Expression of p27kip and XIAP in patients with Hepatocellular carcinoma

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Abstract: Introduction: The increasing incidence of hepatocellular carcinoma (HCC) being a major health problem. Functional alterations of cell cycle regulators (e.g. p27kip) can be observed in HCC as it considered being potent tumor suppressors. Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis. X-linked of apoptotic inhibitors (XIAP) is a regulator of apoptosis, cytokinesis and signal transduction. The aim was to evaluate the expression of p27kip and XIAP in HCC and their clinico-pathological significance. Subjects & Methods: The study was carried on forty patients with newly diagnosed HCC and 10 controls matched for age. Liver function tests, Serum alpha-feto protein, serologic markers for viral hepatitis, abdominal ultrasonography, triphasic computer tomography abdomen, liver biopsy and real-time PCR expression of p27kip and XIAP were done for all cases of the study. Result: There was a significant change in expression of p27kip or XIAP in HCC patients either by decrease (p27kip) or increase (XIAP) as compared to the controls ($p<0.05$). The decreased expression of p27kip or increased XIAP expression were associated with decrease of overall survival, increased incidence of recurrence and associated with more unfavorable prognosis. Conclusion: p27Overexpression may expect good prognosis while overexpression of XIAP suggests poor prognosis for HCC patients and they can be used as an independent prognostic factors for predicting disease-free and overall survival rates of these patients.

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1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem, represents the fifth most common cancer in men and the seventh in women. Most of the burden of disease (85%) is born in developing countries. HCC is associated with a poor prognosis being the third leading cause of oncology-associated death [(1)] making this disease a major challenge that is highly resistant to conventional chemotherapy and radiation [(2)].

HCC pathogenesis has been related to exposure to environment toxins, alcohol and drug abuse, autoimmune disorders, genetic factors, elevated hepatic iron levels, obesity, and infections with hepatotropic viruses especially hepatitis B (HBV) & hepatitis C (HCV) viruses as it has been estimated that chronic infections with HBV and HCV account for up to 80% of HCC [(3)]. Many of liver insults, chronic inflammation, liver regeneration, fibrosis, and cirrhosis are thought to contribute to the development of HCC in these patients [(4)].

Cell division is controlled by a highly conserved group of proteins, the changes in their expression or activity levels are usually observed in human cancer cells and in genetic alterations which lead to cancer formation [(5)].The cyclin kinase inhibitor p27kip1 acts as a tumor suppressor in a variety of human cancers [(6)]. P27kip1 function as an inhibitor of cyclin E or cdk2 (cyclin dependent kinase 2) complexes activities leading to regulating cell progression from a quiescent state into the G1 phase and from the G1 phase into S-phase [(7)]. Several studies have demonstrated that decreased expression levels of p27 in primary cancer tissue correlates with reduced overall and progression free survivalas well as poor response to chemotherapies or targeted treatments. The inverse relations were observed in tumors of breast, prostate, bladder, lung, liver, larynx, ovary, and stomach [(8-10)].

Apoptosis is a physiological mechanism to remove excess cells during liver development and regeneration [(11)]. However, insufficient apoptosis has been involved in development and progression of liver tumors [(12-13)]. Several studies have approved that the increased expression of anti-apoptotic factors like the inhibitors of apoptosis proteins (IAPs) in a variety of solid tumors and cancer cell lines [(14)].

The inhibitor of apoptosis proteins (IAP) include a large family of endogenous caspases inhibitors, which include X-linked IAP (XIAP), c-IAP1, c-IAP2 and Survivin [(15)]. XIAP is the most important and efficient caspases inhibitor as it inhibit caspase initiator activation, caspase-3,-7 and -9 via its interaction through the BIR3 and BIR2 domains [(16)]. XIAP could

inhibit efficiently extrinsic death receptor and the intrinsic mitochondria pathways to generate the effector caspases 2p3. Increased XIAP has been reported in a several human tumors including esophageal carcinoma [17], clear cell renal carcinoma [18], ovarian carcinoma [19], lymphoma [20] and HCC [21]. In some cases, this expression was found to be associated with survival reduction [18].

The aim of this study is to evaluate p27kip and XIAP genes expression in patient with HCC and their clinical importance.

