RESEARCH ARTICLE

Hydroxytyrosol inhibits growth and cell proliferation and promotes high expression of sfrp4 in rat mammary tumours

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Scope: Hydroxytyrosol (a phenolic compound derived from virgin olive oil) has demonstrated an anti-tumour effect in several tumour cell lines in addition to other health-related properties. The aim of this study was to investigate, for the first time in an animal model of experimental mammary carcinoma, the anti-cancer ability of hydroxytyrosol and to discover which pathways are modified by hydroxytyrosol.

Methods and results: Dimethylbenz[α]anthracene-induced mammary tumours were induced in 28 female Sprague–Dawley rats and ten of them were treated with hydroxytyrosol (0.5 mg/kg b.w. 5 days/week for 6 wk). cDNA microarray and quantitative RT-PCR experiments were performed. Hydroxytyrosol was found to inhibit the experimental mammary tumour growth and proliferation rate, with results comparable to those of doxorubicin but better with regard to the histopathological outcome. It also altered the expression of genes related to apoptosis, cell cycle, proliferation, differentiation, survival and transformation pathways.

Conclusions: This study shows that hydroxytyrosol exerts anti-cancer effects in Sprague–Dawley rats with experimental mammary tumours, inhibiting growth and cell proliferation in mammary tumours. Moreover, hydroxytyrosol alters several genes associated with cell proliferation, apoptosis and the Wnt signalling pathway, promoting a high expression of Sfrp4. However, further studies are necessary to better understand the mechanisms of hydroxytyrosol.

Keywords:
Breast Cancer / DMBA / Hydroxytyrosol / Microarray / Rat

1 Introduction

Breast cancer is the most common neoplastic disease and the second cause of death among women. Since the 1940s, the incidence of breast cancer has steadily risen in many industrialised countries, being highest in Western Europe, the United States and Canada, and lowest in Asia [1].

Rodent models of chemical carcinogenesis have provided essential information for precise understanding of the biological, cellular and molecular disturbances involving the

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Abbreviations: b.w., body weight; **DMBA,** 7,12-dimethylbenz[α]anthracene; **DOX,** doxorubicin; **FC,** fold change; **TdT,** terminal deoxynucleotidyl transferase; **TLDA,** TaqMan low density arrays; **TUNEL,** terminal deoxynucleotidyl transferase dUTP-nick-end labelling

development of mammary gland neoplasm [2]. Furthermore, rat breast cancer models exhibit some features resembling human mammary cancer [3], such as the possibility of chemically inducing mammary tumours by 7,12-dimethylbenz[α]anthracene (DMBA). This model is similar to human mammary gland neoplasm from the standpoint of morphology, histology, molecular background and the biochemical markers expressed [4, 5], and in its response to hormones, age, genetic factors [3, 6–8], diet [9], *etc.*, making this a highly valuable model for testing the effects of dietary components on breast cancer [10].

Many studies had been made in animals to test the health benefits of olive oil [11]. Many of the beneficial properties associated with this edible oil have been ascribed to its high content in oleic acid. Today, it is clear that many of the beneficial properties derived from the consumption of virgin olive oil are, in fact, due to some of its minor compounds, among which is hydroxytyrosol (3,4-dihydroxyphenyl-ethanol). The anti-tumour effect of hydroxytyrosol has been studied as a result of its ability to inhibit the proliferation and promote apoptosis in several tumour cell lines, in addition to being chemo-preventive as a result of its high antioxidant activity [12].

To date, all that is known about the anti-tumour effect of hydroxytyrosol comes from *in vitro* studies; none performed *in vivo* have demonstrated as yet that hydroxytyrosol is capable of blocking or diminishing tumour proliferation or growth. To this end, we focused on testing the anti-tumour capacity of hydroxytyrosol in an animal breast cancer model chemically induced by DMBA in rats – the first time such a study has been reported. Furthermore, we sought to identify which genes are altered by hydroxytyrosol intake, using a cDNA microarray platform and its subsequent validation by quantitative RT-PCR.

2 Materials and methods

2.1 Animals

A total of 28 female Sprague–Dawley rats, aged 8 wk and weighing 170 ± 20 g, were purchased from Harlan Interfauna Ibérica S.L (Barcelona, Spain). They were housed four *per* cage with *ad libitum* food (chow diet) and water access and maintained in an environmentally controlled room with a 12-h light/dark cycle. All experiments were performed in accordance with the principles of the Helsinki Declaration, Spanish animal welfare legislation and the Ethical Committee of the University of Granada (CEEA 264-2008) (Spain).

