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RESEARCH ARTICLE

Reversal of EMT by regulation of miR-200c through treatment of metformin and resveratrol in DEN-induced rat HCC.

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Abstract

Epithelial mesenchymal transition (EMT) is a powerful process in which epithelial cells undergo a transcriptional reprogramming and morphological changes that acquire a mesenchymal phenotype which have reported to enhance the migratory properties. The aberrant expression of miR-200 family was reported to play a role in cancer and EMT. The metastatic inhibitory role of the miR-200 family is strongly associated with a pathologic EMT. In the present study, we compared the expression levels of miR-200c between Doxorubicin+ Resveratrol+ Metformin (Dox+Res+Met) and Dox resistant DEN-induced rat HCC, and investigated whether the treatment of rats with resveratrol and metformin could affect the expression of miR-200c. The expression level of miR-200c was significantly down-regulated in Dox-resistant rats that showed EMT characteristics such as lower expression of epithelial marker E-cadherin, and higher expression of mesenchymal markers such as vimentin and ZEB1 which may correlate to Dox resistance. Overexpression of miR-200c in resistant HCC rats restored their sensitivities to Dox, leading to acquisition of mesenchymal-epithelial transition-like phenotypes, including up-regulation of E-cadherin, down-regulation of ZEB1 and Vimentin versus Dox-treated group. These results provided experimental evidence that resveratrol and metformin could function as miRNA regulators leading to the reversal of EMT phenotype, which is likely to be important for designing novel therapies for HCC.

Introduction:

Hepatocellular carcinoma (HCC) is the primary malignancy of the hepatocyte that commonly affects adults. It is the fifth most common solid tumor, affecting over 600,000 people each year worldwide, with major risk factors such as hepatitis B and C infections, alcohol abuse, primary biliary cirrhosis, and diabetes. Currently, there are no effective adjuvant therapies for HCC and control of HCC at initial stage is the most effective therapeutic strategy (Li et al. 2015). Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells undergo a transcriptional reprogramming and morphological changes that acquire a mesenchymal phenotype which have more migratory properties (Panebianco et al. 2014). EMT is a process where epithelial cells lose both polarity and cell- cell contacts (Guo et al. 2014). Cells undergo EMT phenomenon acquire a mesenchymal phenotype, which is characterized by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin and N-cadherin (Lamouille et al. 2013; Guo et al. 2014). A major event in EMT process is inhibition of E-cadherin induced by a number of transcription factors, belonging to the families of SNAIL, TWIST and ZEB (Yang et al. 2013). Down-regulation of E-cadherin leads to the release of β-catenin which translocates into nucleus and activates target transcription factors, promoting cellular adhesion, and cancer progression. Other proteins that mediate EMT include vimentin (Guo et al. 2014). Down-regulated expression of E-Cadherin, and up regulated expression of vimentin, can be used as markers for epithelial cell undergoing EMT (Zeisberg et al. 2009).

MiRNAs target transcription factors like E-cadherin and other EMT regulators. Different molecules act as the repressor of E-cadherin expression including ZEB, Twist, Snail, Slug and TGF-β. The miR-200 family (miR-200a/200b/200c/141/429) has been shown to inhibit cell migration and invasion via targeting ZEB in different types of cancers. MiR-200 inhibition was reported to reduce E-cadherin level while promoting vimentin expression. MiR-200 and ZEB form a reciprocal repression loop where ZEB repressed miR-200 expression while miR-200 targets ZEB (Guo et al. 2014; Chan & Wang 2015).

MiR-200 family composed of 5 miRNA members: miR-200a, miR-200b, miR-200c, miR-141 & miR-429 which were shown to be down-regulated in cancers. Higher levels of miRNA-200 associated with expression of E-cadherin (D´ıaz-L´opez et al. 2014; Feng et al. 2014). Over-expression of miR-200 induced E-cadherin up-regulation and inhibited EMT through targeting ZEB1 and ZEB2 (Zaravino 2015; Patrycja & Bozena 2015).

Resveratrol (3, 5, 4' - trihydroxy –Trans-stilbene) is a dietary polyphenol derived from grapes, peanuts and other plant sources. It also presents in blue berries, and red wine (Shukla & Singh 2011) .Fresh grape skins contain between 50 and 100 mg resveratrol/g (Bishayee & Dhir 2009). There are limited clinical trials with small sample sizes, and animal models. Little evidence exists about resveratrol as an effective anti-cancer property; therefore, the most use of resveratrol is a as cancer preventative agent (Carter et al. 2014). In both early and advanced stages of HCC, resveratrol treatment increased expression of apoptotic markers and decreased expression of anti-apoptotic markers. Resveratrol treatment at both time points also alteration in cellular architecture as well as decreased liver size compared with control rats treated with DENA (Rajasekaran et al. 2011). Recent report indicated that resveratrol treatment up-regulates miR-141 and results in a reduction of invasiveness. Furthermore, resveratrol-induced miR-200c expression induces reversal of EMT through inhibition of Zeb1 and stimulation of E-cadherin. Moreover, the synergistic antitumor activity of resveratrol and miR-200c has been demonstrated in human lung cancer cells (Masaika et al. 2015).

