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Mouse Hepatocellular Carcinoma Sensitivity to Cisplatin and Docetaxel and Analysis of Related Proteins

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Abstract

Hepatocellular carcinoma (HCC) globally ranked fifth common cancer and the third-leading cause of death. This study aimed to characterize the new mouse hepatocellular carcinoma cell line (HCAM) for some of the most important proteins involved in cell cycle regulation P53, HER2/neu, and EGFR by immunocytochemistry. Also, to measure the sensitivity of the cells to some common chemotherapeutic agents such as cisplatin and docetaxel by the MTT cell viability assay. The findings of immunocytochemistry appeared that HCAM cells proven to express the p53 and EGFR positively when compared with the negative control. Furthermore, showing nuclear only low expression for the HER2/neu. For evaluation of the chemotherapeutic agent's efficiency, the cells of hepatocellular carcinoma (HCAM) were treated for 72 hours using different concentrations for Cisplatin and Docetaxel. The IC50 values of docetaxel and cisplatin after 72 h exposure for HCAM was 12.82 and 10.74 respectively. Our in vitro results demonstrate that Docetaxel and Cisplatin are toxic to HCAM cell line in a concentration-dependent manner. In conclusion, our results showed positive expression of p53, EGFR, and weak HER2/neu. Also, HCAM cell line showing to be sensitive to docetaxel and cisplatin, which inhibit cell proliferation.

Key words: *Hepatocellular carcinoma, cisplatin, docetaxel*

Introduction

Over the past several years, tumor cell lines have occupied significant parts in cancer researches to demonstrate molecular characteristics and evolving new therapies together [1] . Drug experimentation in cell lines of cancer is typically some of the initial steps in drug development. It permits the entrance of potential drugs numbers before committing to large scale expensive in vivo experimental approaches [2]. Cancers can develop resistance to particular therapeutics. The most type of primary liver cancer is hepatocellular carcinoma, which is cancer easily acquiring resistance to drug therapy. Therefore, many active anticancer agents available, some are associated with high levels of toxicity. For example, docetaxel agent is beneficial as microtubule depolymerization inhibition, and it has shown a strong influence against cancer [3]. Also, Cisplatin, cisplatinum, or (cis-diamminedichloroplatinum) is a recognized chemotherapeutic agent; it is active against cancers types, including cancer of germ cell, carcinomas, and sarcomas. Cisplatin method of action through to its capability to linking with the purine bases on the DNA; causing DNA damage, interventing with DNA repair mechanisms, and then inducing apoptosis of cancer cells [4]. Immunocytochemistry (ICC) analysis is a beneficial tool not only for cell characterization but in some cases, also for cell lines authentication [5]. The suppressor gene of the tumor (p53) is mutated mostly in 50% of tumors. The p53 ability for various biological roles can be returned to its ability to act as a sequence-specific transcription factor to the regulation of different targets expression, and thus to manage multiple cellular processes including DNA repair, cell cycle arrest and apoptosis [6]. These proteins (EGFR) are found on the surface of some cells of cancer and normal cells types and mediate cell survival, invasion, proliferation, and angiogenesis [7]. A

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type I tyrosine kinase receptor encodes by HER-2/neu proto-oncogene; Also, HER2/neu stimulates the factors that facilitating cell motility and induces cell division [8]. The aim of the work to evaluate proteins expression and analyzed the cytotoxicity assay of docetaxel and cisplatin against the new hepatocellular carcinoma cell line (HCAM).

Methodology

Cell line and Cell culture

The cell line of (HCAM) Murine Hepatocellular Carcinoma Ahmed Majeed was supplied from Iraqi center of cancer, and medical genetics research (ICCMGR) and cultured in vitro in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 µg / ml for ampicillin and streptomycin. The suspension was then transferred to a culture flask for 37°C incubation.

Immunocytochemistry

The Cells of HCAM were grown in RPMI-1640 supplemented 10% FBS to a confluent monolayer on glass coated with coverslips in a six-well plate and sustained in a humidified incubator at 37°C with 5% CO2. Before fixation, monolayer cells were washed using PBS twice. With 4% cold acetone, the cells on a coverslip were fixed for 10 min, rinsed gently in PBS. Endogenous peroxidase activity was neutralized by incubation for 10 min in a solution consisting of 3% H2O2 blocking reagents for 45 min firstly and then primary antibodies selection (anti -Her neu2, anti-P53, and anti-EGFR) (Santa Cruz Biotechnology), for 2 hours at 4°C for detection of markers for hepatocellular carcinoma. The antibodies dilution was (1:50–100) according to the recommendations of manufacturers. The color operation was achieved with the chromogen diaminobenzidine (0.6 mg/ml) in a 0.02% H2O2 solution. Cells were examined with a Leica light microscope after counterstaining of nuclei with hematoxylin [9].

Quantitative image test

ICC images were used for quantitative analysis protocol for the hematoxylin – DAB staining slides were taken by Leica inverted microscope and camera (Leica Microsystems, Germany), three different staining zones of immunocytochemistry images of each slide were analyzed in this study [10]. Firstly, un-mix the DAB by color de-convolution technique, the areas of hematoxylin stained were leaving a complimentary image. As we take three new images. The first image is the hematoxylin stain, the second one is the DAB image, and we quantify the DAB image. The number of pixels of a specific intensity value vs. their respective intensity was raised using "Fiji" version of ImageJ from http://fiji.sc. The intensity numbers in the results window were converted to Optical Density (OD) numbers with the following formula:

OD = log (max intensity/Mean intensity), where max intensity = 255 for 8-bit images [11].

Cytotoxicity assay

Reagents

Cisplatin and Docetaxel were obtained from the pharmacy with a concentration of (cisplatin 50 µg/ml, Docetaxel 100 µg/ml) and stored at 4C°. The preparation of the stock solution was accomplished by dissolving the reagents in distilled water. Before each experiment, this solution was diluted in the free serum medium and immediately used in the tissue culture.

Cell viability assess

The examine of cell proliferation, cells were plated in 96-well plates (Sarstedt, Denmark) with 200 µl of cells in each well and incubated at 37 C°. At monolayer, cells exposed with concentration of Cisplatin (50µg/ml), prepare series dilution (50, 25, 12.5, 6.25 and 3.125) µg/ml, and Docetaxel (100 µg/ml), prepare series dilution (100, 50, 25, 12.5 and 6.25) µg / ml which were diluted with RPMI-1640 free serum medium. Then 200µl of each dilution of chemotherapy described earlier were added to each well. The microplate was incubated at 37C° for 72 hours [12] . After 72 hours of the exposure the viability of cells was assessed and treatment by removing the medium, adding 100 µl of 2 mg/ml solution of MTT and incubating for 2 hours at 37°C, and dissolving of insoluble formazan crystals in 150 µl of dimethyl sulfoxide (DMSO).

The optical density (OD) was measured at 490 nm of the wavelength by a microplate reader. The cell growth inhibition rate calculated using the following formula: **(IR) = (A-B)/A*100**. Where **A**: mean of the optical density of untreated wells, and **B**: optical density of treated wells [13, 14].

Morphological study

The chemotherapy exposed and non-exposed

control hepatocellular cells were photographed at 72h and fixed in 4% paraformaldehyde in phosphate buffer (10 min) at room temperature. The cell was stained with hematoxylin and eosin (H&E) stain. The cells were examined using the Leica inverted microscope. The affected cells were recognized according to cytological features [15] .

Statistical analysis

The data was displayed as mean ± standard deviation. For ICC experiment, n = 3 images were used. One-way (ANOVA) analysis of variance multiple comparisons were done to show variations between groups. The statistical analyses were done using (GraphPad Prism, version 6.07 for Windows, GraphPad Software, San Diego, CA, USA), and $p < 0.05$ as statistically significant.

The Results

Immunocytochemistry assay

The result of immunocytochemical staining for some biomarkers was shown in figure 1. The P53, EGFR, and Her-neu2 expression in the HCAM cell line of hepatocellular carcinoma were noticed using an anti-mouse IgG immunocytochemistry kit. The findings presented that HCAM were positive for EGFR, weakly for Her-neu2, and p53 proteins expression. Her-neu2 showed nuclear expression when compared with control cells. The control cells were exposed to the secondary antibody only, without primary antibody (negative control).