2.2 Experimental design

Mammary tumours were induced with intragastric 7, 12-dimethylbenz[α]anthracene (Sigma-Aldrich, St. Louis, USA), 100 mg/kg body weight (b.w.) dissolved in 1 mL of olive oil. Rats were palpated twice weekly to record the tumour volume with a digital calliper. In each animal, the first tumour of 2 cm^3 volume (called trucut tumour) was core biopsied (initial trucut tumour) twice using a Trucut system (Trucut Bard® Monopty® Biopsy Instrument, C.R. Bard, NJ, USA). One of these biopsies was fixed in 4% buffered formalin, and the other one was stored at −80°C after the overnight treatment with RNAlater™ stabilization reagent (QIAGEN GmbH, Hilden, Germany).

Later, each biopsied animal was randomly assigned to one of three different groups:

(i) CONTROL ($n = 10$): mammary tumours control group.
(ii) Hydroxytyrosol ($n = 10$): 0.5 mg/kg b.w. 5 days/week for 6 wk intragastric of hydroxytyrosol (Cayman Chemical, Ann Arbor, MI, USA). The dose of hydroxytyrosol was fivefold the daily intake of olive oil for a standard man of 70 kg b.w.
(iii) ADR ($n = 8$): tail vein injection of 1 mg/kg b.w. per week for 6 wk of doxorubicin (DOX) (Pharmacia-Upjohn Laboratories, Bridgewater, NJ, USA).

The animals were weighed weekly and palpated twice *per* week. After the treatment schedule, the animals were anaesthetised with intraperitoneal ketamine (150 mg/kg b.w.) and afterwards killed by aortic bleeding. The animal was killed when a tumour exceeded a volume of 10 cm^3. All tumours were harvested and the volumes were measured with a digital calliper: total tumour (whole rat tumours) and final trucut tumour (only the biopsied tumour at sacrifice); then, one half was fixed in 4% buffered formalin and the other half was treated overnight with RNAlater™ stabilization reagent and then stored at −80°C. These frozen tumour tissues were used for gene expression profiling. Tumour volume was measured throughout the study as described by Escrich *et al.* [13]:

$V = 4/3\pi(d_1/2) \times (d_2/2)^2$, where d_1 and d_2 were the two diameters of the tumour ($d_1 > d_2$), and at sacrifice, with the three diameters: $V = 4/3\pi(d_1/2) \times (d_2/2) \times (d_3/2)$; ($d_1 > d_2 > d_3$).

2.3 Histopathology

Formalin-fixed mammary tumour samples (initial and final trucut tumour) were paraffin embedded, sectioned (3 μm thickness) and placed on glass slides. Paraffin-embedded sections of tissue were deparaffinized, rehydrated with graded alcohol and stained with Harris' haematoxylin and eosin (Dako, Glostrup, Denmark) in a Leica Autostainer (Wetzlar, Germany). Histopathological evaluation was performed according to Costa *et al.* [4].

2.4 Proliferation assay

Paraffin-embedded mammary trucut tumours (initial and final) were sectioned (3 μm thickness), placed on a glass

slide, deparaffinized, and rehydrated through a graded alcohol series. After the antigen retrieval procedure at pH 6, primary rabbit antibody Ki-67 (clone SP6. Máster Diagnóstica, Granada, Spain) was applied for 10 min. Sections were blocked and treated with immunodetection solution, consisting of horse radish peroxidase and biotinylated secondary antibody for 30 min. Diluted 1:50 liquid 3,3′-diaminobenzidine (Dako) was used as a chromogenic agent and sections were counterstained in Meyer's haematoxylin (Dako). Negative controls were carried out by the same procedure. Positive exogenous controls were human mammary carcinomas. Tissues were evaluated by a pathologist. Ki-67 proliferation was assessed from the area with the highest mitotic activity. When possible, at least 1000 tumour cells were counted in these areas and a Ki-67 proliferation index was obtained by calculating the percentage of Ki-67-positive tumour nuclei.

2.5 Apoptosis detection

Sections of mammary tumour tissue were stained using the terminal deoxynucleotidyl transferase (TdT) dUTP-nick-end labelling (TUNEL) method using a commercial apoptosis detection kit (Genscript, NJ, USA). Briefly, paraffin-embeded mammary tumour sections were cut at a 5-mm thickness, deparaffinized, hydrated and incubated with a proteinase K for 25 min and blocked with 3% H_2O_2 in methanol for 10 min. After rinsing three times with PBS, a freshly prepared TUNEL reaction mixture containing 92% equilibration buffer, 2% biotin-11-dUTP and 6% TdT was added to the samples for 1 h at 37°C. After rinsing, samples were incubated with a Streptavidin–horse radish peroxidase solution for 30 min at 37°C. Detection was performed with DAB and slides were counterstained with haematoxylin. Negative control samples underwent the same process with omission of the TdT enzyme. Positive control samples were pretreated with 100 U/mL DNase I for 10 min at 15–25°C to induce DNA strand degradation before the labelling procedure.

