School of Sciences and Engineering

Potential Role of Cofactor of BRCA1 in Hepatocellular Carcinoma Pathogenesis

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DEDICATION

To my mother; thanks for making me who I am, my father; thanks for supporting and encouraging me all the way, to my brother (Ahmed) and sister (Engy); thanks for always being there for me, to my beloved husband; this work wouldn’t have been accomplished without your support, to my little daughter (Laila); the joy and happiness of my life

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Abstract

Hepatocellular carcinoma (HCC) is a devastating tumor characterized by poor prognosis. In Egypt, HCC is the first most common cancer in men and the second most common cancer in women in the year 2012. Still, the molecular mechanisms underlying HCC pathogenesis are not fully identified. This leads to the lack of reliable prognostic markers for HCC. COBRA1 is the cofactor of BRCA1, it is one of the four subunits of the negative elongation factor (NELF) referred to as, NELF-B. NELF complex inhibits transcription elongation via stalling RNA polymerase II. COBRA1 was studied in some types of cancer; in breast cancer it was proved to have a tumor suppressor activity meanwhile, it has an oncogenic role in upper gastrointestinal carcinoma (UGC). In UGC, COBRA1 controls the tumor suppressor trefoil factor 1 (TFF1) expression through regulating activator protein-1 (AP-1) complex trans-activation. COBRA1 binds to AP-1 complex subunits (c-Fos and c-Jun) leading to attenuated TFF1 expression.

The main aim of this study was to investigate the role of COBRA1 in HCC progression and whether COBRA1 acts in the studied cell lines through NELF or AP-1 complexes. Four HCC cell lines were studied (HepG2, SNU-449, SNU-398 and SNU-387) in addition to, the immortalized liver cells (MIHA) as a control. The used HCC cell lines are isolated from patients at different stages of the tumor, ranging from early, intermediate and late stage. The expression of COBRA1, the other NELF subunits, c-Fos, c-Jun and TFF1 were examined in the four mentioned cell lines. The mRNAs of COBRA1, NELF subunits (A, C/D and E) and TFF1 were analyzed by semi-quantitative RT-PCR. While the proteins of COBRA1, c-Fos and c-Jun were tested by Western blotting. Our results show that COBRA1 protein was differentially expressed among the tested cell lines. This suggests a potential role of COBRA1 in HCC pathogenesis and development. The other NELF subunits (A, C/D and E) mRNAs were co-dependently expressed in all the tested cell lines. In the three HCC cell lines (HepG2, SNU-449 and SNU-387), c-Fos was expressed in its active phosphorylated form in contrast to c-Jun which was not detected in its active form. TFF1 was only expressed in HepG2 (of early stage HCC) and MIHA cells.

In conclusion, COBRA1 was proposed to play a role in HCC progression and development. TFF1 mRNA expression profile was not correlated to that of AP-1 complex subunits proteins which suggested the involvement of other regulatory pathways in TFF1 expression which need further study.
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List of Abbreviations

AFB1: Aflatoxin B1
AFP: Alpha-fetoprotein
ANOVA: Analysis of Variance
AP: Alkaline Phosphatase
AP-1: Activator Protein-1
AR: Androgen Receptor
A.U.: Arbitrary Unit
BCLC: Barcelona Clinic Liver Cancer
Bp: Base pair
BRCA1: Breast Cancer Susceptibility 1
BZIP: Basic Leucine Zipper
c-Fos: Cellular-Fos (an AP-1 complex subunit)
c-Jun: Cellular-Jun (an AP-1 complex subunit)
ChIP: Chromatin Immunoprecipitation
CLD: Chronic Liver Disease
CLIP: Cancer of the Liver Italian Program
COBRA1: Cofactor of BRCA1
Co-IP: Co-Immunoprecipitation
CT: Computed tomography
DEPC: Diethylpyrocarbonate
DMEM: Dulbecco’s Modified Eagle Medium
DRB: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF: DRB sensitivity-inducing factor
ER-α: Estrogen receptor-alpha
ERK: Extracellular Signal Regulated Kinase
FBS: Fetal Bovine Serum
GI: Gastrointestinal
HBV: Hepatitis B Virus
HBsAg: Hepatitis B Surface Antigen
HCC: Hepatocellular Carcinoma
HCV: Hepatitis C Virus
HDAg: Hepatitis D Antigen
HepG2: Well differentiated human hepatocellular carcinoma cell line
Hr: Hour
IGF-1: Insulin-like Growth factor 1
IHCC: Intrahepatic Cholangiocarcinoma
JNK: c-Jun N-terminal Kinase
kDa: kilo Dalton
LEF-1: Lymphoid Enhancer-binding Factor-1
LR: Liver resection
LT: Liver Transplantation
MIHA: non-tumourigenic immortalized liver cell line
Mins: Minutes
MMP1: Matrix Metalloprotease 1
MRI: Magnetic Resonance Imaging
NELF: Negative Elongation Factor
PBS: Phosphate Buffer Saline
RBST: 0.01% Tween 20 in PBS
pS2: TFF1 synonym
RFA: Radiofrequency Ablation
RNAPII: RNA Polymerase II
RPMI: Roswell Park Memorial Institute medium
RRM: RNA Recognition Motif
RT-PCR: Reverse Transcription Polymerase Chain reaction
SD: Standard Deviation
SDS: Sodium Dodecyl Sulfate
Secs: Seconds
Ser: Serine
SNU: Soeul National University
TACE: Transarterial Chemo-embolisation
Thr: Threonine
TTF1: Trefoil Factor 1
TNM score: HCC scoring system, it depends upon tumor properties (T), presence of nodules (N) and chance of metastasis (M)
UGC: Upper Gastrointestinal Carcinoma
UHMWPE: Ultra High Molecular Weight Polyethylene
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Chapter (1): Literature review

1.1 Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC) is considered the most common type of liver cancer, it is responsible for 70-85% of liver cancer cases (Shibata and Aburatani, 2014; Shaker et al., 2013). Other less common forms of liver cancer are intrahepatic cholangiocarcinoma (IHCC) which is cancer of the bile ducts and hepatoblastoma that is known to affect children (Shibata and Aburatani, 2014; Herzog et al., 2000).

1.1.1 HCC Epidemiology: Incidence and Prevalence

HCC is the sixth most common neoplasm worldwide (Forner et al., 2012; Morise et al., 2014; Cornella`et al., 2011). Surprisingly, it is ranked as the second most common cause of cancer related deaths in the world and accounts for 745,000 deaths in 2012 (Ferlay et al., 2015). Thus, it is one of highly aggressive cancers characterized with poor prognosis.

In Egypt, HCC is the first most common cancer in men and the second most common cancer in women in the year 2012 (according to GLOBOCAN database) (Ferlay et al., 2013). It is worth mentioning that, a rapid increase was observed in the number of HCC patients from approximately 4% in 1993 to 7.3% in 2003 which is almost the double in 10 years. This rising incidence is attributed to the high prevalence of hepatitis C virus (HCV). In fact, Egypt has the highest prevalence of HCV in the world. HCV is important in the etiology of HCC as in most cases HCV patients’ develop liver cirrhosis (Anwar et al., 2008; Omar et al., 2013; Shaker et al., 2013).

1.1.2 Risk Factors and Prevention

It has been reported that most HCC cases (approximately 80%) develop in cirrhotic livers of patients having chronic liver disease (CLD) (Forner et al., 2012; Omar et al., 2013). The most common causes of CLD are viral infections with hepatitis B and C viruses (HBV and HCV). Thus HCC risk factors include chronic HBV infection, chronic HCV infection, aflatoxins and chronic alcohol use. Other less common risk factors are obesity, Diabetes mellitus and non-alcoholic fatty liver disease (Omar et al., 2013). HBV is the most frequent risk factor for HCC
especially in Asia however, in Egypt HCV is more common. Since it has the highest HCV prevalence worldwide, HCV is recognized among around 14% of the total Egyptian population (Anwar et al., 2008; Omar et al., 2013; Shaker et al., 2013). A major cause of liver diseases in Egypt is Schistosomiasis infection which was a major public health problem in the past. A lot of Egyptians suffered from Schistosoma mansoni infection and its complications especially in rural regions. From 1950s to 1980s, a community-wide therapy campaign was carried out using tarter emetic injections to achieve a mass immunization and treatment. But, unexpectedly this helped in the spread of HCV infections among most of the injected patients due to the needle re-use at that time. Moreover, it was found that patients previously infected with schistosomiasis then HCV showed faster progression to liver fibrosis and so a rapid development to HCC compared to others that weren’t infected with the parasite (Omar et al., 2013; Shaker et al., 2013).

Even though HBV and HCV are accused of being the most common risk factors of HCC still there are other reported predisposing factors of HCC such as, aflatoxins (Forner et al., 2012). Aflatoxins are metabolites of certain types of moulds that contaminate some food products for instance maize; the most known one is aflatoxin B1 (AFB1). AFB1 is believed to be toxic and carcinogenic. Some reports postulated that AFB1 may cause mutations in the tumor suppressor gene p53 which may lead to HCC (Anwar et al., 2008). Additionally, alcohol use is considered one of HCC risk factors particularly in developed countries as North America, Europe, and Japan (Forner et al., 2012).

Nowadays, HBV vaccine is available and included to the routine schedule of vaccination from infancy. While, for HCV still there is no available vaccine hence, the proper prevention precautions should be followed when dealing with an HCV patient to avoid blood born transmission. Also, HCV should be treated with the proper antivirals to lessen the progression of the disease to liver fibrosis which may develop HCC (Forner et al., 2012).

1.1.3 Diagnosis

Patients with CLDs should be subjected to routine surveillance to diagnose any possible progression to HCC at early stage of the tumor. This allows early treatment which in turn avoids the patient’s case deterioration and increases the chances of a longer survival after cure (Forner et al., 2012). According to the size of the tumor the suitable diagnostic tool is used. In case of small tumors; ultrasonography is used while, larger tumors are diagnosed by computed
tomography (CT) or magnetic resonance imaging (MRI). Furthermore, determination of α-fetoprotein (AFP) is one of the commonly used serological tests. An AFP level of 400 ng/ml or higher is indicative for HCC however, it is not reliable marker since its diagnostic sensitivity is around 60%. In certain cases imaging techniques are not sufficient to diagnose HCC so a diagnostic biopsy is required (El-Serag, 2011; Forner et al., 2012).

