EXPRESSION OF SOMATOSTATIN RECEPTOR TYPE II AS DIAGNOSTIC AND PROGNOSTIC BIO-MARKER OF HEPATOCELLULAR CARCINOMA

Abdel-hamid NM¹, Atef Abd El-Baky*², Mohamed O¹, and Thabet KM¹.

¹Biochemistry Department, Faculty of Pharmacy, Minia University, Minia, Egypt.
²Biochemistry Department, Faculty of Pharmacy, Port Said University, Port Said, Egypt.

ABSTRACT
Hepatocellular carcinoma is a sequel of chronic liver disease and shows high and increasing prevalence worldwide. In most cases it is associated with the presence of liver cirrhosis and has a poor prognosis. Estimation the expression of somatostatin receptor type II and caspase as diagnostic and prognostic biomarkers in different stages of hepatocellular carcinoma are the aim of this study. METHODS: Thirty two albino mice (20–25 g) were randomly divided into four groups (n=8), the first three groups used for induction of hepatocellular carcinoma, where all mice in the three groups received sub necrotic single dose of DENA 90mg/kg body weight in 0.9% normal saline I.P. The first three groups were sacrificed after 8, 16 and 24 weeks. Fourth group (control group), served as normal healthy group received saline vehicle and was sacrificed after 24 weeks. RESULTS: The mRNA expression of SSTR2 was proportional to each stage of HCC development. The levels of SSTR2 mRNA at 8, 16 and 24 months were 2.71, 4.1, 5.32 fold of that of the control respectively. CONCLUSIONS: Expression of SSTR-II and caspase along with α-fetoprotein (AFP) expression can be used as a useful parameter for diagnosis and to evaluate the prognosis of HCC.

KEYWORDS: Somatostatin, Caspase, Hepatocellular carcinoma, Liver cirrhosis, α-fetoprotein.
INTRODUCTION
Hepatocellular carcinoma is majority of primary liver cancers (Mohammad Hossein Somi., 2005). The growth of HCC is silent in nature, which may delay diagnosis for long period from the time of development (Tanaka et al., 2002).

Hepatocarcinogenesis is a multi-step process involving different genetic alterations leading to malignant transformation of the hepatocyte. While significant progress has been made in recognizing the pathogenesis of other cancers, notably colorectal and certain hematopoietic malignancies, the molecular contribution of the multiple factors in hepatocarcinogenesis are still poorly understood (Ohata et al., 2003).

AFP is the most established tumour marker in HCC and the gold standard by which other markers for the disease are judged. Under physiological conditions, AFP is a fetal-specific glycoprotein with a molecular weight of around 70 kDa. It is synthesized primarily by the embryonic liver, by cells of the vitelline sac and by the fetal intestinal tract in the first trimester of pregnancy. The serum concentration of AFP declines rapidly after birth and its expression is repressed in adults. Pathologically, patients with chronic liver disease, particularly those associated with a high degree of hepatocyte regeneration, can express AFP in the absence of cancer. Also, AFP is elevated in hepatocarcinogenesis, embryonic carcinomas (Grizzi et al., 2007) and in gastric (Chen et al., 2003) and lung cancer (Hiroshima et al., 2002). AFP has been used as a serum marker for HCC for many years. It was first described by Abelev et al, (Abelev et al., 1963). Some patients with cirrhosis and/or hepatic inflammation can have an elevated AFP, even without the presence of a tumor (França et al., 2004).