2.6 RNA extraction and cDNA microarray

Gene expression analysis was performed in paired samples ($n = 5$) as follows: HT final trucut tumour (after treatment) *versus* initial trucut tumour (before treatment) (hydroxytyrosol final *versus* initial). Total RNA from frozen mammary tumours was directly extracted by an RNeasy Mini kit (QIAGEN, TX, USA) and homogenized by QIAshredder (QIAGEN) columns, following the manufacturer's instructions. Microarray analysis was performed using the Affymetrix Rat Genome 230 2.0 GeneChips® array. Chips and results were processed using Gene Chip Operating Software (GCOS 1.4. Affymetrix, USA). Expression values were calculated using the robust multi-array average algorithm (all microarray data have been deposited in the Gene Expression Omnibus public repository: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE15944).

2.7 Quantitative RT-PCR

Differentially expressed genes, FC (fold change) ≥ 1.4 and ≤ 0.6, were validated by quantitative RT-PCR, with a TaqMan® low-density array (Applied Biosystems, CA, USA), using sequence-specific primers/probes on the Applied Biosystems 7900 HT RT-PCR system. Single-stranded cDNA was synthesized from 1 μg of total RNA using random primers in a 20 μL reaction volume using the Applied Biosystem High Capacity cDNA Reverse Transcription kit. Thermal retro-transcription conditions involved two steps: 10 min at 25°C and 120 min at 37°C. PCR mix (100 μL) was composed of cDNA (100 ng) in 50 μL of TaqMan® Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. The total reaction mixture was loaded in the corresponding ports of a TaqMan® low-density array card. Expression value (ΔCt) was calculated by first averaging replicates for each gene and then normalising by subtraction, using an endogenous control gene (18S). Since a lower value of ΔCt indicates a higher expression, a $-\Delta Ct$ was used to correlate with microarray gene expression.

2.8 Statistical, microarray and RT-PCR analysis

All data are expressed as mean ± standard error of the mean. Before any statistical analysis, all variables were checked for normality and homogeneous variance by the Kolmogorov–Smirnov and Levene tests, respectively. All parameters were analysed by a one-way ANOVA; to evaluate mean differences between groups, Bonferroni *post hoc* analyses were used. To evaluate differences between initial and final moments within the same group, Student's t test was performed. All non-normal and anatomopathological variables were analysed by the non-parametrical Kruskall–Wallis test and Mann–Whitney *post hoc* tests. A Wilcoxon test was carried out to elucidate mean anatomopathological differences between initial and final values in the same group. A p-value of less than 0.05 was considered significant. Data were analysed using the SPSS/PC statistical software package (SPSS for Windows, 15.0, 2006, SPSS, IL, USA).

2.8.1 Microarray and functional enrichment

Non-supervised clustering was done using the Pearson correlation. Gene expression statistical significances were identified by Student's t test for paired samples, using GeneSpring GX 7.3 (Agilent Technologies) software.

Over-expression: FC > 1, and Inhibition: FC < 1. The functional enrichment was done by using PANTHER (http://www.pantherdb.org).

2.8.2 RT-PCR validation

Each gene datum was normalised with respect to the normalization factor and to the average expression of baseline genes, thus having gene expression values. The normalization factor was calculated taking gene expression values of housekeeping controls by geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/). Over-expression: values > 1; Inhibition: values < 1. To identify significant gene expression changes between our samples, Student's *t* test was applied for paired samples ($p < 0.05$).

3 Results

3.1 Tumour parameters

The biopsied tumour volume increased in hydroxytyrosol and control group throughout the experiment except in the group treated with DOX although the hydroxytyrosol group showed a significantly ($p < 0.05$) lower mammary Biopsied Tumour Volume than did the Control group at the end of the study (Fig. 1A). However, the hydroxytyrosol group was significantly ($p < 0.01$) higher than the ADR group treated with DOX, which had the lowest biopsied tumour volume. Furthermore, the total tumour volume after hydroxytyrosol and DOX treatments was significantly ($p < 0.01$) lower in both groups compared with the control group and it is important to mention that no statistical differences were found between the hydroxytyrosol and ADR groups (Fig. 1B).

3.2 Histopathology

Pathological parameters (mitosis index, tubular formation, nuclear atypia, Bloom–Richardson grade and morphologic pattern) expressed as a percentage of rats with this grade were measured in mammary-biopsied tumours (Table 1). Differences for the mitosis index, tubular formation and nuclear atypia were found in the control group compared with the other groups at the end of the study. Moreover, the control group consistently reached grade 3 in all these parameters.

