

Transcriptomic study of the cell death-related pathways of cervical cancer HeLa cells in response to *Clinacanthus nutans* treatment

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Abstract

Background

Recent years have witnessed major development of novel therapeutic agents like chemotherapy, targeted therapy and immune checkpoint inhibitors for cervical cancer. However, cervical cancer remains prevalent, leading to a large number of deaths worldwide. A better understanding of the cervical cancer biology and signaling pathways might lead to the development of targeted therapies in reducing the incidence and mortality rate.

Methods

In this study, the RNA-Seq reads of HeLa cells treated with *C. nutans* were compared to the untreated sample. The reads of these two sample groups were firstly aligned to the human reference genome. The results in BAM files format that were generated were then sorted before being assembled. The output of assembly which was in coverage table form was ready for downstream statistical analyses for differential expression. Differentially expressed genes were obtained and the cell-death related pathway were identified by canonical pathway, QIAGEN Ingenuity Pathway Analysis (IPA). The verification of significant genes was carried out using qRT-PCR by including GAPDH as a housekeeping gene

Results

With this, we identified a total of 668 upregulated and 479 downregulated analysis-ready genes across observations upon cut-off setting log2FoldChange at 0.5 and P-value 0.05. A total of 28 cell-death related canonical pathways and 4 activation of cell-death related functions were identified. Upon analyses, we identified four significant genes (Casp9, KAI1, REL and FOXO4) that hold important role in promoting cell death. These findings were also verified against the quantification using qRT-PCR by including GAPDH as a housekeeping gene.

Conclusions

This study provides an insight on the potential role of DCM fraction of *C. nutans* in activating Casp9, KAI1, REL and FOXO4 genes in mediating apoptosis in cervical cancer cells.

Background

Cervical cancer is among the common malignant epithelial tumors that occurs in women (1) and majority of the cases is caused by infection with a high-risk subtype of human papillomavirus (HPV) (2). Even though it is almost completely preventable through HPV vaccination and screening, it is devastating that cervical cancer remains the fourth most common cancer to affect women worldwide.

Current standard treatments include radiotherapy, chemotherapy and surgical resection. However, Farrel et al. (3) believe that against advanced disease, these treatments come with severe side effects and restricted efficiency. Hsiao et al. (4) highlighted that medicinal herbs have been applied to treat many diseases, including cancer. Recently, there are numerous documentations addressing the usage of traditional Chinese medicines in the treatment of cervical cancer (5). Thus, many clinical studies have been carried out and showed positive effects.

According to Alam et al. (6), *Clinacanthus nutans* (*C. nutans*) which is also known as snake grass that belongs to the Acanthaceae family, is believed to be known for its potential therapeutic benefits. Thus, the tropical Asia has been using it as a vital herbal medicine. Some cancer patients have claimed that *C. nutans* leaf consumption has helps in treating their cancer and improving their health (7). Previous studies have reported that *C. nutans* possesses cytotoxicity (8), anti-proliferative, antioxidant (9) and anti-inflammatory (Mau *et al.*, 2013) activities.

In the current study, we leverage the strengths of RNA-Seq pathway analysis to refine our understanding on pathways and genes that potentially promote the cell death of HeLa cells. The effects of *C. nutans* dichloromethane (DCM) fraction on HeLa cell line was determined by identifying the differentially expressed genes (DEGs) upon comparing the treated and untreated cells. Further analyses of the DEGs were conducted to explore the specific mechanism of *C. nutans* action on the HeLa cells.

Methods

RNA-Seq and processing of reads

The samples used in this study were divided into two groups; a HeLa cell line treated with *C. nutans* DCM fraction and untreated HeLa cells. Library construction were performed by Beijing Genomics Institute (BGI, Hong Kong) using Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA) per manufacturer's instructions. The sequencing was done using Illumina HiSeq 4000 platform. Raw read qualities were inspected using FastQC (version 0.11.9) prior to proceeding with the downstream analysis. The filtering was carried out to eliminate any undesired element found during the quality checking carried out. *Fastp* (10) was used to trim off the overrepresented sequences detected within the reads. Then, these clean reads were aligned to human reference genome, hg38 using HISAT2 (11) software. The human reference genome, the hg38 genome, was obtained from the UCSC Genome Browser database (<http://genome.ucsc.edu>). Hg38 was chosen because this genome is the latest and a stable built of human reference genome at the moment. Additionally, this genome is the corrected and improvised version of the previous built, hg19. The results in BAM files format generated by the HISAT2 (12) were sorted using samtools prior to assembling using Stringtie (13). The output of Stringtie, which was in coverage table form, was ready for downstream statistical analyses for differential expression. Read counts per gene per library were summarized by using featureCounts (version 1.4.4) (14) with default settings and the human genome GTF file (GRCh.38, ensembl) as the genome annotation reference file.

The server that was used for the experiments had Intel® Core™ i7-8700 CPU @ 3.20GHz 3.10 GHz and the operating system (OS) was the Ubuntu 18.04 LTS.

Differential expression genes

RStudio/Bioconductor package DESeq2 (15) was used for differential expression analysis. After performing normalization of the raw count data as above, dispersions were estimated using a parametric method with maximum number of iterations of 500. Log-transformed normalized counts per gene were modelled using a generalized linear model. The *estimateSizeFactors* function was used to evaluate the sequencing depth normalization between samples while *estimateDispersions* function was used to estimate gene-wise dispersion across all samples. The *nbinomWaldTest* function was used to estimate and test the significance of coefficients of the covariates. DEGs between conditions were identified by testing the significance of relevant contrasts using the *results* function with the following explicit parameters specified: *alpha* = 0.05, *independentFiltering* = TRUE. Genes with q values less than 0.05 were considered as significant DEGs. The differential expressed gene list obtained by using DESeq2 were validated by performing differential expression using two other reliable tools, egdeR (16, 17) and limma (18).

