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Biological and Physiological Characterization of HCAM Hepatocellular Carcinoma Cell Line

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Abstract

A new liver cancer cell line (HCAM) has been characterized; it is considered as a useful tool in liver cancer research. The HCAM cell line was established from the primary tumor of a white swiss albino male mouse with spontaneous diffuse hepatocellular carcinoma. The current study aimed to physiologically characterize the cells by comparing it to normal liver cells from embryo and adult mice. The eighth passage of HCAM cell line was used in this study. Investigation of liver functions showed high values of liver enzymes (ALT, AST, GGT, LDH, CRP) in HCAM cell line compared with normal liver cells that were taken from healthy mice; these enzymes rates were (52.94, 53.06, 63.63, 390.67, 10.50) U/L respectively ($P \leq 0.05$). High values of tumor marker proteins (AFP, CEA, CA19-9) were also found in HCAM cell line compared with normal hepatocytes; the rates were (24.23, 15.26, 15.54) U/L respectively ($P \leq 0.05$).

The hepatic cancer cell line showing high liver enzyme functional activity, and this makes it useful as a liver cancer model in functional studies.

Keywords: Hepatocellular carcinoma; HCAM; Liver Cancer

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Introduction

Hepatocellular carcinoma (HCC) is the fifth malignant diseases worldwide [1], which ranked second as causes of cancer-related deaths, with more than 600,000 cases of death reported every year internationally [2]. HCC represents about 90% of all liver cancers that occur in males higher than females with a male: female ratio usually averaging between 2:1 and 4:1, and the majority of hepatocellular carcinoma cases occur after the age of 40 [3]. The important risk factors for the development of this type of cancer are toxic (aflatoxins), metabolic (diabetes), obesity, immune-related (cirrhosis and hepatitis), and other factors [4]. In general, cancer cell lines are an important experimental tool in anticancer research; they offer an unlimited supply of a homogeneous self-replicating cell population that can be used in biomedical studies [5]. In addition, these cell lines may retain the hallmarks of primary tumors and may gain mutations during long-term subculture, making them no longer representative of the primary cancers from which they were derived. Because most cancer cell lines were established a long time ago, it is difficult to characterize the degree of which these lines represent their matched primary cancers, and that creates the need to establish new cancer cell lines [6]. Hepatocellular carcinoma cell lines played an important role in cancer studies [7]. The aim of this work was to characterize and understand the physiological properties of newly established mouse hepatic cancer cell line HCAM by comparing it to the characteristics of the normal liver cells, which may help to develop more effective therapies for targeting the disease.

Methods

Primary Culture of Normal Liver Cells

The source of culture was a normal mouse embryo's liver tissues (3-4 days) and normal adult mouse liver tissues (3-6 months). The liver tissue was removed by dissection tools under sterile conditions and washed twice with PBS solution, and then the liver tissues transferred to sterile flasks. The tissues were minced into small fragments by mechanical disaggregation, followed by enzymatic disaggregation by trypsin for 10-15 minutes at room temperature, then the cells were gauze mesh filtered in a sterile flask with 2-3 ml of RPMI 10% media and transferred into tubes for centrifugation at 37°C and 1000 r / min for 5 minutes to precipitate hepatic cells after that PBS solution was added to wash the cells to be debris free, that can be used in liver function tests after counting the cells under a light microscope using Neubauer's chamber slide [8].

HCAM Cells Propagation

When the cells become confluent monolayer, subculture is necessary to maintain healthy cells. RPMI media were poured off, and the cells washed twice with 2 ml of Phosphate buffer solution (PBS), then adding 1 ml of Trypsin-Viersen to the culture flask and incubated for 1-2 minutes at 37°C. After the cells dissociation and dispersion into

a single-cell suspension, they are diluted with 7-10 ml of RPMI with 10% fetal bovine serum (FBS) and transferred into two fresh culture vessels and incubated at 37°C where they will reattach and grow again [8].

Cell Lysate Preparation

HCAM cell line and normal (mouse embryos and adult mice) hepatic cells were studied for liver function tests. One million cells of each type were calculated and transferred into small tubes with 1ml of PBS. Cell lyses done to extract the enzymes through three freeze-thaw cycles, cells kept for five minutes in the Deep freeze with -80 °C and moved to 37°C water bath directly, then put the tubes in a cooled centrifuge for 10 minutes and 1500 rpm/min at 4°C. Finally, the supernatant was used for liver functions and tumor marker analysis.