Digital Image Scoring

Images of ICC stained HCAM hepatocellular carcinoma cells were analyzed using ImageJ software. Figure-1E displays demonstrative pixel and zones intensity analysis of ICC images. The analysis showed that P53, EGFR, and Her-neu2 proteins were significantly expressed when compared to control, not stained cells using ANOVA one-way multiple comparison test.

Morphological study

Cytopathic effect of Docetaxel and Cisplatin on HCAM cell line was studied after 72 hours of exposure. Light microscope unstained and H&E stained images for control untreated cells were relatively uniform in size and shape (spindle) in monolayer culture with and without H&E stain (X20) B, b) Effect of docetaxel 100 µg /ml, (C, c) Effect of Cisplatin 50 µg / ml. The cells tended to grow in clusters and arranged irregularly, cells detached and appeared as oval forms with and without (H&E) stain (X20).

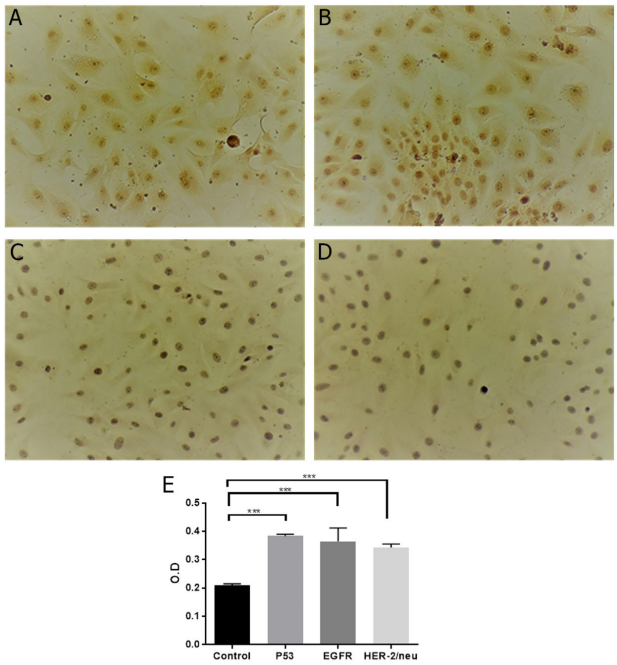

Figure (1): Immunocytochemistry stain of HCAM cell line. A) EGFR-positive staining of HCAM cancer cells (magnification 20×). B) P53-positive staining of HCAM cancer cells (magnification 20×). C) Her/neu2 weak positive nuclear expression of HCAM cancer cells (magnification 20×). D) Negative control of HCAM cancer cells (magnification 20×). E) Digital Image Scoring is showing significant proteins expression when stained with relative mAbs against the markers that analyzed using ImageJ program.

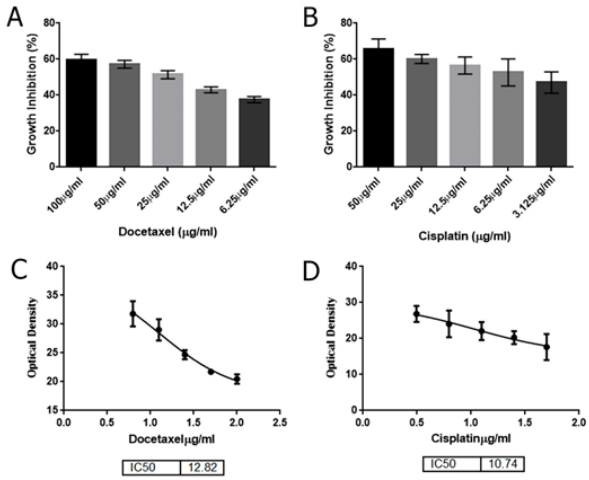

Figure-2: Bar chart for the mean values of growth inhibition rate and ic50 of HCAM cell line (GI%) induced by Docetaxel and Cisplatin after 72 h of exposure.

(A) The IC50 value of docetaxel for HCAM cells.

(B) The IC50 value of Cisplatin for HCAM cells.

(C) The effect of Docetaxel (100 µg /ml) for HCAM cells.

(D) The effect of Cisplatin (50 µg /ml) for HCAM cells.