Functional analyses of significant regulated genes using IPA software

The significantly regulated genes were subjected to functional analyses using QIAGEN Ingenuity Pathway Analysis (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>)(19). The genes were mapped to the Ingenuity Knowledge Base creating molecular networks which were generated *de novo*. The software also determines overrepresented canonical pathways and further identifies the upstream regulators that involve in activation or inhibition of certain pathway. These upstream regulators were used in predicting the effect on biological processes and diseases or functions in the dataset. These analyses were focusing on detecting the cell-death related genes and pathways occurring in the cells being studied.

Data validation using quantitative real-time polymerase chain reaction (qRT-PCR)

Six significantly regulated genes related to cell-death mechanisms were chosen to be validated using qRT-PCR. Primer sets for qPCR were obtained from previous study as mentioned in Additional file 1. Two micrograms of RNA extracted from the control and treated cells were converted to cDNA using High-Capacity RNA-to-cDNA kit (Applied Biosystems, USA). qPCR was carried out using the StepOne Real-Time PCR systems (Applied Biosystems, USA) with 55 amplification cycles. Melting curve analyses were performed for all primers to ensure single amplification product. Each reaction consisted of 20 ng of cDNA, 0.2 mM forward and reverse primers, 2X Fast SYBR® Green Master Mix (Applied Biosystems, USA) in final volume of 20 µl. All qRT-PCR experiments were repeated with 3 biological replicates from different experiments and the reactions were performed in triplicates. Gene expressions were normalized against GAPDH, a housekeeping gene, and fold change was expressed as mean ± SEM.

Results And Discussions

Differential expression genes

The differential expressed gene list obtained using DESeq2 was validated by performing differential expression using two other reliable tools, edgeR and limma. The agreements among these three tools were visualized in basic plot, Venn diagram (Figure 1) and upset plot (Figure 2). The results obtained by three tools capturing DGE showed good agreements as there were 2,097 genes commonly identified in all three tools. While most of the DESeq2 genes were overlapping either with one or both other tools, DESeq2 found a very low number of genes (25 genes) that did not overlap with the other tools. The DESeq2 number was notably low as compared to limmaVoom and edgeR which consisted of 601 and 769 genes, respectively. A review by Costa-Silva et al. (20) specified that DESeq2 showed consistent results and suggested this method is suitable for experiments with a large number of samples and an annotated genome. The high number of uniquely identified DGE in edgeR in this study was parallel with the study by Nookaew et al. (21) which identified more unique DGE using edgeR than Cuffdiff (22), baySeq (23), DESeq (24) and NOISeq (25). Whereas, limmaVoom had already been pointed out in previous works as one of the better results in the DEG ranking and for analyses with more than two samples (21). The upset plot of gene names displays the total size of every set in the bottom left corner, followed by the type of intersection and the size of the intersection using vertical bars.

Pathway enrichment analysis using Ingenuity Pathway Analysis (IPA)

For IPA-based analysis, 2,893 genes could be mapped to IPA identifiers using the Ensembl ID of human genes. A total of 1,147 analysis-ready molecules across observations were obtained upon cut-off setting log2FoldChange at 0.5 and P-value 0.05, in which 668 were found to be upregulated and 479 were downregulated. The genes with positive or negative fold change values are, respectively, upregulated or downregulated genes in treated samples relative to the expression of the untreated samples.

Cell death-related pathway

Any cell death-related pathway among the 107 canonical pathways that involved the genes in our dataset were identified manually regardless of its significant value. The terms that were identified are cell death, apoptosis, ferroptosis, necrosis and autophagy. Thirty cell-death related pathways involved the genes in our dataset (Table 1). The details of the genes involved in the cell-death related pathways with their P-value and Expr Log Ratio values are shown in Additional file 2. Endoplasmic reticulum stress pathway shows a significant activation with the P-value of 4.66E-05 and z-score of 0.378. In this pathway (Figure 3), it is shown in the pathway that the Caspase-9 is activated and leads to apoptosis. Apart from Caspase-9, there were also three other genes activated in this pathway which includes BIP, CALR and GRP4. In contrast, there are three other genes inhibited in this pathway namely CHOP, AFT4 and XBP1.

Validation of significant genes using qRT-PCR

Transcript levels of selected genes were measured by quantitative real-time polymerase chain reaction (qRT-PCR) to validate the gene expressions in the RNA-Seq data results. Three genes were chosen based on their function in the cell-death related pathway. The chosen genes for validation includes Caspase-9, TGFB2, PPP2R2B, REL, CD82 and FOXO4. The results of analyzed qRT-PCR generally show that the results were in good agreement with the RNA-Seq gene expression results in terms of direction and significant value (Figure 4), thus validating the RNA-Seq gene expression result data.

Discussions

C. nutans modulates the cell death mechanisms in HeLa cells

The analysis using IPA shows that the treatment of HeLa cells using *C. nutans* exhibit its ability to modulate cell death-related mechanisms. The extensive regulation of genes involved in apoptosis and cell death in treatments could probably explain that cancer, cell growth and proliferation, as well as cell death-related networks as being the dominant associated network functions in the IPA network analysis.

