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RESEARCH ARTICLE

FOXA2 DNA Binding Domain Mutation and MiRNA-124 Differential Expression in Relation to Sexual Dimorphism of Hepatocellular Carcinoma in Egyptian Patients.

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Abstract

Hepatocellular carcinoma (HCC) is sexually dimorphic in mammals. Forkhead box A2 (FOXA2) binding sites' mutations exhibited impairment in binding between FOXA2, hormonal receptors and their target genes. Recent studies of microRNA (miRNA) expression have demonstrated miRNA-124 dysregulation in hepatocellular carcinoma (HCC). Molecular mechanism underlying sexual dimorphism remains unclear. Here, we investigated FOXA2 DNA binding domain (DBD) polymorphism and the status of miRNA-124 expression in Egyptian patients. **Patients/Methods:** 78 patients were classified into three groups equally matched in sex; HCC, cirrhotic and control subjects. Direct Sequencing of FOXA2 DBD was conducted, followed by mutation frequency calculations. Quantitative Real-time PCR method was used to assess the expression levels of miR-124. **Results:** Two detected significant single nucleotide polymorphisms (SNPs), (c.3699G>T) and (c.3708G>T) were identified in FOXA2 DBD of 14% and 7%, respectively in both HCC and cirrhotic cases. Computationally, these SNPs were found to have no effect on the structure of FOXA2 protein. On the other hand, miRNA results showed that decreased expression of miR-124 was significantly HCC followed by cirrhotic groups versus control group in both sexes. It was additionally documented that reduction in miRNA-124 expression is more prominent in females than males ($P < 0.05$).**Conclusion:** These findings suggest that FOXA2 DBD alterations are not correlated with sexual dimorphism as protein structure is not affected. But, they may be correlated with HCC progression due to DNA repair disorders. On the other hand, miR-124 reduced expression has prognostic value in hepatocellular carcinoma and may serve as a possible prognostic marker for liver cancer.

Introduction

Hepatocellular carcinoma (HCC) represents 83% of all cancer cases worldwide **(Farazi and DePinho, 2006).** In 2010, HCC was listed as the third most diagnosed and the second lethal cancer after lymphoma worldwide. In Egypt, HCC is the first most common cancer in men and the fifth most common cancers in women **(Amal *et al.*, 2010).** HCC is a characterized, global gender disparity disease, with a higher frequency in men than women by two to four folds; subsequently HCC is known to be sexually dimorphic **(Jemal *et al.*, 2011).** This female protection appears to be hormonal-dependent, as indicated by the increase in liver cancer incidence among women who undergo menopause **(Mucci *et al.*, 2001).** Some studies addressed the molecular mechanisms of this dimorphism in a challenge to clarify the underlying causes to this phenomenon, but it is still unclear (**Berasain *et al.*, 2009).**

Transcription factors (TF) are proteins necessary for transcription initiation and regulation in all living organisms. Specific TFs have specific characteristics, such as the presence of a DNA binding domain (DBD), a trans-activation or trans-repression effector region. Such specific TFs are classified according to the structure and homology degree of their DBD **(Benayoun *et al.,* 2011).** Examples of winged helix DBD containing proteins is the eukaryotic families of 'Forkhead' TFs. Functional studies of the winged helix proteins began with the discovery of the hepatocyte nuclear factor-3 (HNF-3) family of liver-specific TFs **(Costa *et al.,* 1989).** According to the homology basis within DBDs, the TFs were classified into related protein families, which include the first identified member, HNF-3α gene known now as FOXA1 **(Lai *et al.,* 1990).** Two other members of this TF family were identified following FOXA1 which are HNF-3β and HNF- 3γ named as FOXA2 and FOXA3, respectively **(Liu *et al.,* 1991).**

Georges *et al.* (2010) revealed that Forkhead factors are cable of identification of a DNA consensus sequence of seven base pairs [5'-(G/A)(T/C)(A/C)AA(C/T)A-3']**.** FOXA2 protein binds to discrete DNA sites and the protein recognition specificity is determined by a 20-amino-acid region, localized adjacent to the DNA H3 helix, such variation in this site could contribute to DNA-binding specificity **(Overdier *et al.,* 1994).** Various functional and genetic analyses have clarified the FOXA proteins role showing that they are also pioneer factors for nuclear hormone receptors in several ways. Previous studies have suggested FOXA factors to be involved in liver development and essential for the sex hormone receptors' binding to their targets, importantly, tumor development and progression **(Kaestner, 2010; Li *et al.,* 2012).** Moreover, in breast and prostate, the estrogen receptor (ER-α) and androgen receptor (AR) recruitment to their target genes depends on FOXA **(Carroll *et al.,* 2005).** Outstandingly, multiple single nucleotide polymorphisms (SNPs) was found in FOXA binding sites that affected FOXA and hormonal receptor occupancy, which were significantly higher in HCC female samples than in their normal livers suggesting that alterations in FOXA binding sites could play a part in increasing the risk of hepatocarcinogenesis in females **(Li *et al.,* 2012).**

MicroRNAs (miRNAs) are highly conserved short RNAs that de-regulate protein expression through binding with the 3'-untranslated region (3'-UTR) of target mRNA. Recent studies in the last few years have demonstrated a functional role of some miRNAs in several biological processes, including dys-regulation of miRNAs in the progression of human cancers **(Macfarlane and Murphy, 2010)**.

