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ORIGINAL ARTICLE

Serum histamine and acetylcholine variations as new noninvasive biochemical markers in staging of experimental hepatocellular carcinoma

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Abstract
Angiogenesis is a major prerequisite for hepatocellular carcinoma (HCC) development and progression. The present study aims to assess the potential role of two endogenous regulators of angiogenesis histamine (His) and acetylcholine (Ach), as possible biochemical markers for staging of HCC. Five groups of rats were used in this study: a control healthy group (I), another 4 intoxicated groups used for the induction of HCC with a high dose of diethyl nitrosamine (DENA, 200 mg/kg, single I.P. dose), (II, III, IV, and V). Groups II, III, IV, and V were killed following 8, 16, 24, and 32 weeks after DENA injection, respectively. Serum level of His and Ach was estimated using high-performance liquid chromatography technique coupled with diode array detector (HPLC–DAD), and alpha-fetoprotein (AFP) was measured using ELISA technique along with liver histological examination for all groups. Progression of HCC was estimated by histopathological examination. The results exhibited prominent increase in serum His and Ach levels during the early stages of HCC in group II, III in comparison with the control, and then His serum level declined to the normal level during the last stage of HCC development (group V).However, Ach elevation continued. AFP serum level showed marked increase, till 32 weeks after hepatocarcinogenesis. The decreased histamine level, combined to elevated AFP, indicates an early stage, while continued elevation of Ach with decreased His levels indicates a later stage of HCC. The combination of these two neurotransmitters to AFP may contribute to a noninvasive biochemical staging for HCC.

Keywords Hepatocellular carcinoma · Histamine · Acetylcholine · AFP · Staging

Introduction

Hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths and the fifth most common tumor worldwide [1]. The incidence of HCC varies widely throughout the world, with rising incidence in Egypt [2]. In Egypt, the high prevalence of hepatitis C virus (HCV) [3, 4] resulted in growing incidence of HCC, which is nearly doubled over the last decade [5–7]. According to the records of National Cancer Registry Program (NCRP) [8], HCC incidence rate occupied the top ranked cancer among Egyptian males and the second top rank among Egyptian females after breast cancer (Fig. 1). The silent growth of HCC may delay the diagnosis for as long as 3 years from the time of development [9].

Early detection of HCC is the most critical step in the management process, so patients with risk factors for HCC should undergo frequent periodical laboratory investigation every 6 months to predict early development [10]. The diagnosis of HCC without pathologic confirmation can be achieved by the assessment of serum alpha-fetoprotein (AFP) level, since its discovery in 1963 [11]. AFP has been regarded as the most useful serum protein for HCC patients [12–14]. However, we still need to improve the early diagnosis of HCC because only 44% of the patients are diagnosed at a localized disease stage and only 30% of HCC patients at the time of diagnosis are candidates for potentially curative treatments [15]. Thus, the discovery of an effective, reliable

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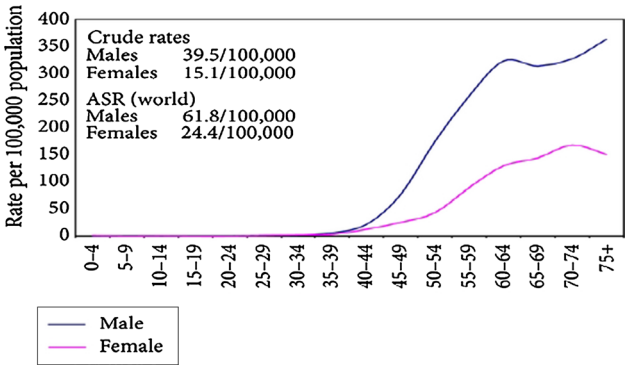

Fig. 1 Calculated age-specific incidence rates for liver cancer in Egypt 2008–2011 [8], *ASR* age-standardized rate

tool for early diagnosis of HCC to increase the number of patients who are suitable for curative treatment improves HCC patients' prognosis.

