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International Journal of Current Research
Vol. 10, Issue, 05, pp.69085-69090, May, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

SSX -1 AND SSX-5 GENES EXPRESSION IN EGYPTIAN HEPATOCELLULAR CARCINOMA PATIENTS: A CASE CONTROL STUDY

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ARTICLE INFO

Article History:
Received 17th February, 2018
Received in revised form
06th March, 2018
Accepted 03rd April, 2018
Published online 23rd May, 2018

Key words:
SSX 1 gene, SSX 5 gene,
Tumor-specific markers,
Hepatocellular Carcinoma.

ABSTRACT

Background: Hepatocellular carcinoma (HCC) has become one of the most prevalent cancers worldwide. With a rising rate, it is a prominent source of mortality. Patients with advanced fibrosis, predominantly cirrhosis and hepatitis B and C infections are predisposed to developing HCC. Genes of Synovial sarcoma X chromosome (SSX) are one of cancer testis associated genes; which are a subgroup of tumor antigens with a restricted expression in testis and malignancies. Limited studies were done about SSX genes expression in Egyptian HCC patients.

Objectives: Therefore this study aims to estimate the SSX-1 and SSX-5 mRNA expression in the blood of Egyptian HCC patients and to evaluate any probable association between the expression of these genes and other clinical and biochemical parameters.

Methods: A case control study was done on 40 patients with HCC, 20 patients with post HCV liver cirrhosis and 20 healthy persons. Assessment of SSX1 and SSX5 mRNA expression in the peripheral blood was done by reverse transcription real-time PCR.

Results: SSX -1 and SSX-5 mRNA were expressed in 47.5 % and 42.5 % of HCC patients respectively. Both genes were not expressed in cirrhotic patients and healthy controls. There was no considerable association between SSX-1 and SSX-5 gene expression and alpha fetoprotein level (P = 0.09& 0.29), size of the tumor (P = 0.56& 0.08) and tumor stage (P = 0.28& 0.22).

Conclusion: SSX-1 and SSX-5 mRNA are exclusively expressed in peripheral blood of patients with hepatocellular carcinoma, thus both genes are considered as a tumor-specific markers to detect HCC in Egypt.

Citation: Sally Abdallah AbdelAziz, Amany Atwa, Iman Elshabrawy, Mahmoud Elbendary and Mohamed A. Zahran, 2018. "Ssx -1 and ssx-5 genes expression in egyptian hepatocellular carcinoma patients: a case control study", *International Journal of Current Research*, 10, (05), 69085-69090.

INTRODUCTION

Hepatocellular carcinoma (HCC) has become one of the most widespread cancers worldwide. It is a major source of mortality due to its rising rate. Patients who have advanced fibrosis, mainly cirrhosis and hepatitis B and C infections are predisposed to developing HCC (Bravi *et al.,* 2013). It is considered the second leading cause of cancer- related deaths (Ferlay *et al.,* 2012). The most frequent underlying risk factors are chronic viral hepatitis, cirrhosis, alcohol abuse, and non-alcoholic steato-hepatitis (NASH) (Maluccio and Covey, 2012). Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide, with rising rates of HCC (Lehman *et al.,* 2008). Hepatocellular carcinoma (HCC) is the second most frequent cancer in men and the 6th most frequent cancers in women, in Egypt the incidence of HCC has been doubled in the past 10 years (Anwar *et al.,* 2008). The SSX gene family, which consists of 9 subtype genes (SSX 1–9) was initially recognized as fusion associates to SYT gene in synovial sarcoma (Smith *et al.,* 2011). The SSX genes exhibit nucleotide homologies ranging from 88 to95% and encode proteins of 188 amino acids that exhibit homologies ranging from 77 to 91% (Soulez *et al.,* 1999). Also, the SSX genes are capable of inducing both humoral and cellular immunity. Both cellular and humoral immune response can kill tumor cells specifically, so that, they are considered useful targets in cancer vaccine immunotherapy (Lu *et al.,* 2007). In normal tissues, SSX-1 and SSX-5 genes expression are found to be restricted to testis, and they are expressed minimally in the tissue of thyroid, on the other hand both genes are found to be expressed at a various range of different cancers (Dos Santos *et al.,* 2000). The expression of the SSX-1 gene is closely related to the occurrence and metastasis of different tumors (Ayyoub *et al.,* 2002). The data about the expression of both SSX1 and SSX5 genes in HCC is limited. The present research intends to investigate expression of SSX-1 and SSX-5 mRNA in the

