The potential involvement of the cofactor of BRCA1 in hepatocellular carcinoma pathogenesis

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Abstract: The cofactor of BRCA1 (COBRA1), which also refers to negative elongation factor polypeptide B (NELF-B), is a negative elongation factor (NELF) subunit that has been implicated in the development and progression of several cancers. While reduced COBRA1 expression has been associated with metastatic breast cancer, COBRA1 negatively regulates the activator protein-1 (AP-1) complex, leading to the down-regulation of trefoil factor-1 (TFF1) expression in gastric cancer cell lines. The involvement of COBRA1 in hepatocellular carcinoma (HCC) tumor formation and progression is not known. Here, we investigated the expression of COBRA1, the AP-1 complex, and TFF1 in several HCC cell lines; ranging from low- to high-grade HCC cell lines generated from patients with different stages of the disease. Our results showed that the COBRA1 protein was highly expressed in the low-grade HCC cell line, while significantly down-regulated in high-grade HCC cell lines. TFF1, previously regarded as a COBRA1 target gene, was only expressed in the low-grade HCC cell line and the control cells. Our results suggest that COBRA1 may play a role in HCC pathogenesis and progression. The TFF1 mRNA expression profile was uncorrelated to that of the AP-1 complex subunit proteins, which suggests the involvement of other regulatory pathways in TFF1 expression; however, this requires further study.

Keywords: COBRA1; HCC; HepG2; SNU; AP-1


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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm worldwide¹⁻³. Surprisingly, it was ranked as the second most common cause of cancer-related deaths in the world in 2012⁴. Thus, it is considered as a highly aggressive cancer with poor prognosis. To date, the molecular mechanisms underlying HCC pathogenesis have not been fully identified, thus resulting in a lack of reliable prognostic markers for HCC⁵⁻⁶.

COBRA1 is the cofactor of BRCA1; it is one of the four subunits of the negative elongation factor (NELF) and is commonly referred to as NELF-B⁷⁻⁸. The NELF complex subunits are recruited to the target promoter proximal region to stall ribonucleic acid polymerase II (RNAPII), thus inhibiting RNA transcription at the elongation step⁹. COBRA1 has been studied in various types of cancer. For example, cell lines and tissues isolated from late-stage metastatic breast cancer tumors showed low expression levels of the COBRA1 protein, which has displayed tumor suppressor activity¹⁰. In contrast, another study reported that COBRA1 was overexpressed in upper gastrointestinal carcinoma (UGC) tissue samples. Intriguingly, this overexpression was accompanied by the decreased expression of a well-known tumor suppressor: trefoil factor-1 (TFF1). COBRA1 controls TFF1 expres-
sion by regulating the activator protein-1 (AP-1) complex trans-activation, leading to attenuated TFF1 expression\cite{11-14} (Figure 1).

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

It is believed that AP-1 is involved in carcinogenesis and oncogenic transformation. It regulates the invasive response in some tumors, mediating metastasis. The c-Fos protein is transiently up-regulated in the early stages of hepatocarcinogenesis and then declines in the later stages of tumor progression. This temporary over-expression is needed for priming hepatocytes for migration and tissue invasion\cite{15}. In contrast, the c-Jun protein is involved in tumor cell survival and apoptosis\cite{16}. A mechanism, by which c-Jun modulates tumorigenesis, is by suppressing the famous cell death regulator p53. Min et al. reported that c-Jun is overexpressed during the early stages of tumor initiation\cite{17}.

TFF1 is a member of the TFF family, which is composed of TFF1, TFF2, and TFF3. These proteins are normally expressed in the upper gastrointestinal (GI) tract\cite{18,19}. The role of TFF1 is to maintain the integrity of the mucus layer that protects the stomach. Moreover, it is expressed in the case of injury or inflammation in the GI tract to restore the protective mucosal layer. This simulates what happens in the UGC cases, wherein TFF1 is elevated in the early stages of tumor in its attempt to repair the damages, but quickly decreases or almost disappears. A number of researchers have proposed that TFF1 is a tumor suppressor gene\cite{11,20}. Aiyar et al. identified TFF1 as one of the genes that is regulated by COBRA1 in breast cancer\cite{19}.

