

Coptidis Rhizome inhibits cell growth in HONE-1 cell line by inducing cell cycle arrest and apoptosis

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Research

Keywords: Nasopharyngeal carcinoma, Huanglian, Xiao Xian Xiong Decoction, Nanostring

Posted Date: May 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-27848/v1>

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Abstract

Background Nasopharyngeal carcinoma (NPC) is among the most common head and neck malignancies seen among adults in Malaysia. Therefore, discovery of novel anti-cancer herbal drugs is of importance. In this study, the cytotoxic effect was conducted on a traditional Chinese herbal prescription (Xiao Xian Xiong Decoction (XXXD) that is made up of 3 Chinese herbal medicines, namely Huanglian (*Coptidis Rhizome*), Banxia (*Pinellia Rhizome*), Gualuo (*Fructus Trichosanthis*).

Methods The cytotoxic effect of the individual herb and in combination of two and three herbs was studied on 8 nasopharyngeal cancer cell lines. Global gene expression analysis was carried on extracted RNA using nCounter XT Gene Expression Assay.

Results TWO-1, TWO-4, HONE-1, SUNE-1, CNE-2, HK-1, CNE-1 and C666-1 treated with Huanglian, the IC₅₀ values obtained were 24.48, 11.77, 4.48, 10.72 6.32, 11.10, 6.77 and 27.30 µg/ml, respectively. For combination of Huanglian and Banxia, the IC₅₀ values obtained were 74.09 µg/ml (TWO-1), 25.80 µg/ml (TWO-4), 38.10 µg/ml (HONE-1), 29.46 µg/ml (SUNE-1), 19.0 µg/ml (CNE-2) and 20.12 µg/ml (HK-1) but did not exert 50% cell killing in CNE-1 and C666-1 cell lines. The IC₅₀ value attained for the combination of Huanglian and Gualuo was 40.70 µg/ml in HONE-1 cell line. The IC₅₀ values obtained for XXXD (triple combination of Huanglian, Banxia and Gualuo)-treated in HONE-1 and CNE-2 cell lines were 88.55 and 92.42 µg/ml, respectively. Out of all these 7 groups of herbal samples, Huanglian showed the highest cytotoxicity against 8 NPC cell lines with the lowest IC₅₀ value of 4.48 µg/ml recorded in HONE-1. Global gene expression showed Huanglian significantly downregulated genes associated with cell cycle arrest and apoptosis, and thus inhibit HONE-1 cell growth.

Conclusions This study suggest that Huanglian could be a potent anticancer herb targeting HONE-1 cancer cell line.

Background

Nasopharyngeal carcinoma (NPC) is classified as a malignant neoplasm, or cancer, arising from the mucosal epithelium of the nasopharynx, most often within the lateral nasopharyngeal recess or fossa of Rosenmüller [1]. Nasopharynx cancer is native to Southeast Asia. The highest incidence and mortality rates are found in Malaysia, Singapore, Indonesia, Vietnam, and Brunei. A total of 68,272 cases (males, 71.02%; females, 28.97%; sex ratio, 2.45) and 40,530 mortalities (males, 71.63%; females, 28.36%; sex ratio, 2.52) were recorded in Asian countries in 2012 [2].

The present treatment options available to treat NPC patients are particularly with regard to liquid biopsies, minimally invasive surgery, and advances in chemotherapy and immunotherapy [3]. However, due to its deep-seated location, the vast majority of affected individuals do not know they have NPC until it has already spread to the lymph nodes in the neck. There are many short- and long-term side effects of radiotherapy and chemotherapy, such as nausea, vomiting, cognitive dysfunction, xerostomia, dysphagia,

and olfactory dysfunction [4]. These side effects limit the use of chemotherapeutic agents despite the high efficacy in treating specific or target malignant cells. Due to the abundance of side effects of the existing medicines, it is wise to look for more alternative medicines for better prognosis. It is believed that natural plants could be the useful sources of new anti-cancer agent [5]. Chinese herbs are now being used as an adjunct to Western medical interventions. Chinese herbal medicine is characterized by “Holistic regulation,” in which the organism is considered as a whole. With the equilibrium of the human body as the guidelines, practitioners of Chinese herbal medicine pay more attention to the diseased patients rather than the suffered diseases itself. Particular western medicine always has a strong effect on a specific disease with focusing on a specific physiological target and neglecting the specific characteristics of each patient [6].

In this study we investigated the cytotoxic effect of single and combination of two and three Chinese herbal medicine on NPC cancer cell lines. Xiao Xian Xiong Decoction (XXXD) is a Chinese Medicine prescription originates from “Shang Han Lun”, a renowned Chinese medical masterpiece written by Master Zhang ZhongJin of ancient Chinese Han Dynasty (216A.C.). The decoction is made up of three Chinese medical herbs derived from natural plants, namely Huanglian (*Coptidis Rhizome*), Banxia (*Pinellia Rhizome*), and Gualuo (*Fructus Trichosanthis*). Huanglian initially recorded in the Shennong Bencao Jing (Emperor Shennong’s Classical Medical Material) and listed as one of the best herbs [7]. Berberine (BBR), an isoquinoline alkaloid, is the major active component found in in Huanglian with multiple pharmacological activities including antimicrobial, antiviral, anti-inflammatory, cholesterol-lowering and anticancer effects [8]. Banxia in the perspective of TCM, it is acrid, warm, and toxic in nature. It is mainly used to treat coughing, dyspnea, dizziness, nausea and vomiting, fullness in chest and palpitation caused by phlegm and fluid retention [9]. Gualuo is a flowering plant and the fruit is used to clear body heat and transform phlegm-heat, unbind the chest, reduces abscesses and dissipate nodules [10]. This plant can be used possibly to dissolve the protein coat on the RNA of the AIDS virus [11].

As decoction XXXD has the effect of clearing away heat, resolving phlegm, relieving stuffiness in the chest and dispersing stagnancy [12]. In the past centuries, it had been used to treat many diseases involving digestion, respiration and cardiovascular disorder. In recent years, the clinical application of XXXD has been widened, especially in the aspects of inhibiting tumour growth and promoting body immunity [13]. The modern pharmacological studies have shown XXXD comprises of distinctive clinical effects, such as anti-inflammation, anti-ulcer, anti-cancer and immunity enhancement [13]. Previous *in-vitro* laboratory research on tumour cell line has shown this decoction consists of inhibitory effect against Ehrlich ascites carcinoma (EAC) and lung cancer (NSCLC) [14, 15].

Previous researches have shown the anti-cancer effect of each individual constituent of XXXD. Nevertheless, the therapeutic value of XXXD on NPC has never been scientifically scrutinized. Overall, this research comprises the preparation of aqueous-extraction of individual and combination extracts of Huanglian, Banxia, and Gualuo and subsequently screening the cytotoxicity of these herbal extracts on a panel of 8 NPC cell lines, namely TWO-1, TWO-4, HONE-1, SUNE-1, CNE-2, HK-1, CNE-1 and C666-1 with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT) assay. Further gene expression

analysis was carried out to quantify transcript levels of 770 genes representing 13 canonical cancer pathways in Huanglian-treated HONE-1 cell line.

Materials And Methods

Material

Roswell Park Memorial Institute 1640 (RPMI-1640) powder purchased from Mediatech, Inc. (Manassas, USA) was prepared at 10.39g/L in ultrapure water and added with sodium bicarbonates 2g/L. Fetal bovine serum (FBS) was purchased from Lonza Inc. (Allendale, NJ, USA). Dulbecco modified Eagle medium (DMEM) supplemented with 4.5 g glucose/L and 300 mg/L glutamine was purchased from Hyclone Laboratories Inc. (Logan, Utah, United States). 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyl-tetrazoliumbromide (MTT) reagent and phosphate buffer saline (PBS) were obtained from Merck (Germany). All other chemicals were of analytical grade and commercially available.

