Biological effects of the olive polyphenol, hydroxytyrosol: An extra view from genome-wide transcriptome analysis

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Abstract

Epidemiological and clinical studies have established the health benefits of the Mediterranean diet, an important component of which are olives and olive oil derived from the olive tree (*Olea Europea*). It is now well-established that not only the major fatty acid constituents, but also the minor phenolic components, in olives and olive oil have important health benefits. Emerging research over the past decade has highlighted the beneficial effects of a range of phenolic compounds from olives and olive oil, particularly for cardiovascular diseases, metabolic syndrome and inflammatory conditions. Mechanisms of action include potent antioxidant and anti-inflammatory effects. Further, accumulating evidence indicates the potential of the polyphenols and potent antioxidants, hydroxytyrosol and oleuropein in oncology. Numerous studies, both *in vitro* and in *vivo*, have demonstrated the anticancer effects of hydroxytyrosol which include chemopreventive and cell-specific cytotoxic and apoptotic effects. Indeed, the precise molecular mechanisms accounting for the antioxidant, anti-inflammatory and anticancer properties are now becoming clear and this is, at least in part, due to high through-put gene transcription profiling. Initially, we constructed phylogenetic trees to visualize the evolutionary relationship of members of the *Oleaceae* family and secondly, between plants producing hydroxytyrosol to make inferences of potential similarities or differences in their medicinal properties and to identify novel plant candidates for the treatment and prevention of disease. Furthermore, given the recent interest in hydroxytyrosol as a potential anticancer agent and chemopreventative we utilized transcriptome analysis in the erythroleukemic cell line K562, to investigate the effects of hydroxytyrosol on three gene pathways: the complement system, The Warburg effect and chromatin remodeling to ascertain relevant gene candidates in the prevention of cancer.
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Introduction

For centuries, the leaves and fruit of the olive tree (*Olea Europaea*) have been used for their medicinal properties. *Olea Europaea* has been the most important and extensively researched species. Native to the Mediterranean region, it is an important representative of the Mediterranean diet, which is rich in organic plant products and is typically centered around virgin olive oil. The earliest evidence of olive cultivation dates back 5000-6000 years ago in Syria, Palestine and Crete and spread into nearby countries from there [1, 2]. Today there are six natural sub-species of the olive tree [3]. *Olea Europaea* belongs to the *Oleaceae* family which comprises of around 24 genera and approximately 600 species of evergreen trees and shrubs including lilacs, jasmine and ash [3, 4]. To make inferences between the medicinal properties of species within the Oleaceae family compared to *Olea Europaea* we explored the evolutionary relationships between 9 species; *Osmanthus fragrans* (fragrant tea olive), *Phillyrea latifolia* (mock privet), *Chionanthus virginicus* (White Fringetree), *Chionanthus retuses* (Chinese Fringetree), *Fraxinus chinensis* (Chinesh Ash), *Fraxinus excelsior* (European Ash), *Syringa vulgaris* (lilac) and *Ligustrum obtusifolium* (border privet).

Recently, the minor phenolic constituents of *Olea Europaea* have been identified as the major derivatives associated with its health benefits, particularly the potent antioxidant hydroxytyrosol [5]. Hydroxytyrosol is found in the fruits and leaves of the olive tree and has been found to contribute to the health benefits associated with cardiovascular diseases, inflammation, atherosclerosis, hypertension, diabetes and platelet aggregation [6-11]. Although the evolutionary relationships of plants in the Oleaceae family has previously been investigated [3], a phylogenetic tree depicting the evolutionary divergence of plants containing hydroxytyrosol is yet to be explored. We visualized the evolutionary relationship between *Olea europaea* to other hydroxytyrosol producing species (Table 1) in order to ascertain potential similarities in the medicinal properties of species evolutionary similar and to identify potential candidates for further exploration for the treatment of disease.