1.1.4 Staging Systems in HCC

The aim of tumor staging systems in HCC is to identify the tumor prognosis and assess patient’s condition to guide the therapeutic approach (Tandon and Garcia-Tsao, 2009; Subramaniam et al., 2013). Different staging systems depend on certain criteria; tumor stage, degree of liver dysfunction, patient’s condition and treatment efficacy (Pons et al., 2005). The most commonly used systems are Okuda classification, Cancer of the Liver Italian Program (CLIP) score, Barcelona Clinic Liver Cancer (BCLC) staging system and the TNM score (Pons et al., 2005; Subramaniam et al., 2013).

The Okuda system classifies HCC cases according to presence of ascites, serum albumin and bilirubin levels. In most cases this system identifies large tumors occupying more than 50% of the liver thus it isn’t suitable for early stage liver tumor classification (Pons et al., 2005; Subramaniam et al., 2013). On the other hand, CLIP score has the advantage of identifying early stage tumors. It considers some parameters as tumor morphology, AFP level, degree of liver impairment and portal vein thrombosis. Also, recurrence can be predicted using CLIP score system (Subramaniam et al., 2013). A frequently used staging system is the BCLC since it correlates between the defined tumor stage and the treatment regimen followed. The patient’s clinical picture is the main guideline of this system. Patients are assigned letters from A to D that indicates the tumor stage from early to late stage. For instance, patients with early HCC are considered stage (A) in which the received therapies are resection, liver transplantation. Finally, the TNM system which is given this name as it depends upon tumor properties (T), presence of nodules (N) and chance of metastasis (M). However, this system doesn’t offer adequate prognosis of the tumor stage (Pons et al., 2005; Subramaniam et al., 2013).

Till now, researchers didn’t agree upon a universal staging system that can be applied on all HCC cases worldwide. This is due to the heterogeneity and complexity of HCC; being initially developed from an impaired liver. Also the diversity of risk factors from one patient to
another hamper the use of a single staging system for all cases (Pons et al., 2005; Subramaniam et al., 2013). As a result, more studies should be conducted to introduce new accurate molecular markers. The expression of these markers depends on the tumor biology so novel staging systems using these reliable markers will be more indicative to the patient’s condition. One of these novel systems is called the genomic signatures in which two identified signatures (G3 and poor survival signature) are associated with recurrence. Another system used insulin-like growth factor-1 (IGF-1) which shows low serum levels in case of liver cirrhosis (Subramaniam et al., 2013).

1.1.5 HCC Treatment

The treatment of HCC is complicated owing to its severity, underlying CLD and the late diagnosis in most cases (Morise et al., 2014). The treatment procedures include surgical treatments which are; liver resection (LR), liver transplantation (LT) while non-surgical treatments are local ablation, transarterial chemoembolisation (TACE) and chemotherapy. Ablation, LR and LT achieved a high extent of response, hence, considered as curative treatments. Other treatments; TACE and chemotherapy are known to be non-curative because they are used for just managing the patient’s symptoms and improving survival (Forner et al., 2012).

Surgical liver resection (LR) is the treatment of choice in case of small sized tumors which are discovered at very early stage and not developed from cirrhotic liver. The expected survival is 5 years after LR surgery for 38%-61% of cases. However, there is a high risk of recurrence in 70% of cases after 5 years as the patient still have CLD that predispose for HCC recurrence (EL-Serag, 2011; Morise et al., 2014). Liver transplantation (LT) is the most effective treatment for HCC since not only the tumor is removed but the injured cirrhotic part is removed as well. This results in low recurrence rates less than 15% after 4 years survival. Patients eligible for this treatment (called Milan criteria) are those with tumor size 5 cm or lower, or with at most 3 nodules with no vascular invasion. Expansion of the recipient criteria to include more HCC patients is under study. Limitations of LT are the risk and cost of the surgical procedure as well as the limited number of donors (Forner et al., 2012; Morise et al., 2014).

In addition to surgical treatments, other non-surgical therapies are widely used such as local ablation. Radiofrequency ablation (RFA) is the most common type. RFA is suitable for
patients with early stage HCC but not eligible to LR and LT (El-Serag, 2011). It is considered as a targeted treatment; by destroying the tumor cells only without affecting neighboring healthy cells (Morise et al., 2014). Besides RFA, transarterial chemoembolisation (TACE) is another available non-surgical therapy. The principle of TACE is injecting chemotherapeutic drugs or radioactive materials into the arteries supplying the tumor (Forner et al., 2012).

1.1.6 HCC Molecular Pathogenesis

HCC is a complicated and devastating tumor that is often diagnosed at late stage due to, the lack of clear reliable diagnostic and prognostic markers. One of the current markers; AFP for instance is not highly sensitive so finding new markers is required (Tandon and Garcia-Tsao, 2008). In fact, several signaling pathways are deregulated in HCC leading to different expression profiles in certain genes. Studying these deregulated players enables the discovery of new molecular markers. Consequently, the molecular mechanisms underlying HCC pathogenesis should be studied in depth (Pons et al., 2005).

From the most frequently interrupted signaling pathways in HCC is the Wnt/β-catenin pathway. Wnt/β-catenin pathway regulates different physiological functions in liver cells. It is involved in liver homeostasis, metabolism and cell proliferation. In normal conditions, β-catenin is attached to the cell membrane. In HCC, mutations may occur in genes coding β-catenin causing an aberrant activation of this pathway (Cornella’ et al., 2011). As a result, β-catenin is transferred to the nucleus and acts as a transcription factor to other genes such as, c-myc which is a well-known proto-oncogene (Yuen et al., 2001). Ultimately, this deregulated pathway along with other mechanisms regulates certain genetic cascades resulting in tumorigenesis (Dahmani et al., 2011).

To sum up, studying the key players of molecular mechanisms underlying HCC pathogenesis allow proper prognosis and assessment of the tumor. In this study, one of these players (COBRA1) is investigated in HCC cell lines. The role of COBRA1 in carcinogenesis is discussed in the next section.
1.2 Cofactor of BRCA1 (COBRA1)

Basically, COBRA1 was identified as the Cofactor of BRCA1. COBRA1 protein is made up of 580 amino acids (Zhong, et al., 2004). Using a yeast two-hybrid assay, it was demonstrated that it binds to breast cancer susceptibility 1 (BRCA1) protein (Aiyar, et al., 2007b). BRCA1 is important in some cell activities such as; transcription and DNA repair (Ye et al., 2001). Later, Narita et al found that COBRA1 structure is identical to that of NELF-B which is one of the four subunits that constitute the negative elongation factor (NELF) complex (Narita et al., 2003). The NELF complex subunits are recruited to the target promoter proximal region to stall RNA polymerase II (RNAPII) and hence, inhibit RNA transcription at the elongation step (Aiyar et al., 2007a). COBRA1 partially initiates the chromatin unfolding activity in a similar way to BRCA1. BRCA1 is involved in the chromatin decondensation which serves as an initiation step for various cell activities including transcription, DNA replication, repair, and recombination (Ye et al., 2001). Additionally, a study reported the absence of inner cell mass and embryonic death upon COBRA1 knock out in mouse embryo. This revealed that COBRA1 plays a crucial role during early embryogenesis (Amleh et al., 2009).

Besides the mentioned physiological roles of COBRA1, its role in cancer has been elucidated in certain cancer types. Levels of COBRA1 expression were controversial according to the tumor type due to its potential tissue specific behavior. In breast cancer, COBRA1 shows low expression levels in cell lines and tissues from late stage of the tumor. Reduced expression is linked to presence of metastasis. Subsequently, COBRA1 has a tumor suppressor role in breast cancer development (Sun et al., 2008). In contrast, another study postulated that COBRA1 is overexpressed in upper gastrointestinal carcinoma (UGC) tissue samples. Intriguingly, this overexpression was accompanied with decreased expression of a well known tumor suppressor trefoil factor-1(TFF1). Therefore, it has a potential oncogenic role in UGC (McChesney et al., 2006).

In HCC, the role of COBRA1 is still largely unexplored. To the best of our knowledge, the first study conducted on COBRA1 in HCC was done in Dr Amleh’s lab. COBRA1 was examined on both the RNA and protein levels, in tissue specimens collected from 16 HCC Egyptian patients. The results revealed that COBRA1 was upregulated in nearly half of the tested samples and downregulated in other half relative to the normal tissue samples (Kamel, 2012).
Another in-silico study came from Dr Amleh’s lab, reported a significant overexpression of COBRA1 mRNA in HCC tumor tissues versus their normal tissue counterparts. This was done by analyzing three different microarray datasets available on Oncomine Cancer Microarray Database (El Zeneini, 2015). Nevertheless, the exact role of COBRA1 in the molecular mechanism underlying HCC pathogenesis is not fully understood yet.

It should be noted that, COBRA1 does not possess a DNA binding domain so it cannot bind solely on the promoter site of its target genes. Accordingly, it should be associated in a complex to regulate its targets. Consequently, COBRA1 actions are mediated through the negative elongation factor (NELF) complex (Aiyar et al., 2004; Aiyar et al., 2007b; Sun et al., 2008). Furthermore, COBRA1 may be associated to other complex known as, the activator protein-1 (AP-1) complex (Zhong et al., 2004; McChesney et al., 2006). The next subsections explain the mechanisms of COBRA1 binding to NELF and AP-1 complexes as well as their structures in detail.