Aqueous-extraction of the individual and combination herbs

Standalone Chinese herbs (Huanglian, Banxia, Gualuo) were bought from TCM Medical Hall (Tongrentang (M) Sdn. Bhd.) at Kuala Lumpur. Table 1 shows the combination of the extracts for the extracts preparation. The amount of Huanglian, Banxia and Gualou used is based on a ratio of 1:2:5 as stated in Shan Han Lun. All the herbs were soaked in 500ml of deionized water for an hour. Then, the herbs were boiled for half hours in a 3L customized boiling device. After cooling, the supernatant was collected and filtered with whatman paper. The filtrates were lyophilized and kept in -20°C prior use.

Cultivation of NPC cancer cell lines

A total of 8 NPC cancer cells were used in this study (Table 2). HK-1 was obtained from Professor S.W. Tsao from the Department of Anatomy, University of Hong Kong, Hong Kong, China. C666-1 was obtained from Professor K.W. Lo from Prince and Wales of Hospital, Hong Kong, China. TWO-4, HONE-1 TWO-1, SUNE-1, CNE-1 and CNE-2 were obtained from Dr. Yap Lee Fah from the Department of Oral Biology and Biomedical Sciences, Faculty of Dentistry, University of Malaya, Malaysia. TWO-4, HONE-1, SUNE-1, CNE-2, HK-1 and C666-1 cells were cultured in RPMI-1640 medium whereas TWO-1 and CNE-1 were cultured in DMEM. All these mediums were supplemented with 10% FBS. Cells were grown as monolayers and were maintained in a humidified CO₂ incubator at 37°C.

Determination of Optimal Cell Seeding Concentration

MTT assay was used to determine the optimum cell seeding density of each cell line. A total of 2.5×10^3 , 5×10^3 , 10×10^3 , 20×10^3 and 40×10^3 and 80×10^3 cells/ml were seeded in a 96-well plate and incubated for 72 hours prior to cytotoxicity evaluation by MTT assay. Each well was read at 570 nm for absorbance. Absorbance versus cell density graph was plotted. The optimum cell concentration was determined approximately at the middle point of exponential phase.

MTT assay

All the herbal extracts were tested for their cytotoxicity effect on a panel of NPC cell lines. A total of 100µL of cells at the optimum cell concentration were seeded into each well of 96-well plate. Three wells containing 100µL medium only were included as a negative control and three more wells containing 100µL cell solution (untreated with herbs) serve as positive control. After 24 hours of incubation, each well was added with 10µL mixture of fresh medium containing respective herbs named as L1 to L7 at the concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml via MTT assay and then topped up with 90 µL of medium. The cytotoxicity of the herbal extract was determined at 72 hours of incubation. After 72 hours, the solution was discarded and refilled with 200 µL of fresh medium. 20 µL of MTT was then added into each well and incubated for 3 hours. After 3 hours, the plate was centrifuged in 3000 rpm for 5 minutes in 5°C. Then, 180 µL of the solution was removed and 200 µL of DMSO was added. The mixture was swirled gently and kept in dark for 15 minutes. The assays were conducted in triplicate and repeated in three independent experiments. The average absorbance against number of cells/mL graph was plotted and IC₅₀ value was calculated.

RNA extraction

Total RNA was extracted with Direct-zol™ RNA Miniprep Plus (Zymo Research, CA, USA) following the recommendations of the manufacturer. Briefly, 600µl of TRI-Reagent® was added to pellet and homogenized completely. 600µl of ethanol 95% was directly added to the solution and homogenized. The sample was added to Zymo-Spin IIC™ column with the collection tube and centrifuged at 16,000xg for 30 seconds at 4°C. The column was moved to a new collection tube and the collection tube containing the filtrate was discarded. The RNA samples were treated with DNase. Next, 400µl Direct-zol™ RNA, a prewash was added to the column, and centrifuged. Subsequently, the filtrate was discarded and this step was repeated. 400µl RNA wash buffer was added to the column and it was centrifuged.. The column was carefully transferred from the collection tube into the RNase-free tube, 50µl DEPC-treated nuclease-free water was added to it and it was centrifuged. Extracted RNA was stored frozen at <-70°C. Purity and concentration of the total RNA were determined by spectrophotometry using the 260 nm/280 nm ratio (NanoDrop 1000, Thermo Scientific, USA) and 1% agarose gel electrophoresis.

Nanostring sample preparation

The preparation of hybridization was carried out using nCounter XT Gene Expression Assays (NanoString Technologies, Seattle, WA). Initially, 70 µL of Hybridization Buffer was added to Reporter CodeSet to create a Master Mix. Secondly, the hybridization reaction was set up by adding 5 µL of pre-prepared RNA samples to 8 µL of Master Mix to make up 13 µL of total volume in a tube. Thereafter, 2 µL of Capture ProbeSet was added to the tube before making the final volume of 15 µL. The tube was then capped, well mixed and briefly spinned. Finally, a total of 12 tubes (triplicate at time point of 0, 4, 8, 12 hours) were incubated in thermocycler at 65°C for about 16 hours. The next steps was purification and immobilisation by using the nCounter MAX/FLEX system which was carried out in the nCounter Prep Station to remove

excess probes and immobilize target-probe complexes onto nCounter Cartridges. The cartridges were scanned on the nCounter Digital Analyzer for 2.5 hours. Reporter Code Count (RCC) file was an output file generated by this nCounter instruments. One RCC file was produced for each sample tested; one file contains the barcode counts from each gene and control in the CodeSet. A Reporter Library File (RLF) is a file specific to the CodeSet. It provides nCounter instruments and the nSolver 4.0 software application with valuable information about the CodeSet, such as the assignment of probe to gene.

After importing to nSolver 4.0 software (NanoString Technologies), the RCC data files will be stored under the corresponding RLF file CodeSet on the Raw Data tab. All samples were normalized using the geometric mean of the housekeeping genes RBM45-mRNA, SLC4A1AP-mRNA, AGK-mRNA, FCF1-mRNA, MRPS5-mRNA, DDX50-mRNA, HDAC3-mRNA, EIF2B4-mRNA, NUBP1-mRNA. Differentially expressed genes between the treated and the untreated control cells were given as fold change expression. Gene expression data were mapped onto KEGG pathway graphs by Path view function of the PCPAA, providing intuitive views of regulation at the pathway level. Regulated KEGG pathways were identified via Nanostring Advance Analysis software. The common differentially expressed genes at the different time point, in both the upregulated and the downregulated gene lists, were identified by using Venny online tool.

Results

1. Optimal cell density

After being incubated for 72 hours, each cell line was tested for its optimal cell density. Table 3 shows the results obtained for the optimum cell seeding density of each NPC cell line. The optimum cell seeding density of each cell line is ranging from 2.5×10^3 to 5×10^3 cells.

2. Cytotoxicity of the herbal extract L1 to L7 via MTT assay

The values of IC_{50} ($\mu\text{g/mL}$) attained for cytotoxic effects of L1, L2, L3, L4, L5, L6, L7 against 8 NPC cell lines are summarised in Table 4 and illustrated in Figure 1. The herbal sample L1 (Huanglian) exhibited most distinctive cytotoxic effects among other extracts. The IC_{50} values of L1 against 8 NPC cell lines ranging from 4.48 to 27.30 $\mu\text{g/mL}$ in which it is the lowest and sensitive towards HONE-1 cell line. L2, L3 and L6 did not show cytotoxic effects towards all NPC cell lines tested. The IC_{50} value of L4 marked at 19.00 $\mu\text{g/mL}$ towards CNE-2 that was the lowest among 8 NPC cell lines. L5 did not show any cytotoxic effects towards NPC cell lines tested except HONE-1 with IC_{50} value of 40.70 $\mu\text{g/mL}$. L7 only exhibited its cytotoxic effects on HONE-1 and CNE-2 with IC_{50} values of 88.55 and 92.95 $\mu\text{g/mL}$, respectively.