The Bloom–Richardson grade percentage is shown in Table 1. All groups presented mammary trucut tumours with grade 1 at baseline. As expected, the control group developed more tumours with grade 2 than the other groups and this was the only group which developed more malignancy towards grade 3. The hydroxytyrosol group had the lowest histopathological grade, even lower than that of the ADR group.

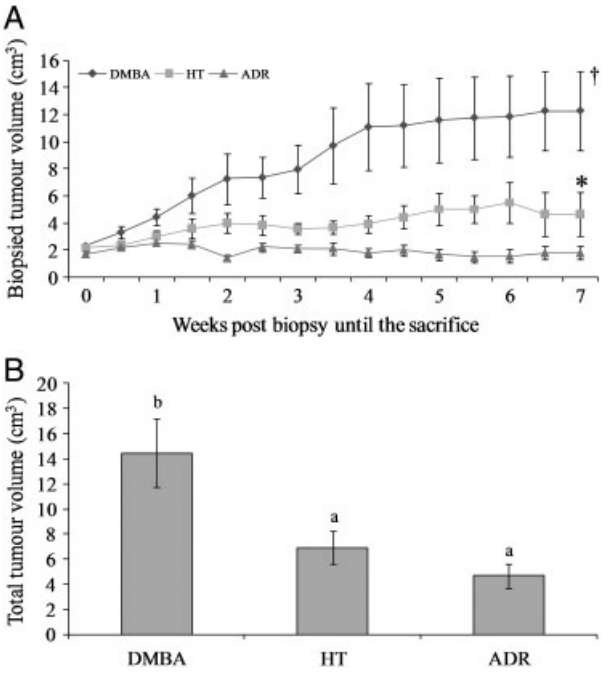

Figure 1. (A) Progress of the trucut mammary tumour volume; (B) Total tumour volume at the end of the study. Values are expressed as mean ± SEM. Bars with different letters significantly differ among groups, $p < 0.05$. Symbols indicate significant differences between baseline and final within the same group ($^{*}p < 0.05$; $^{\dagger}p < 0.001$). DMBA: breast cancer control group, HT: group of rats treated with hydroxytyrosol, ADR: group treated with adriamycin.

Finally, Table 1 shows the morphological pattern of the mammary trucut tumours. This indicates that cribriform was the major histological pattern at the baseline and at the end of the study, even though the hydroxytyrosol group exhibited more variation at the baseline. At the end of the study, the cribriform pattern was the most common, followed by mixed and papillary patterns. Moreover, hydroxytyrosol and ADR exhibited a very similar development to that of papillary patterns. Only the control group registered other types of patterns, such as solid or tubular (Fig. 2).

3.3 Breast tumour cell proliferation and apoptosis

Ki-67 immunostaining (Fig. 3) showed that hydroxytyrosol group significantly reduced (18%; $p < 0.01$) the nuclear staining of this antibody in mammary cells, to the same degree as in the ADR group (23%; $p < 0.05$) with respect to the control group. The highest percentage of nuclear staining of Ki-67 was shown by the control group (42%). Apoptosis was performed as a TUNEL assay but no detected values were found in the experimental groups (data not shown).

Table 1. Pathological study in rat with mammary tumours induced by DMBA and treated with doxorubicin or hydroxytyrosol at the beginning and at the end of the study[a)]

	Control		Hydroxytyrosol		ADR	
General pathologic parameters	Baseline	End	Baseline	End	Baseline	End
Mitosis index (%)						
I	44.4	11.1	50	22.2	50	25
II	33.3	11.1	30	44.4	30	37.5
III	22.2	77.7	20	33.3	20	37.5
Mean ± SEM	1.78 ± 0.3	2.6 ± 0.2*	1.6 ± 0.3	2.1 ± 0.3	1.3 ± 0.2	2.1 ± 0.3
Tubular formation (%)						
I	88.8	33.3	90	77.7	85.7	50
II	11.1	55.5	10	22.2	14.3	50
III		11.1				
Mean ± SEM	1.1 ± 0.1	1.78 ± 0.2*	1.1 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	1.5 ± 0.2
Nuclear atypia (%)						
I	77.7	33.3	80	55.5	80	62.5
II	22.2	55.5	20	44.4	20	37.5
III		11.1				
Mean ± SEM	1.2 ± 0.1	1.78 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.0 ± 0.0	1.3 ± 0.2
Bloom Richardson grade (%)						
I	90	22	90	55.5	100	50
II	10	67	10	44.4	0	50
III	0	11	0	0	0	0
Mean ± SEM	1.11 ± 0.1	1.89 ± 0.2*	1.1 ± 0.1	1.4 ± 0.2	1.0 ± 0.0	1.5 ± 0.2
Morphological pattern (%)						
Cribriform	100	67	80	60	100	89
Mixed	0	22	10	30	0	11
Papillary	0	0	10	10	0	0