COBRA1 regulates a number of clustered genes (including TFF1) and its deregulated expression has been reported in various types of cancers\cite{8,9,11,21,22}. In this study, we hypothesized that COBRA1 is involved in HCC pathogenesis. We examined this hypothesis indirectly by investigating the expression of the NELF complex subunits A, B (COBRA1), C/D, and E at the RNA and/or protein levels in four HCC cell lines (HepG2, SNU-449, SNU-398, and SNU-387). These cell lines were generated from patients with different grades of HCC, ranging from early to advanced. They were classified according to Edmondson-Steiner’s grading into four grades from I to IV on the basis of histological differentiation\cite{23}. The HepG2 cells were isolated from well-differentiated tumor that represents low-grade tumor\cite{24}. Meanwhile, the grade II SNU-449 cells represent tumor of intermediate grade, whereas the SNU-398 and SNU-387 cells (originating from less differentiated grade III and IV tumors) are considered as high-grade HCC (Table 1)\cite{24,25}

We also inspected the levels of AP-1 complex subunits (c-Fos and c-Jun proteins) in these cells. Finally, we assessed the AP-1 complex action by noting the levels of the downstream target, TFF1. To the best of our knowledge, this is the first study to evaluate the mRNA expression levels of COBRA1 and its protein in cell lines representing advanced stages of HCC.

**Materials and methods**

**Cell culture**

A total of five cell lines (MIHA, HepG2, SNU-449, SNU-398, and SNU-387) were used in this study. MIHA cells were used as control. The HCC cell lines were originally isolated from patients with different tumor stages, ranging from low-grade (HepG2) to intermediate (SNU-449) to high-grade (SNU-398 and SNU-387). The characteristics of these cell lines and the growth media are explained in Table 1. The MIHA cell line was a kind gift from Dr. Jayanta Roy-Chowdhury at the Albert Einstein College of Medicine. Meanwhile, the HepG2, SNU-449, and SNU-398 cell lines were a generous gift from Dr. Mehmet Ozturk at the Department of Molecular Biology and Genetics, Bilkent University, Turkey. The SNU-387 cell line was a kind gift from Dr. Habiba Bougherara at Ryerson University, Toronto, Canada. The cells were incubated at 37 °C and supplied with 5% CO₂ in a humidified CO₂ incubator.

**Semi-quantitative reverse transcription on polymerase chain reaction (RT-PCR)**

Total RNA was isolated from each cell line, involving around 7 × 10⁶ cells from a T-75 flask, using Trizol reagent (Invitrogen, USA) as per the manufacturer’s protocol. First strand cDNA was synthesized from 1 µg of the
Table 1. Clinico-pathological characteristics of liver biopsies used to derive the studied cell lines and culturing specifications

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clinical data</th>
<th>HBV DNA detected</th>
<th>Tumorigenicity</th>
<th>Patient received treatment prior to cell line isolation</th>
<th>In vivo description</th>
<th>Doubling time</th>
<th>Growth media</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIHA</td>
<td>59 years old, male</td>
<td>Undetermined</td>
<td>Non-tumorigenic</td>
<td>Undetermined</td>
<td>-</td>
<td>-</td>
<td>72 h</td>
<td>DMEM supplemented with 10% FBS and 5% Penicillin-streptomycin antibiotic (Lonza, USA)</td>
</tr>
<tr>
<td>HepG2</td>
<td>15 years old, Caucasian male</td>
<td>No viral infection</td>
<td>Non-tumorigenic</td>
<td>No treatment received</td>
<td>Well differentiated</td>
<td>Early grade</td>
<td>24–30 h</td>
<td>RPMI supplemented with 10% FBS and 5% Penicillin-streptomycin antibiotic (Lonza, USA)</td>
</tr>
<tr>
<td>SNU-449</td>
<td>52 years old, 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>
<td>36 h</td>
<td>[24, 25]</td>
</tr>
<tr>
<td>SNU-387</td>
<td>41 years old, Asian, female</td>
<td>HBV detected</td>
<td>Tumorigenic</td>
<td>TACE + Doxorubicin + mitomycin C</td>
<td>Single nodular</td>
<td>III–IV</td>
<td>61 h</td>
<td>[25]</td>
</tr>
<tr>
<td>SNU-398</td>
<td>42 years old, Asian, male</td>
<td>HBV detected</td>
<td>Tumorigenic</td>
<td>TACE + Doxorubicin + mitomycin C</td>
<td>Single nodular with perinodal extension</td>
<td>IV</td>
<td>39 h</td>
<td>[24, 25]</td>
</tr>
</tbody>
</table>