There are three different AFP variants, differing in their sugar chains (AFP-L1, AFP-L2, AFP-L3). AFP-L1 is the main glycoform of AFP in the serum of patients with non-malignant chronic liver disease. In contrast, Lens culinaris-reactive AFP, also known as AFP-L3, is the main glycoform of AFP in the serum of HCC patients (Zhou et al., 2006). Elevated levels of AFP-L3 were associated with a shorter tumor doubling time in comparison with those with low levels of AFP-L3. Moreover, AFP-L3 acts as a marker for clearance of HCC after treatment and as a predictor of recurrence as failure to decline to the normal level indicates residual disease. Recurrence of HCC is expected when AFP-L3 levels increased (Yuen et al., 2003). Thus, this biomarker may be able to predict advanced tumor stage and a worse prognosis. (Wright et al., 2007).
Somatostatin {also known as growth hormone inhibiting hormone (GHIH) or somatotropin release-inhibiting factor (SRIF)} is a peptide hormone peptide that regulates the endocrine system and affects neurotransmission and cell proliferation via interaction with G-protein-coupled somatostatin receptors and inhibition of the release of numerous secondary hormones. The neuropeptide somatostatin is widely distributed in the central and peripheral nervous system and it play a very important role in the endocrine, autocrine and paracrine functions in living organisms (Ferjoux et al., 2000). Originally characterized as a hypothalamic regulator of growth hormone (GH) secretion, somatostatin also modulates the secretion of multiple pituitary, pancreatic and gastrointestinal hormones like thyroid stimulating hormone, insulin and glucagon. Somatostatin inhibits intestinal motility, absorption of nutrients and ions, vascular contractility and cell proliferation (Lewin, 1992). It also functions as a neurotransmitter, modulating locomotor activity and cognitive functions (Epelbaum et al., 1994). In recent years, attention has focused on its role in the progression and control of neoplastic disease as well as its function in certain areas of the central nervous system like the cortex, hippocampus and striatum (Ferjoux et al., 2000). Disorders in somatostatin metabolism have been proposed to contribute to pathogenesis of Alzheimer’s disease, epilepsy, Parkinson’s syndrome and gastrointestinal motility disorders. On a more basic level, studies on somatostatin metabolism have unified diverse concepts in intracellular signal transduction and eukaryotic gene expression (Ferjoux et al., 2000). There are predominantly two biologically active forms of somatostatin: somatostatin-14 and somatostatin-28. In mammals, these 2 products are generated by endoproteolytic processing of the prohormone prosomatostatin, which is in turn, generated from a 116-amino acid precursor called preprosomatostatin (Barnett, 2003). Downregulation of SSTR transcription may result in loss of a tumor suppressive (Li et al., 2012).

Somatostatin acts on its multiple cell targets via a family of six receptors that originate from five genes: SSTR1, SSTR2a, SSTR2b, SSTR3, SSTR4 and SSTR5. SSTR2 is alternatively spliced at its C-terminus producing the SSTR2a and the SSTR2b variants that have a somewhat different tissue distribution. The different SSTRs are expressed throughout the central nervous system (CNS) as well as in peripheral tissues like pancreas, stomach, small intestine, etc. Several hormones such as estrogen and thyroid hormone can regulate SSTRs expression in several tissues at the transcriptional level (Patel 1999). Somatostatin analog improves survival in patients with advanced hepatocellular carcinoma by interacting with its specific receptor 2 and 5 (Li et al., 2012).
Besides their expression in normal tissues, SSTRs have been identified in tumor cell lines of different etiology including pituitary, pancreatic, breast and hematopoietic. Moreover, the majority of human tumors do express SSTRs, often more than one receptor subtype. In general, SSTR2 is the most common SSTR subtype found in human tumors (Reubi 2003). The different signaling pathways activated by the various SSTR subtypes vary according to the receptor subtype and tissue localization. However, all SSTR subtypes inhibit adenylate cyclase and cAMP production upon ligand binding (Olias et al., 2004).

Somatostatin and analogs can promote apoptosis in normal and tumor cells (Bousquet et al., 2006). Somatostatin has been reported to control the growth of fibroblastic-like cells in both physiological and pathological conditions (Guillermet-Guibert et al., 2007). Somatostatin may have no direct effect on apoptosis but it may indirectly act through TNF induced apoptosis (Georgiadou et al, 2011).

The major risk factor for the development of HCC is cirrhosis of the liver. However, the major known risk factors for HCC are viral (chronic hepatitis B and hepatitis C), toxic (alcohol and aflatoxins), metabolic (diabetes and non-alcoholic fatty liver disease, hereditary haemochromatosis) and immune-related, primary biliary cirrhosis and autoimmune hepatitis (Parikh and Hyman, 2007).

Diehylnitrosamine (DENA) is a well known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics and pharmaceutical products (Brown 1999). DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models (Bhosale et al., 2002).

The present study aims mainly to estimate SSTRII and caspase expression as diagnostic and prognostic bio-marker in different stages of hepatocellular carcinoma, along with AFP expression and histological examination of liver sections in experimental albino mice with HCC.

MATERIALS AND METHODS
2.1. Material
Thirty two albino mice (20–25 g) were selected for the present study. They were obtained from the National Research Centre Cairo, Egypt. They were kept under constant experimental
conditions with free access to food and water. They were left for one week for accommodation before starting the study. They were monitored for body weight once a week and the doses were adjusted weekly according to body weight. They were fed with basic diet and allowed to free access of tap water and kept under constant environmental conditions at room temperature. During the period of experiment; animals were kept at 12 h light/12 h dark cycle.