a) General Pathological Parameters: values are expressed as % of rats with this grade and mean ± sem. Control: breast cancer control group, Hydroxytyrosol: group of rats treated with hydroxytyrosol, ADR: group treated with adriamycin. Mitosis Index: I:0-9/10HPF (high power fields), II:10-19/10HPF, III: > 19/10HPF; Tubular Formation: I: < 25%, II: 25–75%, III: > 75%; Nuclear Atypia: 1–3 grades. Morphological Pattern: Cribriform (> 70 of this pattern). Mixed (30–70% of cribriform pattern and the rest of papillary). Papillary (< 30% of cribriform pattern). *Significant differences ($p < 0.05$) between baseline and end for each group by the Wilcoxon test.

3.4 Differentially expressed genes

A total of 595 sequences were identified ($p < 0.05$). Ninety-nine genes were significantly upregulated (FC ≥ 1.4) ($p < 0.05$) (32 unknown and 67 known genes). Among the upmodulated known genes we found some related to apoptosis (death receptor Fas or Tnfrsf6 or TNF receptor superfamily, member 6; epithelial membrane protein 2, Emp2), cell proliferation, differentiation and cell cycle (secreted frizzled-related protein 4, Sfrp4; four and a half LIM domains 1, Fhl1; WNT1 inducible signalling pathway protein 2, Wisp2; cyclin-dependent kinase inhibitor 2a, Cdkn2a; quiescin Q6, Qscn6; c-mer proto-oncogene tyrosine kinase, Mertk; fibroblast growth factor receptor 2, Fgfr2; cadherin 13, Cdh13; SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 2, Smarca2), inflammation (interleukin 6 signal transducer, Il6st or gp130), or response to stress (crystallin, αB, Cryab; glutathione *S*-transferase, mu2, Gstm2). Seventy-four genes were downregulated (FC ≤ 0.6) ($p < 0.05$) (7 unknown and 67 known genes). Important known genes were found downmodulated with a role in apoptosis (activating transcription factor 3, Atf3; growth arrest-specific 5, Gas5; immediate early response 3, Ier3; basic helix-loop-helix domain containing, class B2, Bhlhb2; Sequestosome 1, Sqstm1), cell cycle and proliferation and differentiation (ubiquitin-conjugating enzyme E2B, RAD6 homolog (*Saccharomuces cerevisiae*), Ube2b; chemokine (C-X-C motif) ligand 1, Cxcl1; Jun oncogene; high mobility group nucleosomal-binding domain 2, Hmgn2), or inflammation (mast cell proteases 8, 9 and 10, Mcpt8, Mcpt9 and Mcpt10) (see Supporting Information – Suppl_Data1 – for complete signal intensity and annotation data).

About 354 gene sequences were fit for functional classification, out of which 209 genes were classified by the PANTHER biological process. The gene ontology of the sequences found modulated after exposure to hydroxytyrosol revealed that 28 biological processes were altered by this phenolic compound. The more representative functions are related to protein metabolism and modification, nucleoside,