Several studies demonstrated dysregulation of miRNA expression in HCCs. Moreover, several deregulated miRNAs (eg, miR-21, miR-26, miR-29, miR-101, miR-151, miR-221 and miR-223) have shown to regulate HCC cell growth, apoptosis, migration and invasion **(Gramantieri *et al.,* 2008)**. These findings suggest that miRNA dysfunction may be associated with hepatocarcinogenesis. Clearly, further investigations are required to clarify the role of miRNAs in the progression of HCC and to identify those miRNAs that may serve as a prognostic and diagnostic biomarker and/or therapeutic targets for HCC **(Giordano and Columbano, 2013).**

MiRNA expression studies have documented down regulation of several miRNAs, including miR-124, in HCC tissues. It suggested that ectopic expression of miR-124 in HCC cells inhibits cell growth. Additionally, miR-124 has been reported to regulate the differentiation of stem cells and neural development, to inhibit the proliferation of glioblastoma multiform cells and to induce differentiation of brain tumor stem cells **(Li *et al.,* 2013)**. More recently, Hunt and colleagues reported that miR-124 could suppress the motility of oral squamous cell carcinoma. These data suggest a potential tumor suppressive function of miR-124 **(Lv *et al.,* 2011).** To date, however, the role of miR-124 in hepatocarcinogenesis and the molecular mechanisms by which miR-124 exerts its functions and modulates the malignant phenotypes of HCC cells remain largely unknown. Our study aimed to investigate the possible alterations in FOXA2 DBD and the status of miRNA-124 differential expression in correlation with HCC Egyptian patients in view of sexual dimorphism.

Patients and Methods

The study was approved by the local institutional ethical committee and patients' consents were obtained according to the regulations of the Egyptian National Cancer Institute (NCI). Patients were recruited from the out-patient Clinics of the Oncology Centre at NCI. Three milliliter (ml) blood was collected on EDTA from 78 subjects, they were classified into three groups, **Group I:** A group of 26 HCC Egyptian patients, 13 males (50%) and 13 females (50%) with ages ranged between 40-80 years. **Group II:** A group of 26 Egyptian cirrhotic patients, matching age and sex with HCC group, without any evidence of HCC. **Group III:** A group of 26 healthy controls, 13 males and 13 females matching age and gender, without any clinical evidence of any pathological disorder.

Clinico-pathological parameters were obtained from medical records to create a patient-level longitudinal database including serum alanine aminotransferase (ALT), aspartate transaminase (AST), serum alpha-fetoprotein (AFP), albumin (Alb), etc. Hepatitis viral antigen (HCV-Ag) was conducted to confirm the clinical evidence of each case.

FOXA2 DNA binding domain mutation detection

Genomic DNA extraction from lymphocytes was performed using Wizard® Genomic DNA Purification Kit (Promega, Cat.A1120, USA) according to manufacturer's protocol. DNA samples' quality and quantity were characterized using a UV spectrophotometer (Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer, USA) by measuring absorbance at 260/280 nm followed by on 0.8% ethidium bromide visualized gel electrophoresis. Ratios between 1.8 and 2.3 were considered satisfactory.

- ***Amplification of FOXA2 DNA binding domain region***

Forward and Reverse PCR primers were designed to flank the region of interest which is FOXA2 DNA binding domain using Integrated DNA technology (IDT) Primer quest tool **(Owczarzy *et al.*, 2008)**. All the required criteria including length, GC content, internal secondary structure and specificity were confirmed using IDT Oligo-analyzer tool and MFE primer tool **(Qu *et al.*, 2012)** as presented in **table (1)**. This sequence was retrieved from NCBI (Accession: NC_000020.11 Ref.GRCh38.p2). The underlined sequence expresses the sequence of forward and reverse primers. Bold part represents Fork head domain region including DNA binding sites of FOXA2.

GCGACCCCAAGACCTACAGGCGCAGCTACACGCACGCA**AAGCCGCCCTACTCGTACATCTCGCTCA TCACCATGGCCATCCAGCAGAGCCCCAACAAGATGCTGACGCTGAGCGAGATCTACCAGTGGAT CATGGACCTCTTCCCCTTCTACCGGCAGAACCAGCAGCGCTGGCAGAACTCCATCCGCCACTCG CTCTCCTTCAACGACTGTTTCCTGAAGGTGCCCCGCTCGCCCGACAAGCCCGGCAAGGGCTCCT TCTGGACCCTGCAC**CCTGACTCGGGCAACATGTTCGAGAACGGCTGCTACCT

One hundred ng of genomic DNA was subjected for amplification using GoTaq Flexi DNA Polymerase (Promega, 100u (units), Cat.M8291, USA). 25µl reaction mixtures were prepared consisting of 1 unit of GoTaq® Flexi DNA Polymerase, 5µl of 5X Colorless GoTaq® Flexi Buffers, 1.5µl of 25 mmol/l Magnesium Chloride Solution, 0.5µl of 10 mmol/l dNTPs and 0.5µl of (20 µmol/l) specific forward and reverse primers ordered from Invitrogen, USA. 35 cycles were performed of: 95 °C for 45 seconds, at 57°C for 1 minute and at 72°C for 1 minute. PCR products were visualized on 1.5% stained agarose gel which was photographed using gel documentation system after 30 minutes of electrophoresis at 120 volt.

Purification of PCR product from gel was performed by High Pure PCR Product Purification Kit (Cat.732668001, Roche Applied Sciences, Germany). Purified DNA quality and quantity were determined using a UV spectrophotometer (Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer, USA) by measuring absorbance at 260/280 nm.

- ***Sequencing of target region of FOXA2 and Sequence Data Analysis:***

Sequencing procedure started with preparing the reactions, which was done by BigDye® Terminator v3.1 Cycle Sequencing Kit, Cat.4337455, SABiosciences, QIAGEN 53711-5399, USA followed by purifying Extension Products by Ethanol/EDTA/Sodium Acetate precipitation, After that, the samples were subjected to the Analyzer Electrophoresis plate where the first two samples were control sequence of pGEM-3Zf(+) followed by the samples of the three studied groups which were loaded to the plate sequentially with specific forward and reverse primers for FOXA2 DBD (bi-directional sequencing).