Liver Functions Analysis

Cell lysate from cancer and normal cells of 100000 cells were used to measure liver function's parameters to study the differences between the new cancer cell line and the normal adult and embryonic hepatic cells. The tests were: alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), Lactate dehydrogenase (LDH) and C-reactive protein (CRP) were measured in the hepatic cells lysate by CYANsmart semi-automated biochemistry analyzer (Cypress diagnostics, Langdorp, Belgium).

ALT, AST, and GGT Tests

Normal and cancer cell lysates were measured in this test by using CYANsmart semi-automated biochemistry analyzer (Cypress diagnostics, Langdorp, Belgium). mixed 1 ml of working reagent with 0.1 ml of the samples into a Cuvette 1 cm light path and waited 1 min, then started the stopwatch and readied absorbance every minute for 3 min in Spectrophotometer for different wavelengths (340 nm for ALT and AST enzymes and 405 nm for GGT enzyme) at 37°C, then calculate the difference between the absorbance and the average absorbance differences per minute (Δabs⁄min), according to the following equations:

- ALT or AST (U⁄l) = Δabs⁄min x 1750
- GGT (U⁄l) = Δabs⁄min x 1190

LDH Test

The same liver lysate cells were measured using the same ALT kit, mixed 1 ml of working reagent with 20 µl of samples into a Cuvette 1 cm light path. After 25 second incubation, measured the change of absorbance per minute (Δabs⁄min) during 3 minutes in Spectrophotometer for 340 nm at 37°C, according to the following equation:

- Activity (U⁄l) = Δabs⁄min x 8095

CRP Test

The hepatic cells lysate was diluted with TRIS buffer as a 1:20 ratio (i.e., added 10 µL of the sample with 190 µL of TRIS buffer). Added 10 µl of diluted sample to 100 µl FITC Label and 20 µl Nanomagnetic microbeads, then incubated for 5 minutes and washed with buffer (400 µl cycle washing), after that added 200 µl ABEI and incubated for 10 minutes, then washed with 400 µl cycle washing and finally liver samples were measured by automatic electrochemical immuno-analyzer (Maglumi 800, SnibeCo., Ltd, Shenzhen, China).

Tumor Marker Proteins Levels

The same cell lysates previously prepared were used to measure some tumor markers to study the transformation nature between the HCAM cancer cell line and the normal adult and embryonic hepatic cells. The tests were: Alpha-fetal protein (AFP), carcinoembryonic antigen (CEA) and Cancer Antigen 19-9 (CA19-9) in the hepatic cell lysates were measured by automatic electrochemical immuno-analyzer (Maglumi 800, SnibeCo., Ltd, Shenzhen, China).

AFP and CEA Tests

Cells samples that prepared above were measured in these tests by mixing 100 µl FITC label with 20 µl nanomagnetic microbeads and 40 µl of cell samples and incubated for 10 min, then washed with 0.9% NaCl (400 µl cycle washing), and added 200 µl ABEI and incubated for 10 min, then washed with 400 µl cycle washing and measured by using automatic electrochemical immuno-analyzer (Maglumi 800, SnibeCo., Ltd, Shenzhen, China).

CA19-9 Test

Normal and cancer, liver cell lysate were used and measured by adding 20 µl of nanomagnetic microbeads with 100 µl of buffer and 50 µl of the samples and incubated for 10 min, then washed with 400 µl of 0.9% NaCl, and added 200 µl ABEI and incubated for 10 min, then washed with 400 µl NaCl and measured with automatic electrochemical immuno-analyzer (Maglumi 800, SnibeCo., Ltd, Shenzhen, China).

Statistical Analysis

Liver function's data were statistically analyzed using an ANOVA table in one way, with standard error (Std.) and least significant difference (LSD) at ($P \leq 0.05$).

Results

Liver Function Analysis

The results showed that all the liver enzymes (ALT, AST, GGT, LDH, and CRP) concentrations in cancer cells were elevated significantly (P≤0.05) (Table 1) in comparison to normal hepatic cells, the rates of these enzymes in mouse embryo cells significantly lower than their levels in adult mouse cells were (± 0.40). The highest increment in the levels of these enzymes was observed in the HCAM cancer cell line (P≤0.05).

Table 1: Biochemical liver functions.

HCAM	Normal liver cells of adult mice	Normal liver cells of mice embryos	Biochemical liver functions
Cell line (passage 8)	(3-6 months)	(3-4 days)	
c	**b**	**a**	ALT
52.94 (± 0.76)	18.90 (± 0.64)	07.35 (± 0.50)	
c	**b**	**a**	AST
53.06 (± 0.47)	26.23 (± 0.72)	16.27 (± 0.37)	
c	**b**	**a**	GGT
63.63 (± 0.48)	37.67 (± 0.97)	09.07 (± 0.69)	
b	**a**	**a**	LDH
390.67 (± 1.46)	136.77 (± 1.22)	133.33 (± 1.86)	
c	**b**	**a**	CRP
10.50 (± 1.47)	01.93 (± 0.40)	00.25 (± 0.02)	

Where: ± Standard error
Similar letters indicate the absence of significant differences P≤0.05
Different letters indicate significant differences P≤0.05

Tumor Marker Proteins Levels

HCAM cancer cells showed significantly high levels of the tested markers (AFP, CEA, and CA19-9) when compared to the normal non-cancer cells. The presence of significant differences between groups (P≤0.05). Embryonic and adult mouse cells expressing less marker protein with no significant differences between them (Table 2).

Table 2: Tumor marker proteins levels.

HCAM cell line (passage 8)	Normal liver cells of adult mice (3-6 months)	Normal liver cells of mice embryos (3-4 days)	Tumor marker proteins
b	**a**	**a**	AFP
24.23 (± 0.77)	00.71 (± 0.02)	00.65 (± 0.07)	
b	**a**	**a**	CEA
15.26 (± 0.61)	01.25 (± 0.03)	01.06 (± 0.11)	
b	**a**	**a**	CA19-9
15.54 (± 0.94)	01.14 (± 0.07)	02.78 (± 0.27)	

Where: ± Standard error

Similar letters indicate the absence of significant differences P≤0.05

Different letters indicate significant differences P≤0.05

Discussion

The current work results revealed up-regulation in all liver function enzymes tested (ALT, AST, GGT, LDH, and CRP); elevation in liver enzyme levels is one of the most common problems encountered in clinical practice in hepatocellular carcinoma patients [9]. We analyzed the enzymes in hepatic cells lysate and found that ALT and AST significantly elevated compared with the lysate of the same cell numbers of normal hepatocytes taken from the normal embryo and normal healthy adult mice. ALT and AST enzymes found to be released in large amounts from damaged hepatocytes into the blood, and their activities have been widely recognized as effective tools to detect hepatic cancer [10]. These enzyme levels may be raised because of an increase in pyridoxal-5'-phosphate (vitamin B6) [11]. The AST/ALT ratio in HCAM was one while in normal cells were higher than one (2.2 for the embryo and 1.3 for the Adult hepatocytes), Liu XE, et al. (2007) [12], found that the AST/ALT ratio was significantly lower in patients with HCC than in patients without cancer, these results confirm our findings.

A significant increase in GGT liver enzyme level was noticed in our new cell line (HCAM). High expression of GGT, is often reported in tumors, where it plays a role in tumor progression, invasion, and drug resistance as GGT is a cell surface enzyme involved in cellular glutathione homeostasis [13], GGT is located on the outer aspect of the plasma membrane of liver cells and is often released at high levels into the bloodstream in hepatocellular tumors [14].

Our results showed notable excess of the LDH enzyme level in hepatic cancer cell line compared with normal liver cells. LDH is typically released in a high amount from hepatic tumor cells. Cancer cells rely on anaerobic respiration for convert glucose to lactate even under oxygen-sufficient conditions, so this mechanism allows tumor cells to convert the majority of their glucose stores into lactate regardless of oxygen availability, shifting use of glucose [15]. Many authors reported the usefulness of serum LDH levels as a prognostic marker for various solid tumors, including HCC. They evaluated the role of LDH, assuming that malignant cells in a tumor would have a low oxygen supply and would be the main source of increased LDH [16,17].

