

The Impact of EVI1 on Carbonic Anhydrase III Expression and the Sensitivity of Rat1 Fibroblasts to H₂O₂-induced Apoptosis

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

EV11 is a transcriptional factor with two distinct zinc finger domains and encoded in human chromosome 3q26. In human, the protein is believed to have role in cell proliferation, organogenesis and it was detected in kidneys and lungs. EV11 overexpression has been associated with various conditions such as myelodysplastic syndrome (MDS), juvenile myelomonocytic leukaemia (JMML) and acute myeloid leukaemia (AML). In the present study, Carbonic Anhydrase III (CaIII) was expressed at much lower levels in EV11 overexpressing fibroblast cells compared to the wild type indicating an inhibitory role of EV11 against CaIII. Our results were further confirmed by western blot in which CaIII protein in normal fibroblast cells was more abundance than that in cells with EV11 enforced expression. Furthermore, Luciferase reporter assay showed repressed promotor activity in EV11 overexpressing cells resulted from EV11 interfering with CaIII promotor by direct or indirect binding. Fibroblast cells with repressed CaIII showed higher sensitivity to apoptosis induced by hydrogen peroxide, this is attributed to the downregulation of CaIII gene which has a defensive role against the oxidative damage. It has been reported that the CaIII overexpression increases the cell

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resistance against the oxidative stress caused by exposure to H₂O₂. Thus, elevated EVI1 levels repress CaIII expression leading to increased cell sensitivity to H₂O₂-induced apoptosis. These findings seem to be constant with previous studies, and might introduce a novel approach to treat leukaemia depending on H₂O₂-induced apoptosis.

Keywords: Zinc finger; carbonic anhydrase III; plasmid DNA; PVDF.

1. INTRODUCTION

1.1 Overview

EVI1 (Ecotropic Viral Integration site 1) is a locus in the human chromosome 3q26 which encodes EVI1 protein functioning as a transcriptional factor with two distinct zinc finger domains at both c-terminal and N-terminal regions [1]. The gene was first described as an integration site of the retrovirus during a study investigating the myeloid transformation caused by retroviral insertion into the EVI1 locus. The expression of *EVI1* gene is normally limited and its protein exists at low levels in the cells [2], it has been established that the gene is associated with various chromosomes abnormalities. The viral insertion process to this locus leads to abnormal expression of the gene, which mainly caused by the long terminal repeats LTR of the retrovirus, resulting in cell proliferation disruption and dangerous cellular transformation [3].

1.2 *EVI1* Gene

As aforementioned, the *EVI1* gene is located on the chromosome 3 band 26, and it spans 100 kb encompassing 16 exons, ten of which are coding exons [4,5]. The activation of the gene in mice starts when the retroviral insertion occurs either in between the exons 1 and 2 or upstream the gene *EVI1*, both insertion sites are located at the 5' end [3,6]. Moreover, the insertion might occur at Cb-1/Fim3 locus which located upstream the exon 1 by 90 kb, and it is suggested that the activation, in this case, is mediated by the viral enhancer [4]. On the other hand, the gene transcription in human is induced by chromosomal translocation or inversion and it has not been reported to occur through retroviral insertion [7,8].

The gene sequences in both species human and mice have homology of 91% and they differ in the initial 78bp of their genes [4], other difference is represented in 27 bp sequence inserted at the seventh exon [2] The gene is transcribed into different mRNA variants and spliced transcripts

[5], the main form results from the gene translation is 145 kDa EVI1 protein. One of the well-studied example of the variants is the alternative spliced variant $\Delta 324$ transcript which give rise to 88 kDa protein [9]. It is well established that the translation initiation starts at the third exon which codes for methionine [4].

1.3 EVI1 Protein and Its Function

The EVI1 protein is believed to function as a transcription factor belongs to SET/PR zinc finger family [10,11]. The protein is coded for by *EVI1* gene which translated into 1051 amino acids forming 145 kDa phosphoprotein, the protein is nuclear DNA binding protein and there is no evidence of its existence in the cytoplasm [12,13]. The protein is constituted from two sets of discrete Cys₂Hys₂ zinc fingers domains, one set consists 7 fingers at N-terminus and 3 near to the acidic C-terminus, and moreover, it contains a repression domain at the centre (Fig. 1) [14,15,16].

Since the protein is working as a transcription factor, it binds to a specific sequence of DNA as shown in Fig. 1, the binding occurs through the zinc fingers on both terminals, the multiple zinc motifs are of high importance as it provides high affinity to the binding sites. It is believed that the zinc fingers 4-7 are the only fingers involved in binding in the amino terminal, whereas, the ninth finger on the carboxyl is the most essential one in the binding procedure within this domain [17].

The EVI1 protein was detected in both mice and human. In mice the gene expression was detected, at different levels, in Mullerian system, liver and urinary system. It is suggested that the protein is involved in the cell differentiation, mouse development, cell growth and cell migration [18,14]. In human, the protein was observed in various regions including kidney and lung and at lower levels in other organs, and the protein role in cell proliferation, organogenesis and differentiation [3,5].

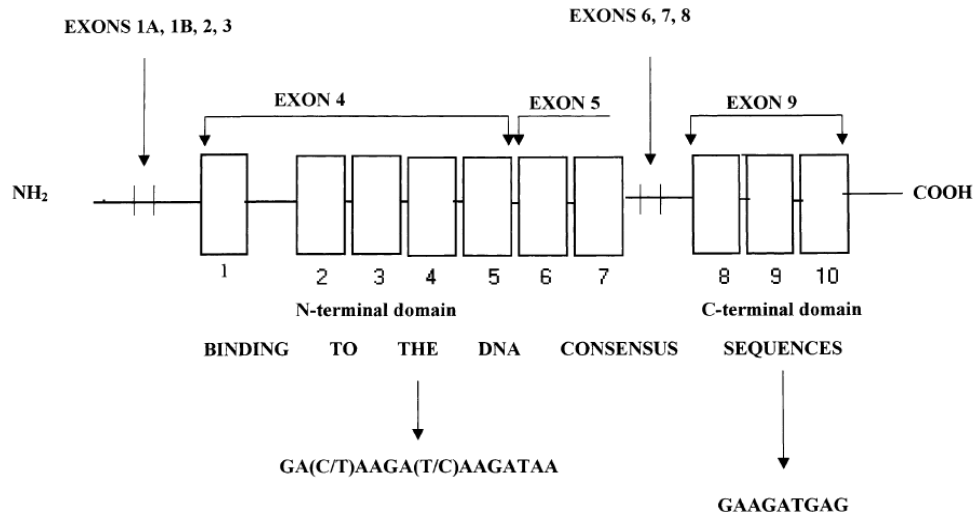


Fig. 1. Schematic drawing of EVI1 protein, the numbered boxes indicates the zinc fingers, the figure shows the DNA binding sequence at each terminus [4]

The transcriptional factor EVI1 interferes with different signalling pathways in the human body including Transforming growth factor- β (TGF- β) (Fig. 2) and c-Jun N-terminal kinase (JNK) [19]. EVI1 protein interact with Smad3 mediator through the zinc fingers causing an inhibition to its function, which is the inhibition of cell growth. What is more, the second zinc fingers domain has a role in increasing the activity of AP-1 transcription factor, both interactions results in raised cell proliferation [1].