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blood of Egyptian HCC patients and to evaluate any probable association between the expression of these genes and other clinical and biochemical parameters.

MATERIALS AND METHODS

Subjects: The present case-control study included 60 patients (40 HCC patients, 20 post hepatitis C Cirrhosis) and 20 controls and was conducted during the time period from December 2016 to May 2017 at Medical Biochemistry Department and Tropical Medicine Department, Mansoura Faculty of Medicine. They were divided into three different group: The initial group contained 40 HCC patients who were collected from Tropical Medicine Department, Mansoura University Hospitals. The diagnosis of HCC was made according to European Association Study of Liver Disease (EASL) guidelines (Easl eortc, 2012). Patients with cancers rather than liver cancer, as well as those with persistent heart disease, chronic inflammatory diseases and septicemia were excluded from this study. The second group contained 20 patients with post hepatitis C liver cirrhosis as confirmed by clinical examination, serological markers of viral hepatitis and radiological investigations were collected from Tropical Medicine Department, Mansoura University Hospitals. The third group incorporated 20 normal individuals. They have normal liver function tests with negative hepatitis B and C markers. All 60 patients incorporated in this research were verified to have normal thyroid gland standed on clinical assessment and typical thyroid function tests. Male subjects appeared clinically to have normal testicles. This study was approved by ethical committee of Mansoura Faculty of medicine. A written consent was obtained from all subjects before their participation in this study. We collected clinical records and biochemical results from medical reports of patients.

Methods

All studied groups were *subjected* to:

Blood sampling and extraction of RNA

We collected (5 mL blood samples in EDTA). 1 volume of human whole blood was added to 5 volumes of erythrocyte lysis(EL Buffer) in an appropriately sized tube. The tube was incubated for 10–15 min on ice and mixed by vortexing briefly two times during incubation, the tube was centrifuged for 400 x g for 10 min at 4°C and completely remove and discard the supernatant. Leukocytes will form a pellet after centrifugation. In accordance with the manufacturer's directions, via Mini Kit miRNeasy (Qiagen, Valencia and USA), the resulting cell pellets were stored at -80 C until RNA extraction. Total RNA was extracted from the nucleated cells. We determined the concentration of RNA by measuring the absorbance at(A260/A280 nm)in addition to we stored RNA at -80 ˚C. The integrity of RNA was detected by agarose gel electrophoresis as well as staining with ethidium bromide.

Reverse transcription polymerase chain reaction (RT-PCR)

The primer sequence of the B-actin and chosen genes are demonstrated in Table 1. Primer sets for SSX1 gene, SSX5 and B-actin were designed using Primer 3 software (version 0.4.0). Primers were designed to span exon - exon junction to preclude amplification of genomic DNA(Primers were chosen and designed so that one half hybridizes to the 3 end of one exon and the other half to the 5 end of the adjacent exon of the target genes). Two Micrograms (ug) of RNA were added to reverse transcription with random primers using Gene- specific PCR primers were purchased from Vivantis Technologies Sdn Bhd in proportion to the manufacturer's directions. We checked the integrityof cDNA using amplification of b-actin simultaneously as a control gene. A CR reaction components were prepared by adding 10ul of2X SYBR Green PCR Master Mix, 0.8of forward Primer, 0.8 reverse Primer, 8.4ul of cDNA template. The PCR amplifications were performed using a thermo cycler (Applied Biosystem7500) and under the following conditions: The products of PCR were appeared on 2.0% Ethidium bromide-staining we visualized agarose gel on ultra-violate transilluminator as shown in Figure (1), (2) and (3)

Statistical Analysis

Data were tabulated, coded then analyzed using SPSS (Statistical Package for Social Science) version 23.0. One way ANOVA followed by post-hoc tukey was used for data expressed as mean ± SD, Mann-Whitney test was used for data expressed as median (IQR) andChi-square test has been used for data expressed as frequency. P value was measured statistically significant once it was below 0.05.