To date, the antioxidant capabilities of hydroxytyrosol have been relatively well-characterised in both *in vitro* and *in vivo* investigations [6, 12, 13]. However, more recently numerous studies have also shown that hydroxytyrosol may be a potential anti-cancer agent [14-17]. In vitro studies in promyelocytic leukemic cells and colon adenoma, hydroxytyrosol has been shown to induce apoptosis, cause cell cycle arrest and limit cell growth and proliferation [18, 19]. *In vivo* investigations have reported hydroxytyrosol inhibits mammary tumor growth and proliferation rates by altering expression of genes related to apoptosis, cell cycle, proliferation, differentiation and transformation pathways [20]. Using genome-wide mRNA-Seq, we previously explored the molecular mechanisms and major genetic pathways altered by hydroxytyrosol in human keratinocytes and erythroleukemic K562 cell lines [21]. We reported alterations in numerous transcription factors in K562 cells following treatment with 100μM hydroxytyrosol which suggested possible mechanisms of action for its anti-cancer effects. In the present study, using transcriptome analysis and the online program MetaCore (GeneGo Inc.) for gene pathway analysis, we highlight the effects of hydroxytyrosol on the complement system, The Warburg effect and further explore epigenetic alterations involved in K562 cells to ascertain relevant gene candidates in the prevention of cancer.

Table 1. *Hydroxytyrosol containing plants used to reconstruct phylogenetic tree*

Scientific name	Common name	Common Gene sequence found for tree construction
Origanum dictamnus	Greek oridanum	matK, rbcL, trnL-trnF IGS
Teucrium polium	Felty Germander	matK, rbcL, trnL-trnF IGS
Ceratonia siliqua	Carob tree	matK, rbcL, trnL-trnF IGS
Ligustrum lucidum	Privet	matK, rbcL, trnL-trnF IGS
Ligustrum vulgare	Privet	matK, rbcL, trnL-trnF IGS, rps16
Olea europea	Olive	matK, rbcL, trnL-trnF IGS, rps16
Olea paniculata	Native Australian Olive	matK, rps16, trnT-trnL
Phoenix dactylifera	Dates	matK, rbcL, trnL-trnF IGS
Elaeodendron australe	Red Olive Berry	matK, trnL

Materials and methods

Phylogenetic tree construction

Six genera involving nine species collectively from the Oleaceae family were used for phylogenetic analyses as follows: *Olea Europaea* (olive tree), *Osmanthus fragrans* (fragrant tea olive), *Phillyrea latifolia* (mock privet), *Chionanthus virginicus* (White Fringetree), *Chionanthus retuses* (Chinese Fringetree), *Fraxinus chinensis* (Chinesh Ash), *Fraxinus excelsior* (European Ash), *Syringa vulgaris* (lilac) and *Ligustrum obtusifolium* (border privet). We compared the evolutionary divergence of the species based on their sequences collected from NCBI (www.ncbi.nlm.nih.gov). In addition nine hydroxytyrosol containing species were analysed to depict evolutionary descent of different genes form a common ancestor. Hydroxytyrosol species were characterised using the *matK* DNA region and included: *Origanum dictamnus* (Gree oridanum), *Teucrium polium* (Felty Germander), *Ceratonia siliqua* carob tree), *Ligustrum lucidum* (privet), *Ligustrum vulgare* (privet), *Olea europea* (olive), *Olea paniculata* (native australian olive), *Phoenix dactylifera* (dates) and *Elaeodendron australe* (red olive berry) (Table 1). Sequences were aligned using CLUSTAL W [22], phylogenetic trees where constructed using the UPGMA (Unweigthed Pair Group Method with Arithmetic Mean) method and MEGA 5 software [23]. The reliability of the tree was evaluated with a Bootstrap analysis.

