ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Doxorubicin (Dox) is an anthracycline antibiotic used as a single chemotherapeutic agent for HCC. The present work was conducted to study the immunohistochemical alterations in HCC patients treated with Dox. Thirty cases (24 males and 6 female) with a confirmed diagnosis of hepatocellular carcinoma (HCC) were used. They were divided into 3 groups, group 1. Ten specimens of HCC were taken before Dox treatments, group 2. Ten specimens HCC patients were taken one week after Dox treatment and group 3. Ten specimens of HCC patients were taken two weeks after Dox treatment. Hepatic biopsies were obtained from the three groups and prepared for histological, immunohistochemical (p53, Bcl-2 and CD34) and molecular studies. Histological examination of the specimen of HCC patients, before and after Dox treatment, showed trabecular appearance, cytoplasmic vacuolation of the hepatocytes, fatty degeneration and necrosis. Cirrhosis appeared in 40% of the patients before treatment and 40% and 30% after one week and 2 weeks of treatment, respectively. Immunohistochemical results revealed an increase in expression of p53, CD34 and Bcl-2 in HCC patients. Overexpression of p53, decrease of Bcl-2 and mild degree of expression of CD34 was recorded in patients treated with Dox. Significant increase in DNA fragmentation was recorded in HCC patients treated by Dox in comparison with untreated HCC.

Keywords: Bcl-2, Doxorubicin, Hepatocellular Carcinoma, Immunohistochemistry, P53

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide [1], ranks fifth in frequency worldwide among all malignancies and the third most common cause of cancer related death cause, 1 million deaths annually. [2] It accounts for more than 90% of all primary hepatic tumors [3] its incidence increases with age and is five times more common in men than in women. [4] Nonetheless, the incidence of HCC in Western countries is expected to increase owing to the high prevalence of hepatitis C virus infection that represents a high risk factor for HCC [5] the male and female ratio for HCC ranges from 2:1 to 6:4:1 and mostly 4:1. [6] In Egypt, male predominance may be explained by a high prevalent of hepatitis B surface antigen (HBsAg) carrier state, high sociability to environment carcinogenic factors and greater exposure to them. Chronic liver disease is more frequent in men than in women and this tendency become increasingly apparent as the disease progresses from chronic hepatitis to cirrhosis to HCC. [7] Median age at diagnosis is in the fifth decade of life in high incidence areas in Egypt, it presents at a somewhat older age in other regions. [8]

HCC is widely considered to be chemotherapy resistant. Response rates for single-agent chemotherapy are low, and durable remission is rare. The most commonly used single agents for HCC are the anthracyclines and anthraquinones doxorubicin [9], 4’- epidoxorubicin [10], and mitoxantrone. [11] These drugs consistently produce response rates of approximately 15% to 20%. Complete remissions have been described but are seldom durable. [12] Doxorubicin is the first generation anthracycline antibiotic of wide spectrum of action. At the cellular level, it is incorporated in between two nitric bases of double DNA helix, thus causing the inhibition of DNA
dependent DNA and RNA polymerases. [13] It is used as a single chemotherapeutic agent for HCC and has been shown to produce a response rate of about 10–15% but with no proven survival benefits. [14] The overall response rate from 13 published trials is approximately 20%, with a median survival of 4 months. [15]. The present work was conducted to study the immunohistochemical alterations in HCC patients treated by doxorubicin.

MATERIALS AND METHODS

Patients: The present study was performed at the National Cancer Institute, Cairo University, and Cairo, Egypt. The study met the criteria of the Ethics Committee of the National Cancer Institute, Cairo University, Cairo, Egypt. The study included 30 cases (24 males and 6 female) had a confirmed diagnosis of hepatocellular carcinoma (HCC). They were divided into 3 groups.

Group 1. Ten specimens of hepatocellular carcinoma patients were taken before doxorubicin treatments.

Group 2. Ten specimens of hepatocellular carcinoma patients were taken one week after doxorubicin treatment.