Endoplasmic reticulum (ER) stress-induced cell death

In this study, upon analysis on the canonical pathway results, we proposed that *C. nutans* treatment had significantly triggered the endoplasmic reticulum (ER) stress pathway within HeLa cell line with z-score activation rate of 0.378 and p-value 4.66E-05. ER is a vital organelle that have multiple roles in protein folding, biosynthesis of phospholipids, maintaining calcium homeostasis (26), autophagy, redox regulation and apoptosis (27). The ER has the capability to discard the unfolded or misfolded proteins from the secretory pathway and exports only the properly folded proteins to their final destinations (26, 28).

Numerous pathological conditions and chemical compounds are implicated in the disruption of ER functions and thereby can lead to the activation of ER stress conditions within cells. One of the major causes of ER stress is decreased oxygen levels, which refers to hypoxia. According to Rozpedek *et al.* (26) hypoxia is a physiologically important dynamic hallmark, which is present in all solid tumours. This theory is aligned with our study as our sample, cervical cancer cell line is a type of solid tumour. This condition is supported by our canonical pathway result which shows the activation of Hypoxia-inducible factor 1-alpha (HIF1- α) signalling pathway. In contrast, previous accumulative evidence and recent advances in cancer metabolism research reviewed by Hayashi *et al.* (29) suggest that hypoxia-independent mechanisms for HIF signalling activation is a hallmark of cancer.

As a consequence for the hypoxic condition in the cell, the signalling platform of mitochondria is triggered to emit the hypoxic stress responses. Missiroli *et al.* (27) believed that mitochondria and ER

were described as tight contacts where they were structurally and functionally modulated through a series of molecular bridges formed at specific subdomains of the ER membrane, defined mitochondria-associated membranes (MAMs). MAMs have been widely described as an essential hub regulating the most important cellular pathways controlling cell fate (27, 30). Krols, Bultynck and Janssens (31) suggested that the close connections between ER and mitochondria also establish transmission that plays a crucial role in several processes including calcium (Ca^{2+})-transfer and apoptosis. Many oncoproteins and tumor suppressor proteins can themselves directly modulate Ca^{2+} signalling by interacting with Ca^{2+} channels, pumps and exchangers localized at the plasma membrane and various intracellular compartments (32). Interestingly, the Bcl-2 family of oncoproteins has been the most extensively studied in this respect and found to regulate Ca^{2+} signalling in ways that complement their roles as apoptotic regulators (33).

However, the stress in ER may affect cells in many ways depend on the severity of the stress conditions. First, it induces the activation of Unfolded Protein Response (UPR) pathways resulting in the promotion of a pro-adaptive signalling pathway. But if the stress prolonged, the pro-adaptive responses will fail to cope with the condition and this will trigger the second response, the apoptotic cell death. This switch can happen if the ratio of the unfolded or misfolded proteins to the rate of their degradation exceeds a certain threshold (34, 35). In the result data of this research, UPR pathway was found to be significantly inhibited with the z-score of -0.728. This indicated that the treatment on HeLa cell used in this study is causing the occurrence of ER stress in the cell. But the inhibition of the UPR pathway in the canonical pathway result data might indicate that the cell was not compromising the stress by not allowing the cell to adapt and survive. In fact, the ER stress condition had forced the UPR to activate the pro-cell death role via apoptosis.

There are three apoptosis pathways identified in this apoptotic event. The pathways are the transcriptional activation of the gene for C/EBP homologous protein (CHOP), the activation of the c-JUN NH2-terminal kinase (JNK) pathway and the third one is the activation of ER-associated caspase-12. Caspase-12 is activated by ER stress, but apparently not the death receptor-mediated or mitochondrial-targeted apoptotic signals. Our result in this study shows the upregulation of Caspase-9 in ER stress pathway and downregulation of CHOP gene. This means that the apoptosis in our study was using the apoptosis pathway that involves activation of caspase and not involving CHOP.

The major molecular components of the apoptotic machinery are represented by the family of cysteine proteases termed caspases, which are essential for the regulation of apoptotic signalling (26, 36). The caspase activation pathways resulting in the apoptosis can be divided into two main pathways, the intrinsic pathway and the extrinsic pathway. The former is generally engaged by stresses that come from outside the cells while the latter involves the stresses within the cell. The stresses within the cells are including DNA damage, metabolic stress and endoplasmic reticulum stress (37). Numerous studies were undertaken to explain the mechanism associated with the activation of caspases under ER stress condition. Based on the pathway analysis of our data, caspase-9 was identified to be upregulated in not just ER stress pathway, but also in other twelve pathways. These pathways are; a) role of PKR in

interferon induction and antiviral response, b) endocannabinoid cancer inhibition pathway, c) dilated cardiomyopathy signalling pathway, d) HER-2 signaling in breast cancer, e) death receptor signaling, f) induction of apoptosis by HIV1, g) TNFR1 signaling, h) ENOS signalling, erthropoitein signalling pathway, i) prostate cancer signalling, j) pancreatic adenocarcinoma signalling and k) endothelin-1 signaling.