Total RNA for each sample using random primers of RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) in accordance to the manufacturer’s instructions.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was carried out for all the tested cell lines’ synthesized cDNAs to assess the mRNA steady-state levels of the NELF subunits (A, B, C/D, and E) and TFF1, and the housekeeping gene (β-actin) was used as an endogenous control. The sequences of the primers and their optimized annealing temperatures are given in Table 2.

The PCR conditions used were as follows: 94°C for 5 min, followed by the optimized number of cycles (i.e., 94°C for 30 s, optimized annealing temperature for 45 s, and 72°C for 45 s) and finally, an extension at 72°C for 7 min. The amplified PCR products were run on 1.5% agarose gel electrophoresis, followed by visualization using the 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 RT-PCR, their optimized annealing temperatures, and amplicon sizes

<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′-ACATCCAACAGACAGAAC-3′ R: 5′-GATCCAGCTGTCCAGCTTC-3′</td>
<td>59.5°C</td>
<td>366 bp</td>
</tr>
<tr>
<td>TFF1[^1]</td>
<td>F: 5′-TTTGGAGCAGAGGACAG-3′ R: 5′-TTGGATTAGGATAGACCCAG-3′</td>
<td>60°C</td>
<td>240 bp</td>
</tr>
<tr>
<td>NELF-A</td>
<td>F: 5′-GTCGACGTAAGGTCAATT-3′ R: 5′-GATCCAGCTGTCCAGCTTC-3′</td>
<td>60°C</td>
<td>250 bp</td>
</tr>
<tr>
<td>NELF-C/D</td>
<td>F: 5′-GGAGAGGAGGACAGCCAGC-3′ R: 5′-GATCCAGCTGTCCAGCTTC-3′</td>
<td>56°C</td>
<td>443 bp</td>
</tr>
<tr>
<td>NELF-E</td>
<td>F: 5′-TTGGTAAAGTCAACAGGAC-3′ R: 5′-GATCCAGCTGTCCAGCTTC-3′</td>
<td>63°C</td>
<td>565 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′-GCAAGGACCTGTACGCCAAC-3′ R: 5′-GAGACCCAAAAGCCTTCATC-3′</td>
<td>58°C</td>
<td>777 bp</td>
</tr>
</tbody>
</table>

(F: forward primer; R: reverse primer; bp: base pair)
Western blotting

Harvested cells were lysed in Laemmli buffer (50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS) and 10% glycerol), enriched with 1X Halt Protease 17 Inhibitor Cocktail (ThermoScientific, USA). Insoluble cellular debris was removed by centrifugation, while the supernatant was processed using a Pierce® BCA Protein Assay Kit (Pierce, Cat. No.: 23225) to determine the protein concentration by following the manufacturer’s protocol.

A total of 20 μg of protein from each sample was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane (GE Healthcare). Each blotted membrane was blocked for 2 h at room temperature in phosphate buffered saline + 0.05% Tween 20 (PBST), containing 5% of non-fat dry milk. Blocked blots were incubated overnight at 4 °C with the protein of interest’s primary antibody. By the end of the hybridization period, the membrane was extensively washed in tris-buffered saline + Tween 20 (TBST), incubated with secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) (KPL, Cat. No.: 4751-1516), and detected using the CDP-Star® Chemiluminescent Phosphatase Substrate (PhosphaGLO, KPL, Cat. No.: 50-60-05). The primary antibodies used were: rabbit monoclonal to COBRA1 antibody (anti-COBRA1, Abcam, Cat. No.: ab167401), used with a 1:1000 dilution (v/v) in 3% non-fat dry milk in 1X PBST; rabbit polyclonal to c-Fos antibody (anti-c-Fos, Abcam, Cat. No.: ab53036), used with 1:500 dilution (v/v) in 5% non-fat dry milk in 1X PBST; rabbit polyclonal to c-Jun antibody (anti-c-Jun, Abcam, Cat. No.: ab32137), used with 1:1000 dilution (v/v) in 5% non-fat dry milk in 1X PBST; and rabbit polyclonal to α-tubulin antibody (anti-α-tubulin, ThermoScientific, Cat. No.: PA1-20988), used with 1:800 dilution (v/v) in 5% non-fat dry milk in 1X PBST.