2. Subjects and Methods:

Patients

The study was carried out on forty patients suffering from HCC diagnosed from inpatients of Internal Medicine, Tropical and Oncology departments, Tanta University Hospital. They were 31 males and 9 females. Their ages ranged from 40-62 years.

Methods

All subjects in this study underwent the following

1. Complete history taking including disease duration.
2. Full clinical examination.
3. Doppler abdominal Ultrasonography.
4. Ultrasound and\or CT-guided liver biopsies .
5. Triphasic computer tomography (CT) abdomen.
6. Chest X-ray.
7. Bone scan if there is bone pain or elevated alkaline phosphatase.

Laboratory Investigation

- Complete blood count.
- Fasting and post prandial blood sugar
- Kidney function tests
- Liver function tests were done, including serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and prothrombin time and activity.
- Hepatitis B surface antigen was assayed using enzyme immunoassay kits {SURASE B-96 (TMB) from General Biologicals Corporation, Innovation First Road, Science-Park, Hsin Chu,Taiwan,R.O.C.}
- Circulating anti-HCV antibodies were detected using the Murex enzyme immunoassay kit (Innotest® HCV Ab. From Innogenetics N.V. Belgium) (18).
- Serum alpha-feto protein by immunoassay method.
- Routinely Hematoxylin-Eosin-stained sections of HCCs and surrounding tissue were reviewed by the same histopathologists.
- The tumors were staged according to according TNM staging system [22].

None of the patients received chemotherapy or radiation before enrollment in the study or suffered from any other malignancies.

The Different treatment modalities of the patients with HCC are shown below (table 4).

The follow-up period ranged from 1 to 30 months.

Normal control tissues of the liver were taken from ten HCV patients who underwent liver biopsy before start HCV treatment and have Ishak fibrosis stages 0–1(F=0-1) [23]

Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) version I7 (SPSS Inc., Chicago, IL, USA) and the Graph Pad Prism software (GraphPad Prism Software Inc. San Diego, California, USA).Descriptive statistics were done. Categorical data were presented as number and percent. Statistical significance was defined as a P value of <0.05.Chi-square test was used to compare between the significant differences and match between the studied groups and some other qualitative variables. Comparison of continuous data between two groups was made by using Mann-Whitney test. Overall survival was measured from the date of random assignment until death. Patients who had not died or who were lost to follow-up were censored for overall survival when they were last known to be alive. The survival charts were depicted on Kaplan Meier plots. Analysis of the disease prognostic factors was done using the log rank test. To investigate whether expression levels of each gene and protein were associated to patient survival, HCC samples were categorized in high or low expressor groups if the target level wasabove or below the median expression value, respectively.

Expression of XIAP and p27kip mRNA was assessed by real-time PCR in liver tumor specimens.

Isolation of total RNA from liver tissues specimen using **PAXgene** Tissue RNA Kit (Qiagen, Germany). Purification of total RNA from tissues fixed and stabilized in PAXgene Tissue Containers according to the manufacturer's instructions and quantified by spectrophotometry (Pharmacia Biotech) at 260 nm. Total RNA was stored at -80°C until molecular investigation was performed.

Synthesis of cDNA from total RNA samples is the first step in using TaqMan Gene Expression Assays. The process involves the following procedures:1. Preparing the RT master mix. 2. Preparing the cDNA archive reaction plate.3. Performing reverse transcription.

Preparing RT Master Mix:

To prepare RT master mix:

The Total volume 50 µL: 10 µL Reverse Transcription Buffer, 4µL dNTP's, 10µL random primer,5 µL Multiscribe Reverse Transcriptase ,50 U/µL and 21µL Nuclease – free H_2O. Mix 50µL of the RT Mix with 50µL of isolated RNA (High-Capacity cDNA Archive Kit; Applied Biosystem, Foster City, CA, USA) under standard conditions (Table 2).