Generally, caspases are initially translated as inactive procaspases that are activated by numerous cell stresses. Caspases are split into two types based on their location within the apoptotic signalling pathway, the initiator caspases and also the effector caspases. The initiator caspases, which include caspases 8, 9 and 12 play a pivotal role within the activation of the caspases cascade that rapidly leads to apoptosis (38). Li et al. (39) suggested that the fate of cells to decide apoptosis and survival is not only determined by caspase-9 but also by the relative abundance and interactions of various crucial apoptotic proteins. Furthermore, caspase-9 is essential to eliminate cells by executing apoptotic death early in developmental stage as it is indispensable to inhibit proliferative diseases through the continual removal of irreparable cells within the lifecycle. The splicing of caspase-9 gene produces two isoforms caspase-9a and caspase-9b/9S. The former bears the full cysteine protease activity, whereas the latter is only a part of the central catalytic domain (39–41). Caspase-9b acts as an apoptotic inhibitor by opposing caspase-9a for attaching to the apoptosome and subsequently suppressing caspase enzyme cascade (39, 42). The overrepresentation of the pathway associated with caspase-9 also are involving HER-2 signalling in breast cancer pathway, prostate cancer signalling pathway and pancreatic adenocarcinoma signalling pathway. These results indicated that caspase-9 is also found to be related to with multiple varieties of cancers. In fact, the upregulation of caspase-9 within these pathways might contribute to the apoptosis of the cell.

Apart from caspase-9, KAI1, which is also referred as CD82 was found to be upregulated in UPR pathway. As a member of tetraspanin superfamily, KAI1 is a metastasis suppressor gene located on chromosome 11p11.2 (43, 44) which are reported to be downregulated during tumor progression and metastasis in several cancers. KAI1 expression is decreased in malignant tumors and is closely associated with malignant progression, metastasis, and prognosis, containing breast, colon, lung, ovarian, nasopharyngeal, liver and pancreatic cancer (44–47). A study to see the results of the overexpression of KAI1 by using xenograph tumor model by Chai et al. (48) reveals that the overexpression of KAI1 had an inhibitory effect on *in vitro* cell growth and invasion. Additionally, the results of this study also indicated that the overexpression of KAI1 accelerated the apoptosis of OSCC-15 cells. These studies supported our finding in which the upregulation of KAI1 will cause the apoptosis of cell as shown UPR pathway.

In the tumor microenvironment pathway, there are three genes related to the cell death, including FOXO4 and REL. Both genes were found to be activated in this pathway. Tumor microenvironment is a complicated system that comprises of multiple cells and cytokines (49). The tumor microenvironment contains the non-cancerous cell present within the tumor, which encompasses immune cells, fibroblasts, and cells that comprise the blood vessels (50–52). FOXO4 is a member of the FOXO family. According to Liu et al (2020), this family member is mostly involved in regulating genes which are responsible for metabolism, cell cycle, apoptosis and cellular homeostasis through transcriptional activity. Clinically,

FOXO4 has been considered to be a novel tumor suppressor in many human cancers, including leukemia, neuroblastoma, cervical, colorectal, pancreatic and lung cancers (53–55).

Disease analysis related to cell death

To further understand the functional working mechanism related to cell death, disease and function analysis was also performed. The analysis was performed using IPA through IPA database focusing on the 'Cell death and survival' category. Based on the results, four out of eighteen diseases or functions annotation were predicted to be activated. The functions are 'Apoptosis of endothelial cells', 'Necrosis of epithelial tissue', 'Apoptosis of vascular endothelial cells' and 'Apoptosis of vascular cells'. The most significant among the activated functions was 'Apoptosis of endothelial cells' annotation which involves 29 molecules. Among 29 molecule or genes, the upregulated are caspase-9, CXCR4, LRP1, OLR1, APP, HSPA5, THBS1, COL4A3, FOXO4, MCAM, PROCR, TNRRSF11B, ATP1B3, NR4A3, SERPINB5 and TNFSF15. These results support our interpretation on the cell death-related genes in the canonical pathways.

Conclusions

In conclusion, the present study shows that the *C. nutans* has the potential in inhibiting cervical cancer proliferation and regulating the apoptosis process in HeLa cells. Subsequently, a set of DEGs between the treated and untreated cells was obtained using RNA-Seq technique. Using the RNA-Seq data, the potential canonical signalling pathways and the associated diseases and functions involving the DEGs as a results of the treatment were enriched and analyzed using the IPA system. The treatment was found to regulate a few cell-death related pathways, including unfolded protein response pathway, tumor microenvironment pathway and endoplasmic reticulum stress pathway. Upon analysis on the canonical pathways, four significant genes involved in promoting apoptosis were identified. Each gene that has changed the expression level plays a role in regulating various pathways that lead to cell death. In addition, data from the present study also suggests that this treatment serves a role in the 'disease and functions' analysis in which the most significant among the activated functions in 'Cell death and survival' category was 'Apoptosis of endothelial cells'. The present study uncovered some of the previously unknown capabilities of *C. nutans* on cervical cancer cells.

In this study, there are some limitations that need to be considered. Firstly, rather than the data from real cases of cervical cancer, we only used sample from cell line. Experiment using the samples from real case data should be considered to further verify these results. Secondly, according to the IPA analysis, further research is needed to focus on the mechanisms involving differential signalling pathways that are specific to cell death. Lastly, while there is rapid accumulation of databases in public repositories that we can use to define the functional categories and implement pathway analysis, in this study we only used single database, which is the IPA database. Although this article has these limitations, this study is able to provide a set of important pathways and genes for further investigation into the molecular mechanisms involved in cell death of cervical cancer cells. The main challenge encountered was in setting an optimum cut-off value of the datasets in IPA. Despite the limitations and challenge faced in this study, the objectives were achieved and it is expected that this study will provide a new avenue for

the clinical treatment of cervical cancer and a theoretical basis for the clinical application of *C. nutans* DCM.