Metformin is anti-hyperglycemic agent which is derived from hypoglycemic galegine. It is found naturally in goat's rue (*Galega officinalis*), and considered as the front-line therapy for type II diabetes (T2D) (Hung et al. 2015). The anti-cancer effect of metformin was associated with activation of adenosine monophosphate-activated protein kinase (AMPK). In addition to, metformin may also inhibit HCC by regulating cell-cycle proteins, such as cyclin E & cyclin D1 (Cheng et al. 2014). C-myc was suggested as a critical mediator in HCC. However, no study among animal studies discussed such mechanism underlying specific HCC stage (Li et al. 2015).

Several works revealed that metformin can exert anticancer effects through miRNA modulation. It has been shown that metformin increased the number of murine embryonic fibroblasts entering a senescent stage in response to doxorubicin treatment. This occurs through metformin-induced modulation of mir-200. Pulito and his colleagues (2014) showed that pancreatic cell lines treated with metformin re-expressed some miRNAs that are usually switched off in pancreatic cancer, such as miR-200 family which plays a role in EMT and in maintaining the stem cell state. They also reported that metformin inhibited tumor sphere formation by deregulating CSC markers (CD44, Notch-1, Nanog, and Oct4) through the up regulation of miRNAs **(**Pulito et al. 2014).

Herein we report the efficacy of a new strategy to inhibit EMT through modulation of mir-200c combined treatment using resveratrol and metformin in addition to conventional chemotherapy. HCC in rats were established and the target signaling pathways of miR-200c which is involved in MET by regulation of the targets E-cadherin, vimentin and ZEB1 were investigated.

Experimental design and methods:

Animals and HCC induction:

Sixty male Sprague Dawley rats were used in the current study. Rats were purchased from the laboratory animal facility of the Medical Research Institute at Alexandria University at the age of four weeks. Rats were housed in wire cages under controlled conditions of humidity (50-60%), lighting (12h light/dark cycles) and temperature of 25ºC with free access to water and basal diet (ad libitum and corn). Animals fed palatable, non-contaminated and nutritionally adequate food daily according to their particular requirements. The rats were treated and handled according to the ethical committee recommendations for the handling regulations of experimental animals at Medical Research Institute.

Rats were divided into four major groups; 1- mock-treated control rats, that received an empty vehicle of the different administered chemicals. Control rats for DEN injection that received either single intra-peritoneal injection (I.P) of 0.9% NaCl or for resveratrol treatment, received 100mg daily by oral gavage. 2- Induced group, were prepared by single I.P of N-diethyl nitrosamine (DEN) at a dose of 200mg/Kg. body weight (in 0.9% NaCl; Sigma, St Louis, Missouri, USA). Tumor initiation and induction were performed as described before by administration of DEN (Afzal et al. 2012; Alwahaibi et al. 2010; Muzio et al. 1999). Two weeks from DEN injection, at the age of 10 weeks, tumor promotion was achieved by subcutaneous injection of CCl_4 (0.2ml/100g) twice/week continuously for five months after DEN injection. 3- Chemo-preventive group that received resveratrol daily by oral gavage. Resveratrol was administered in a dose of 50 mg that was increased gradually up to 100 mg as previously described (Bishayee et al. 2010; Bishayee & Dhir 2009). Treatment with resveratrol was initiated four weeks prior to DEN injection and continued for 20 consecutive weeks. The fourth group represents the treated rats. Treatment was established using doxorubicin (Dox) (purchased from Sigma- Aldrich) as a conventional chemotherapy. Other induced rats were treated by addition of resveratrol and metformin to Dox. Treatment regimen was performed according to the following protocol; Metformin was administered in reduced dose of 125 mg/kg (3 times weekly for 3 weeks) based on previous study by Afzal et al. (2012) both in treated and protected groups and resveratrol in a dose of 100mg/kg body weight in addition to Dox.