Cell culture and treatment

Human chronic myelogenous leukaemia K562 cells, obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in complete-Royal Park Memorial Institute (RPMI) 1640 medium supplemented with 20mmol/L HEPES (pH 7.4; GIBCO-Invitrogen, Carlsbad, CA, USA), 10% (v/v) fetal bovine serum (FBS; In vitro Technologies, VIC, AUS), 2mM/L L-glutamine (GIBCO-Invitrogen), and 20 μg/mL gentamicin (GIBCO-Invitrogen). Cells were cultured in suspension in a humidified atmosphere of 5% (v/v) CO_2 at 37°C and were maintained in exponential growth phase. For maintenance, cells were passaged twice per week and seeded at ratios 1:10. For experiments, cells were seeded at cell densities of $5x10^5$ cells/mL in T75cm^2 vented culture flasks and treated 20μM or 100μM hydroxtyrosol (3,4-Dihydroxyphenyl ethanol; Cayman Chemical, Michigan, USA) for 24h at 37°C, 5% (v/v) CO_2 prior to experiments.

Cell viability

Cells seeded at densities of 7,500 cells/ in black flat bottom 96-well plates (Nalge Nunc, Penfield, NY, USA) were treated with 100μM hydroxytyrosol 24h at 37°C, 5% (v/v) CO_2. Following incubation with the test samples, Cell-Titer Blue reagent (20μl per 100μL) was added to each well and mixed gently for 5sec prior to 4h incubation at 37°C in a humidified atmosphere of 5% CO2. Fluorescence intensity (λex = 550nm and λem = 615nm) was determined using a Perkin Elmer Victor3 multilabel microplate counter (PerkinElmer, Waltham, MA, USA).

Apoptosis

Apoptosis was determined following 24h incubation with 100μM hydroxytyrosol prior to cell lysis and measuring caspase-

3/7 enzymatic activity with Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, WI, USA), according to the manufacturer's instructions. The fluorescence intensity (λex = 485nm and λem = 525nm) was determined on a Victor3 multilabel plate counter (PerkinElmer, Waltham, MA, USA).

Flow cytometric analysis

Flow cytometry was utilized for cell cycle analysis. Briefly, K562 cells were treated 100μM hydroxytyrosol for 24h before centrifugation to pellet the cells. Cells were washed with ice-cold PBS containing 2% (v/v) FBS twice and fixed overnight with continuous rotation in 70% (v/v) ethanol at 4°C. Cells where then stained in 100 μg/mL propidium iodide containing 1μg/mL RNase A (Qiagen Inc., Valencia, CA), overnight whilst on rotation at 4°C in the dark. Samples were transferred to FACS tubes and analysed by flow cytometry using a FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of cells in each phase of the cell cycle was analysed using Flowing Software (version 2.5.1, Perttu Terho, Turku Bioimaging, Finland).

Statistical analysis

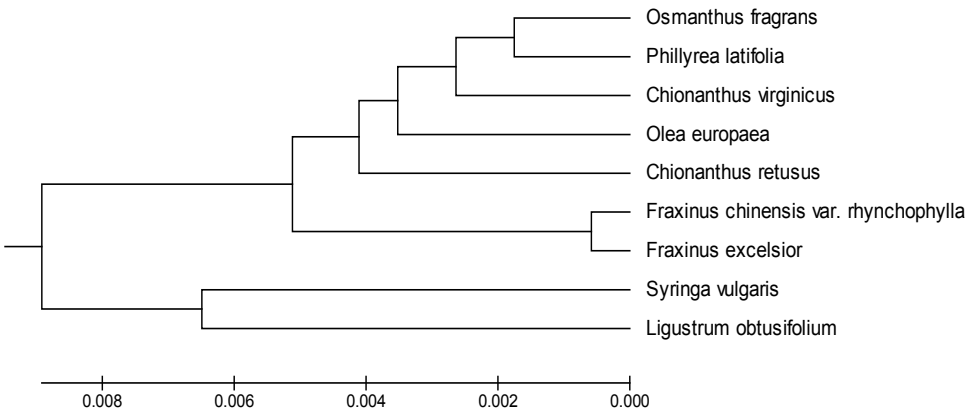
Statistical analysis was measured using Prism (version 6, GraphPad Software, San Diego, CA, USA). A student's t-test was employed to determine the statistical significance between untreated cells to cells treated with hydroxytyrosol. The level of significance was accepted at *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

