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Effects of Resveratrol on Gene Expression in Renal Cell Carcinoma

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Abstract

Studies have shown that Resveratrol (RE) can inhibit cancer initiation, promotion, and progression. However the gene expression profile in renal cell carcinoma (RCC) in response to RE treatment has never been reported. To understand the potential anticancer effect of RE on RCC at molecular level, we profiled and analyzed the expression of 2059 cancer-related genes in a RCC cell line RCC54 treated with RE. Biological functions of 633 genes were annotated based on biological process ontology and clustered into functional categories. Twenty-nine highly differentially expressed genes in RE treated RCC54 were identified and the potential implications of some gene expression alterations in RCC carcinogenesis were identified. RE was also shown to inhibit cell growth and induce cell death of RCC cells. The expression alterations of selected genes were validated using reverse transcription polymerase chain reaction. In addition, the gene expression profiles under different RE treatments were analyzed and visualized using singular value decomposition. The findings from this study support the hypothesis that RE induces differential expression of genes that are directly or indirectly related to the inhibition of RCC cell growth and induction of RCC cell death. In addition, it is apparent that the gene expression alterations due to RE treatment depend strongly on RE concentration. This study provides a general understanding of the overall genetic response of RCC54 to RE treatment and yields insights into the understanding of the cancer preventive mechanism of RE in RCC.

Keywords

microarray; resveratrol; renal cell carcinoma

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‡These authors made an equal contribution. L.L. conceived, initiated and helped plan the project, P.S. performed most of the cell-based experiments and T.S. analyzed the gene expression data, performed RT-PCRs and prepared the manuscript.

NOTE ADDED IN PROOF

Liou L, et al., submitted has now been accepted for publication and is in press at BMC Urology. The correct reference is:

Liou LS, Shi T, Duan ZH, Sadhukhan P, Der SD, Novick AC, et al. Microarray gene expression profiling and analysis in renal cell carcinoma. BMC Urol 2004; In press.

INTRODUCTION

Cancer is the largest single cause of death in human, claiming over 6 million lives each year worldwide. Renal cell carcinoma (RCC) accounts for 3% of all malignancies with about 30,000 new cases and 12,000 deaths each year in the United States. RCC is the most common cancer in adult kidney and the most lethal cancer in urinary system.¹ Previous studies have indicated that RCC is one of the most therapy-resistant cancers. It responds very poorly or not at all to chemotherapy, hormonal therapy and radiation therapy.^{1,2} Even for the immunotherapy, regarded to be the best therapy for RCC, the response rate is only 10–15% and the response is mostly partial.¹

Chemoprevention emerged as a new strategy to fight cancer, seeks to prevent and reduce cancer risk by ingestion or administration of natural or synthetic chemicals. The chemicals are able to delay, suppress or reverse the initiation, promotion and progression that are associated with carcinogenesis and are of low toxicity to human. In the search for new cancer chemopreventive agents, many plant extracts have been evaluated for their chemopreventive activities against cancers over the past few years.^{3–10}

Resveratrol (RE) is a polyphenolic compound (trans-3, 5, 4'-trihydroxystilbene). The chemical structure of the compound is shown in Figure 1. Recent study has shown that RE can serve as an effective chemopreventive agent to inhibit the initiation, promotion and progression of many different cancers.¹¹ RE is found primarily in the skin of grapes and is relatively abundant in red wines. The high levels of RE in the grape skin are synthesized in response to fungal infections. It has been reported that the chemopreventive effect of RE is partly due to its antioxidant activities and its inhibitory effect on two cyclooxygenases COX1 and COX2.^{12,13} The cyclooxygenases catalyze the conversion of arachidonic acid to pro-inflammatory substances such as prostaglandins that can stimulate tumor cell growth and suppress immune surveillance.

The emergence of DNA microarray technology made it possible to investigate the expression of thousands of genes simultaneously^{14–17}. Recently, microarray gene expression profiling has been performed to identify gene expression patterns for many solid and hematological malignancies such as colon cancer, breast cancer, kidney cancer, prostate cancer, leukemia, and lymphoma.^{18–24} However, only a few studies have been carried out to investigate the alterations of gene expression profiles in cancers such as breast cancer,²⁵ colon cancer,²⁶ leukemia,²⁷ ovarian cancer,²⁸ and prostate cancer cells²⁹ in response to chemopreventive agents. To the best of our knowledge, the gene expression profile in RCC in response to RE has never been reported.

To understand the chemopreventive effect of RE on RCC at molecular level, we profiled the expression of 2059 cancer-related genes in a RCC cell line RCC54 in response to different RE treatments using oligonucleotide arrays.

MATERIALS AND METHODS

Growth and RE Treatment of RCC Cell Line

The metastatic RCC cell line RCC5430 was obtained from Memorial Sloan-Kettering Cancer Center. Primary RCC culture at passage two was isolated from a primary RCC tumor. RCC cells were grown in RMPI with glutamine and containing 10% FBS. Cells were treated with high performance liquid chromatography purified RE by adding stock solutions of RE in ethanol to RMPI 1640 to the concentration of 25 μ M or 50 μ M and incubating for 12 hours or 24 hours. Control RCC54 cells were treated by adding ethanol alone to RMPI 1640. For cell growth experiments, RCC cells and normal renal cells of proximal tubule

origin were treated with ethanol (0.2%) only or RE at 50 μ M for 72 hours. A monolayer of treated and nontreated cells was visually monitored and photographed.