3. Gene expression and pathway analysis

NanoString PanCancer Pathways Panel gene expression analysis was used to quantify transcript levels of 770 genes representing 13 canonical cancer pathways in HONE-1 cells upon treatment with L1, Huanglian. The gene expression was analysed at 0 vs 4, 0 vs 8 and 0 vs 12 hour treatment. Total RNA extracted from HONE-1 cells at different time points were hybridized to the code set with approximately one million raw counts tallied for all genes in each sample. Raw data were processed and normalized using nSolver 4.0 following manufacturer's guidelines (details in Materials and Methods).

0 vs 4 hrs gene expression

Among the 770 genes analysed, 66 up regulated genes show a positive fold change whereas 114 down regulated genes show a negative fold change ($P < .05$) as shown in Figure 2a. The top 10 most statistically significant genes were shown in Volcano plot (Figure 2b). Genes involved in a number of cancer pathways were enriched among the top differentially expressed genes as shown in Table 5. Among upregulated gene set, genes in MAPK signalling pathway appeared 3 times in the top 10 list (Table 5), followed by the TGF-beta, Wnt and driver gene (2 times each) and JAK-STAT signalling pathway (1 time). Among downregulated gene sets, genes in cell cycle appeared 5 times with mostly in apoptosis pathway, followed by JAK-STAT and driven gene with 2 times, each. Gene set for DNA damage appeared once. Gene expression data were mapped onto KEGG pathway graphs by Pathview function of the PanCancer pathway software, providing intuitive views of both up and deregulation at the pathway level. Representative graphs for the MAPK signalling pathway and cell cycle pathway are shown in Figures 3a and 3b, respectively.

0 vs 8 hrs gene expression

Among the 770 genes determined, 35 up regulated genes shows a positive fold change whereas 114 down regulated genes show a negative fold change ($P < .05$) as shown in Figure 4 a. The top 10 most statistically significant genes are shown in Volcano plot (Figure 4b). Genes involved in a number of cancer pathways were enriched among the top differentially expressed genes as shown in Table 6. Among upregulated gene set, genes in WNT pathway appeared 4 times in the top 10 list followed by the MAPK, JAK-STAT (2 times each) and PI3K, as well as PI3K, transcriptional misregulation pathway and chromatin modification, cell cycle – apoptosis pathway (1 time). Among downregulated gene sets, genes in cell cycle appeared 3 times with mostly in apoptosis pathway, followed by DNA damage-repair, WNT, JAK-STAT gene with 2 times each as well as Ras, Hedgehog, PI3K genes appeared once each. Gene expression data were mapped onto KEGG pathway graphs by Pathview function of the PanCancer pathway software (PCPAA), providing intuitive views of both up and deregulation at the pathway level. Representative graphs for the WNT signalling pathway and cell cycle pathway are shown in Figures 5 and 3b, respectively.

0 vs 12 hrs gene expression

Among the 770 genes identified, 77 up regulated genes show a positive fold change whereas 91 down regulated genes show a negative fold change ($P < .05$) as shown in Figure 6a. The top 10 most statistically significant genes are shown in Volcano plot (Figure 6b). Genes involved in a number of cancer pathways were enriched among the top differentially expressed genes as shown in Table 7. Among upregulated gene set, genes in MAPK pathway appeared 5 times in the top 10 list (Table 6), followed by the P13K (4 times), RAS and cell cycle (2 times) as well as JAK-STAT, WNT and transcriptional misregulation pathway (1 time). Among downregulated gene sets, genes in cell cycle appeared 5 times with mostly in apoptosis pathway, followed by P13K (4 times), MAPK and RAS (2 times) as well as DNA damage-repair, TGF-beta, transcriptional misregulation pathway (1 time) (Table 7). Gene expression data were mapped onto KEGG pathway graphs by Pathview function of the PanCancer pathway software (PCPAA), providing intuitive views of both up and deregulation at the pathway level. Representative graphs for the MAPK signalling pathway and Cell Cycle pathway are shown in Figures 3a and 3b, respectively.

The common differentially expressed genes of HONE-1 at three different treatment times such as 4, 8 and 12 hrs after exposure to L1, in both downregulated and upregulated gene lists, were identified by using Venny online tool. As shown in Figure 7, a total of 319 genes were downregulated and 178 genes were upregulated in HONE-1 cells at the three time points. In the case of downregulated genes, a total number of 43 genes (18.1%) were found between 4 and 8 hrs treatment time; 24 genes (10.1%) found between 8 and 12 hrs treatment time and 4 genes (1.7%) was found between 4 and 12 hrs treatment time whereas 5 genes (2.1%) in between three treatment time. In the case of upregulated genes, a total number of 15 genes (18.4%) were found between 4 and 8 hr treatment time; 8 genes (5.6%) found between 8 and 12hrs treatment time and 7 genes (4.9%) found between 4 and 12 hrs treatment time whereas 2 genes (1.4%) found in between three treatment time.

Discussion

This study was designed to uncover alterations in gene expression patterns and pathway mechanisms involved in nasopharyngeal carcinoma and subsequent comparison at different time point upon treated with Huanglian.

Nearly, 80% of the world's population depend on traditional medicines and more than 60% of clinically approved anticancer drugs are derivatives of these medicinal plant [16]. Many types of secondary metabolites including alkaloids, lignans, phenylpropanoids, flavonoids, phenolic compounds, saccharides, and steroids have been isolated from Huanglian. Among them, protoberberine-type

alkaloids, such as berberine (30–50%) [17, 18], palmatine (4–25%), coptisine (20–30%), epiberberine, jatrorrhizine, columamine, are the main components with cytotoxicity effect [19, 20].

Methods of extraction of medicinal plants include solvent extraction, distillation method, pressing and sublimation according to the extraction principle and etc. In this experiment, aqueous-extraction of Huanglian was adopted. It was a simple, economical and effective method for oral consumption and faster absorption in body for curing diseases.

This study reports the cytotoxic activity of Xiao Xian Xiong Decoction (XXXD), two combination herbs and individual herb of Huanglian (*Coptidis Rhizome*), Banxia (*Pinellia Rhizome*) and Gualuo (*Fructus Trichosanthis*) on human nasopharyngeal carcinoma cancer cell lines. Among these extract samples, the IC_{50} values attained for Huanglian against 8 NPC cell lines of TWO-1, TWO-4, HONE-1, SUNE-1, CNE-2, HK-1, CNE-1 and C666-1 showed the strongest cytotoxicity, which were ranging from 4.48 to 27.30 $\mu\text{g/mL}$, in which the lowest IC_{50} value at 4.48 $\mu\text{g/mL}$ was obtained for HONE-1. It is clear that HONE-1 cells exposed to Huanglian lose their capability to proliferate in a time- and dose-dependent manner. This finding suggests that Huanglian has the potential to suppress the growth of the nasopharyngeal carcinoma cancer cells irreversibly.