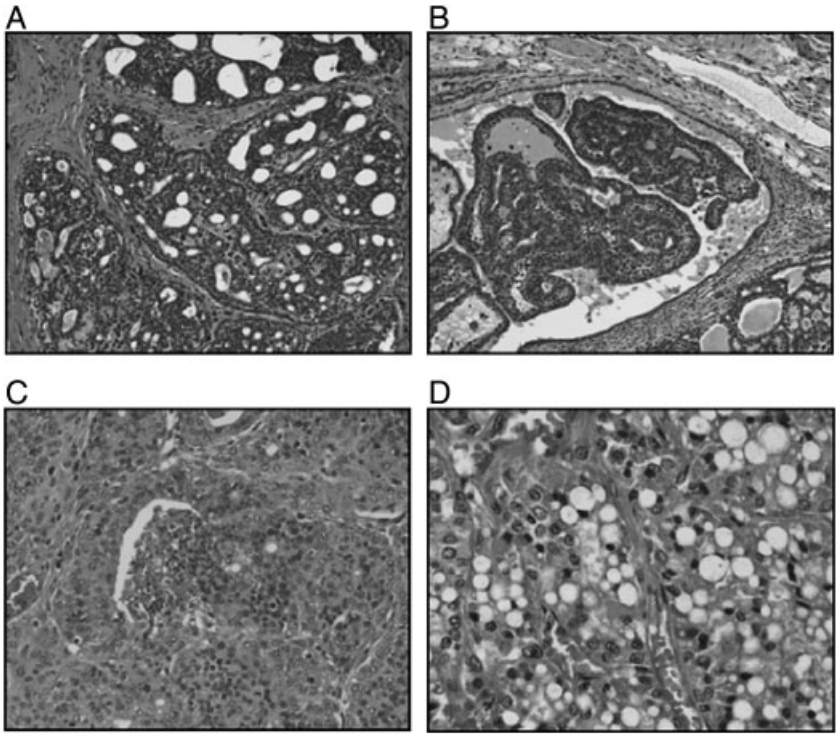

Figure 2. Examples of different morphological patterns found in the mammary tumours. (A) Cribriform (100 × magnification); (B) Papillary (100 × magnification); (C) Comedocarcinoma (200 × magnification); (D) Secretor (200 × magnification).

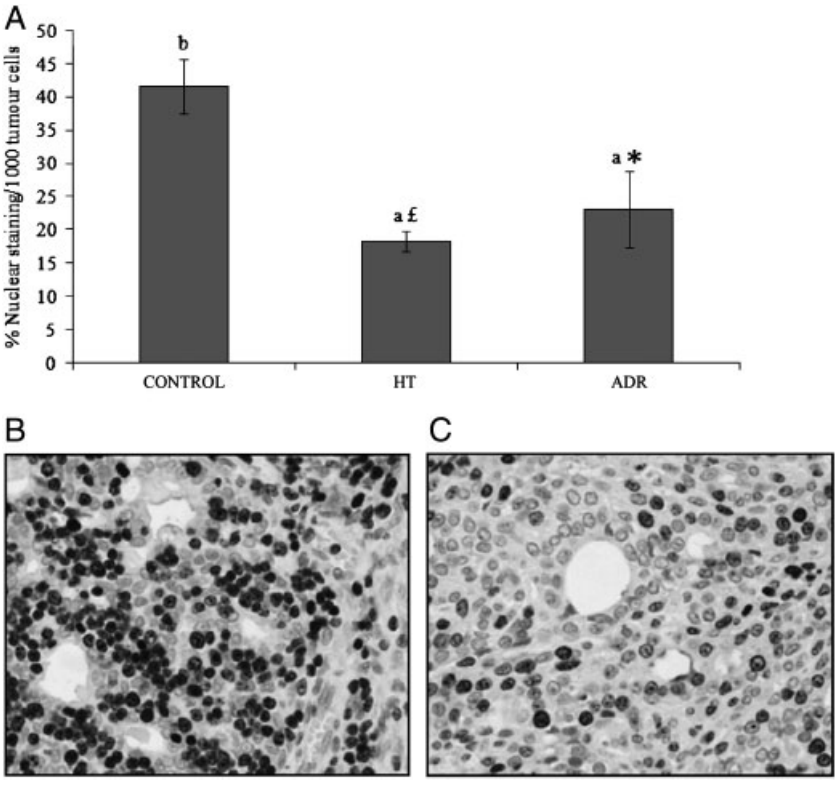

Figure 3. (A) % Ki-67 positive staining normalised *per* 1000 tumour cells. Bars with different letters significantly differ among groups (*$p<0.05$ ADR *versus* control; £ $p<0.01$ HT *versus* control); (B) Example of high proliferative index area (400 × magnification); (C) Example of medium proliferative index area (400 × magnification).

nucleotide and nucleic acid metabolism, signal transduction, biological process unclassified, developmental processes, cell proliferation and differentiation, intracellular protein traffic, transport, cell cycle, cell structure and motility, immunity and defence, lipid, fatty acid and steroid metabolism, apoptosis and oncogenesis (Table 2). Supporting Information, Table S1 shows all genes classified by PANTHER into different clusters of biological processes.

3.5 Differential gene expression pattern validation

For the validation of the cDNA microarray results, we performed a quantitative RT-PCR analysis of a list of 70 selected genes found to be overregulated ($FC \geq 1.4$) and downmodulated ($FC \leq 0.6$) after the treatment with hydroxytyrosol (Supporting Information, Suppl_Data2). Thirteen genes were confirmed after this validation with a statistically significant expression ($p < 0.05$). A total of six genes involved in apoptosis, cell proliferation, cell cycle, cell survival and stress response were overexpressed (Sfrp4, Fas receptor or Tnfrsf6, Cdkn2a, Cryab, chaperone ABC1 activity of bc1 complex like or Cabc1, and Il6st). Among the downregulated genes, Ier3, JunB and c-Jun are involved in several processes such as apoptosis, cell cycle, cell proliferation, cell survival and oncogenesis. Other downmodulated genes were Per2 (period homolog 2 (Drosophila)), Ccnl2 (cyclin L2), Apbb3 (amyloid β (A4) precursor protein-binding, family B, member 3), and Car11 (Carbonic anhydrase 11) (Table 3).