Group 3. Ten specimens of hepatocellular carcinoma patients were taken two weeks after doxorubicin treatment.

Treatment Schedule: Treatment schedule consists of i.v. injection of doxorubicin at a dose of 15 mg/m² weekly for 3 weeks. The cycle is repeated as long as the condition permits and the total dose of 500 mg/m² are not exceeded.

Histopathological study: Hepatic cases biopsies were obtained from HCC cases by a surgeon after computed tomography (CT) or magnetic resonance imaging (MRI) studies. A preoperative clinical diagnosis of primary liver cancer was made on the basis of an evaluated serum AFP level and characteristic features of the disease that were visible in the CT or MRI scans. For histological examination samples were fixed in 10% formalin. Following fixation, specimens were dehydrated through ascending series of alcohol, cleared in xylene and embedded in molten paraplast. Sections of 5 micron thickness were cut using rotary microtome, mounted on clear glass slides and stained with hematoxylin and counter stained with eosin.

Immunohistochemical studies: Immunohistochemical reaction was performed using an avidin biotin complex immunoperoxidase technique on paraffin sections. [16] p53, Bcl-2 and CD34 were detected using an anti human p53, Bcl-2 and CD34 monoclonal antibody (Dako A/S, Glostrup, Denmark), respectively. The mean percentage of p53 and Bcl2-positive tumor cells in all major foci of cancer was used as immunohistochemical scoring system.

Molecular Studies: DNA was isolated from the liver tissue of HCC patients according to Sato et al.[17] Liver tissue were digested by 600 µl of digestion buffer and incubated at 50°C for 12-18 hr then 3 µl RNase were added and incubated for one hr at 37°C. The samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation at 10000 rpm for 10 min, DNA was precipitated by adding 0.5 volume of ammonium acetate and 2 volume of 100% ethanol and centrifuged at 5000 rpm for 2 min. DNA was washed several times in 70% ethanol. The ethanol was then removed and the pellets dried. DNA was resuspended in 100 µl of TE buffer. The DNA was electrophoresed on agarose gel (1.3%) stained with ethidium bromide, then visualized by placing the gel on a UV transilluminator and photographed directly by a Polaroid camera. The UN-SCAN-IT gel documentation software was used for the quantitative of DNA bands.

Statistical Analysis: Sigma plot system was used for data analysis. Mean and standard error were used as descriptive measures. ANOVA was used for comparing means of independent groups. P value is significant if <0.05.

RESULTS

The mean age of patients with HCC was 65.1±12.1, 63±13.4 and 58.6±12.5 years in the first, second and third group, respectively.

Histopathological results: Histological examination of the specimen of HCC patients, before treatment showing trabecular and acini of polyhedral cords cells. Several features as degenerative hepatocytes accompanied by vacuolated and granular cytoplasm were observed. Most of the malignant cells having deeply basophilic or pyknotic nuclei (Fig.1a). Other lesions exhibit small karyolitic nuclei. Mitotic figure was detected in areas of the liver tissue which appeared with fat droplets (Fig.1b). Congested blood sinusoids are also identified. Moreover, a mild infiltrate of lymphocytic inflammatory cells in liver tissues are visible. Cirrhotic liver were observed in 4 patients of these group (table 1). The HCC consists of the tumor cells growing in sheets or small trabeculae that are
Examined the specimen of HCC patients after one week of Dox treatment showing hepatocytes with cytoplasm vacuolation and extensive areas of necrosis was evidenced. Moreover, giant hepatocytes were occasionally presented with granular cytoplasm and nuclei are polymorphic. The tumor cells showed trabecular structure. The cells are polymorphic with mild amount of weekly stained cytoplasm while, many other cells became swollen and hydropic (ballooning degenerative) (Fig 2a). Also, numerous intracytoplasmic inclusions are occasionally observed. In some parts of the lobule, few mitotic figures were noticed. The hepatic sinusoids are dilated in between trabecular pattern of the hepatic cells tissue. Cirrhotic nodule on abundant sheet was observed in 4 out of 10 patients. Specimens examined after 2 weeks of treatment with Dox showed severe degenerative changes of hepatocytes. The hepatic cells were necrotic, but others showed sign of hydropic degeneration or ballooning feature. Necro-inflammatory cells were seen in sinusoids (Fig 2b). The neoplastic cells showed acini formation and cells with pyknotic nuclei. Cirrhotic liver were observed in 3 out of 10 patients in this group (Fig 2c).