This study investigated the differences between CRP level in the liver cancer cell line, and it's level in normal liver cells, high expression of CRP was included in our hepatic tumor cells. CRP has been identified as an inexpensive, simple prognostic marker for patients with HCC [18]. The highest level of CRP was significantly related to exit vascular invasion, multiple tumors, and larger tumor size in HCC patients. Also, increase CRP levels was tended to be associated with tumor differentiation, though not significantly [19]. The molecular mechanism of tumor-related CRP elevation in HCC cells was complicated. One possible explanation was the proinflammatory cytokine, IL6, which usually highly expressed in the tumor microenvironments. The principal regulator of CRP (IL6) has been shown to be related to hepatocellular carcinoma progression and metastasis [20].

On the other hand, this study conducted an analysis of high AFP expression in HCAM cancer cells in our experience compared with the low expression of this marker in normal embryonic and normal adult mice. Many studies have demonstrated that higher levels of AFP are an independent risk factor for developing HCC, and improved that alpha-fetoprotein levels decline gradually after birth, reaching low levels in normal adults; these results confirm with our findings [21]. Despite the fact that AFP is still the golden standard among diagnostic markers for HCC, its diagnostic value is more questioned due to poor sensitivity and specificity of the assay. In addition, given that AFP levels can be increased in benign conditions such as chronic hepatitis or acute hepatitis, so it is difficult to determine the appropriate value at which sensitivity and specificity are maximized [22]. Many researchers have indicated that elevated alpha-fetoprotein may not be indicative, or be only suggestive of HCC [23]. The value of such a test may be improved by the parallel monitoring of other markers [24]. Levels of AFP often correlate with HCC tumor size, an increase in this marker may indicate tumor growth [25].

The augmentation in the level of the CEA enzyme was noticed in our hepatic cancer cell line. Elevated CEA might be associated with HCC overall survival. The higher CEA level is an independent prognostic factor for hepatocellular carcinoma [26]. The greatest value of CEA is a positive marker for HCC cancer when the proper pattern of staining is identified. Although the highest value of CEA leads to a shortcoming of this antibody, which is the occasional difficulty in interpret of the pattern of staining [27]. Carcinoembryonic antigen levels are not specific for various neoplasms including hepatocellular carcinoma, because CEA produces a specific pattern of canalicular staining in HCCs: this may be combined with non-canalicular membrane and cytoplasmic staining which are not specific for this cancer [28]. The concentrations of CEA were significantly higher in patients with HCC than in the control group. The elevation of CEA may be due to, at least in part, it's drainage from a tumor to blood vessels [29]. Carpelan-Holmstrom M, et al. (2002) believed that the difference in the values of the CEA marker is at least partially due to its different sensitivity [30].

In the present study, we investigated the elevated values of CA19-9 tumor maker in our HCAM cell line, in addition to its low levels in normal liver cells, an increase in CA19-9 level in HCAM cell line, which has been associated with increased mortality in patients with HCC [31]. An elevated CA19-9 level is most obvious in patients with stage I HCC as the predictor of shorter long-term survival for HCC patients, and this marker may reflect liver cancer cells' growth, differentiation, invasion, and metastasis to some degree [32]. An increment in CA19-9 serum level is often seen in biliary obstruction [33]. CA19-9 is synthesized by normal biliary epithelium, so that, a high CA19-9 level was detected in normal bile [34]. Local compression of the biliary tree by the tumor mass may cause obstruction of small bile ducts and hence

produce an increase in the serum level of CA19-9 marker [35]. These observations may partially explain the prognostic implication of serum CA19-9 levels [32]. HCAM cell line found to have weak nuclear HER2/neu expression, while express p53 and EGFR proteins. Furthermore, HCAM cells are susceptible to docetaxel and cisplatin [36].

In conclusion, we observed increased levels of all liver enzymes increased in the cells lysate of HCAM cancer cell line. In addition, the expressions of tumor marker protein levels (AFP, CEA, and CA19-9) were found to be elevated, and these properties make the HCAM cell line good model of liver cancer disease to study the physiological features.

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