Scratch wound healing assay

HepG2 and SNU-449 cell lines were seeded in a 6-well plate and cultured up to 70%–80% confluence. The cell monolayer was scraped using a sterile 10 μL pipette tip to form a cross-shaped wound. The cells were then washed with fresh medium and incubated for 48 h. Cell pictures were taken using phase contrast at 10X magnification at 0 and 48 h. The TScratch program (Computational Science and Engineering Laboratory, Zurich, Switzerland) was used to analyze the wound areas.[26]

Statistical analysis

All presented data were calculated as mean ± standard deviation (SD) of at least three independent experiments. The bands’ intensities for the levels of the steady-state mRNA and protein products of the tested genes were measured (as indicated by PCR and Western blot data), and normalized using the internal control of the ImageJ software. The resulting data were loaded onto the statistical analysis program RStudio® (version 0.97.336, The R Foundation for Statistical Computing, ISBN 3-900-051-07-0, and Platform: i386-pc-mingw32/i386 32-bit)). P-values for statistical significance were computed using analysis of variance (ANOVA) followed by Tukey’s honest significant differences test. P-value less than 0.05 was considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

mRNA expression levels of NELF subunits A, B, C/D, and E in different HCC cell lines including a non-cancerous hepatocyte cell line

The steady-states of the NELF subunits’ mRNA expression levels were examined using RT-PCR in the five tested cell lines (Figure 2). NELF-C/D is the translation variants of the same mRNA; thus, they were detected using a common primer set named NELF-C/D[27]. All NELF subunits’ mRNA steady-state levels were expressed in all HCC cell lines. Maximum mRNA expression was observed in MIHA cell lines for all the NELF subunits except for the NELF B (COBRA1) mRNA, which showed a maximum expression in the low-grade HCC cell line, i.e., HepG2. The increase in mRNA expression of COBRA1 in HepG2, SNU-449, and SNU-398 was significant (P < 0.001) compared to that of MIHA. Moreover, mRNA expression of COBRA1 was significantly higher in the low-grade HCC cell line (HepG2) compared to the high-grade HCC cell lines (SNU-398 and SNU-387) (P < 0.05 and P < 0.001, respectively).

COBRA1 protein levels correlate with cell line grade

COBRA1 protein levels were determined via Western blot analysis for the same samples that were analyzed in RT-PCR. There was a maximum COBRA1 expression in low-grade HCC cell line (HepG2) of >1.1 folds when compared to the control (MIHA) (Figure 3). It decreased gradually among the other higher grade HCC cell lines (SNU-449 and SNU-398), showing a minimum expression of <0.1 folds in SNU-387 relative to MIHA. The P-values shown in Figure 3b revealed significant differences in the expression of the cell lines. The decrease in COBRA1 protein expression in SNU-387 was
Figure 2. RT-PCR analysis of mRNA expression of NELF subunits in MIHA and HCC cell lines. (a) RT-PCR analysis of COBRA1 mRNA steady-state levels in all cell line samples. (b) RT-PCR analysis of NELF subunits A, C/D, and E in all cell line samples. The negative control contained water instead of the cDNAs of the tested samples; it did not show any bands. The housekeeping gene (β-actin) bands (777 bp) appeared in the gel (lower lane), which act as an endogenous control. (c) Graphical representation of mRNA relative expression of NELF subunits A, B, C/D, and E in MIHA and the four HCC cell lines. The intensity of the bands was measured and then normalized to the corresponding bands of the endogenous control (β-actin) using ImageJ. The mRNA relative expressions of the NELF subunits were expressed as fold change to MIHA as well as the calculated P-values, except those represented as horizontal lines. The P-values for the statistical significance were computed using Tukey’s honest significant differences test, (**P < 0.01, *P < 0.05). The data were presented as the mean of three independent experiments (mean ± SD).

significant (P < 0.05) compared to MIHA. There was also a significant difference in the protein expression observed in HepG2 versus those of SNU-398 and SNU-387 (P < 0.05 and P < 0.001, respectively). The COBRA1 protein expression reflected a similar pattern to the one observed for the mRNA, but with different magnitudes relative to MIHA.