**Grading of tumor:** Three grades were detected in patients with HCC grade I, II and III. Patients with HCC before treatment contain 3 patients with grade I, 6 patients with grade II and 1 patient with grade III. While patients with HCC after one week of treatment contain 4 patients with grade I, 3 patients with grade II and 3 patients with grade III. The patients with HCC after two weeks of treatment contain 3 patients with grade I, 5 patients with grade II and 2 patients with grade III (table1).

**Immunohistochemical observations:** Microscopic examination of the specimens of hepatocellular carcinoma patients before treatment showed low nuclear expression of P53 (Fig 3a). Over expression of p53 was recorded in patients after one and two weeks of chemotherapy treatment (Fig3b). Figure 4 showed mean percentage of p53 immunostaining cells in HCC of different group. High expression of Bel-2 was detected in HCC patients (Fig 5a). This expression decreased significantly (p<0.05) after treatment with Dox (Figs.5b&6). Microscopic examination of endothelial sinusoidal cells of HCC patients before treatment showed over expression of CD34 (Fig 7a). Mild degree of expression of CD34 was recorded in HCC patients treated with Dox (Fig 7b).

**DNA fragmentation:** DNA isolated from HCC patients of different groups showed degradation into oligonucleotide fragments forming a clear laddering pattern of apoptosis (Fig 8). The data of DNA fragmentation of HCC patients after one week treatment showed a significant increase (37.2%), when compared with DNA fragmentation of HCC before treatment (21.9%). Highly significant (P<0.001) DNA fragmentation of HCC was recorded after two weeks of treatment with Dox (45.3%) (Fig 9).

**DISCUSSION**

Histological examination of the HCC patients before treatment showed that tumour masses are arranged in trabecular pattern and acinar formation. This histological frequency is consistent with what described by Kond [18] who found that HCC has been observed in different histological types, trabecular pattern was the most common type, followed by acinar formation and then solid and small cell type. The malignant hepatocytes have pyknotic nuclei, small prominent nucleoli and few mitotic figures associated with pale cytoplasm. Masamichi [19] reported that HCC revealed variable pattern formed by malignant hepatocytes with different grades of anaplasia with marked nuclear pleomorphism, bizarre cells, mild degree of mitosis, prominent nucleoli associated with scanty cytoplasm. Cirrhotic nodules surrounded by fibrous sheets were observed in HCC patients. Similarly, Roncalli et al. [20] reported that the explanted livers have more fibrosis so that any well differentiated HCC is surrounded by cirrhotic septa with distinct margin.

In the present work, after Dox treatment, the liver specimens of HCC patients showed extensive areas of necrosis, polymorphic cells, swollen or ballooning hepatocytes and accumulation of inflammatory cells. Moreover, intracytoplasmic inclusion was observed. These observations are in agreement with Fong et al. [21] who showed that Dox induced cells injury, most injury was confined to the centrilobular zone and characterized by: (i) abnormally swollen or ballooned hepatocytes, (ii) increased numbers of inflammatory cells in the areas of injury, (iii) necrotic cells and apoptotic bodies, and (iv) many cells showed disintegrated organelles. Some cases of the current study showed cirrhotic nodule on abundant sheet after Dox treatment. Similarly, Miliward-Sadler et al. [22] reported that cirrhotic areas with abundant fibrous stroma separating cords of tumor cells are...
most often seen following radiation, chemotherapy or infarction with subsequent scar formation.