RNA Extraction and Microarray Experiments

Cell culture media RPMI 1640 was removed from cell monolayers by aspiration and rinsed three times with phosphate-buffered saline. Total RNA was immediately isolated by TRIzol reagent following the manufacturer's procedures (Invitrogen, Carlsbad, CA). Double-stranded cDNAs were synthesized from 10 μ g of total RNA using SuperScript Choice double-stranded cDNA synthesis kit from Invitrogen following the manufacturer's protocol. cDNAs were purified by phenol/chloroform extraction and ethanol precipitation. Biotin-labeled cRNAs were synthesized by an in vitro transcription reaction using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). cRNAs were purified from the in vitro transcription reaction using RNeasy Mini kit (Qiagen, Valencia, CA). The fragmentation of biotin-labeled cRNAs and hybridization of the fragments to the Human Cancer G110 Oligonucleotide Arrays (Affymetrix, Santa Clara, CA) were carried out by the DNA Expression Array Core Facility at Case Western Reserve University (Cleveland, OH). Hybridization was done following standard Affymetrix protocols. The chips were washed and stained according to the Affymetrix protocol Midi-3 Euk2v3, and scanned using a Hewlett-Packard GeneArray scanner (Hewlett-Packard, Palo Alto, CA) with a 570 nm filter and a pixel size of 3 μ M. The same total RNA that had been used for the microarray experiments was used for RT-PCR experiments.

Data Preprocessing

Raw data were acquired using Microarray Suite 5.0 software of Affymetrix and normalized following a standard practice of scaling the trimmed mean of all gene signal intensities to a common arbitrary value, defaulted to 1000. The default parameters for Human Cancer G110 were used throughout the analyses. Only genes that were not labeled as "Absent" in all experimental conditions using the default parameter were considered for further analysis. The data was renormalized so that the trimmed mean, previously 1000, was rescaled to 1.00.

Functional Clustering Analysis

To analyze the expression profiles of genes in different biological functional categories, six hundred and thirty-three genes were annotated for biological process using the software GeneSpring 5.1 from Silicon Genetics (Redwood City, CA). The ontology is based on the description of the Gene Ontology Consortium.³¹ The annotated genes were then categorized into functional categories and analyzed based on the gene expression levels.

RT-PCR

cDNAs were synthesized by reverse transcription of 2.5 μ g total RNA using oligo(dT)₁₂₋₁₈ and SuperScript II RNA H⁻ reverse transcriptase (Invitrogen). Using the same cDNA preparation as template, DNA fragments of CXCR4, CYP1B1, IGFBP5, MDM2-A, GADD45, RelB, TNFAIP and TRAF-1 were amplified by 30 PCR cycles using Tag polymerase (Invitrogen). Normalization was made using α -tubulin. Primers used for the PCR amplifications are shown in Table 2. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized using the FluorChem 8000 imaging system (version 2.0) (Alpha InnoTech, San Leandro, CA). The intensities of the PCR product bands were quantified using ImageQuant version 1.2 (Molecular Dynamics). The intensities of individual PCR product bands for different genes were first normalized by dividing their intensities by the intensities of their corresponding α -tubulin bands. These normalized intensities of individual PCR product bands from RCC54 treated with RE at 50

μg were then divided by the normalized intensities of the corresponding PCR product bands from control RCC54 to obtain fold changes in mRNA levels in RE treated RCC54.

Singular Value Decomposition

Singular value decomposition is a very powerful method to analyze and compare the subspaces associated with a matrix. It has been widely used in data compression and visualization.³² Recently there have been many applications of SVD to analyze microarray gene expression data.^{33–37} Following the notation of Golub and van Loan, the SVD of a real m -by- n ($m \geq n$) matrix A can be written as:

$$A=U\Sigma V^T,$$

where $U = [u_1, u_2, \dots, u_n] \in \mathbb{R}^{m \times n}$ and $V = [v_1, v_2, \dots, v_n] \in \mathbb{R}^{n \times n}$ are column-orthogonal matrices and $\Sigma = \text{diag}(\sigma_1, \dots, \sigma_n) \in \mathbb{R}^{n \times n}$ is a diagonal matrix and $\sigma_1 \geq \dots \geq \sigma_r > \sigma_{r+1} = \dots = \sigma_n = 0$. The vectors u_i and v_i are the i th left and right singular vectors respectively, σ_i are the singular values of A , and r is called the rank of A . Based on the structure of the decomposition, the SVD expansion can be readily obtained

$$A = \sum_{i=1}^r \sigma_i u_i v_i^T.$$

The magnitudes of singular values indicate how close a given matrix A is to a matrix of lower rank. In the gene expression data analysis, each column of A represents the expression profile of a corresponding sample and each row represents the transcriptional response of a specific gene. The singular values indicate how well a lower dimensional linear projection of the expression data can represent the original data. The projection onto a subspace spanned by the first p left/right singular vectors can be described by

$$A_p = \sum_{i=1}^p \sigma_i u_i v_i^T.$$

Analyzing and visualizing a resulting lower dimensional projection can provide a great insight into understanding the inherent structure of the original data. In this study, the gene expression data was projected onto a 2-D subspace spanned by the first two left singular vectors.