1.2.1 Negative Elongation Factor (NELF) Complex

NELF complex consists of four subunits NELF- (A, B, C/D and E). It is proved that the NELF complex subunits stall RNAPII and thus pause transcription elongation (Yamaguchi et al., 1999; Narita et al., 2003). Transcription elongation is the rate limiting step through which transcription is controlled (Jonkers and Lis, 2015). NELF-A is similar to hepatitis delta antigen (HDAg) which is a viral protein that binds and activates RNAPII. As mentioned NELF-B subunit is identical to COBRA1. NELF C/D are translation variants of the same mRNA (Narita et al., 2003). NELF-E binds to the RNA during transcription since it has an RNA recognition motif (RRM) (Gilchrist et al., 2008). NELF cooperates with DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity-inducing factor (DSIF) /human Spt4-Spt5 to stall RNAPII (Yung et al., 2009). Narita and colleagues, proposed a model for the assembly of NELF subunits and the mechanism of transcription pausing (Figure 1). As shown, NELF subunits are associated to DSIF and RNAPII. NELF-B and NELF-C/D represent the core of the complex by bringing NELF-A and NELF-E together. NELF-A binds to RNAPII directly while, NELF-E is attached to the RNA. Transcription is repressed due to the assembly of the NELF subunits in this site. On the other hand, absence of any of the subunits of NELF complex results in the complex
disassembly leading to the release of RNAII polymerase and thus the progression of transcription.

Figure 1. Hypothetical model of NELF subunits binding to RNAPII: binding of NELF to DSIF caused transcription pausing (top) in comparison to transcription mediated by HDag (bottom). NELF subunits are depicted in light grey while DSIF is presented in dark grey. The black filled boxes indicate proven interactions and the question marks denote hypothetical interactions between NELF and DSIF (Narita et al., 2003).

Another study, Aiyar et al. (2004) speculated that, NELF-B (COBRA1) together with the other NELF subunits interacts with estrogen receptor-α (ER-α) and results in attenuation of ER-α mediated activation of its target genes. This bound NELF complex acts by interfering with RNAPII movement and thus represses ER-α dependant transcription. Moreover, silencing of one of the NELF subunits in breast cancer cells results in, retaining ER-α mediated transcription. Interestingly, the addition of exogenous estrogen recruits NELF subunits to the promoter site which illustrates a possible mechanism of hormonal regulation (Aiyar et al., 2004). It was noticed that, depletion of any of the NELF subunits abolishes the inhibitory action of NELF complex. As a result, a functional co-dependence of the NELF subunits is observed. This was proved by conducting a knock down experiment for each NELF subunit in breast cancer cells then examining the influence of this knock down on the other subunits protein expression levels. The experiment showed the decrease in any of the NELF subunits results lower protein expression of the other three subunits (Sun et al., 2008). Despite this co-dependent decrease in NELF subunits proteins, it was not reflected on the mRNA levels which proposed that the regulation of these molecules occurs at the post-transcriptional level (Sun and Li, 2010).
In HCC, NELF-E was examined in tissue samples on both the mRNA and protein levels. A significant high expression was observed in the HCC versus the non-HCC tissue. In addition, an increased expression was noticed in cases having portal vein invasion (PVI). Accordingly, it was proposed that the overexpressed NELF-E may predict the possibility of metastasis (Iida et al., 2012).

Not only COBRA1 binds to ER-α receptor, but it can also binds to androgen receptor (AR) and possibly affect the alternative splicing of genes regulated by androgen stimulated promoter (Sun et al., 2007).

1.2.2 Activator Protein-1 (AP-1) complex

The Activator Protein-1 (AP-1) complex is a multifunctional eukaryotic transcription factor. It is involved in many cell functions such as, cell cycle proliferation, development and apoptosis (Lopez-Bergami et al., 2010). The AP-1 complex is made up of members of the Fos and Jun families, two of them are cellular Fos and Jun (c-Fos and c-Jun). c-Jun can form homodimers with other c-Jun subunits while c-Fos only heterodimerize with c-Jun (Ozanne et al., 2007). AP-1 complex is characterized by a basic leucine zipper (bZIP) domain; it contains five consensus of leucine amino acids arranged as a helix in addition to a basic region. Upon dimerization the leucines form a coiled conformation and the basic region binds to the promoter region of the target DNA sequence (Vogt, 2001).

c-Fos and c-Jun proteins are activated through phosphorylation by certain kinases. This activation is crucial for AP-1 complex formation and facilitates their nuclear translocation (Vogt, 2001). The main phosphorylated sites of c-Fos are Thr325, Thr331 and Ser374 that are phosphorylated by extracellular signal regulated kinase (ERK). Other sites are Ser362 and Thr232 which are phosphorylated by other kinases. c-Jun is phosphorylated by c-Jun N-terminal kinase (JNK) on Ser63 and Ser73 residues (Lopez-Bergami et al., 2010).

It is believed that AP-1 is involved in carcinogenesis and oncogenic transformation. It regulates the invasive response in some tumors and hence, mediates metastasis. Among the regulated AP-1 target genes is the matrix metalloprotease 1 (MMP1) which is linked to tumor invasion (Ozanne et al., 2007). c-Fos is transiently upregulated in early stage of hepatocarcinogenesis then declined at late stage of tumor progression. This temporary
overexpression is needed for priming hepatocytes to migration and tissue invasion. Notably in
mammary epithelial cells, c-Fos downregulates the tumor suppressor E-cadherin involved in cell
adhesion. This leads to nuclear translocation of β-catenin and activation of Wnt signaling
pathway and thus, promotes carcinogenesis (Mikula et al., 2003). On the other hand, c-Jun is
involved in tumor cell survival and apoptosis (Fu et al., 2011). One mechanism by which c-Jun
modulates tumorigenesis is, suppressing the famous cell death regulator p53. Also it was
reported that, c-Jun is overexpressed during the early stages of tumor initiation (Min et al.,
2012).

In breast cancer, Zhong et al., found that COBRA1 inhibits AP-1 transcriptional
activation activity via physical binding to c-Fos. This means that, COBRA1 regulates the
previously stated roles of AP-1 in proliferation, apoptosis and oncogenic transformation. Even
though, the mechanism by which COBRA1 inhibits AP-1 trans-activation is not clear yet, they
raised the possibility that COBRA1 may be performing this action by recruiting the other NELF
subunits to inhibit AP-1 transcriptional activity (Zhong et al., 2004). A different assumption was
proposed by McChesney et al., in upper gastrointestinal carcinoma (UGC), they demonstrated
that COBRA1 inhibits AP-1 trans-activation of TFF1 independently of NELF-E. They found
that, upon NELF-E knock down, TFF1 expression was not stimulated indicating that COBRA1 is
probably exclusively responsible for preventing AP-1 binding to the promoter of TFF1
(McChesney et al., 2006).

### 1.2.3 Trefoil Factor 1 (TFF1)

Trefoil factor 1 (TFF1) is a member of the TFF family; composed of TFF1, TFF2 and
TFF3. They are normally expressed in the upper gastrointestinal (GI) tract. Meanwhile, TFF1
was also identified in breast cancer cell lines and previously referred to as pS2 (Xiao et al., 2015;
Im et al., 2013). The role of TFF1/pS2 is to keep the integrity of the mucus layer protecting the
stomach. Moreover, it is expressed in case of injury or inflammation in the GI tract to restore the
protective mucosal layer. This simulates what happens in case of UGC where TFF1 is elevated at
early tumor stage in an attempt to repair the damage occurred but soon, it is decreased or almost
disappeared. Accordingly TFF1 is proposed as a tumor suppressor gene (Buache et al., 2011;
McChesney et al., 2006). The possible causes for this low expression are; TFF1 gene mutation,
methylation of TFF1 promoter or the GI cancerous glands are not able to produce TFF1 (Buache
et al., 2011; Xiao et al., 2015). While TFF1 shows low expression in UGC conversely, TFF3 is elevated (Im et al., 2013).

Increased TFF1 expression was reported in breast cancer. In addition, TFF1 was identified in cases that developed metastasis in bone. TFF1 is an estrogen receptor (ER) regulated gene that is stimulated by estrogen response element (Buache et al., 2011). TFF1 was one of the genes identified by Aiyar and colleagues to be located downstream to COBRA1 in breast cancer (Aiyar et al., 2007a). Similarly, in liver cancer the regulation of TFF1 is mediated by ER-α, estrogen responsive element and activator protein 1 (AP-1) response element (Barkhem et al., 2002).

1.3 Cell Culture

In this work we used cell lines as a model to study HCC pathogenesis. Cell culture is considered a powerful technique to study molecular mechanisms taking place in different cells in vitro. The studied cell lines were commercially available HCC cell lines each of them is originally cultured from a single liver of HCC patient at certain stage of the tumor then maintained and propagated to represent a cancer model system. As a result, we can understand more about the molecular mechanisms involved in HCC pathogenesis through examining the expression of COBRA1 and its regulated genes in the tested cell lines.

Cell culture has diversity of applications; it is used to monitor cell growth, viability and physiology. Moreover, some techniques that help in cells characterization utilize cell culture such as flow cytometry (Kuystermans & Al-Rubeai, 2012). Also, cell culture is used in biopharmaceutical industry to produce some biopharmaceuticals and vaccines on large scale such as, influenza vaccine (Milián & Kamen, 2015).

The applications of cell culture are extended to test the cytotoxicity of different biomaterial orthopedic implants. To our interest, it was reported that some orthopedic prostheses may lead to cancer development (Keel et al., 2001). We did a previous work on fibroblast cell line L929 to evaluate the biocompatibility of a biomaterial known as ultra high molecular weight polyethylene (UHMWPE) (Firouzi et al., 2014). In appendix 2 we represent the data related to testing the biocompatibility of this biomaterial. In future, we intend to study the involvement of this biomaterial in cancer development.
Study Objectives and Experimental Design

In fact, COBRA1 is one of the NELF complex subunits and a cofactor for AP-1 complex thus, it regulates a number of clustered genes, one of them is TFF1 (Aiyar et al., 2007a; Zhong et al., 2004; McChesney et al., 2006). Also, it was reported to have deregulated expression in certain types of cancer for instance in breast cancer, it is down-regulated at late stage of the tumor (Aiyar et al., 2004; Aiyar et al., 2007b). However, in HCC a preliminary study suggested its overexpression in a number of HCC tissue specimens but still the exact role of COBRA1 in HCC is not fully determined (Kamel, 2012). Based on these reported roles of COBRA1, we hypothesize that COBRA1 may be involved in HCC pathogenesis. To address this hypothesis, the present study has the following objectives.