The expression of p53 was significantly increased in HCC patients before treatment. In agreement with this result Mei et al. [23] found that the expression of p53 was increased in patients with HCC when compared to that of normal individuals. Meanwhile, this finding is in go parallel with Atta et al.[24] who found that p53 of HCC patients in Egypt were significantly higher than both liver cirrhosis patients and healthy control groups. Over expression of p53 was recorded in patients after one and two weeks of Dox treatment. Expression of p53 protein was seen in 12 cases out of 25 HCC patients treated with doxorubicin. [25] Elevation of p53 is seen with fatty liver in humans and during drug and chemically induced cellular injury as doxorubicin. [26] P53 tumor suppressor gene plays a major role in HCC. It is well known that inactivation of p53 is the most common genetic alterations in human cancer including HCC. [27] The p53 has a critical role in regulation of cell cycle, DNA repair and synthesis as well as in apoptosis. [28] The prevention of cancer is profoundly dependent on the p53 tumor suppressor protein. The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multicellular organisms. [29] Expression of Bcl-2 was highly significant in HCC patients before treatment. Frommel et al. [30] stated that Bcl-2 expression was significantly elevated in HCC patients as compared with normal healthy individuals. Osama et al. [31] reported that the expression of Bcl-2 protein in HCC patients is higher than in cirrhotic patients. On other hand, our results recorded significant decrease of Bcl -2 in patients who received Dox. This result is in agreement with Zhang et al. [32] who observed that rapamycin induce apoptosis via activation of caspase-3 and disruption of mitochondrial membrane potential, as well as by down regulation of antiapoptotic protein Bcl-2 and up-regulation of proapoptotic protein Bcl-xl on HCC cells. The anti-apoptotic role of Bcl-xL relates to its sequestration of the pro-apoptotic family members and prevention of the oligomerization required for the initiation of apoptosis. [33] Bcl-2 family members are membrane bound two Ca2+-sensitive organelles, the mitochondria and ER. BclXL regulates the inositol 1,4,5-triphosphate receptor Ca2+-release channel in the ER to antagonize apoptosis [34] and is up to 10 times more potent than Bcl-2 in antagonizing cell death. Endothelial sinusoidal cells of HCC patients before treatment showed marked expression of CD34. Ma-Jeeet al.[35] concluded that CD34 is a useful marker for distinguishing HCC from non cancerous liver tissue; and HCC showed a diffuse capillarization with over expression.Yang et al.[36] found that CD34 was expressed in the vascular endothelial cells of normal liver, paracarcinomatous tissue and HCC tissue in the following proportions of specimen: 86.7% , 93.8% and 100 % respectively . However, they found expression of CD34 in both normal and tumor tissue indicating that it was not a reliable marker for HCC. Examination of the specimen of HCC patients after Dox treatment revealed that CD34 was decreased gradually from moderate to mild degree. Wang et al. [37] have also shown that rapamycin, significantly reduced microvessel density and inhibited tumor angiogenesis in HCC.

Angiogenesis is an essential mechanism for HCC growth and metastasis, and its inhibition is an attractive target for investigators. Vascular endothelial growth factor (VEGF) and its tyrosine kinase receptor are the principal molecules involved in endothelial cell proliferation, survival, formation of new blood vessels, and vascular permeability. [38] mTOR proteins regulate the phosphorylation of p70S6 kinase and the translational repressor protein PHAS- 1/4E-BP. Both proteins regulate the translation of proliferation and angiogenesis-relevant proteins, such as c-myc, cyclin-D1, ornithine decarboxylase, HIF-a, and are indirectly involved in the expression of vascular endothelial growth factor. [39] The present findings strongly supported this theory because we found a significant inhibition of tumor angiogenesis after Dox treatment.

