Abstract. Background/Aim: Wild yam extract [Dioscorea villosa, (WYE)] is consistently lethal at low IC₅₀s across diverse cancer-lines in vitro. Unlike traditional anti-cancer botanicals, WYE contains detergent saponins which reduce oil-water interfacial tensions causing disintegration of lipid membranes and causing cell lysis, creating an interfering variable. Here, we evaluate WYE at sub-lethal concentrations in MDA-MB-231 triple-negative breast cancer (TNBC) cells. Materials and Methods: Quantification of saponins, membrane potential, lytic death and sub-lethal WYE changes in whole transcriptomic (WT) mRNA, miRNAs and biological parameters were evaluated. Results: WYE caused 346 differentially expressed genes (DEGs) out of 48,226 transcripts tested; where up-regulated DEGs reflect immune stimulation, TNF signaling, COX2, cytokine release and cholesterol/steroid biosynthesis. Down-regulated DEGs reflect losses in cell division cycle (CDC), cyclins (CCN), cyclin-dependent kinases (CDKs), centromere proteins (CENP), kinesin family members (KIFs) and polo-like kinases (PLKs), which were in alignment with biological studies. Conclusion: Sub-lethal concentrations of WYE appear to evoke pro-inflammatory, steroid biosynthetic and cytostatic effects in TNBC cells.

Dioscorea villosa is a North American native plant within the genus Dioscorea, and the roots and rhizomes of this species are known as wild yam (1). This plant has been widely used as a botanical dietary supplement to treat menopause-related hot flashes, muscular cramps, arthritis, upset stomach, coughs, problems related to childbirth, and in cosmetic topical ointments (1). Research on the medicinal value of wild yam root extract (WYE) has shown evidence suggesting anticancer properties in particular for breast cancer and in both hormone receptor-positive and triple-negative breast cancers (TNBC), where it alters epigenetic 5-hydroxymethylcytosine DNA patterns, induces toxicity, halts cell cycle, inhibits fatty acid synthase and modifies the activity of estrogen and progesterone hormone receptors (1-5). Because TNBC is characterized by the lack of estrogen, progesterone, and human epidermal growth factor receptor 2 receptors, treatment options are limited, leading to highly aggressive metastatic cancers, with poor clinical outcomes in terms of treatment relapse and life expectancy. For this reason, a good deal of research has been focused on finding effective alternative treatments for TNBC, such as the case for WYE, which contains hundreds of constituent saponins such as deltonins, dioscoreavillosides, diarylheptanoids (6), diosgenin, and dioscin, the latter two alone can slow breast tumor growth, migration, deter stem cell phenotype and cause cell death in various models (7-11).

There is a unique element of saponin-rich plants, which vastly differs from most naturally derived plant-based chemotherapies like taxol (Taxus brevifolia), having inherent emulsification properties and a capacity to destroy fats on contact, including those housed within biological membranes (12, 13). The "on-contact" cell lytic nature of saponins was first observed in red blood cells (RBCs) in the 1920s, likened to taurocholic acid (12), which disrupts cholesterol or phosphatidylcholine rich triglycerides causing pore formation, micellar structures, lytic permeability, and cell death (13). Steroidal saponins in WYE, such as dioscin and diosgenin target phosphatidylcholine-rich membranes while triterpene saponins tend to destroy cholesterol-rich membranes (14-18);
the former can induce membrane lytic destruction within minutes (19-21).

Given that WYE and those of the Dioscorea species, to our knowledge, are of the most consistently cytotoxic herbs in vitro across diverse cancer cell lines by saponification/lytic membrane-mediated lysis (22, 23), the question remains as to effects that are occurring at concentrations (sub-lethal) that precede saponin induced lytic membrane destruction. In this work, we evaluate whole transcriptomic patterns induced by WYE at sub-lethal concentrations in MDA-MB-231 triple-negative breast cancer (TNBC) cells, where both immune stimulation and cell-cycle ablation are confirmed.

Materials and Methods

Wild yam extract (WYE) preparation. Wild Yam powder was purchased from Mountain Rose Herbs (Eugene, OR, USA). A crude WYE was prepared by dissolving the powder in absolute ethanol at 50 mg/ml, followed by vortexing and storage in the dark at –20°C. Serial dilutions of WYE were prepared in sterile HBSS.

Cell culture. MDA-MB-231 HTB-26™ cells were purchased from ATCC. The cells were cultured in 75 cm² flasks with high glucose [4,500 g/l] DMEM supplemented with 7% FBS and 100 U/ml penicillin G sodium/100 µg/ml streptomycin sulfate. Cells were grown at 37°C in 95% atmosphere 5% CO₂ and sub-cultured every three to five days. Experimental studies involving monolayers were conducted in growth media (as described above) in 96 well plates or 75 cm² flasks. Experimental cultures involving 3D spheroids were seeded in culture media, using low-adhesion spheroid forming 96 well plates, and pelleted by centrifugation at 1,800 × g for 3 min, daily for the first three days. The spheroids were grown at 37°C in 95% atmosphere 5% CO₂ for 7 days prior to experimental treatment. Changes in morphology and live-cell imaging with FDA were captured using an inverted fluorescence microscope.

Human cytokine antibody array. Human cytokine antibody arrays (Cat# AAM-CYT-1000) (Raybiotech Inc, Peachtree Corners, GA, USA) were used to profile supernatant cytokine content. Briefly, antibody-coated array membranes were first incubated for 30 min with 1 ml of blocking buffer. After 30 min, the blocking buffer was decanted and replaced with 1 ml supernatant. Supernatants were pre-diluted in sample buffer to 20% to detect signals in highly expressed control proteins. Membranes were allowed to incubate for 5 h with shaking. Membranes were then washed with the prepared washing buffer and then incubated with 1 ml biotin-conjugated antibodies. After incubation, the mixture of biotin-conjugated antibodies was removed, and membranes were incubated with HRP-conjugated streptavidin (2 h). Detection of chemiluminescent spots was captured on Quantity One software installed on a Bio-Rad Versadoc (Bio-rad, Hercules, CA, USA), followed by densitometry processing from CEL to CHP files were conducted using an expression console, prior to data evaluation using the Affymetrix transcriptome analysis console, String Database (String Consortium 2020) and DAVID functional annotation microarray tools (28-30), n=3. The dataset has been deposited to NIH Gene Expression Omnibus located at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180621.

Expression arrays. Briefly, RNA was synthesized to the first-strand cDNA, then second-strand cDNA, followed by a subsequent transcription to cRNA. cRNA was purified and assessed for yield prior to 2nd cycle single-stranded cDNA synthesis, hydrolysis of RNA, and purification of 2nd cycle single-stranded cDNA. cDNA was then quantified for yield and equalized to 176 ng/ml. Subsequently, cDNA was fragmented, labeled, and hybridized onto the arrays prior to being subjected to fluids and imaging using the Gene Atlas (Affymetrix, ThermoFisher Scientific). The array data quality control and initial processing from CEL to CHP files were conducted using an expression console, prior to data evaluation using the Affymetrix transcriptome analysis console, String Database (String Consortium 2020) and DAVID functional annotation microarray tools (28-30), n=3. The dataset has been deposited to NIH Gene Expression Omnibus located at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180621.

ELISA for IL-8. Supernatants from MDA-MB-231 cells (Control vs. WYE Lo 15 µg/ml) were collected and centrifuged at 1,000 × g for 5 min at 4°C. Specific ELISA was performed using IL-8 ELISA kit
following manufacturer’s instructions (Human IL-8/CXCL8 ELISA) (Millipore-Sigma, Saint Louis, MO, USA). The sample was diluted in buffer 20% supernatant/buffer, and 100 μl of prepared supernatant from samples was added to 96 well plates pre-coated with the capture antibody. After incubation, 100 μl of prepared biotinylated antibody mixture was added to each well. After 1 h, the mixture was decanted, and 100 μl streptavidin solution was placed in each well and incubated. Substrate reagent (100 μl) was then added to each well for 30 min followed by a 50 μl stop solution. Plates were read at 450 nm using a Biotek H.T.X. Synergy-multi-mode microplate reader.

Statistical analysis. Statistical analysis was performed for the basic studies using GraphPad Prism (version 3.0; Graph Pad Software Inc., San Diego, CA, USA). The significance of the difference between the groups was assessed using either a student’s t-test or a one-way ANOVA followed by Tukey post hoc analysis.