The *EVI1* gene overexpression in hematopoietic cells leads to myeloid leukaemias in both models human and mice. In human, the gene activation is connected to myelodysplastic syndrome MDS,

juvenile myelomonocytic leukaemia JMML and acute myeloid leukaemia AML [3].

1.4 The Carbonic Anhydrase III Enzyme (CaIII)

Carbonic anhydrase III is a metalloenzyme which belongs to active α class enzymes, it is present in different tissues and it is abundant in liver and brain tissues which have high rate of oxygen consumption. Even though, the CaIII is one of poorly understood enzymes, it has been established that the enzyme has a defensive role against the oxidative stress and preventing cells' damage. The enzyme catalyses the hydration of carbon dioxide to bicarbonate interaction,

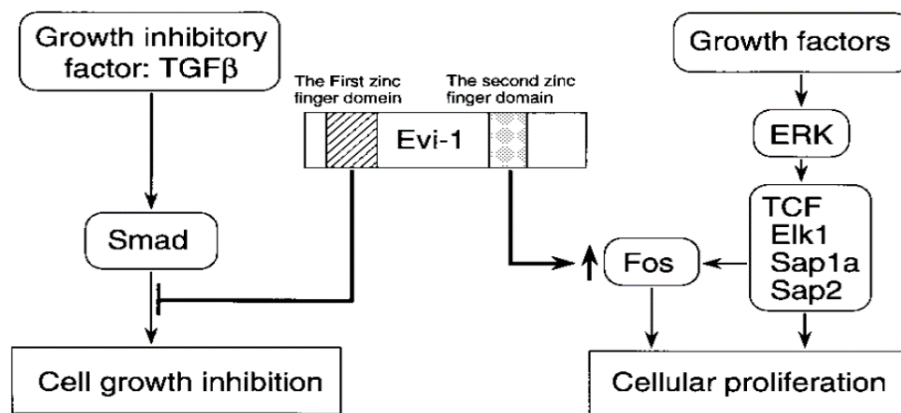


Fig. 2. Diagram illustrates the role of zinc fingers domains in interrupting the TGF- β signalling pathway and with the growth factors resulting in increased cellular proliferation [1]

therefore, the elevated levels of hydrogen peroxide H_2O_2 does not induce reactive oxygen species ROS [8,20].

Interestingly, a study by Roy et al. [21] showed that the overexpression of the *EVI1* gene, which has role in apoptosis evasion, in Rat 1 cells increased the sensitivity to H_2O_2 -induced apoptosis. Such effect is attributed to the down regulation of *CaIII* gene, thus, increased susceptibility to H_2O_2 , the *CaIII* gene repression in *EVI1* overexpressing cells was confirmed by microarray technique.

1.5 Aim

The study aims to investigate the role of *EVI1* transcription factor on *CaIII* expression, and the increased sensitivity of *EVI1* overexpressing Rat1 cells to H_2O_2 induced apoptosis, therefore, its potential application in treating human diseases.

2. MATERIALS AND METHODS

2.1 Samples and Reagents Preparation

The Rat1 fibroblast cell lines used in this experiment were provided in the laboratory at Glasgow Caledonian University. These cell lines were designated as Rat1neo1, which are cells containing an empty expression vector i.e. control sample, and Rat15.61 that contains expression vector encoding *EVI1* gene resulting in overexpressing *EVI1* gene in these cells. *Escherichia coli* DH5 α Competent Cells, as well as other different kits and reagents, were supplied at the lab, however, some other materials and reagents were prepared as following.

2.1.1 LB Broth containing ampicillin

To prepare 100 ml of Luria-Bertan (LB-broth) with 100 μ g/ml ampicillin, 2.5 g of media were dissolved in 100 ml of deionised water. 10 ml of the suspension was transferred into a glass universal, the two bottles were then autoclaved. After cooling, 90 μ l of ampicillin were added to the bottle containing 90 ml dissolved media and stored at the room temperature.

2.1.2 LB-ampicillin plates

Four grams of LB-agar were dissolved in 100 ml of deionised water and autoclaved, after cooling to a temperature of 60°C, 100 μ l of ampicillin were added aseptically and poured into 6 plates. The plates were stored until required.

2.1.3 10X TBS-T (Tris-buffered saline, tween 20) preparation

This blocking buffer is an ideal solution to be used in different applications such as western blotting and immunoassays. 1M Tris-HCl, pH 7.6 and 4M NaCl were prepared in order to make up the required 10X TBS-T. The former was prepared by dissolving 12.1 g of Tris-HCl in 100 ml of deionised water, the latter was prepared by adding 46.75 g of NaCl into 200 ml deionised water. The required 60 ml of 10X TBS-T were prepared by adding 12ml of 1M Tris-HCl, pH 7.6, 20.6 ml of 4 M NaCl, and 0.6 ml of 20 tween (100%) in 100 ml glass bottle. In order to make up 60 ml of the solution, 26.8 of deionised water was added to the previously prepared solution. The solution bottle was then labelled accordingly and stored in the room temperature.

2.1.4 50XTAE (Tris-acetate EDTA) buffer preparation

24.2 g of Tri-acetate EDTA base were transferred into 100 ml beaker and double-distilled water (dd H_2O) was added to make up 70ml of the solution and mixed properly using a magnetic bar. In a fume hood 5.71 ml glacial acetic acid were added to the solution, after that, 10 ml 0.5 M EDTA were dissolved in the solution and then transferred to 100 ml measuring cylinder. By using dd H_2O the solution volume was adjusted to 100 ml. the final product bottle was labelled accordingly and stored in the room temperature for future use.

2.2 DH5 α Competent Cells Transformation with Plasmid DNA's

Four different plasmids, namely pGL3basic, pBluescriptKSII, pRLCMV and pCAIII α luc, were provided in small quantities (1.5 ml microfuge tubes), these plasmids encodes a resistance against the antibiotic ampicillin. The supplied DH5 α competent cells vial was thawed and 12ml snap cap Falcon tubes were labelled and placed on ice. 1 μ l of the four plasmids were added to the corresponding Falcon tube, 2.5 μ l of the pUC19 control plasmid DNA, and no DNA was added to the negative control tube.

The Falcon tubes were incubated on ice for 30 minutes then at 42°C for 20 seconds before placing them again on ice for 2 more minutes. 950 μ l of pre-warmed LB-broth was added to each tube before placing them in 37°C shaking incubator for an hour, after that, 100 μ l of each

transformation were spread on LB-ampicillin plates and incubated overnight at 37°C.

For obtaining bacterial cultures of transformed bacteria after the incubation period, a single colony was aseptically picked using a wire loop from transformation plates to correspondingly labelled 12.5 ml Falcon tubes containing 3 ml of LB-ampicillin broth. These tubes were incubated overnight at 37°C in 225 rpm orbital shaker.

2.3 Total Cellular RNA and Protein Extraction from Mammalian Cells

2.3.1 Total RNA preparation

Two confluent monolayers of both Rat1 neo and Rat1 5.6 were provided in a six-well dish, which was then placed in ice for 5 minutes. 350 µl of lysis buffer RP1 and 3.5 µl of 1M dithiothreitol DTT were added to one of each pair of monolayers. By using a scraper the cells were scraped and the lysate was transferred to the duplicate monolayer, the cells in these wells were also scraped before transferring their lysate into accordingly labelled 1.5 µl microfuge tube. After mixing by vortex, the cell lysate was transferred into Nucleospin®Filter that placed in a collection tube, it was then centrifuged at highest speed for 1 minute. The filter was discarded afterwards and the 350µl of 70% ethanol were added to the collection tube and mixed by pipetting. The cell lysate was transferred into Nucleospin®RNA/Protein column attached to collection tube and centrifuged at maximum speed for 30 seconds. The collection tube was retained for protein extraction procedure.