Preparing the PCR Master Mix for Each sample:

The cDNA of tumor tissue was used as a template to amplify the studied genes and cDNA of normal tissue was used to amplify GAPDH using the primers listed in Table 1. The assay identification numbers of target and housekeeping genes are as follow: p27kip gene Hs00153277_m1,XIAP gene Hs00236913_m1, and cDNA of normal tissue was used to amplify GAPDH gene Hs99999905_m1using the primers listed in Table 1. The reaction mixture was performed in a final volume of 25 µL: containing 5 µL cDNA, 1.25 µL Forward primer, Reverse primer, TaqMan Probe (FAM Dye) Target Gene (p27kip,XIAP genes), 1.25 µL Forward primer, Reverse primer, TaqMan Probe of reference Gene (GAPDH) , 12.5 µL TaqMan® Universal PCR Master Mix with UNG Supplied AS a 2 x concentration (Applied Biosystems,USA)which consist of: AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference ROX dye was used for the PCR analysis and optimized buffer, and 5 µL nuclease-free water.

Table 1: Nucleotide sequence of the primers and probes used in the study were generated by using Primer 3 software (http://fokker.wi.mit.edu/cgi-bin/primer3/ primer3_www_slow.cgi)

Oligonucleotides	Nucleotide Sequence
P27kip-forward	5`- AGCACACGCATTTGGTGGA-3`
P27-reversed	5`- TAGAAGAATCGTCGGTTGCAGGT-3`
P27kip-TaqMan probe	FAM- 5`-AAAGACTGATCCGCGGACAGCCAGA-3`TAMRA
	5`-AGTGGTAGTCCTGTTTCAGCA-TCA-3`
XIAP- forward	5`- CCGCACGGTATCTCCT-TCA-3`
XIAP-reversed	FAM 5`- CACTGGCACGAGCAGGGTTTCTTTATACTG-3`TAMRA
XIAP- TaqMan probe	
	5`- GAAGGTGAAGGTCGGAGT-3`
	5`- GAAGATGGTGATGGGATTTC-3`
	FAM 5`-CAAGCTTCCCGTTCTCAGCC-3`TAMRA
GAPDH-forward	
GAPDH-reversed	
GAPDH- TaqMan probe	

Table 2: Times and Temperatures (two-step RT-PCR)

Times and Temperatures (Two-steps RT-PCR)				
1.RT step	Hold	Hold	Hold	
	10 min@25°C	120 min@37°C	5 sec@85°C	
	Initial Steps		PCR(Each of 40 cycles)	
2.PCR Step	AmpErase® UNG Activation	AmpliTaq Gold® DNA polymerase,	Melt	Anneal/Extend
	Hold	Hold	°Cycle	
	2 min@50°C	10 min@95°C	15sec@95°C	1 min@60°C

By using real time PCR Step One instrument and software (Applied Biosystems, Foster City, CA). Calculation of the results were done by application of comparative CT method for relative quantitation , $2^{-\Delta\Delta CT}$ $\Delta CT_{unknown} = CT_{target} - CT_{reference}$

The difference in threshold cycles for target and reference gene.

$\Delta\Delta CT = \Delta CT\ CT_{unknown} - \Delta CT_{Calibrator}$.

(RQ) Relative Quantitation = $2^{-\Delta\Delta CT}$

The Fold Change (FC) was calculated, defined as the ratio between averaged normalized expression level of targets in neoplastic and corresponding non-neoplastic samples. Normalized RQ were log 2-transformed for statistical analysis.

There is a significant increase in expression of both p27 kip and XIAP genes in HCC patients as compared to the control (p =0.02, 0.04 respectively) as fold change of p27expression in the patients ranged from 0.02 to 5.0 while in the control ranged from0.01 to 0.9. Fold change of XIAP expression in the patients ranged from 0.32 to 5.99 while in the control ranged from 0.21 to 1.3.

(A)

