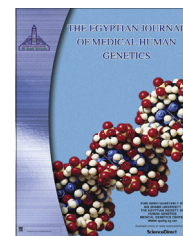




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ORIGINAL ARTICLE

Expression of SSX-1 and SSX-5 genes in the peripheral blood of patients with hepatocellular carcinoma

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KEYWORDS

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Abstract *Background:* Liver cancer is the fifth most common cancer in men and the seventh in women. Hepatocellular carcinoma (HCC) is responsible for significant morbidity and mortality in patients with liver cirrhosis and accounts for 90% of primary liver cancer. Synovial sarcoma X chromosome (SSX) genes belong to cancer testis antigens (CTA) family; expressed only in germ cell tumors. There have been some studies about the SSX genes expression in the HCC. To the best of our knowledge no reports included these genes expression in the Egyptian patients with HCC.

Aim: This study aims to evaluate the SSX-1 and SSX-5 mRNA expression in tumor cells circulating in the peripheral blood (PB) of a cohort of Egyptian patients with HCC and to find out any possible associations between these genes expression and different clinical/laboratory parameters.

Subjects and methods: This study included 100 subjects; 52 HCC cases, 25 with post viral hepatitis liver cirrhosis and 23 apparently healthy controls. Expression of SSX-1 and SSX-5 mRNA in PB was tested by reverse transcription polymerase chain reaction (RT-PCR).

Abbreviations: NCI, national cancer institute, SSX, synovial sarcoma X chromosome, HCC, hepatocellular carcinoma, CTA, cancer testis antigen, CTAs, cancer testis antigens, HCC, hepatocellular carcinoma, HCV, hepatitis C virus, HBV, hepatitis B virus, AFP, alpha fetoprotein, RNA, ribonucleic acid, mRNA, messenger ribonucleic acid, RT-PCR, reverse transcription polymerase chain reaction, EDTA, ethylenediaminetetraacetic acid, dNTP, deoxyribonucleotides triphosphate, DNA, deoxyribonucleic acid, cDNA, cyclic deoxyribonucleic acid, PBS, phosphate buffer saline, bps, base pairs, SPSS, statistical package for social science program, n, number, CTC, circulating tumor cells

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Results: SSX-1 and SSX-5 mRNA were expressed in 40.4% and 36.5% of the HCC patients, respectively. SSX-1 and/or SSX-5 were not detected in healthy controls or cirrhotic patients. Neither SSX-1 nor SSX-5 expression showed an association with Alfa-Fetoprotein (AFP) levels, tumor size, tumor differentiation, hepatitis B infection and Bilharziasis ($P > 0.05$).

Conclusion: SSX-1 and SSX-5 mRNA are specifically expressed in tumor cells circulating in PB of HCC patients and thus could be used as easy access, simple method molecular markers for early diagnosis of HCC patients in Egypt.

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1. Introduction

Liver cancer is the fifth most common cancer in men and the seventh in women [1]. During the past 20 years, the incidence of HCC has tripled while the 5-year survival rate remained below 12%. HCC is responsible for significant morbidity and mortality in patients with liver cirrhosis and accounts for 90% of primary liver cancer [1,2]. Most of HCCs in the world occur in the setting of cirrhosis and over half-million people develop liver cancer every year and an almost equal number die of it [3]. HCC is one of the most aggressive cancers. Less than 10% of the patients, who show progression to the terminal stage, have one year survival [4]. In Egypt, liver cancer is considered a major health problem. According to the Egyptian NCI registry, held from January 2002 to December 2003, liver cancer ranked the 2nd most common cancer site, after bladder cancer, in males (11.3% of the newly diagnosed cases) and ranked the 7th cancer site, 3.7% of the newly diagnosed female cases. Ninety percent of these liver cancers were diagnosed as HCC [5]. This may be attributed to the high prevalence rate of HCV infection in Egypt [6,7]; the main etiological factor in this population.