RNA isolation, mRNA sequencing and pathway analysis

Total RNA was isolated from the cells using Trizol (Invitrogen) preparation following manufacturer's protocol. RNA integrity and concentration was measured with the RNA kit and MultiNA capillary electrophoresis system (Shimadzu). RNA sequencing library construction and sequencing was performed as previously described [24] Data from bioinformatic analysis was displayed as a fold change in mRNA expression. The online program MetaCore (GeneGo Inc. St. Joseph, MI, USA) was used for pathway analysis.

Results

Initially we collected DNA sequences from the NCBI database of species in the Oleaceae family and hydroxytyrosol containing species characterized by the *matK* gene and aligned them using CLUSTAL W to construct a phylogenetic tree using the UPGMA method with Bootstrap analysis to test statistical reliability (Fig. 1). Given the minor constituent of the olive tree hydroxytyrosol has been found to have therapeutic potential in various diseases including cancer we investigated the effects of 100μM of hydroxytyrosol on the erythroleukemic K562 cell line. We found hydroxytyrosol decreases cell viability and proliferation, increase caspase 3/7 induction of apoptosis and alters the cell cycle following a 24h incubation (Fig. 2). Finally we utilized genome wide next generation sequencing (mRNA-Seq) analysis to identify the top ten regulated pathways in K562 cells following 100μM hydroxytyrosol treatment. We found the pathways with highest relevance and lowest p-values for K562 cells treated with 100μM hydroxytyrosol were the Lectin-induced and classical complement complement pathways (Fig. 3). In addition, the alternative complement pathway was also found to be top ten most affected pathways (Fig. 3). We found 45% of genes had differential expression following hydroxytyrosol treatment, with 77% of these genes being up-regulated opposed to 23% being down-regulated. Of the genes being differentially expressed, complement component 2 was most highly up-regulated 9.19-fold followed by complement component 5, up-regulated 5.79-fold (Table 2).



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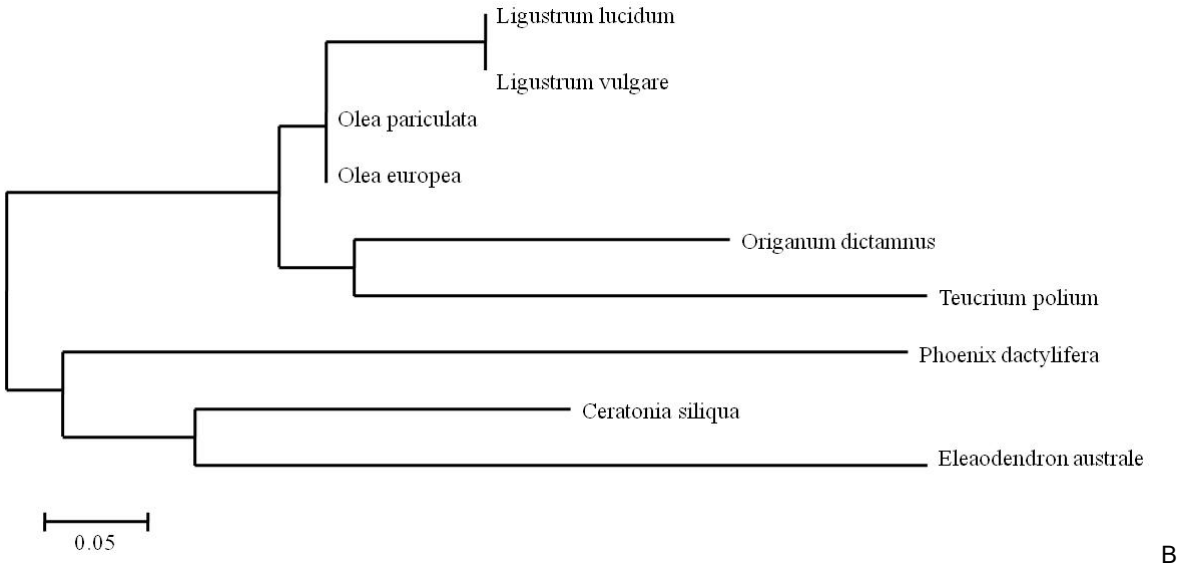