RESULTS AND DISCUSSION

The gene ontology tree that describes the patterns of gene expression alterations in RCC cell line treated with RE at 50 μM for 24 hours is shown in Figure 2. Six hundred and thirty-three genes were annotated for their biological functions based on gene ontology. These annotated genes were associated with 62 functional categories. There are three numerical numbers following the name of each gene category. The first integer represents the number of genes in the category. The first number in parentheses stands for the number of genes in the category that are at least two-fold upregulated after RE treatment and the second number represents the number of genes in the category that are at least two-fold downregulated after RE treatment. As shown in Figure 2, about 18.5% of the 633 annotated genes were

differentially expressed by at least two-fold after RE treatment, and some interesting alterations in expression patterns can be clearly observed.

To identify the gene categories on upregulation/downregulation dominated pathways, we performed binomial tests on the gene categories at the third level of the gene ontology tree. In the tests, we assumed that each gene had a 50% chance to be upregulated by two-fold and the same chance to be downregulated by two-fold. The cell growth category was found to be significantly downregulated with a significance level of 0.03. The protein kinase cascade group was found to be significantly upregulated with a significance level of 0.008. In addition, necrosis appears to be in the upregulation dominated pathways with a significance level of 0.1. On the other hand, in many other groups such as metabolism, proliferation and cell cycle, the numbers of upregulated genes and downregulated genes are roughly the same, revealing diverse variations (likely including some random variations) in the gene expression levels even within a functional category. The fact that RE treatment of RCC cells results in diverse variations as opposed to random variations is probably best exemplified by the observations that RCC cells but not normal renal cells are potentially affected by the compound (see Fig. 4).

Table 1 presents twenty-nine genes that were highly differentially expressed in RCC54 treated with RE at 25 μM and 50 μM with a fold change greater than five at either concentration. Among them, eighteen were upregulated while eleven were downregulated. The twenty-nine highly differentially expressed genes belong to many different biological functional categories including signal transduction, metabolism, cell proliferation, cell adhesion, and cell growth. Furthermore, as shown in (Table 1), the expression levels of all the eighteen upregulated genes increase as the concentration of RE increases, and the expression level of all but one of the eleven downregulated genes decrease as the concentration of RE increases. These results demonstrate a dose effect of RE on the regulation of gene expression in RCC.

Vitamin D receptor (VDR) is one of the upregulated genes in the signal transduction category. VDR is a nuclear receptor that is closely related to the thyroid hormone receptors.³⁸ Once activated by the binding of vitamin D, VDR interacts with target DNA in a sequence-specific manner and activates target gene expression to produce a variety of biological effects. Our previous study (Liou L, et al., submitted) has shown that the expression of VDR was downregulated in the RCC tissue by more than 10-fold compared with that in the normal kidney tissue (Liou L, et al., submitted). This difference between downregulation in RCC tissue and upregulation in RCC cell line after treatment with RE reveals some anti-tumor activities of RE on RCC. Another interesting upregulated gene in this category is Epstein-Barr virus-induced protein (TRAF-1). TRAF-1 is a member of TNF receptor associated factors. The hetero-dimerization of TRAF-1 and TRAF-2 is required for TNF- α -mediated activation of NF- κB . It has been reported that RE has very interesting concentration-dependent effects on NF- κB activities in prostate cancer. RE treatment inhibited the activation of NF- κB at nM concentrations, but activated NF- κB at μM level concentrations.⁵ The RE concentrations used in this study are at μM level. It appears that the effect of TRAF1 upregulation in RCC by RE agrees with the NF- κB activation found in prostate cancer exposed to RE at the same concentration level.

The two downregulated genes in the signal transduction category are glial cell line-derived neurotrophic factor (GDNF) family receptor alpha 2 (GFRA2) and hemopoietic cell protein-tyrosine kinase (HCK). GFRA2 is a cell surface receptor for GDNF and mediates the activation of the Ret tyrosine kinase receptor and is important for the survival of central dopaminergic neurons, motor neurons, and several other neurons in the central and peripheral nervous systems.³⁹ HCK is a protein-tyrosine kinase expressed mainly in

hematopoietic cells. It plays a major role in activating the respiratory burst. Our previous study has shown that HCK was upregulated by about ten-fold in the RCC tissue (Liou L, et al., submitted). The downregulation of HCK in RCC54 may suggest the anti-tumor activities of RE on RCC.

A gene involved in the metabolism of drugs, dioxin-inducible cytochrome P450 (CYP1B1), was repressed by RE. CYP1B1 encodes a monooxygenase, which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. It has been reported that CYP1B1 is over-expressed in a wide variety of human tumors including leukemia, RE undergoes metabolism by CYP1B1 to give a metabolite known as anti-leukaemic agent piceatannol.⁴⁰ Furthermore, our previous work has shown that CYP1B1 is upregulated by more than six-fold in RCC tissue (Liou L, et al., submitted). The downregulation of CYP1B1 suggests a potential role of RE in maintaining the expression level of CYP1B1 at basal expression levels.