The main objective of this study was to examine the role of COBRA1 in HCC progression and whether it acts via NELF or AP-1 complexes. To achieve this objective we had 2 specific aims:

1- To determine the expression of NELF complex subunits A, B (COBRA1), C/D, and E at the RNA and/or protein levels in the four HCC cell lines.

2- To examine the levels of expression of AP-1 complex subunits (c-Fos and c-Jun) at the protein level in the four HCC cell lines. The AP-1 complex action would be assessed, by examining the levels of the downstream target, TFF1.

We designed a set of experiments including RT-PCR and Western blot to determine the expression profiles of the tested molecules (Figure 2). RNA and protein samples were extracted from the four HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) as well as the control (MIHA) cell line. The extracted RNA samples were used for semi-quantitative RT-PCR experiments using COBRA1, NELF-A, NELF-C/D, NELE-E and TFF1 primers. Meanwhile, the protein samples were employed in Western blot experiments to determine COBRA1, c-Fos and c-Jun proteins. The obtained data were measured and normalized to their corresponding housekeeping gene by ImageJ software followed by statistical analysis.
Figure 2. Experimental design schematic diagram: HCC cell lines and control cells were well cultured and propagated. Then RNA and Protein samples were extracted and quantified. RT-PCR and Western blots were conducted for the genes of interest. The obtained data were analyzed and normalized to the internal control using ImageJ software followed by statistical analysis.
Chapter (2): Materials and Methods

2.1 Cell culture

Five cell lines (MIHA, HepG2, SNU-449, SNU-398 and SNU-387) were used in this study. The characteristics of these cell lines are explained in (Table 1). The first four cell lines were a kind gift from Dr. Mehmet Ozturk from the Department of Molecular Biology and Genetics, Bilkent University, Turkey. The SNU-387 cell line was a kind gift from our collaborator Dr. Habiba Bougherara from Ryerson University, Toronto, Canada. MIHA and HepG2 cell lines were cultured and maintained in media composed of; DMEM (Dulbecco’s Modified Eagle Medium) (Lonza, USA) supplemented with 10% FBS (Fetal bovine serum) (Lonza, USA) and 5% Penicillin-streptomycin antibiotic (Lonza, USA). While, the used media for SNU-449, SNU-398 and SNU-387 cell lines composed of; RPMI-1640 medium (Roswell Park Memorial Institute medium) (Lonza, USA) supplemented with 10% FBS (Fetal bovine serum) (Lonza, USA) and 5% Penicillin-streptomycin antibiotic (Lonza, USA). Cells were incubated at temperature 37 °C and 5% CO₂ in humidified CO₂ incubator (SHEL LAB, USA). Cells were observed by using the inverted microscope (Olympus 1X70, USA) for monitoring morphologic changes.
Table 1: Characteristics and differentiation of the studied cell lines
(Park et al., 1995; Jung et al., 2012; Brown et al., 2000)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clinical data</th>
<th>HBV DNA detected</th>
<th>Tumorigenicity</th>
<th>Patient received treatment prior cell line isolation</th>
<th>In-vivo description</th>
<th>Doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIHA</td>
<td>59 years, male</td>
<td>Not determined</td>
<td>Non-tumorigenic</td>
<td>Not determined</td>
<td></td>
<td>72 hrs</td>
</tr>
<tr>
<td>HepG2</td>
<td>15 years, Caucasian American, male</td>
<td>No viral infection</td>
<td>Non-tumorigenic</td>
<td>No treatment received</td>
<td>well differentiated</td>
<td>Early grade</td>
</tr>
<tr>
<td>SNU-449</td>
<td>52 years, Asian, male</td>
<td>HBV detected</td>
<td>Tumorigenic</td>
<td>No treatment received</td>
<td>Single nodular with perinodal extension</td>
<td>II-III</td>
</tr>
<tr>
<td>SNU-398</td>
<td>42 years, Asian, male</td>
<td>HBV detected</td>
<td>Tumorigenic</td>
<td>Transcatheter arterial embolization + Doxorubicin + mitomycin C</td>
<td>Single nodular with perinodal extension</td>
<td>IV</td>
</tr>
<tr>
<td>SNU-387</td>
<td>41 years, Asian, female</td>
<td>HBV detected</td>
<td>Tumorigenic</td>
<td>Transcatheter arterial embolization + Doxorubicin + mitomycin C</td>
<td>Single nodular</td>
<td>III-IV</td>
</tr>
</tbody>
</table>
2.2 RNA extraction, quantification and cDNA synthesis

RNA was extracted from each cell line after confluency from a T-75 flask (7 x 10^6 cells) using Trizol reagent (Invitrogen, USA) as per the manufacturer's protocol. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC) treated water then quantified by measuring A260nm using UV spectrophotometer (Shimadzu, Japan) and the concentration of each RNA sample was calculated.

cDNAs were synthesized for each RNA sample using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) from 1µg of the extracted RNA according to the manufacturer’s instructions.

2.3 RT-PCR

Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) was carried out for all the synthesized cDNAs of the tested cell lines to test for the mRNA steady state levels of COBRA1, TFF1 and NELF subunits (A, C/D and E) relative to the house keeping gene β-actin as an internal control. The sequences of the used primers and their optimized annealing temperatures are mentioned in table (2). The PCR reaction was carried for each tested gene using a mixture consisting of 1 µl cDNA template, 2.5 µl 10X DreamTaq Green Buffer (Thermo Scientific), 1 µl dNTP mix (10 mM) (Thermo Scientific), 0.25 µl DreamTaq DNA Polymerase (Thermo Scientific) and 0.75 µl of each forward and reverse primers to reach a final volume 25 µl. The PCR conditions were run as follows: 94°C for 5 mins, followed by optimized number of cycles of (94°C for 30 secs, annealing temp for 45 secs and 72°C for 45 secs) and finally by an extension at 72°C for 7 mins. The annealing temperatures and number of cycles were optimized for each primer.

The amplified PCR products were run on 1.5% agarose gel electrophoresis then visualized using Gel Doc EZ System (Bio-Rad, USA). The RNA extractions and PCR experiments were performed in triplicates for all the tested genes and for each cell line.
Table 2: Sequences of primers used in the RT-PCR, their optimized annealing temperatures and amplicon size (F: forward primer, R: reverse primer, bp: base pair)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing temperature</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>COBRA1</td>
<td>F: 5’-ACATCACCAAGCAGAGGAA-3’ R: 5’-GATCCAGCTGTCCAGCTTC-3’</td>
<td>59.5 °C</td>
<td>366 bp</td>
</tr>
<tr>
<td>TFF1 (Gillesby et al., 1999)</td>
<td>F: 5’-TTTGGAGCAGAGGGAGCAATGG-3’ R: 5’-TGTTATTAGGATAGAAGCACCAGGG-3’</td>
<td>60 °C</td>
<td>240 bp</td>
</tr>
<tr>
<td>NELF-A</td>
<td>F: 5’-GTCGGCAGTGAAACTCAAGT-3’ R: 5’-TTCACACTCACCCACCTTTTCT-3’</td>
<td>60 °C</td>
<td>250 bp</td>
</tr>
<tr>
<td>NELF-C/D</td>
<td>F: 5’-GAAGAAGGAGGAGGAGGAC-3’ R: 5’-GTGCCCAAGGCTAGTGTGAT-3’</td>
<td>56 °C</td>
<td>443 bp</td>
</tr>
<tr>
<td>NELF-E</td>
<td>F: 5’-GTTGAAGTCAGGAGGAC-3’ R: 5’-CGCCGTTCAAGGGAATGATC-3’</td>
<td>63 °C</td>
<td>565 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5’-GCAAAGACCTGTACGCCAAC-3’ R: 5’-GAGACCAAAAGCCTTCATACATCTC-3’</td>
<td>58 °C</td>
<td>777 bp</td>
</tr>
</tbody>
</table>

2.4 Protein extraction and quantification

The tested cells were rinsed with 1 X PBS then whole cell proteins were extracted using Laemmli buffer (50mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), and 10% glycerol), enriched with 1X Halt Protease 17 Inhibitor Cocktail (ThermoScientific, USA). Then the samples were incubated at 4°C for one hr followed by centrifugation at 12,000 rpm at 4°C for 20 mins. The supernatant containing the protein of interest was collected. It was quantified using Pierce® BCA Protein Assay Kit (Pierce Cat. No: 23225) following the manufacturer’s protocol.

2.5 Western blotting

The concentrations of the extracted cell lysates were adjusted by lysis buffer to equal concentrations before loading. The cell lysates were mixed with 6x loading dye (60% Glycerol, 360 mM Tris-HCl pH 6.8, 12% SDS, 0.06% bromophenol blue, 30% β- mercaptoethanol) then boiled at 99 °C for 10 mins. 20 µg from each sample was loaded on 12% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). The gel was allowed to run until the frontal dye ran off and bands separate. It was then blotted on a nitrocellulose membrane (GE Healthcare) at 120 V for 90 mins on cold. The membrane was blocked for 1 hr by incubating
with 5% non-fat dry milk in 1 X PBST (0.01% Tween 20 in 1 X PBS) at room temperature. Each membrane was incubated overnight at 4 °C with the primary antibody of the protein of interest. The membrane was exposed to the primary antibody overnight at 4°C. The primary antibodies used were; rabbit monoclonal to COBRA1 antibody (anti-COBRA1, Abcam Cat. No. ab167401) used with dilution 1:1000 in 3% non-fat dry milk in 1 X PBST. Rabbit polyclonal to c-Fos antibody (anti-c-Fos, Abcam Cat. No. ab53036) used with dilution 1:500 in 5% non-fat dry milk in 1 X PBST. Rabbit polyclonal to c-Jun antibody (anti-c-Jun, Abcam Cat. No. ab32137) used with dilution 1:1000 in 5% non-fat dry milk in 1 X PBST. Rabbit polyclonal to α-tubulin antibody (anti-α-tubulin, ThermoScientific Cat. No. PA1-20988) used with dilution 1:800 in 5% non-fat dry milk in 1 X PBST. After incubation the membrane was washed 3 times with 1 X PBST 5 mins each. Then it was incubated for 1 hr with secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) (KPL Cat. No.: 4751-1516) diluted to 1:20,000 in 5% non-fat dry milk in 1X PBST. The membrane was washed again 3 times with 1 X PBST 5 mins each and once with Tris base pH 9-9.5. Afterwards, the membrane was incubated for 1 min with phosphatase chemiluminescent substrate (PhosphaGLO, KPL, Cat. No.: 50-60-05). Finally the membrane was exposed to X-ray film in the dark and developed using developer and fixer solutions.