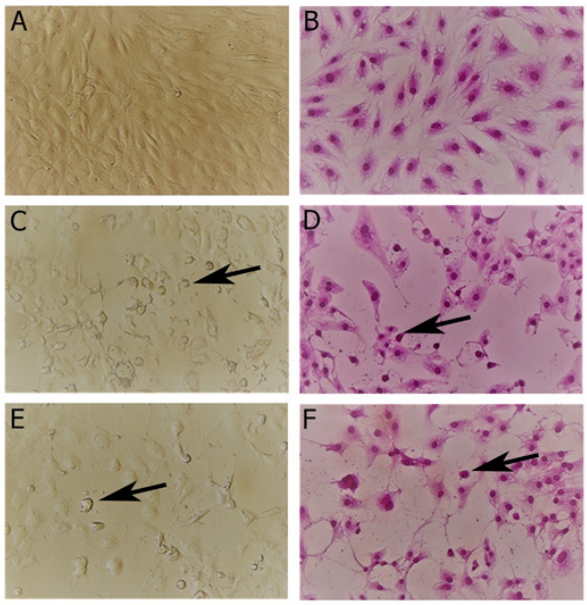

Figure (4): cytotoxicity effect of Docetaxel and Cisplatin on HCAM cell line after 72 hours.

(A, a) Untreated cells, HCAM cells were relatively uniform in size and shape (spindle) in monolayer culture with and without H&E stain (X20).

(B, b) Effect of docetaxel 100 µg /ml, **(C, c)** Effect of Cisplatin 50 µg / ml.

The cells tended to grow in clusters and arranged irregularly, cells detached and appeared as oval forms with and without (H&E) stain (X20).

Discussion

The ICC study presented that most of the HCAM cells were positive for EGFR, HER2/neu, and P53 proteins. The p53, EGFR, and HER2/neu genes expression impact the management of cancer cells due these genes provide both prognosis and therapeutic information [16] . HCAM cells were positive for P53 and EGFR as nuclear and cytoplasmic expression while for HER2/neu was very weak nuclear expression only. Positive expression of the cytoplasm and nucleus suggests mutation in p53, HER2/neu, and EGFR because it has important role in cell cycle control and DNA repair [(17-19)]. Several epigenetic and genetic alterations involved in the molecular pathogenesis of HCC as a somatic mutation of the tumor suppressor gene (p53) [20] . In addition, [21] shown that HCC models in both human and mouse, EGFR are upregulated in macrophages of the liver, where it acts as a tumor-promoting function. HER2/neu expressed weakly in non-neoplastic normal epithelia [22]. Otherwise, some researchers showed that development and modulation of HER2/neu oncogene in hepatocellular carcinoma is a rare event [23]. In agreement with our findings of HER2/neu nuclear expression in our HCAM cell line, another researcher [24] found that hepatocytes with altered metabolic and cell cycle may have nuclear HER2/neu expression.

In the current study, MTT viability test was used to evaluate the cytotoxic effect of docetaxel and cisplatin on the growth rate of the cell line (HCAM) in vitro. Docetaxel and cisplatin can inhibit the growth of HCAM cells in a concentration-dependent manner. Docetaxel enhances the assembly of tubulin into stable microtubules and prevents their assembly that leads to a reduction of free tubulin and cancer death [25]. Our results demonstrated that docetaxel showed a cytotoxic effect of HCAM growth rate with high concentration 100, 50, and 25 µg/ ml at 72 hours. Docetaxel with high concentration level, can effects on cell cycle kinetics, apoptosis, or increased ROS level. [26] Our data presented that cisplatin significantly inhibited the growth of HCAM cell with concentration (50, 25 and 12.5 µg/ ml) at 72 hours, which is consistent with a previous study [30] that found hepatocellular carcinoma treated with cisplatin significantly inhibited the growth rate and induced apoptosis.

Conclusion

Our results suggest that HCAM cell line express p53 and EGFR proteins in both nuclear and cytoplasm, but weak nuclear HER2/neu expression. Also, HCAM cell line is sensitive to cisplatin and docetaxel that induces a significant cytotoxic effect. HCAM is a new important mouse model that can be used for anti-hepatic cancer drug development.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the Faculty of Dentistry, University of Babylon, Hillah city, Iraq and all experiments were carried out in accordance with approved guidelines.

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