Figure 1. Phylogenetic relationships in the Oleaceae family (A) and species containing hydroxytyrosol (B). The scale shows the number of nucleotide substitutions/site. The rate of the substitution was considered to be constant.

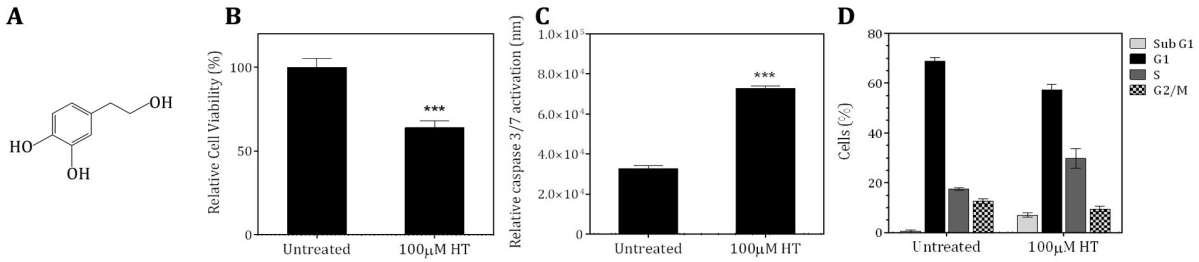

Figure 2. Biological effects of 100μM hydroxytyrosol in human leukemic K562 cells following 2h treatment. Structure of hydroxytyrosol, a minor phenolic constituent of the olive tree (A). Hydroxytyrosol induces cell death (B), apoptosis (C) and alters the cell cycle (D) in malignant erythrolekemic K562 cells. Mean ± standard deviations from a single experiment performed in duplicate are shown; total of 3 independent experiments tested.

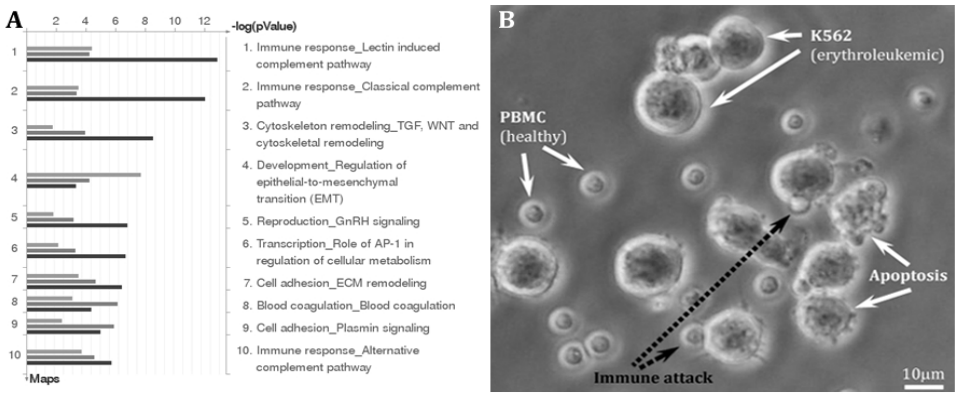

Figure 3. Top 10 gene pathways affected by 100μM hydroxytyrosol treatment for 24h in K562 cells (black bar) (A). Photomicrograph of human erythroleukemic K562 cells co-incubated with normal human peripheral blood mononuclear cells in ratios of 1:20 and treated with 20μM hydroxytyrosol for 24h highlights immune attack of malignant cells by natural killer cells (B).