The Nucleospin®RNA/Protein column was placed in a new collection tube and 350 µl MDB buffer was added and then the centrifuged for 1 minute. The collection tube contents were discarded and reattached to the column, then a mixture of 10µl rDNase and 90 µl rDNase reaction buffer was added to the Nucleospin® RNA/Protein column and incubated for 15 minutes at the room temperature. 200 µl of RA2 buffer were added to the column and then centrifuged for 30 seconds, the collection tube contents were disposed and the tube was attached back to the column. The sample was treated with 600 µl of RA3 buffer at the same conditions, and then treated with 250 µl of RA3 buffer and centrifuged for 2 minutes. The collection tube was discarded and the column was applied to a sterile microfuge tube, 60 µl of

RNAse-free H₂O was added to the column and incubated at the room temperature for 1 minute before being centrifuged for 1 minute at the highest speed. The microfuge tube containing the total cellular RNA was labelled and stored at -70°C.

2.3.2 Total protein preparation

The retained collection tube from the previous procedure was treated with 700 µl of PP buffer and incubated for 10 minutes at the room temperature. The tube was then centrifuged for 5 minutes and the supernatant was discarded and the intact pellet was then treated by 500 µl of 50% ethanol and centrifuged for 1 minute. The supernatant was discarded and the pellet was dried by leaving it for 10 minutes in the room temperature, the pellet was then dissolved in 100 µl PSB-TCEB buffer. The tube was incubated at 95°C for 3 minutes and then cooled down to the room temperature before centrifuging it for 1 minutes, the contents was then transferred into sterile labelled microfuge tube and stored -20°C.

2.4 Purification of Plasmid DNA

1 ml of overnight transformed bacterial culture was transferred into 1.5 ml microfuge tube and centrifuged for 30 seconds. The supernatant was decanted and another 1 ml of culture was added and the same procedure was repeated, and any supernatant was removed. The sample was then treated with 250 µl A2 buffer and mixed by inverting the tube several times and then incubated for 5 minutes in room temperature. Following the same steps the sample was treated with 300 µl of A3 buffer and then centrifuged for 5 minutes. After that 750 µl of cell lysate was transferred into Nucleo Spin® plasmid column attached to collection tube and centrifuged for 1 minute.

The flow through was discarded and the lysate remainder was added to the column and centrifuged again, the flow through was discarded and 500 µl of AW buffer was added and the sample was centrifuged. The flow through was discarded and before treating the sample with 600 µl of A4 buffer, and centrifuged, the flow through was discarded and the column was placed again in the same collection tube. The sample was then centrifuged for 2 minutes, and the collection tube with its contents was discarded. The Nucleo Spin® plasmid column was placed in labelled 1.5 ml microfuge tube, 50 µl buffer AE were added to the column,

incubated at room temperature for 1 minute, and then centrifuged for 1 minute. The microfuge containing the DNA plasmid was retained and stored at -20°C and the NucleoSpin® plasmid column was discarded.

2.5 Quantification and Restriction Endonuclease Digestion of Plasmid DNA

It is important to assess the quantity and quality of the previously extracted plasmids DNA. The assessment was conducted using the Nanodrop spectrophotometer in which the plasmids optical density was measured at 260nm. The DNA concentration was then adjusted to 200ng/μl using nuclease free water for diluting the samples.

In terms of plasmids identification, KpnI restriction endonuclease digestion for the plasmids (pGL3basic, pBluescriptKSII, pRLCMV pCAI1lluc, pUC19 and the negative control) retained in 1.5 ml microfuge tubes was applied. The restriction digests were used as shown in the Table 1.

The tubes were then incubated at 37°C for 2 hours, 20 μl of each sample along with 5μl of hyperladder I were loaded into 1% agarose after being mixed with 5 μl of loading buffer. After the gel electrophoresis running the gel was visualised.

2.6 Agarose Gel Electrophoresis

It is a tool by which macromolecules such as DNA and RNA are separated on basis of size and visualised.

2.6.1 1% agarose gel preparation

The preparation of 100 ml of 1% agarose was achieved by microwaving a flask containing 1 g of agarose in 60 ml of 1X TAE in order to melt the agarose. After that the volume was made up to 100 ml by adding 40 ml of 1X TAE. After the mixture is cooled to almost 60°C, 2 μl of midior green. The molten agarose was then poured in the gel tray and the comb was inserted in its place and was left to set for about 25 minutes. 1X TAE was used to fill the apparatus tank to cover the gel. 1 μl of loading dye (6X dye) is mixed with each 5 μl of samples to be loaded in the gel wells.

2.6.2 Analytical agarose gel electrophoresis of extracted mammalian RNA

RNA samples were prepared for the analysis by mixing 6μl with 14μl of supplied sample buffer in 1.5 ml microfuge tubes in the fume hood. The samples were heated at 65°C for 5 minutes, and 2 μl of RNA loading buffer was added to the samples. The gel; electrophoresis was then run at 100V for 20 minutes before visualising and photographing the gel.

2.7 Determination of Protein Concentration

At this stage, the protein concentration of the extracted total protein from the mammalian cells (Rat1neo1, Rat15.61) was measured on basis of standard curve. This curve was produced using a reference protein bovine serum albumin (BSA). Microfuge tubes of dilution series of BSA stock solution were prepared as shown in the Table 2.

Table 1. Volumes of plasmids and endonuclease enzyme used for digestion preparation

	Plasmid	Kpn1	10X Kpn1 buffer	H ₂ O
Digested	DNA1 3 μl	1 μl	2 μl	14 μl
Undigested	DNA1 3 μl	-	2 μl	15 μl

Table 2. Showing quantities used for protein dilutions using PBS stock solution.

Tube	Protein Solving Buffer	BSA	BSA conc	BSA protein in 20μl
1	-	100 μl	1 μg/μl	20 μg
2	50 μl	50 μl of tube 1	0.5 μg/μl	10 μg
3	50 μl	50 μl of tube 2	0.25 μg/μl	5 μg
4	50 μl	50 μl of tube 3	0.125 μg/μl	2.5 μg
5	50 μl	50 μl of tube 4	0.063 μg/μl	1.25 μg
6	50 μl	50 μl of tube 5	0.031 μg/μl	0.625 μg
7	50 μl	-	0 μg/μl	0 μg

After setting up the dilutions, 20 µl aliquots of each tube was dispensed into 2 microfuge tubes to obtain 14 tubes, the same process conducted for the protein samples i.e. two tubes for each protein. Each tube was treated with 40 µl of protein solving buffer (PSB), then with 40 µl quantification reagent (QR). All tubes were subjected to shaking until colour changed from blue to yellow. By centrifugation, the tube contents were pelleted and then the whole 100 µl were transferred into 96 well microtiter plate, the samples were labelled and the plate was incubated for 30 minutes before measuring the absorbance by plate reader at 600 nm.