Membranes incubated with primary antibodies COBRA1, c-Fos and c-Jun are then stripped to be incubated with α-tubulin primary antibody which served as a loading control. This was done by incubating the membrane with stripping buffer (10% SDS, 0.5M Tris HCl and β-mercaptoethanol) at 65 °C for 15 mins. Then, the membrane was washed with water and blocked with 5% non-fat dry milk in 1X PBST. Afterwards, the membrane is washed with 1 X PBST, incubated with α-tubulin primary antibody and developed following the previously mentioned steps.

2.6 Statistical analysis

All the presented data were averages ± standard deviation (SD) of at least three independent experiments. For the PCR and Western blot data, the intensities of the bands were measured then normalized to the used internal control using ImageJ software. All the data represent the mean ± standard deviation (SD) from three independent experiments. The resulted
data were loaded onto the statistical analysis program RStudio® version 0.97.336, The R Foundation for Statistical Computing, ISBN 3-900051-07-0, and Platform: i386-pc-mingw32/i386 (32-bit). P-values for significance were computed using ANOVA (analysis of variance) followed by the Tukey honest significant differences test. A P-value less than 0.05 was considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).
Chapter (3): Results

3.1 The morphology of studied cell lines:

The cultured cell lines were routinely examined microscopically to monitor their growth. Cell photos were taken to show the difference in the morphology between the tested cell lines (Figure 3). It was observed that all the tested cell lines were apparently epithelial in shape. HepG2 cells appeared as epithelial adherent cells. While SNU-449 cells, were observed as polygonal, adherent cells. In case of SNU-398 cells, they were round, spindle cells but mixed adherent and floating. At last, SNU-387 cells were identified as polygonal, spindle adherent cells. The observed HCC cell lines morphology was consistent with that reported in previous researches (Park et al., 1995). The four studied HCC cell lines were characterized according to their origin, differentiation, grade and tumorigenicity (Table 1).

Figure 3. Cell morphology of the studied cell lines at approximately 70-80% confluency (200x magnification). (A) MIHA (B) HepG2 (C) SNU-449 (D) SNU-398 (E) SNU-387
3.2 Molecular analysis results:

3.2.1 Levels of COBRA1 mRNA expression in different cell lines:

The mRNA level of COBRA1 gene expression was examined using RT-PCR for each of the HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) relative to the control (MIHA cells). These HCC cell lines were originally isolated from patients at different stages of the tumor; HepG2 from early stage, SNU-449 from intermediate stage while, SNU-398 and SNU-387 from late stage. Thus they represent different stages of HCC aggressiveness; early, intermediate and late stages. As shown in figure 4 COBRA1 mRNA was differentially expressed in all the HCC cell lines. All the levels of expression were compared to the MIHA (control). HepG2 exhibited the highest expression (1.6 folds) relative to MIHA. While SNU-387, showed the lowest expression (0.7 folds) relative to MIHA. The other two HCC cell lines (SNU-449 and SNU-398) showed lower expression than HepG2 but still higher than MIHA cells. The increase in expression of COBRA1 mRNA in HepG2, SNU-449 and SNU-398 was significant (P < 0.001) compared to that of MIHA. The difference in expression between HepG2 and SNU-387 was significant (P < 0.001). Moreover, the difference in expression between SNU-387 and that of SNU-449 and SNU-398 was significant (P < 0.001). In addition, the high expression of COBRA1 mRNA in HepG2 and SNU-449 was significant (P < 0.05) compared to SNU-398. The β-actin housekeeping gene was used as an endogenous control in which the bands showed comparable intensity across the tested cell lines.
Figure 4. RT-PCR for COBRA1 mRNA in MIHA and HCC cell lines: The figure showed the appearance of COBRA1 mRNA bands (366 bp) in all the samples and its absence in the negative control (-ve). Negative control contained water instead of the cDNAs of the tested samples. The housekeeping gene β-actin bands (777 bp) appeared in the gel (lower lane) which acted as an endogenous control. b) Graphical representation for the relative expression of COBRA1 mRNA in MIHA and 4 HCC cell lines. The intensity of the bands were measured then normalized to the corresponding bands of the internal control β-actin by ImageJ. Relative expression of COBRA1 mRNA was expressed as fold change to MIHA. HepG2 showed the highest expression 1.6 folds more than MIHA (the control) while, SNU-387 showed the lowest expression 0.7 folds less than MIHA. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (*** P < 0.001 and *P < 0.05).

3.2.2 Levels of COBRA1 protein expression in different cell lines:

COBRA1 protein was determined by Western blot for the same samples mentioned above in the mRNA analysis. COBRA1 showed maximum expression in HepG2 1.1 folds compared to the control (MIHA) (Figure 5). It decreased gradually among the other cell lines (SNU-449 and SNU-398) to reach minimum expression in SNU-387 < 0.1 folds relative to MIHA. P values were calculated and shown in the chart below to reveal significant differences in the expression
between some cell lines. The decrease in COBRA1 protein expression in SNU-387 was significant (P < 0.05) compared to MIHA. Also, there was a significant difference in expression between HepG2 and that of SNU-398 and SNU-387 (P < 0.05 and P < 0.001) respectively. The COBRA1 protein expression reflected similar pattern to that observed for the mRNA but with different values of fold change relative to MIHA. α-tubulin was used as loading control to detect if the samples were loaded equally across all wells and ensure the successful transfer of protein on the membrane while blotting.

**Figure 5. Western blot analysis for COBRA1 protein in MIHA and HCC cell lines:** a) Western blot results for COBRA1 (61 kDa) in MIHA (control) and 4 HCC cell line samples. The house keeping gene α-tubulin bands (55 kDa) appeared in the blot which acted as loading control. b) Graphical representation for the relative expression of COBRA1 protein in MIHA and 4 HCC cell line samples. The intensity of the bands were measured then normalized to the corresponding bands of the internal control α-tubulin by ImageJ. Relative expression is expressed as fold change to MIHA. HepG2 showed the highest expression 1.1 folds more than MIHA (the control) while, SNU-387 showed the lowest expression <0.1 folds less than MIHA. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (** P < 0.01 and *P < 0.05).
3.2.3 **Levels of NELF subunits A, C/D and E mRNA in different cell lines:**

As previously mentioned COBRA1 is one of the NELF complex subunits referred to as NELF-B. The NELF-B subunit (COBRA1) expression was previously examined at both the mRNA and protein levels (Figure 4 and 5). Accordingly, it was important to test the expression of the other three NELF subunits (A, C/D and E). The mRNA levels of NELF subunits were examined using RT-PCR in the five tested cell lines (Figure 6). NELF-C/D are translation variants of the same mRNA thus they were detected by one primer named NELF-C/D (Gilchrist et al., 2008). All the levels of expression were represented as fold change of MIHA cell line (lane 1) as the control. MIHA showed the highest expression of all the NELF subunits compared to the four HCC cell lines. Whereas, among the HCC cell lines comparable levels of expression were noticed (Afify, 2014).

In case of NELF-A expression, there was a significant difference in expression between MIHA and each of the HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) (P < 0.01). Regarding NELF-C/D, the difference in expression between MIHA and SNU-449 was significant (P < 0.01). Eventually in case of NELF-E, there was a significant difference in expression between MIHA and each of the HCC cell lines (SNU-449, SNU-398 and SNU-387) (P < 0.05).
Figure 6. RT-PCR for NELF subunits A, C/D and E mRNA in MIHA and HCC cell lines: a) Gel electrophoresis results for NELF subunits mRNA in MIHA (control) and 4 HCC cell line samples. NELF-A (250 bp), NELF-C/D (443 bp) and NELF-E (565 bp) were expressed in all the samples whereas no expression in the negative control (-ve). Negative control contained water instead of the cDNA. The housekeeping gene β-actin bands (777 bp) appeared in the gel which acted as an endogenous control. b) Graphical representation for the relative expression of NELF subunits mRNA in MIHA and HCC cell lines. The intensity of the bands were measured then normalized to the corresponding bands of the internal control β-actin by ImageJ. Relative expression of NELF mRNA was expressed as fold change to MIHA. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (** P < 0.01 and * P < 0.05).

3.2.4 Levels of c-Fos protein expression in different cell lines:

c-Fos and c-Jun are members of the AP-1 complex (Lopez-Bergami et al., 2010; Mikula et al., 2003). Some reports found that COBRA1 may be associated with AP-1 complex via physical
binding to c-Fos on the promoter proximal region of its target genes in breast cancer and UGC (Zhong et al., 2004; McChesney et al. 2006). Therefore, examining the c-Fos protein expression level may indicate the possible COBRA1/c-Fos interaction. Western blot was carried out to determine the c-Fos protein in the four HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) and the control (MIHA). It was reported that c-Fos should be activated by phosphorylation in order to dimerize to c-Jun subunit and form AP-1 complex (Ozanne et al., 2007; Lopez-Bergami et al., 2010). Consequently, multiple bands were observed for c-Fos protein at (50, 60, 65 and 70 kDa) corresponding to the phosphorylated and unphosphorylated forms of the protein. The unphosphorylated c-Fos was detected at 50 kDa while the phosphorylated c-Fos was apparently observed at higher molecular weights (60, 65 and 70 kDa) (Figure 7a).