Transmembrane carcinoembryonic antigen (BGP_a) in cell adhesion category was expressed at a significant higher level (more >24-fold when the concentration of RE was at 50 μ m). BGP_a is a main antigen in the carcinoembryonic antigen cluster that mediates the binding of granulocytes to endothelial E-selectin. It has been reported that the loss or reduced expression of the BGP_a adhesion molecule is a major event in colorectal carcinogenesis.⁴¹ BGP_a was also shown to exhibit antiangiogenic properties in in vitro and in vivo angiogenesis assays.⁴² The upregulation of BGP_a in RCC54 treated with RE suggests that a potential inhibition effect of RE on RCC carcinogenesis through anti-angiogenesis. Mucin 1 (MUC1) in cell adhesion is a membrane-associated glycoprotein. It was downregulated in RCC54 by about 6 fold. MUC1 is known to interfere with both cell-cell and cell-matrix adhesion. The over-expression of MUC1 has been associated with poor prognosis of many different cancers including RCC.^{43,44}

In the cell growth category, more genes were downregulated as expected. Among them, the chemokine (C-X-C motif) receptor 4 (CXCR4) has been intensively studied.⁴⁵ CXCR4 encodes a G-protein-coupled chemokine receptor, which has been implicated in invasive tumor growth. It has been reported that this gene was also downregulated by wild type tumor suppressor gene von Hippel-Lindau (VHL) in normal kidney, but was over-expressed in clear cell RCC, where VHL was inactivated due to mutations. High level CXCR4 expression has also been associated with poor survival outcomes of RCC patients, possibly through its influence on the metastatic behavior of RCC. The down-regulation effect of RE on CXCR4 appears to be consistent with the inhibitory effect of RE on cell growth and suggests that RE has the suppressive effect on the tendency of RCC metastasis.

GADD45 in the cell growth category encodes a growth arrest and DNA-damage-inducible protein. It is upregulated by more than 14 fold in RCC54 treated with RE at 50 μ M. The encoded protein responds to environmental stresses by mediating activation of the p38/Jun N-terminal Kinase pathway. The stressful growth arrest conditions and DNA damage-induced transcription of GADD45 are mediated by both p53-dependent and p53-independent mechanisms.⁴⁶ The observed upregulation of GADD45 reveals the inhibitory effect of RE on the growth of cells in RCC. Activating transcription factor 3 (ATF3) is another cell growth associated gene that was significantly upregulated in RCC54 treated with RE at 50 μ M. It has been reported that the over-expression of ATF3 protein repressed cell growth by slowing down progression of HeLa cells from G₁ to S phase.⁴⁷ These results reveal the role of RE in inducing cell growth inhibition and demonstrate the potential of RE as a chemopreventive agent.

To validate the gene expression alterations obtained, reverse transcription polymerase chain reaction (RT-PCR) was performed on eight selected genes including CXCR4, CYP1B1, insulin-like growth factor-binding protein 5 (IGFBP5), mouse double minute 2-A (MDM2-A), GADD45, v-rel reticuloendotheliosis viral oncogene homolog B (RelB), tumor necrosis factor α inducible protein 3 (TNFAIP3) and TRAF-1. As shown in Figure 3, the results were largely consistent with the microarray results except for RelB for which the RT-PCR results did not show a clear difference at the mRNA level between RE-treated and control RCC54.

In addition, effects of RE on RCC cell growth were also evaluated and one example is shown in Figure 4. The results reveal significant anti-proliferation and cell death induction effects of RE on both the primary RCC cells and RCC cell lines including RCC54. One set of the cell growth data was shown in Figure 4. The effect of RE on the growth of normal renal cells derived from proximal tubule cells (common origin of RCC) was not significant. Similar results were also observed with other RCC cell lines (RCC45 and RCC13) and another primary RCC culture (data not shown). These results suggest the chemotherapeutic potential of RE in the treatment of human renal cell carcinoma.

To further investigate the RE effect on the gene expression of different functional categories and the expression of individual genes, the overall gene expression profiles were studied and visualized using singular value decomposition (SVD). SVD is a very powerful visualization method to analyze and compare the subspaces associated with a matrix and has been widely used in data compression and visualization (see Materials and Methods for detail). Recently SVD has been increasingly applied to analyze microarray gene expression data.

In this study, the gene expression data of RCC54 treated with RE at five different conditions were first preprocessed and the genes with very low intensity levels (labeled as “Absent”) across all the samples were eliminated. As a result, 1230 genes remained. Furthermore, the expression levels of each gene were normalized to zero mean and unit variance across the samples. The resulting expression matrix was then decomposed using SVD. The singular values {0.3329, 0.2607, 0.2171, 0.1894, 0.0000} form a spectrum (the last zero was a result of the normalization process). It is apparent from the magnitude of the values that the first two singular vectors account for about 60% of the total variance in the expression data. The projections of the five expression profiles onto the first two singular vectors are displayed in Figure 5. It appears that the first singular vector represents the genes whose expression levels reflect the effect of different RE concentration. The second singular vector seems to represent the group of genes whose expression levels changes to a certain degree with the duration of RE treatment. As shown in Figure 5, the sample treated with RE for 24 hours at 25 μ M is clustered together with the sample treated for 24 hours with ethanol alone, indicating a weak effect of RE on gene expressions at this concentration. The result is consistent with the result obtained from Table 1 about the dose effect of RE on gene expressions.