Neurotransmitters act as powerful upstream regulators that orchestrate numerous cell and tissue functions, by releasing angiogenic mediators, pro-inflammatory cytokines, growth factors, arachidonic acid, metastatic factors and local neurotransmitters from cancer cells and their microenvironment. Moreover, they modulate angiogenesis, proliferation, apoptosis and metastasis directly by intracellular signaling downstream of neurotransmitter receptors [16].

Histamine is a biogenic amine that is released throughout the entire body of an organism [17–21], with high concentration in the lymph nodes, stomach and thymus. The lowest concentrations of histamine are found in the liver, brain, intestines and lung [22]. It is an important chemical mediator that regulates different pathophysiological functions such as cellular invasion, migration, angiogenesis, differentiation, apoptosis and different immune responses [18]. There have been numerous studies involving histamine in liver cancers. Histamine-induced effects were attenuated by the inhibition of either H1 or H2 His receptors [23]. Although His was proposed since 1960s to have a probable role in carcinogenesis [21], it remains under discussion. It can produce different and, sometimes, contradictory effects on tumor cell growth through the activation of its four membrane-specific receptors, H1, H2, H3 and H4. Moreover, most of the physiological and pathological actions of His have been shown to strictly depend on both its endogenous/exogenous concentration and the tumor cell type under study [24, 25]. Acetylcholine (Ach) functions in regulation of cell fate, such as cellular proliferation, differentiation and apoptosis. Cholinergic system, including acetylcholine esterase and acetylcholinic receptors, has been detected in HCC, assuming that Ach promotes HCC cell proliferation [26]. It enhanced cell migration and invasion but suppressed apoptosis in HCC [27]. Looking for noninvasive biochemical markers for staging HCC instead of histological examination was tried at an experimental level [28].

The present work was conducted to assess a possible role of two endogenous regulators of angiogenesis, His and Ach, as possible noninvasive biochemical markers for staging of HCC, in addition to AFP.

Materials and methods

Animals

Forty male Sprague–Dawley albino rats about 250 g were used in the present experiment. They were purchased from the animal house, Asyut University, Asyut, Egypt. The animals were housed under standardized environmental conditions, fed with standard diet and left to acclimatize at 22 °C ± 2 °C and 12/12 h. light/dark cycle for 1 week prior to inclusion in the experiment.

Chemicals

Histamine and acetylcholine standards were obtained from Santa Cruz Technology, Inc., UK, diethylnitrosamine (DENA) from Sigma chemical company, St Louis, MO, USA. Rat alpha-fetoprotein (AFP) ELISA kit was obtained from WKEA Med Supplies Corp, China (code no. WAR-348). Other chemicals were obtained either from Sigma chemical company or commercial suppliers, unless otherwise mentioned.

Basic experimental design

Diethylnitrosamine (DENA) was used to induce HCC in rats [29, 30] as follows: Animals were divided randomly into five groups (8 rats/group) as follows: I, a control healthy group (injected with saline), II, III, IV, and V: HCC model groups (injected with DENA, 200 mg/kg single intra-peritoneal dose). Groups II, III, IV, and V were killed following 8, 16, 24, and 32 weeks after DENA injection, respectively. Rats were initially anesthetized with 3% halothane before they were sacrificed to collect blood and livers for experimental analyses. Blood samples were collected on the last day of the experiment after 12 h. Fasting blood samples were left for 15 min for clotting, then centrifuged at 3000×g for 15 min for serum collection. Sera were kept at − 80 °C for biochemical measurements.

Liver tissue preparation

Livers were dissected, fixed and embedded in paraffin block for histopathological examination. Microscopical

examination was performed on liver samples, stained with hematoxylin and eosin.

Biochemical investigations

1. Measurement of serum AFP

   Serum AFP was measured by ELISA kit following manufacturer's manual.
2. Measurement of serum histamine and acetylcholine levels

   Histamine and acetylcholine have been determined by HPLC using diode array detector (DAD) [31–33]. In our study, we used HPLC–DAD (Agilent Technologies 1200 series, Germany), histamine and acetylcholine were separated on 300SB-C18 (250 mm × 4.6 mm, 5 µm) column (Merck, Darmstadt, Germany). The mobile phase of phosphate buffer (25 mM, pH 3) and acetonitrile (5:95) was run on isocratic at a flow rate of 1 ml min^{-1}.