RESULTS

This research incorporated three different groups. The initial group contained 40 HCC diseased persons. They were 27 males and 13females and the mean of their ages was 58.12 ±5.93 years. The second group contained 20 patients with post hepatitis C liver cirrhosis. They were 12 males and 8 females and the mean of their ages was 58.35 ± 7.53 years. The third group contained 20 normal individuals. They were 10 males and 34 females and the mean of their ages was 57.45± 7.06 years. The characteristics of HCC patients involved in this study are summarized in Table (3). In this study as shown in Table (4), neither SSX1 gene nor SSX5 gene could be expressed in the blood samples of 20 cirrhotic patients or 20 healthy controls On the other hand, SSX1 gene was expressed in 19 patients (47.5%) and SSX5 gene was expressed in 17 patients (42.5%).

Both genes were co-expressed in 11 patients (27.5%) in the blood samples of 40 patients with HCC. In the current study, SSX1 gene was expressed in 11patients with tumor size less than 5 cm (57.9 %) and was expressed in 8 patients with tumor size more than 5 cm (42.1%). Also, it was expressed in 11 patients with stage I HCC (57.9%), 3 patients with stage II HCC (15.8%), 4 patients with stage III HCC (21.1%) and only in one patient with stage IV HCC (5.3%). On the other hand, SSX5 gene was expressed in 8 patients with tumor size less than 5 cm (47.1%) and was expressed in 9 patients with tumor size more than 5 cm (52.9%). Also, it was expressed in 6 patients with stage I HCC (35.3%), 8 patients with stage II HCC (47.1%), 2 patients with stage III HCC(11.8%) and only in one patient with stage IV HCC (5.9%). As shown in table (5), there was no significant association between SSX1 gene expression, SSX 5 gene expression and size of the tumor (P = 0.56 and 0.08 respectively), tumor stage (P = 0.28 and 0.22 respectively) and Alfa fetoprotein level (P = 0.09 and 0.29 respectively).

Table 1. Primer sequences and product size for PCR amplification

Gene	Primer sequences	Refrence sequences	PCR product size (bp)
B-actin	F : 5 GTGGCCGAGGACTTTGATTG 3 R 5 GTGGGGTGGCTTTTAGGATG R	NM-001101.3	104bp
SSX-1	F 5 GCTCCACAGAATCATCCCGA3 R 5 CTGTGGGTCCAGGCATGTTT3	NM_001278691.1	192
SSX-5	F 5 ACGGAGACGATGCCTTTGTA3 R 5 TTCCACGGTCACAGACTTGT3	NM_021015.3	96

Cycles	Temp.	Time	Notes
1	95˚ C	2sec	PCR activation
40	95	5 sec	Denaturation
	60-65	10 sec	Annealing
	72	5-20 sec	Extension

2-step cycling

Cycles	Temp.	Time	Notes
1	95˚ C	2sec	PCR activation
40	95	5 sec	Denaturation
	60-65	15-30 sec	Annealing,Extension

Table 3. Description of characteristics of Hepatocellular carcinoma patients

Variable	Total number = 40	Percentage
Age	Mean 58.12 SD ± 5.93	
Sex		
Male	27	67.5 %
Female	13	32.5 %
Tumor Size		
< 5 cm	25	62.5%
> 5 cm	15	37.5%
Tumor Stage		
Stage I	20	50%
Stage II	12	30%
Stage III	6	15%
Stage IV	2	5%
Lymph node Metastasis	Present 2	95 %
	Absent 38	5%
Distant Metastasis	Present 2	95 %
	Absent 38	5%
Barcelona Clinic Liver Cancer Staging (BCLC)	A 20	50%
	B 18	45%
	C 2	5%
Alpha fetoprotein (AFP)	Median 39 Range (1.5 – 1423)	