In case of phosphorylated c-Fos expression, the most prominent band was identified at 70 kDa (referred as phosphorylated c-Fos1) in all the tested cell lines. HepG2 showed the maximum expression then the level of expression decreased gradually to reach its minimum in SNU-387 (Figure 7b). There was a significant difference in phosphorylated c-Fos expression between HepG2 and each of SNU-398 and SNU-387 (P < 0.05 and P < 0.01 respectively). Another band was observed at nearly 65 kDa (referred as phosphorylated c-Fos2) expressed in all the samples following the same pattern of phosphorylated c-Fos1 but without showing a significant difference (p > 0.05). At about 60 kDa a very faint band was recognized in SNU-398 but barely seen in MIHA and HepG2 (wasn’t included in the statistical analysis). In case of unphosphorylated c-Fos, an apparent band was detected at 50 kDa in MIHA cell line only (Figure 7b).

The total c-Fos protein (both the phosphorylated and unphosphorylated forms) expression was represented in Figure 7b in which c-Fos was differentially expressed in all the tested samples. The maximum expression was found in MIHA then it showed a gradual decrease in the other three cell lines (HepG2, SNU-449 and SNU-398) with the SNU-387 cell line showing the lowest c-Fos expression. There was a significant difference in expression between MIHA and that of SNU-398 and SNU-387 (P < 0.01 and P < 0.001) respectively. Moreover, the decrease in expression in SNU-387 was significant compared to HepG2 and SNU-449 (P < 0.001 and P < 0.05) respectively.
Figure 7. Western blot analysis for c-Fos protein in MIHA and HCC cell lines: a) Western blot results for c-Fos in MIHA (control) and 4 HCC cell line samples. Three bands were visible in the gel; representing the phosphorylated c-Fos protein (65 kDa and 70 kDa) in all the tested cell lines and the unphosphorylated (50 kDa) in MIHA only. The house keeping gene α-tubulin bands (55 kDa) appeared in the blot which acts as loading control. b) Graphical representation for the relative expression of total c-Fos protein, its two phosphorylated forms and unphosphorylated form in MIHA and 4 HCC cell line samples. The data were represented after measuring the intensity of the bands in the Western blot and normalization to the corresponding bands of the loading control α-tubulin by ImageJ. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (**P < 0.01, *P < 0.05). The first phosphorylated c-Fos protein which appeared at 70 kDa (blue), the second phosphorylated c-Fos protein which appeared at 65 kDa (red), the total c-Fos protein (green) and the unphosphorylated form which appeared at 50 kDa (violet).

The percentage of the phosphorylated form of c-Fos was different in each one of the different cell lines. Assuming total c-Fos protein was 100%, the different forms of phosphorylated and unphosphorylated proteins were calculated as percentage from the total c-Fos protein (Figure 8). The four cell lines (HepG2, SNU-449, SNU-398 and SNU-387) were totally phosphorylated with similar percentages representing the different phosphorylated isoforms. Meanwhile, the MIHA cell line was approximately 60% phosphorylated, with the remaining percentage existing in the unphosphorylated form.
Figure 8. Chart representing the percentage of phosphorylation of c-Fos protein in MIHA and HCC cell lines: The amount of c-Fos existing in the phosphorylated or unphosphorylated forms was expressed as percentage of the total c-Fos protein. While c-Fos in the MIHA seemed to exist in both phosphorylated and unphosphorylated forms, in the remaining cell lines c-Fos was present in only phosphorylated forms. The first phosphorylated c-Fos protein (blue), the second phosphorylated c-Fos protein (red) and the unphosphorylated form (green).

3.2.5 Levels of c-Jun protein expression in different cell lines:

After examining the level of expression of c-Fos, the c-Jun protein was also determined to obtain the total AP-1 complex expression profile. Western blot analysis was done for the same samples mentioned above to test for c-Jun protein. c-Jun was only expressed in three cell lines (HepG2, SNU-449 and SNU-387). No bands were observed for either MIHA or SNU-398. HepG2 had the highest expression while SNU-387 exhibited the least expression (Figure 9). There was a significant difference in c-Jun expression between HepG2 and each of the following cell lines (MIHA, SNU-449, SNU-398 and SNU-387) (P < 0.001). It is worth mentioning that only the unphosphorylated form of c-Jun (36 kDa) was expressed. The phosphorylated form of c-Jun (43 kDa) was not observed.
Figure 9. Western blot analysis for c-Jun protein in MIHA and HCC cell lines: a) Western blot results for c-Jun in MIHA (control) and 4 HCC cell line samples. The unphosphorylated c-Jun protein (36 kDa) was expressed only in HepG2, SNU-449 and SNU-387 cell lines, while no bands appeared for either MIHA or SNU-398 cell lines. The housekeeping gene α-tubulin bands (55 kDa) appeared in the blot which acted as a loading control. b) Graphical representation for the relative expression of c-Jun protein in the three HCC cell line samples. The intensity of the bands were measured then normalized to the corresponding bands of the internal control α-tubulin by ImageJ. HepG2 had the highest expression while, SNU-387 exhibited the lowest expression. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (*P < 0.001).

3.2.6 Levels of TFF1 mRNA expression in different cell lines:

The last molecule tested in this study was the TFF1. TFF1 was reported as a downstream target to COBRA1 in breast cancer and UGC (Aiyar et al., 2007a; Aiyar et al., 2007b; McChesney et al. 2006). So, by testing the TFF1 mRNA in HCC cell lines, we can elucidate any correlation between COBRA1 and TFF1 in HCC. RT-PCR was carried out for the five cell lines to test for TFF1 mRNA (Figure 10). TFF1 was only expressed in MIHA and HepG2 cell lines. No bands were observed for the remaining cell lines (SNU-449, SNU-398 and SNU-387). It was
highly expressed in HepG2 (> 5 folds) compared to MIHA revealing a significant difference (P < 0.001).

**Figure 10. RT-PCR for TFF1 mRNA in MIHA and HCC cell lines:** a) Gel electrophoresis results for TFF1 mRNA in MIHA (control) and 4 HCC cell line samples. The figure showed the appearance of TFF1 bands (240 bp) in only 2 samples and its absence in the negative control (-ve). Negative control contained water instead of the cDNAs of the tested samples. TFF1 was expressed only in MIHA and HepG2 while no bands appear in the other 3 HCC cell lines. The housekeeping gene β-actin bands (777 bp) appeared in the gel which acted as an endogenous control. b) Graphical representation for the relative expression of TFF1 mRNA in MIHA and HepG2. The intensity of the bands were measured then normalized to the corresponding bands of the internal control β-actin by ImageJ. Relative expression was expressed as fold change to MIHA. HepG2 showed higher expression 5.5 folds more than MIHA. The data were presented as a mean of three independent experiments (mean±SD). The P values for statistical significance were computed using the Tukey honest significant differences test. (*** P < 0.001).
Chapter (4): Discussion

Hepatocellular carcinoma (HCC) is ranked as the second most common cause of cancer related deaths in the world (Ferlay et al., 2014). It is one of the tumors characterized by poor prognosis (Dahmani, et al., 2011). Consequently, the introduction of new diagnostic and prognostic markers for HCC is very essential. The signaling pathways involved in HCC are still not fully characterized. Accordingly, the study of the molecular mechanisms underlying the pathogenesis of HCC is considered a cornerstone of finding these new markers. COBRA1 is the cofactor of BRCA1 a well-known transcription factor involved in different tumors pathogenesis; it acts as a tumor suppressor gene in breast cancer unlike its oncogenic role in the upper gastrointestinal adenocarcinoma (UGC) (McChesney, et al., 2006). Recently this player (COBRA1) has been identified in HCC using diseased Egyptian liver specimens (Kamel, 2012). Kamel’s study tested 16 HCC tissue specimens for COBRA1 expression; it was found that COBRA1 protein was overexpressed in approximately 50% of the cases and downregulated in the other 50%. Hence, more studies are required to explain the role of COBRA1 in HCC.

To the best of our knowledge this is the first study to investigate COBRA1 in this group of HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) in addition to immortalized normal liver cells (MIHA) as control (Table 1). These cell lines were chosen since they were initially isolated from patients suffering from HCC at different stages of the tumor. Therefore, the level of expression of COBRA1 in different tumor stages could be tracked. Furthermore, the role of COBRA1 in molecular mechanisms of HCC pathogenesis could be investigated. Cell lines are considered a powerful tool to this study because these cells are first isolated from a primary tumor of a single patient. Then cultured and propagated in vitro to divide into numerous cells exhibiting the same properties. As a result, cell lines are believed to be more homogenous environment to examine molecules involved in cancer progression compared to tissue samples.

It was reported that COBRA1 does not have a DNA binding domain so it must complex with other protein(s) to bind to the promoter site of its downstream targets. Thus, it could be either a part of the negative elongation factor (NELF) referred to as NELF-B, or it could bind to activator protein 1 (AP-1) complex or even to other not yet identified complexes (McChesney et al., 2006; Zhong et al., 2004). In breast cancer, COBRA1 (NELF-B) binds to the estrogen
receptor (ER) α and attracts other NELF complex subunits to pause the transcription elongation of downstream target genes via stalling RNA polymerase II (RNAPII). It can also bind to the AP-1 complex subunits (c-Fos and c-Jun) to inhibit AP-1 transactivation (Zhong et al., 2004). While in upper gastrointestinal adenocarcinomas (UGC), it was found that COBRA1 binds to c-Fos independent on ER-α and NELF-E. As a result, it inhibits the AP-1 complex transactivation at the trefoil factor 1 (TFF1) promoter site and so decreases TFF1 expression (McChesney et al. 2006). Consequently, they introduced the following model (Figure 11).

Figure 11. Schematic diagram of the model proposed in UGC: The model suggested by McChesney and his colleagues in which COBRA1 interacts with AP-1 complex through physical binding to c-Fos on the promoter proximal region of TFF1 in order to inhibit TFF1 expression. The black bars represent the exons of TFF1.

This work aimed to study the two possible scenarios by which COBRA1 binds to the promoter proximal region of TFF1 gene to control its expression, it either binds to the AP-1 complex or it is a part of the NELF complex. This was done by; first, measuring the levels of RNA and protein expression of COBRA1. Second, the expression of the other NELF subunits (A, C/D and E) was examined on the mRNA level. Third, the expression pattern of c-Fos and c-Jun proteins was determined. Finally, testing the levels of mRNA steady state levels of TFF1 as a downstream target to COBRA1 then correlates all these results together.