Migratory potential of SNU-449 cells relative to HepG2 cells

One of the most important indicators of cancer prognosis is the migratory potential of cancer cells. The migratory ability of HepG2 cell lines showed high level of COBRA1’s migration whereas SNU-449 cell lines revealed relatively lower migratory level of COBRA1. The migratory ability was analyzed using a scratch wound healing assay, while wound areas were analyzed using the TScratch program (Computational Science and Engineering Laboratory, Zurich, Switzerland)[26]. The migration ability of SNU-449 was shown to be significantly higher than that of HepG2 (Figure 4), indicating a possible correlation between the low level of COBRA1 and tumor cell invasion, and thus poor prognosis.

mRNA expression levels of TFF1 in different cell lines

Several researchers have reported that TFF1 was a downstream target to COBRA1 in breast cancer and UG-C[9,11,22]. By testing the mRNA levels of TFF1 in HCC cell lines, we attempted to elucidate the correlation between COBRA1 and TFF1 in HCC. RT-PCR was carried out in the four mentioned HCC cell lines and the non-cancerous hepatocyte cell lines to test for TFF1 mRNA steady-state levels (Figure 5). TFF1 was only expressed in the MIHA and low-grade HCC cell line (HepG2). The absence of bands was observed in the remaining cell lines of higher grade HCC (SNU-449, SNU-398, and SNU-387). TFF1 showed significantly high mRNA expression levels in HepG2 (>5 folds) compared to MIHA (P < 0.001).
Figure 3. Western blot analysis of COBRA1 protein in MIHA and HCC cell lines. (a) COBRA1 protein (61 kDa) was detected in MIHA (control) and the four HCC cell lines. (b) Graphical representation of the relative expression of COBRA1 protein. It was differently expressed in all cell lines. The protein bands of α-tubulin (55 kDa) appeared in the blot, which act as loading control. The data was presented after measuring the intensity of the bands in Western blot and normalizing it to the corresponding bands of the loading control (α-tubulin) using ImageJ. The data was presented as the mean of three independent experiments (mean ± SD). The P-values for statistical significance were computed using Tukey’s honest significant differences test (**P < 0.01, *P < 0.05).

Figure 4. Scratch wound healing assay. (a) Images were taken using phase contrast at 10X magnification at 0 and 48 h after scraping the cell monolayer using a sterile 10 μL pipette tip. (b) Images were analyzed by the TScratch program[26]. The migratory ability of SNU-449 was shown to be significantly higher than that of HepG2 (*P < 0.05). Data represented as the mean ± SD from three experiments.
Figure 5. RT-PCR analysis for TFF1 mRNA expression in MIHA and HCC cell lines. (a) Gel electrophoresis results for TFF1 mRNA in MIHA (control) and the four HCC cell line samples. The figure shows the appearance of the TFF1 bands (240 bp) in only two of the samples and their absence in the negative control. The negative control contained water instead of the cDNAs of the tested samples. TFF1 was expressed only in MIHA and HepG2, while the other three HCC cell lines showed absence of bands. The housekeeping gene (β-actin) bands (777 bp) appeared in the gel, which act as the endogenous control. (b) Graphical representation of the mRNA relative expression of TFF1 in MIHA and HepG2. The intensity of the bands was measured and then normalized to the corresponding bands of the internal control (β-actin) using ImageJ. The relative expression was expressed as fold change to MIHA. HepG2 showed higher mRNA expression (5.5 folds) than MIHA. The data were presented as the mean of three independent experiments (mean ± SD). The P-values for statistical significance were computed using Tukey’s honest significant differences test (***P < 0.001).