Animals were randomly divided into four groups (n=8), the first three groups used for induction of hepatocellular carcinoma, where all mice in the three groups received sub necrotic single dose of DENA 90mg/kg body weight in 0.9% normal saline I.P. (Amarjit et al., 2007). The first group was sacrificed after 8 weeks. The second group was sacrificed after 16 weeks and the third group was sacrificed after 24 weeks. Fourth group (control group), served as normal healthy group received saline vehicle and was sacrificed after 24 weeks.

2.2. Sample Preparation
At the end of time point for each group mice were anaesthetized with diethyl ether and sacrificed. The livers were excised rapidly and divided into three parts. The first part was subjected for RNA preparation, the second part was homogenized in 0.25M sucrose solution and stored at -80°C and the last part of liver was fixed in 10% neutral buffered formalin for histopathological examination.

2.3. Preparation of liver homogenate
Briefly the liver tissues were homogenized in hexane/ propanol (3:2 v/v) and centrifuged. The extract was collected and washed with aqueous sodium sulfate. Supernatant was evaporated and the precipitate was weighed, dissolved in 10 ml hexane and stored at -20°C prior to analysis (Hara and Radin 1978).

Reduced glutathione (GSH) and malondialdehyde (MDA) were determined in liver homogenate. GSH was determined according to Ellman (Ellman 1959) and MDA was determined according to Uchiyama and Mihara (Uchiyama and Mihara 1978).

2.3. RNA extraction: using total RNA kit (Omega bio-tek).
30 mg of liver tissue was homogenized in 400µl of TRK lysis buffer (20µl of 2- mercaptoethanol per 1 ml of TRK lysis buffer was added directly before use). Homogenization was done using variable speed homogenizer (Glas-Col, cat No 099CK6424)
which is the most preferred method for disrupting and homogenizing tissue samples. The lysate was centrifuged at 13000rpm for 5 min at room temperature and then mixed with 400µl of 70% ethanol by vortex. This mixture was applied into the hibind RNA spin column and centrifuged at 10000 rpm for 15 second at room temperature, then 300µl of RNA wash buffer-I were added and centrifuged. This step were repeated three times and in the last one using 500 µl of RNA wash buffer-II diluted with ethanol. The columns were dried by centrifugation at full speed for 1 min to completely dry the hibind matrix. Finally, the RNA was eluted by adding 100µl of pre heated at 70°C DEPC-treated water and centrifugation for 1 min at maximum speed.

2.4. RT-PCR procedure

Using RT/PCR preMix kit (Biorom). The premix tubes have all the components necessary for cDNA synthesis and PCR amplification. The first strand cDNA was synthesized from mouse liver total RNA by reverse-transcription as following: 1µg of total RNA and 20pmol of the reverse primer were mixed in a sterile tube then incubated at 70°C for 5 min and rapidly chilled on ice. The content was transferred into PCR PreMix tube and 20 pmol of the forward primer was added. The volume was completed with DEPC-H2O to 50 µl final volume. The cDNA synthesis reaction was performed by incubating the PreMix tube at 42°C for 60 min, followed by incubation at 94°C for 5 min for inactivation of reverse-transcriptase. The PCR was carried out using the same PreMix tubes that contain the DNA polymerase, amplification was carried out in 28 cycle using Bio metra as follows: Denaturation for 30 Sec at 94°C, annealing for 30 Sec at 55°C and extension for one mint at 72°C, after the initial denaturation at 95°C for 5 min.

2.5. Oligonucleotides used for amplifications

Sequences of mouse AFP, caspase-3 and SSTR-2 were obtained from Gene Bank®. These sequences (coding sequence) were used to design the primer pairs and the distance between the two primers was 500 bases. Primers were chosen to contain a GC content of 40-60% and TM 62°C. The primer sets as following:

**Somatostatin receptor type 2 primers (SSTR-2)**

Upper primer
5'-GGG TGT CCT CTC CAT TTG AC-3'

Lower primer
5'-CCG GCG TAT ATC ATG ATG GG-3'
Alpha feto-protein gene primer (AFP)
Upper primer
5'-GAC GGA GAA GAA TGT GCT TAG-3'
Lower primer
5'-CGA CCT TGT CGT ACT GAG CA-3'

Caspase primer
Upper primer
5'-GAC CAT GGA GGA CAA CAA AAC-3'
Lower primer
5'-GGC AGG CCT GAA TGA TGA AG-3'

2.6. Determination of protein concentration
Protein concentration was determined using Bradford assay (Bradford 1976).
1µl of diluted (1:10) liver homogenate was added to 200µl of Bradford reagent (Coomassie brilliant blue G250 dissolved in 95% ethanol and phosphoric acid).