Furthermore, given the emerging importance of the Warburg effect in cancer metabolism we investigated the Warburg-related gene expression changes in K562 cells treated with 100 μM hydroxytyrosol (Table 3). Pre-treatment of cells with hydroxytyrosol resulted in differential expression of 30% of these genes. In accordance with the experimental data a pro-apoptotic effect was observed following treatment with hydroxytyrosol. Finally, we explored the differential changes of transcription factors and enzymes related to epigenetic processes in K562 cells following hydroxytyrosol treatment. We show equal proportions of up- and down-regulated genes mainly involved in histone post-translational modifications (Table 4).

Table 2. *Genes involved in the complement system- significant up- and down-regulated genes in malignant erythroleukemic K562 cell treated with 100μM hydroxytyrosol*

Gene symbol	Gene name	Fold change
Up-regulated		
C2	complement component 2	9.19
C5	complement component 5	5.79
CLU	clusterin	3.03
ITGAM	alpha-M/beta-2 integrin(complement component 3 receptor 3 subunit)	2.16
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	2.04
CR2 (CD21)	complement component (3d/Epstein Barr virus) receptor 2	2.04
ITGAM	alpha-X/beta-2 integrin (complement component 3 receptor 3 subunit)	1.90
C1S	complement component 1, s subcomponent	1.75
C3	complement component 3	1.63
CD55 (DAF)	CD55 molecule decay accelerating factor for complement (Cromer blood group)	1.53
SERPING1 (CR1)	serpine peptidase inhibitor, clade G (C1 inhibitor) member 1	1.53
C5AR1	complement component 5a receptor 1	1.51
Down-regulated		
C4A	complement component 4A (Rodgers blood group)	1.53
C9	complement component 9	1.96
C1qRp	CD93 molecule	1.96
C1QL3	complement component 1, q subcomponent-like 3	4.06

Table 3. *Genes involved in the Warburg effect- significant up- and down-regulated genes in malignant erythroleukemic K562 cell treated with 100μM hydroxytyrosol*

Gene Symbol	Gene Name	Fold change
Up-regulated		
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	4.28
C12orf5	chromosome 12 open reading frame 5	3.57
BBC3	BCL2 binding component 3	2.66
VEGFA	vascular endothelial growth factor A	2.65
TP53	tumor protein p53	2.30
CASP7	caspase 7, apoptosis-related cysteine peptidase	2.27
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	2.22
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	2.04
CASP4	caspase 4, apoptosis-related cysteine peptidase	1.87
CASP8	caspase 8, apoptosis-related cysteine peptidase	1.62
Down-regulated		
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	-1.53
ACLY	ATP citrate lyase	-1.60
SREBF1	sterol regulatory element binding transcription factor 1	-1.69
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	-1.79
TFR2	transferrin receptor 2	-1.90

Table 4. *Genes related to epigenetic processes - the top up- and down-regulated genes in malignant erythroleukemic K562 cell treated with 100μM hydroxytyrosol*

Gene Symbol	Gene Name	Fold Change
UP		
SETD7	SET domain containing (lysine methyltransferase) 7	3.41
HDAC9	histone deacetylase 9	2.05
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	2.00
PRDM12	PR domain containing 12	2.00
PRDM9	PR domain containing 9	1.85
KDM4A	lysine (K)-specific demethylase 4A	1.71
KDM6B	lysine (K)-specific demethylase 6B	1.70
SMYD3	SET and MYND domain containing 3	1.60
SMYD3	SET and MYND domain containing 3	1.57
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	1.56
HDAC9	histone deacetylase 9	1.50
DOWN		
NCOA2	nuclear receptor coactivator 2	1.50
GTF3C4	general transcription factor IIIC, polypeptide 4, 90kDa	1.55
SETD1A	SET domain containing 1A	1.56
EHMT1	euchromatic histone-lysine N-methyltransferase 1	1.63
KDM4C	lysine (K)-specific demethylase 4C	1.66
MLL3	myeloid/lymphoid or mixed-lineage leukemia 3	1.68
HDAC7	histone deacetylase 7	1.76
PRDM15	PR domain containing 15	1.78
HDAC7	histone deacetylase 7	1.78
PRMT8	protein arginine methyltransferase 8	1.89
PRDM10	PR domain containing 10	1.92
PRDM6	PR domain containing 6	2.00
NPM2	nucleophosmin/nucleoplasmin 2	3.33