Table 4. Expression of SSX1 gene and SSX5 gene in the studied groups

			Control group	Cirrhosis group	HCC group	P	P1	P2	P3
SSX 1 gene expression	Negative	No	20	20	21	<0.001***	1.00	<0.001***	<0.001***
		%	100.0%	100.0%	52.5				
	Positive	No	0	0	19				
		%	0.0%	0.0%	47.5%				

SSX 5 gene expression	Negative	No	20	20	23	<0.001***	1.00	0.001**	<0.001***
		%	100.0%	100.0%	57.5%				
	Positive	No	0	0	17				
		%	0.0%	0.0%	42.5%				

P:Probability
P1: significance between Control& Cirrhosis groups
P2: significance between Control& HCC groups
P3: significance between Cirrhosis& HCC groups

Table 5. Association of SSX 1 gene and SSX5 gene expression and different HCC characteristics

		SSX 1 gene expression				P
		Negative		Positive		
		No	%	No	%	
Size of tumour	<5cm	14	66.7	11	57.9	0.567
	>5cm	7	33.3	8	42.1	
Tumor stage	Stage I	9	42.9	11	57.9	0.287
	Stage II	9	42.9	3	15.8	
	Stage III	2	9.5	4	21.1	
	Stage IV	1	4.8	1	5.3	
AFP	Median(IQR)	41 (1.5 – 1423)		19 (4.1 – 1996)		0.095

		SSX 5 gene expression				P
		Negative		Positive		
		No	%	No	%	
Size of tumour	<5cm	17	73.9	8	47.1	0.083
	>5cm	6	26.1	9	52.9	
Tumor stage	Stage I	14	60.9	6	35.3	0.221
	Stage II	4	17.4	8	47.1	
	Stage III	4	17.4	2	11.8	
	Stage IV	1	4.3	1	5.9	
AFP	Median(IQR)	39 (1.5 – 1423)		110 (4.10 – 1996)		0.297

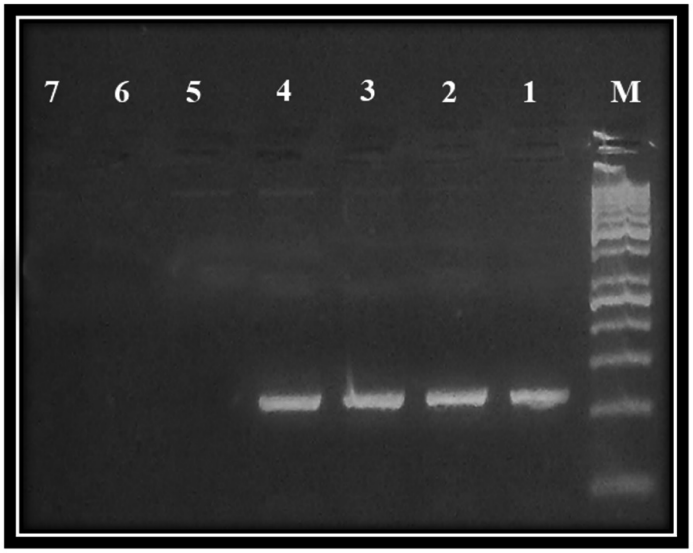

Fig. 1. PCR products of B actin (104bp) in Lanes 1,2,3,4 M Lane: DNA molecular weight 50 base pairs

