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Gene Expression on Rat1 Fibroblast Cells After Transformation by EVI1

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AIM

This research aimed to demonstrate the gene expression of Rat1 Fibroblast cells overexpressing EVI1 as well as the relation between the levels of expression of the EVI1 transcription factor and CAIII gene expression in the transformed cells and looking at the molecular and cellular mechanisms of EVI1.

MATERIAL AND METHODS

To assess CAIII gene expression in relation to cellular environment EVI1 level, real-time quantitative polymerase chain reaction (qPCR) was performed followed by Western blot to confirm the gene and protein expression in Rat1neo and Rat15.6 cells, including the effect of CAIII gene in Rat1neo after hydrogen peroxide (H_2O_2) treatment and silencing the expression of CAIII in these cells by siRNAs.

To confluent monolayers of Rat1neo and Rat15.6 fibroblasts on 6 well tissue culture dishes was added RP1 Buffer and 1M of dithiothreitol (DTT) and transferred to Nucleospin®Filter Nucleospin®RNA/Protein (Macherey-Nagel Products, Neumann-Neander Str. Germany) rDNase was added, and the buffers RA2, RA3 washed the filters. RNAse-free Water extracted the total cellular RNA, and PP buffer, ethanol 50%, and PBS-TCEB buffer were used to extract the protein. The RNA levels were measured and analysed in nanodrop spectrophotometer Epoch device. The RNA concentration was adjusted to 200ng/µl for analytical agarose gel electrophoresis. The RNA samples were added sample buffer of 10XMOPS, formaldehyde and formamide and loading buffer in a fume hood. The samples were pre-heated at 65°C followed by a brief centrifuge and were run at 100V on a gel for 20 minutes then photographed with a Benchtop 3UV™ Transilluminator device.

cDNA was obtained by mixing from InvitrogenTM kit composed of nuclease free water, RT enzyme mix, 2XRT reaction mix and RNA in a 0.2ml dome capped PCR tube and incubated in DNA thermal cycler in a different time before and after mix with RNAseH.

RNA from cultured Rat1neo and Rat15.6 cells was obtained and an analytical agarose gel electrophoresis was performed followed by cDNA preparation for qPCR. 2µl of cDNA of Rat1neo and Rat15.6 diluted in a quantity of 1 in 5 was prepared and mixed with 5' and 3' CAIII and GAPDH primers and probes and Nuclease free water added. 30µl of PerfeCta FastMix II Hot Start QPCR (Quanta BioScienceTM Manchester, UK), and this was finally amplified in the MJ QPCR DNA thermal cycler. The resulting data was processed in Microsoft Excel where mean and standard deviation of Ct values (threshold cycle) were calculated.

All antibodies and blocking were diluted in blotto (0.5 marvel and 10ml of 1XTBST) to prevent non-specific binding. The primer and second antibodies were diluted in 1/1000 and 1/15000 respectively. Finally, the images. were acquired in Li-Cor Odyssey image analyser using image studio software version 2.0 in a black and white background

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RESULTS

The protein absorbance obtained from the cell lysates showed a moderate difference between Rat1neo (0.147ng/µl) and Rat15.6 (0.1665ng/µl) as revealed in the spectrophotometer and for Western blot analysis, both samples had to be adjusted to same concentration for 20µl.

In the figure 1 the proteins analysed by Western blot are presented. This shows the expression of EVI1 protein in Rat1neo and Rat15.6 with a migration band around 149 KDa.

GAPDH protein showed the same thickness bands between Rat1neo and Rat15.6 samples, whereas, for CAIII the bands were thicker in Rat1neo and thinner for Rat15.6.

The absorbance was made in duplicate, so a mean was calculated between the two values. The results from protein absorbance and RNA quantification are shown on Table 1.