In summary, we profiled the expression of 2059 cancer-related genes in RE treated RCC54. The biological functions of 633 genes were annotated based on biological process ontology and clustered into functional categories. The analysis of the expression profiles of genes in the annotated functional categories provides insights into biological pathways of RCC that are affected by RE treatment. Twenty-nine highly differentially expressed genes in RE-treated RCC54 were identified and the potential implications of some significant gene expression alterations in RCC carcinogenesis were discussed. The results indicate the significant effect of the RE concentration on gene expression profiles. Results also show that RE inhibits RCC cell growth and induce cell death. The expression alterations of selected genes were verified using reverse transcription polymerase chain reaction. Although the specific roles of some individual RE regulated genes involved in cell growth, cell cycle

arrest and apoptosis remain to be elucidated, this study provides a substantial glimpse into human cancer-related genes and a general understanding of their overall genetic response to RE treatment in RCC54, and yields insights into the elucidation of the cancer preventive mechanism of RE in RCC. In addition, the gene expression profiles under different RE treatments were analyzed and visualized using singular value decomposition.

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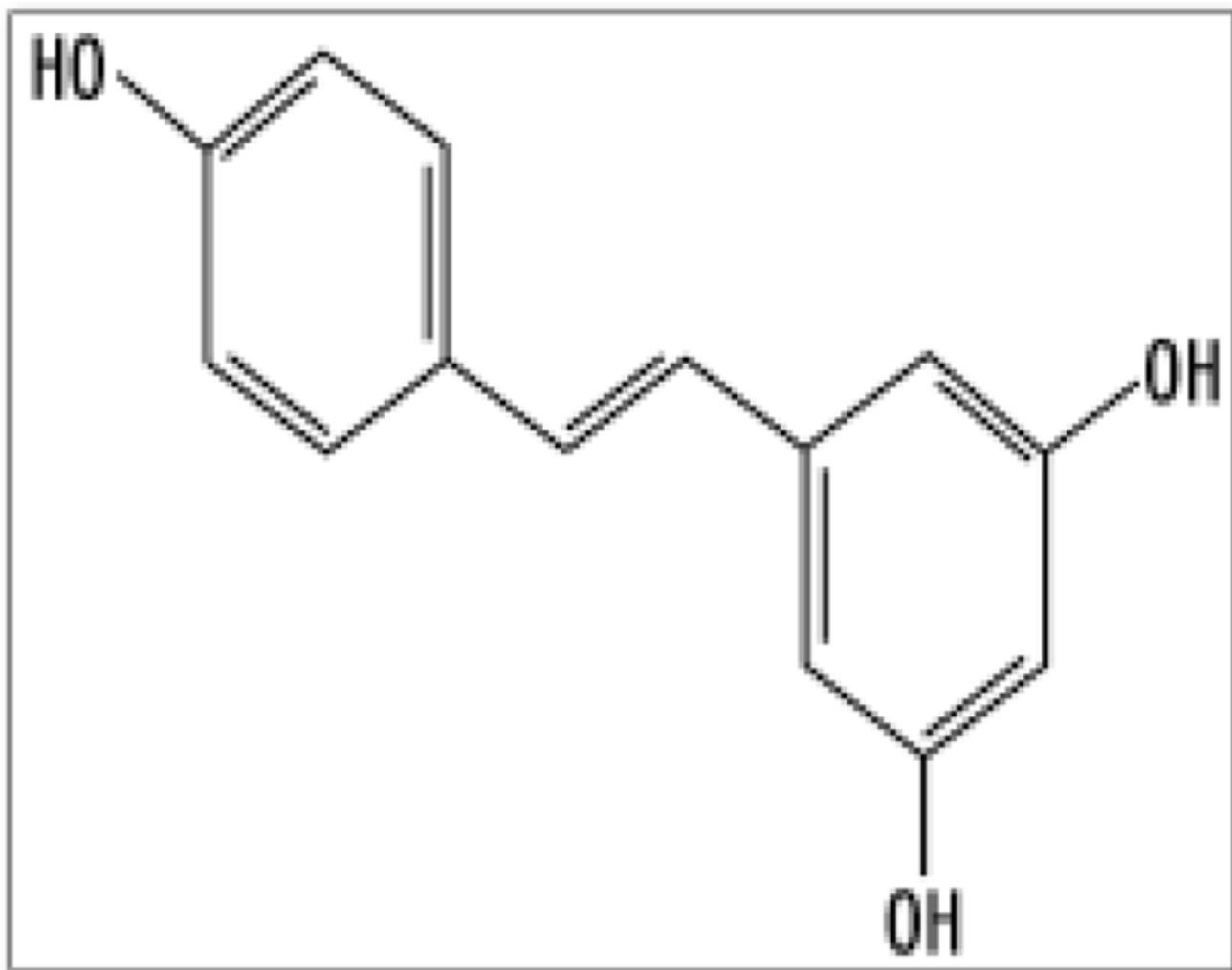


Figure 1.
Chemical structure of the polyphenolic compound resveratrol.