The data of DNA fragmentation of HCC patients after one week treatment showed significant increase (37.2%), when compared with DNA fragmentation of HCC before treatment (21.9%). But DNA fragmentation of HCC recorded a high score after two weeks of Dox treatment (45.3%). Huang et al. [40] showed that cleavage of DNA into large fragments may be an initial event that is critical for drug induced apoptosis.Deders et al. [41] indicated that cleavage of cellular DNA at the intranucleosomal sites has been observed in cells undergoing apoptosis induced by many agents and physiological conditions, this cleavage produced DNA fragmentation. Induction of apoptosis in HCC by Dox was reported by Fong et al. [21] The mechanisms of anthracycline cytotoxicity, particularly of Dox, in cancer cells include: (i) intercalation into DNA with inhibition of DNA replication and RNA transcription; (ii) generation of free radicals with DNA damage and lipid
peroxidation; (iii) DNA binding and arylation; (iv) DNA crosslinking; (v) interference with DNA unwinding, DNA strand separation, and helicase activity; (vi) direct membrane damage due to oxidation of lipids, and (vii) inhibition of topoisomerase II.

Dox is metabolized in microsomes by cytochrome P-450, lead to generation of semichinon, which is the free radical responsible for cytotoxic activity of Dox.

In conclusion, our results suggest that Dox metabolism triggered production of ROS and reactive intermediates in liver resulting in oxidative stress and genomic injury followed by apoptosis which was associated with down regulation of Bcl-2, release of cytochrome c from mitochondria into the cytosol and increases p53 protein levels.

CONCLUSION

Our results suggest that Dox metabolism triggered production of ROS and reactive intermediates in liver resulting in oxidative stress and genomic injury followed by apoptosis which was associated with down regulation of Bcl-2 and increases p53 protein.

Conflict of interest statement
We declare that we have no conflict of interest.

Fig.1.a). Section of HCC patient before treatment showing trabecular (T) and acini formation (A),(H&EX400).b).Section of HCC patient showing invasive hepatic carcinoma with few mitotic figure (M) and fat droplets (FD) (H&EX400).c).Section of HCC patient showing cirrhotic areas (C) and fibrous sheath (F) (H&E 200).
Fig. 2: a). Section of HCC patient after one week of Dox treatment showing neoplastic cells in trabecular form with giant cells (G). Intracytoplasm inclusions and mitotic figure (M) were seen. b). Section of HCC patient after two weeks of Dox treatment showing hepatocellular acini and sinusoids with inflammatory cells (I). c). Section of HCC patient after two weeks of Dox treatment showing cirrhotic nodules (C) (H&E X400)
Fig. 3. a). Immunostained of hepatocellular carcinoma section before treatment showing expression of p53. b). Over expression of p53 in HCC patient treated with Dox. (X 400).

Fig. 4. Mean percentage of p53 immunostaining cells in HCC of different group
Fig. 5. **a)** Immunostained of hepatocellular carcinoma section before treatment showing marked expression of bcl-2. **b)** Low expression of bcl-2 in HCC patient treated with Dox, (X 400).

Fig. 6. Mean percentage of bcl-2 immunostaining cells in HCC of different group.
Fig. 7. a). Immunostained of hepatocellular carcinoma section before treatment showing high expression of CD34. b). Immunostained of hepatocellular carcinoma section after Dox treatment showing mild degree of CD34 expression (X400).

Fig. 8. Gel electrophoresis of DNA fragments derived from HCC before treatment (lane 1), after one week of Dox treatment (lane 2) and after 2 weeks of Dox treatment (lane 3), M: Marker.
Fig. 9: Mean percentage of DNA fragmentation in HCC of different group

Table 1: Patients with cirrhosis and HCC grades in different group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of patients with cirrhosis</th>
<th>Grade of tumour</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>HCC</td>
<td>4</td>
<td>3</td>
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<tr>
<td>HCC+1 week Dox</td>
<td>4</td>
<td>4</td>
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<tr>
<td>HCC+2 weeks Dox</td>
<td>3</td>
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