2.8 Protein Gel Electrophoresis

2.8.1 Running buffer preparation

To prepare the running buffer, 50 ml of 20XSDS was diluted with 950 ml of deionised water and was mixed in measuring cylinder. 120 ml of the mixed buffer was transferred to a beaker to be used for the inner chamber of apparatus and 800 ml for the outer chamber.

2.8.2 Assembling pre-cast gel in electrophoresis chamber

The NuPAGE gel was removed from its pouch and the gel cassette was rinsed using deionised water. The tape on the bottom was removed and the comb was pulled off, the sample wells within the gel were rinsed three times using the running buffer prepared above. The two gels were assembled in Biorad former and it was placed in the electrophoresis with the notched side facing the inwards. The outer and inner chambers were filled with the previously prepared buffer ensuring the wells are submerged with the running buffer.

2.8.3 Samples preparation and loading

The concentration of the mammalian protein samples was adjusted by diluting them using PSB-TCEB buffer to 200 µg/µl. The volume was made up to 20 µl in 1.5 ml microfuge tubes. The samples were then heated for 10 minutes at 70°C, 300 µl of anti-oxidant were added to the inner chamber before loading the protein samples. To pellet the contents the tubes were centrifuged and then 20 µl of the samples were loaded to the gel as well as 10 µl of protein standard marker. The gel was run for 50 minutes at 200V.

2.9 Western Blotting

This technique is used for identifying proteins of interest after being separated by the gel electrophoresis and transferred into a membrane, after that the proteins are detected using a specific analyser.

2.9.1 Protein transfer to PVDF membrane

The procedure was carried out using the iBlot™ semi-dry blot device. In the sandwich box, the Polyvinylidene fluoride (PVDF) membrane was soaked in methanol followed by deionised water for one minute. The anode stack bottom was placed on the surface of the open lid iBlot™ device, the two pre-run gel with the PVDF membrane were placed on the anode stack followed by the pre-soaked filter paper. After that, the cathode stack was placed over these layers, and any bubbles were removed by blotting roller, a disposal sponge was placed over the cathode before running the device that was set on 2 programme for 6 minutes. The lid was opened, the cathode, anode, filter papers and the sponge were disposed, and the PVDF membrane was transferred to 50 ml conical tube ensuring the gel contact side facing the tube centre.

2.9.2 PVDF membrane with antibodies incubation

At this stage of western blotting, the transferred proteins would be incubated with different antibodies specific to the proteins EVI1, CaIII and gapdh prior to the detection step using the Li-Cor Odyssey FC image analyser. 600 ml 1XTBST was prepared for the incubation by mixing 60 ml 10XTBST with 540ml of de-ionised water. The previously papered PVDF membrane was incubated overnight at 4°C with 10 ml fresh blotto, which was prepared by dissolving 0.5 g of marvel in 10 ml of 1XTBST.

After the incubation period, the blotto was discarded, and the membrane was incubated in the tube with a certain volume of the primary antibody (dilution of 1/1000) specific to the protein of interest and 3 ml of blotto. The blotto at this step was prepared by dissolving 0.75 of marvel in 15 ml of 1XTBST. The set was incubated in a roller at room temperature for 1 hour, the blotto with the primary antibodies were discarded and replaced with 25 ml of 1XTBST and left at room temperature for 15 minutes, and then discarded, this step was repeated two more

times. After washing, the membrane was treated with 15 ml blotto and 1 μ l of appropriate secondary antibody (1/15000), which is light sensitive, thus the tube must be wrapped in aluminium foil. In a roller, the tube was incubated for 1 hour at room temperature. After the incubation, the membrane was washed three times with 1XTBST, and washed one more time using 25 ml of PBS and left at room temperature for 15 minutes. The same procedure was applied for incubating each protein with its specific antibodies. Anti-EV11 primary antibodies and HRP-conjugated anti-rabbit secondary antibody used for EV11 protein detection, anti-gapdh antibody as the primary antibody and IRD680RD-conjugated anti-mouse as secondary antibody were used for glyceraldehyde 3 phosphate dehydrogenase (gapdh) protein detection, whereas, anti-carbonic anhydrase III antibody as the primary antibody and IRDYE800CW-conjugated anti-goat as secondary antibody were used for Ca11 protein.

2.9.3 Proteins detection

The PVDF membrane was drained from the washing buffer, and then transferred into a tray containing sufficient amount of PBS that submerge the membrane. The tray was then placed in the Li-Cor Odyssey FC image analyser in order to obtain the black on white background using image studio software version 2.0, the image was saved.

2.10 cDNA Preparation

This procedure was conducted in order to produce complementary DNA of the previously extracted RNA from the mammalian cells, the cDNA synthesis is important before subjecting the samples to quantitative polymerase chain reaction Q-PCR which measure the gene expression. The procedure was carried out on a cleaned area with ethanol 70% and using filter tip pipette to avoid any contamination and the RNA samples concentration was adjusted to 0.2 μ g/ μ l. 10 μ l of 2XRT reaction mix, 3 μ l of nuclease free H₂O and 2 μ l RT enzyme mix was mixed in sterile 0.2 ml dome capped PCR tube placed on ice, the tube was then spun to locate the contents at the tube bottom. 5 μ l of RNA was added to the tube and then incubated in a DNA thermal cycler under the following conditions, 10

minutes at 25°C, then 30 minutes at 50°C, then 5 minutes at 85°C and finally held at 0°C. The samples were placed on ice and then treated with 1 μ l RNaseH before returning the accordingly labelled tubes to the DNA thermal cycler for 20 minutes at 37°C. The cDNA samples were then stored at -20°C.

2.11 Real-time Quantitative PCR (QPCR)

This procedure was carried out in order to measure Ca11 gene expression. On a cleaned work area with ethanol 70%, by using filter tip pipette the previously prepared cDNA samples were diluted by pipetting 2 μ l cDNA with 8 μ l of nuclease-free water in accordingly labelled microfuge tubes. Six of 0.2 ml dome capped PCR tubes were labelled and set as shown in the Table 3 underneath, 3 of them for the Ca11 samples and the rest for the mRNA housekeeping standard (GAPDH).

The tubes contents were pelleted before adding 20 μ l of PerfeCta FastMix II Hot Start QPCR mix to each tube and then their contents were pelleted once again. In 96 well PCR plate, 3 wells for each tube, 20 μ l for each well were transferred i.e. 18 wells for the 6 tubes previously set. The samples were then transferred into MJ QPCR DNA thermal cycler for amplification under the following conditions, 1 cycle at 95°C for 15 minutes, and then 40 cycles at 95°C for 15 seconds and at 60°C for 60 seconds.

2.12 Sub-culturing of Mammalian Cells (Rat1neo and Rat15.6 cells)

In this procedure, the previously provided T-25 flasks containing confluent monolayers of both cell lines Rat1neo and Rat15.6 were subcultured. The process was carried out in a clean work area and using aseptic technique by working in close proximity to a Bunsen burner. The cells monolayer was firstly examined using inverted microscope to ensure mosaic appearance of the cells. The culture medium was discarded from the flasks using 10 ml pipette and then the cells were rinsed using 5 ml of PBS, the buffer was then discarded and the cell monolayers were rinsed with 2 ml trypsin-EDA and discarded afterwards before incubating the flasks for 5 minutes at 37°C.