The SSX gene family, which was originally identified as fusion partners to the SYT gene in synovial sarcomas, consists of 9 subtype genes (SSX 1–9). The known SSX family members share high homology at the protein and DNA level. Also, naturally occurring serologic responses mounted by cancer patients against one SSX family member cross-react with other members of the family [8]. They are also capable of eliciting spontaneously humoral and cellular immune responses in cancer patients, and are potentially useful targets in cancer vaccine-based immunotherapy [9]. The expression of the SSX-1 and SSX-5 genes in normal tissues is found to be limited to testis, and they are expressed at a low level in the tissue of thyroid, but expressed at a different frequency in many other kinds of tumors [9,10]. Other researchers suggested that there is a close relationship between the expression of the SSX-1 gene and the occurrence of metastasis, recurrence and prognosis of tumors [11]. There have been some reports about the expression of SSX-1 and SSX-5 genes in HCC [9]. Mean-while, up to our knowledge, none of these studies included any data about the Egyptian patients with HCC. Therefore, the aim of this work was to investigate the SSX-1 and SSX-5 mRNA expression in tumor cells circulating in the peripheral blood of a cohort of Egyptian patients with HCC. Also, the study aimed to find out any possible associations between these genes expression and different clinical/laboratory parameters.

2. Subjects and methods

2.1. Subjects

This study included 100 subjects collected during the time period from October 2012 to February 2013. They were divided into three groups; the first group included 52 patients who were recruited from the oncology outpatient clinic at the National Cancer Institute – Cairo University. Similar to previous studies these 52 patients were preliminary diagnosed as HCC according to noninvasive diagnostic criteria obtained by imaging modalities [12–14] and, whenever possible, were confirmed by the gold standard histopathological examinations. Patients with any cancer other than HCC, as well as those with septicemia, chronic inflammatory disorders or chronic heart disease were excluded from this study. The second group included 25 patients who were recruited from the National Liver Institute – Menoufiya University and were suffering from post HBV and/or HCV liver cirrhosis as confirmed by their clinical examinations, radiological findings and serological tests for hepatitis viral markers. The third group included 23 apparently healthy normal subjects who were considered as the control group. These subjects had normal liver function tests and were sero-negative for hepatitis B and C markers. All 100 subjects included in this study were proved to have healthy thyroid gland based on free clinical examination and normal routine thyroid function tests. Male patients and healthy male subjects, in addition, appeared clinically to have normal testicles. This study was approved by the local institutional review board (ethics committee) as this work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Also written informed consents were obtained from all participating subjects before they were enrolled into the study. The clinical data and laboratory results (like serum AFP levels, tests for Bilharziasis and PCR for viral hepatitis B and C) were all collected from the patient's medical files.

2.2. Methods

All patients and control groups were submitted to:-

2.2.1. Sampling and RNA extraction

Peripheral blood samples (10 mL in EDTA), were collected. Nucleated cells were isolated by the osmotic red blood cell lysis method and the resulting cell pellets were stored at -80°C until RNA extraction. Total RNA was extracted from the

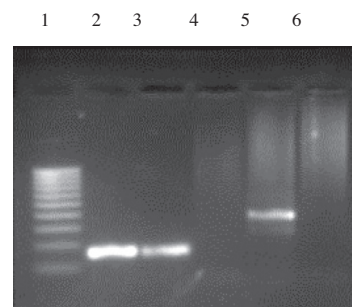
nucleated cells according to the manufacturer's instructions, using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, and USA). RNA concentration was determined by measuring the absorbance at 280 nm and stored at -80°C . RNA integrity was checked by gel electrophoresis and ethidium bromide staining.

2.2.2. Reverse transcription polymerase chain reaction (RT-PCR)

The primer sequence of the selected genes and β -actin (Promega, USA) are illustrated in Table 1. To avoid a false positive outcome resulting from contaminating genomic DNA, we amplified by primers selected the different exons as described by Lu et al. [9]. Two μg of RNA was subjected to reverse transcription with random primers using the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, Carlsbad, CL, USA) according to the manufacturer's instructions. The cDNA integrity was checked using β -actin amplification as a control gene. The PCR amplification reaction contained 10 μL of a 1:5 dilution of reverse-transcribed products, 2 μL each of 15 $\mu\text{mol/L}$ specific primers (sense and antisense), 2 μL of 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 2 μL each of 10 $\mu\text{mol/L}$ β -actin, 2.5 U Taq DNA polymerase and PCR-buffer solution. The total volume was brought to 50 μL using distilled water. For confirmation, the PCR amplifications were performed in duplicates for each sample; using a thermocycler (T-personal Biometra) and under the following conditions: For SSX-1: denaturation (94°C for 45 s), annealing (54°C for 45 s) and extension (72°C for 60 s). For SSX-5: denaturation (94°C for 45 s), annealing (52°C for 45 s), and extension (72°C for 45 s). Products were subjected to 35 cycles of amplification, followed by a final extension of 15 min at 72°C . The PCR products were resolved on Ethidium bromide-stained 2.0% Agarose gel and photographed under ultra-violet light illumination, (Figs. 1 and 2). The length of the PCR products of SSX-1 and SSX-5 were 422 and 314 base pairs (bps), respectively.