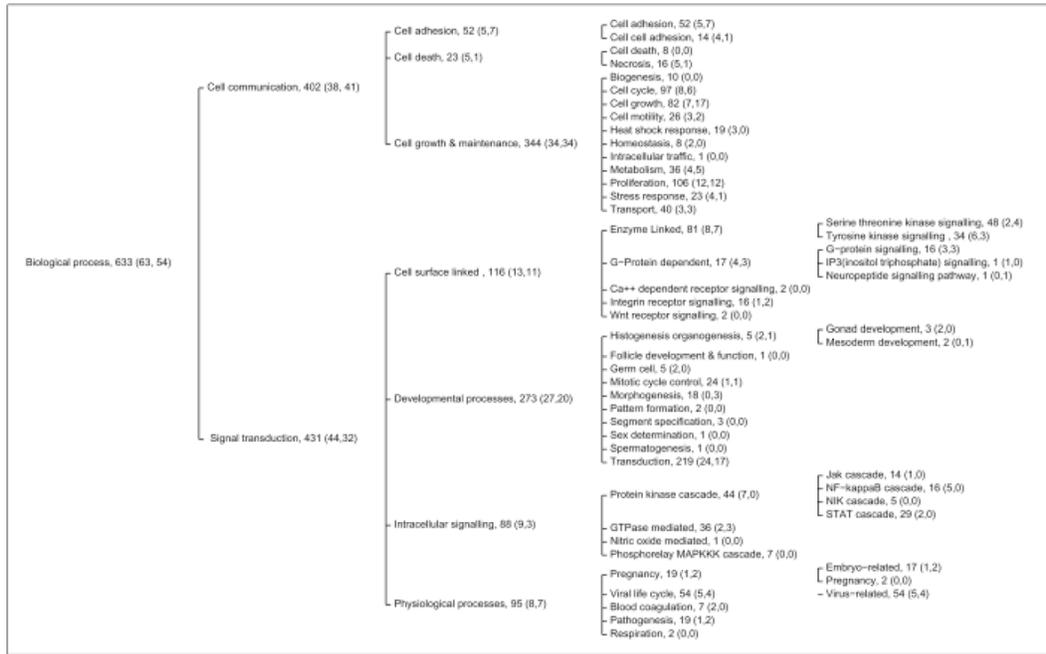


Figure 2. Biological process ontology tree including 633 genes associated with RE treated RCC54 cell line. The first integer following the name of each functional category represents the number of genes associated with the category. The first number in the parentheses stands for the number of genes in the group that are at least two-fold up regulated after RE treatment at 50 μ M for 24 hours. The second number in the parentheses is the number of downregulated genes after RE treatment.

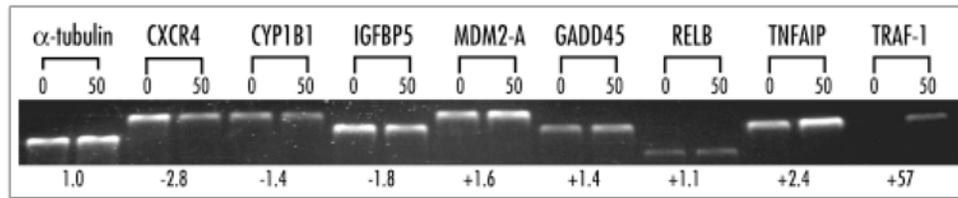


Figure 3.

Results of semi-quantitative RT-PCR of selected genes in the RCC 54 cell line. The band showing the mRNA level in the control RCC 54 is labeled with 0 and the bands indicating the mRNA level in RCC54 treated with RE at 50 μ M for 24 hours is labeled with 50. The amplification of DNA fragment of α -tubulin was used as the quantitative control. The decimal numbers below the image represent fold changes in the mRNA levels for different genes in RE treated RCC54 after normalization with the mRNA levels of α -tubulin. +, upregulation; -, downregulation.