Table 3. Showing the volumes of probes and primers used for tubes set preparation prior to Q-PCR assay

Tube	5 μ M CaIII 5' primer	5 μ M CaIII 3' primer	1 μ M CaIII probe	5 μ M gapdh 5' primer	5 μ M gapdh 3' primer	1 μ M gapdh probe	Rat1neo diluted cDNA	Rat1 5.6 diluted cDNA	Nuclease free H ₂ O
1	3.9 μ l	3.9 μ l	6 μ l	-	-	-	3 μ l	-	13.2 μ l
2	3.9 μ l	3.9 μ l	6 μ l	-	-	-	-	3 μ l	13.2 μ l
3	3.9 μ l	3.9 μ l	6 μ l	-	-	-	-	-	16.2 μ l
4	-	-	-	3.9 μ l	3.9 μ l	6 μ l	3 μ l	-	13.2 μ l
5	-	-	-	3.9 μ l	3.9 μ l	6 μ l	-	3 μ l	13.2 μ l
6	-	-	-	3.9 μ l	3.9 μ l	6 μ l	-	-	16.2 μ l

After the incubation, the flasks were banged and examined under inverted microscope to ensure detachment of cells, 5ml of complete medium (CM) was then added to cell containing flasks. 2 ml of the cell and CM suspension was transferred to each of new T-25 flasks containing 4 ml of CM. the flasks were then closed tightly with caps and returned to the incubator. Moreover, different dilutions of cells (1:2, 1:3, 1:5) were prepared in 12 well plate by pipetting 1 ml aliquots to the wells. This plate was used for the transfection procedure below.

2.13 Transfection of siRNA's and Plasmid DNA into Mammalian Cells

2.13.1 Transfection of siRNA's into Rat1 cells

The previously prepared 12 well plate containing cell monolayer was incubated overnight at 37°C, 5% CO₂, after that the CM was discarded aseptically. In this procedure the cell dilution of 1:3 was chosen for treatment, and were subcultured in 6 wells plate (two wells were treated with the inhibitory siRNA, two wells with control siRNA, and two for control). After incubation and CM discard, 600 μ l CM was added and again incubated at the aforementioned conditions. CaIII siRNA mix was prepared as following, two tubes were prepared, a tube A containing 48.5 μ l of serum free medium (DMEM) and 1.5 μ l of Silentfect was incubated at room temperature for 10 minutes. And a tube B containing 46.5 μ l of serum free medium and 3.5 μ l of stock siRNA (2 μ M). The tube B contents were added to tube A and incubated at room temperature for 20 minutes. This mix was added to the retrieved plate to the wells labelled with siRNA, the two adjacent wells were treated with siRNA control and the other well were left untreated. One set was treated with H₂O₂ then subjected to Caspase-Glo 3/7 assay, and the

other set was then subjected to RNA extraction, quantification, cDNA synthesis and QPCR assay to measure the gene expression post siRNA transfection.

2.13.2 Transfection of mammalian cells with plasmid DNA

At this stage, both cells Rat1 Neo1 and Rat1 5.61 were transfected with plasmid DNA in order to subject them to dual luciferase assessment by which the gene promotor activity was measured. The cells were subcultured from T-25 flasks into 96 well plate, 20 wells for each cell monolayer were pipetted with different dilutions (undiluted, 1:2, 1:3, and 1:5), and the plate was then incubated overnight under the described conditions.

After the incubation period, the dilution 1:3 was chosen for treatment due to its required mosaic tissue structure which was visualised by inverted microscope. Dilutions of provided plasmid DNA was prepared and stored at -20°C, and transfection mixture was prepared as following. Tubes labelled A were prepared aseptically by mixing 31.3 μ l of serum free medium (SF) and 2 μ l of FuGENE®6 and incubated at room temperature. While incubating, tubes labelled B containing diluted plasmids DNA were prepared as shown in Table 4 and incubated for 5 minutes. After incubation, the tubes B contents were mixed with their corresponding A tubes, and this mixture was incubated for 30 minutes at room temperature.

The Rat1 Neo1 and Rat1 5.61 monolayers in the 96 well plate were treated with 5 μ l of AB mixture for each well, and then incubated at 37°C, 5% CO₂ for 48 hours before proceeding to the Dual Luciferase Reporter Assay.

Table 4. Volumes of plasmids used in DNA plasmids transfection into mammalian cells.

Tube B	Firefly luc	Renilla luc	pBluescript	pEGFP-N1
Neo1	pCaIIla 1.7 µl	pRLCMV 1.7 µl	pBSKSII 1.4 µl	-
5.61	pCaIIlb 1.7 µl	pRLCMV 1.7 µl	pBSKSII 1.4 µl	-

2.14 Hydrogen Peroxide Treatment and Caspase-Glo 3/7 Assay

The treatment with hydrogen peroxide induce the apoptosis of mammalian cells, at this stage the treated and untreated cells with H₂O₂ was subjected to Caspase-Glo 3/7 Assay in order to measure the apoptosis extent through measuring the activity of caspase enzyme within the cells. The retrieved plate containing siRNA transfected Rat1 Neo1 cells (prepared in procedure 2.13.1) was subcultured using 0.5 ml PBS with 0.5 ml trypsin-EDTA for each well. These trypsinised cells were suspended in 1 ml of CM, from which 100 µl into 96 well plate and labelled accordingly before incubating them at the same conditions. After that, the wells containing siRNA and siRNA control was subjected to 100 µl of CM with 750 µM of H₂O₂ and the untreated well was left as it was.

The plate was incubated then for 16 hours at 37°C, 5% CO₂. After the incubation period the plate was left for 10 minutes at room temperature before discarding 50 µl of each from CM. 50 µl of Caspase-Glo reagent was added to each well and the plate was incubated for 30 minutes at room temperature. The contents of the incubated plate was transferred into white 96 well plate and labelled accordingly using specific sheet to memorise the samples location. Finally, the plate was read using plate-reading luminometer.

2.15 Dual Luciferase Reporter Assay

The medium in the 96 well plate containing transfected cells with plasmid DNA prepared in (2.13.2) was discarded and replaced with 100 µl of PBS in each well. After washing with PBS it was discarded and the wells were treated with 20 µl 1X PLB lysis buffer and incubated on orbital shaker for 5 minutes before other incubation period of 30 minutes at room temperature. After the incubation, 20 µl of each well were transferred into opaque 96 well plate and notes of their locations were taken. Finally, the luciferase activity measurement was taken using Fluostar OPTIMA luminometer (BMG LABTECH).

3. RESULTS

3.1 Identification of DNA Plasmids

3.1.1 DH5α competent cells transformation with plasmid DNA's

After the *E. coli* DH5α competent cells were transformed using different plasmids, and their growth on amp plates indicates the success of transformation process since these plasmids encodes resistance to this antibiotic. The cells count was noted for each plate containing transformed bacterial cells with plasmids DNA as shown in Table 5.

Table 5. Presenting the number of colonies of *E. coli* DH5α competent cells recovered on amp-plates after transformation with DNA plasmids

DNA plasmid	Growth	No. of colonies
pGL3basic	+ve*	98
pBluescriptKSII	+ve	133
pRLCMV	+ve	171
pCaIIlluc	+ve	235
pUC19 (+ve control)	+ve	47
(-ve control)	-ve**	0

*+ve = positive, **-ve = negative

3.1.2 The quality and quantity of purified DNA plasmids using the Nanodrop spectrophotometer

The assay was to determine the quality of the extracted DNA form transformed *E. coli* cells, the optical density of the samples were measured and presented in Table 6.