The output sequences (abi files) were visualized and aligned on BioEdit v7.2.5 **(Hall, 1999)**. Scanning the chromatogram isn't enough if the aim of the project to realize SNPs. The DNA sequencing investigations and analysis of DNA variants, including: SNPs, insertions and deletions (INDELS) were performed by Mutation Surveyor® software version 5.0 **(Minton *et al.*, 2011)**. This was done by introducing all the sequence output files to Mutation Surveyor® software in refer to the reference sequence of Homo sapiens forkhead box A2 (FOXA2) gene, complete cds from GeneBank on NCBI (Accession: EU402966). The frequency of the each mutation occurrence was statistically analyzed using PASW Statistics 20, SPSS Inc**.** Moreover, the effect of each SNP on the intrinsic aggregation propensity the stability of the protein was performed by SNPeffect 4.0 **(De Baets *et al.*, 2012)**, Switch Laboratory Department of Cellular and Molecular Medicine, 3000 Leuven, Belgium. The software focuses on the effect of mutation on aggregation propensity (TANGO), amyloid propensity (WALTZ) and chaperone binding (LIMBO).

MiRNA Isolation and Quality Estimation

Sera samples were used to isolate microRNAs using the miRNeasy Mini Kit (Cat. #217004, QIAGEN, USA) which combines phenol/guanidine-based lysis of samples and silica membrane–based purification of total RNA. MicroRNA samples' quantity and quality were characterized using UV spectrophotometer (Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer, USA) by measuring absorbance at 260/280 nm. Ratios between 1.8 and 2.3 were considered satisfactory.

- ***cDNA Synthesis for miRNAs***

cDNA Synthesis from miRNA samples was performed by miScript II RT Kit (Cat.#218161, QIAGEN, USA). 5X miScript HiSpec Buffer was used to prepare cDNA for subsequent mature miRNA profiling using miScript miRNA PCR arrays. 100 ng of template RNA for each sample was added to reverse-transcription master mix which is composed of 2µl 5x miScript HiSpec Buffer, 1 µl 10x nucleics mix and 1µl miScript Reverse Transcriptase Mix and then completed with Nuclease free water to 10 µl total volume. The samples were incubated for 60 minutes at 37ºC (optimum temperature of reverse transcriptase) then incubated for 5 minutes at 95ºC to inactivate miScript Reverse Transcriptase and finally placed on ice. The reverse transcription reactions were diluted with 45µl nuclease free water and transferred to a –20ºC freezer to be ready to proceed with real-time PCR.

- ***Real Time PCR for microRNA-124 assay expression and array profiling***

Real-Time PCR was performed to evaluate miRNAs relative expression. Assays for miRNA profile analysis and for the internal controls were carried out according to Applied Biosystem protocols. cDNA prepared in a reverse-transcription reaction using miScript HiSpec Buffer serves as the template for real-time PCR analysis using a miScript miRNA PCR Array (Cat.#MIHS-989Z, QIAGEN, USA). MiScript miRNA PCR Array contains miRNA-124 specific miScript Primer (forward primer) and the miScript SYBR Green Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. qPCR was done in an Applied Biosystem 7500 Fast Real-time PCR system in 96 well ABgene® PCR Plates (Thermo Fisher Scientific, USA), in duplicates. Normalization was performed using housekeeping miRNA (SNORD68).

Software for each miScript miRNA PCR Array is available where both the miScript miRNA PCR Array Web-based software and the miScript miRNA PCR Array Data Analysis Excel® Template can be accessed. This software automatically performs normalization with the endogenous housekeeping microRNA (SNORD68) and quantification using the ΔΔCT method of relative quantification and interpretation of the control assays. The results were then statistically analyzed using GraphPad Prism version 5 for Windows and $P < 0.05$ was considered to be statistically significant. Differences in miR-124 expression between the diseased groups versus control group were analyzed using the Student's t-test. Hierarchical clustering was performed on all samples and miRNAs investigated using the PermutMatrix clustering tool.

Results

Statistical analysis was performed using the SPSS software version 20 (SAS Institute, Cary, NC). Values were expressed as the mean ± standard deviation (SD) and being compared using the student's t-test or analysis of variance (ANOVA). The results showed significant difference for liver function parameters including ALT, AST, Alb and AFP data of the three groups as presented in as presented in **table (2).**

Sequencing Analysis of Amplified FOXA2 Forkhead region

- ***Sequences Visualization and Mutation Detection***

The presence study demonstrated three different SNPs among the samples as shown in **table (3)**. Two of them were statistically significant. The 1st polymorphism occurred between G/T in position 498 (c.3699G>T) and the 2nd polymorphism was between G/T in position 3708 on coding sequence (c.3708G>T). Both polymorphisms don't result in any amino acid change. As for the 1st SNP, proline (P) amino acid didn't change as its DNA codon can be CCT, CCC, CCA, and CCG. For the 2nd SNP, serine (S) amino acid didn't change as its DNA codon can be TCT, TCC, TCA, TCG, AGT and AGC. Finally as for the 3rd polymorphism, it occurred in one sample only among all groups and it occurred as an insertion for TT at position 3796-3797 on coding sequence which resulted in nucleotide frame shift coding for the amino acid sequence leading to change in primary structure of the resulted FOXA2 protein.

Sequence analysis and alignment for sequences with any alterations was carried out using NCBI/BLAST/blastn suite as in **figure (1 A and B)**. It shows that the 1st polymorphism occurred between G/T in position 3699 (c.3699G>T) and the 2nd polymorphism was between G/T in position 3708 on coding sequence (c.3708G>T). The frequency of the each mutation occurrence among 78 samples, these results was as follows, (c.3699G>T) represents about 14%, (c.3708G>T) represents about 7% and the (3796-3797 insTT) represents 0.78%. Whilst, in each of the three studied groups, these results showed that about 54%, 36% and 11% of the alterations occurred in HCC, cirrhotic and control cases, respectively with significant p value < 0.05.