2.7. Animal Approval Committee
An approval was taken from the University committee resident in College of Medicine/Minia University. The groups were classified and dosed as mentioned above.

RESULTS
3.1. RT-PCR analysis
3.1.1. Relation between HCC development and SSTR2:
The mRNA expression of SSTR2 in response to hepatocellular carcinoma development was measured at three different time points (8, 16, and 24 weeks) from day one of DENA injection; the results were measured in comparison to normal healthy group. The results exhibited that the mRNA expression of SSTR2 was proportional to each stage of HCC development. The levels of SSTR2 mRNA at 8, 16 and 24 months were 2.7, 4.1, 5.3 fold of that of the control respectively.

3.1.2. Relation between SSTR2 and apoptosis
Caspase-3 as apoptotic factor was selected to give an idea about the process of apoptosis which occurs during the expression of anti-growth hormone receptors in response to hepatocellular carcinoma development. The mRNA expression of caspase-3 was measured at
three time points (the same time points used for SSTR2 measurements, 8, 16 and 24 weeks) in comparison to normal healthy control group. The results exhibited that the mRNA expression of caspase-3 was proportional to each stage of HCC development. The levels of caspase-3 mRNA at 8, 16 and 24 weeks were 1.9, 2.1, 2.4 fold in comparison to that of the control respectively.

3.1.3. Expression of Alpha-Feto-Protein (AFP)

AFP is the gold tumor marker for HCC. In the present study we measured the expression of AFP mRNA during the development of HCC. The results exhibited that there is a high expression of AFP mRNA at all time points of HCC development in comparison to the normal control group, but this increase in AFP expression is not proportional with each stage. The levels of AFP mRNA at 8, 16, 24 weeks were 4.4, 2.8, 6.3 fold of that of the control respectively.

3.2. Western blotting analysis

3.2.1. Relation between HCC development and SSTR2.

By using western blot technique, the level of SSTR2 protein expression that had been synthesized by liver tissue in response to HCC development was measured at three different time points (2, 4 and 6 months from day one of DENA injection) in comparison to normal healthy control group. The results exhibited that the SSTR2 protein synthesized by liver tissue was proportional to each stage of HCC development. The levels of SSTR2 protein at 2, 4 and 6 months were 3.2, 3.9And 5 fold of that of the control respectively.

![Figure (1): Expression of SSTR type 2 during different stages of HCC development.](image)

*CONTROL*: group of mice injected with normal saline only.

*8, 16 and 24 weeks*: the time from DENA injection till sacrificing the animals.
The figure shows Marked increase in the expression of SSTR type 2 in HCC induced model than the control. The increase reflects the stage of HCC.

*Control: group of mice injected with normal saline only.

*8, 16 and 24 weeks: the time from DENA injection till sacrificing the animals.

The figure shows Marked increase in the expression of caspase-3 in HCC induced model than the control, the increase is proportional with the stage of HCC.

*Control: group of mice injected with normal saline only.

*2, 4 and 6 Months: the time from DENA injection till sacrificing the animals.

The figure shows Marked increase in the expression of AFP in all HCC induced model than the control, but the increase not proportional with the stage of HCC.
Figure (9-A): SSTR 2 protein synthesized in liver tissue during different stages of HCC development.

*Control*: group of mice injected with normal saline only.

*8, 16 and 24 weeks*: the time from DEN injection till sacrificing the animals.

The figure shows Marked increase in the SSTR type 2 protein synthesis in HCC induced model than the control, the increase reflects the stage of HCC.

Fig (9-.B): Percentage change the synthesis of SSTR2 in response to hepatocellular carcinoma development.

Percentage change The synthesis of SSTR2 in response to hepatocellular carcinoma development at three different time points (8, 16, and 24 weeks from day one of DENA injection), in comparison to normal control.

The hepatic tissue content of GSH was significantly decreased by 34%, 52% and 68% at 2, 4, and 6 months respectively, but the hepatic tissue content of MDA was significantly increased
by 3, 5 and 6.2 fold at 2, 4, and 6 months respectively in comparison to that of the normal healthy control (Table 1).