Samples collection and HCC development follow up:

Following DEN injection, at the intervals of 6 weeks, two to three rats from each group were sacrificed and AFP test was assessed as well as the histo-pathological examination to monitor HCC development. Biochemical (data not shown) and molecular analyses confirmed the tumor development at the first week of the sixth month. A group of HCC-induced rats were sacrificed at the end of induction duration and treatment group were continued to receive the different proposed treatment regimen. A blood sample was collected by cardiocentesis under anesthesia and serum was separated for biochemical analyses. Liver tissues were removed, washed with sterile saline and dissected then stored at -80^{o}C for future analyses.

Histological examination of Hepatic Tumors:

Sections from the dissected tissues were collected and fixed immediately in 10% formalin buffered with 0.1M phosphate buffer (pH 7.2). Each paraffin section (4µm thickness) was prepared and stained with hematoxylin and eosin stain (H & E).The specimens were microscopically examined for the presence of HCC nodules.

RNA extraction and quantitative real time PCR assay

Part of the dissected frozen liver tissues (adjacent normal tissues and HCC tissues) were chopped on sterile glass plate, and transferred to an Eppendorf tube containing Biozol reagent (Invitrogen CA, USA) (Alizadeh et al. 2010; Mou et al. 2013). Extraction protocol was applied as indicated by the manufacturer guidelines. In brief, tissue samples were homogenized and incubated on ice for 15 min. Glycogen was added to each homogenate to a final amount of 1ug. Chloroform was added, vortexed and the mixture was incubated on ice for 15 min. Aqueous supernatant was collected after centrifugation and transferred to nuclease free Eppendorf tube. Equal volume of cold isopropyl alcohol was added to sample tube to precipitate the RNA content. The precipitated RNAs were washed and treated with DNAase whenever it was necessary to obtain pure preparation. Prepared RNA samples were stored as aliquots at -80^{o}C for future analyses.

Quantitative real time PCR (qRT-PCR) was applied to measure the alterations in the expression of mir-200c. CDNA synthesis was carried out using miScript II RT Kit (Qiagen) according to the manufacture guidelines. The primers for mir-200c: F-GCTTGGATTGGGTAAAGGA, R-TTCTTTGTCTGATGGAGCTG, and U6 (internal control): F-GGAACGATACAGAGAAGATTAGC, R- AAATATGGAACGCTTCACGA. The expression level of mir-200c was normalized to U6. Results were expressed as fold difference from control that is calculated as described by Livak and Schmittgen (2001).

Western blotting:

Western blotting analysis of ZEB1, E-cadherin, Vimentin and β-actin were performed according to Xu et al (2013) method on prepared total cell extracts. Briefly, 75µg protein lysates from each sample was mixed with 2x loading buffer (130 mM Tris-HCl (pH 8), 30% (v/v) glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, 2% DTT), boiled for 5 min then cooled at 4 C for 5 min. Samples were separated on 12% SDS-PAGE mini gel and run at 120 V. proteins were transferred plotted to nitrocellulose membrane using wet transfer system. The membrane was washed then incubated in blocking buffer (containing 5% nonfat dry milk) for 1 hour at room temperature and then

incubated overnight in primary antibody to Zeb1, E-cadherin, Vimentin or β-actin. Antibody binding was detected by using the appropriate HRP-conjugated secondary antibody. Bands were detected after probing the membrane based on chemiluminescence detection method, and equal loading was confirmed by probing with β-actin monoclonal antibody (sc-81178).

Statistical analysis:
All obtained and represented data are the results of at least three independent experiments for each condition. SPSS16.0 analysis software was used for the statistical analysis preparation. Statistical evaluation of data was tested by one-way ANOVA and t-test for comparison of differences between the two groups where equal variances are not assumed. A value $p<0.05$ set to evaluate a statistical difference. Data were presented as the mean ± standard deviation (SD).

Results:

Histopathological analysis using H&E stain indicated that liver of control rats revealed normal parenchymal cells with granulated cytoplasm and small nuclei arranged around central vein. Animals subjected to DEN/CCl_4 regimen showed marked loss of normal architecture with oval or irregular–shaped hepatocytes then transformed hepatocytes of foci and nodules become enlarged, vesiculated and binucleated. Many nuclei were hyperchromatic (basophilic) with central nucleoli. Furthermore, vacuolation was observed in cytoplasm around nucleus with acidophilic (eosinophilic) material. Sinusoids were greatly dilated with hyperplastic cells. Three months after DEN –induction, liver tissues showed hyper mitotic activity, irregular sinusoids and hyperchromatosis, where after 4 months of DEN, liver tissues showed nodule trabeculae of malignant cells in the form of regeneration with abnormal mitosis and hyperchromatosis, hyperplasia. Six months of DEN administration, liver tissues showed diffuse and well differentiated malignant cells in the form of bizarre neoplastic cells. Hepatocellular carcinoma showing diffuse malignant fossci cells with multinucleated cells, abnormal mitosis hyperchromatosis were developed after seven months. Damage to hepatocyte structure integrity induced by DEN is supported by our histopathological findings, where hepatic hemorrhage associated with hyperchromatic stain, hyperplasia, necrosis, proliferating hepatocytes were observed. Representative photos are shown in fig 1.