3.1.3 The Analytical agarose gel for DNA plasmids

The DNA fragments pattern for the plasmids digested with Kpn1 was examined using analytical gel electrophoresis which visualise the size of these fragments and the distance they migrated in comparison to the DNA marker Hyperladder I as shown in Fig. 3.

Table 6. Shows the optical density of DNA plasmid samples at 260 nm by which the purity of DNA was assessed

DNA plasmids sample	Absorbance	OD ₂₆₀ /OD ₂₈₀	OD ₂₆₀ /OD ₂₃₀
pGL3basic	7.8826	1.77	1.42
pBluescriptKSII	13.176	1.90	1.62
Prlcmv	14.567	1.877	1.53
pCAIIIuc	4.925	1.813	3.75

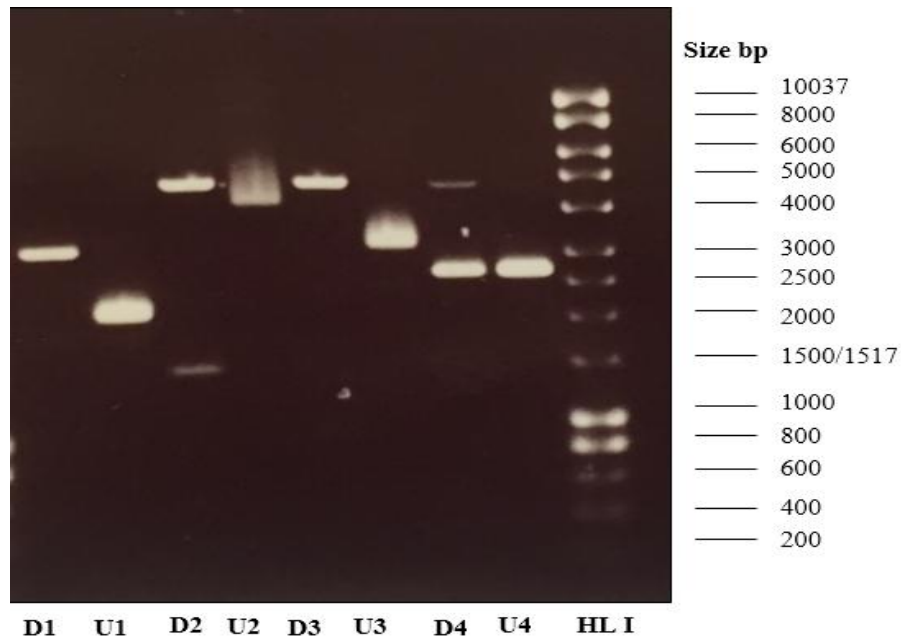


Fig. 3. DNA plasmids in 1% gel electrophoresis image for plasmids identification based on their size after digest enzyme treatment. (D) Digested samples, (U) undigested samples. Lanes: (1) pBluescriptKSII, (2) pCAIII luc, (3) pRLCMV, (4) pGL3basic. (HLI) hyper ladder I

3.2 Quantification and Analysis of Total RNA

3.2.1 Quantification of cellular RNA extracted from Rat1 Neo1 and Rat1 5.6 cells

The extracted RNA from the mammalian cells were subjected to Nanodrop spectrophotometer to determine the quality of the RNA. The optical density obtained is shown in Table 7.

3.2.2 The analytical agarose gel for the mammalian cells RNA

The total RNA of both cells was extracted and subjected to gel electrophoresis in order to detect the bands representing ribosomal RNA S18 and S28 which indicates intact RNA samples. The obtained results are shown in Fig. 4.

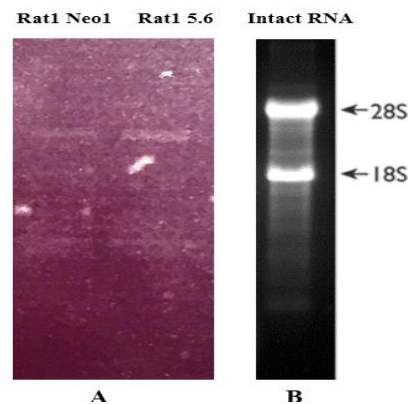


Fig. 4. (A) RNA samples of both cell types, the RNA samples appear low molecular weight smear indicating degraded RNA samples compared to (B) which represent the bands should be obtained that indicates intact RNA samples

3.3 Real-time Quantitative PCR (QPCR)

The quantitative PCR was performed to measure the *CaIII* gene expression in both cell lines, therefore, obtaining an indication of the effect of overexpressed *EV11* gene in Rat1 5.6 cells compared to the normal cell Rat1 Neo1. The gene expression finding is presented in Fig. 5. From the histogram, it is seen that the expression of the gene is much higher in the normal cells Rat1 Neo1.

3.4 Detection of Proteins by Western Blot Analysis

The western blotting assay used to detect the proteins of interest in this experiment by detecting bands and measure their sizes. The protein gel electrophoresis is presented in Fig. 6.

The *CaIII* protein band intensity in the control cells refers to the abundance of the protein compared to *EV11* overexpressing cells, in which *EV11* protein is more abundant than that in control cells *EV11 neo1*.

3.5 Dual Luciferase Assay Analysis

The assay was performed to measure the luciferase activity by which the activity of the promoter responsible for *CaIII* gene expression was measured. This assay give an indication whether the *CaIII* inhibition occur at the transcription level or not. The findings are presented in Fig. 7. It is seen that Rat 1 Neo1 has higher *CaIII* gene expression than that in Rat15.61 indicating the inhibition occur by intervening with the promoter activity.

Table 7. Shows the optical density of total RNA extracted from the mammalian cells at 260 nm, using Nanodrop spectrophotometer for quality assessment

Sample	Absorbance	OD ₂₆₀ /OD ₂₈₀	OD ₂₆₀ /OD ₂₃₀	RNA concentration
Rat1 Neo1	5.20	2.10	21.20	276.7
Rat1 5.6	5.60	2.09	1.00	223.4

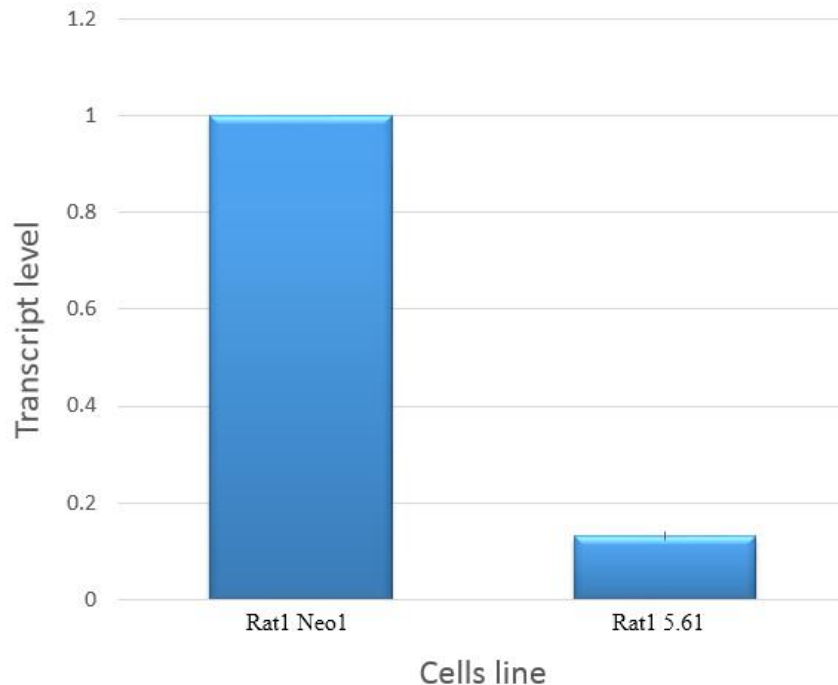


Fig. 5. Real-time quantitative data presented in a histogram. Showing the transcript levels of *CAIII* in both cell lines Rat1 Neo1 and Rat15.61