- ***Prediction of the SNP effect on the Protein Structure and Stability***

p.Pro166Pro *and* ***p.Ser169Ser***

Based on TANGO, WALTZ, LIMBO and FoldX in SNPeffect 4.0, the mutation does not affect aggregation tendency of the protein, amyloid propensity of the protein or chaperone binding tendency of the protein respectively. Also the mutation has no effect on the protein stability as shown in **figure (2).**

p.Phe199fs

It was not statistically significant in refer to total number of subjects. In this alteration, insertion of TT at position 3796-3797 on DNA level resulted in a frame shift affecting the amino acid (phenylalanine) between the first (initiation, ATG) and last codon (termination, stop), replacing the normal C-terminal sequence with one encoded by another reading frame encountering a translation termination (stop) codon resulting in non-functional protein.

- **Normal C-terminal sequence:**
  FPFYRQNQQRWQNSIRHSLSFNDCFLKVPRSPDKPGKGSFWTLHPDSGNMFENGCYLRRQKRFKCEK QLALKEAAGAAGSGKKAAAGAQASQAQLGEAAGPASETPAGTESPHSSASPCQEHKRGGLGELKGT PAAALSPPEPAPSPGQQQQAAAHLLGPPHHPGLPPEAHLKPEHHYAFNHPFSINNLMSSEQQHHHSHH HHQPHKMDLKAYEQVMHYPGYGSPMPGSLAMGPVTNKTGLDASPLAADTSYYQGVYSRPIMNSS
- **Frame shift C-termianal sequence: FS**PSTGRTSSAGRTPSATRSPSTTVS **Stop**

Real-time PCR for microRNA-124

The results exhibited differential expression among three groups with $p < 0.05$. The result showed that the expression levels of miR-124 in HCC group were significantly reduced followed by cirrhotic group compared with control group as presented in **figures (3 and 4)**. **Figure (5)** is the melting curves resulted from Real-time PCR run of one random sample from each group. The results showed that miR-124 expression was significantly lower in HCC patients compared with either cirrhotic or control subjects ($P < 0.005$). As regards control subjects, miR-124 expression was significantly higher versus cirrhotic and HCC patients. **Figure (3)** showed that the highest expression exhibited for control followed by cirrhotic then finally HCC subjects in both sexes. The present study also documented that miRNA-124 expression was greatly reduced in HCC females than the respective male subjects ($P < 0.05$).

Discussion

Hepatocarcinogenesis is a slow, multistep and complex process. The leading causes of HCC are chronic infection with HBV, HCV, consumption of AFB1 and/or alcohol, which may lead to the injury of liver cells resulting in chronic hepatitis and cirrhosis **(McGlynn and London, 2005).** HCC is one of the most highly lethal and malignant cancers of the world. HCC is the second deadliest cancer globally. Very recently, it was estimated that there are 782,000 new cases with HCC **(Wu *et al.,* 2014)**. Cirrhosis often, but not always, precedes cancer. Even after controlling for known risks, it is the fifth most common cancer in men and ninth in women **(El-Serag and Rudolph, 2007).** HCC is of particular concern in Egypt because of the high prevalence of Hepatitis C Virus (HCV). The development and progression of HCC is a complicated process that involves the dysregulation of multiple genes that are essential for different cellular activities. Due to its poor prognosis, HCC is the leading cause of cancer-related deaths in Egypt **(Amal *et al.,* 2010)**. The Egyptian population was found to have the world's most elevated prevalence of liver cirrhosis because of HCV infection **(Darwish *et al.,* 2001)**.

The liver is a sex-hormone sensitive or in other words a sexual dimorphic organ. Although sex hormones function in sexual organs, such as the breast, they also affect many mammalian liver functions **(Eagon *et al.,* 1985)**. Both male and female livers contain androgen receptors (AR), as well as high-affinity, low-capacity estrogen receptors (ER) **(Nagasue *et al.,* 1985).** Gender differences exist with regard to gene expression, mitochondrial function, microsomal enzyme activity, membrane lipid composition, and immune responses **(Rogers *et al.,* 2007).** Liver-gender differentiation commences early in development, but the greatest divergence occurs at puberty **(Waxman and O'Connor, 2006).** A gender disparity is also observed in liver cancer, with higher prevalence in men by three to five folds **(El Bakry, 2015)**. This sex bias is even more pronounced in mouse models of HCC, which was found to be sex hormone-dependent. Some studies have attempted to state the molecular mechanisms of this disparity; but with inconclusive and sometimes contradicting outcomes **(El Bakry, 2015).**

Two recent breakthroughs have increased the interest in the study of estrogen action in liver cancer: one was the discovery of the protective role of ERα in HCC **(Naugler *et al.,* 2007)**. According to the same author, estrogens have shown to prevent against HCC through inhibition of IL-6 expression. The other was discovery cited by **Li *et al.* (2012)** who claimed that ERα-mediated signaling seems to protect against the development of HCC in mice via interaction with Foxa factors, Foxa1 and Foxa2**.** The studies on the roles of FOXA factors in carcinogenesis have

been mainly focused on human prostate and luminal subtype-A breast cancers **(Bernardo *et al.*, 2013).** Both FOXA1 and FOXA2 are required for AR-mediated signaling in promoting the growth of prostate cancer cells **(Barbieri *et al.*, 2012).** In addition, ERα, FOXA1, and GATA3 may form a transcription factor network to regulate the growth of breast cancer cells **(Nakshatri and Badve, 2009).** Moreover, FOXA1 has also been shown to modulate the proliferation of lung cancer, brain cancer, and endometrial cancer cells **(Qiu *et al.*, 2014).** Genomic distribution analysis showed that FOXA factors and ERα or AR frequently bound to adjacent cis-regulatory elements in the genome. The recruitment of ERα or AR to their binding sites seems to be dependent on FOXA factors in breast and prostate cancer cells, respectively **(Grasso *et al.*, 2012; Bernardo *et al.*, 2013).** Thus, these studies suggest that FOXA-dependent genomic landscapes of steroid hormone signaling exist in the human genome, which provides a solid foundation for the understanding of sex hormone regulation in liver cancer. Genetic mutations at FOXA and/or ERα binding sites are highly correlated with the incidence of human HCC, indicating that individual estrogen targets could play essential roles in hepatic tumorigenesis. Genetic variants at FOXA or ERα binding sites that caused loss of estrogen signaling were also found to play a key role in breast cancer **(Cowper-Sal lari *et al.*, 2012).**