Statistical analysis

The statistical analysis of results was done using Graph Pad Prism 5 (Graph pad Software, San Diego, California, USA). The results were expressed in terms of mean ± SEM, and differences between the mean values for individual groups were assessed by one way ANOVA.

Results

Biochemical investigations

In this study, we measured serum AFP during the development of HCC. The results showed an increase in serum AFP level in all experimental groups (II, III, IV, and V) than control group (I), but this increase was significant only during moderate and late stages of HCC (Groups IV and V, p value ≤ 0.03 and 0.01, respectively). There was no significant difference between group IV and V, so the change in AFP level cannot reflect the stage of HCC, as well as, its level started to decline by 32 weeks (Fig. 2). AFP mean values were multiplied by 10 in the figure to clarify the column lengths.

Our results show a highly significant increase in serum His level than the control during early stages of HCC development (groups II and III, p value < 0.001), while its level for moderate stage of HCC development (group IV) showed significant increase ($p < 0.01$) than the control and significant decrease (p value < 0.001) than early stage groups (II and III), and finally group V which represents the late stage of HCC development showed decline in serum His to normal level without any

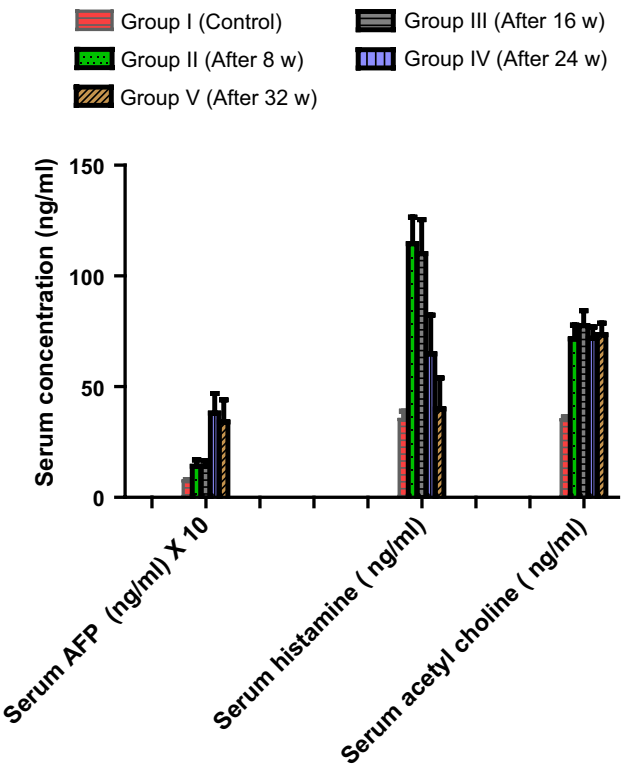

Fig. 2 Serum levels of AFP and histamine during the different time points of HCC progression. (Values are expressed as M ± SE, $n = 8$.) Actual AFP levels are multiplied by 10 to be more apparent on graph

significant change than the control. A significant increase in serum Ach level during all stages of HCC development was seen (groups II, III, IV and V, with $p < 0.001$) in comparison with the control group (Fig. 2).

Histopathological examination of hepatic tissues

In this study, we found no morphological changes in hepatic tissues of control groups, normal cellular shape with no sinusoidal growth pattern, cellular and nuclear pleomorphism, prominent nucleoli, or increased nuclear/cytoplasmic ratio (N/C ratio). Eight weeks after administration of DENA, an increased N/C ratio with high-grade sinusoidal pattern was shown. Multinuclear giant cell formation with increased mitotic figures was seen after 16 weeks of administration of DENA. Multinuclear giant cell formation and increased width of cord cells and poorly differentiated HCC were seen following 24 weeks post-administration of DENA. After 32 weeks of DENA injection, cellular and nuclear pleomorphism increased the width of cord cells, microacinar formation was more apparent (Fig. 3).