Abbreviations

μl: microliter

°C: degree celsius

AFT4: Activating Transcription Factor 4

APP: Amyloid Beta Precursor Protein

ATP1B3: ATPase Na⁺/K⁺ Transporting Subunit Beta 3

BAM: Binary Alignment Map

bp: base pair

Bcl-2: B-cell lymphoma 2

BGI: Beijing Genomics Institute

BIP: Binding immunoglobulin protein

CA: California

Ca²⁺: calcium

CALR: Calreticulin

CASP9: caspase-9, cysteine-aspartic acid protease

Caspase-9b: cysteine-aspartic acid protease beta

Caspase-9S: small variant of cysteine-aspartic acid protease

CD82: Cluster of Differentiation 82

cDNA: complementary DNA

CHOP: CCAAT-enhancer-binding protein homologous protein

COL4A3: Collagen Type IV Alpha 3 Chain

CPU: Cental processing unit

CXCR4: C-X-C Motif Chemokine Receptor 4

DCM: Dichloromethane

DEG: Differential Expression Gene

DNA: Deoxyribonucleic acid

ENOS: Endothelial nitric oxide synthase

Ensembl ID: identifier for a gene as per the Ensembl (European Bioinformatics Institute and the Wellcome Trust Sanger Institute) database

ER: endoplasmic reticulum

F: Forward

Fastp: FASTQ data pre-processing tool

FOXO4: Forkhead Box O4

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GHz: gigahertz

GRP4: glycine-rich RNA binding protein

GTF: Gene transfer format

HER-2: human epidermal growth factor receptor 2

Hg38: Homo sapiens (human) genome assembly GRCh38

HIF1- α : hypoxia-inducible factor subunit alpha

HIV1: Human Immunodeficiency Virus type 1

HPV: human papillomavirus.

HSPA5: Heat Shock Protein Family A (Hsp70) Member 5

IPA: Ingenuity Pathway Analysis

JNK: c-JUN NH2-terminal kinase

KAI1: Kangai 1

LRP1: lipoprotein receptor-related protein 1

LTS: long term support

MAMs: mitochondria-associated membranes

MCAM: Melanoma Cell Adhesion Molecule

mM: millimolar

ng: nanogram

NR4A3: Nuclear Receptor Subfamily 4 Group A Member 3

OSCC: Oral Squamous Cell Carcinoma

OLR1: Oxidized low-density lipoprotein receptor 1

PKR: protein kinase R

PPP2R2B: Protein Phosphatase 2 Regulatory Subunit B beta

PROCR: Protein C Receptor

qRT-PCR: quantitative reverse transcription Polymerase chain reaction

R: Reverse

REL: proto-oncogene c-Rel

RNA-Seq: RNA sequencing

SEM: standard error of the mean

SERPINB5: Serpin Family B Member 5

TGFB2: transforming growth factor beta-2

THBS1: Thrombospondin 1

Tm: melting temperature

TNFR1: Tumor necrosis factor receptor 1

TNFSF15: Tumor necrosis factor superfamily member 15

TNRRSF11B: tumor necrosis factor receptor superfamily, member 11b

UCSC: University of California, Santa Cruz

UPR: Unfolded Protein Response

XBP1: X-box binding protein 1

Declarations

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Contributions

Kristine Sandra Pey Adum conducted all the experimentation, bioinformatics analysis and wrote the manuscript. Hasni bin Arsad conceived the study. Nor Hasyimah binti Haron provided the data. Zaleha binti Toha helped in experimental design. All authors agreed to be accountable for all aspects of the work.

Availability of data and materials

The RNA-seq data of raw and processed files have been deposited in NCBI Gene Expression Omnibus, accession number GSE207454 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207454>).

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Tables

TABLE 1

The cell-death related canonical pathways with their P-value and Expr Log Ratio values

No.	Pathway	Cell-death mechanism involve	Cell-death related genes		P- value	z- score
			up	down		
1.	Unfolded protein response	Apoptosis	KAI1	-	1.09E-08	-0.728
2.	Tumor microenvironment pathway	Apoptosis of tumor cells	FOXO4, REL	RELB	2.68E-05	0
3.	Endoplasmic reticulum Stress Pathway	Apoptosis	CASP9	-	4.66E-05	0.378
4.	Role of PKR in interferon induction and antiviral response	Apoptosis	CASP9	IRF1	3.01E-04	-1.069
5.	Role of Tissue Factor in cancer	Apoptosis	PIK3R3	-	5.83E-04	NaN
6.	Aryl Hydrocarbon Receptor Signaling	Apoptosis	TGFB2, TGFB-a2M	-	1.05E-03	-2.121
7.	Ferroptosis signaling pathway	Ferroptosis	-	CHAC1, AFT4, ANGPTL4	3.06E-04	-0.905
8.	Ceramide signaling	Apoptosis	PPP2R2B, PPM1L	PPP2R2C	1.65E-03	-0.333
9.	BEX2 Signaling Pathway	Mammary tumor cell apoptosis	-	BEX2,PP2A	1.98E-03	-1

TABLE 1 (continue)

No.	Pathway	Cell-death mechanism involve	Cell-death related genes		P-value	z-score
			up	down		
10.	HGF Signaling	Anoikis	COX2 (PTGS2), PIK3R3	-	2.22E-03	0.816
11.	PTEN Signaling	Apoptosis	FOXO4	-	3.33E-03	-1.134
12.	Natural killer cell signalling	Apoptosis	REL	RELB	3.63E-03	-0.728
13.	Inhibition of angiogenesis by TSP1	Apoptosis	-	Fyn, MAPK11, c-Jun	4.70E-02	NaN
14.	Endocannabinoid cancer inhibition pathway	Apoptosis	CASP9	AFT3, NUPR1, DDIT3, TRIB3	2.89E-02	-0.707
15.	Neuroinflammation signaling pathway	Neurons apoptosis	COX2	-	5.49E-03	-0.688
16.	Role of pattern recognition receptors in recognition of bacteria and viruses	apoptosis	PIK3R3	-	6.22E-03	0.816