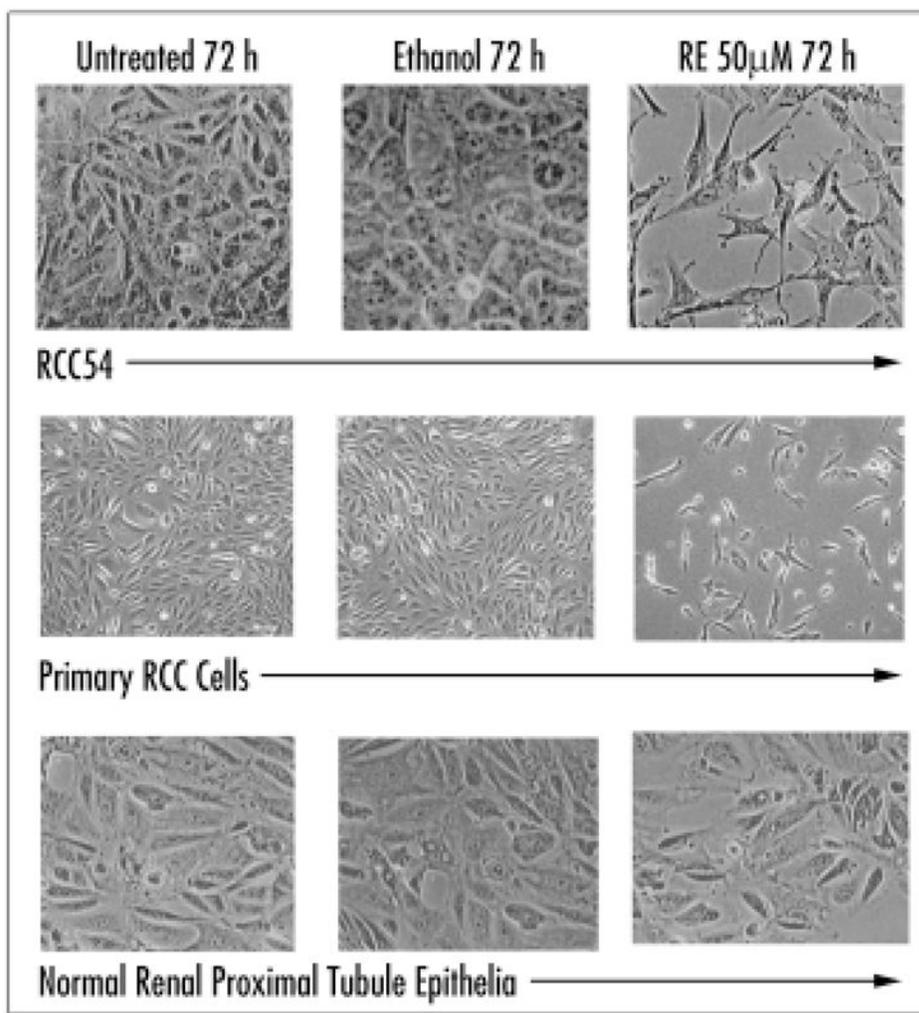


Figure 4. REinduced cell growth inhibition and apoptosis at 50 μ M for 72 hours on the RCC 54 (A), primary RCC cells (B), and normal renal proximal tubule epithelia (C). The images show the significant changes in cell numbers and morphology of RCC cells when treated with RE (A and B). No obvious change can be observed for normal cells (C).

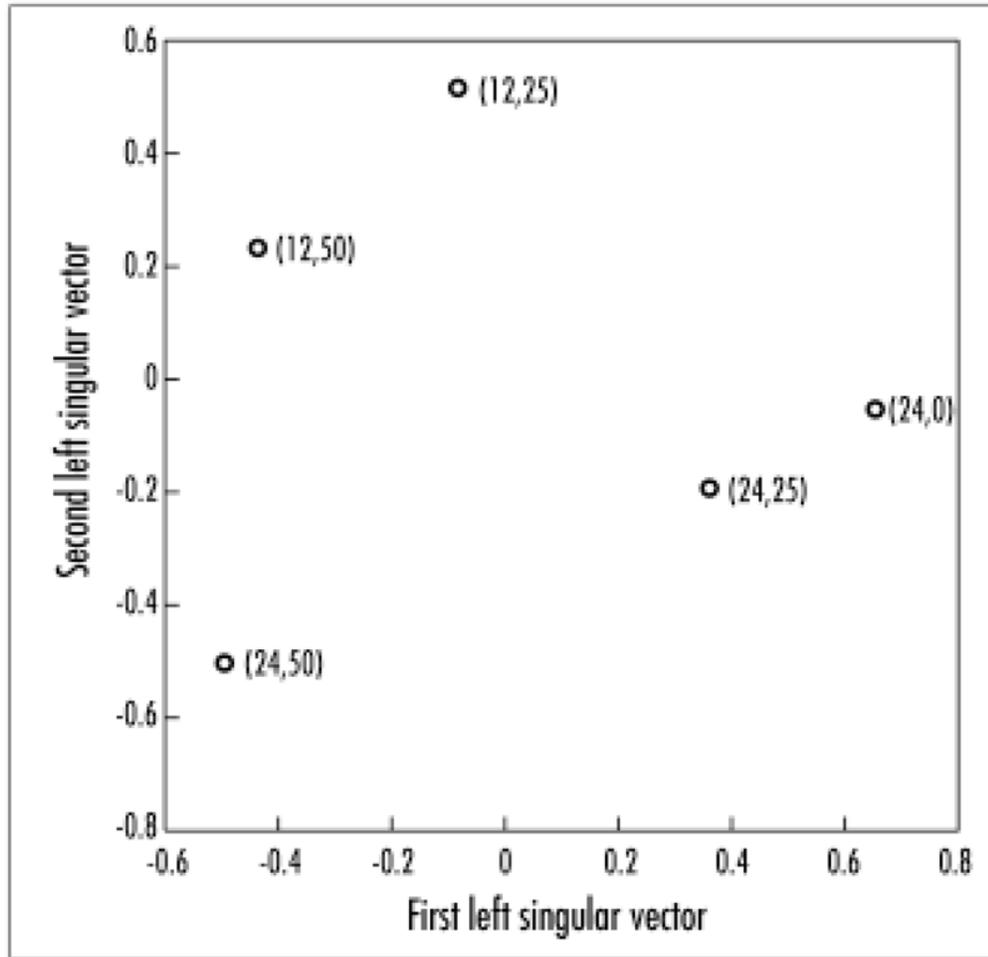


Figure 5.

SVD projection of the five gene expression profiles onto a space spanned by the first two left eigenvectors. Profiles of RCC 54 treated with RE at 50 μ M are clustered together while the profiles RCC54 treated with 25 μ M are clustered into a distinct group. The profile of control RCC54 treated with ethanol only is well separated from the RE treated profiles.