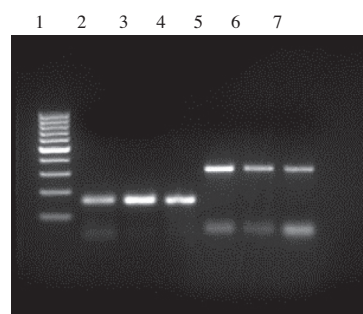
2.3. Statistical analysis

Data have been collected and entered into the computer using SPSS (Statistical Package for Social Science) program for statistical analysis (version 17; Inc., Chicago, IL). Chi-square test has been used to measure the association between qualitative variables. Fisher exact test has been used for 2×2 qualitative variables when more than 25% of the cells had an expected count less than 5. P -value was considered statistically significant when it is less than 0.05.



Lane 1: DNA molecular weight 100 base pairs (Promega, USA)
Lanes 2 & 3: β -actin positive (160 base pairs)
Lane 4: SSX-1 negative
Lane 5: SSX-1 positive (422 base pairs)
Lane 6: Negative control for SSX-1

Figure 1 PCR products of SSX-1 in HCC patients.



Lane 1: DNA molecular weight 100 base pairs (Promega, USA)
Lanes 2, 3 & 4: β -actin positive (160 base pairs)
Lanes 5, 6 & 7: SSX-5 positive (314 base pairs)

Figure 2 PCR products of SSX-5 in HCC patients.

3. Results

This study included three groups. The first group included 52 patients with HCC. They were 39 males and 13 females and their ages ranged from 35 to 90 years with a median of 59.5 years. The second group included 25 patients with liver cirrhosis. They were 20 males and 5 females and their ages ranged from 34 to 68 years with a mean of 54.7 ± 7.1 years. The third group included 23 healthy control subjects; 15 were males and 8 were females. Their ages ranged from 28 to 53 years with a mean of 51.65 ± 5.2 years. The characteristics of the HCC patient's group are demonstrated in Table 2.

Table 1 Primer sequence and product size for PCR amplification.

Gene		Primer sequence	Product size (base pair)
SSX-1	Sense	5'-CTAAAGCATCAGAGAAGAGAAGC-3'	422
	Antisense	5'-AGATCTCTTATTAATCTTCTCAGAAA-3'	
SSX-5	Sense	5'-GTT CTC AAA TAC CAC AGA AGA TG-3'	314
	Antisense	5'-CTC TGC TGG CTT CTC GGG CG-3'	
β -Actin	Sense	5'-CTT TGA TTG CAC ATT GTT GT-3'	160
	Antisense	5'-GAA AGC AAT GCT ATC ACC TC-3'	

The source of primer sequence of the selected genes and β -actin is Promega, USA.

Table 2 Description of characteristics of Hepatocellular carcinoma patients.

Studied variables	Total number = 52	Percent
<i>Age (years)</i>		
Median	59.5	
Range	35–90	
<i>Gender</i>		
Male	39	75.0
Female	13	25.0
<i>Tumor size:</i>		
≤5 cm	21	40.4
> 5 cm	31	59.6
<i>Stage</i>		
II	14	26.9
III	32	61.6
IV	6	11.5
<i>Tumor differentiation</i>		
Well differentiated	10	19.2
Moderately differentiated	34	65.4
Poorly differentiated	8	15.4
<i>Hepatitis B</i>		
Positive	5	9.6
Negative	47	90.4
<i>Hepatitis C</i>		
Positive	11	21.2
Negative	41	78.8
<i>SSX-1*</i>		
Positive	21	40.4
Negative	31	59.6
<i>SSX-5*</i>		
Positive	19	36.5
Negative	33	63.5
<i>Bilharziasis</i>		
Positive	11	21.2
Negative	41	78.8
<i>AFP</i>		
Mean ± SD	5557.8 ± 30520.0	
Range	2.6–218000.0	

* SSX = Synovial sarcoma X chromosome gene.