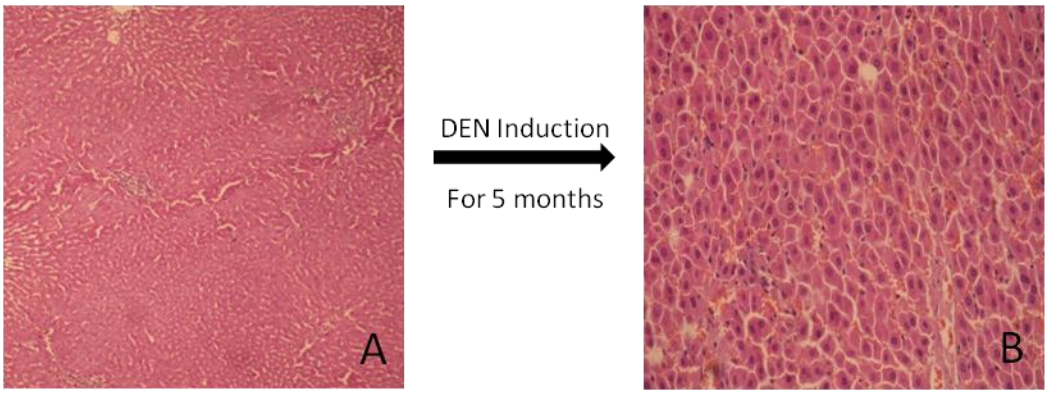

Fig (1): Demonstrate the microscopic morphological differences in the appearance of normal liver and tumor nodules. Hepatic histopathological malignant features induced by DEN (at the end of month-5) is represented in Figure 1-B, compared to mock-treated that is shown in figure 1-A.

Effect of resveratrol and metformin combination addition to Doxorubicin in DEN-induced HCC
In the current study, resveratrol and metformin (Res+Met) combination were added to the conventional chemotherapy doxorubicin (Dox) to test their possible synergistic effect as chemo-preventive and chemo-therapeutic agents. Microscopic histological analysis (H&E stain at 200X resolution) of DEN-induced rats` tissues that were treated with resveratrol and metformin combination as chemo-preventive agents, before and co to DEN-induction, showed dense lymphocytic infiltration of portal tract by inflammatory cells (fig 2-B), which indicate their hepato-protection effect compared to induced rats (fig 2-A). Treatment with doxorubicin (Dox) as chemotherapeutic alone showed apoptotic cell death and inflammation of normal cells and necrosis of cancer cells (fig 2-C). The administration of Res-Met combination with Dox for three weeks as therapeutic agents against DEN-induced HCC, showed that the hepatocytes preserving or maintaining near-to-normal architecture, moderate necrosis, marked

disappearance of tumor tissue with infiltration of lymphocytic cells (fig 2-D). The Res+Met positive control group, that received the combination for the duration of the study showed the presence of clear cell foci rather than basophilic/eosinophilic foci. Generally liver tissue maintained near normal architecture (data not shown).