This study was intended to characterize the temporal dynamics of gene expression in HONE-1 when treated with Huanglian at 0, 4, 8, 12 hrs to illuminate the series of progressive biological mechanisms involved in HONE-1 proliferation. The genes expressed in MAPK signalling pathway was upregulated at 4 and 12 hours while the genes expressed in WNT signalling pathway was upregulated at 8 hours. The cell cycle was downregulated at all time point starting from 0 until 12 hours. The MAPK pathways relay, amplify and integrate signals from a diverse range of stimuli and elicit an appropriate physiological response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis in mammalian cells. MAPK pathway activation has ambiguous effect on tumour cells fate depending on cell type or cancer stages [21]. Upregulation of ERK activators *NGF* and *BDNF* was observed in HONE-1 cell at 4 hours. MAPK/ERK pathway activation is a common feature of NPC and these pathways are stimulated by the EBV-encoded LMP1 and LMP2A genes to promote the migration and invasion of NPC cells [22, 23]. Furthermore, high expression of phosphorylated ERK in primary NPC is related to lymph node metastasis and radioresistance [24]. These information suggest activation of MAPK/ERK from 4 to 12 hours in this study. Similarly, activation of WNT signalling pathway also correlated with nature of NPC cell lines. Upregulation of *MMP7*, *PLCB4*, *LEF1* and *FOSL1* genes were observed at 8 hours post treatment. WNT signaling pathway is one of the essential signaling pathways regulating cell proliferation and differentiation. It is composed of various signal molecules, ligands and receptors such as Wnt protein and β -catenin, and is very conservative in evolution [25]. Zeng et al in 2007, concluded Wnt signaling pathway may be abnormally activated in NPC due to abnormal accumulation of WNT5A and nuclear β -catenin [26].

The cell cycle is the series of events that takes place in a cell leading to its division and replication. Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and

repair of genetic damage as well as the prevention of uncontrolled cell division. One of the most important tumour suppressors is tumour protein p53, which plays a key role in the cellular response to DNA damage [27]. In this experiment, Huanglian downregulated genes involved in cell cycle pathway in HONE-1. p53 could be activated and bind with DNA that further resorted to DNA repair and triggered to cell apoptosis. Huanglian decreased the expression of *HDAC1*, *SMC1A*, *RAD*, *AKT3*, *CDC7*, *CCNA2*, *CCNE1/2* and *CDC6* that induces cell cycle arrest and apoptosis, and thus subsequently inhibited cell proliferation of HONE-1.

Conclusion

In a nutshell, the findings of this study indicate that Huanglian induces dose-dependent responses in HONE-1 that involve the up-regulation of a large group of genes associated with MAPK and WNT signaling pathways and the downregulation of genes associated with cell cycle arrest and apoptosis. This reciprocal regulatory mechanism provides clues to further understand the mechanism driving growth inhibition in NPC HONE-1 cancer cells treated with the Huanglian.

Abbreviations

XXXD :	Xiao Xian Xiong Decoction
TCM :	Traditional Chinese Medicine
NPC :	Nasopharyngeal carcinoma

Declarations

- **Ethics approval and consent to participate**

Not applicable.

- **Consent for publication**

Not applicable.

- Availability of data and materials

The datasets analyzed during the current study available from the corresponding author on reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

- **Funding**

The authors express their gratitude to Universiti Tunku Abdul Rahman for the financial support to this project (IPSR/RMC/UTARRF/2017-C1-W01).

- Acknowledgements

We would like to record our utmost appreciation to Professor Dr. Lo Kwok Wai, Professor S.W. Tsao and Associate Professor Dr. Yap Lee Fah for providing NPC cell lines for this study.

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KFL (Kim Fey Leu) Project development, Data collection, Bioinformatics analysis, Manuscript writing/ editing;

SM (Subramaniam Menaga) Data collection, Bioinformatics analysis, Manuscript editing;

XHW (Xing Hua Wang) advised on the use of XXX decoction stated in the “Shang Han Lun or known as *Treatise on Cold Damage Disease*;

ZY (Zao Yang) provided the knowledge on the nasopharyngeal carcinoma from the TCM philosophical understanding;

LFY (Lee Fah Yap) provided the NPC cell lines (TWO-4, HONE-1 TWO-1, SUNE-1, CNE-1 and CNE-2) and technical support and advices for the study pertaining to cell lines handling;

KWL (Kwok Wai Lo) provided the C666-1 cell line and technical support and advices for the study pertaining to cell line handling;

YML (Yang Mooi Lim) Project development, Data analysis and interpretation, Manuscript writing/ editing and conceived the entire study.

All authors read and approved the final manuscript.

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Tables

Table 1: Name and weight of herb and combination

Group No	Name of herb	Weight (g)
L1	Huanglian	60
L2	Banxia	120
L3	Gualuo	300
L4	Huanglian + Banxia	60 + 120
L5	Huanglian + Gualuo	60 + 300
L6	Banxia + Gualuo	120 + 300
L7	Huanglian + Banxia + Gualuo	60 + 120 + 300

Table 2: Morphology of human NPC cell lines

Cell line	Cell type	Medium
TWO-1	EBV-negative epithelial NPC	DMEM
TWO-4	Epithelial NPC	RPMI 1640
HONE-1	poorly differentiated squamous NPC	RPMI 1640
HK-1	differentiated squamous carcinoma	RPMI 1640
C666-1	Undifferentiated squamous	RPMI 1640
SUNE-1	undifferentiated epithelial carcinoma.	RPMI 1640
CNE-1	Lymphoblast differentiated squamous carcinoma (EBV- negative)	DMEM
CNE-2	poorly differentiated epithelial carcinoma.	RPMI 1640

Table 3: Optimum cell seeding density of 8 cancer cell lines

Cell lines	Optimum cell density (cells/well)
TWO-1	4.0 x 10 ³
C666-1	4.0x 10 ³
CNE-1	5.0 x 10 ³
CNE-2	5.0 x 10 ³
HONE-1	3.0 x 10 ³
HK-1	4.0 x 10 ³
SUNE-1	5.0 x 10 ³
TWO-4	2.5 x 10 ³

Table 4: IC₅₀ values (µg/mL) attained for the cytotoxic effects of Huanglian, Banxia, Gualuo as standalone and in combination against 8 cancer cell lines

Cell lines	L1	L2	L3	L4	L5	L6	L7
TWO-1	24.46	NA	NA	74.09	NA	NA	NA
C666-1	27.30	NA	NA	NA	NA	NA	NA
CNE-1	6.77	NA	NA	NA	NA	NA	NA
CNE-2	6.32	NA	NA	19.00	NA	NA	92.95
HONE-1	4.48	NA	NA	38.10	40.70	NA	88.55
HK-1	11.10	NA	NA	20.12	NA	NA	NA
SUNE-1	10.72	NA	NA	29.46	NA	NA	NA
TWO-4	11.77	NA	NA	25.80	NA	NA	NA

*NA denotes values of IC₅₀ not obtained. L1 denotes Huanglian; L2 denotes Banxia; L3 denotes Gualuo; L4 denotes Huanglian+Banxia; L5 denotes Huanglian+Gualuo; L6 denotes Banxia+Gualuo; L7 denotes Huanglian+Banxia+Gualuo.