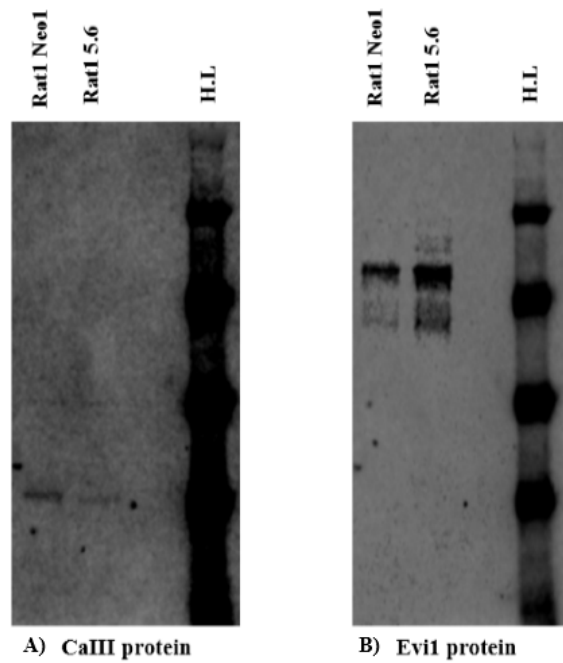


Fig. 6. Western blot analysis for proteins carbonic anhydrase III and EVI1 in both cell lines Rat1 Neo1 and Rat1 5.6. (A) CaIII cytosolic protein detection which has a molecular weight size of ~30-kDa. (B) EVI1 protein detection which has a molecular weight size of 145-kDa

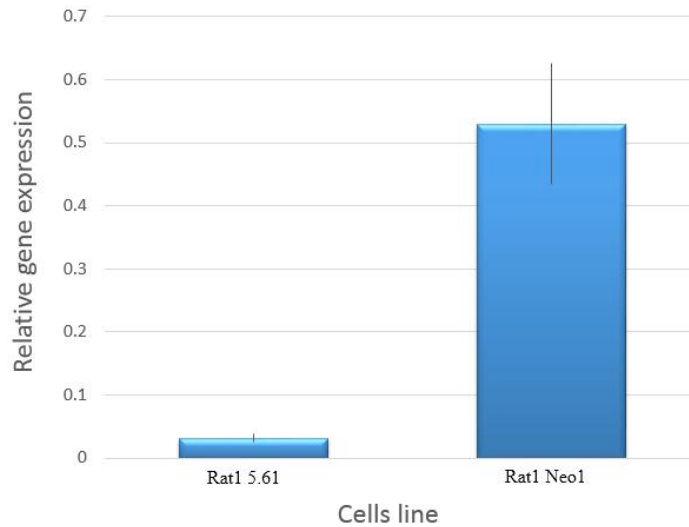


Fig. 7. Luciferase assay histogram. Showing the CaIII gene promoter firefly luciferase reporter activity in both cell lines Rat15.61 and Rat1 Neo1

3.6 Caspase Activity Analysis

The assay was conducted to measure the extent of the cell apoptosis induced by H_2O_2 , the assay was conducted on Rat1 Neo1 cells transfected with siRNA and compared to untreated sample.

The findings were presented in Fig. 8. It is seen from the histogram that the highest level of apoptosis occurred in the cells in which CaIII gene is inhibited, this indicates a fundamental role of CaIII protein in defence against the oxidative stress resulting from H_2O_2 treatment.

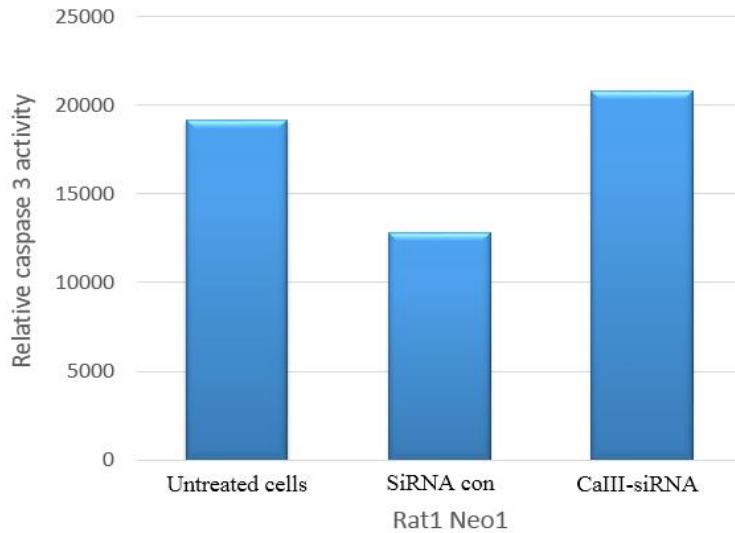


Fig. 8. Caspase-Glo 3/7 assay histogram. Showing the apoptosis extent in three samples of Rat1 Neo1, untreated cells, siRNA control, CaIII-siRNA. The findings indicates highest apoptosis level in cells transfected with CaIII siRNA

3.7 Quantification and Analytical Agarose Gel for Isolated RNA from Rat1 Neo1 Transfected with siRNA

The assay was used to assess the quality, purity and confirm obtaining intact RNA samples from Rat1 Neo1 cells. The quality of RNA was assessed using Nanodrop spectrophotometer, the findings showed good quality of extracted

RNA since all values was ranging between 1.8 and 2.0.

The analytical agarose gel assay was used to detect the ribosomal RNA S18 and S28, both of which indicating the success of purifying an intact RNA samples from Rat1 Neo1 cells. These cells are either transfected with CaIII siRNA, siRNA control or untreated cells. The analytical gel image for these samples is presented in Fig. 9.