MiRNAs regulate various physiological and pathological processes by modulating the expression of their target mRNAs, which play important roles in diverse cellular processes including differentiation, proliferation, growth, migration and survival **(Cullen, 2004).** More than 50% of miRNA genes are located at fragile sites of chromosomes or in cancer-associated genomic regions, indicating that they are cancer-related and could be used as new diagnostic and prognostic cancer markers as well as potential molecular targets **(Bartel, 2004).**

The genes and proteins underlying the development and progression of HCC have been extensively investigated in the past decades and miRNAs have only recently been found to be frequently dysregulated in HCC. This dysregulation is related to HCC progression and specific miRNAs were found to be associated with the metastasis, recurrence, and prognosis of HCC **(Ji *et al.*, 2009).** The expression profiles of microRNAs are associated with the initiation and progression of human tumors **(Lu *et al.*, 2005).**

Depending on the before mentioned information about HCC development and sexual dimorphism as well as their correlation with the functional significance of FOXA factors and differential expression of miRNAs, the present study has been undertaken to expose the effect of FOXA2 DNA binding domain alterations and miR-124 differential expression among Egyptian HCC, cirrhotic patients with reference to control subjects in both sexes.

We recruited cirrhotic and HCC patients with reference to control subjects since they represent two major stages of hepatocarcinogenesis. Both diagnostic, phenotypic characteristics and medical data have confirmed the clinical evidence of each case. The studied patients were the out-patient Clinics of the Oncology Centre, NCI, Egypt. Subjects of each group spanned a wide age range (from 40 – 80 years) in both males and females. As regards, female subjects spanned a wide range of reproductive age, premenopausal (between 40 and 50 years) and postmenopausal (aged above 55 years).

As regards FOXA2 DBD mutations, the present study has identified two statistically significant somatic SNPs in FOXA2 DNA binding region, namely (c.3699G>T) and (c.3708G>T) in cirrhotic and HCC samples. The first SNP (c.3699G>T) was detected in eighteen Egyptian patients (cirrhotic and HCC), the second (c.3708G>T) was detected in six cirrhotic patients. However three of control subjects (two males and one female subjects) have shown to have the second SNP (c.3708G>T). These recent findings might suggest a correlation between FOXA2 DBD alterations and HCC progression and incidence. On the other hand, occurrence of the second SNP (c.3708G>T) among both cirrhotic and control subjects might suggest an impairment in DNA repair capability. This postulation might provide evidence that DNA repair detects is considered a predisposing factor of cancer development as supported by **Teoh *et al.* (2008).** According to the author, impairment of DNA repair pathway has been proposed to participate in the disruption of cell cycle checkpoints leading to chromosomal instability and accelerated development of HCC **(Teoh *et al.*, 2008).**

Based on computational analysis of the SNPs effect on the structure of FOXA2 protein, we propose that these two SNPs are silent alterations, which do not affect amino acid sequence and hence have no effect on the protein structure. Proline in the first SNP (c.3699G>T) and serine in the second SNP (c.3708G>T) have not been changed because both of them have four different DNA codes which have not been changed by those point mutations. In addition, this is also confirmed by the fact that computational analysis has shown no effect on intrinsic aggregation propensity, amyloid propensity, chaperone binding and the stability of the protein.

It is worth mentioned here that our study has shown a third insertional polymorphism (insTT 3796-3797) in a single female cirrhotic patient with good quality and intense output file peaks. Despite the fact that this polymorphism was not frequent among the studied patients, but it should be considered in further studies with larger number of samples because those types of frame shift mutations are usually associated with expression of nonfunctional protein.

Taking together, the present data concerning FOXA2 DBD polymorphism suggests no ultimate alterations in target gene(s) expression and/or impaired binding with ER. Accordingly, we assumed that FOXA2 DBD polymorphisms, studied so far in Egyptian population, are not correlated with HCC sexual bias. However, it seems necessary to conduct further functional genomic and proteomic studies to validate the activity and frequency of FOXA2 DBD alterations, if any, in correlation to hepatocarcinogenesis in Egyptian or other population in view of gender bias.

Now concerning miRNA-124 differential expression in the three studied groups of the present study, data revealed statistically significant down-regulation in both sexes of HCC and cirrhotic patients. It was additionally documented that this reduction is more prominent in HCC female than their respective male subjects ($P < 0.05$). This down-regulation might be attributed to hypermethylation-mediated silencing of miR-124 and some other miRNAs during tumorigenesis through the activation of target oncogenic pathways as supported by previous studies **(Lujambio *et al.*, 2007; Furuta *et al.*, 2010)**. In this context, **Jiang *et al.* (2008)** has confirmed down-regulation of miR-15b, miR-122a, miR-124, miR-199a, miR-199b and miR-203 regulates multiple genes during HCC progression. In addition, **Lujambio *et al.* (2008)** demonstrated miR-124 silencing through methylation of CpG islands in several types of human cancer, such as colon, breast and lung cancers and suggested CDK6 to be a possible target for this miRNA.