Table 5: Top 10 genes with increased and decreased linear log2 fold change expression between up regulated genes and down regulated genes with pathways in 4 vs. baseline of 0 hour

Up-regulated genes	Log2 fold change	Pathway	Down-regulated genes	Log2 fold change	Pathway
FST-mRNA	1.75	TGF-beta	MLH1-mRNA	-0.136	Driver Gene
NGF-mRNA	1.68	Cell Cycle - Apoptosis, MAPK, PI3K, Ras	HDAC1-mRNA	-0.145	Cell Cycle - Apoptosis, Chromatin Modification, Notch, Transcriptional Misregulation
FZD8-mRNA	1.32	Wnt	SMC1A-mRNA	-0.167	Cell Cycle - Apoptosis
DUSP4-mRNA	1.17	MAPK	H3F3A-mRNA	-0.178	Driver gene, Transcriptional Misregulation
BDNF-mRNA	1.12	MAPK	RAD21-mRNA	-0.18	Cell Cycle - Apoptosis
TNFAIP3-mRNA	1.02	Driver Gene	AKT3-mRNA	-0.181	Cell Cycle - Apoptosis, JAK-STAT, MAPK, PI3K, Ras
INHBA-mRNA	0.93	TGF-beta	STAT1-mRNA	-0.182	JAK-STAT
IL24-mRNA	0.9	JAK-STAT	STAT3-mRNA	-0.196	JAK-STAT
SOCS1-mRNA	0.891	Driver Gene, JAK-STAT	MNAT1-mRNA	-0.211	DNA Damage - Repair
DKK1-mRNA	0.869	Wnt	CDC7-mRNA	-0.222	Cell Cycle - Apoptosis

Table 6: Top 10 genes with increased and decreased linear log2 fold change expression between up regulated genes and down regulated genes with pathways in 8 vs. baseline of 0 hour

Up-regulated genes	Log2 fold change	Pathway	Down regulated genes	Log2 fold change	Pathway
NR4A1-mRNA	1.28	MAPK, PI3K	STAT3-mRNA	-0.15	JAK-STAT
IL6-mRNA	0.886	JAK-STAT, PI3K, Transcriptional Misregulation	XRCC4-mRNA	-0.191	DNA Damage- Repair
MMP7-mRNA	0.767	Wnt	CDC7-mRNA	-0.205	Cell Cycle - Apoptosis
SOCS3-mRNA	0.765	JAK-STAT	MDC1-mRNA	-0.205	DNA Damage - Repair
DUSP4-mRNA	0.585	MAPK	STAT1-mRNA	-0.214	JAK-STAT
PLCB4-mRNA	0.466	Wnt	IDH2-mRNA	-0.23	Driver Gene
LEF1-mRNA	0.45	Wnt	SHC1-mRNA	-0.236	Ras
FOSL1-mRNA	0.445	Wnt	GSK3B-mRNA	-0.243	Cell Cycle - Apoptosis, Hedgehog, PI3K, Wnt
HELLS-mRNA	0.385	Chromatin Modification	CCNA2-mRNA	-0.244	Cell Cycle - Apoptosis
E2F1-mRNA	0.371	Cell Cycle - Apoptosis	TBL1XR1-mRNA	-0.254	Wnt

Table 7: Top 10 genes with increased and decreased linear log2 fold change expression between up regulated genes and down regulated genes with pathways in 12 vs. baseline of 0 hour

Up-regulated genes	Log2 fold change	Pathway	Down regulated genes	Log2 fold change	Pathway
NR4A1-mRNA	2.76	MAPK, PI3K	CASP3-mRNA	-0.171	Cell Cycle - Apoptosis, MAPK
MMP7-mRNA	1.98	Wnt	CCNE1-mRNA	-0.178	Cell Cycle - Apoptosis, PI3K
FASLG-mRNA	1.84	Cell Cycle - Apoptosis, MAPK, PI3K, Ras	PPP2CB-mRNA	-0.183	PI3K, TGF-beta
DDIT3-mRNA	1.58	MAPK, Trans-criptional Misregulation	SHC1-mRNA	-0.186	Ras
DDIT4-mRNA	1.51	PI3K	CCNE2-mRNA	-0.188	Cell Cycle - Apoptosis, PI3K
ANGPT1-mRNA	1.36	PI3K, Ras	XRCC4-mRNA	-0.195	DNA Damage - Repair
MAP3K8-mRNA	1.32	MAPK	CDC6-mRNA	-0.196	Cell Cycle - Apoptosis
CDKN1C-mRNA	1.29	Cell Cycle - Apoptosis	U2AF1-mRNA	-0.198	Driver Gene
MAP2K6-mRNA	1.27	MAPK	RELA-mRNA	-0.199	Cell Cycle - Apoptosis, MAPK, PI3K, Ras, Transcriptional Misregulation
SOCS3-mRNA	1.16	JAK-STAT	H3F3C-mRNA	-0.203	Transcriptional Misregulation

Figures

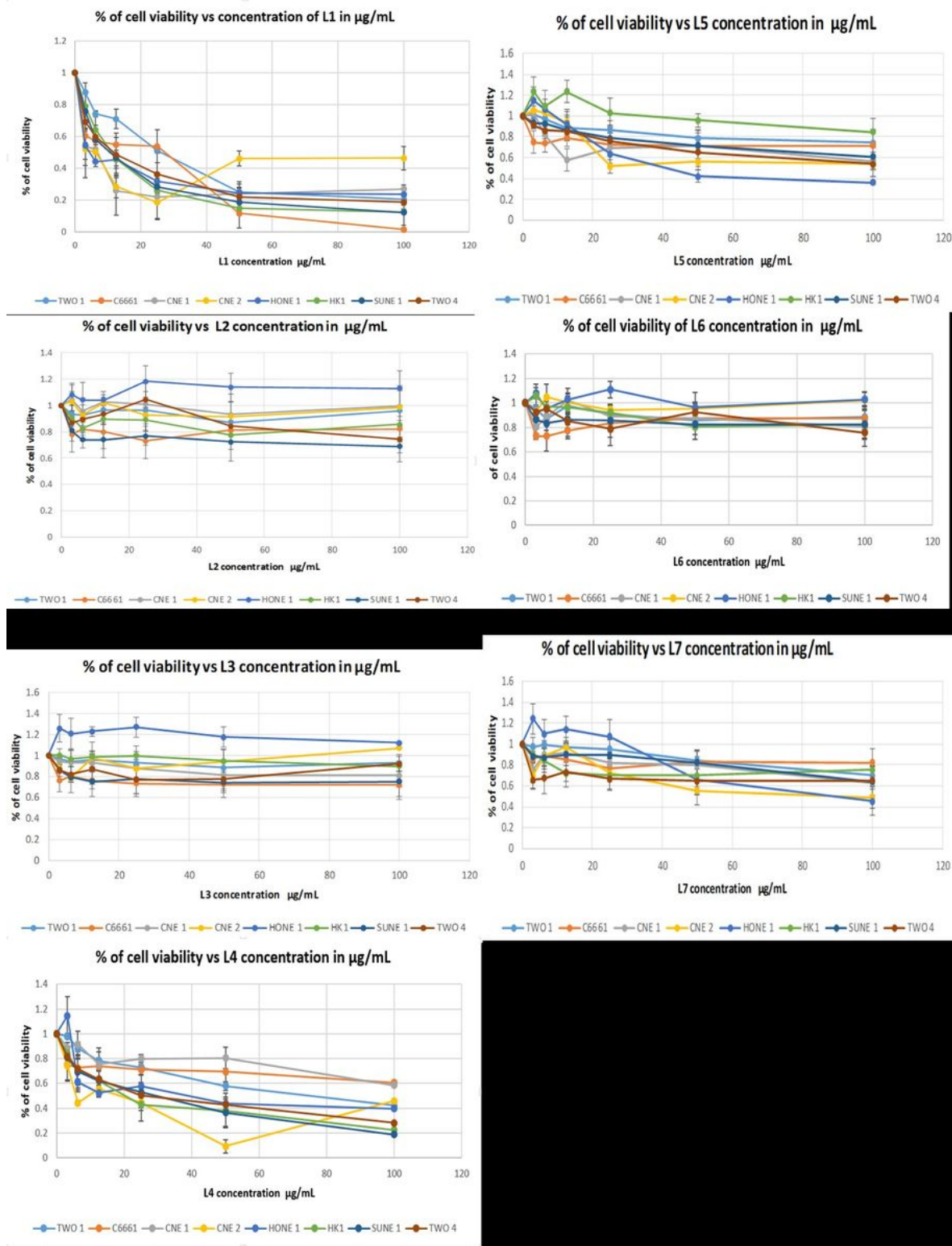


Figure 1

Graph of cell viability (%) versus treatment concentration (μg/mL) for the cytotoxicity study on 8 cancer cell lines: TWO-1, TWO-4, HONE-1, SUNE-1, CNE-2, HK-1, CNE-1 and C666-1.