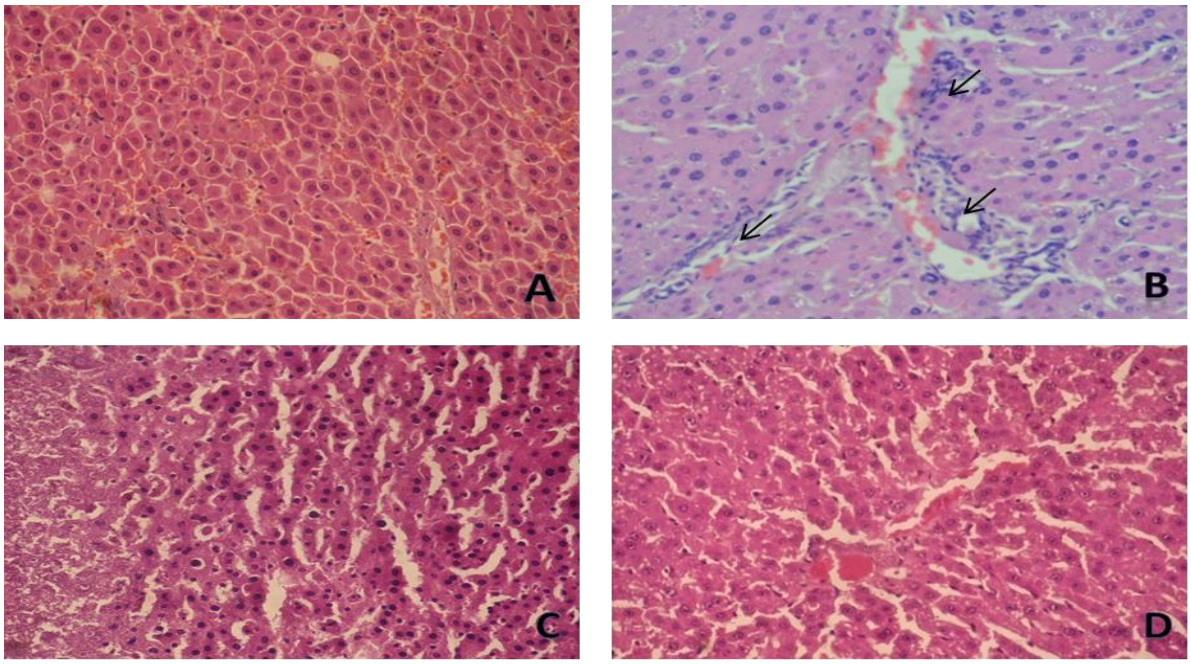

Fig (2): Representative microscopic photos of H&E stained liver sections of the different experimental groups. Hepatic histopathological malignant features induced by DEN in rats at resolution of 200X. A) Demonstrates DEN-treated rats, showing diffuse and well differentiated malignant cells in the form of bizarre neoplastic cells. B) Show lymphocytic infiltration in the chemo-preventive group that was administered Res-Met before and co to DEN administration. C) Demonstrates Liver tissues showing apoptotic cell death and inflammation of normal cells and necrotic cells of cancer in Dox treated group. D) Show marked disappearance of tumor with lymphocytic cells in Res-met treated group which indicate that resveratrol and metformin combination enhanced the therapeutic effect of Dox.

Mir-200c expression profile in induced and treated rats:

The results showed that the expression level of miR-200c in DEN-group was significantly decreased (** $p<0.01$) with around 40 percent reduction compared to mock-treated rats at week-4 of DEN induction. Mir-200c expression was continued to reduce significantly in a time-dependent manner over the period of 20 weeks versus control (fig 3). On the other hand, the expression level of miR-200c in Dox treated-group was not significantly increased ($p>0.05$) compared to DEN-group at week-20. Dox resistance was encountered by co-addition of Res and Met that significantly induced the expression of mir-200c (***$P<0.01$) compared to induced rats at week 20.