TABLE 1 (continue)

No.	Pathway	Cell-death mechanism involve	Cell-death related genes		P- value	z- score
			up	down		
17.	Toll-like receptor signaling	apoptosis	-	IL-1	1.41E-02	-1.633
18.	Dilated cardiomyopathy signaling pathway	CM apoptosis	CASP9	-	1.43E-02	0.302
19.	HER-2 Signaling in breast cancer	Apoptosis	COX2, REL, CASP9	RELB	1.61E-02	0.229
20.	Death Receptor signaling	Apoptosis	ARHGBIB, ACTG2, ACTC1, CASP9, BIRC3, TNFSF15	TNFR2	1.84E-02	0.707
21.	Sirtuin signaling pathway	Autophagy	ATG (GABARAPL1)	-	2.72E-02	-1.29
22.	Corticotropin Releasing hormone signaling	Apoptosis	-	c-Jun	2.91E-02	-0.378
23.	Autophagy	Autophagy	-	SQSTM1, ULK1	3.50E-02	-1.213
24.	Induction of apoptosis by HIV1	Apoptosis	CASP9, CXCR4	-	3.64E-02	0.447
25.	TNFR1 signaling	Apoptosis	CASP9	-	3.69E-02	NaN

TABLE 1 (continue)

No.	Pathway	Cell-death mechanism involve	Cell-death related genes		P-value	z-score
			up	down		
26.	eNOS signaling	Apoptosis	CASP9	-	4.03E-02	0
27.	Erythropoitein signaling pathway	Apoptosis	BIRC3	-	4.12E-02	0.577
28.	Prostate cancer signaling	Apoptosis	CASP9	-	4.51E-02	NaN
29.	Pancreatic adenocarcinoma signaling	Apoptosis	CASP9	-	4.52E-02	-0.378
30.	Endothelin-1 signaling	Apoptosis	CASP9	-	4.53E-02	1.732

Figures

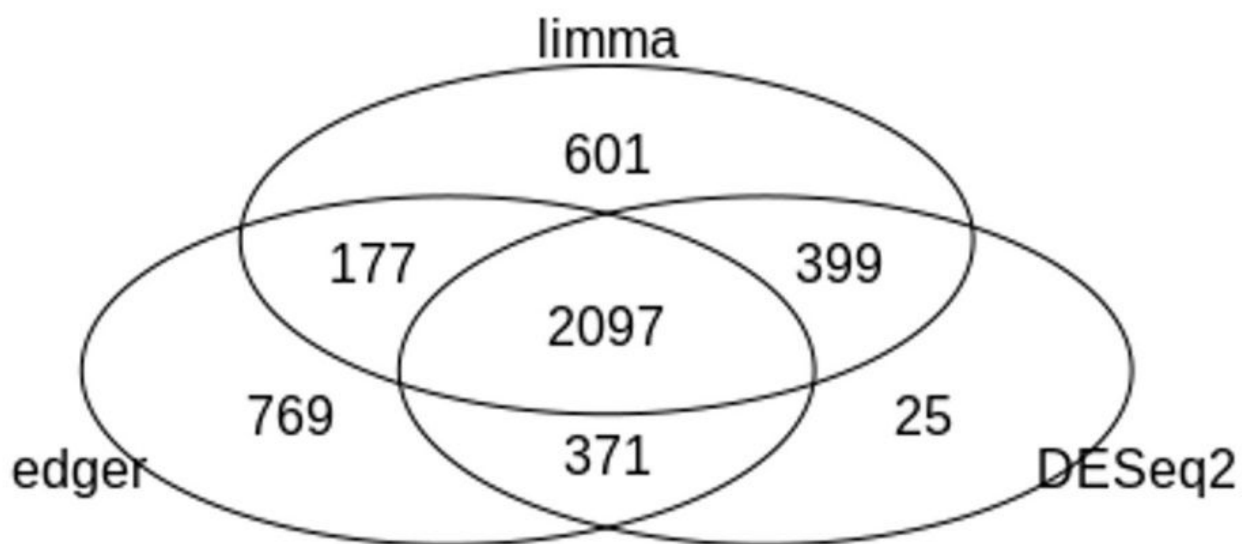


Figure 1

Venn diagram comparing DESeq2, edgeR and limma DEGs using 'gplots'.