Recent reports showed that some miRNAs can modulate the major proliferation pathways through interacting with critical cell cycle regulators such as cyclin-dependent kinase enzyme (CDK) complexes, cell cycle inhibitors of the Cip/Kip family, the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling cascade, and other cell growth regulatory genes. Cyclins are a family of proteins that control the cell cycle progression by activating CDKs. Both cyclins and CDKs, the positive regulators of the cell cycle, are found to be targeted by miRNAs in HCC **(Xu *et al.*, 2009)**. In addition, miR-124 can mediate HCC cell growth arrest by directly targeting vimentin, SET, and MYND domain, and IQ motif containing GTPase activating protein 1 **(Furuta *et al.*, 2010).** This indicates that the miRNA differential expression in HCC is due to several interactions and pathways.

The present investigation can also postulate a possible correlation between miR-124 expression and sexual dimorphism in HCC which in fact needs further investigations on larger sample size and a narrower age range. Although the present study was not targeting sex hormonal display in both sexes but it seemed that female sex hormones play a crucial role in HCC protection. And since our samples spanned a wide age range (pre- and postmenopausal), our findings in postmenopausal females was supposed to have lowered levels of sex hormones. This critical point could be discussed in view of the argument of **Ahmed *et al.* (2015)** in which they claimed elevated levels of female sex hormones in both pre- and postmenopausal of HCC and HCV infected patients.

On the other hand, very recent studies have claimed a possible interaction between miRNAs and FOXA2. **Lin *et al.* (2014)** suggested that miRNAs can have effects on FOXA2 in HCC. They found that FOXA2 protein levels were up-regulated in HCC tissues compared with respective normal adjacent tissues. However, FOXA2 mRNA levels varied in random tissues, suggesting that a post-transcriptional mechanism was involved in its regulation. Bioinformatic analyses were done to search for miRNAs that could potentially target FOXA2 and specific targeting sites for miR-141 were identified in the 3′-untranslated region (3′-UTR) of the FOXA2 gene. By over-expressing miR-141 in HepG2 cells, they experimentally validated that miR-141 directly regulates Foxa2 expression **(Lin *et al.*, 2014).**

Liver-enriched transcription factors namely, FOXA1 and FOXA2 have shown to play central regulatory role in loss of miR-122 in HCC (**Coulouarn *et al.*, 2009).** According to **Gramantieri *et al.* (2007),** miR-122 can suppress HCC cell growth by directly targeting cyclin G1 expression**.** In another report, the expression of the miR-124 (the one measured in the present study) analyzed in adult mouse organs and in the developing mouse pancreas has shown to be increased at embryonic stage e18.5 compared with stage e14.5. Computationally, Foxa2 gene product was identified as a potential miR-124 target and it was revealed that increasing the level of miR-124 negatively regulates the level of Foxa2 protein **(Baroukh *et al.*, 2007).** A recent study identified a targetable pro-inflammatory loop consisting of miR-24/miR-629/HNF-4α/miR-124/STAT3, and revealed that HNF-4α transient silencing in cell xenografts maintained low levels of HNF-4α in liver cancers. Furthermore, the study demonstrated that miR-124 delivery was sufficient to limit tumor growth **(Hatziapostolou *et al.*, 2011).** Therefore, it seems to be possible to block essential feedback loops in hepatocarcinogenesis with specific miRNA delivery.

As mentioned before, prevention of liver cancer through ERα-dependent gene regulations in female and AR-mediated promotion of liver cancer in male depend on FOXA1 and FOXA2. Binding of the steroid hormone receptors to those of their targets that are near FOXA binding sites is abolished in the absence of the FOXA factors **(Zhao *et al.*, 2014).** One can suggest a possible scenario of miRNA-124 targeted therapy to limit tumor growth where it may function as a tumor suppressive miRNA in HCC cells through the inhibition of FOXA2 translation in males. This, in turn, decreases the level of the downstream targets' expression of FOXA2 resulting in decreasing their suitability to HCC. In conclusion, the interaction between miRNAs and gene expression is a complicated regulatory network that requires further investigation.

Tables

Table 1: Demographic and Clinical Data of HCC and Cirrhotic Patients versus Control Subjects

	Item/ Group	Control Group (n=26)		Cirrhotic Group (n=26)		HCC Group (n=26)		P value
		Female (n=13)	Male (n=13)	Female (n=13)	Male (n=13)	Female (n=13)	Male (n=13)	
Liver function parameters	ALT (IU/L)	27.54 ± 2.625	36.62 ± 3.814	41.10 ± 7.482	47.92 ± 7.776	68.85 ± 9.391	55.77 ± 5.621	
		32.08 ± 2.443		44.51 ± 5.330		62.31 ± 5.519		0.0001
	AST (IU/L)	23.31 ± 2.017	27.85 ± 2.063	54.00 ± 8.746	64.08 ± 8.041	101.3 ± 13.43	75.77 ± 11.00	
		25.58 ± 1.484		59.04 ± 5.907		88.54 ± 8.881		0.0001
	Alb (g/dL)	3.908 ± 0.2197	3.823 ± 0.2473	3.538 ± 0.1666	3.392 ± 0.2263	3.208 ± 0.1222	3.100 ± 0.1373	
		3.865 ± 0.1623		3.465 ± 0.1384		3.154 ± 0.09068		0.0015
	AFP (ng/ml)	8.523 ± 1.087	8.431 ± 1.177	26.63 ± 3.988	24.64 ± 3.887	453.6 ± 101.1	406.9 ± 61.94	
		8.477 ± 0.7853		25.63 ± 2.736		430.3 ± 58.28		0.0001

Table 2: Forward and Reverse Primers

Primer	Forward Primer	Reverse Primer
Sequence	5’ GCGACCCCAAGACCTACAG 3’	5’ AGGTAGCAGCCGTTCTCGAA 3’
Length	19	20
GC Content	63.20%	55%
Tm	60.4ºC	61.8ºC
ΔG (kcal/mol)	No secondary structures	Hairpin ΔG = -0.19
Product Size	310 base pairs (bp)	