Table 2. Biological processes altered by hydroxytyrosol in trucut mammary tumours

Biological process	Microarray genes	Panther ID
Protein metabolism and modification	43	BP00060
Nucleoside, nucleotide and nucleic acid metabolism	38	BP00031
Signal transduction	37	BP00102
Biological process unclassified	33	BP00216
Developmental processes	22	BP00193
Cell proliferation and differentiation	22	BP00224
Intracellular protein traffic	22	BP00125
Transport	20	BP00141
Cell cycle	15	BP00203
Cell structure and motility	14	BP00285
Immunity and defence	14	BP00148
Lipid, fatty acid and steroid metabolism	14	BP00019
Apoptosis	14	BP00179
Oncogenesis	9	BP00281
Neuronal activities	6	BP00166
Muscle contraction	5	BP00173
Carbohydrate metabolism	5	BP00001
Electron transport	5	BP00076
Protein targeting and localization	5	BP00137
Other metabolism	4	BP00289
Cell adhesion	4	BP00124
Amino acid metabolism	4	BP00013
Homeostasis	3	BP00267
Coenzyme and prosthetic group metabolism	2	BP00081
Phosphate metabolism	2	BP00095
Sensory perception	2	BP00182
Nitrogen metabolism	1	BP00090
Miscellaneous	1	BP00211

4 Discussion

The anti-proliferative and proapoptotic properties of hydroxytyrosol have been reported previously in tumour cell lines [12]. However, to the best of our knowledge, no experiments have been performed to test the anti-tumour activity of hydroxytyrosol *in vivo*.

In this report, we establish for the first time that hydroxytyrosol inhibits mammary tumour growth in rats to a degree similar to that of DOX, and not only of biopsied mammary tumour but also of the whole tumour volume. These results have not been previously reported in an animal model of cancer. This anti-tumour capacity of hydroxytyrosol is supported by the decrease in cell proliferation, associated with a less nuclear Ki-67 immunostaining, demonstrating a powerful anti-proliferative activity similar to that of DOX. Recent studies report the ability of hydroxytyrosol for inhibiting the proliferation, cell viability and the induction of apoptosis of MCF-7 human breast cancer cells [14, 15].

The histopathologic analysis and the classification of rat mammary lesions followed in this paper were first reported by Costa *et al*. [4]. Such a protocol is similar to that applied in human pathology, as it allows more information to be obtained about tumour characteristics, and shows the DMBA model to be a useful tool for studying human carcinogenesis of the breast from a histopathologic viewpoint. Thus, the DMBA model remains topical among the current studies of breast carcinogenesis in rats [16–18]. Several studies have reported that an olive oil rich diet is associated with mammary adenocarcinomas with a predominantly low histologic grade (Bloom–Richardson grade I and II), a lower degree of morphological malignance, and a predominantly cribiform structural pattern with respect to a high corn oil diet and the control group [4, 9, 19]. In one of these studies [19] the authors described results similar to our results finding than in the olive oil group the total tumour volume were lower even than this of the control group indicating a negative effects of that diet on the growth of the DMBA-induced mammary tumours. These results could, at least in part, be explained as suggest theses studies by the olive oil phenolic fraction and oleic acid. Our results reflect a better histopathologic outcome after hydroxytyrosol administration, providing slightly better results than in the DOX-treated group.

Table 3. Validated gene expression data in rats with experimental breast cancer treated with hydroxytyrosol: microarray *versus* RT-PCR data[a)]

			Microarray		RT-PCR	
ProbeID	Gene symbol	Description	FC	$p<0.05$	FC	$p<0.05$
1388587_at	*IER3*	Immediate early response 3	0.53	0.0077	0.51	0.0005
1368303_at	*PER2*	Period homolog 2 (Drosophila)	0.48	0.0004	0.53	0.0013
1393213_at	*CCNL2*	Cyclin L2	0.68	0.0048	0.50	0.0046
1368394_at	*SFRP4*	Secreted frizzled-related protein 4	6.54	0.0085	12.51	0.0065
1384842_s_at	*TNFRSF6*	TNF receptor superfamily, member 6	1.62	0.0018	1.64	0.0111
1369194_a_at	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A	2.39	0.0476	2.54	0.0138
1370026_at	*CRYAB*	Crystallin, αB	2.03	0.0046	2.40	0.0161
1372536_at	*CABC1*	Chaperone, ABC1 activity of bc1 complex like	1.78	0.0133	1.71	0.0262
1387185_at	*APBB3*	Amyloid β (A4) precursor protein-binding, family B, member 3	0.61	0.0363	0.49	0.0331
1387788_at	*JUNB*	Jun-B oncogene	0.77	0.0308	0.56	0.0334
1374275_at	*CAR11*	Carbonic anhydrase 11	0.70	0.0125	0.55	0.0337
1389528_s_at	*JUN*	Jun oncogene	0.60	0.0047	0.48	0.0337
1370957_at	*IL6ST*	Interleukin 6 signal transducer	1.39	0.0485	1.57	0.0373

a) Gene expression is shown as FC (fold change) (Trucut tumour final *versus* initial). Data are tabulated by descendent *p*-value of RT-PCR.