Results

Both cytostatic and cytotoxic curves were generated over a dose-response of WYE, where saponins were quantified by a simple foam test (Figure 1). The data show a close correlation between foam and cell death, with the (anti-proliferative) cytostatic effects being in close proximity to but slightly preceding sub-lethal concentrations of WYE. At this point, it is uncertain whether the loss of the cell cycle is related to basic cytotoxicity given the proximity of these curves, which will be further elucidated in this work. Next, the data show that greater saponin content in higher concentrations of WYE are associated with cell death, which coincides closely to damage to lipid membrane bio-layers. In Figure 2, we show basic fluorescent cell imaging of plasma membrane integrity/viability (left panel) and mitochondrial membrane (right) using fluorescent probes FDA and TMRE, respectively. The data show complete loss of both with greater saponin foam content in WYE. To determine if WYE saponins would also destroy a small 3D tumor spheroid, we evaluated cell survival (FDA) and morphological changes with increasing concentration of WYE (Figure 3). The data show spheroid tumors to be slightly more resistant to WYE, however, at concentrations exceeding 148 μg/ml there was near complete death of the entire spheroid.

For microarray studies, we chose to conduct experimentation at 2 sub-lethal concentrations denoted as Low (15 μg/ml); sub-lethal and High (30 μg/ml); sub-lethal/cusp of death (Figure 4).
Cells for microarray studies were prepared in 75 cm² flasks and monitored to ensure no morphological structure or attachment changes occurred over 24 h of treatment prior to cell pellet collection. Both cell pellets and supernatant were collected from the same samples and stored at –80°C. While we provide whole transcriptomic data on both sets, we focus the analysis within this article on only the WYE (low vs. control) data set. In both sets, less than 0.7% of the whole transcriptome showed differentially expressed genes (DEGs), with criteria set at -2<x>+2-fold change, \( p \)-Value, and FDR \( p \)-Values <0.05. The data for DEGs in the WYE sub-lethal (low) vs. control groups are presented in Table I, with both sets, including sub-lethal WYE (high) vs. control provided at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180621.

Using Stringdb, all down-regulated genes meeting criteria for WYE (low) were entered, and relational networks were identified (Figure 5). The data show a statistical loss in gene elements that transcribe for cell cycle and mitosis, showing a network FDR value for biological processes analysis (gene ontology) \( p \)-Value <0.235e-83. High significance for changes in the chromosome, cell division, DNA replication, and cross strand repair using local network cluster (STRING) also show FDR \( p \)-values up to \( p<4.48e-63 \). All database platforms pickup up this differential as significant, including the Reactome and Kyoto Encyclopedia of Genes and Genomes (Kegg) pathways, where the latter was also found in the WIKIpathway report of Affymetrix/applied Biosystems transcriptome analysis console report (including up and down DEGs) (Figure 6). Similarly, stringdb analysis was performed on up-regulated DEGs for WYE (Low) (Figure 7), where most significant changes centered on steroid synthesis and cytokine signaling, specifically affecting the TNF-alpha pathway, changes also reflected in the component Kegg overlap map (Figure 8).

The supernatant of the samples matching the microarray was tested for the presence of cytokines using antibody arrays where the largest up-regulated differential DEG by WYE was for CXCL8/ IL-8, being observed in densitometry values within the anti-body array itself (Figure 9A,B), the Affymetrix microarray (Figure 10) and confirmed by Elisa (Figure 11). Figure 9A,B shows the relative comparison of cytokines released in the supernatant and the mRNA in cells, where the criteria for gene analysis was lowered to less than 2-fold, with a significant \( p \)-Value <0.05 and no filter on FDR.

Figure 2. Effect of WYE on cell membrane potentials (plasma/viability) obtained with FDA (left panel) and mitochondrial TMRE (right panel). (A) Control, WYE Treatment (B) 18.5 μg/ml, (C) 37 μg/ml, (D) 74 μg/ml, (E) 148 μg/ml, (F) 296 μg/ml.
Figure 3. Effect of WYE on 3D tumor spheroids. Cell viability and morphological changes in MDA-MB-231 tumor spheroids with increasing concentration of WYE are shown. The data represent basic changes in spheroid structure (main image: black and white), with fluorescent FDA viable cell staining (green) in the lower left section of each main image. (A) Control, WYE Treatment (B) 4.62 μg/ml, (C) 9.25 μg/ml, (D) 18.5 μg/ml, (E) 37 μg/ml, (F) 74 μg/ml, (G) 148 μg/ml, (H) 296 μg/ml.
p-Values. There was a high degree of matching values between proteins released in the supernatant and the mRNA transcription for those proteins in the same pellet sample. In summary, this provides an overall snapshot of the effects of WYE in MDA-MB-231 cells at sub-lethal concentrations.

Discussion

In this study, we evaluate the biological and transcriptomic effects of WYE on TNBC cells at sub-lethal concentrations, well below the point involving saponin-mediated cell lysis. WYE shows antiproliferative effects at sub-lethal concentration tantamount to severe downregulation of gene transcripts involved in mitosis and cell division, including transcripts of the following classes: cell division cycle (CDC), cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma overexpressed (CCN), cyclin-dependent kinases (CDK), centromere proteins (CENPs), kinesin superfamily transcripts (KIFS), and polo-like kinases (PLK). These effects were concurrent with single gene up-regulation of the p21 gene CDKN1A, all of which are likely responsible for the observed cytostatic effects of WYE in a 6-day proliferation study. Our findings in this aspect support much of the existing literature for WYE and saponins from the *Dioscorea* species having the capacity to induce cell-cycle arrest (G2/M) across diverse cancer cell lines with observed downregulation of a similar list of cyclins and cell cycle regulatory transcripts (Cdc25C, Cdk1) (31-34).

While WYE was able to halt cell division effectively at low concentrations (15 μg/ml), the data in this work show an apparent rise in a series of genes that demarcate immune stimulation. As to the compounds responsible for these effects, they could be one or more of the known hundreds of compounds present in the root, such as diterpenes, phenolics, cyanidins, quinones, methyl parvifloside, trigofenoside A-1, protodeltonin, deltonin, glucosidodeltonin, zingiberensis I, methylprotodioscin, zingiberensis, dioscin, prosapogenin, dioscoreavillosides A and B, diarylheptanoids or possibly lipidated steroid saponins (6).
Table I. Effects of WYE on the transcriptome in MDA-MB-231 cells. Selection criteria include greater than or less than 2 fold change and both p-Values and FDR p-values <0.05. The data is presented as the effects of WYE on transcript by Fold Change, Gene Symbol, Gene Description, and significance.

Transcriptome changes: Control vs. WYE (Low 15 μg/ml).