Table 1- Protein Absorbance and RNA Quantification for Rat1neo and Rat15.6

Conc.	Rat15.6	Rat1neo	20µg BSA	10µg BSA	5µg BSA	2.5µg BSA	1.25µg BSA		0.625µg BSA	0µg BSA
RNA (ng/µl)	137.401	404.145	-	-	-	-	-		-	-

The RNA quality was checked by agarose gel electrophoresis and two bands of ribosomal RNA, 28S and 18S, were observed which indicated the RNA quality obtained for this experiment.

The qPCR results of CAIII gene expression in Rat1neo and Rat15.6 cells. The CAIII was highly expressed on Rat1neo whereas in Rat15.6 it was expressed at a reduced level because of the EVI1 gene overexpressing and the CAIII gene is repressed.

After bacteria culture for transformation, there was no bacterial growth on some plates as indicated in Table 2 and to complete the experiment, samples for Falcon tubes 1 and 2 were taken from classmates.

Table 2- Number of colonies counted for bacterial transformation

Plate	Plasmid	#colonies
1	1	0
2	2	0
3	3	118
4	4	141
5	pUC19	16
6	Control	0

The absence of bacteria growth for plasmid 1 and 2 may be related to the quantity of plasmid to give antibiotic resistance to the DH5α *E. coli* strain, coinciding with the negative control (no plasmid- no bacterial growth in presence of ampicillin).

Plasmid DNAs were amplified and identified in bacterial growth *E. coli* strain DH5α and 1µl of Kpn1 enzyme (Fermentas-Life Science) was used to digest different plasmid DNAs in different fragments in 1% agarose gel electrophoresis.

According to the DNA fragmentation and compared to the hyperladder, plasmid 1 corresponded to pBluescriptKSII because it migrates at around 3100bp band, plasmid 2- pCAIIIUC with two fragments at 5500-1750bp; plasmid 3- pGL3 and plasmid 4- pRLCMV. The digest enzyme Kpn1 cut different plasmid DNAs in different fragments as observed in agarose gel electrophoresis.

Approximately 40 percent of pEGFP-N1 transfection was achieved in Rat15.6 fibroblast cells and about 1 percent for Rat1neo fibroblast cells.

A comparison between Rat1neo and Rat15.6 according to the enzyme Luciferase activity as CAIII gene can drive the expression of firefly luciferase from its promotor and expression of renilla luciferase by the cytomegalovirus (CMV) also from its promotor, renilla luciferase was used to normalise the results as it is permanently present, without vary, in transfected cells with plasmids carrying both exotic genes but the expression of firefly may alter and this variation can be measured according to the intensity of luciferase activity in the gene promotor region and for this work it was higher in Rat1neo and lower for Rat15.6.

Luciferase activity was higher for Rat1neo and it may indicate that CAIII is more expressed in Rat1neo cells than in Rat15.6 where EVI1 gene is expressed.

Caspase 3 activity in Rat1 cells was measured after hydrogen peroxide (H_2O_2) treatment to evaluate apoptosis. Therefore, siRNA was used to target the expression of CAIII gene (siRNA CAIII) in Rat1neo cells by transfection in distinction to siRNA control and untreated cells. After, the role of CAIII gene in cell protection against oxygen-stress induced apoptosis was observed by caspase 3 activity among untreated, siRNA control and siRNA CAIII.

The Caspase 3 activity was higher in siRNA CAIII as the protection factor against H_2O_2 was knocked down by the dicer silencing targeting CAIII RNA and apoptosis raised up.

CONCLUSION

So, caspase 3 activity was higher in Rat1neo cells treated with siRNA CAIII and H_2O_2 compared to the untreated which prove the repression of CAIII by EVI1. Moreover, the luciferase activity demonstrated that EVI1 overexpression, at the molecular level, represses CAIII expression by acting on its promotor region. Consequently, cells, where CAIII is knocked out, are no longer protected against H_2O_2 and elevated caspase 3 activity increases the level of apoptosis. Following these principles, it is suggested that in leukaemia if drugs containing related H_2O_2 agents are used, they may selectively target transformed cells with EVI1 gene overexpression and these could be candidates for the cancer therapy.

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