Table (1): Effect of HCC development on hepatic content of malondialdehyde (MDA) and reduced glutathione (GSH).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GSH (µmol/g tissue)</th>
<th>MDA (µmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.30 ± 0.18</td>
<td>29.10 ± 1.73</td>
</tr>
<tr>
<td>HCC 8 weeks</td>
<td>2.18 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.9 ± 7.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>stage 16 weeks</td>
<td>1.61 ± 0.12&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>146.2 ± 9.81&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>after 24 weeks</td>
<td>1.31 ± 0.09&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>161.0 ± 11.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE
* Control: group of mice injected with normal saline only.
* 8, 16 and 24 weeks: the time from DENA injection till sacrificing the animals - Multiple comparisons were done using one-way ANOVA followed by Tukey-Karmer as post ANOVA test.

a: Highly significantly different from control group at P < 0.001.
b: Highly significantly different from 8 weeks group at P < 0.001.
c: The difference is non significant from 16 weeks group at P < 0.05
d: Significantly different from 8 weeks group at P < 0.05

3.3. Histopathological examination of hepatic tissues
Morphological changes in hepatic tissues of male albino mice were qualitatively assessed using tissue sections stained with Hematoxylin and Eosin.

Normal hepatic tissues showed normal cellular shape with no sinusoidal growth pattern, cellular and nuclear pleomorphism, prominent nucleoli, or increased nuclear/cytoplasmic ratio (N/C ratio) (Photo 1).

Eight weeks after administration of DENA in a dose of (150 mg/kg I.P.) were resulted in mild pleomorphism with low grade sinusoidal growth pattern (Photo 2).

After 16 weeks of DENA administration caused an increase in cellular and nuclear pleomorphism with giant cells formation and increased N/C ratio (Photo 3).
While after 24 weeks of DENA administration caused multinuclear giant cell formation and increased width of cord cells more than two cells, with high grade sinusoidal pattern (Photo 4).

<table>
<thead>
<tr>
<th>Photo (1): Normal hepatic tissue sections showing normal cellular shape with no sinusoidal growth pattern, or increased N/C ratio (H &amp; E x 100)</th>
<th>Photo (2): Hepatic tissue section after 8 weeks from DENA injection, showing mild pleomorphism with low grade sinusoidal growth pattern.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photo (3): Hepatic tissue section after 16 weeks from DENA injection, showing cellular and nuclear pleomorphism with giant cells formation and increased N/C ratio. (H &amp; E x 100)</td>
<td>Photo (4): Hepatic tissue section after 24 weeks from DENA injection, showing multinuclear giant cell formation and increased width of cord cells more than two cells, with high grade sinusoidal pattern. (H &amp; E x 100)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Hepatocellular carcinoma a third largest cause of cancer related death, with an estimated mortality rate of about one million deaths annually and incidence to mortality ratio very close to one (Parkin et al., 2001). Unlike many malignancies, HCC has well defined risk factors that include chronic viral hepatitis, alcohol-associated liver disease, non-alcoholic fatty liver disease and exposure to chemicals like aflatoxins and diethylnitrosamine (DENA) (Leong et al., 1999).

DENA is known to induce damage in DNA repair enzymes and so it was used to induce liver cancer in experimental animal models (Bhosale et al., 2002). Cancer formation after DENA and other hepatocarcinogens is generally considered to result from genotoxicity that lead to...
mutation which is tumor initiation and cytotoxicity resulting in regenerative cell proliferation which occurs in initiated hepatocytes (Jang et al., 1992). The electrophilic species formed from the activation of DENA bind covalently to multiple target sites in DNA, proteins and other cell constituents resulting in DNA damage and mutations as well as in cell death and subsequent regenerative proliferation (Gonzalez 2001). DENA administration caused multinuclear giant cell formation and increased width of cord cells more than two cells, with high grade sinusoidal pattern of liver cells.

AFP is the most established tumor marker in HCC and the gold stander by which other markers for the disease are judged. AFP was synthesized by hepatocellular carcinoma cells where it is a fairly specific but insensitive marker for HCC but, may used to speculate whether a tumor has been completely resected to evaluate the efficacy of therapy and to monitor the recurrence of cancer (Koteish and Thuluvath 2002). Sensitivity of HCC detection by blood markers was improved by combining other markers in addition to AFP (Dusheiko 1988).