Discussion

Phylogenetic analysis

Given the importance of olives and in particular, hydroxytyrosol, we undertook brief phylogenetic analyses for two different purposes: 1) to identify hydroxytyrosol producing species and determine their phylogenetic relationship and 2) to evaluate the medicinal properties of members of the *Olecaecae* family to explore similarities and differences. We identified a number of diverse species of plants that produce hydroxytyrosol (Table 1, Fig. 1). Further, we explored the medicinal properties of members of the *Olecaecae* family (Fig. 1). Our findings indicated both distinct and complementary properties between species. *Osmanthus fragrans* from the genus Osmanthus is a sweet, fragrant olive, native to Asia from the Himalayas through southern China to Taiwan and southern Japan. *Osmanthus* tea has been used in Chinese medicine for the treatment of menopathies and in vitro assays using the dried leave and fruit extracts have shown neuroprotective, free-radical scavenging and antioxidant effects. *Chionanthus virginicus* (White Fringetree) is a tree native to the Eastern United States where the dried roots and bark are known to have been used to treat skin inflammations by Native Americans and crushed bark was used to treat sores of skin lesions. *Fraxinus chinensis* (Chinesh Ash) native to central and east Asia and *Fraxinus excelsior* (European Ash), native to western palearctic are from the genus Fraxinus and studies have shown plants extracts to have anti-bacterial, antu-allergic, anti-cancer and antioxidantr properties. *Syringa vulgaris* (lilac), native to the Balkan Peninsula is an aromatic ornamental plant with recorded anti-inflammatory properties. Finally, *Ligustrum obtusifolium* is a species of privet native to Japan, Korea and China and has reported to be antihyperglycemic. Overall, our findings indicate that hydroxytrosol is present in a number of species with distinct medicinal properties. Similarly, different members of the Oleacae family have a range of medicinal properties. In this context it will be interesting to explore the therapeutic benefits of combinations of antioxidants. For example, combinations of hydroxytyrosol and resveratrol from vitis Vinifera (grapes), a well known SIRT1 agonist, may provide additive beneficial health effects.

Complement system regulation

Adequate regulation of the complement system is essential, as uncontrolled complement activation can cause injury to host cells. Control of the complement system is maintained by a group of cell surface and circulating proteins referred to as complement regulatory proteins. Specific cell types can upregulate or downregulate the expression of genes for these proteins in response to a variety of signals. In the complement cascade, the complement regulatory proteins can act at different points. One important consequence of this is that tissues can modulate local activation of the complement system through alternations in the density of complement regulatory proteins. In response to hydroxytyrosol, the regulatory protein DAF was up-regulated 1.53-fold, altering the cells ability to respond to infection (Table 2).

Complement component C2 functions as a key regulator in the early activation phase of the classical pathway and participates in the formation of the classical pathway C3 convertase C4b2a [25]. C2 is also a critical component of the lectin pathway [26]. Activation of component C3 is central to the three complement pathways and results in inflammation. In this study, K562 cells treated with hydroxytyrosol were found to have elevated C2 (9.19-fold) and elevated C3 (1.63-fold) (Table 2). These pathways converge in the formation of C3 convertase, and the protease activity of the short-lived C3 convertase complexes determines opsonisation of pathogens and altered host cells, playing a pivotal role in raising complement-mediated immune responses [25].