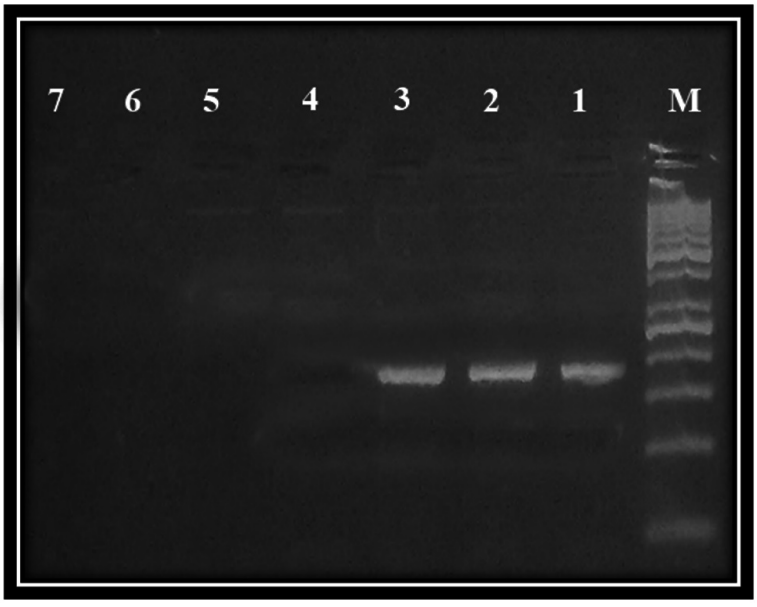

Fig. 2. SSX-1 gene PCR products in HCC patients (192bp) in Lanes 1,2,3. M Lane: DNA molecular weight 50 base pairs

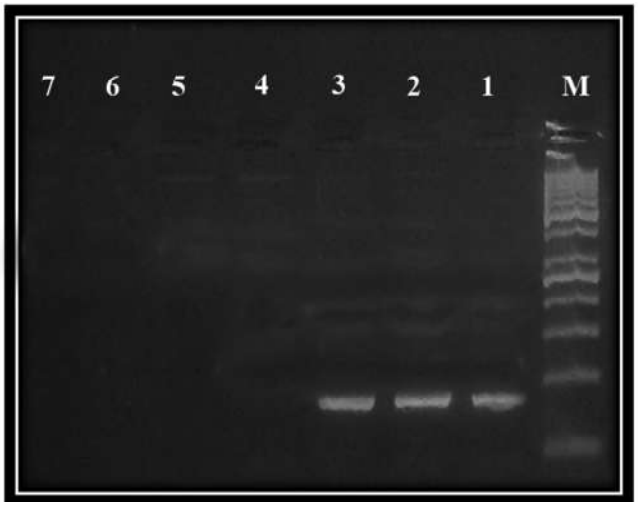

Fig. 3. SSX-5 PCR products gene in HCC patients (96 bp) in Lanes1,2,3. M Lane : DNA molecular weight 50 base pairs

DISCUSSION

Liver cancer is considered one of the most widespread malignant tumors worldwide. Liver cancer occurred mostly in patients who had chronic parenchymatous liver diseases for instance chronic hepatitis C as well as chronic hepatitis B infections. The early detection of HCC is very important to improve the prognosis of those patients. The pathogenesis of liver cancer is a multifaceted process. Different circulating and prognostic markers are required for proper management of those patients who were at initial phase of the disease (Yao *et al.,* 2007). Cancer testis associated genes are considered as tumor associated antigens that normally expressed in male germ cells but not in adult somatic tissue (Chiappini, 2012). They are beneficial for tumor immunotherapy due to their tumor-specific expression and effectiveness in inducing immune responses. These antigens are expressed in numerous malignant tumors at different frequencies including melanoma, lung cancer and hepatocelluar carcinoma (MashinoK, Sadanaga *et al.,* 2001). A number of cancer testis associated antigen have been found to be expressed specifically in HCC. The current study aimed to evaluate appearance of the SSX1 and SSX5 mRNA in the blood of HCC Egyptian patients. We found that high rates of SSX1 and SSX5 mRNA expression in the blood of liver cancer patients although their expression was absent in the peripheral blood of patients with cirrhosis and normal individuals.