4.1 Role of COBRA1 in HCC progression and pathogenesis:

COBRA1 was differentially expressed in all the tested HCC cell lines relative to the control on both the mRNA and protein levels. On the mRNA level HepG2 showed the highest
expression while SNU-387 exhibited the lowest expression relative to the control (MIHA) (Figure 4). In case of COBRA1 protein, it nearly paralleled the mRNA expression levels; where the highest expression was present in HepG2 and the lowest in SNU-387 (Figure 5). However, the decrease in COBRA1 protein expression in the three cell lines (SNU-449, SNU-398 and SNU-387) was more prominent compared to that of corresponding mRNA. This may be due to post transcriptional modification in the transcripts prior to translation that led to lower protein levels.

HCC is usually diagnosed at late stage of the tumor in which treatment is of limited efficacy. Thus, prognosis and follow up is necessary for patients’ condition regular assessment to predict any risks before condition deterioration. Among the well-known prognostic markers in HCC are α-fetoprotein (AFP), tumor size and bilirubin. However, these markers are still insufficient due to their variable sensitivity. For instance, some patients might have similar AFP profile level although their tumor aggressiveness is different (Tandon and Garcia-Tsao, 2008). Accordingly, introducing more accurate prognostic markers for HCC is highly demanded. In particular, molecular markers are highly sensitive since their expression levels depend on the tumor biology. In this study, the results showed a gradual decrease in COBRA1 protein expression with increasing the aggressiveness of HCC. It was upregulated in early cancer stages represented by HepG2 and decreased to its minimum expression in SNU-387 representing late HCC stage. Therefore, COBRA1 was suggested to have a tumor suppressor activity and can be proposed to have a potential role in HCC progression.

4.2 NELF (A, C/D and E) subunits co-dependence expression:

NELF is a transcription elongation factor made up of four subunits NELF (A, B, C/D and E). COBRA1 was identified as NELF-B subunit of the complex (Narita et al., 2003; Aiyar et al., 2004; Yung et al., 2009). As discussed, COBRA1 was differentially expressed in the tested cell lines. Given that, these cell lines represent different stages of HCC. Hence, we need to find if the other NELF subunits follow similar expression pattern to COBRA1 or not. Therefore, the NELF subunits (A, C/D and E) were analyzed on the mRNA level. A semi-quantitative RT-PCR was carried out utilizing the cDNAs of the four HCC cell lines (HepG2, SNU-449, SNU-398 and SNU-387) as well as the control (MIHA) cells (Figure 6). It was found that, the three NELF subunits (A, C/D and E) showed comparable expression in all the tested samples.
This finding was consistent with previous researches that proved the simultaneous expression of the NELF subunits to bind on ER-α regulated targets (Aiyar et al., 2004). In addition, Sun et al., postulated that on knock down of any of the NELF subunits a co-depletion occurred for the other NELF members on the protein level. However, this co-depletion was not observed on the mRNA level which proposed that the deregulation of these subunits took place at a post-transcriptional step. This emphasized the co-dependence nature of NELF subunits expression (Sun et al., 2008; Sun and Li, 2010). Given that, this study tested the NELF subunits on the mRNA level only, so in order to get the complete picture a further study on the protein level is recommended.

4.3 AP-1 complex subunits (c-Fos and c-Jun) expression relative to COBRA1:

AP-1 is a dimeric transcription factor that consists of members of the Fos and Jun gene families including c-Fos and c-Jun. It is involved in oncogenesis since it controls some downstream oncogenes (Ozanne et al., 2007). It participates in some cellular activities such as cell proliferation, differentiation, apoptosis and metastasis (Watanabe et al., 2013). c-Fos and c-Jun subunits must be activated through phosphorylation by certain kinases to associate in the form of AP-1 complex. c-Fos is activated through phosphorylation of certain serine and threonine residues by extracellular signal regulated kinase (ERK). While c-Jun is activated by phosphorylation of its serine residues by c-Jun N-terminal kinases (JNKs) (Lopez-Bergami et al., 2010). Besides, a study demonstrated that there is a correlation between the expression of protein kinase R (PKR) and that of c-Fos and c-Jun. The tested human HCC specimens that exhibited high PKR had greater expression of phosphorylated c-Jun and phosphorylated c-Fos which indicates that PKR is involved in c-Fos and c-Jun activation (Watanabe et al., 2013).

In the present study both c-Fos and c-Jun proteins were examined by Western blot. We observed that the active phosphorylated forms of c-Fos and c-Jun could not be detected simultaneously in the tested cell lines. This might suggest that the AP-1 complex is not formed under the tested experimental conditions, as will be outlined in the following subsections.

4.3.1 c-Fos protein expression:

Regarding the levels of the total c-Fos protein (both phosphorylated and unphosphorylated forms); the highest expression was observed in (MIHA). For (HepG2, SNU-449 and SNU-398)
lower expression was noticed and eventually (SNU-387) exhibited minimum expression. On the other hand, the phosphorylated active form of c-Fos protein was differentially expressed in all the tested cell lines while its unphosphorylated protein was only expressed in (MIHA). HepG2 showed the maximum expression of phosphorylated c-Fos then the level of expression decreased gradually to reach its minimum in SNU-387 (Figure 7b). These findings demonstrate that, the total c-Fos protein expression decreased with increasing the aggressiveness of the tumor in the tested cell lines. This finding is supported by a previous study performed on immortalized Met murine hepatocytes which revealed that c-Fos is temporarily upregulated in early stage of the tumor then declined at late stage carcinogenesis. This variable expression enables c-Fos to direct the cell activities towards tissue invasion and metastasis (Mikula et al., 2003).

Accordingly, the detected expression level of both phosphorylated and total c-Fos would preliminarily imply the formation of the AP-1 complex through its association with the phosphorylated form of c-Jun. However, this assumption was challenged by the expression profile of c-Jun subunit.

4.3.2 c-Jun protein expression

It was essential to test the other AP-1 complex subunit (c-Jun) in order to obtain the complete expression profile of the AP-1 complex. Similar to c-Fos, in order to dimerize c-Jun should be first activated by phosphorylation (Lopez-Bergami et al., 2010). As shown in Figure 9, the active phosphorylated form of c-Jun could not be observed in any of the cell lines. While, the c-Jun unphosphorylated form could only be detected in three cell lines (HepG2, SNU-449 and SNU-387) in which HepG2 exhibited the highest expression while the least expression was observed in SNU-387. Paradoxically to what could be concluded from the c-Fos expression profile, this suggests that AP-1 complex could not be formed in the cell lines (HepG2, SNU-449 and SNU-387) despite the phosphorylation of the c-Fos. Similarly, the absence of detectable level of c-Jun in (MIHA and SNU-398) suggests that the formation of the AP-1 complex is not likely. However, the presence of the phosphorylated form of c-Jun cannot be completely ruled out due to the reported labile nature of the active form of c-Jun protein. It has a short half life of approximately 2 hours so it is rapidly degraded by the cell machinery (Lopez-Bergami et al., 2010).
According to the obtained data compiled in Figure 12, c-Fos was expressed in all the studied cell lines in the phosphorylated active form. In contrast, c-Jun was only detected in three cell lines (HepG2, SNU-449 and SNU-387) in the unphosphorylated inactive form. Due to the absence of the active form of c-Jun, we suggest that the AP-1 complex might not be assembled in the studied cell lines. However, further assays are required to investigate the possibility of AP-1 complex formation in these cell lines.

A previous report studied the expression of both c-Fos and c-Jun in HCC patients’ tissue specimens using immunohistochemistry. It postulated that c-Fos expression levels were higher in tumor tissue relative to non-tumor tissue. In addition, high c-Jun expression levels were reported in tumor tissue. Accordingly, there is an association between the expression of both c-Fos and c-Jun in tumor tissue (Yuen et al., 2001). These assumptions are not in line with our findings for c-Fos expression. But, this may be due to some differences between the two studies. First, the tumor stage of the majority patients involved in the study was moderately differentiated while in our study different stages (early, intermediate and late) of the tumor were studied. Second, they used tissue specimens while we worked on cell lines. Finally, the different assays used; they carried out immunohistochemistry while in our study Western blot was performed.

4.3.3 AP-1 complex expression in correlation to COBRA1:

Our results showed that the expression of the three proteins COBRA1, c-Fos and c-Jun in the four HCC cell lines followed nearly a similar pattern. In which, maximum levels are observed in HepG2 and minimum levels in SNU-387 (Figure 12).

These findings revealed that the three proteins were differentially expressed among the tested cell lines. They were high at early HCC stage and decreased gradually till reached their least levels of expression in advanced HCC stage. Therefore, this study suggested that not all the HCC cell lines behaved the same in expressing the 3 tested proteins proposing the possible use of these molecules in characterization of HCC cell lines on the molecular level.
Figure 12. Graphical representation for the relative expression of COBRA1, c-Fos and c-Jun proteins (AP-1 complex subunits) in MIHA and 4 HCC cell lines: COBRA1 and c-Fos were expressed in all the samples while c-Jun was expressed in all samples except MIHA and SNU-398. The cell line (MIHA) that express c-fos in unphosphorylated form don’t express c-Jun and thus most probably the AP-1 complex might not be formed.

4.4 Trefoil Factor 1 (TFF1) in correlation to AP-1 complex:

TFF1 is normally found in the stomach as it helps in formation of the mucus layer. In most upper gastrointestinal carcinoma (UGC) cases, TFF1 is downregulated suggesting a tumor suppressor role (Buache, et al., 2011). In this study, the TFF1 mRNA expression level was examined in HCC cell lines by RT-PCR. TFF1 mRNA was only expressed in two cell lines (MIHA and HepG2) in which, HepG2 exhibited significant higher expression level compared to MIHA (P < 0.001). Given that, HepG2 represents early tumor stage so this supports the tumor suppressor character of TFF1 that rises at early stages of the tumor in order to protect the affected tissue (Tanaka et al., 2013). While, in SNU-449, SNU-398 and SNU-387 which represent intermediate and advanced tumor stages, TFF1 mRNA was not detected which indicates absence of TFF1 protein and supports its tumor suppressor role. This result was in agreement with a previous study conducted on intrahepatic cholangiocarcinoma (IHCC) tissue specimens in which TFF1 expression was high in non-invasive IHCC but significantly decreased
in invasive IHCC (Sasaki, et al., 2003). Therefore, these results support a tumor suppressor role of TFF1 in HCC.