The complement component C5 is cleaved to give rise to C5a, a potent pro-inflammatory molecule and C5b, which participates in the formation of the membrane attack complex (MAC). C5a is essential for the recruitment and activation of inflammatory cells such as granulocytes and it mediates its effect primarily by binding a G-protein coupled receptor (C5AR) [27]. In response to hydroxytyrosol, C5 and C5AR were up-regulated 5.79 and 1.51-fold respectively in K562 cells (Table 2). By acting as an anaphylatoxin, C5a promotes local inflammation around the cell [27]. Its upregulation in the K562 cancer cell line suggests an increased likelihood for the cancer cell to be cleared away by the inflammatory response.

Alpha-X/beta-2 integrin and alpha-M/beta-2 integrin were also upregulated in the K562 cell line when treated with 100μM Hydroxytyrosol, 2.04 and 2.16-fold respectively (Table 2). Integrins such as this, promote adherence to phagocytes, ultimately enhancing phagocytosis of the cell [28]. The selective increase in these integrins in the K562 line may imply the role of hydroxytyrosol in activating the innate immunity to remove the cancer cell selectively. Ultimately, this study indicates that, hydroxytyrosol is suggestive of the formation of the MAC as the end product of all three complement pathways, leading to the lysis and death of the cancer cell. To further these findings, in future studies, more hydroxytyrosol affected pathways need to be studied with detailed studies on complement components associated with diseases or associated with other pathways. In particular, investigation into the MAC inhibitors such as Clusterin as well as the effect of hydroxytyrosol in normal cell lines may be required.

The Warburg effect and post-translation modifications

Typically normal cells metabolize glucose by glycolysis and oxidative phosphorylation. In contrast, even in the presence of oxygen, proliferating and cancer cells exhibit an increased uptake of glucose and increased rate of glycolysis and predominantly undergo lactic acid fermentation. This phenomenon is known as the Warburg effect in honor of Otto Warburg who first made these observations in the 1920s. After a period of general lack of interest, the field has recently become an intense focus of research. Indeed it is becoming evident that there is a strong correlation between gene expression and cancer metabolism. We have utilized mRNA-Sequencing and explored a “Warburg gene-set” which we derived from the publicly available Cell Signalling Technology pathway map http://www.cellsignal.com/reference/pathway/warburg_effect.html). We evaluated changes in the expression of Warburg-related genes in human erythroleukemic K562 cells following treatment with 100μM hydroxytyrosol for 24h.

In accordance with the experimental data (Fig. 1) a pro-apoptotic effect was observed following treatment with hydroxytyrosol. Preliminary interrogation of the data shows significant up-regulation in apoptosis regulator C12orf5 (chromosome 12 open reading frame 5) by 3.57-fold in K562 cells following treatment with hydroxytyrosol. In addition, a strong pro-apoptotic affect was observed via the up-regulation of caspases 4, 7 and 8 and the p53 up-regulated modulator of apoptosis BCL2 binding component 3 (BBC3), by a fold change of 1.87, 2.01, 1.62, and 2.29 respectively. Further, metabolic regulators PFKFB4 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4), PFKB2 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) and ACLY (ATP citrate lyase) were down-regulated by 1.79, 1.53 and 1.59 fold change respectively (Table 3). Importantly, down-regulation of the iron-transporter (transferrin receptor 2; TRF2), which is involved in iron hemostasis, growth and proliferation, was significantly down-regulated by hydroxytyrosol (1.9-fold). Further, investigations into the differential expression of transcription factors and enzymes involved in post-translation modifications in K562 following treatment with 100μM hydroxytyrosol found equal proportions of up- and down-regulated genes (Table 4). SET domain containing lysine methyltransferase 7 (SETD7), SET and MYND domain containing 3 (SMYD3) and lysine(k)-specific demethylase 4A and 6B (KDM4a, KDM6b) were all significantly up-regulated 3.41, 1.6, 1.71 and 1.7 -fold respectively. This may suggest that the SET domain, or changes in histone methylation, is target for hydroxytyrosol. Overall, phylogenetic and transcriptome investigations into hydroxytysol provide insights into the mechanism of its action as an anti-cancer agent. Our findings will be explored further in a variety of normal, transformed and malignant cell lines

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