| Gene Symbol | Description                                | Fold change | p-Value    | FDR p-Value |
|-------------|--------------------------------------------|-------------|------------|-------------|
| CXCL8       | Chemokine (C-X-C motif) ligand 8            | 9.09        | 3.79E-10   | 1.83E-05    |
| KLF2        | Kruppel-like factor 2                      | 7.82        | 4.67E-07   | 1.90E-03    |
| RHOB        | Ras homolog fam. mem. B                    | 7.64        | 1.08E-09   | 2.60E-05    |
| IL1A        | Interleukin 1 alpha                        | 7.53        | 1.15E-05   | 6.50E-03    |
| PTGS2       | Prostaglandin-endoperoxide synthase 2 [COX-2] | 7.01        | 1.59E-06   | 2.80E-03    |
| HMOX1       | Heme oxygenase 1                           | 5.79        | 1.75E-06   | 2.80E-03    |
| NR1D1       | Nuclear receptor subfam. 1, group D, mem. 1 | 5.45        | 5.65E-07   | 2.10E-03    |
| HKDC1       | Hexokinase d.c. 1                          | 5.36        | 5.50E-05   | 1.29E-02    |
| GEM         | GTP b.p. overexpressed in skeletal muscle  | 5.29        | 1.46E-07   | 7.00E-04    |
| HMGCS1      | 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) | 5.25        | 9.36E-08   | 5.00E-04    |
| INSIG1      | Insulin induced gene 1                     | 5.25        | 2.24E-08   | 3.00E-04    |
| AT3F        | Activating transcription factor 3          | 4.94        | 4.95E-06   | 5.00E-03    |
| DUSP10      | Dual specificity phosphatase 10            | 4.91        | 6.61E-06   | 5.70E-03    |
| GDF15       | Growth differentiation factor 15           | 4.90        | 6.16E-07   | 2.10E-03    |
| KDM7A       | Lysine (K)-specific demethylase 7A         | 4.88        | 8.70E-08   | 5.00E-04    |
| EGR1        | Early growth response 1                    | 4.74        | 1.99E-05   | 8.00E-03    |
| TNFAIP3     | Tumor necrosis factor, alpha-induced protein 3 | 4.65        | 6.86E-07   | 2.10E-03    |
| LOC105378662| Uncharacterized LOC105378662; LOC105378663 | 4.32        | 7.74E-06   | 5.70E-03    |
| TM4SF19     | TM4SF19; TCTEX1D2; TM4SF19-TCTEX1D2         | 4.26        | 2.18E-06   | 3.00E-03    |
| RAD         | Ras-related associated with diabetes       | 4.21        | 5.85E-08   | 5.00E-04    |
| STC1        | Stanniocalcin 1                            | 4.09        | 7.30E-08   | 5.00E-04    |
| FBXO32      | F-box protein 32                           | 3.86        | 1.00E-04   | 1.80E-02    |
| KLHL24      | Kelch-like fam. mem. 24                    | 3.85        | 1.62E-06   | 2.80E-03    |
| BACH1-IT2   | BACH1 intronic transcript 2                | 3.75        | 1.64E-05   | 7.50E-03    |
| CREB5F      | CREB regulatory factor                     | 3.71        | 1.87E-05   | 7.90E-03    |
| ZFPM2-AS1   | ZFPM2 antisense RNA 1                      | 3.69        | 1.71E-05   | 7.50E-03    |
| ABTB2       | Ankyrin repeat and BTB (POZ) d.c. 2        | 3.64        | 2.89E-05   | 9.70E-03    |
| LOC105373713| Uncharacterized LOC105373713              | 3.55        | 6.22E-05   | 1.33E-02    |
| YPEL2       | Yippee like 2                              | 3.53        | 7.57E-07   | 2.10E-03    |
| EID3        | EP300 interacting inhibitor of differentiation 3 | 3.48        | 3.00E-04   | 3.26E-02    |
| CDKN1A      | Cyclin-dependent kinase inhibitor 1 A (p21, Cip1) | 3.46        | 6.19E-05   | 1.33E-02    |
| ZNF114      | Zinc finger protein 114                    | 3.40        | 9.59E-06   | 6.00E-03    |
| PLEKHM1     | Pleckstrin homology, fam. M (w.RUN domain) mem. 1 | 3.37        | 6.46E-06   | 5.70E-03    |
| SLC2A3      | Solute carrier fam. 2 (facilitated glucose), m 3 | 3.37        | 8.34E-07   | 2.20E-03    |
| KL7F        | Kruppel-like factor 7 (ubiquitous)         | 3.34        | 1.00E-04   | 1.73E-02    |
| IL11        | Interleukin 11                             | 3.33        | 3.00E-04   | 3.09E-02    |
| KPN7A       | Karyopherin alpha 7 (importin alpha 8)     | 3.24        | 9.69E-06   | 6.00E-03    |
| SIK1        | Salt-inducible kinase 1                    | 3.22        | 3.00E-04   | 3.18E-02    |
| ZBTB10      | Zinc finger and BTB d.c. 10                | 3.21        | 4.00E-04   | 3.96E-02    |
| IRAK2       | Interleukin 1 receptor associated kinase 2 | 3.19        | 2.00E-04   | 2.15E-02    |
| KL9F        | Kruppel-like factor 9                      | 3.18        | 8.04E-06   | 5.90E-03    |
| RASGEF1B    | RasGEF domain fam. mem. 1B                | 3.14        | 4.89E-06   | 5.00E-03    |
| TMEM159     | Transmembrane protein 159                 | 3.11        | 9.17E-06   | 6.00E-03    |
| HIST1H2BD   | Histone cluster 1, H2bd                 | 3.10        | 4.00E-04   | 3.83E-02    |
| MIR4315-1   | MicroRNA 4315-1:2 PLEKHM1                 | 3.09        | 1.06E-05   | 6.30E-03    |
| CBLB        | Cbl proto-oncogene B, E3 ubiquitin protein ligase | 3.05        | 1.60E-05   | 7.50E-03    |
| ICAM1       | Intercellular adhesion molecule 1          | 3.05        | 3.00E-04   | 2.87E-02    |
| GEPT2       | Glutamine-fructose-6-phosphate transaminase 2 | 3.03        | 9.73E-06   | 6.00E-03    |
| UAP1L1      | UDP-N-acetylglucosamine pyrophosphorylase 1 like 1 | 3.03        | 2.00E-04   | 2.15E-02    |
| NPC1        | Niemann-Pick disease, type C1             | 3.01        | 1.90E-05   | 7.90E-03    |
| GAB2        | GRB2-associated b.p 2                     | 2.98        | 1.20E-05   | 6.70E-03    |
| JUNB        | Jun B proto-oncogene                      | 2.98        | 6.79E-05   | 1.39E-02    |
| DSC2        | Desmocollin 2                             | 2.97        | 8.50E-06   | 5.90E-03    |
| MXD1        | MAX dimerization protein 1                | 2.96        | 5.60E-06   | 5.20E-03    |

Table 1. Continued
Table I. Continued

| Gene symbol | Description                                      | Fold change | p-Value    | FDR p-Value |
|-------------|-------------------------------------------------|-------------|------------|-------------|
| GABARAPL1   | GABA(A) receptor-associated protein like 1      | 2.95        | 1.39E-05   | 7.20E-03    |
| FRZB        | Frizzled-related protein                        | 2.93        | 1.38E-06   | 2.80E-03    |
| TP53NIP1    | Tumor protein p53 inducible nuclear protein 1   | 2.91        | 3.34E-07   | 1.50E-03    |
| RAGC        | Ras-related GTP binding C                      | 2.89        | 2.07E-06   | 3.00E-03    |
| GRB10       | Growth factor receptor bound protein 10        | 2.87        | 1.74E-05   | 7.60E-03    |
| KDM6B       | Lysine (K)-specific demethylase 6B              | 2.87        | 1.00E-04   | 1.90E-02    |
| JUND        | Jun D proto-oncogene                            | 2.84        | 2.87E-05   | 9.70E-03    |
| LSS         | Lanosterol synthase                             | 2.84        | 2.00E-04   | 2.19E-02    |
| TCP11L2     | T-complex 11, testis-specific-like 2            | 2.82        | 1.75E-06   | 2.80E-03    |
| HBP1        | HMG-box transcription factor 1                 | 2.80        | 6.98E-05   | 1.40E-02    |
| ABL2        | ABL proto-oncogene 2, non-receptor tyrosine kinase | 2.75    | 2.28E-05   | 8.50E-03    |
| FLCN; PLD6  | Golliculin; phospholipase D fam., mem. 6        | 2.75        | 4.37E-05   | 1.17E-02    |
| BACH1       | BTB and CNC holomorphy 1, BLZTF 1               | 2.73        | 6.29E-05   | 1.33E-02    |
| KLF6        | Kruppel-like factor 6                           | 2.73        | 2.10E-05   | 8.10E-03    |
| DENND2C     | DENN/MADD d.c. 2C                               | 2.72        | 6.70E-06   | 5.70E-03    |
| TRAF1       | TNF receptor-associated factor 1                | 2.71        | 1.00E-04   | 2.03E-02    |
| MSMO1       | Methylsterol monoxygenase 1                    | 2.70        | 1.56E-06   | 2.80E-03    |
| PGM2L1      | Phosphoglucosamutase 2-like 1                  | 2.70        | 2.00E-04   | 2.68E-02    |
| C3orf52     | Chromosome 3 open reading frame 52             | 2.69        | 2.00E-04   | 2.23E-02    |
| LIPPL2      | Lipoma HMGIC fusion partner-like 2             | 2.69        | 7.62E-06   | 5.70E-03    |
| CXCL2       | Chemokine (C-X-C motif) ligand 2               | 2.68        | 2.00E-04   | 2.75E-02    |
| FNIP1       | Follucin interacting protein 1                 | 2.68        | 7.67E-06   | 5.70E-03    |
| TLR8AS1     | TLR8 antisense RNA 1                            | 2.67        | 1.00E-04   | 1.86E-02    |
| PLIN2       | Perilipin 2                                    | 2.61        | 5.37E-06   | 5.10E-03    |
| SMOX        | Spermine oxidase                               | 2.61        | 4.00E-04   | 3.36E-02    |
| ZFYVE1      | Zinc finger, FYVE d.c. 1                       | 2.59        | 6.51E-05   | 1.36E-02    |
| FTH1        | Ferritin, heavy polypeptide 1                  | 2.58        | 7.07E-06   | 5.70E-03    |
| USP53       | Ubiquitin specific peptide 53                  | 2.58        | 7.49E-06   | 5.70E-03    |
| TRPV3       | Transient receptor potential cation channel, sub-V, mem. 3 | 2.57    | 2.00E-04   | 2.09E-02    |
| SLC43A2     | Solute carrier fam. 43 (amino acid), mem. 2     | 2.56        | 3.00E-04   | 3.35E-02    |
| DDT3        | DNA-damage-inducible transcript 3              | 2.54        | 4.22E-06   | 4.60E-03    |
| SOD2        | Superoxide dismutase 2, mitochondial            | 2.53        | 5.88E-06   | 5.30E-03    |
| PIM1        | Pim-1 proto-oncogene, serine/threonine kinase  | 2.52        | 2.60E-05   | 9.30E-03    |
| CD83        | CD83 molecule                                  | 2.51        | 4.00E-04   | 3.93E-02    |
| HSPA1A; HSPA1B | Heat shock 70kDa protein 1A; 1B                | 2.50        | 1.85E-05   | 7.90E-03    |
| CRYM-AS1    | CRYM antisense RNA 1                           | 2.49        | 1.38E-05   | 7.00E-03    |
| DUSP8       | Dual specificity phosphatase 8                 | 2.49        | 7.99E-05   | 1.48E-02    |
| SPDYA       | Speedy/RINGO cell cycle regulator fam. mem. A  | 2.47        | 2.45E-05   | 8.90E-03    |
| KIAA1551    | KIAA1551                                      | 2.46        | 3.67E-05   | 1.07E-02    |
| ERN1        | Endoplasmic reticulum to nucleus signaling 1   | 2.45        | 7.36E-06   | 1.42E-02    |
| NPPA-AS1    | NPPA antisense RNA 1                           | 2.45        | 3.12E-05   | 1.00E-02    |
| PNR1C1      | Proline-rich nuclear receptor coactivator 1    | 2.44        | 1.00E-04   | 1.81E-02    |
| LOC101929125 | Uncharacterized LOC101929125              | 2.44        | 4.32E-06   | 4.60E-03    |
| CSF1        | Colony stimulating factor 1 (macrophage)       | 2.41        | 1.00E-04   | 1.86E-02    |
| ZC3H12C     | Zinc finger CCC-type containing 12C            | 2.41        | 3.00E-04   | 2.95E-02    |
| ITGB3       | Integrin beta 3                                | 2.40        | 4.23E-05   | 1.16E-02    |
| NOV         | Nephroblastoma overexpressed                   | 2.40        | 2.00E-04   | 2.09E-02    |
| TBC1D7      | TBC1 domain fam., mem. 7                      | 2.40        | 2.70E-05   | 9.40E-03    |
| HMGCRC      | 3-hydroxy-3-methylglutaryl-CoA reductase       | 2.39        | 2.66E-06   | 3.50E-03    |
| IL4R        | Interleukin 6 receptor                         | 2.39        | 1.00E-04   | 1.81E-02    |
| MMP1        | Matrix metalloproteinase 1                    | 2.38        | 5.01E-06   | 5.00E-03    |
| HSPA1B; HSPA1A | Heat shock 70kDa protein 1B; 1A              | 2.37        | 9.02E-05   | 1.60E-02    |
| JUN         | Jun proto-oncogene                             | 2.37        | 2.25E-05   | 8.50E-03    |
| GAREM1      | GRB2 associated regulator of MAPK1 1          | 2.34        | 9.89E-05   | 1.68E-02    |
| MIR616; DDT3 | microRNA 616; DNA-damage-inducible transcript 3 | 2.33    | 4.72E-05   | 1.22E-02    |
| PER1        | period circadian clock 1                      | 2.33        | 2.00E-04   | 2.61E-02    |
| NEU1        | Sialidase 1 (lysosomal sialidase)              | 2.33        | 7.43E-05   | 1.42E-02    |
| DHRS9       | dehydrogenase/reductase (SDR fam.) mem. 9     | 2.32        | 3.52E-05   | 1.05E-02    |
| Gene symbol | Description | Fold change | p-Value | FDR p-Value |
|-------------|-------------|-------------|---------|-------------|
| LRRC37B     | Leucine rich repeat containing 37B | 2.32 | 4.00E-04 | 3.74E-02 |
| ATP6V0D2    | ATPase, H+ tr, lysosomal 38kDa, V0 su. d2 | 2.31 | 3.49E-05 | 1.05E-02 |
| FOXC1       | Forkhead box C1 | 2.31 | 2.00E-04 | 2.49E-02 |
| SQLE        | Squalene epoxidase | 2.30 | 5.31E-05 | 1.28E-02 |
| JARID2      | Jumonji, AT rich interactive domain 2 | 2.29 | 2.00E-04 | 2.27E-02 |
| UGDH        | UDP-glucose 6-dehydrogenase | 2.29 | 1.25E-05 | 6.80E-03 |
| LOC105379177| Uncharacterized LOC105379177 | 2.29 | 6.29E-05 | 1.33E-02 |
| LDLR        | Low density lipoprotein receptor | 2.28 | 6.00E-05 | 1.32E-02 |
| NFKBIA      | NFK light polypeptide gene enh B-cells inhibitor, alpha | 2.28 | 4.29E-05 | 1.16E-02 |
| AHNK2       | AHNK nucleoprotein 2 | 2.27 | 2.00E-04 | 2.68E-02 |
| CEBPB       | CCAAT/enhancer b.p (C/EBP), beta | 2.27 | 8.36E-05 | 1.53E-02 |
| DTNA        | Dystrobrevin, alpha | 2.26 | 5.00E-04 | 4.10E-02 |
| SPAG9       | Sperm associated antigen 9 | 2.26 | 2.06E-05 | 8.00E-03 |
| CYTH1       | Cytohesin 1 | 2.25 | 2.00E-04 | 2.34E-02 |
| FAM102A     | Fam. with sequence similarity 102, mem. A | 2.25 | 1.00E-04 | 1.74E-02 |
| RNF19B      | Ring finger protein 19B | 2.25 | 4.00E-04 | 3.72E-02 |
| ZBTB43      | Zinc finger and BTB d.c. 43 | 2.25 | 5.62E-05 | 1.30E-02 |
| IDH1        | Isopentenyl-diphosphate delta isomerase 1 | 2.24 | 8.46E-06 | 9.09E-03 |
| CHD2        | Chromodomain helicase DNA b.p 2 | 2.23 | 6.97E-05 | 1.40E-02 |
| NDRG1       | N-myc downstream regulated 1 | 2.23 | 3.94E-05 | 1.13E-02 |
| BHLHE40     | Basic helix-loop-helix fam., mem. e40 | 2.22 | 3.29E-05 | 1.04E-02 |
| IER3        | Immediate early response 3 | 2.21 | 1.00E-04 | 1.70E-02 |
| LIF         | Leukemia inhibitory factor | 2.21 | 1.00E-04 | 1.80E-02 |
| ZNF165      | Zinc finger protein 165 | 2.21 | 2.00E-04 | 2.14E-02 |
| BTG1; LINC01619 | B-cell translocation gene 1, LINE RNA 1619 | 2.20 | 6.00E-04 | 4.57E-02 |
| PMP2        | Peripheral myelin protein 22 | 2.19 | 1.65E-05 | 7.50E-03 |
| THEMIS2     | Thymocyte selection associated fam. mem. 2 | 2.19 | 8.30E-05 | 1.53E-02 |
| CD55        | CD55 molecule, decay accelerating factor | 2.17 | 1.51E-05 | 7.40E-03 |
| DUSP16      | Dual specificity phosphatase 16 | 2.17 | 1.00E-04 | 1.70E-02 |
| SOX4        | SRY box 4 | 2.17 | 2.00E-04 | 2.39E-02 |
| LOC105369949| Uncharacterized LOC105369949 | 2.17 | 4.00E-04 | 3.87E-02 |
| LOC105379695| Uncharacterized LOC105379695 | 2.17 | 6.02E-05 | 1.32E-02 |
| FZD8; MIR4683| Frizzled class receptor 8; MicroRNA 4683 | 2.16 | 4.00E-04 | 3.74E-02 |
| CLDN4       | Claudin 4 | 2.14 | 4.00E-04 | 3.64E-02 |
| LINC-PINT   | Long intergenic non-protein coding RNA, p53 | 2.13 | 2.00E-04 | 2.17E-02 |
| IRS2        | Insulin receptor substrate 2 | 2.12 | 2.00E-04 | 2.34E-02 |
| MCOLN3      | Mucolipin 3 | 2.12 | 5.50E-05 | 1.29E-02 |
| STAROD      | StAR-related lipid transfer d.c. 4 | 2.12 | 4.09E-05 | 1.16E-02 |
| CTSL        | Cathepsin L | 2.11 | 1.00E-04 | 1.89E-02 |
| RHOF        | Ras homolog fam. mem. F (in filopodia) | 2.11 | 6.00E-04 | 4.93E-02 |
| TIPARP      | TCDD-inducible poly(ADP-ribose) polymerase | 2.11 | 2.00E-04 | 2.17E-02 |
| STX3; OR10Y1P| Syntaxin 3; olfactory receptor, Fam. 10, subfam. Y, mem. 1p | 2.10 | 2.00E-04 | 2.43E-02 |
| PEL1I       | Pellino E3 ubiquitin protein ligase 1 | 2.08 | 5.16E-05 | 1.27E-02 |
| YOD1        | YOD1 deubiquitinating | 2.08 | 3.00E-04 | 3.16E-02 |
| CYP51A1; LRRD1 | Cytochrome P450, 15f1, subfam. A, 1; LRR and DDC 1 | 2.07 | 3.16E-05 | 1.00E-02 |
| IRF9        | Interferon regulatory factor 9 | 2.07 | 4.00E-04 | 3.43E-02 |
| AGPAT4      | 1-acetylglcerol-3-phosphate O-acyltransferase 4 | 2.05 | 2.34E-05 | 8.60E-03 |
| HDAC9       | Histone deacetylase 9 | 2.05 | 3.00E-04 | 2.97E-02 |
| ZCCHC14     | Zinc finger, CCHC d.c. 14 | 2.05 | 6.00E-04 | 4.95E-02 |
| JMD1C       | Jumonji d.c. 1C | 2.02 | 6.00E-04 | 4.96E-02 |
| SQSTM1      | Sequestosome 1 | 2.02 | 1.00E-04 | 1.89E-02 |
| KLF3        | Kruppel-like factor 3 (basic) | 2.01 | 3.00E-05 | 9.80E-03 |
| TFPI2       | Tissue factor pathway inhibitor 2 | 2.01 | 9.34E-05 | 1.64E-02 |
| AJUBA       | Ajuba LIM protein | -2.00 | 5.00E-04 | 3.97E-02 |
| KNTC1       | Kinetochore associated 1 | -2.00 | 1.00E-04 | 1.70E-02 |
| POLR3B      | Polymerase (RNA) III (DNA directed) polypeptide B | -2.00 | 2.00E-04 | 2.25E-02 |
| SASS6       | SAS-6 centriolar assembly protein | -2.00 | 1.00E-04 | 1.95E-02 |

Table 1. Continued
Table I. Continued

| Gene symbol | Description                                                                 | Fold change | p-Value       | FDR p-Value |
|-------------|------------------------------------------------------------------------------|-------------|---------------|-------------|
| HMGB2       | High mobility group box 2                                                     | -2.01       | 3.00E-04      | 3.26E-02    |
| BIRC5       | Birculoviral IAP repeat containing 5                                         | -2.02       | 7.84E-05      | 1.46E-02    |
| GLMN        | Glomulin, FKBP associated protein                                            | -2.02       | 3.00E-04      | 2.94E-02    |
| HIST1H2AJ   | Histone cluster 1, H2aj                                                      | -2.02       | 3.00E-04      | 2.87E-02    |
| ZNF738      | Zinc finger protein 738                                                      | -2.02       | 5.76E-05      | 1.31E-02    |
| CHRNA5      | Cholinergic receptor, nicotinic alpha 5                                      | -2.03       | 4.47E-05      | 1.18E-02    |
| NCAPD3      | Non-SMC condensin II complex su. D3                                          | -2.03       | 7.60E-05      | 1.43E-02    |
| RMI1        | RecQ mediated genome instability 1                                           | -2.03       | 4.00E-04      | 3.93E-02    |
| CDCA7L      | Cell division cycle associated 7-like                                         | -2.04       | 2.00E-04      | 2.17E-02    |
| FAM111A     | Fam. with sequence similarity 111, mem. A                                    | -2.04       | 9.96E-05      | 1.68E-02    |
| FANCB       | Fanconi anemia complementation group B                                        | -2.04       | 2.00E-04      | 2.44E-02    |
| MYBL1       | V-myb avian myeloblastosis viral oncogene homolog-like 1                     | -2.04       | 1.62E-05      | 7.50E-03    |
| CEP192      | Centrosomal protein 192kDa                                                   | -2.06       | 5.00E-04      | 4.23E-02    |
| CCNF        | Cyclin F                                                                    | -2.07       | 3.44E-05      | 1.05E-02    |
| STIL        | SCL/TAL1 interrupted locust                                                  | -2.07       | 2.00E-04      | 2.27E-02    |
| DHRF        | Dihydrofolate reductase                                                      | -2.08       | 2.00E-04      | 2.34E-02    |
| E2F8        | E2F transcription factor 8                                                   | -2.08       | 9.40E-06      | 6.00E-03    |
| RAD54B; FSBP| RAD54 homolog B, fibrinogen Silencer b.p                                   | -2.08       | 2.00E-04      | 2.34E-02    |
| SPRY1       | Sprouty RTK signaling antagonist 1                                           | -2.08       | 4.00E-04      | 3.62E-02    |
| TPX2        | TPX2, microtubule-associated                                                  | -2.08       | 5.58E-05      | 1.30E-02    |
| DSN1        | DSN1 homolog, MIS12 kinetochore complex component                            | -2.09       | 3.00E-04      | 2.87E-02    |
| CHAC2       | ChaC, cation transport regulator homolog 2 (E. coli)                         | -2.10       | 3.44E-05      | 1.05E-02    |
| PLK1        | Polo-like kinase 1                                                           | -2.10       | 4.13E-05      | 1.16E-02    |
| PLK2        | Polo-like kinase 2                                                           | -2.10       | 1.60E-05      | 7.50E-03    |
| CASC5       | Cancer susceptibility candidate 5                                            | -2.11       | 2.00E-04      | 2.56E-02    |
| CEP55       | Centrosomal protein 55kDa                                                    | -2.11       | 1.00E-04      | 1.93E-02    |
| PRIMPOL     | Primase and DNA directed polymerase                                          | -2.11       | 5.00E-04      | 4.01E-02    |
| YEATS4      | YEATS d.c. 4                                                                 | -2.11       | 6.21E-05      | 1.33E-02    |
| HSPA14      | Heat shock 70kDa protein 12                                                  | -2.12       | 9.58E-05      | 1.66E-02    |
| PRC1        | Protein regulator of cytokinesis 1                                           | -2.12       | 8.98E-05      | 1.60E-02    |
| CENPO       | Centromere protein Q                                                         | -2.13       | 2.00E-04      | 2.49E-02    |
| MTBP        | MDM2 b.p                                                                    | -2.13       | 2.00E-04      | 2.16E-02    |
| RFC5        | Replication factor C su. 5                                                   | -2.13       | 8.72E-05      | 1.56E-02    |
| SLCO4A1     | Solute carrier organic anion transporter fam., mem. 4A1                      | -2.13       | 1.00E-04      | 2.02E-02    |
| SKA1        | Spindle and kinetochore associated complex su. 1                             | -2.13       | 2.00E-04      | 2.55E-02    |
| TICRR       | TOPBP1-interacting checkpoint and replication regulator                      | -2.13       | 1.00E-04      | 1.70E-02    |
| EZH2        | Enhancer of zeste 2 polycomb repressive complex 2 su.                        | -2.14       | 6.16E-05      | 1.33E-02    |
| RAD51       | RAD51 recombinase                                                            | -2.14       | 2.19E-05      | 8.30E-03    |
| STAMBPL1    | STAM b.p.1                                                                   | -2.14       | 4.40E-05      | 1.17E-02    |
| WDHD1       | WD repeat and HMG-box DNA b.p 1                                              | -2.14       | 2.00E-04      | 2.49E-02    |
| ZNF93       | Zinc finger protein 93                                                       | -2.14       | 1.00E-04      | 1.70E-02    |
| HAUS8       | HAUS augmin like complex su. 8                                               | -2.15       | 9.87E-05      | 1.68E-02    |
| RBBP8       | Retinoblastoma b.p.                                                          | -2.15       | 5.02E-05      | 1.25E-02    |
| RNU6-57P    | RNA, U6 small nuclear 57, pseudogene                                         | -2.15       | 4.00E-04      | 3.80E-02    |
| ANLN        | Anillin actin b.p                                                            | -2.16       | 8.51E-05      | 1.55E-02    |
| FIGN        | Fidgetin                                                                    | -2.16       | 2.00E-04      | 2.14E-02    |
| GSG2        | Germ cell associated 2 (haspin)                                              | -2.16       | 1.00E-04      | 1.89E-02    |
| HIST1H3B    | Histone cluster 1, H3b                                                       | -2.16       | 4.24E-05      | 1.16E-02    |
| CDC25A      | Cell division cycle 25A                                                     | -2.18       | 3.00E-04      | 3.16E-02    |
| CCNB2       | Cyclin B2                                                                   | -2.18       | 2.00E-04      | 2.48E-02    |
| G2E3        | G2/M-phase specific E3 ubiquitin protein ligase                              | -2.18       | 6.00E-04      | 4.94E-02    |
| KIF14       | Kinesin fam. mem. 14                                                         | -2.18       | 2.00E-04      | 2.56E-02    |
| TEMPO       | Thymopoietin                                                                | -2.19       | 4.43E-05      | 1.17E-02    |
| PAK6; BUB1B | p21 protein (Cdc42/Rac)-Activated kinase 6, BUB1                             | -2.19       | 4.23E-05      | 1.16E-02    |
| RFC3        | Replication factor C su. 3                                                   | -2.19       | 1.70E-05      | 7.50E-03    |
| CCNE2       | Cyclin E2                                                                    | -2.20       | 7.59E-06      | 5.70E-03    |
| PSIP1       | PC4 and SFRS1 interacting protein 1                                          | -2.20       | 6.44E-05      | 1.36E-02    |

Table I. Continued
| Gene symbol | Description                                      | Fold change | p-Value  | FDR p-Value |
|-------------|--------------------------------------------------|-------------|----------|-------------|
| RBL1        | Retinoblastoma-like 1                            | -2.20       | 2.00E-04 | 2.15E-02    |
| SMC4        | Structural maintenance of chromosomes 4          | -2.20       | 2.14E-05 | 8.20E-03    |
| XRCC2       | X-ray repair cdc in Chinese hamster cells 2      | -2.20       | 9.50E-05 | 1.65E-02    |
| FANCI       | Fanconi anemia complementation group I           | -2.21       | 1.00E-04 | 1.80E-02    |
| FAM216A     | Fam. with sequence similarity 21f, mem. A        | -2.22       | 3.00E-04 | 3.26E-02    |
| HELLS       | Helicase, lymphoid-specific                      | -2.22       | 2.99E-05 | 9.80E-03    |
| NEK2        | NIMA-related kinase 2                            | -2.22       | 3.00E-04 | 3.29E-02    |
| NACPG       | Non-SMC condensin I complex su. G               | -2.22       | 5.94E-05 | 1.32E-02    |
| NCPH        | Non-SMC condensin I complex su. H               | -2.22       | 1.45E-05 | 7.40E-03    |
| SDPR        | Serum deprivation response                      |             | 1.00E-04 | 1.87E-02    |
| UBE2C       | Ubiquitin-conjugating enzyme E2C                 | -2.22       | 2.00E-04 | 2.05E-02    |
| LOC100507516| Uncharacterized LOC100507516                    | -2.22       | 2.00E-04 | 2.44E-02    |
| CIT; MIR1178| Citron rho-interacting Ser/thrkinase; microRNA 1178| -2.23       | 2.78E-05 | 9.70E-03    |
| CENPI       | Centromere protein 1                             | -2.24       | 4.00E-04 | 3.62E-02    |
| GINS1       | GINS complex su. 1 (Psf1 homolog)               | -2.24       | 1.91E-05 | 7.90E-03    |
| GINS4       | GINS complex su. 4 (Sld5 homolog)               | -2.25       | 5.88E-06 | 5.30E-03    |
| KIF20B      | Kinesin fam. mem. 20B                           | -2.25       | 3.00E-04 | 3.08E-02    |
| HIST2H2AB   | Histone cluster 2, H2ab                         | -2.26       | 1.27E-05 | 6.90E-03    |
| SLFN13      | Schlafen fam. mem. 13                           | -2.26       | 3.54E-05 | 1.05E-02    |
| UBE2T       | Ubiquitin-conjugating enzyme E2T                 | -2.26       | 2.40E-06 | 3.20E-03    |
| PCNA-AS1    | PCNA antisense RNA 1                             | -2.27       | 7.19E-05 | 1.42E-02    |
| TIMELESS    | Timeless circadian clock                        | -2.27       | 6.12E-05 | 1.33E-02    |
| MMS22L      | MMS22-like, DNA repair protein                   | -2.29       | 2.94E-05 | 9.70E-03    |
| PARPB       | PARP1 b.p.                                       | -2.29       | 3.00E-04 | 3.03E-02    |
| C1orf54     | Chromosome 18 open reading frame 54             | -2.30       | 9.02E-05 | 1.60E-02    |
| CCDC138     | Coiled-coil d. 138                              | -2.30       | 6.00E-04 | 4.55E-02    |
| MKI67       | Marker of proliferation Ki-67                   | -2.30       | 1.14E-05 | 6.50E-03    |
| VRK1; LINC00618| Vaccinia related kinase 1; LIN RNA 618      | -2.30       | 1.00E-04 | 1.81E-02    |
| ZNF215      | Zinc finger protein 215                          | -2.31       | 2.63E-05 | 9.30E-03    |
| GMNN        | Geminin, DNA replication inhibitor               | -2.31       | 5.80E-05 | 1.31E-02    |
| NUSAP1      | Nucleolar and spindle associated protein 1       | -2.31       | 3.97E-05 | 1.13E-02    |
| CCNB1       | Cyclin B1                                        | -2.32       | 5.24E-05 | 1.27E-02    |
| RAD54L      | RAD54-like (S. cerevisiae)                      | -2.32       | 5.00E-04 | 4.16E-02    |
| ARHGAP11A   | Rho GTPase activating protein 1A                 | -2.32       | 3.16E-05 | 1.00E-02    |
| TMPO-AS1    | TMPO antisense RNA 1                             | -2.32       | 5.44E-06 | 5.10E-03    |
| ASPM        | Aabnormal spindle microtubule assembly           | -2.33       | 2.00E-04 | 2.17E-02    |
| ATAD2       | ATPase fam., AAA d. c. 2                        | -2.34       | 7.06E-05 | 1.41E-02    |
| FOXM1       | Forkhead box M1                                 | -2.34       | 8.37E-05 | 1.53E-02    |
| FIGNL1      | Fidgetin-like 1                                 | -2.35       | 3.79E-05 | 1.09E-02    |
| LOC105376603| Uncharacterized LOC105376603                    | -2.35       | 6.61E-05 | 1.37E-02    |
| Ctorf112    | Chromosome 1 open reading frame 112             | -2.36       | 2.91E-05 | 9.70E-03    |
| KIF18B      | Kinesin fam. mem. 18B                           | -2.36       | 3.59E-05 | 1.05E-02    |
| BLM         | Bloom syndrome, RecQ helicase-like               | -2.37       | 4.97E-05 | 1.25E-02    |
| KIF4A       | Kinesin fam. mem. 4A                            | -2.39       | 5.00E-04 | 4.24E-02    |
| MND1        | Meiotic nuclear divisions 1                      | -2.39       | 1.30E-05 | 6.90E-03    |
| NUF2        | NUF2, NDC80 kinetochore complex component        | -2.39       | 5.00E-04 | 4.01E-02    |
| C5orf34     | Chromosome 5 open reading frame 34              | -2.40       | 5.60E-05 | 1.30E-02    |
| CDRN3       | cyclin-dependent kinase inhibitor               | -2.41       | 9.46E-05 | 1.65E-02    |
| FANCD2      | Fanconi anemia complementation group D2         | -2.41       | 7.18E-05 | 1.42E-02    |
| PLK4        | Polo-like kinase 4                               | -2.41       | 2.00E-04 | 2.17E-02    |
| BARD1       | BRCA1 associated RING domain 1                   | -2.44       | 1.44E-05 | 7.40E-03    |
| CENPE       | Centromere protein E                            | -2.44       | 4.00E-04 | 3.42E-02    |
| MAD2L1      | MAD2 mitotic arrest deficient-like 1 (yeast)     | -2.44       | 2.00E-04 | 2.17E-02    |
| SHCBP1      | SHC SH2-domain b.p 1                            | -2.45       | 1.10E-05 | 6.50E-03    |
| WDR76       | WD repeat domain 76                             | -2.45       | 1.00E-04 | 1.83E-02    |
| FBXO43      | F-box protein 43                                 | -2.46       | 4.96E-05 | 1.25E-02    |
| CDC6        | Cell division cycle 6                           | -2.47       | 2.00E-04 | 2.61E-02    |
| ESPL1       | Extra spindle pole bodies like 1, separate       | -2.47       | 5.09E-05 | 1.26E-02    |

Table I. Continued
| Gene symbol | Description                                                                 | Fold change | p-Value   | FDR p-Value |
|-------------|-----------------------------------------------------------------------------|-------------|-----------|-------------|
| DEPDC1B     | DEP d.c. 1B                                                                   | -2.48       | 1.00E-04  | 2.00E-02    |
| HMMR        | Hyaluronan-mediated motility receptor (RHAMM)                                 | -2.48       | 3.00E-04  | 3.09E-02    |
| KIF1C       | Kinesin fam. mem. C1                                                          | -2.48       | 3.34E-05  | 1.04E-02    |
| RAD51AP1    | RAD51 associated protein 1                                                    | -2.48       | 1.00E-04  | 1.69E-02    |
| SPC24       | SPC24, NDC80 kinetochore complex component                                    | -2.48       | 7.12E-06  | 5.70E-03    |
| CENPU       | Centromere protein U                                                         | -2.49       | 1.50E-05  | 7.40E-03    |
| KIF11       | Kinesin fam. mem. 11                                                          | -2.49       | 3.63E-05  | 1.06E-02    |
| NEIL3       | Nei-like DNA glycosylase 3                                                   | -2.49       | 8.28E-06  | 5.90E-03    |
| FBXO5       | F-box protein 5                                                               | -2.50       | 2.00E-04  | 2.48E-02    |
| KIF2C       | Kinesin fam. mem. 2C                                                          | -2.50       | 1.06E-04  | 6.30E-03    |
| CEP295      | Centrosomal protein 295kDa                                                   | -2.51       | 1.00E-04  | 1.81E-02    |
| TOP2A       | Topoisomerase (DNA) II alpha                                                  | -2.51       | 6.59E-05  | 1.37E-02    |
| SUV39H2     | Suppressor of variegation 3-9 homolog 2 (Drosophila)                         | -2.52       | 2.00E-04  | 2.37E-02    |
| ARHGAP11B   | Rho GTPase activating protein 11B                                             | -2.54       | 1.05E-05  | 6.30E-03    |
| DLGAP5      | Discs, large (Drosophila) homolog-associated protein 5                       | -2.55       | 1.00E-04  | 1.89E-02    |
| BRC2        | High mobility group nucleosomal binding domain 2                             | -2.55       | 6.66E-05  | 7.18E-02    |
| ZGRF1       | Zinc finger, GRF-type containing 1                                            | -2.55       | 8.46E-05  | 1.56E-02    |
| TRIP13      | Thyroid hormone receptor interactor 13                                       | -2.57       | 1.00E-04  | 1.93E-02    |
| BRIP1       | BRC1 interacting protein C-terminal helicase 1                               | -2.59       | 1.67E-05  | 7.50E-03    |
| BUB1        | BUB1 mitotic checkpoint serine/threonine kinase                              | -2.59       | 2.01E-05  | 8.00E-03    |
| ESCO2       | Establishment of sister chromatid c.N-acetyltransferase 2                    | -2.61       | 1.93E-06  | 3.00E-03    |
| GEN1        | GEN1 Holliday junction 5 flap endonuclease                                    | -2.61       | 6.76E-05  | 1.39E-02    |
| SPCC5       | SPCC5, NDC80 kinetochore complex component                                    | -2.61       | 9.19E-06  | 6.00E-03    |
| TACC3       | Transforming, acidic coiled-coil containing protein 3                         | -2.61       | 4.29E-06  | 4.60E-03    |
| DEPDC1      | DEP d.c. 1                                                                    | -2.62       | 3.00E-04  | 2.99E-02    |
| HJURP       | Holliday junction recognition protein                                         | -2.63       | 3.58E-06  | 4.40E-03    |
| EXO1        | Exonuclease 1                                                                 | -2.65       | 1.92E-05  | 7.90E-03    |
| KIF18A      | Kinesin fam. mem. 18A                                                         | -2.65       | 8.64E-05  | 1.56E-02    |
| KIAA1524    | KIAA1524                                                                     | -2.67       | 2.00E-04  | 2.65E-02    |
| LMNB1       | Lamin B1                                                                      | -2.67       | 2.37E-05  | 8.60E-03    |
| CDK1        | Cyclin-dependent kinase 1                                                     | -2.68       | 7.24E-05  | 1.42E-02    |
| SERTA4      | SERTA d.c. 4                                                                  | -2.68       | 4.00E-04  | 3.72E-02    |
| MCM10       | MCM 10 replication initiation factor                                         | -2.70       | 1.66E-05  | 7.50E-03    |
| KIF15       | Kinesin fam. mem. 15                                                          | -2.71       | 9.35E-06  | 6.00E-03    |
| KIF20A      | Kinesin fam. mem. 20A                                                         | -2.73       | 1.81E-05  | 7.80E-03    |
| POLE2       | Polymerase (DNA directed), epsilon 2, accessory su.                          | -2.75       | 9.01E-07  | 2.30E-03    |
| OIP5        | Opa interacting protein 5                                                     | -2.77       | 2.00E-05  | 8.00E-03    |
| SKA3        | Spindle and kinetochore Associated complex su. 3                             | -2.77       | 9.33E-06  | 6.00E-03    |
| CDCA7       | Cell division cycle associated 7                                             | -2.78       | 6.94E-06  | 5.70E-03    |
| DNA2        | DNA replication Helicase/nuclease 2                                           | -2.79       | 1.71E-05  | 7.50E-03    |
| NDC80       | NDC80 kinetochore complex component                                          | -2.82       | 1.00E-04  | 1.73E-02    |
| CENPK       | Centromere protein K                                                          | -2.83       | 1.48E-05  | 7.40E-03    |
| CDC7        | Cell division cycle 7                                                         | -2.86       | 1.61E-06  | 2.80E-03    |
| ATAD5       | ATPase fam., AAA d.c. 5                                                       | -2.87       | 1.15E-05  | 6.50E-03    |
| FANCM       | Fanconi anemia complementation group M                                        | -2.88       | 1.23E-05  | 6.80E-03    |
| FAM111B     | Fam. with sequence similarity 111, mem. B                                     | -2.89       | 1.68E-06  | 2.80E-03    |
| PBK         | PDZ binding kinase                                                            | -2.89       | 4.74E-05  | 1.22E-02    |
| CCNA2       | Cyclin A2                                                                    | -2.91       | 5.24E-06  | 5.10E-03    |
| SGOL1-AS1   | SGOL1 antisense RNA 1                                                         | -2.92       | 1.00E-04  | 1.80E-02    |
| BRCA2       | Breast cancer, early onset                                                   | -2.94       | 6.66E-05  | 1.37E-02    |
| CDCA8       | Cell division cycle associated 8                                             | -3.00       | 8.82E-06  | 6.00E-03    |
| PRM1        | Primase, DNA, polypeptide 1 (49kDa)                                           | -3.01       | 6.46E-07  | 2.10E-03    |
| CDCA8       | Cell division cycle associated 8                                             | -3.06       | 1.63E-06  | 2.80E-03    |
| GXP8        | Glutathione peroxidase 8 (putative)                                          | -3.17       | 6.00E-04  | 4.93E-02    |
| FAM83D      | Fam. with sequence similarity 83, mem. D                                     | -3.20       | 2.77E-06  | 3.50E-03    |
| POLQ        | Polymerase (DNA directed), theta                                              | -3.30       | 1.00E-04  | 1.70E-02    |
| BORA        | Bora, aurora kinase A activator                                              | -3.53       | 4.12E-05  | 1.16E-02    |
| DCC1        | DNA replication and sister chromatid cohesion 1                              | -3.60       | 2.02E-06  | 3.00E-03    |
| SGOL1       | Shugoshin-like 1 (S. pombe)                                                   | -3.61       | 1.29E-05  | 6.90E-03    |
| MIR924      | MicroRNA 924                                                                  | -3.68       | 2.00E-04  | 2.17E-02    |
Figure 5. Stringdb relational network analysis of down-regulated DEGS caused by WYE (Low) 15 μg/ml vs. untreated controls after meeting selection criteria: -2<x>+2 fold change, p-Value <0.05 and FDR p-Value <0.05. The data in the corresponding table: represent database used, ID #s within a database, description of system altered, count in the network for system, strength of relationship and the false discovery rate (FDR) p-Value. Color codes indicate genes (by symbol) in the string diagram corresponding to the table class description.
To our knowledge, this is the first scientific report describing the pro-inflammatory immune-stimulating properties of WYE in a cancer cell line, amongst what appears to be an absence in the literature of similar type studies. Meanwhile, for over 60 years, the research literature has been well established for describing the effects of low-dose saponins as immune-stimulating vaccine adjuvants, which carry out the sole purpose of stimulating a strong, long-lasting learned immune response to administered vaccine antigens. Saponin adjuvants bear a similar molecular similarity to known saponins in WYE, some of which include Quil A, QS-21 from *Quillaja*, and saponins in the *Momordica* (35), *Glycyrrhiza* (36), and the *Dioscorea* botanical species itself (37). Botanically derived saponin adjuvants boost the immune system by increasing the Th1/Th2 cell-mediated response, antibody production, targeted cytotoxic T-CD4+ cell response with concurrent stimulation of a variety of cytokines [IL-2, IL-10, IL-12 (p70) and IFN-γ] (20, 38-44). Yet, interestingly, most of all of the studies on saponin adjuvants have been carried out only in the presence of the antigenic substance containing the vaccine. To add to this gap in the literature, only a few studies have explored the immune-stimulating properties of saponin-rich herbs, which describe an effect on the innate immune response by crude extracts or compounds within *Dioscorea* species. These changes include capacity to stimulate phagocytosis in macrophages, enhance natural killer (NK) cell activity, activate Toll-like receptor 4 (TLR4) and activate downstream

Figure 6. Cell cycle Wikipathway analysis report for DEGS caused by WYE (Low) 15 μg/ml vs. untreated controls after meeting selection criteria; -2<Δ>+2, p-Value <0.05 and FDR p-Value <0.05. DEGS are color coded (red=down), (green=up) with color intensity darker with fold higher fold change. Gene symbol denoted by cross bars /// were not altered.
Figure 7. Stringdb relational network analysis of up-regulated DEGS caused by WYE (Low) 15 μg/mL vs. untreated controls after meeting selection criteria: -2<x>+2, p-Value <0.05 and FDR p-Value <0.05. The data represent database used, ID #s within a database, description of system altered, count in the network for system, strength of relationship and the false discovery rate (FDR) p-Value. Color codes indicate genes (by symbol) in the string diagram corresponding to the table class description.
signaling pathways (ERK/JNK, and p38) involving cytokine release (e.g., IL-2, IFN-γ, TNF-α, IL-1β, and IL-6) (45-47). Similarly, Dioscorea glycoproteins alone can trigger infiltration and recruitment of macrophages, lymphocytes, neutrophils, and monocytes while at the same time augmenting NK cytotoxic cell response (48, 49). There is some remote similarity in the manner in which biological responses to the FDA approved saponin adjuvant used for the zoster vaccine (50) AS01 (the QS-21 saponin) works to activate the immune system, such as acting on TLR4 signaling, however, reports central around activation of the adaptive immune response (38, 51-53). In turn, there are also studies showing that other types of saponin-rich plants can stimulate both the innate and adaptive immune response by activation of TLR4, NK cells, humoral and cell-mediated immunity, macrophage phagocytosis, cytokine and immunoglobulin production, and T CD8+ cell-mediated anticaner immune response, such as the case for Codonopsis pilosula (54, 55) and Astragalus (56-58).

Figure 8. TNF signaling Kegg Pathway report for up-regulated DEGS caused by WYE (Low) 15 μg/ml vs. untreated controls after meeting selection criteria: -2<x>+2 fold change, p-Value <0.05 and FDR p-Value <0.05. Up-regulated DEGS are demarcated by a red star.

While data in this work show WYE to evoke some immune response in the cancer cells itself, the impact of this remains speculative. We cannot exclude the possibility that it could also worsen existing cancers with an inflammatory component. In brief, the immune system is a double-edged sword as it plays a role in both the destruction of and pathological advancement of cancer. Initiation of cancer can occur from many injurious environmental elements, including; chronic persistent inflammation, exposure to pathogens, irritants, toxins with greater vulnerability occurring in immunocompromised individuals. There is a delicate balance between immunosuppression (under-active) and inflammation (over-active) which controls the initial susceptibility to cancer and various infections.

A healthy immune system will recognize and destroy malignant cells on demand. In established cancers, the host immune system no longer recognizes malignant cells (immune escape) but instead supports the survival,
proliferation, and metastasis of cancer. This is carried out by the formation of a protective inflammatory barrier within the tumor microenvironment (TME) (59, 60) which fosters cancer growth while at the same time suppresses the body’s immune system to recognize and target cancer (61). The elevation of cytokines released by the tumor tissue itself could worsen pre-existing cancers, driving inward trafficking of leukocyte subpopulations such as monocytes toward the tumor (62), which along with acidity, can polarize and mature into M2- tumor-associated macrophages (TAMS) (63, 64). The M2 tumor-promoting phenotype is largely immunosuppressive, aligning with the increased presence of cytokines (IL-6, IL-8, IL-10), COX2, cathepsins, IL-1A, and MMPS within the TME, creating a highly inflammatory aggressive breast cancer with poor clinical outcome (65-72). In the current work, we showed that WYE at very low concentrations, in theory, could perpetuate the M2 phenotype by establishing...
tumor cell production of COX2, and the release of cathepsins, MMP1, IL-8, IL-6, IL-1A, and CCL2, the consequences of which would be counter-indicated and harmful for cancer patients.

While on the one hand, the WYE used in this study provokes a rise in aggravating tumor cytokines, paradoxically compounds in the Dioscorea species are known to augment the body’s natural capacity to target and destroy tumor cells, as described above. The ability to redirect the body to destroy its own tumor cells is the aim of current-day research in developing immune-modulating anticancer drugs. These therapies aim to restore the body’s natural immune killing capacity by a) penetrating through the TME and b) activating tumor recognition and destruction by the body’s tumor CD8+ T cytotoxic and NK. Cells, both being used in adoptive immune therapies (AIT) (73, 74). While there are most certainly natural compounds unidentified to date that can do this, at the current time, AITs involves using the patient’s blood to derive peripheral blood mononuclear cells (PBMCs), which are genetically engineered or modified then reinfused back into the same patient to target cancer (59, 75). However, it would suffice to say that any agent, drug, or process can mimic the effect of AITs to boost the body’s own NK cells (76) cytotoxic T (CD8+)/ Th (CD4+), antigen-presenting dendritic cells (DCs)/cytokine-induced killer (CIK) cells (DC-CIK cells therapies) (77-79) would, in theory, establish long term immunity and positive patient outcome including for individuals with stage IV breast and TNBC cancers (80-83).

In conclusion, this work provides an overall transcriptomic analysis of WYE at sub-lethal concentrations, corroborating existing evidence by demonstrating its cytostatic effects and establishing a unique immune-stimulatory response. This work emphasizes the need for future research to investigate the immune stimulatory effects of saponin-rich herbs as it relates to cancer prevention and/ or treatment.

Availability of Data and Material

The dataset has been deposited to NIH. Gene Expression Omnibus located at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180621.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors’ Contributions

EM and AA conducted studies on cell viability, saponins, antibody arrays, ELISA, and microarray. SDR and KS were involved with troubleshooting, literature review, manuscript preparation, and KS oversaw and guided this project.

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