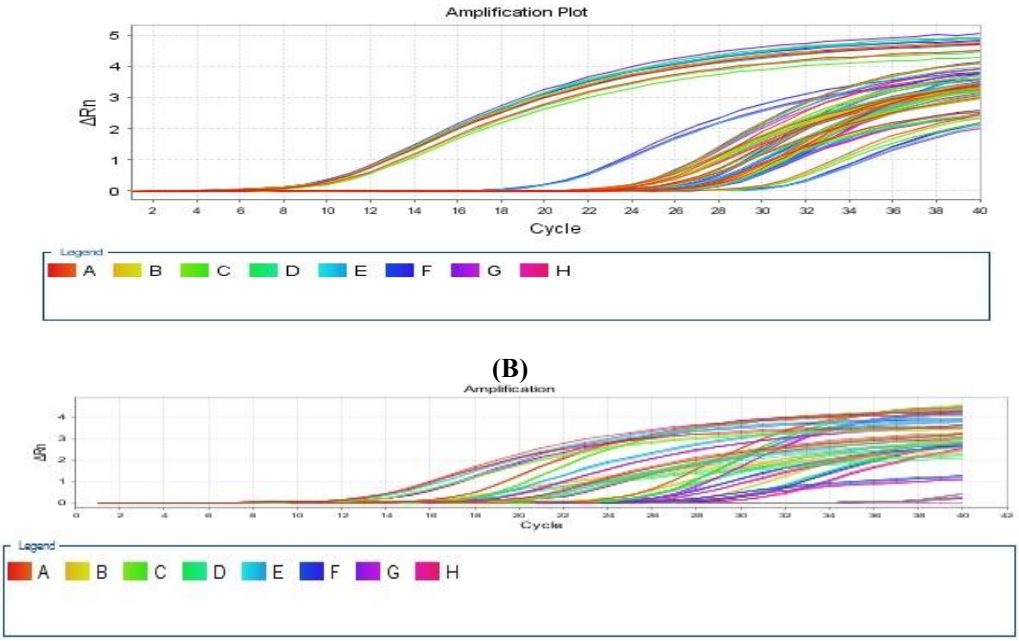


Figure 1. Amplification plots of p27kip and XIAP using real time PCR. A represents the amplification plot of p27kip expression and B represents that of XIAP. 3. Results

p27Kip expression was significantly increased with cirrhosis (p=0.02), portal venous invasion (p=0.02), histologic grade (p=0.00), TNM staging(p=0.00), tumour recurrence (p = 0.00), and metastasis (p = 0.00),whereas no significant change was found between p27Kip1 expression and other clinicopathologic variables. Table3. On the other hand, XIAP expression was significantly increased with portal venous invasion (p=0.02), TNM staging(p=0.00), tumour recurrence (p = 0.00), and metastasis (p = 0.00), whereas no significant change was found between XIAP expression and other clinicopathologic variables. Table 3.

Correlation of the expression of p27kip and XIAP with the patients' survival

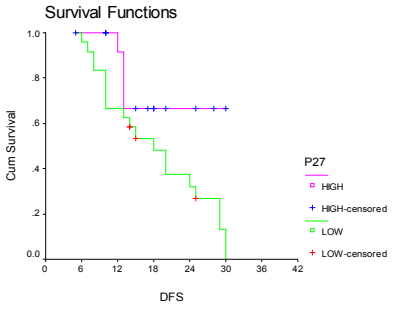
By using the Kaplan-Meier analysis, the patients with statistical analysis also indicated that both the disease free survival and overall survival rate of the p27kip overexpression group were significantly higher than that of the low expression group (P<0.05) (Fig. 1A,B).While their data of the XIAP overexpression group were significantly lower than that of the low expression group (P<0.05) (Fig. 1C,D).

Discussion

The prognosis of HCC has been significantly improved in the past few years due to earlier diagnosis and more effective treatments. However, tumor recurrence and metastasis are still the major problems for long term survival [(24-25)].

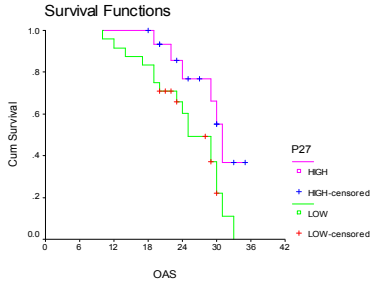
There is a wide agreement that liver cirrhosis caused by HBV or HCV infection is an important risk factor for the development of HCC as they develop a microenvironment of chronic liver injury, inflammation, and regeneration [(1,26)].