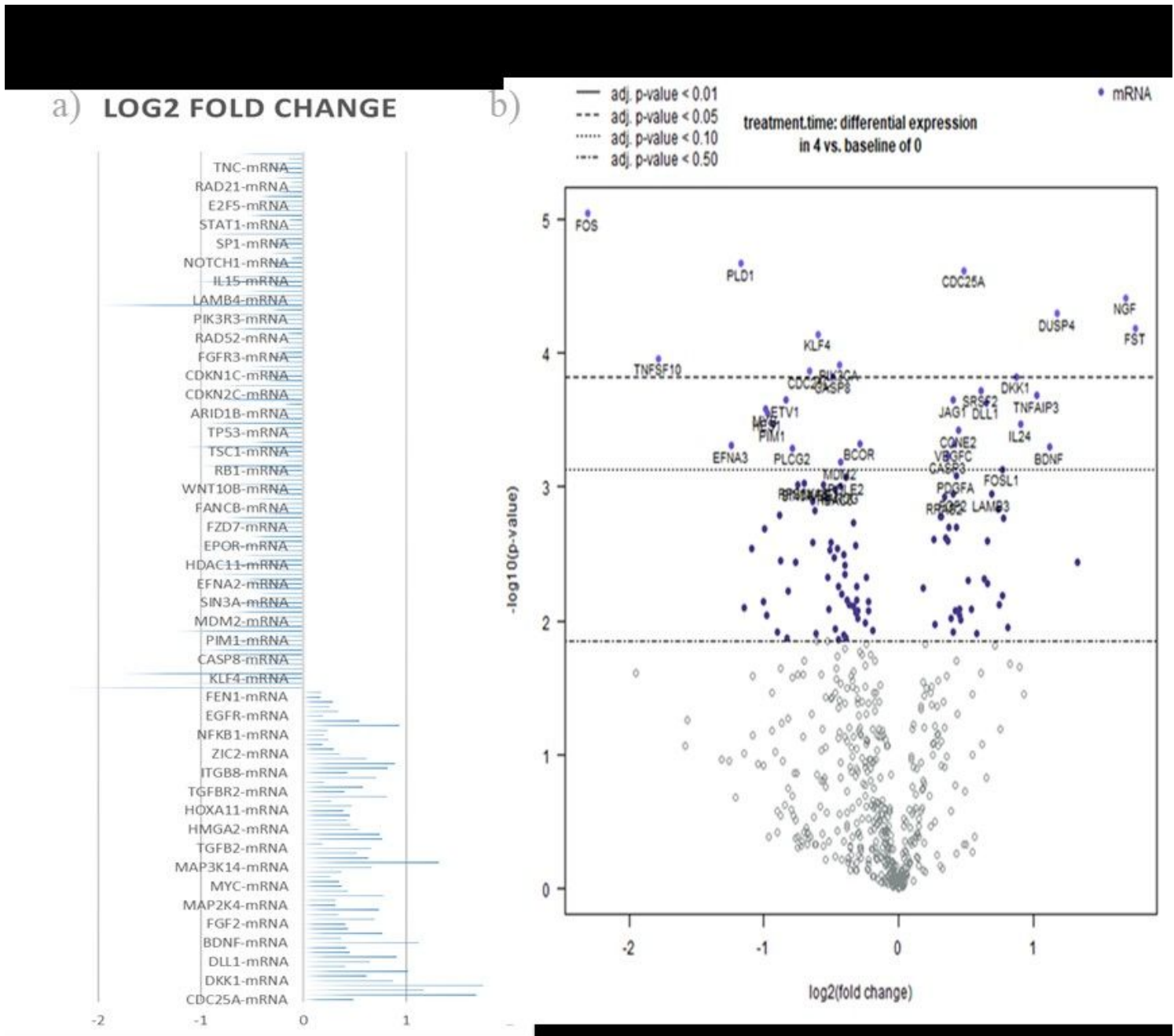


Figure 2

a) Log2 fold change difference between 4 vs. baseline of 0 in HONE-1 cells treated with L1 (Huanglian) with differential expression comparing statistically significant differences ($P < .05$) in mRNA expression in the up and down regulated genes separately. A total of 66 up regulated genes show a positive fold change whereas 114 down regulated genes show a negative fold change. b) Volcano plot.

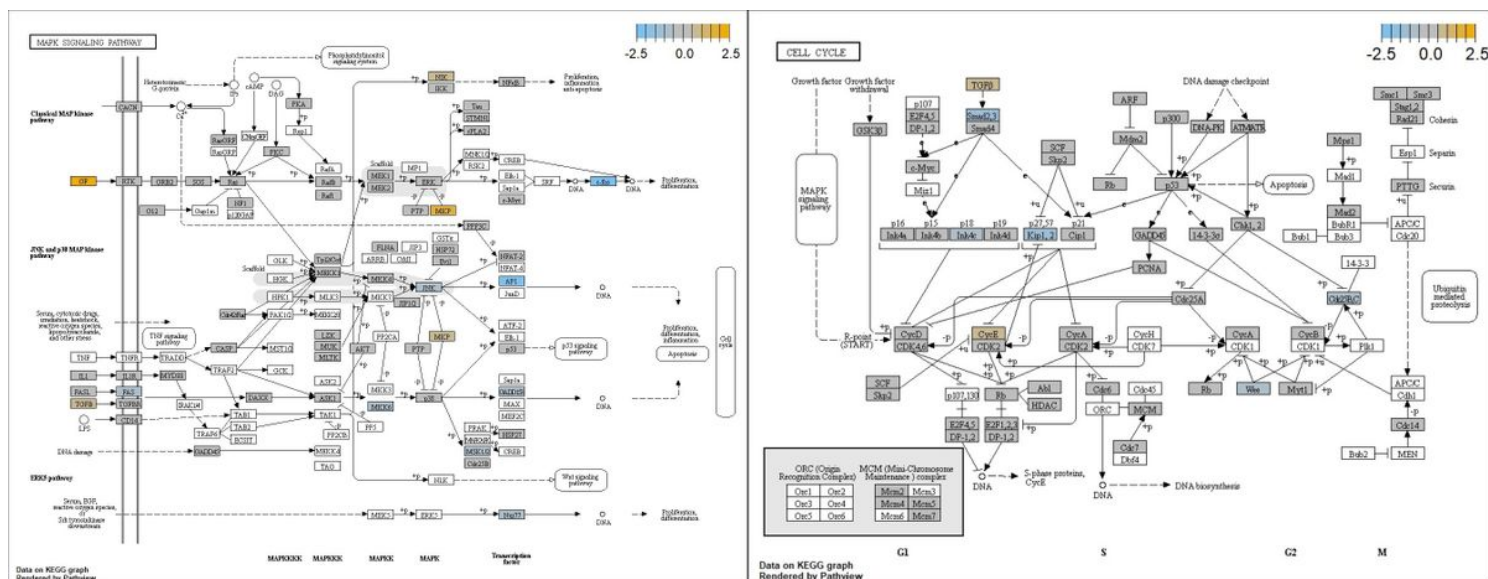


Figure 3

a: MAPK signalling pathway (treatment time: differential expression in 4vs. baseline of 0) b: Cell cycle pathway (treatment time: differential expression in 4 vs. baseline of 0)