These results were similar to another study conducted in Egyptian patients with the same methods by Amal Fawzy *et al.,* (2013) indicating that these genes are specific for liver cancer and can propose their significance for identifying cancerous cells. Also, these results are in harmony with (Zhao *et al.,* 2010; Wu *et al.,* 2006; Zhao *et al.,* 2004 and Lu *et al.,* 2007). Thus these genes can reveal blood spreading of cancerous cells more particularly than other traditional methods and can play a complementary task in diagnosis of liver cancer (Zhao *et al.,* 2010).In this study, SSX1mRNA expression in the peripheral blood of patients with HCC was 47.5% of while it was 40.4 % in the previous Egyptian study conducted by Amal Fawzy *et al.,* 2013. And our results are approximately similar to previous different studies. Chien *et al.,* 2001 found that SSX-1 mRNA was detected 80% of the HCC tissues. Zhao L *et al.,* 2004 found thatSSX-1 was expressed in 72.4% of HCC tissues. Zhao L *et al.,* 2010 found that SSX-1 was expressed in 73.3% of 90 HCC tissue samples and expressed in 34.4% of HCC patients in Peripheral blood mononuclear cell (PBMC) samples. Lu *et al.,* 2007 found that SSX-1 mRNA was expressed in 61.1% in HCC cancer tissues and 38.9% in blood samples patients with HCC.

The different rate of expression of this gene may be due to racial distribution of the patients, different sample size as well as different technical methods of detection. Also, they revealed that there was no expression of SSX1 gene in tissues of cirrhosis, healthy liver tissues as well as the blood of healthy persons (Lu *et al.,* 2007). Thus, SSX1 is considered as a tumor-specific markers in the peripheral blood of HCC patients. As regard SSX5 gene expression, it was expressed in 42.5 % of the patients with HCC in our study while it was 36.5 % in the previous Egyptian study (15). Our results are in accordance with Chien *et al*., 2001 (19) who found that SSX-5 gene was detected in 33.3% of the HCC tissues. Wu *et al*.,2006 also found that SSX-5 was expressed in 25% of blood samples from HCC patients. This study revealed that there was no significant association between mRNA expression of SSX1 and SSX 5 and tumor size, tumor staging and AFP level (P value > 0.05) and this is similar to the previous study conducted by Amal Fawzy *et al.,* 2013 and these results also are confirming data of the previous studies (Chien *et al.,* 2001), (Wu *et al*., 2006) (17) and (Lu *et al*., 2007).

This study revealed that there was no significant association between mRNA expression of SSX1 and SSX5 and tumor stage but the expression of both genes was higher in the early stages of the disease indicating earlier circulating tumor cells in the peripheral blood and the potential utility of both genes as molecular markers in HCC patients and these results are similar to the previous Egyptian study conducted by Amal Fawzy *et al.,* 2013. The current study contains 20 patients in stage I HCC with higher level of both mRNA gene expression indicating the utility of both genes as molecular markers for early detection of HCC in Egyptian patients and it differs from the previous study conducted by Amal Fawzy *et al.,* 2013 (15) as it lacks the presence of patients with stage I HCC. Moreover, our results showed that SSX-1mRNA was detected in 57.9%, 15.8% of HCC patients at stage I and stage II respectively and 21.1%, 5.3% of HCC patients at stage III,IV respectively. In contrast to(Zhao L *et al.,* 2010)(16)who found that SSX-1 mRNA was detected in PBMC from 51.4% of HCC patients at stages I and II and from 77.9% of HCC patients at stages III and IV, showing that advanced stages of HCC are correlated with the higher expression frequency of SSX-1. This variation between the results of both studies may be due to different sample size, different number of patients in each group as well as different technical methods of detection. Potential imitations of the present study include the followings : i) It is a small sample, single institution study; ii) only patients with post hepatitis C cirrhosis and subsequently HCC were recruited, Therefore, a large-scale validation study is required to confirm these results.

Conclusion

In consummation SSX-1 and SSX-5 mRNA are exclusively expressed in peripheral blood of patients with hepatocellular carcinoma, thus both genes are considered as a tumor-specific markers for initial detection of HCC in Egyptian patients.

Acknowledgements: Not Applicable.

Conflict of interest: No conflict of interest.

Funding: No funding sources.

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