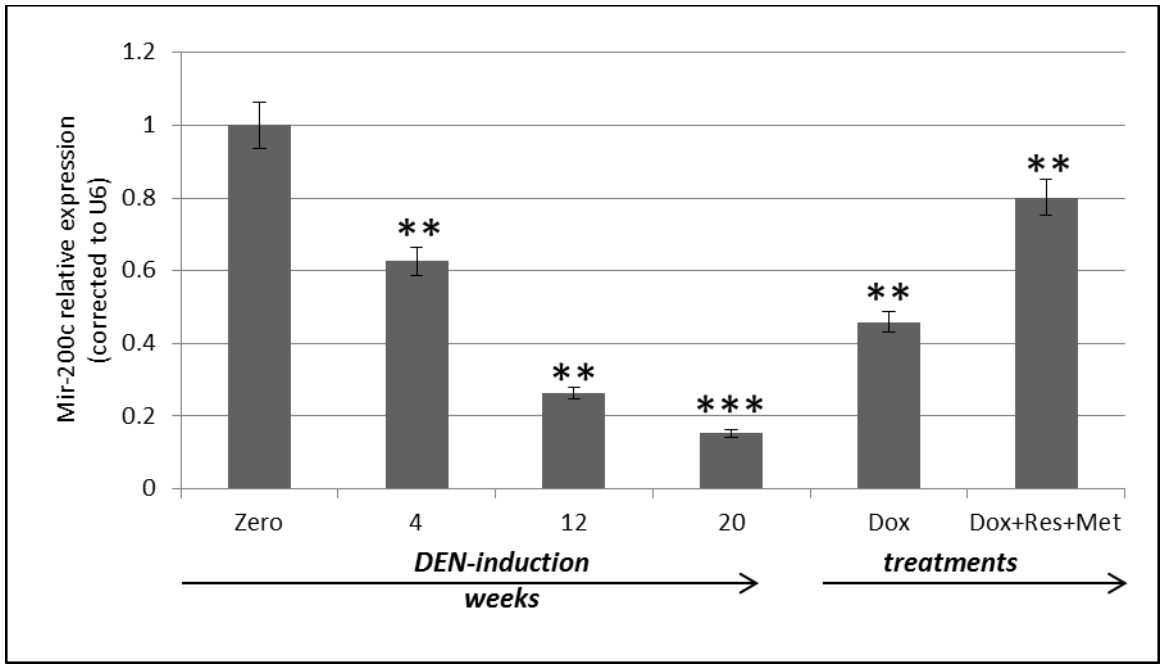

Fig (3): Illustrate the relative expression level of mir-200c, using quantitative real time-PCR. Expression profile in the different experimental groups indicated that Dox injection with resveratrol and metformin induced significant ($p<0.01$) expression of mir-200c that is implicated, in part, in the process by which cancer cells overcome resistance.

Expression of Epithelial mesenchymal transition (EMT) markers:

Expression profile of Epithelial-mesenchymal and mesenchymal-epithelial transitions markers (EMT and MET, respectively) including E-cadherin, ZEB1 & Vimentin were detected using western blotting in the prepared tissue homogenates of tumor and adjacent (non-tumor) tissues. Representative blots are shown in figures 4 and 5. A follow up analyses from zero time (control) over 20 weeks of DEN-induction indicated the involvement of E-cadherin down-regulation in hepatocytes carcinogenesis. As shown, a marked reduction in its protein level is observed overtime (fig 4). Furthermore, the expression levels of the positive EMT markers, ZEB1and Vimentin, were shown to be markedly detected with carcinogenesis progression from weeks 4 to 20 compared to control rats (fig 4).

Treatment of HCC with Dox alone did not induce strong shift in the expression profile of EMT versus MET markers compared to tumor samples (fig 5). On the contrary, a stronger expression of ZEB1 and Vimentin proteins were observed. Adding Res and Met to Dox enhanced tumor cell death and differentiation of normal cells, as indicated by marked expression of E-cadherin and reduction in ZEB1 and Vimentin in a time-dependent manner (fig 5).

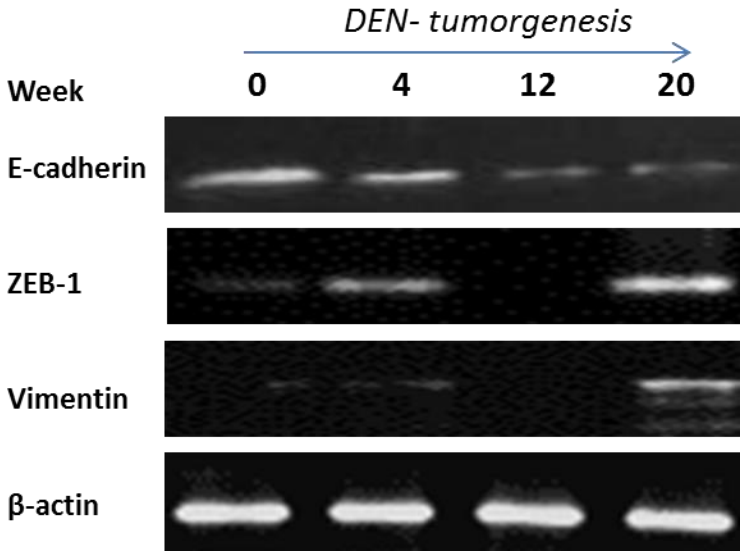

Fig (4): Protein expression of E-cadherin as a marker of MET and of ZEB1 & Vimentin as EMT markers. Western blotting analysis at weeks zero, 4, 12 and 20 of DEN induction indicated that E-cadherin protein level is down-regulated in a time-dependent manner. ZEB1 and vimentin expressions were detected over time and markedly at week 20