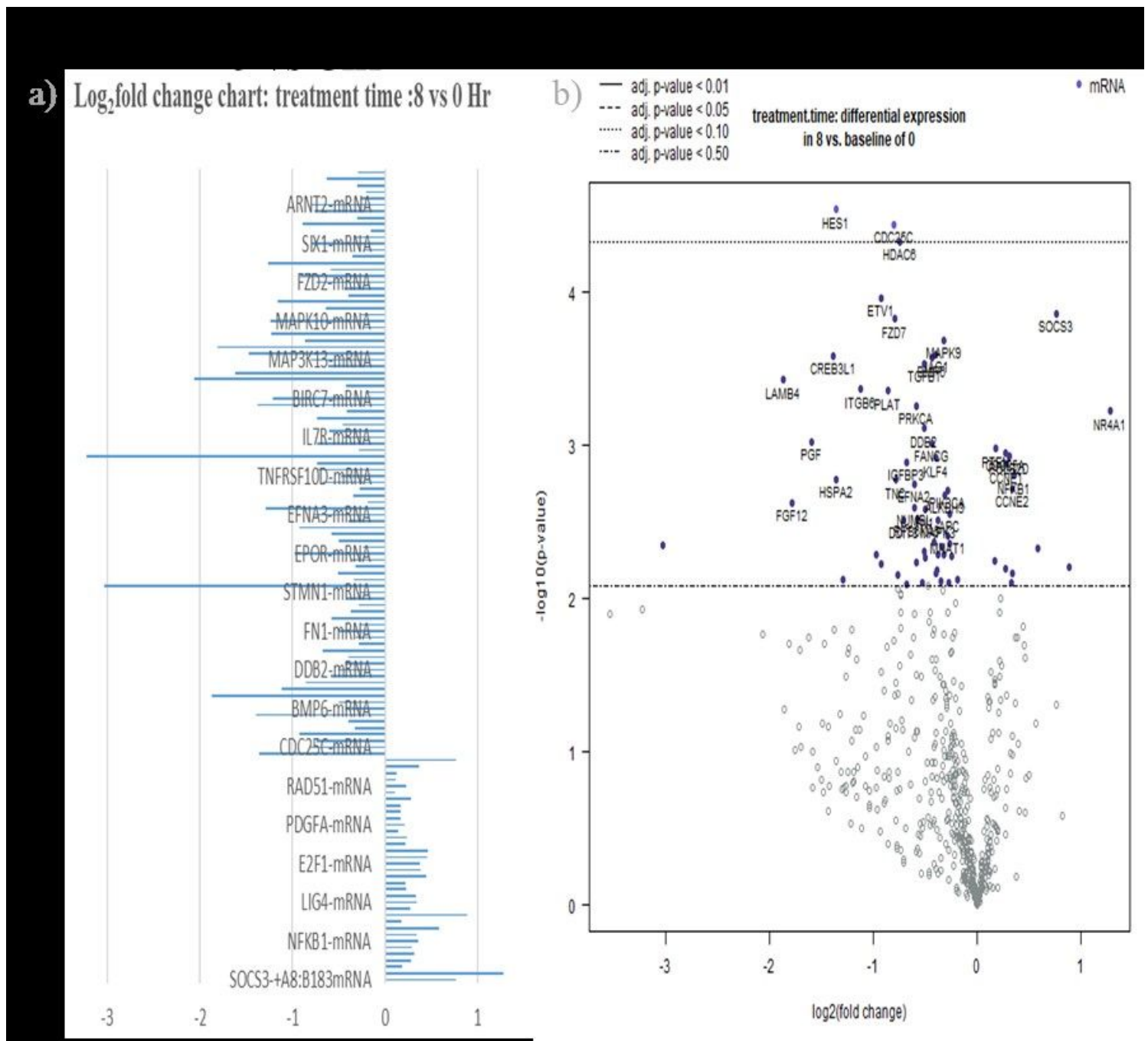


Figure 4

a) Log₂ fold change difference between 8 vs. baseline of 0 in HONE-1 cells treated with L1 (Huanglian) with differential expression comparing statistically significant differences ($P < .05$) in mRNA expression in the up and down regulated genes separately. A total of 35 up regulated genes show a positive fold change whereas 114 down regulated genes show a negative fold change. b) Volcano plot.

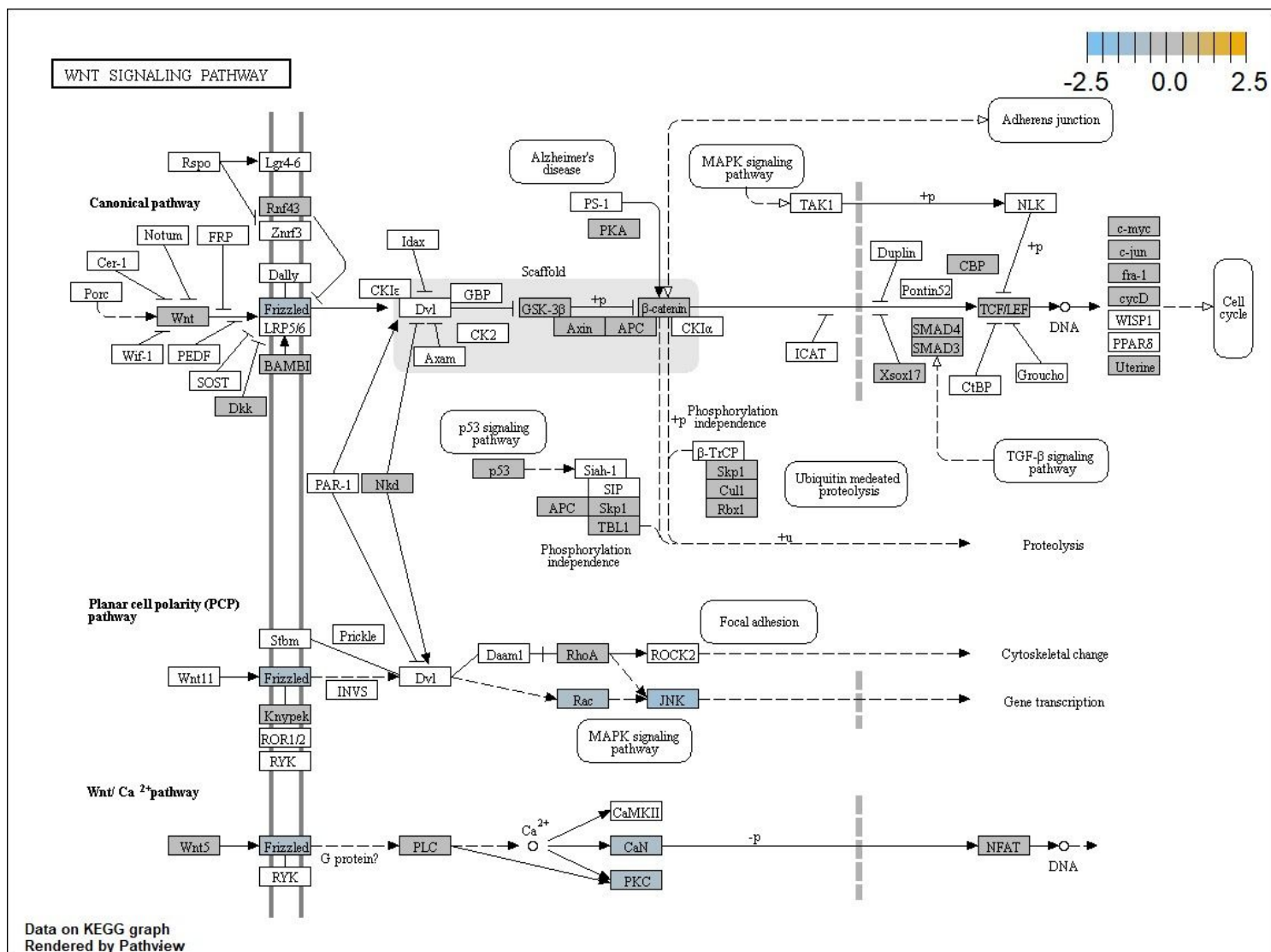


Figure 5

WNT signalling pathway (treatment time: differential expression in 8 vs. baseline of 0)