The present study exhibited markedly increase in RNA expression of AFP as tumor marker for HCC development at different stages of experiment without significant difference that can reflect HCC stages. These results were in harmony with other studies that indicated a positive rate of AFP was not stage-dependant (Kew 2000; Johnson 2001).

Somatostatin receptors were not expressed in normal hepatocytes in contrast to HCC hepatocytes, where these receptors were frequently expressed with SSTR-2 subtype being predominant (Kouroumalis et al., 1998).

Our results showed marked increase in mRNA expression and protein synthesis of SSTR-2 in response to HCC development at three different stages of experiment when matched with normal in agreement with others (Xie et al., 2007; Yun et al., 2009), who reported that an increased in mRNA expression and protein synthesis of SSTR-2 was not significantly different from normal but also, there was a significant difference in SSTR-2 mRNA and protein expressed in each stage of HCC development. SSTR expression can be used as a useful parameter to evaluate the prognosis of HCC (Li et al., 2012).

A comparison between the expression pattern of both SSTR-2 and AFP at different stages of HCC development, we found both SSTR-2 and AFP were highly expressed during all stages
of HCC in comparison to the normal, and this result was matched with a previous study (Xie et al., 2007).

Apoptosis was a selective, controlled and genetically programmed cell death process that would occur as a result of normal cellular differentiation and development. Defects in apoptosis contributed in many diseases including cancer, autoimmune and neurodegenerative disorders. This process was mediated by a complex mechanism involving intracellular proteases, the caspases and activators and inhibitors of these cell death proteases (Reed 2000). The differential effect of somatostatin on apoptosis may be due to its different effect on TNFR1 expression in the two cell lines. It may have no direct effect on apoptosis but it may indirectly act through TNF induced apoptosis (Georgiadou et al., 2011).

Caspase play an important role in regulating cancer cell death both induced by activated lymphocytes and by chemotherapeutic agents (Kidd 1998). Caspase-3 is the key member of effectors caspases (Kitada et al., 1998). Positive expression of caspase-3 by HCC did not affect the apoptotic indexes, this suggests that other factors may exist in regulating normal cell apoptosis (Bao et al., 2000). Loss of caspase-3 expression was associated with resistance to apoptosis and resistance to therapy in a variety of tumors and cell lines like renal cell carcinomas (Kolenko et al., 1999). Resistance to apoptosis by a variety of apoptotic stimuli including chemotherapeutic agents due to lack of expression or activation of caspase-3 had been demonstrated in breast cancer, leukemia and classic Hodgkin's disease cell lines (Devarajan et al., 2002). Resistance to cisplatin, tamoxifen and ionizing radiation induced apoptosis was dependent on caspase-3 in breast, lymphoblast and HeLa cell lines (Devarajan et al., 2002).

Our results exhibited marked increase in mRNA expression of caspase-3 during HCC development at all stages of experiment in comparison to normal. Also, there was a significant difference in caspase-3 mRNA expressed in each stage of HCC development by a way that reflects the stage. This result is matched with previous study who reported that the expression of caspase-3 was correlated with HCC differentiation (Bao et al., 2000).

Damage due to oxidative stress and free radicals is one of the important factors in carcinogens and these compounds must be detoxified in order to avoid DNA damage and lipid peroxidation. GSH is a very important factor in detoxification, while MDA is a sign of
lipid peroxidation (Reed 2000). In the present study, oxidative stress was assessed by measuring the MDA, a product of lipid peroxidation and the intracellular antioxidant GSH. Our data of this study revealed that DENA was significantly increased TBAR and decreased GSH in liver cells, suggesting that reactive oxygen species induces by DENA would play an important role in DENA-induced hepatic carcinogenesis. Increased generation of ROS and decreased antioxidant enzymes in liver tissues had been reported in many models of DENA-induced hepatocellular carcinoma (Yadav and Bhatnagar 2007; Sivaramakrishnan et al., 2008). It has been reported that ROS play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA, DNA repair system and other enzymes (Kensler and Trush 1984).

CONCLUSION
SSTRII and caspase-3 expression in liver tissues were considered as a marker of HCC developed stages in combination with AFP in order to increase the sensitivity of AFP. Also, changes in hepatic tissue contents of GSH and MDA during HCC induction and management, suggest that oxidative stress induced by DENA play an important role in hepatic carcinogenesis.

REFERENCES


43. Tanaka Y., Hanada K., Mizokami M. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA, 2002; 99: 15584-15589.