4.1. Expression of both SSX-1 and SSX-5 in peripheral blood

In our study neither SSX-1 nor SSX-5 mRNA could be detected in the peripheral blood of the 25 patients with liver cirrhosis or the 23 normal control subjects.

In the peripheral blood samples of the 52 HCC patients, 34/52 cases (65.4%) showed positive expressions of SSX-1 and/or SSX-5 (Table 3), positive rates were (40.4%) and (36.5%) for SSX-1 and SSX-5 expression, Figs. 1 and 2, respectively. SSX-1 and SSX-5 were co-expressed in 6 (11.54%) out of 52 cases of HCC (Table 3).

4.2. Peripheral blood expression of SSX-1 and SSX-5 in relation to clinical/laboratory parameters

In this study, no significant associations were found between SSX-1 and SSX-5 expression in peripheral blood, on one side, and most of the clinical/laboratory parameters studied, on the

other side. These parameters include age, gender, tumor staging, tumor size, degree of tumor differentiation, different AFP serum levels, hepatitis B viral infection and Bilharziasis (P -values were > 0.05), (Table 4).

Also, our results revealed that 9/14 stage II HCC cases (64.3%) were positive for at least one of the SSX genes expression (2 positive for SSX-1, 6 for SSX-5 and 1 for both), meanwhile, 20/32 stage III HCC cases (62.5%) were positive for at least one of the SSX genes expression (11 positive for SSX-1, 6 for SSX-5 and 3 for both) and 5/6 stage IV HCC cases (83.3%) were positive for at least one of the SSX genes expression (2 positive for SSX-1, 1 for SSX-5 and 2 for both).

Among the twenty-one HCC patients who had SSX-1 gene transcripts and the nineteen HCC patients who had SSX-5 gene transcripts detected in their peripheral blood, only 6 patients 28.6% and 31.6%, respectively, showed AFP levels > 400 ng/ml, in their serum (Table 4); consistent with the general standard levels of AFP required for diagnosis of HCC [15].

In the present study, 27/52 of our HCC cases (51.9%) had prior liver cirrhosis; among which 11 were positive for HCV, 5 were positive for HBV and 11 were positive for Bilharziasis. From the 11 positive HCV, 7 cases (63.6%) showed positive expression of one/or both SSX genes, while, all the 5 positive HBV cases (100%) showed positive expression of one/or both SSX genes. Also, out of the 11 cases with post Bilharzial cirrhosis 7 cases (63.6%) showed positivity of one/or both SSX genes.

Table 4 shows a significant association between SSX-1 mRNA expression, in peripheral blood of HCC cases, and hepatitis C infection (P -value = 0.02), while, it shows no association between SSX-5 mRNA expression and hepatitis C infection (P -value = 0.16).

5. Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. It occurs mainly in patients with chronic liver disease such as hepatitis B and C infection. These high-risk patients are closely followed up, and increasing numbers of small equivocal lesions are detected by imaging diagnosis. They are now widely recognized as a precursor or early stage of HCC and are classified as dysplastic nodules or early HCC. It is considered that early HCC is a key step in the process of HCC development and progression. However, the molecular mechanisms of early hepato-carcinogenesis are far from clear. Recent progress in comprehensive analysis of gene expression is shedding some light on this issue [16]. The carcinogenesis of HCC is a multifactorial, multistep and complex process. Its prognosis is poor and early diagnosis and monitoring metastasis of HCC are of the utmost importance. Circulating diagnostic and prognostic molecular markers could be used in proper postoperative treatment of patients at an early stage of HCC development [17].

Cancer testis antigens represent a category of tumor-associated antigens normally expressed in male germ cells but not in adult somatic tissues [18]. A number of cancer testis antigens have been found expressed with high percentage and specificity in HCC. The expression of SSX mRNA was investigated by Wu et al., in HCC tissues and corresponding peripheral blood of 37 patients with HCC, 15 samples of cirrhotic tissues and 15

Table 3 Association between the expressions of SSX-1 and SSX-5 among the studied group of Hepatocellular carcinoma patients.