Although surgical resection and liver transplantation are the only treatment modalities that enable prolonged survival in patients with hepatocellular carcinoma (HCC), the majority of HCC patients presents with advanced disease and do not undergo resective or ablative therapy. Transarterial chemoembolization (TACE) is indicated in intermediate/advanced stage unresectable HCC even in the setting of portal vein involvement (excluding main portal vein). Sorafenib has been shown to improve survival of patients with advanced HCC in two controlled randomized trials. Yttrium 90 is a safe microembolization treatment that can be used as an alternative to TACE in patients with advanced liver disease or in case of portal vein thrombosis. External beam radiation can be helpful to provide local control in selected unresectable HCC. These different treatment modalities may be combined in the treatment strategy of HCC and also used as a bridge to resection or liver transplantation. Patients should undergo formal multidisciplinary evaluation prior to initiating any such treatment in order to individualize the best available options [(27)].



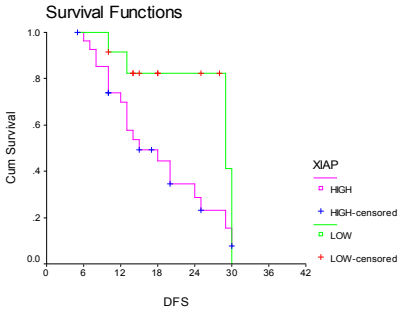
A

P=0.03



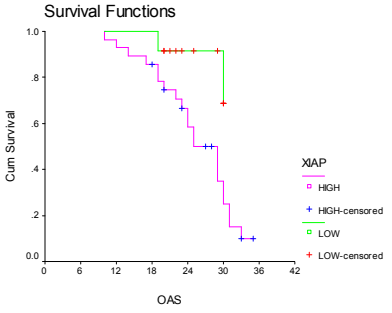
B

P=0.02



C

P=0.04



D

P=0.02

Fig 2. Kapalan-Meier survival curves of patients with HCC. (A,B) Disease and Overall survival rate are significantly higher in patients with p27kip overexpression. (C,D) Disease and Overall survival rate are significantly lower in patients with XIAP overexpression.

Deregulated apoptosis has a major role in pathogenesis of cancer, abnormality of cell differentiation, and chemotherapy resistance.

Caspase inactivation is contributed to enhanced survival and reduced apoptosis in tumor cells. X-linked inhibitor of apoptosis proteins (XIAP) is a principal inhibitor of apoptosis through its ability to inhibit caspase-3 and caspase-7 [(28)].

In this study, there was an increase in XIAP expression in patients with HCC and this was significantly associated with TNM stage, recurrence and metastasis. While, there was no relation with gender, age, presence of cirrhosis or histological grade. There was a decrease in overall and disease free survival in patients with high expression of XIAP.

These results in agreement with the results of Shiraki et al., [(29)]who found that XIAP is mainly expressed in all HCC cell lines and in approximately 70% of HCC tissue, whereas little or no expression is seen in chronic hepatitis or cirrhotic tissue and XIAP expression was inversely correlated with apoptosis.

And with Shi et al.,[(30)] who found that the level of XIAP expression in the three established HCC lines correlated with their metastatic capability by providing survival advantage to the metastatic cells via its anti-apoptosis effect. They suggested that XIAP could be an important factor in determining clinical outcomes of HCC patients. As majority of HCC samples were found to be highly positive for XIAP. In contrast, less than half of the adjacent liver tissues expressed XIAP, suggesting that high XIAP expression was characteristic of the HCC cells. They also noticed that increased XIAP expression had significantly shorter disease-free survival indicating that XIAP expression could provide additional prognostic factor. They also noticed that suppression of XIAP could be beneficial in cancer therapy and anti-sense oligonucleotides against XIAP have been developed and found to possess therapeutic effects in both in vivo and vitro studies.

Additionally, Notarbartolo et al.,[(31)] observed that XIAP high expression in HCC samples was associated with a more unfavorable prognosis and inhibitors of apoptotic proteins might play in the adverse biology of hepatocellular carcinoma. Augello et al.,[(32)] also found that XIAP high expression correlated with HCC recurrence and shorter overall survival while there was no association with other clinicopathological parameters.

Cyclin/cyclin-dependent kinase (CDK) complexes are integrators of multiple signals from both the extracellular and intracellular environment for efficient regulation of cell cycle progression [(33)].