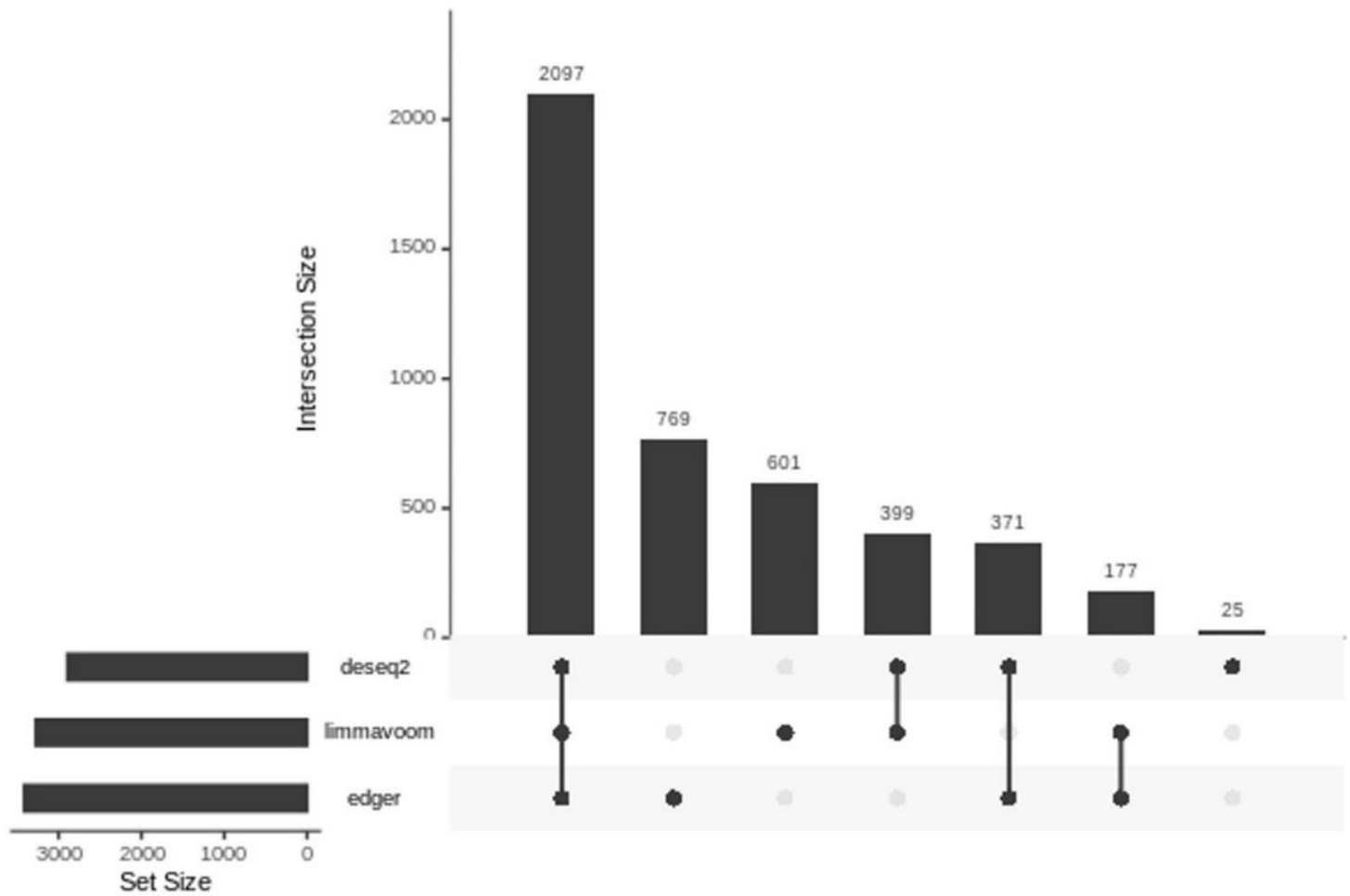


Figure 2

Upset plot comparing DESeq2, edgeR and limmaVoom DEGs using 'gplots'.