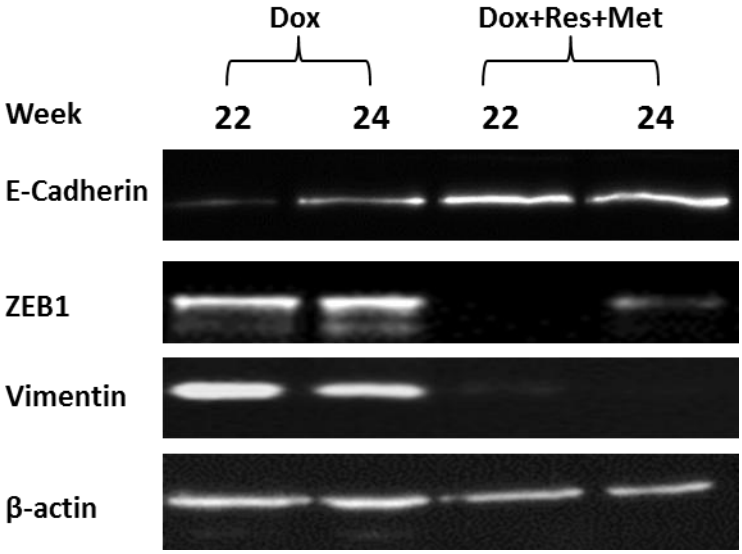

Fig (5): expression of EMT and MTE markers in the treated groups. Dox treatment did not induce significant inhibition in the expression of ZEB1 and vimentin over 4 weeks. Addition of Res and Met to Dox inhibited markedly the expression of both proteins and induced E-cadherin markedly.

Discussion:

HEPATOCELLULAR CARCINOMA (HCC) is an aggressive hepatic neoplasm and one of the most frequent primary cancer of the liver at which its incidence rate has been highly increased to become the fifth highest mortality rate and the most common malignancy worldwide (Zhong et al. 2014). HCC pathogenesis is multi factorial, highly associated with several risk factors, although it is mainly developed after exposing of cellular machinery to mutations resulting in cell proliferation rapidly and avoiding apoptosis (Younis et al. 2013).

The main therapy of HCC is surgical resection and liver transplantation (Zhu et al. 2013). For non-resectable HCC, several therapies were developed such as chemotherapies by 5-Flouro-uracil, doxorubicin (Dox) which directly applied or delivered through drug encapsulation technology (Ali et al. 2015). Recent studies have displayed that Dox is one of the most widely and successfully used anti-cancer drugs. Despite of its well therapeutic effects, its clinical applications are still limited because of its cumulative and dose-dependent cardiac toxicity (Ali et al. 2015).

In the present study we aimed to investigate the protective and therapeutic effect of resveratrol (Res) and metformin (Met) against DEN-induced HCC in rats. The initiation–promotion or two-stage HCC model resembles the early events of period of human HCC (Bishayee A & Dhir 2009). In this study, we analyzed the inhibitory effect of resveratrol and metformin on the appearance of early pre-neoplastic events in liver, employing two-stage HCC model combining DEN/ CCl_4. The histopathological examination of liver tissues, displayed the hepatic nodules or HCC lesions in rats administered DEN. The same observation was documented by different studies (Zhao et al. 2014). The histopathological features evaluation revealed that resveratrol and metformin were effective in reducing DEN- induced hepato-carcinogenesis as strong chemo-preventive agents in a dose-dependent manner for long period.

The inhibition of nodule growth and enhancement of their regression by Res+Met as evaluated in our study may be important for HCC chemoprevention. Histological findings clearly showed that the normal architecture of liver tissue was damaged due to DEN/CCl_4 treatment. The hyperplastic nodular hepatocytes were shown to form aggregates of cellular thickness with hyperbasophilic foci around portal vein. Acidophilic cells form altered hepatocyte foci, represent pre neoplastic focal lesions leading to malignant transformation in later stages of HCC with formation of neoplastic nodules and finally HCC nodules. Exposures to long period resveratrol and metformin treatment lead to a reduced hepatocyte aggregation and basophilicity with a reversal of heterogeneity towards normal cellular architecture (Bishayee A & Dhir 2009; Afzal et al. 2012).

Metastases are the main cause of 95% of human cancer deaths. An important characteristic of metastasizing cells is their transition from an epithelial phenotype to a mesenchymal phenotype (Książkiewicz et al. 2012). Cells undergo epithelial–mesenchymal transition (EMT) phenomenon acquire a mesenchymal phenotype, which is characterized by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin and N-cadherin (Lamouille et al. 2013; Guo et al. 2014). A major event in EMT process is inhibition of E-cadherin induced by a number of transcription factors, as SNAIL, TWIST and ZEB (Yang et al. 2013). Down-regulated expression of E-cadherin, and up-regulated expression of vimentin, can be used as markers for epithelial cell to undergo EMT (Zeisberg et al. 2009). The miR-200 family (miR-200a/200b/200c/141/429) has been shown to inhibit cell migration and invasion via targeting ZEB in several cancer types. MiR-200c inhibition was reported to reduce E-cadherin level while promoting vimentin expression.