Variable	SSX-5 negative patients (<i>n</i> = 33)		SSX-5 positive patients (<i>n</i> = 19)		<i>P</i> -value
	No.	%	No.	%	
SSX-1 negative patients (<i>n</i> = 31)	18	54.5	13	68.4	0.33
SSX-1 positive patients (<i>n</i> = 21)	15	45.5	6	31.6	

SSX = synovial sarcoma X chromosome gene, *n* = number.

Table 4 Associations of expression of SSX-1 gene and SSX-5 gene in peripheral blood of hepatocellular carcinoma patients and studied variables.

Studied variables	SSX-1 gene expression				<i>P</i> -value	SSX-5 gene expression				<i>P</i> -value
	Negative patients (<i>n</i> = 31)		Positive patients (<i>n</i> = 21)			Negative patients (<i>n</i> = 33)		Positive patients (<i>n</i> = 19)		
	No	%	No	%		No	%	No	%	
<i>Age</i>										
< 40	2	6.5	2	9.5	0.68	1	3.0	3	15.8	0.13
≥ 40	29	93.5	19	90.5		32	97.0	16	84.2	
<i>Gender</i>										
Male	22	71.0	6	28.6	0.42	25	75.8	14	73.7	0.87
Female	9	29.0	15	71.4		8	24.2	5	26.3	
<i>Tumor size</i>										
≤ 5 cm	15	48.4	6	28.6	0.15	13	39.4	8	42.1	0.85
> 5 cm	16	51.6	15	71.4		20	60.6	11	57.9	
<i>Stage</i>										
II	11	35.5	3	14.3	0.138	7	21.2	7	36.8	0.281
III	18	58.1	14	66.7		23	69.7	9	47.4	
IV	2	6.4	4	19.0		3	9.1	3	15.8	
<i>Tumor differentiation</i>										
Well differentiated	7	22.6	3	14.3	0.34	6	18.2	4	21.1	0.96
Moderately differentiated	21	67.7	13	61.9		22	66.7	12	63.2	
Poorly differentiated	3	9.7	5	23.8		5	15.2	3	15.8	
<i>AFP</i>										
≤ 20	9	29	3	14.3	0.45	9	27.3	3	15.8	0.62
> 20 ≤ 400	14	45.2	12	57.1		16	48.5	10	52.6	
> 400	8	25.8	6	28.6		8	24.2	6	31.6	
<i>Hepatitis B</i>										
Negative	30	96.8	17	81.0	0.14	31	93.9	16	84.2	0.34
Positive	1	3.2	4	19.0		2	6.1	3	15.8	
<i>Hepatitis C</i>										
Negative	21	67.7	20	95.2	0.02*	28	84.8	13	68.4	0.16
Positive	10	32.3	1	4.8		5	15.2	6	31.6	
<i>Bilharziasis</i>										
Negative	24	77.4	17	81.0	0.76	25	75.8	16	84.2	0.73
positive	7	22.6	4	19.0		8	24.2	3	15.8	

SSX = synovial sarcoma X chromosome gene, *n* = number.

* Significant association.

normal tissues with the same method. Two cancer testis antigens (SSX-2 and SSX-5) showed in this study high specific and high frequent expression only in HCC tissues. In corresponding peripheral blood of HCC patients, the positive expression rate of these two cancer testis antigens mRNA was not very high [19]. However, a later study on another two cancer testis antigens (SSX-1 and NY-ESO1) in the same group of patients and with the same methods showed that

SSX-1 can be potentially used as a molecular marker in peripheral blood, with short-term recurrence rate at 46% in patients whose peripheral blood expressed SSX-1 mRNA, while the recurrence rate in patients with negative SSX-1 mRNA was 28.6% [9].

In a different study, specific expression of cancer testis antigens was observed in AFP negative HCC, suggesting the application of their mRNA as tumor markers to detect circulating

HCC cells [20]. Also FATE/BJ-HCC-2 (another cancer testis antigen) mRNA expression was detected in the peripheral blood mononuclear cells (PBMCs) of 46.67% patients, whose HCC tissue samples were positive for FATE/BJ-HCC-2 mRNA, which implicated tumor cell dissemination in blood circulation and related to the metastasis of HCC [21]. These studies suggest that cancer testis antigen expressions can be used in peripheral blood to detect HCC circulating cells.

In our study, the expression of SSX-1 and SSX-5 genes in the peripheral blood, among a cohort of Egyptian HCC patients, was detected by RT-PCR and up to our Knowledge this is the first study in Egypt investigating the expression of both genes in the peripheral blood samples rather than the tumor tissues. Our study showed positive rates of expression of SSX-1 and SSX-5 mRNA in the peripheral blood of HCC patients, while, their expression was not detected in the peripheral blood of the cirrhotic patients' group or the healthy controls; therefore, indicating a considerable degree of their specificity for HCC tumor and may suggest the importance of detecting HCC circulating tumor cells (CTC) as recently highlighted by some other authors [18,22]. Detecting these transcripts in PB of early HCC patients can demonstrate hematogeneous dissemination of tumor cells more specifically than conventional methods, thus playing a supplementary role in the diagnosis of HCC [23].

Chinese studies with SSX-1 expression in the peripheral blood of HCC patients showed positive expression rates of 34.4% [24] and 38.9% [9]. These rates might be considered fairly close to ours (40.4%) because of the difference between the Chinese and Egyptian populations as well as that between their sample sizes and ours. Also, they revealed that no SXX-1 expression was detected in cirrhotic liver tissues, normal liver tissues or the peripheral blood of control patients [9].

As for the SXX-5 expression, our rate (36.5) was considerably higher than that (23.1%) revealed by others [25]; who also found that SXX-5 was not detected either in patients with hepatic cirrhosis or in normal controls.

This study revealed no significant associations between mRNA expression of SSX-1 and SSX-5 and many clinical/laboratory parameters in HCC patients; including age, gender, tumor staging, tumor size, extent of differentiation, serum α -fetoprotein (AFP) level and infection with hepatitis B virus (all *P*-values were >0.05). Therefore, our results were confirming some of the previously published studies [9,24,25].

In this study, the statistically significant association encountered between SSX-1 gene expression and hepatitis C infection was indirect or negative; suggesting a possibility of specific inhibitory effect of the HCV on the SSX-1 gene expression among the HCC cases. This effect might be related to certain characteristics specific for the HCV species prevalent in Egypt; however we could not explain this possibility in this study. Therefore, extensive future studies are highly recommended to verify, explain or prove this suggestion.

In our study 51.9% of the HCC cases had prior liver cirrhosis and positivity of one/or both SSX genes was detected in 63.6% of the hepatitis C positive cases, 100% of the hepatitis B positive cases and 63.6% of cases with post Bilharzial cirrhosis. These results may indicate that this group of molecular markers seems promising as early detectors of HCC conversion in cirrhotic patients but further studies have to be done.

Our results revealed no significant association between the SSX genes expression in the peripheral blood and the HCC

tumor stages, yet, 64.3%, 62.5% and 83.3% of cases with stages II–IV, respectively, were positive for at least one of the SSX genes expression. The latter two rates of SSX genes expression might have been expected to be high among the late (more advanced) stages (III and IV), but, the former high rate of positive expression among the early stage (II) was striking, indicating that CTC probably appear early in circulation and it is safe to assume that they can be detected as early as stage I. However none of our cases was stage I so further study of this stage is required. Also, it definitely suggests that SSX-1 and/or SSX-5 expressions in the peripheral blood of HCC patients have the potential to be used as early indicators for the tumor development. Furthermore, up to 83.3% of stage IV cases were positive for one or both genes confirming its reliability in detection of CTCs. In our study, Lack of statistical significance in relation between peripheral blood SSX genes expression and HCC stages can be attributed to the small number of patients in each stage.

The SSX proteins are a highly homologous group of CT-X antigens with demonstrated immunogenicity in patients with cancer, and a number of characteristics that make them attractive targets for tumor therapy. SSX2 in particular may be a high priority target for cancer therapy based upon certain pre-defined criteria for prioritization of tumor antigens, such as tumor specificity, oncogenicity, expression level, and number of identified epitopes [26,27].

6. Conclusion

ZThe SSX-1 and SSX-5 mRNA are specifically expressed in tumor cells circulating in the peripheral blood of patients with HCC and could be used as easy access, simple method molecular markers for early diagnosis of HCC patients in Egypt.

Application of SSX genes as targets for specific immunotherapy could be a highly interesting point in future studies on HCC patients in Egypt.

Conflict of interest

The authors declare no conflict of interest. There is no financial or personal relationship with other people or organizations that could inappropriately influence their work.

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