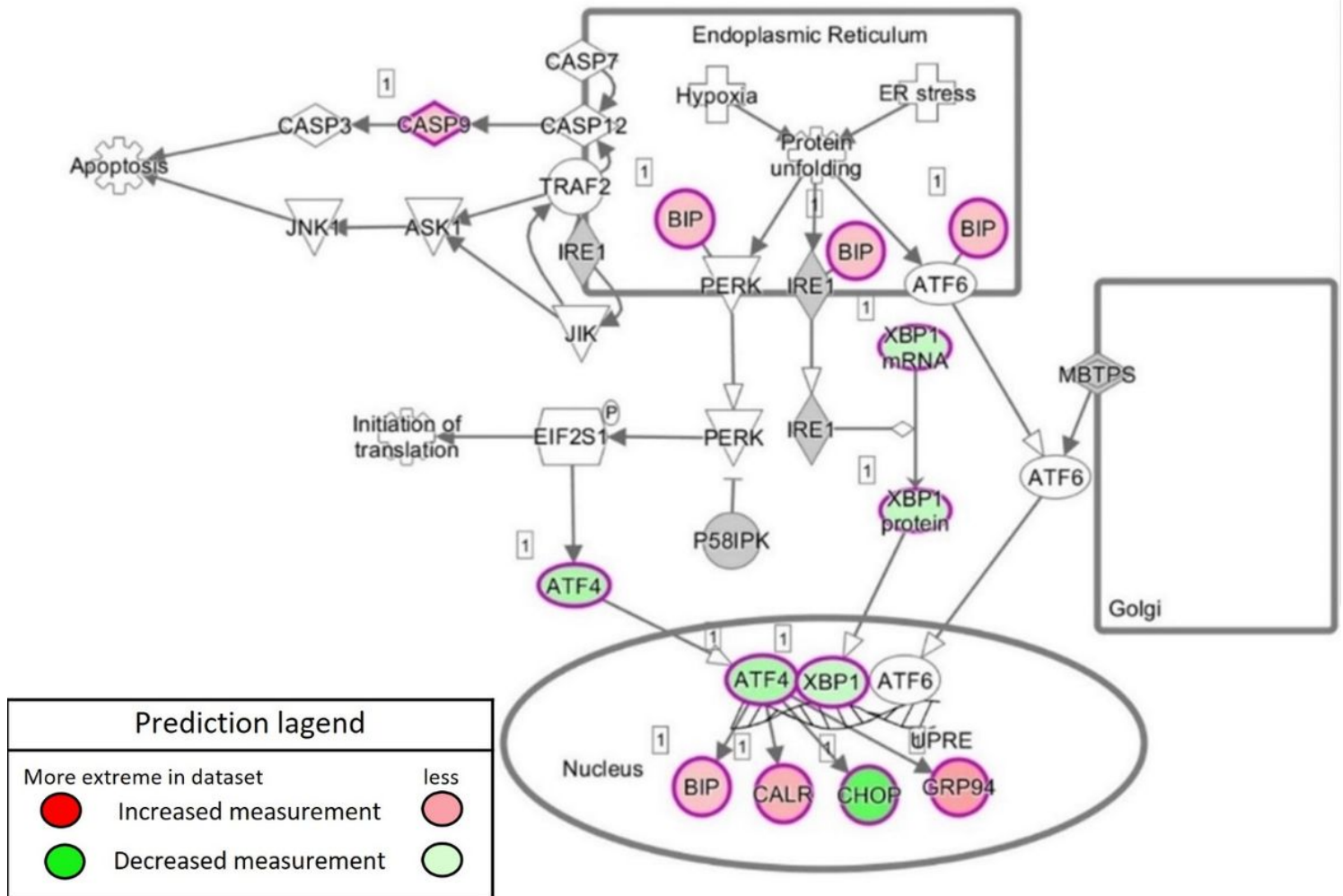


Figure 3

The 'Endoplasmic reticulum stress pathway' highlighting differentially expressed genes in HeLa cell treated with *C. nutans*. Canonical pathways were identified and depicted by IPA software. This pathway shows the 'Endoplasmic reticulum stress pathway' as related to significant DE genes of our sample. Genes that are highlighted in green are considered as downregulated while the genes that are highlighted in red are considered as upregulated.

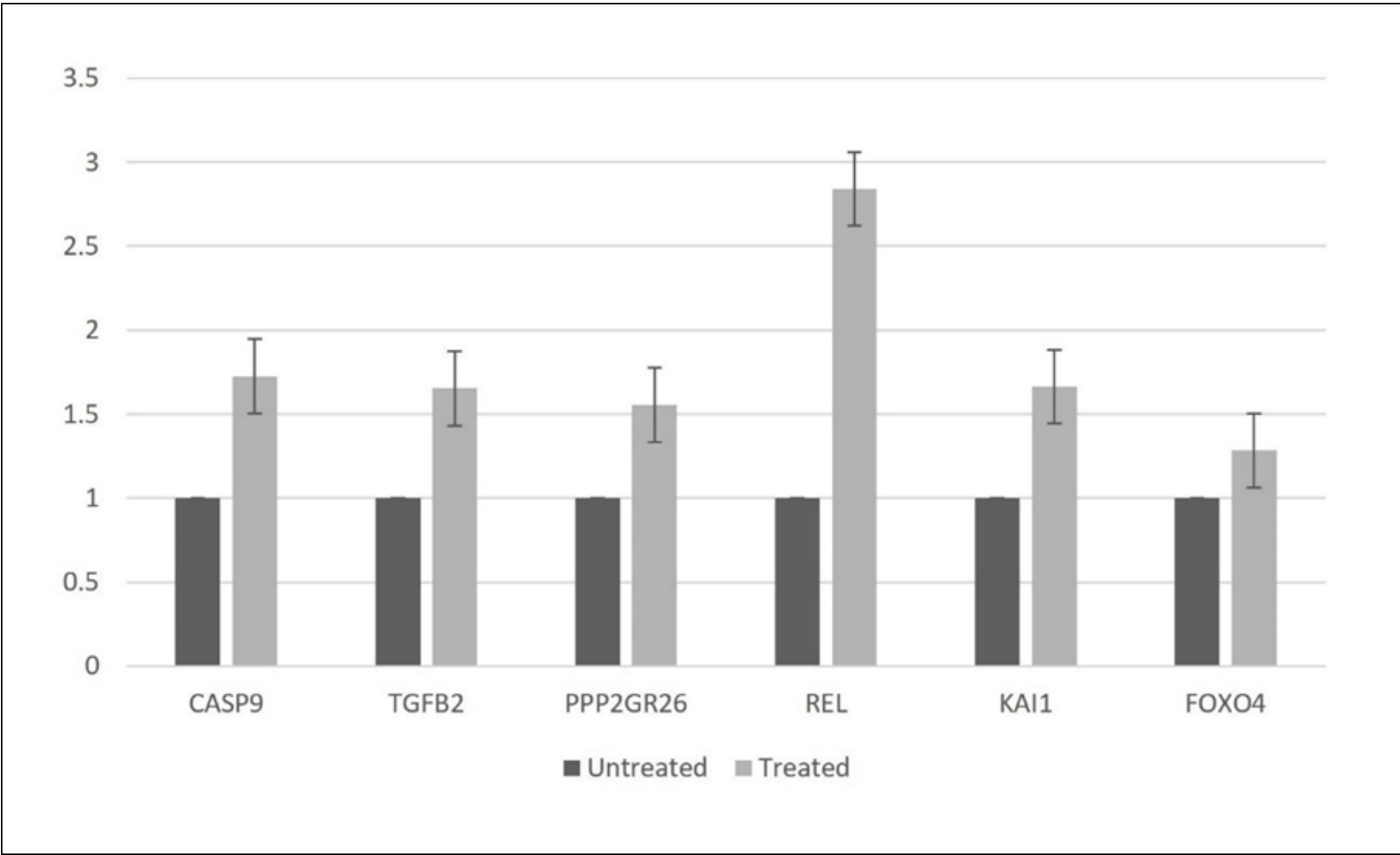


Figure 4

Bar graph showing the fold change of the genes in qRT-PCR compared to control.

Supplementary Files

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