Dietary olive oil and corn oil differentially affect experimental breast cancer through distinct modulation of the p21Ras signaling and the proliferation–apoptosis balance

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Extra-virgin olive oil (EVOO) has been hypothesized to have chemopreventive effects on breast cancer, unlike high corn oil (HCO) diets that stimulate it. We have investigated mechanisms of these differential modulatory actions on experimental mammary cancer. In 7,12-dimethylbenz(a)anthracene adenoscinoma of rats fed a high EVOO, HCO and control diets (n = 20 for each group), we have analyzed the expression and activity of ErbB receptors, p21Ras and its extracellular signal-regulated kinase (ERK) 1/2, Akt and RalA/B effectors by immunoblotting analyses. We explored the Ha-ras1 mutation status by Southern blot, mismatch amplification mutation assay and sequencing, and the 3-hydroxy-3-methylglutaryl-coenzyme A reductase and squalene synthase messenger RNA expression by real-time polymerase chain reaction. We analyzed the tumor mitotic index, proliferating cell nuclear antigen (PCNA), and apoptosis through Caspase-3 analysis and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assays. Finally, we measured the 8-oxo-2'-deoxyguanosine levels. Non-parametrical statistics were used. The EVOO diet decreased Ras activation, downregulated the Ras/phosphatidyl inositol 3-kinase/Akt pathway and upregulated the Raf/Erk pathway, compared with the control. In contrast, the HCO diet did not modify Ras activity but rather enhanced the Raf/Erk pathway. The EVOO diet decreased the cleaved ErbB4 levels, compared with the HCO diet, increased apoptosis and diminished the mono-ubiquitylated PCNA levels, which is related to DNA damage. Tumors from rats fed the EVOO diet displayed a more benign behavior and histopathological features of rat 7,12-dimethylbenz(a)anthracene-induced mammary adenocarcinomas, according to their stimulating and protective effects, respectively (19,20). We have also shown that changes in tumor fatty acid composition and in the expression of cell proliferation and differentiation related genes account for these differential modulatory effects (10,21,22). To gain insight into the complex mechanisms underlying these effects, in this study, the possible involvement of the ErbB/Ras pathway, as well as its three main effectors, i.e. ERK1/2, Akt and RalA/B, have been investigated. The results obtained have led us to explore the tumor modulation status of Ha-ras1 and the messenger RNA (mRNA) expression of two key enzymes of the mevalonate pathway, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and squalene synthase (SQS). In addition, changes in cell proliferation and apoptosis, as well as in levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG), were also investigated.

Materials and methods

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; EVOO, extra-virgin olive oil; GTP, guanosine triphosphate; HCO, high corn oil diet; HFCO, high-fat corn oil diet group; HFOO, high-fat olive oil diet group; HMGC-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LA, linoleic acid; LF, low-fat diet group; mRNA, messenger RNA; Ubi, proliferating cell nuclear antigen, mono-ubiquitylated proliferating cell nuclear antigen; OA, oleic acid; 8-oxo-dG, 8-oxo-2'-deoxyguanosine; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PUFA, polyunsaturated fatty acids; SQS, squalene synthase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

Introduction

Breast cancer is the most common cancer in women of Western countries (1). Its association with dietary fat has been examined for a long time and it remains controversial (2,3). The main epidemiological evidence of a chemopreventive effect of EVOO, there are conflicting results (7,10). The specific mechanisms of the dietary lipid effects on breast cancer have not been well elucidated but alterations in hormonal status, structure and function of cell membranes, cell signaling transduction pathways, oxidative stress, gene expression and the immune system have been reported (10).

Ras proteins function as molecular switches of many signaling pathways that regulate cell growth, differentiation and survival (11). Mutational-activated Ras as well as the upregulation of normal Ras have been shown to play a role in mammary tumorigenesis and metastasis (12). The ErbB family of receptor tyrosine kinases is essential in triggering Ras signaling cascades, and alterations of its members are all linked to carcinogenesis (13,14). The most highly characterized effector pathways of activated Ras are Raf-1/extracellular signal-regulated kinase (ERK) 1/2, phosphatidylinositol 3-kinase (PI3K)/Akt and RalGDS/RalA/B. They modulate a broad array of cellular functions including proliferation, survival, apoptosis, motility, transcription, metabolism and differentiation (15–17). While Raf-1/ERK1/2 and PI3K/Akt pathways are known to play a key role in breast cancer growth and apoptosis, the contribution of the Ral guanosine triphosphatases in mammary tumorigenesis is still unclear (18).

We have previously demonstrated that high corn oil (HCO), rich in LA, and high EVOO diets differentially modulate the clinical behavior and histopathological features of rat 7,12-dimethylbenz(a)anthracene-induced mammary adenocarcinomas, according to their stimulating and protective effects, respectively (19,20). We have also shown that changes in tumor fatty acid composition and in the expression of cell proliferation and differentiation related genes account for these differential modulatory effects (10,21,22). To gain insight into the complex mechanisms underlying these effects, in this study, the possible involvement of the ErbB/Ras pathway, as well as its three main effectors, i.e. ERK1/2, Akt and RalA/B, have been investigated. The results obtained have led us to explore the tumor modulation status of Ha-ras1 and the messenger RNA (mRNA) expression of two key enzymes of the mevalonate pathway, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and squalene synthase (SQS). In addition, changes in cell proliferation and apoptosis, as well as in levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG), were also investigated.

Materials and methods

Animals and experimental design

Mammary adenocarcinomas were obtained in a previous work from 60 female Sprague–Dawley rats induced with a single dose of 5 mg of...
dimethylbenz(x)anthracene (Sigma–Aldrich, St. Louis, MO) and fed different semi-synthetic diets, following a design of carcinogenesis promotion. Rats (n = 20 for each group) were given a 3% corn oil diet (control-low-fat diet group, LF), a high, 20% corn oil diet (high-fat corn oil diet group, HFCO), and a high EVOO diet—containing 3% corn oil and 17% EVOO (high-fat olive oil diet group, HFO) (18). The fatty acid composition of the corn oil was palmitic acid (16:0), 11.2%; palmitoleic acid (16:1), 0.2%; stearic acid (18:0), 2.1%; OA (18:1n-9), 28.2%; LA (18:2n-6), 57.1%; linoleic acid (18:3n-3), 0.9% and arachidonic acid (20:4), 0.3%. The virgin olive oil contained palmitic acid, 7.89%; palmitoleic acid, 0.49%; stearic acid, 2.64%; OA, 79.03%; LA, 8.24%; linoleic acid, 0.6% and arachidic acid, 0.5%. At necropsy, microscopic mammary lesions were rapidly removed and measured. A sample of each one of the samples was fixed in formalin for histopathology and total deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) analysis, whereas the rest was flash-frozen in liquid nitrogen and stored at –80°C for molecular analyses.

Protein extraction

Tumor tissue was homogenized in 250 mM Tris–HCl pH 7.2, 250 mM sucrose, 50 mM NaF, 100 mM Na3VO4, protease inhibitor cocktail (Sigma–Aldrich), 10 mM β-mercaptoethanol, 5 mM MgCl2 and 1% Triton X-100. Samples were then centrifuged at 13 000 g for 20 min at 4°C and the supernatant saved as the total tumor extract. For p21Ras and RalA/B analysis, Tumor tissue was homogenized in 250 mM Tris–HCl pH 7.2, 250 mM sucrose, 10 mM β-mercaptoethanol, 5 mM MgCl2 and 1% Triton X-100, following a protocol described elsewhere (23). Protein concentration was measured using a Lowry protein assay.

Western blot analyses

Total or subcellular extracts were resolved by 8–15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Primary antibodies used were epidermal growth factor receptor (EGFR) (1:1000) and Ras B (1:5000) from Upstate Biotechnology (Lake Placid, NY); phospho-EGFR (Tyr1173, 1:200), phospho-Neu (Tyr1248, 1:200) and phospho-ERBB4 (Tyr1056, 1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-EGFR (Tyr1068, 1:2500), phospho-ERBB3 (Tyr1289, 1:1000), p44/42 mitogen-activated protein kinase (1:2000), phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204, 1:2500), Akt (1:2500), phospho-Akt (Ser473, 1:2500), Caspase-3 (1:2000), phospho-EGFR (Tyr1068, 1:2500), phospho-EGFR (Tyr1068, 1:2500), phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204, 1:2500), Akt (1:2500), phospho-Akt (Ser473, 1:2500), Caspase-3 (1:2000), proliferating cell nuclear antigen (PCNA) (1:5000) and Ubiquitin (1:1000) from Cell Signaling Technology (Danvers, MA); c-ErbB2 (1:267) and c-ErbB3 (1:400) from Thermo Fisher Scientific (Fremont, CA); c-ErbB4 (1:500) from Abcam (Cambridge, UK); pan-Ras (1:1000) from Calbiochem (Darmstadt, Germany) and Ras A (1:5000) from BD Biosciences (San Jose, CA). Immunoreactive protein bands were detected using the ECLTM Detection Reagents (GE Healthcare, Buckinghamshire, UK). Densitometric values of bands were normalized to the mean value of three replicates of a control tumor sample and then to the microgram of protein loaded.

Ras-guanosine triphosphate and RasB/A-guanosine triphosphate pulldown assays

The capacity of Ras-guanosine triphosphate (GTP) to bind Ras-binding domain of Raf-1 was used to analyze the active p21Ras. Three-hundred micrograms of total or cytosolic extracts and 150 μg of the membrane extract were incubated for 2 h at 4°C with glutathione sepharose-4B beads (GE Healthcare) pre-coupled with glutathione S-transferase (GST)–Ras-binding domain of Raf-1. Beads were washed in the above-described buffer with (total and membrane extracts) or without Triton X-100 (cytosol extract). For RasB/A-GTP measurement, their ability to bind to the activated form of RALBP1 was used. Two-hundred and fifty micrograms of subcellular extracts were incubated with GST-RALBP1 protein bound to agarose beads (Upstate Biotechnology) for 2 h at 4°C. Proteins were boiled off the laemmli sample buffer and analyzed by western blot.

Immunoprecipitation

To identify mono-ubiquitylated PCNA (Ub-PCNA), immunoprecipitation of total PCNA protein was performed, followed by immunodetection with the ubiquitin antibody. Briefly, 300 μg of total extract without β-mercaptoethanol were incubated overnight at 4°C with 3 μl of PCNA antibody. The resulting pellet was washed, solubilized, incubated together with the previously prepared Protein A–Agarose Fast Flow (Sigma–Aldrich) for 2 h at 4°C and analyzed by western blot.

DNA extraction and detection of c-Ha-ras1 point mutation

Genomic DNA was isolated by phenol–chloroform extractions. The codon 61 c-Ha-ras1 mutation was detected by Southern blot, as described previously (24), using a digoxigenin-labeled v-Ha-ras probe (Oncor, Gaithersburg, MD) synthesized through the PCR DIG Probe Synthesis kit (Roche Applied Science, Mannheim, Germany). It was also assessed by allele-specific mismatch amplification mutation assays according to Cha et al. (25). The primers used were as follows: 5’-GGTTAGGCCCTTAAAGCTGTG-3’ (forward); 5’-CATGGCATTAGCTCCTCC-3’ (reverse). One microgram of genomic DNA was amplified using Ready-To-Go Polymerase Chain Reaction (PCR) Beads (GE Healthcare) for 30 cycles [94°C for 1 min, 55°C for 1 min and 72°C for 1 min] followed by a final extension at 72°C for 5 min. A 120 bp fragment was detected by agarose gel electrophoresis in mutated allele-bearing samples.

To confirm the presence of the mutation, a 256 bp fragment of c-Ha-ras1 second exon was amplified, using the primers 5’-GGTGGACCCCTAAAGCTGTG-3’ (forward) and 5’-CCTTCCTCCCATACATGGCAT-3’ (reverse) and one-hundred nanogram of DNA in the reaction conditions described above. PCR products were then 2% gel purified and automatically sequenced.

RNA extraction, reverse transcription and real-time PCR

mRNA expression of HMG-CoA reductase and SQS was analyzed by real-time PCR. Total RNA was isolated using the guanidinium thiocyanate method, and genomic DNA was removed by DNase treatment. RNA integrity and quantity were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two micrograms of total RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Twenty-five nanograms of complementary DNA were amplified by PCR with the fluorescent TaqMan methodology (Applied Biosystems) in the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Reactions were prepared with the TaqMan Universal PCR Master Mix and the suitable TaqMan assay (Hmgcr: Rn00565598_m1; Sqs: Rn0057323_m1 and Hprt: Rn152640_m1). Complementary DNA was denatured for 10 min at 95°C and amplified for 40 cycles of 15 s at 95°C and 60 s at 60°C. The gene expression was normalized to Hprt as a control transcript using ΔCt methodology (Ct as PCR cycle crossing the threshold).

Apoptosis detection

Apoptosis was assessed by western blot of cleaved Caspase-3. It was also detected through the TUNEL assay using the ApopTag Plus Peroxidase In Situ Detection Kit (Chemicon, Concord Road, MA) according to the manufacturer’s protocol. Apoptotic cells were visualized under an Axiosstar Plus microscope (Carl Zeiss Microimaging, S.L., Barcelona, Spain).

DNA enzymatic digestion and 8-oxo-dG assay

DNA enzymatic digestion and 8-oxo-dG assay were performed according to Oltra et al. (26). The amount of 8-oxo-dG and deoxyguanosine in the DNA digest was measured by electrochemical and ultraviolet absorbance detection, respectively.

Characterization of tumor phenotype

Based on the modified Scarff-Bloom-Richardson grading scheme (20), carcinomas were classified as well (Grade I), moderately (Grade II) or poorly (Grade III) differentiated. Mitotic activity was classified into five classes, defined on the basis of the number of mitoses in 10 high-power fields, at ×400 magnification as follows: (i) ≤3; (ii) 4–6; (iii) 7–9; (iv) 10–19 and (v) ≥20. The tumor growth was characterized through the following statistics: tumor volume at sacrifice, Vs; average area of the tumor growth curve, