4.46	80.00%	77.78%	0.877

AUC, area under curve; the results according to the ROC curve analysis.

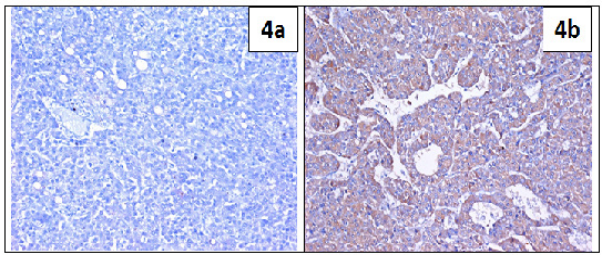

Figure 4. a, Section in Viral Hepatitis C Negative Expression of SOX2 in Hepatocytes within the Regenerating Nodules. (IHC for SOX2 with DAB detection, X200). b, Section in Viral Hepatitis C Showing HCC, with Diffuse Cytoplasmic Brownish Positivity for SOX2 in Malignant Hepatocytes. (IHC for SOX2 with DAB detection, X200).

dysplasia and HCC showed significantly high percentage of cases with positive expression of all studied markers (p<0.05) (Table 2).

Generally, there was an increase in the percentage of cases showing positive expression of all studied markers from low to high grades of hepatitis activity except for TTF-1. However, this relation was not statistically significant (p>0.05). Regarding the differential study of fibrosis versus cirrhosis, it was found that cirrhosis showed lower percentage of cases with positive expression of TTF-1, RAGE and SOX2 as well as equal percentage of GLUT-1 positive expression compared to fibrosis, however, this relation was statistically non-significant (p>0.05) (Table3).

SOX2 expression showed significant increase with increasing grade of HCC. On the other hand, RAGE expression showed inverse relation with grade of HCC. GLUT-1 expression and TTF-1 expression showed irrelevant relation to HCC grades. Liver cell dysplasia showed significantly higher percentage of cases with positive expression of all studied markers (p<0.05). (Table 4).

Biochemical Results

Relative expression of TTF-1, RAGE, GLUT-1 and Sox-2 genes in tissue and serum samples

TTF-1, RAGE, GLUT-1 and SOX-2 genes expressions were quantified in HCC and non-tumor HCV hepatitis tissues and their corresponding serum using qRT-PCR and compared with control group as shown in Figure 1. In liver tissue samples, SOX-2 and RAGE genes were significantly down-regulated in tumor tissues compared to non-tumor HCV hepatitis tissues (p=0.022 and p=0.031 respectively). However TTF-1 gene (P=0.045) was significantly up-regulated in tumor tissues compared to non-tumor tissues. The expression of GLUT-1 gene was approximately the same in both tumor and non-tumor tissues in cases of HCV hepatitis. Also in serum samples RAGE, GLUT-1 and SOX-2 were significantly down-regulated in HCC serum samples compared to control samples from healthy volunteers (p=0.027, p=0.019 and p=0.023 respectively). However TTF-1 gene was significantly up-regulated in HCC serum samples compared to controls (p=0.030).

Correlation between TTF-1, RAGE, GLUT-1 and SOX-2 genes expression levels with fibrosis and tumor grade

There was significant positive correlation of the expression levels of GLUT-1 (p=0.0346*), a highly significant positive correlation of RAGE (p=0.0040**) and very highly significant correlation of TTF-1 and SOX2 (p<0.0001***) with tumor grades. Only TTF-1 gene expression in serum showed positive significant correlation with the fibrosis score (p=0.022*), however no correlation was found between the expression of RAGE, GLUT-1 and SOX-2 genes in serum with the fibrosis score Table 5.

Table 6 shows the Receiver Operator Characteristic (ROC) analysis which used to determine the optimum cut-off value, sensitivity, specificity and area under curve of the qRT-PCR of TTF-1, RAGE, GLUT-1 and SOX-2

genes in the serum.

Discussion

HCV is a major global cause of liver disease. It is the main cause of liver cirrhosis and liver cancer (Gomaa et al., 2014). Egypt has the highest burden of the HCV in the world with prevalence rate as high as 14.7%, however, a recently published Egypt Health Issues Survey (EHIS) in 2015 on a nationally representative sample showed that 10% of Egyptians between 15-59 years of age had been infected with HCV infection, while 7% are chronic active hepatitis C patients (Ministry of Health and Population [Egypt], El-Zanaty and Associates [Egypt], and ICF International, 2015). HCC constitutes 70.48% of all liver tumors among Egyptians (Mokhtar et al., 2007) that is usually detected at advanced stage at which no treatment may be effective. Early detection of HCC provides the best chance for a curative treatment (El-Tayeha et al., 2012).

TTF-1 is initially identified as a mediator of thyroid-specific gene transcription. Its expression was characteristically seen in the lung and thyroid epithelia and their tumors (Compérat et al., 2005). In non-tumor hepatitis tissue, TTF-1 immuno-histo-positivity was seen in 75% of cases. This is parallel to that documented by Lei et al., (2006) who found high TTF-1 expression in their studied hepatitis and cirrhosis cases. A higher percentage (99%) of TTF-1 positive cases was found also by Coa et al (2011) in biopsies from the adjacent non-neoplastic hepatic tissue. Low grades of hepatitis activity showed high percentage of positive cases compared to high grades with non- significant statistical difference. On the other hand, cirrhosis showed non-significantly lower percentage of cases exhibiting positive expression of TTF-1 compared to non-cirrhotic liver sections. TTF-1 was found in all dysplastic and HCC lesions. This is similar to the result of Chen et al., (2001a) who found TTF-1 in all their studied HCCs, and close to that detected by Lei et al., (2006) who found TTF-1 immunoreactivity in 97% of HCC cases. Meanwhile, Gokden and Shinde (2005) and Coa et al., (2011) detected TTF-1 immunopositivity in 77% and 80% of their studied HCC cases respectively. However, we found no significant relation between TTF-1 expression and HCC grades.

Similarly our biochemical study showed significant up-regulation of TTF-1 gene in tumor tissue more than in non-tumor tissue. Also, TTF-1 gene expression was significantly up-regulated in HCC patient's serum compared to normal samples.

RAGE is a multi-ligand receptor classified as an immunoglobulin superfamily cell-surface molecule. It is recognized to be responsible for cancer progression in several human cancers (Vlassara et al., 1989). Our immunohistochemical results showed a significantly lower RAGE expression in non-tumor tissue compared to dysplasia and HCC groups. RAGE score increased with increased intensity of inflammation. This was in accordance with the findings of Hiwatashi et al., (2007). who provided a new hypothetical concept that hepatic RAGE expression may be relevant to the stage or severity of inflammation. However, no significant relations were achieved between RAGE expression and grades of hepatitis activity or cirrhosis. High scores of RAGE expression was found in most cases of dysplasia and HCC and most of the studied HCC sections whether cirrhotic or non-cirrhotic showed high scores of RAGE expression. This finding was in accordance with the results of Ito et al., (2014) who showed that there was no significant relationship between RAGE positivity in tumor and background liver status. In addition, the percentage of HCC cases with high scores of RAGE expression is higher in low grades of malignancy compared to high grade HCC. This goes with Hiwatashi et al., (2007) who reported that RAGE messenger RNA expression was high in well and moderately differentiated tumors but declined in poorly differentiated HCC.

Our biochemical study showed also that the expression of RAGE was down-regulated in tumor tissue in comparison with the non-tumor samples. Also, HCC serum samples showed significant down-regulation of RAGE expression level in comparison to the control. Lung cancer showed a results that parallel to ours of HCC in that the level of RAGE was down-regulated compared to the normal lung and that there was a correlation between the down regulation of RAGE and the stage of disease (Waghray et al., 2013). On the contrary, in Prostate cancer, it was reported that RAGE was up-regulated and over-expressed in the prostate cancerous tissue than the normal tissue (Nedić et al., 2013). In colon cancer there was conflict of results in the relevancy of RAGE to the cancer. According to Duke's classification it was reported that there was over-expression in RAGE levels while on other studies it showed no significant change in the RAGE level (Pusterla et al., 2013).

GLUT-1 is a key rate-limiting factor in the transport and metabolism of glucose. It is over-expressed in a significant proportion of human carcinomas (Airley and Mobasheri, 2007). Cancer cells show increase of uptake of glucose compared to normal tissue and GLUT-1 is responsible for the passive transport of glucose across the cell membrane (Chen et al., 2001b). In our immunohistochemical study, GLUT-1 expression was found in half of non-tumor tissues. Its expression in these lesions can be related to the role played by hypoxia in the pathogenesis of HCV and in turn hypoxia results in an increased transcription of the GLUT-1 gene (Czech et al., 2014). No correlation was found between GLUT-1 expression and both the grade of hepatitis activity or the stage of fibrosis. GLUT-1 immunopositivity was more frequently over-expressed in dysplastic and HCC tissues compared to non-tumorous tissues; this is in accordance with results of Czech et al., (2013) and Smith (1999). This finding could point to the important role of GLUT-1 in the uptake of glucose by HCC cells, being the main energetic source of this neoplastic growth. On the contrary, Amann et al., (2009) reported that GLUT-1 was detectable in only 13.2% of their HCC cases, and in none of the non-cancerous liver tissues. We found also that the percentage of GLUT-1 expression progressively but non-significantly increases with increasing grades of HCC. This goes with Gatenby et al., (2007) and Krzeslak et al., (2012) who reported a correlation between GLUT-1 expression and tumor grade

in breast and endometrial carcinomas cases respectively.

In serum samples of HCC patients, the expression of GLUT-1 was down-regulated compared to controls, while in tissue extracts, the expression was approximately the same in tumor and non-tumor specimens. In a study by Kim et al., (2000), their results showed that GLUT-1 was not largely expressed in normal gastric tissues but was expressed in gastric cancerous tissues. In addition, Kang et al., (2002) reported high expression of GLUT-1 in only 47% of breast cancerous tissue, however in the normal tissue the expression was not detected. Another study by Shaker et al., (2013) for prediction of prognosis of HCC showed that GLUT-1 expression was significantly higher in HCC than in the adjacent non-tumor tissue. All previous data indicates that GLUT-1 expression was up-regulated in malignancy compared to controls, which agree with our immunohistochemical findings but disagree with our biochemical results of serum and tissue extracts.

SOX2 is a key player in the maintenance and self-renewal of embryonic as well as adult stem cells (Boyer et al., 2005). It participates in oncogenesis and progression of various cancers by regulation of multiple cell signaling pathways, including HCC (Sun et al., 2013; Zhao et al., 2015). We found that -immunohistochemically- SOX2 expression was low in non-tumor tissue, with non-significant lower expression in association with cirrhosis than with fibrosis, but without significant relation to the activity grade of hepatitis. Similarly, Sun et al., (2013) found no significant correlation between SOX2 expression and the stage of hepatic fibrosis or cirrhosis. We found also that SOX2 expression was more frequently over-expressed in dysplasia and HCC tissue, compared to non-tumor tissue. Additionally, we found an increase of SOX2 expression with increasing grade of HCC. This expression profile suggests a role of SOX2 in the progression of HCC. These findings match to results of Sun et al., (2013) who found that SOX2 expression was low in non-tumor liver tissues, increased in noninvasive HCC, and reached the highest level in invasive HCC and also they found a significantly higher SOX2 expression in high grades of HCC compared to low grade ones.

Our study on serum and tissue extract showed that SOX2 expression in serum of control samples from healthy volunteers was significantly higher than expression in serum of HCC patients, and in tissue extract, the expression of SOX2 in non-tumor tissue was significantly higher than that in tumor tissue. This is on contrary to results of Yin et al., (2013) using qPCR and Fang et al., (2010) using reverse transcription (RT) PCR analyses, who reported higher expression levels of SOX2 in HCC and colorectal cancer specimens respectively, compared to those in the corresponding adjacent non-tumor tissues.

In all the studied markers, liver cell dysplasia showed significantly higher percentage of positive cases in relation to non-tumor tissue, this may be an indicator for role of these markers in initiation of hepatocarcinogenesis.

In conclusion, immunohistochemical and biochemical results showed some discrepancy. This was also evident between serological and biochemical assessment of these markers in tissue extract. However, lower expression of TTF-1, RAGE, GLUT-1 and SOX2 in non-tumor tissue compared to its higher expression in malignant and dysplastic tissues indicated that up-regulation of these markers represent early events during the development of HCV-related hepatocellular carcinoma. Accordingly, these markers could be a reliable tool in the diagnosis of premalignant changes associated with HCV infection, assisting in tumor classification, grading and monitoring the treatment response.

Declaration

No conflict of interest.

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