Table 3. Expression of p27Kip1& XIAP genes and clinicopathological parameters in HCC patients

	No of patients	P27kip Low expression	P27 high expression	X^2	XIAP Low expression	XIAP high expression	X^2
Gender							
Males	31	19	12	0.75	11	20	0.16
Females	9	5	4		1	8	
Age							
<45years	13	8	5	0.89	3	10	0.50
≥45years	27	16	11		9	18	
Viral status							
HB	7	3	4	0.49	3	4	0.06
HC	29	19	10		6	23	
HB&HC	4	2	2		3	1	
Cirrhosis							
Presence	32	22	10	**0.02***	11	21	0.22
Absence	8	2	6		1	7	
No. of liver Tumors							
1-5	31	18	13	0.66	12	19	0.19
>5	9	4	5		3	6	
Tumor size							
<5 cm	26	14	12	0.27	7	19	0.56
≥5 cm	14	10	4		5	9	
Bilobar disease							
Yes	17	8	9		10	7	
No	23	10	13	0.33	12	11	0.44
Portal vein invasion							
Yes							
No	19	15	4	**0.02***	9	10	**0.023***
	21	9	12		3	18	
Histological grade							
Well							
Moderate							
Poor	8	4	4	**0.00***	2	6	0.66
	19	16	3		7	12	
Serum AFP (ng/ml)	13	4	9		3	10	
<100							
≥ 100							
TNM staging	24	15	9	0.69	5	19	0.12
I-II	16	9	7		7	9	
III-IV							
Recurrence							
Yes	29	21	8	**0.00***	6	23	**0.03***
No	11	3	8		6	5	
Metastasis							
Presence	24	10	14	**0.00***	10	14	
Absence	16	14	2		2	14	**0.04***
	6	4	9	**0.00***	7	6	
	34	20	7		5	22	**0.02***

Table 4. Different treatment modalities of forty patients with HCC

Radiofrequency ablation (RF)	**11**
Transarterial chemoembolization (TACE)	**16**
Cheomtheraputic &targted agents	**4**
Supportive therapy	**9**

There is an obvious relation of CDKs inhibitors and disease pathogenesis. p27kip1 is a CDK inhibitor that controls CDK activity throughout the cell cycle. As a CDK inhibitor, p27KIP1 has tumor suppressor activity [34].

In this study, there was a decrease in p27kip1 expression in patients with HCC and this was

significantly associated with presence of cirrhosis, histological grade, TNM stage, metastasis and recurrence. While, there was no relation with gender or age. There was a decrease in overall and disease free survival in patients with low expression of p27kip1.

These results with the results of Huang et al., [35] who reported that p27Kip1 expression correlated significantly with histological grade, venous invasion and cirrhosis, whereas no significant correlation was found between p27Kip1 expression and gender or age. The low expression of p27Kip1 was associated with short survival rate.

Chen et al.,[36] also found in their study on patients with HCC that the low expression of p27 Kip1 was inversely significantly related to cancer differentiation and tumor metastasis. There was no significantly difference between low expression and over expression in terms of age and gender. The survival rate of the p27 overexpression group was significantly higher than that of the low expression group. Multivariate analysis using the Cox's proportional hazards model showed that p27 Kip1 protein are independent prognostic indicators for patients' overall survival. They explained that as p27 Kip1 regulate cell proliferation as CDK inhibitor by inhibiting cell cycle progression from G1 to S phase in a dose dependent fashion.

El Bassiouny et al., [37] noticed in their study that the expression of p27Kip1 significantly decreased in HCC cases. Matsuda et al., [38] found that p27 is abundantly expressed in quiescent cells and is downregulated in many aggressive cancers.

It has been found [39], that p27 is frequently inactivated in HCC, and is considered to be a potent tumor suppressor as it is a negative regulator of G1-S phase transition through inhibition of the kinase activities of Cdk2/Cyclin E. in addition to other studies that reported the decreased expression of p27Kip1 is related to tumor progression and could be used as a potential predictor for HCC [40-41].

In conclusion, the decreased p27kip and increased expression of XIAP in HCC is involved in cancer cell behavior including proliferation, differentiation, and metastasis. Abnormal expression of them either separate or in conjunction may expect prognosis for patients with HCC. Moreover, effective therapy directed against them can be used in treatment of HCC, so, further studies about their used as a tool of treatment should be evaluated on wide scale of patients.

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