Protein expression levels of c-Fos and c-Jun in different HCC cell lines, including a non-cancerous hepatocyte cell line

C-Jun and c-Fos are members of the AP-1 complex[12,15]. A number of studies found that COBRA1 may be associated with the AP-1 complex via physical binding to c-Fos on the promoter proximal region of its target genes in breast cancer and UGE[8,11]. Therefore, examining c-Fos protein expression levels may indicate the possible COBRA1/c-Fos interaction. Western blot analysis was carried out to study c-Jun and c-Fos protein levels in the four HCC cell lines (HepG2, SNU-449, SNU-398, and SNU-387) and in the control cell line (MIHA). In the case of c-Jun, the protein was only expressed in three of the cell lines (HepG2, SNU-449, and SNU-387). The absence of bands was observed either in MIHA or SNU-398 cell lines. HCC low-grade cell line (HepG2) revealed the highest protein expression level, while the HCC high-grade cell line (SNU-387) exhibited the lowest expression level (Figure 6a). There was a significant difference between c-Jun protein levels in HepG2 and each of the following cell lines: MIHA, SNU-449, SNU-398, and SNU-387 (P < 0.001) (Figure 6b). It is worth mentioning that only the unphosphorylated form of c-Jun (36 kDa) was detected; the phosphorylated form of c-Jun (43 kDa) was not observed.

In the case of c-Fos, multiple bands were observed for c-Fos protein at 50 kDa, 60 kDa, 65 kDa, and 70 kDa corresponding to the phosphorylated and unphosphorylated forms of the protein[28,29]. The unphosphorylated
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Figure 6. Western blot analysis of c-Fos and c-Jun protein expressions in MIHA and HCC cell lines. (a) The unphosphorylated c-Jun protein (36 kDa) was only detected in the HepG2, SNU-449, and SNU-387 cell lines, while there was an absence of bands in either the MIHA or SNU-398 cell lines. The phosphorylated c-Fos protein was expressed in all of the tested cell lines in two forms (65 kDa and 70 kDa), while the unphosphorylated form of the protein (50 kDa) only appeared in MIHA. The “house keeping gene” (α-tubulin) bands (55 kDa) were used as the loading control. (b) Graphical representation of the relative expression of c-Fos and c-Jun proteins (AP-1 complex subunits). c-Fos and c-Jun proteins were expressed in all samples, with an exception that c-Jun protein expression was absent in MIHA and SNU-398. (c) Graphical representation of the relative expression of total c-Fos protein, its two phosphorylated forms, and its unphosphorylated form in MIHA and four HCC cell line samples. The data was presented after measuring the intensity of the bands in Western blot and normalizing it to the corresponding bands of the loading control (α-tubulin) using ImageJ. The data were presented as the mean of three independent experiments (mean ± SD). The P-values for the statistical significance were computed using Tukey’s honest significant differences test (**P < 0.01, ***P < 0.001, and *P < 0.05).

c-Fos was detected at 50 kDa, whereas the phosphorylated c-Fos was observed at higher molecular weights (60 kDa, 65 kDa, and 70 kDa) (Figure 6a). The total c-Fos protein (both the phosphorylated and unphosphorylated forms) levels are presented in Figure 6c, in which c-Fos was differentially expressed in all the tested samples. Its maximum expression level was observed in MIHA; it then decreased gradually in the other three cell lines (HepG2, SNU-449, and SNU-398), with the SNU-387 cell line showing the lowest c-Fos level. There was a significant difference in the protein expression of c-Fos in MIHA and that of SNU-398 and SNU-387 (P < 0.01 and P < 0.001, respectively). Moreover, the decrease in the expression of c-Fos in SNU-387 was significant compared to HepG2 (P < 0.001) and SNU-449 (P < 0.05).

Discussion and conclusion

HCC tumors are characterized by poor prognosis; consequently, the introduction of new diagnostic and prognostic markers for HCC is essential[30]. However, the signaling pathways involved in HCC have not been fully described to date. Accordingly, studying the molecular mechanisms underlying the pathogenesis of HCC is considered to be crucial in finding these new markers. COBRA1 is the cofactor of BRCA1, a well-known transcription factor involved in different tumors’ pathogenesis. COBRA1 acts as a tumor suppressor gene in breast cancer, unlike its oncogenic role in UGC[11]. However, the involvement of COBRA1 in HCC pathogenesis has not yet been examined. This study sheds light on the po-
Potential involvement of COBRA1 in HCC progression and pathogenesis