Moreover, the TFF1 mRNA steady state level was examined in this study to assess the AP-1 complex role in regulating TFF1. It was reported that TFF1 is a downstream target to COBRA1 (Aiyar et al., 2007a). In UGC, COBRA1 controls TFF1 expression through regulating AP-1 complex trans-activation (McChesney, et al., 2006). We aim to elucidate whether COBRA1 and AP-1 complex play a role in regulating TFF1 expression. Our findings showed a discrepancy in TFF1 mRNA expression; it was only expressed in two cell lines (MIHA and HepG2) and was not detected in the other three cell lines (SNU-449, SNU-398 and SNU-387). While for the AP-1 complex subunits (c-Fos and c-Jun) protein expression, the phosphorylated active form of c-Jun was not detected in all the tested cell lines which most likely excludes the possibility of AP-1 complex formation. Therefore, even though AP-1 complex subunits are not present in their active forms in all the studied cell lines TFF1 mRNA is expressed in only two cell lines. This indicates that, in the tested HCC cell lines the TFF1 transcription is not only regulated by AP-1 but, may be other regulatory pathways are involved.

4.5 Differential expression of the tested molecules and etiology of cell lines

It was evident from the discussed results that HepG2 cell line exhibited the highest expression levels of all the tested molecules. The different patterns of expression of the examined molecules in the four HCC cell lines used in this study may be attributed to the different etiologies of these cell lines. As all the tested HCC cell lines in this study, except HepG2, were isolated from tumors of chronic HBV patients (Costantini et al., 2013). HBV is one of the risk factors of HCC. A study postulated that there was a simultaneous high expression of Hepatitis B surface antigen (HBsAg) and lymphoid enhancer-binding factor 1 (LEF-1) in tumor liver cells. The LEF-1 upregulation stimulate hepatocytes proliferation through the Wnt pathway which may lead to malignancy and HCC development (Tian et al., 2009). Moreover, another study stated that HBV X peptide activated both c-Fos and c-Jun expression in which this mechanism was involved in liver carcinogenesis (Yuen et al., 2001). Taken together, these cell
lines are different in the molecular mechanisms underlying carcinogenesis which in turn affected the expression profiles of the tested molecules in this study.
Chapter (5): Conclusion

As far as we know, this is the first study to examine COBRA1 in this group of HCC cell lines representing different tumor stages.

According to our findings we can conclude that; COBRA1 is differentially expressed among the tested cell lines suggesting a potential role of COBRA1 in HCC pathogenesis and development.

The high expression of TFF1 RNA in early stage HCC (HepG2) and its decrease to undetectable levels in late stage (SNU-387), suggests a tumor suppressor role of TFF1. Nevertheless, more studies are required to examine the correlation between COBRA1, AP-1 complex and TFF1 in HCC before a clear conclusion can be drawn here.

This preliminary work serves as a foundation and starting point for further investigation of COBRA1 role in HCC development.
Future Recommendations

The studied HCC cell lines in this work (except HepG2) are isolated from patients infected with HBV. However, in Egypt, the most prevalent risk factor for HCC is HCV. Thus, this work can be expanded to include more HCC cell lines of HCV etiology to compare the influence of different etiologies on COBRA1 expression.

Examining the NELF subunits (A, C/D and E) on the protein level in the tested HCC cell lines is recommended since their RNAs are subjected to post-transcriptional modifications.

Co-immuno precipitation (Co-IP) assay should be performed to test for COBRA1/AP-1 binding, as well as chromatin Immunoprecipitation (ChIP) to confirm their association on the TFF1 promoter site in HCC.

The c-Jun primary antibody used in this study didn’t detect the phosphorylated active form of c-Jun. Despite, the same antibody was used in a previous research and detected the phosphorylated c-Jun (Kan and Tabin, 2013). Therefore, we suggest purchasing either a new c-Jun antibody from different batch or a specific antibody for phosphorylated c-Jun to confirm the presence or absence of its phosphorylated form.
References


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Appendix 1: Copyright Form

Name: Aya Atef Youssef

Address: American University in Cairo, Biotechnology Master’s Program. AUC Avenue, P.O. Box 74. New Cairo 11835, Egypt.

Date: 13/10/2015

I, Alaa Abfy, hereby authorize Aya Atef Youssef to include my data related to RNA expression of NELF (A,C,D,and E) subunits downstream using her cDNA samples in her Master’s thesis.
Permission for Reference

Aya Youssef <aya.youssef@aucegypt.edu>
to hhanda

Dear Dr. Handa,

I hope this email finds you fine. I would like to ask your permission to refer to figure (8b) from your paper “Human Transcription Elongation Factor NELF: Identification of Novel Subunits and Reconstitution of the Functionally Active Complex” in my MSc thesis. I will add it as a figure from the paper with the proper citation in reference to the paper.

Looking forward to your reply.

Thanks a lot,

Regards,

---

hhanda@tokyo.med.ac.jp
to me

Dear Aya,

Thank you for your email. We agree that you use the figure. I appreciate your integrity.

Best regards,

Hiroshi
Appendix 2: Previous Research

We participated in research project studying the cytotoxic effect of a biomaterial used in orthopedic implants that was published under the name:

“A new technique to improve the mechanical and biological performance of ultra high molecular weight polyethylene using a nylon coating.”


Summary of work:

The study suggested a new technique using nylon coating onto a biomaterial called ultra high molecular weight polyethylene (UHMWPE) to improve its mechanical and biological properties. Cytotoxicity of the nylon coated material was examined using MTT assay in comparison to the neat material. Cytotoxicity studies have demonstrated significant improvement in cell viability using the nylon coated UHMWPE over the neat one (72.4% vs 54.8%) for 48h and (80.7 vs 5%) for 72h (P<0.01). Therefore, this study suggests that UHMWPE coated with nylon could be used as a novel material in clinical applications with lower cytotoxicity, less wear debris-induced osteolysis, and superior mechanical properties compared to neat UHMWPE.

Background:

Ultra high molecular weight polyethylene (UHMWPE) was involved in many orthopedic applications due to number of advantages; light weight, good wear resistance, chemical stability and lubricity. However, leachable substances from the material may induce cytotoxicity and limit the use of the material in orthopedic implants. One common problem that leads to implant failure is osteolysis. It is an inflammatory reaction induced by materials characterized by high stiffness that causes bone-lysis at the bone-implant interface. Therefore, implants should exhibit high strength and low stiffness to overcome osteolysis. Several attempts were made to improve the UHMWPE properties such as; gamma irradiation cross linking, using fillers as glass and thermal processing. Other studies reported a decrease in the material cytotoxicity when coating it with titanium or hydroxyapatite. In this study a new technique is introduced to decrease the cytotoxic and osteolysis effect of UHMWPE using nylon coating.
Materials and Methods:

1. Cell culture:

L929 fibroblasts cell line was used to examine the cytotoxicity of the coated and neat UHMWPE material. They were grown in DMEM supplemented with 10% FBS and 5% penicillin/streptomycin. The cells were seeded in 96-well plate and incubated for 24 hrs at 37 °C and 5% CO₂. Then, the culture media was replaced by the conditioned media of both the nylon coated and the neat material. Some of wells were left with the cells untreated to act as a control. The cells were incubated at 37 °C and 5% CO₂ for 24, 48 and 72 hrs.

2. Cytotoxicity assay by MTT:

The Methylthiazol Tetrazolium (MTT) assay was used to determine the cytotoxicity of the material on the L929 cells. The MTT assay is based on the ability of living cells to convert a water-soluble yellow dye, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide into violet formazan crystals by the action of mitochondrial dehydrogenase enzymes present in living cells. As a result, the cell viability can be assessed by the intensity of the violet color formed. Then, the conditioned media were discarded and the MTT reagent was added and incubated for 4hrs. Afterwards, the formed formazan crystals were solubilized and the color intensity was measured. Cytotoxicity was calculated as the percentage of control cell viability where the control cells were considered to be of 100% survival.

Results and Discussion:

Recently, significant attention has been given to the field of biomaterials in bone prosthesis due to the increasing number of accidents and bone fractures that need the surgical implantation of these biomaterials into the body. As a result, testing the cytotoxicity of these biomaterials first in vitro is considered a good approach to judge the biocompatibility of the proposed material. In this study, the cytotoxicity of the nylon coated UHMWPE material is tested in comparison to the neat UHMWPE material using MTT assay. This assay indicated the degree of cell survival of the L929 fibroblasts cell line after incubating them with the conditioned media of both the nylon coated and uncoated UHMWPE material and the results of the assay reflected the cytotoxicity of the tested materials so that the effect of coating the
material with nylon can be evaluated. MTT assay results indicated that the neat material induced significant cell death compared to the control with the same P-value (P < 0.001) in the three incubations (Figure 13). While, the nylon coated material induced significant cell death compared to the control with different P-values in the three incubations which means that, the uncoated UHMWPE material caused a more significant decrease in cell survival than the nylon coated UHMWPE material in relation to the control. Consequently, the nylon coated UHMWPE material was proved to be less cytotoxic and that the nylon coat improved the UHMWPE material biocompatibility.

Figure 13. Effect of neat and Nylon coated UHMWPE extracts on the viability of L929 fibroblast cells at 24, 48 and 72 hours determined by MTT assay. It is presented as percentage of the negative control which is considered to be 100% viability (*P < 0.05, **P < 0.01, and ***P < 0.001).

Conclusion:

Coating the UHMWPE material with nylon coating improved the mechanical properties and biocompatibility of the material. This resulted in less cell death and cytotoxicity effect. Therefore, the nylon coated UHMWPE was proposed as a promising biomaterial candidate in bone prosthesis.