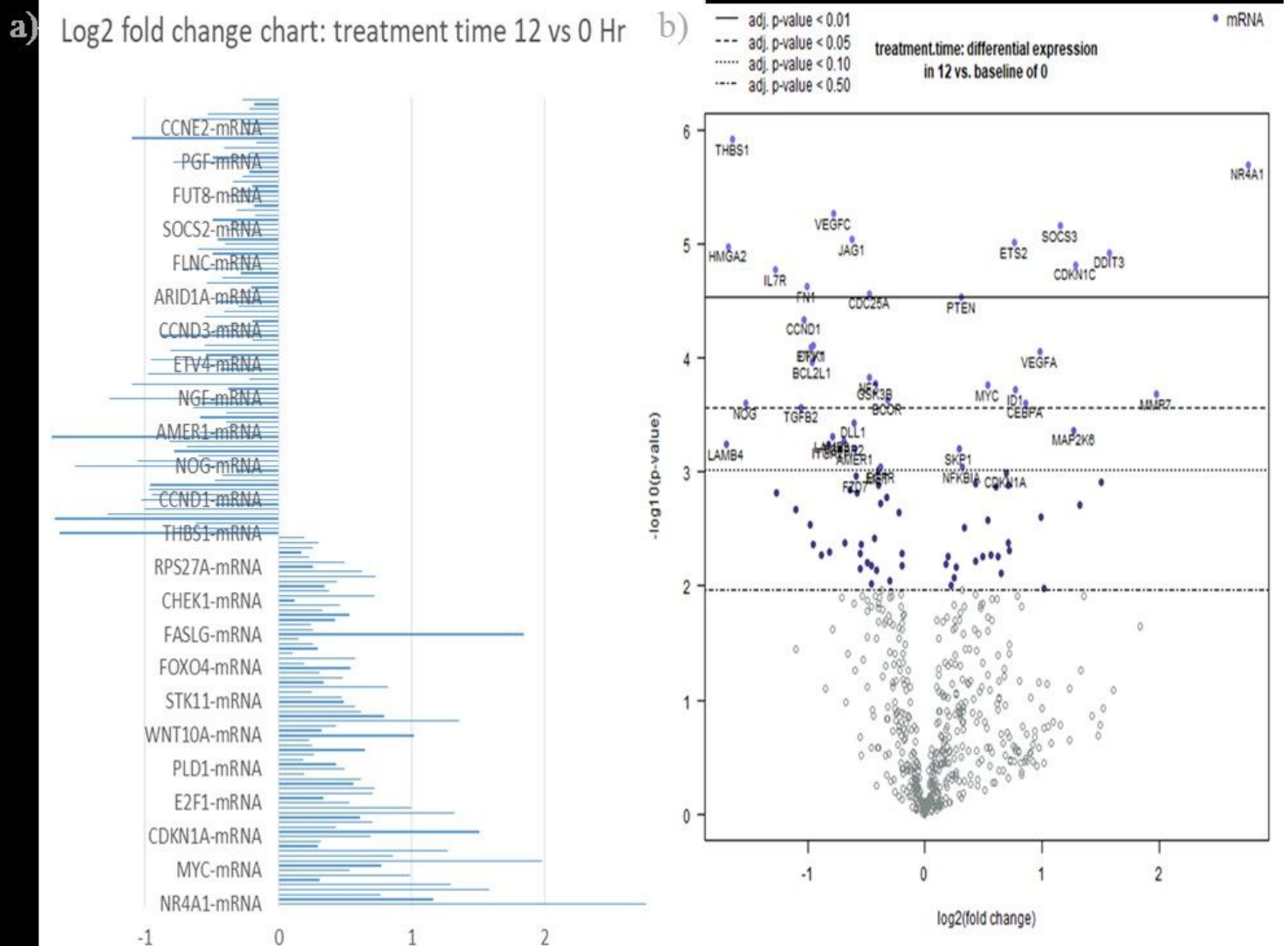


Figure 6

a) Log2 fold change difference between 12 vs. baseline of 0 in HONE-1 cells treated with L1 (Huanglian) with differential expression comparing statistically significant differences ($P < .05$) in mRNA expression in the up and down regulated genes separately. A total of 77 up regulated genes show a positive fold change whereas 91 down regulated genes show a negative fold change. b) Volcano plot

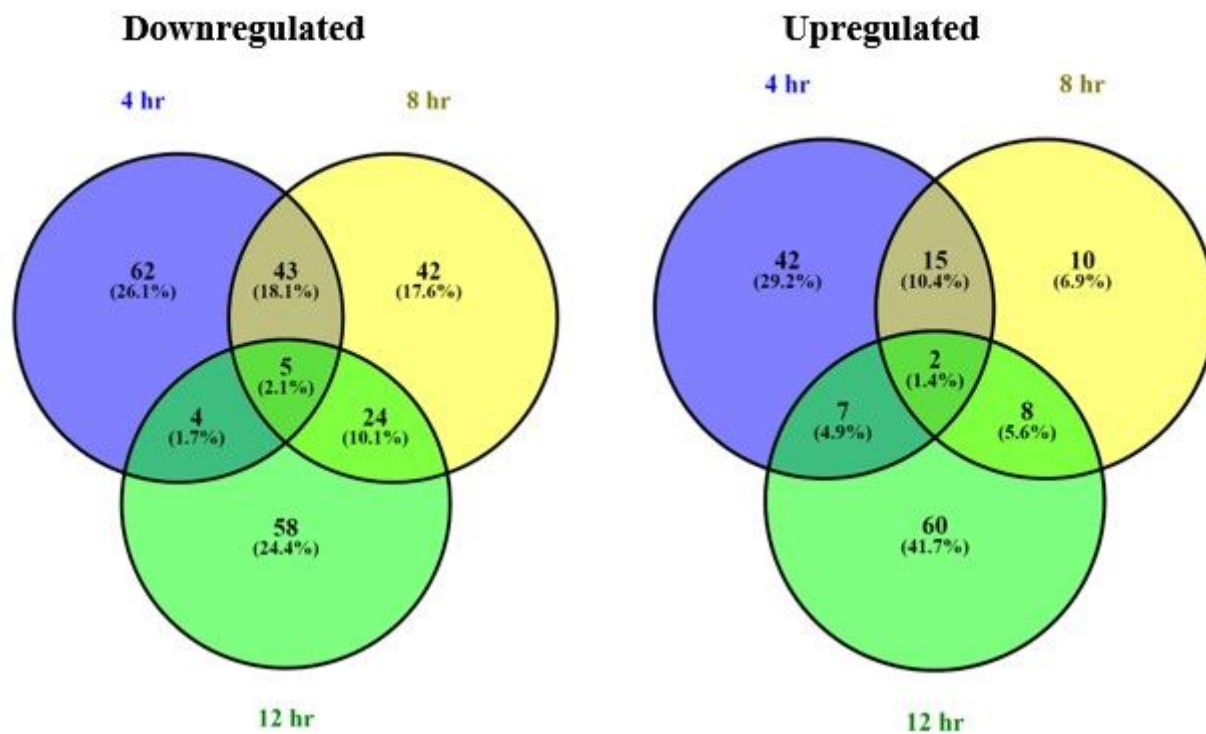


Figure 7

Venn diagram depicting the common genes differentially regulated in HONE-1 cells after treatment at different time point. Downregulated and upregulated genes in HONE-1 cells after exposure to L1.