COBRA1 was differentially expressed in all the tested HCC cell lines relative to its control, both at the mRNA and protein levels. At the mRNA level, the HCC low-grade cell line (HepG2) showed the highest expression level, whereas the HCC high-grade cell line (SNU-387) exhibited the lowest expression level relative to their control (MIHA) (Figure 2). Similarly, in the case of the COBRA1 protein, the highest expression was evident in HepG2 while the lowest was in SNU-387 (Figure 3). However, the decrease in COBRA1 protein expression in three cell lines (SNU-449, SNU-398, and SNU-387) was more prominent compared to that in the corresponding mRNAs. These lower protein levels could be due to post-transcriptional modification in the transcripts, or due to post-translational regulation

The migratory ability of tumor cells is a crucial factor in tumor invasion and progression. Thus, the capability of cancer cells to migrate from the primary tumor is a critical indicator of cancer prognosis. Studying tumor cell motility and the factors involved in regulating the migration process could provide a clear view on cancer progression and metastasis. In order to investigate the possible association of COBRA1 levels with HCC cell migration, we carried out the scratch wound healing assay to mimic the in vivo migratory behavior of HCC cells. The migratory ability of SNU-449 that had relatively lower COBRA1 levels was significantly higher than that of the HepG2 cell lines with high COBRA1 expression (Figure 4), which suggests a possible correlation between the low protein level of COBRA1 and tumor cell migration and thus, poor prognosis. This is in agreement with a previous study reporting the association between the lack of COBRA1 expression and poor prognosis in breast cancer.

HCC is usually diagnosed in the later stages of the tumor where, at some point, treatment is of limited efficacy. Thus, prognoses and follow-ups are necessary to regularly assess the patients and to predict any risks before the deterioration of patients’ condition. Among the well-known prognostic markers in HCC are \( \alpha \)-fetoprotein (AFP), tumor size, and bilirubin; however, these markers are insufficient owing to their variable sensitivity. For instance, some patients might have similar AFP profile levels even though their tumor aggressiveness is different. As such, introducing much more accurate prognostic markers for HCC is crucial. In particular, molecular markers are highly sensitive as their expression levels depend on the tumors’ biology. In this study, the results showed a gradual decrease in COBRA1 protein expression with the increased aggressiveness of the HCC. It was up-regulated in the early stages of cancer (represented by HepG2), before decreasing to a minimum expression level in SNU-387 (representing late HCC stages). Therefore, we suggest that COBRA1 displays tumor suppressor activity and may have a potential role in HCC progression.

We noticed a different pattern of expression when comparing the mRNA expression levels of COBRA1 and the other three NELF subunits’ mRNAs among the tested HCC cell lines. COBRA1 showed a significant differential expression in the HCC cell lines, unlike the NELF subunits A, C/D, and E which showed comparable expression with no significant difference in the examined cell lines. Even though previous research has reported simultaneous co-dependent expressions of all NELF subunits to bind to estrogen receptor-\( \alpha \) (ER-\( \alpha \))-regulated target, this study proposes a different expression profile for COBRA1.

Although cancer cell lines are regarded as a powerful tool in studying genetic alterations associated with cancer formation, they do not recapitulate the endogenous gene interactions, which may affect the overall genetic alterations. Therefore, it is important to complement the knowledge derived from established cancer cell lines using other model systems, including genetically modified cells. It remains to be determined whether the consequences of silencing the expression of COBRA1 mediated by siRNA of COBRA1 would re-enforce the findings reported in this study.

TFF1 correlates with HCC cell line’s grading

TFF1, normally found in the stomach, helps in the formation of the mucus layer. In most UGC cases, TFF1 is down-regulated, suggesting its tumor suppressor role. In this study, the mRNA of TFF1 was only expressed in two cell lines (MIHA and HepG2), in which HepG2 exhibited a significantly higher expression level than MIHA \( (P < 0.001) \). Given that HepG2 represents the early stages of tumor, it supports the tumor suppressor characteristic of TFF1 that arises in the early stages of the tumor in order to protect the affected tissue. In SNU-449, SNU-398, and SNU-387 (which represent intermediate and advanced tumor stages), the mRNA of
**TFF1** was not detected, indicating the absence of TFF1 protein and supports its role as tumor suppressor. This result is in agreement with a previous study conducted on intrahepatic cholangiocarcinoma (IHCC) tissue specimens. The authors found that TFF1 expression was high in non-invasive IHCC but significantly lower in invasive IHCC\(^{36}\). Therefore, these results support the tumor suppressor role of TFF1 in HCC.