Table 1

Differentially Expressed Genes in RE Treated RCC 54

Accession No.	Gene Name	Major Function	Fold change ^a	
			25 μ M	50 μ M
X16354	Transmembrane carcinoembryonic antigen (BGP α)	Cell adhesion	+5.15	+24.35
U33199	Mouse double minute 2-A (MDM2A)	Proliferation	+3.45	+18.12
M60974	Growth arrest and DNA-damage-inducible protein (GADD45)	Cell growth	+3.07	+14.34
M92424	p53-associated mRNA (MDM2)	Proliferation	+1.45	+6.97
U33203	Mouse double minute 2-E (MDM2E)	Proliferation	+1.11	+7.57
S62138	Oncogene TIs/Chop, fusion activated	Oncogene	+1.93	+7.50
S57153	Retinoblastoma binding protein 1 isoform I (RBP1)	Tumor suppression	+4.73	+6.94
U27193	Protein-tyrosine phosphatase	Proliferation	+1.40	+5.53
J03258	Vitamin D receptor (VDR)	Transduction	+1.26	+14.16
M13207	Granulocyte-macrophage colony-stimulating factor (CSF1)	Transduction	+1.31	+7.47
M28130	Interleukin 8 gene (IL8)	Transduction	+1.67	+5.37
J00117	Chorionic gonadotropin (HCG) β subunit	Transduction	+1.38	+5.13
M97815	Retinoic acid-binding protein II (CRABP-II)	Transduction	+3.28	+6.01
U19261	Epstein-Barr virus-induced protein (TRAF-1)	Transduction	+1.58	+7.81
J03764	Plasminogen activator inhibitor-1	Cell adhesion	+1.53	+5.33
M59465	Tumor necrosis factor α inducible protein 3 (TNFAIP3)	Cell death	+3.72	+15.44
M83221	V-rel reticuloendotheliosis viral oncogene homolog B (RelB)	Transduction	+2.01	+6.21
L19871	Activating transcription factor 3(ATF3)	Cell growth	+3.24	+131.38 ^b
U02390	Adenylyl cyclase-associated protein 2	Structure regulation	-9.20	-1.33
M16592	Hemopoietic cell protein-tyrosine kinase (HCK)	Transduction	-2.22	-12.96
AF002700	GDNF family receptor α 2 (GFR α 2)	Transduction	-1.57	-6.43
M65062	Insulin-like growth factor binding protein 5 (IGFBP-5)	Cell growth	-2.57	-6.20
L27559	Insulin-like growth factor binding protein 5 (partial exon 4) (IGFBP-5)	Cell growth	-1.84	-15.15
X16323	Hepatocyte growth factor (HGF)	Cell growth	-1.68	-5.66
M83667	NF-IL6- β protein	Immune response	-1.32	-5.79
U03688	Dioxin-inducible cytochrome P450 (CYP1B1)	Metabolism	-1.40	-7.49
X58840	Nuclear factor 1, variant hepatic	Cell growth	-1.57	-7.11
X52229	Mucin 1, epithelial, alt. splice 9 (MUC1)	Cell adhesion	-1.53	-5.61
L06797	Chemokine (C-X-C motif) receptor 4 (CXCR4)	Cell growth	-1.20	-8.66

^aData indicate the fold change in the expression level in RCC54 treated with RE at 25 μ M and 50 μ M comparing with the expression level in the control RCC54. +, upregulation; -, downregulation).

^bThe transcripts of ATF3 in control RCC54 and in RCC54 treated with RE at 25 μ M were marked as absent (undetected) but the signal intensity of the transcript at 50 μ M was at least 5 fold above the background.

Table 2

Primers Used for the PCR Amplifications

Gene	Primer Sequence
RELB	sense: 5'-CCACTGACACTGGACTCGTACCAGG-3' antisense: 5'-AACCCGAGCAACGAGCTTCTCGCC-3'
TNFAIP3	sense: 5'-ACTGCTTGCATAAACTCAACCAGCT-3' antisense: 5'-AGAGGCATTAACAGAACACAGAGTA-3'
MDM2A	sense: 5'-GGCTTTGATGTTCCCTGATTGTAAAA-3' antisense: 5'-TGTTTGGCCATGGACAATGCAACCA-3'
GADD45	sense: 5'-AGAGGTGAAAATGAAGAAGGAAGCT-3' antisense: 5'-TTTATTGATGGTATTGTGTTTCAA -3'
IGFBP5	sense: 5'-GACCTACTCCCCAAGATCTTCCGG-3' antisense: 5'-TTCATCCCGTACAAGTCCACGCACC-3'
CYP1B1	sense: 5'-TGCCAGTATTTTTAAAGGCATTAGA-3' antisense: 5'-TTCCATATAAACACAGCTTTCTTTT-3'
TRAF-1	sense: 5'-TGGACTCTCACCAAATGAGAAGAAA-3' antisense: 5'-AATGTTCCAGAACCCCTGTAGCTC-3'
CXCR4	sense: 5'-AGGAAAGCGAGGTGGACATTCATCT-3' antisense: 5'-GGGCTAGTTATATCAAATAAATACT-3'