With the aim of determining the profile of altered genes after hydroxytyrosol administration, we performed a microarray assay and quantitative RT-PCR on the biopsied mammary carcinomas. The results obtained show that hydroxytyrosol promotes a high expression of Sfrp4, which is a family member of secreted antagonists of the Wnt pathway. These are considered tumour suppressors in several kinds of cancer, binding directly to Frizzled receptors (Fzd) or to Wnt ligands, antagonizing Wnt signalling. Moreover, they exhibit biphasic actions, increasing Wnt activity at low levels and antagonizing it at high ones. These actions block the non-canonical and canonical β-catenin pathways [20, 21]. Sfrp4 competes with Fzd5 and/or 10 to bind Wnt7a, suppressing the activation of Wnt7a signalling in both an autocrine and a paracrine manner, and inhibiting the proliferation of endometrial cancer cells [22]. In addition, Sfrp4 spurs physiologic apoptosis in mammary involution [23].

On the other hand, hydroxytyrosol also intensifies the expression of Cabc1. This gene encodes a protein responsible for synthesising the coenzyme Q_{10} [24], a powerful anti-oxidant and an essential component of the mitochondrial respiratory chain [25, 26]. Moreover, this phenolic compound overexpresses the mRNA of Il6st. High levels of interleukin (IL)-6 and sIL6R (a soluble form of the IL6 receptor) have been reported in several chronic inflammatory and autoimmune diseases as well as in cancer [27]. Il6st triggers the caspase-dependent apoptotic cascade after binding with the complex IL6–sIL6R–IL6R (the mechanism of IL6 trans-signalling) [28]. Han *et al.* [14] have recently pointed that hydroxytyrosol (25 μg/mL for 24 h) is able to increase the MCF-7 cell number in G_0/G_1 phase. This raising of cell number is translated into a block of G_1 to S phase transition on MCF-7 human breast cancer cells. Our data manifested that the treatment of mammary tumour-bearing rats with hydroxytyrosol could modulate the expression of some genes with role in proliferative events. A very recent study has been carried out with the aim of investigating whether hydroxytyrosol and oleuropein can affect the proliferation stimulated by oestradiol through interfering with oestradiol-induced molecular mechanisms on MCF-7 breast cancer cells [15]. This paper shows that both hydroxytyrosol and oleuropein interfere with the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) by oestradiol. It is widely known that ERK1/2 activation regulates cell growth, cell proliferation and it is necessary for the G_0/G_1 to S phase transition [29].

This study reveals an upregulation of the small heat shock protein Cryab with constitutive expression in breast cancer diseases [30]. It has been recently reported that Cryab triggers cyclin D1 ubiquitination and increases its turnover, preventing the G_1/S phase progression in breast carcinogenesis [31]. A downmodulation after hydroxytyrosol has also seen for Cdkn2a, which encodes two different tumour suppressors, $p16^{INK4A}$ and $p14^{ARF}$ [32]. $p14^{ARF}$ stabilizes to the p53 tumour suppressor, leading to G_1 cell cycle arrest, and $p16^{INK4A}$ promotes quiescence and irreversible senescence in tumour cells, causing permanent G_1-phase arrest [33]. Hydroxytyrosol downregulates c-Jun and JunB oncogenes. It has been shown in melanoma-derived B16-F10 cancer cells that the downexpression of both c-Jun and JunB leads to G_2/M arrest [34]. Hydroxytyrosol also downmodulates the mRNA expression of Ier3, which encodes a protein that may be proapoptotic or anti-apoptotic depending on the conditions [35].

In conclusion, we have shown that hydroxytyrosol exerts anti-tumour properties in Sprague–Dawley rats with experimental mammary tumours, inhibiting the growth and cell proliferation (associated with a less nuclear Ki-67 immunostaining) in these tumours. Moreover,

hydroxytyrosol alters several genes associated with cell proliferation, apoptosis and the Wnt signalling pathway, promoting a high expression of Sfrp4, although further studies must be made to better understand the mechanisms of hydroxytyrosol.

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