Table 3: Estimation of number of samples in each group per SNP

Samples’ number	Group	Position (N)	Ref (N)	Var (N)	Position (A)	Ref (A)	Var (A)
18 samples	9 HCC male 6 HCC female 3 Cirrhotic female	3699	G	T	166	P	P/P
9 samples	2 Control male 1 Control female 4 Cirrhotic female 2 Cirrhotic male	3708	G	T	169	S	S/S
1 sample	1 Cirrhotic female	3796-3797 (insTT)					

Figures

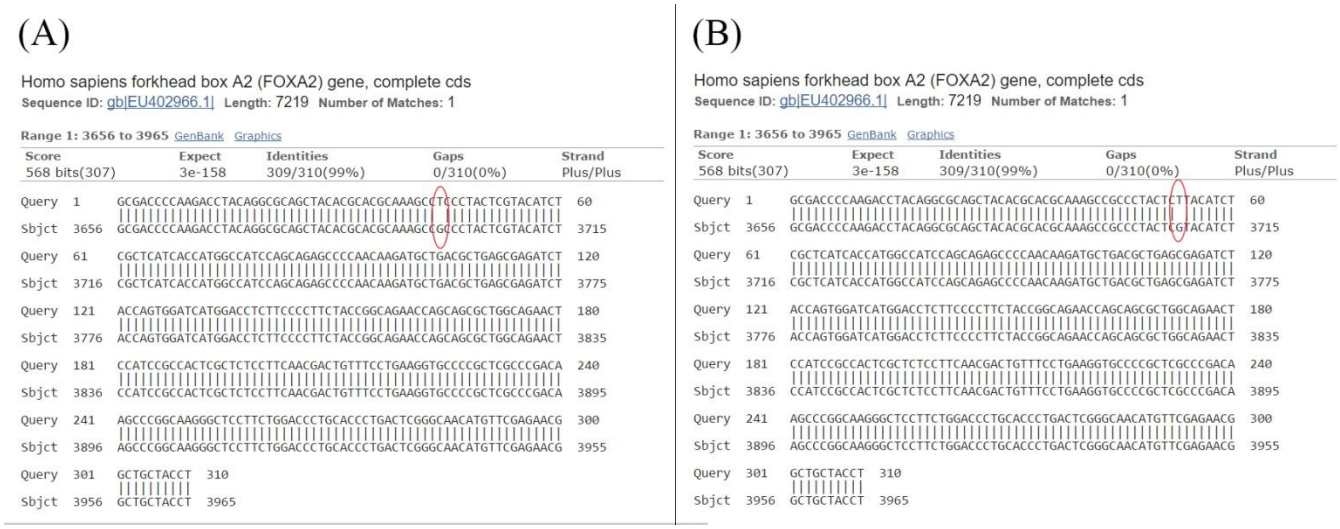

Figure 1: (A) Sequence alignment of FOXA2 reference gene on NCBI with sequence having alteration of c.3699G>T. (B) Sequence alignment of FOXA2 reference gene on NCBI with sequence having alteration of c.3708G>T.

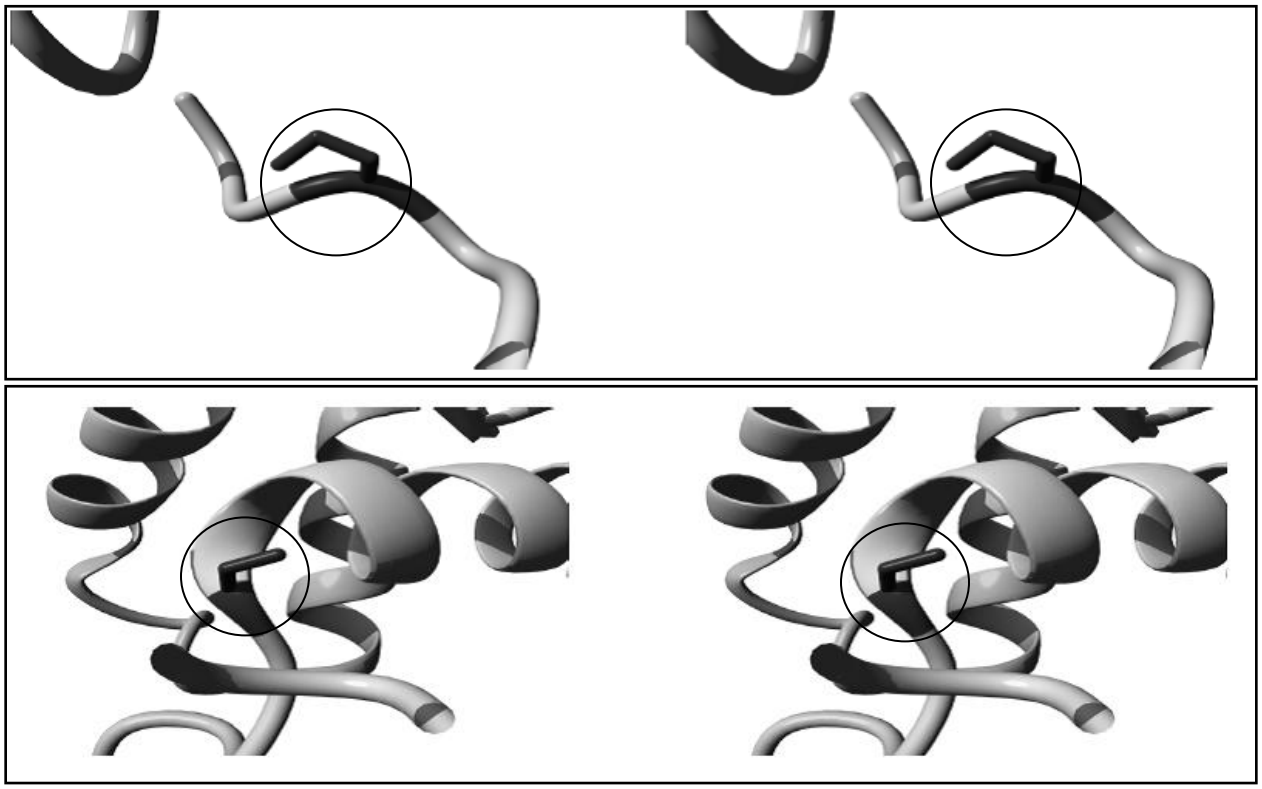

Figure 2: (A) Molecular visualization of the wild type (left) and variant (right) amino acid. The circled residue represents the wild type (PRO) and variant residue (PRO). (B) Molecular visualization of the wild type (left) and variant (right) amino acid. The circled residue represents the wild type (SER) and variant residue (SER).

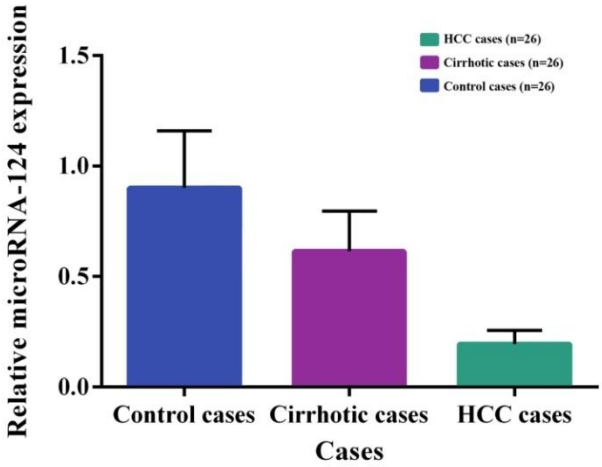

Figure 3: MiR-124 expression profile showing significant gradual decrease of miR-124 in cirrhotic cases and HCC, respectively as compared to control cases (n = 78; P < 0.05).

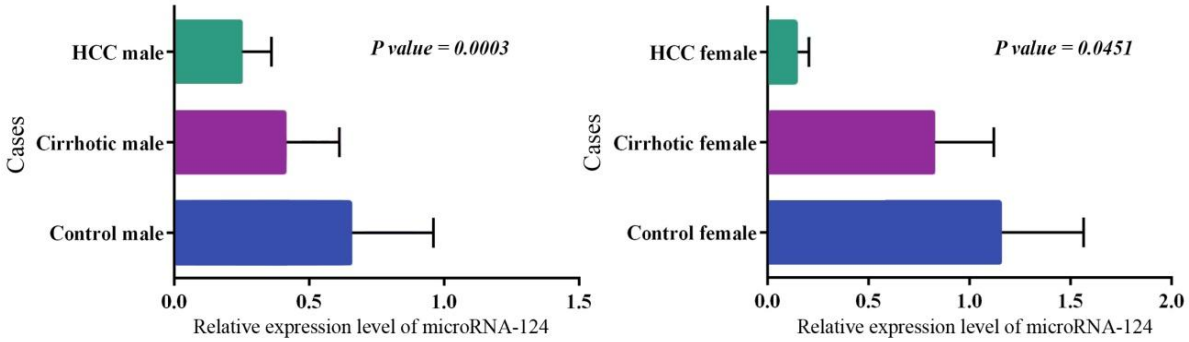

Figure 4: MiR-124 expression profile showing significant gradual decrease of miR-124 in both male and female samples of cirrhotic cases and HCC, respectively in compare with control cases (n = 39 for each gender, P-value < 0.05).

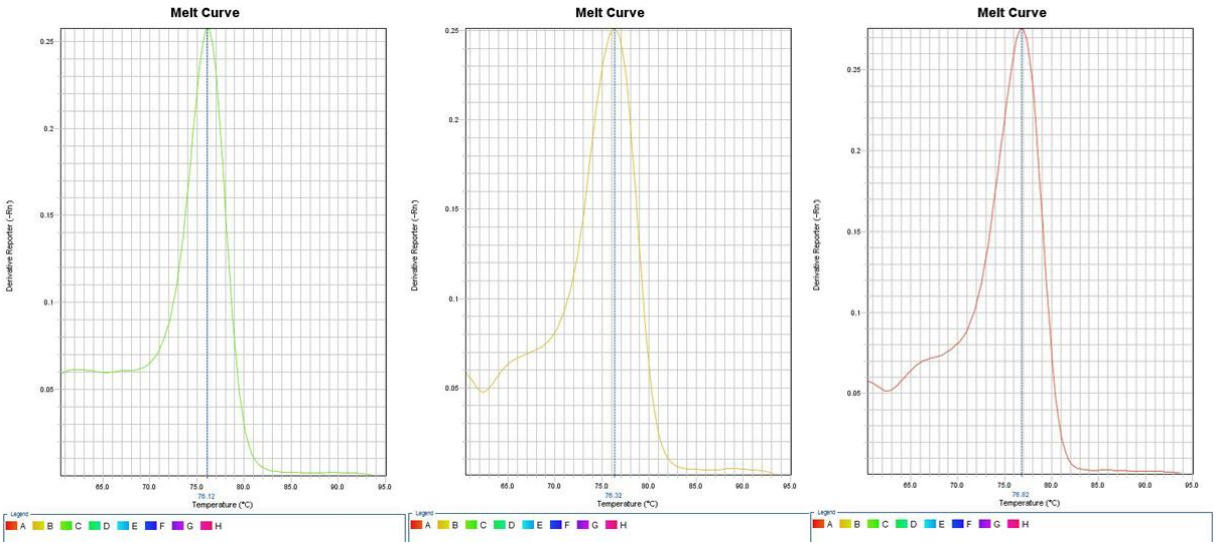

Figure 5: Melting curves resulted from Real-time PCR run for miR-124 of one random sample in HCC, cirrhotic and control groups.

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