The miR-200 family, are known for their role in suppressing EMT, and proved to play an additional role in mediating cancer cell response to traditional therapeutic regimens. MiR-200 and ZEB form a reciprocal repression loop where ZEB repressed miR-200 expression while miR-200 targets ZEB (Guo et al. 2014; Chan & Wang 2015). Our study exhibited the same observations where we investigated the role of miR-200c in HCC and its relation with E-cadherin, vimentin and ZEB1 through qRT- PCR analysis of mir-200C expression profile in different experimental groups as shown in the different experimental groups. These findings indicated the tumor suppressor functions of this miRNA. These findings are in agreement with previous reports (D´ıaz-L´opez et al. 2014; Feng et al. 2014; Zhang et al. 2015; Shao et al. 2015) that confirmed down-regulation of miR200c in many types of cancer such as HCC. Protein expression of EMT markers ZEB1 and Vimentin were detected in HCC tumor lesions over 20 weeks' period. As tumor development progresses there was an increase in their expressions associated with marked reduction in E-cadherin. These findings suggested that ZEB1 and Vimentin were up-regulated whereas E-cadherin was down regulated during HCC development which increases HCC progression and metastasis through reversal of epithelial phenotype to mesenchymal phenotype (EMT) that involved in doxorubicin resistance (fig 6). These findings are consistent with Lamouille et al. (2014) and Nickel & Stadler (2015) studies.

MiR-200c functions as a strong antagonist to self-renewing capabilities as it targets BMI1, an oncogene necessary for self-renewing cell divisions that is also a suppressor of p53 activity (Ahmad 2013). Soubani et al (2012) found that re-expression of miR-200C was consistent with up regulation of PTEN expression, suggesting that miR-200c could regulate PTEN expression. Other studies have reported that over expression of PTEN could inhibit VEGF expression through antagonizing PI3K/Akt pathway (as reviewed in Tian et al. 2010).

In conclusion, our results indicate positive correlation between resveratrol/metformin administration and the induction of miR-200c expression. Novel therapeutic approaches that can potentiate the effect of the conventional chemotherapeutic agents that act to overcome cancer resistance to drugs. In this study, our findings documented that miR-200 is a potent protector against DOX-induced toxicity probably via regulating the expression of MET. Further studies are needed to investigate the functional roles of Dox-met-res in regulation of the growth and other cellular processes of liver cells.

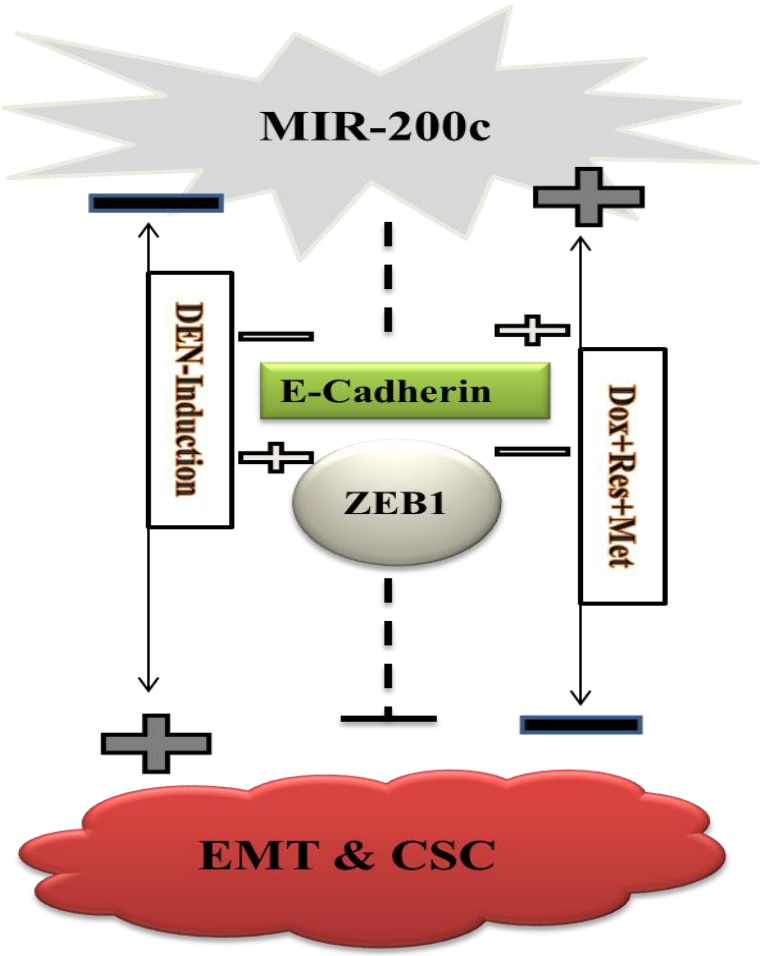

Fig (6): Representative illustration of the possible mechanism by which resveratrol and metformin potentiate HCC sensitivity to conventional chemotherapy treatment.

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