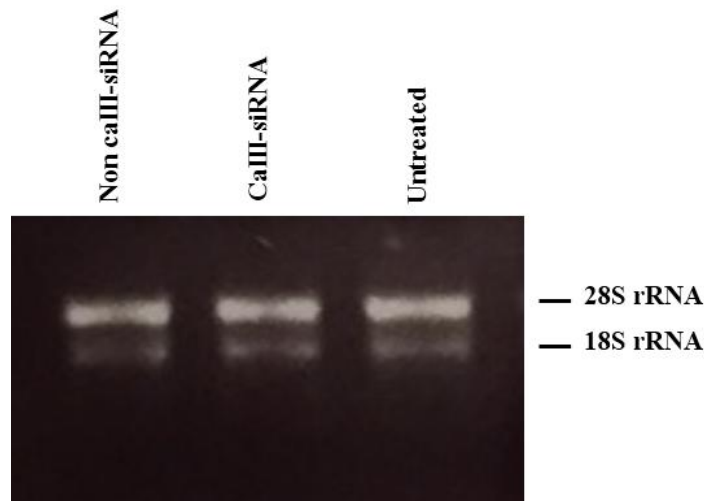


Fig. 9. Analytical gel electrophoresis image for different RNA samples extracted from Ra1 Neo1 as annotated in the figure. From the image it is clear that all purified RNA samples are intact due to 18S and 28S rRNAs detection

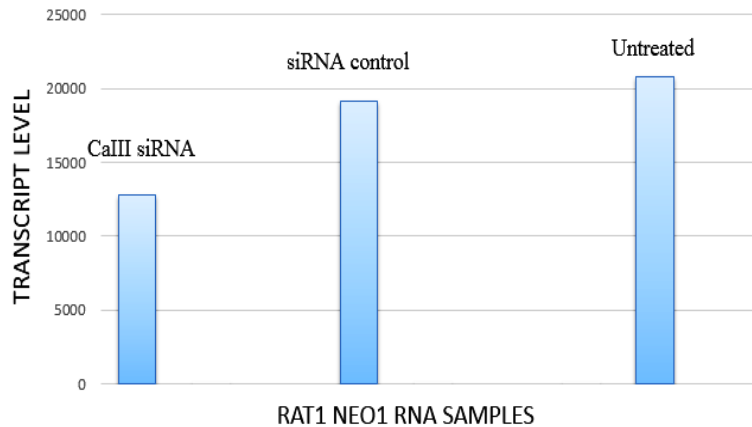


Fig. 10. QPCR data presented in a histogram. Showing the transcript levels of CaIII in Rat1 Neo1 cells. The cells transfected with siRNA had highly reduced CaIII gene expression

3.8 QPCR of Rat1 Neo1 RNA Samples

The assay conducted in order to examine the inhibited CaIII gene expression after being treated with CaIII sRNA. The findings are presented in Fig. 10.

4. DISCUSSION

In this experimental study, the main aim was to validate the down regulatory effect of EVI1 on the expression of the carbonic anhydrase III, and the role of EVI1 in protecting the cell from apoptosis. Even though the role of CaIII enzyme is poorly understood, it is well established that the protein has a cellular antioxidant role. This suggestion arose from the fact that the CaIII overexpressing cells blocks the rapid increase in reactive oxygen species (ROS), which leads to a significant damages to the cell, unlike other enzyme from the same family such as call. This observation might explain the enzyme abundance in high oxidation potential regions such as the skeletal muscles [22]. Furthermore, the study investigated the extent of H₂O₂-induced apoptosis in Rat1 Neo1 fibroblast cells post-inhibition of CaIII gene, which allow us to observe the protective role of CaIII protein against the oxidative effect of the elevated ROS which finally lead to the cell death.

The early stage of this experiment was to transform the *E. coli* strain DH5 α competent cells, which is used for cloning experiments and plasmids transformation, with different plasmids all of which encoding ampicillin resistance. The success of transformation was observed as the transformed bacterial cells managed to grow on

LB-ampicillin plates as shown in Table 5. The transformation process was carried out to enlarge the amount of the provided DNA plasmids, which was then further treated with KpnI restriction endonuclease enzyme to determine their identity. The findings of restriction enzymes action was visualised by gel electrophoresis as presented in (Fig. 3). KpnI restriction map was used to identify the treated plasmids sizes and compared with hyperladder I, in lane D1, which represent the digested pBluescriptKSII plasmid, the obtained cut plasmid resulted in linear plasmid with size of (3000 bp) and that perfectly matches the map. Lane D2 represent digested pCAIII_{luc} which has two linear DNA fragment, the expected sizes according to the restriction map are 1430 bp and 3388bp, in our findings the former matches the expectation whereas, the latter appeared to have higher molecular weight. The pRLCMV plasmid was not cut by the enzyme which resulted in intact plasmid with size of ~ 4079bp, this size comply with the expected size shown in the map. pGL3basic plasmid shows two bands, the plasmid is expected to have one linear fragment DNA sized 4818 bp which matches the size of one of these fragment. The meaningless results could be attributed to minor errors while conducting the experiment.

In the present study, the CaIII gene expression in both cell lines (Rati Neo1 fibroblast, which represent the normal cell model, and Rat1 5.6, which represent the EVI1 overexpressing cells) was measured using quantitative PCR and presented in (Fig. 5). From the histogram, it is clear that the transcript level in Rat1 Neo1 line is much higher than that in Rat1 5.6 line, these

findings indicate an inhibitory impact of EVI1 which is overexpressed in the second cell line. The outcomes were further confirmed by the western blotting analysis by which the abundance of CaIII and EVI1 proteins was estimated, from (Fig. 6-A), it is seen that the CaIII protein band in Rat1 Neo1 has higher intensity than the corresponding band in Rat1 5.6.

In respect of the CaIII promoter activity, it was measured for both cell lines using dual luciferase assay was carried out, this process was to assess whether the EVI1 inhibition occurs at the transcription level. The (Fig. 7) shows that the promoter activity and the gene expression was higher in Rat1 Neo1 cells indicating the action of EVI1 as a transcription inhibitor for CaIII gene. Therefore, the CaIII gene can be listed with an array of genes regulated by EVI1 such as Gadd45 g, SnoN and other genes [23], and these results are consistent with previous study conducted by Roy et al. [21].

It has been reported that the transcriptional factor EVI1 has an enhancement effect on c-Jun and c-Fos gene and inhibitory effect on JNK by acting indirectly on their promoters, on the other hand the EVI1 might directly bind to a specific gene promoters [24]. In the case of CaIII down regulation by EVI1, further investigations are required to determine the precise mechanism either direct or indirect binding by which the EVI1 repress CaIII expression.

Additionally, the caspase 3 is a protein playing a crucial role along with other cysteine proteases and a complex cascade in programmed cell death [25,26]. Hence, the caspase 3 catalytic activity of Rat1 Neo1 cells with knocked down CaIII by introducing siRNA and treated with H₂O₂ was measured to determine the apoptosis extent resulting from the oxidative stress. The anti-oxidative role of CaIII upon the exposure to endogenous or exogenous H₂O₂ has been reported, the enzyme provides resistance against elevated levels of ROS. The response to such stress is initiated through a process termed S-glutathiolation at which two cysteines forms disulfide link with glutathione (GSH) [27]. Our results [Fig. 8] support these observations since the highest H₂O₂-induced apoptosis was in the sample in which CaIII gene was inhibited. The apoptosis extent in untreated sample was much lower than that in CaIII knocked off sample confirming the protective role of this isozyme in conditions of oxidative stress.

5. CONCLUSION

The EVI1 protein was associated with increasing the cell survival and conveying a resistance to TGF β -mediated cell death by intervening with phosphoinositide-3-kinase (PI3K) and the effector AKT. Furthermore, it is well established that EVI1 provides resistance against the therapeutic apoptotic agent Taxol [28]. However, a study by Roy et al. [21] showed that the EVI1 overexpressing cells increased the sensitivity of these cells to H₂O₂-induced apoptosis, and that is attributed to the inhibited CaIII expression. Our experimental study appeared to be constant with previous studies in such regard. Future studies are needed to determine the exact mechanism by which CaIII is inhibited as well as more studies about the development of therapeutic potential of H₂O₂ as a novel agent in treating leukaemias.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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