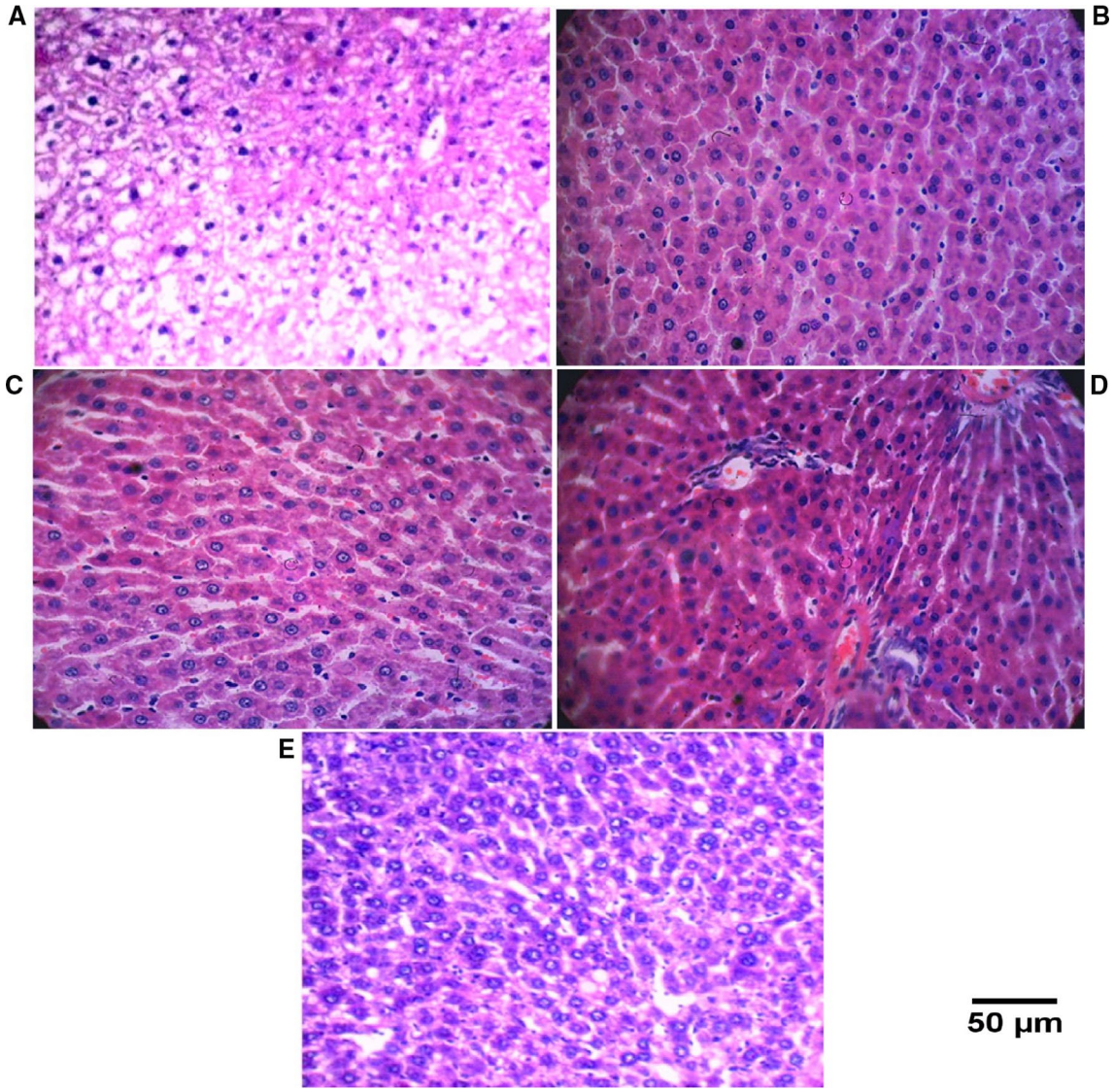

Fig. 3 Histopathological photographs of HCC during the different time points of the experiment: **a** control hepatic tissue sections showing normal cellular architecture (H&E × 100). **b** Hepatic tissue section after 8 weeks from DENA injection, showing increased nuclear/cytoplasm (N/C) ratio with high-grade sinusoidal pattern (H&E × 100). **c** Hepatic tissue section after 16 weeks from DENA injection, showing multinuclear giant cell formation with increased mitotic figures (H&E × 100). **d** Hepatic tissue section after 24 weeks from DENA injection, showing multinuclear giant cell formation and increased width of cord cells more than two cells and poorly differentiated HCC (H & E × 100). **e** Hepatic tissue section after 32 weeks from DENA injection, showing cellular and nuclear pleomorphism, increased width of cord cells more than two cells, with microacinar formation (H&E × 100)

Discussion

Histamine was shown to induce a multitude of effects on various cellular pathologies, although growing evidence suggests that His and its receptors may be involved in tumor growth and/or depletion [34, 35]. Our results show a highly significant increase in serum His level than the control during early stages of HCC development (groups II and III), with a decline in group IV and significant decrease near to normal control after 32 w. His can behave as a proliferative or an anti-proliferative factor within the same tumor cells, depending on its concentration and the receptor subtype to which it binds [36]. Similar results have been reported for HCC cells [23]. Histopathological results for our experiment show slow rate for HCC growth during the first 16 weeks (group II and III), and these two groups also show high serum His level, while the rapid cancer growth was noticed in groups IV and V showing significant decrease in serum His level, which means possibly that, high His level in group II &III may contribute to the tumor growth.

Histamine release can also be catalyzed from L-histidine by the enzyme L-histidine decarboxylase (HDC) [37, 38]. So we have two probable reasons for change in serum histamine

level, change in HDC activity and/or change in mast cell number.

Accumulated evidence points to a direct relationship between up-regulation of HDC activity and growth of several types of human tumors. Overexpression of HDC at both the mRNA and protein levels and increased levels of His have been shown in melanoma [39], small-cell lung carcinoma [40], breast carcinoma [41], endometrial cancer [42] and colorectal carcinoma [43]. Mast cells, where His is stored, are increased in number during many types of malignant tumors [44–46]. HCC tissues with different histological grades showed variable numbers of mast cells, with well-differentiated HCC showing the highest number. They tended to decrease in less-differentiated HCC, suggesting a possible role in the early stage of HCC development [47], so this may explain the significant perturbations in His level which we observed in the early and last stages of HCC development.

The literature linking the level of Ach and Ach esterase to cancer still is very scarce. It was proved that there is an association between cholinergic system in human hepatocytes and HCC. Thus, Ach degradation enzyme (Ach esterase) is down-regulated, leading to an increase in Ach availability and thereby activates the Ach receptors which promotes HCC proliferation and counteracts the drug-induced apoptosis [26]. In our study, serum Ach level was elevated just after HCC induction and kept up-regulated till the 32nd week of DENA hepatocarcinogenesis. Increased nicotinic Ach receptor activation was attributed to the up-regulation of Ach and the decreased Ach esterase. Ach receptors were known to mediate cell signaling that stimulates the growth and angiogenesis, mediating oncogenic signal transduction during cancer development in a cell type-specific manner [48]. The persistent elevation of Ach with AFP in this work means a contribution to cancer progression and invasion. This action was mostly first studied in experimental HCC.

In summary, measurement of both His and Ach serum levels, in addition to AFP, may have an important diagnostic value for biochemical staging of HCC. The elevation of the 3 parameters simultaneously indicates an early stage of liver cancer. However, the elevated Ach and AFP, with decreased His levels, assume an advanced stage of HCC. Separately, inhibition of both His and Ach up-regulation may open a new therapeutic line in the HCC management.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the animal experiments were conducted in accordance with the guide for the care and use of laboratory animals of the Faculty of Pharmacy, Kafrelsheikh University, Egypt.

Informed consent The experiment is not a clinical study, so not applicable.

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