**In UGC, COBRA1 regulates TFF1 through AP-1: Is it the same in HCC?**

AP-1 is a dimeric transcription factor that consists of both Fos and Jun genes’ family members, which include c-Fos and c-Jun. It is involved in oncogenesis because it controls some of the downstream oncogenes\(^{11}\). The c-Fos and c-Jun subunits must be activated by certain kinases through phosphorylation in order to associate in the form of an AP-1 complex\(^{12,24,25}\). In this study, both c-Fos and c-Jun proteins were examined via 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.

The phosphorylated active form of c-Fos protein was differentially expressed in all of the tested cell lines while its unphosphorylated protein was only expressed in MIHA. 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 the c-Jun subunit.

As shown in Figure 6a, the active phosphorylated form of c-Jun was absent in all of the cell lines. The unphosphorylated form of c-Jun was only detected in three of the cell lines (HepG2, SNU-449, and SNU-387), in which the HCC low-grade cell line (HepG2) exhibited the highest protein expression of the unphosphorylated form of c-Jun, whereas the HCC high-grade cell line (SNU-387) exhibited the lowest expression. Paradoxical to what could be concluded from the c-Fos expression profile, this suggests that the AP-1 complex could not be formed in the HepG2, SNU-449, and SNU-387 cell lines despite the phosphorylation of the c-Fos. Similarly, the absence of a detectable level of c-Jun in MIHA and SNU-398 suggests the unlikely formation of the AP-1 complex. However, the presence of the phosphorylated form of c-Jun could not be completely ruled out owing to the reported labile nature of the active form of c-Jun protein. It has a short half-life of approximately two hours, thus the cell machinery rapidly degrades it\(^{12}\).

According to the data compiled in Figure 6b, c-Fos was expressed in all of the studied cell lines in its active phosphorylated form. In contrast, c-Jun was only detected in three of the cell lines (HepG2, SNU-449, and SNU-387) in its unphosphorylated inactive form. Owing 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.

Moreover, the steady-state level of **TFF1** mRNA was examined in this study to assess the role of AP-1 complex in regulating TFF1. Aiyar et al. reported that TFF1 was a downstream target of COBRA1\(^{9}\). In UGC, COBRA1 controls TFF1 expression by regulating the AP-1 complex trans-activation\(^{11}\). Our findings showed a discrepancy in mRNA expression of **TFF1**: 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). Moreover, the absence of the active forms of the AP-1 complex subunits in the studied cell lines excluded the possibility of AP-1 complex formation. Therefore, the **TFF1** transcription is not only regulated by AP-1 but also involves other regulatory pathways.

Our results showed that the expression of the three proteins (COBRA1, c-Fos, and c-Jun) in the four HCC cell lines followed similar patterns: the maximum levels were observed in low-grade HCC cell line (HepG2) and the minimum levels were observed in high-grade HCC cell line (SNU-387) (Figures 3b and 6b). These findings revealed that the three proteins were differentially expressed among the tested cell lines. The expressions are high in the early stages of HCC and decrease gradually until the proteins reach their lowest levels of expression in the advanced stages of HCC. Therefore, this study suggests that not all HCC cell lines behave the same way in terms of expressing the three tested proteins. We propose that these molecules could be used in the characterization of HCC cell lines at the molecular level.

**Conclusion**

Based on our findings, we could derive the following fact: COBRA1 is differentially expressed in all the tested cell lines, suggesting its potential involvement in the pathogenesis and development of HCC. High mRNA expression of **TFF1** in the early-stage HCC (HepG2) and its reduction to undetectable levels in the late-stage HCC (SNU-387) suggest its role as tumor suppressor. Nonetheless, further studies are required to examine the correlation between COBRA1, the AP-1 complex, and TFF1 in HCC before a clear conclusion can be drawn.
Author contributions

Asma Amleh conceived the initial idea. Asma Amleh, Aya Youssef, and Heba Shawer designed the experiments. Aya Youssef, Heba Shawer, and Alaa Afify performed the experiments. All authors were involved in analyzing the data. The paper was written by Aya Youssef, Heba Shawer, and Asma Amleh.

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Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

References

The potential involvement of the cofactor of BRCA1 in hepatocellular carcinoma pathogenesis


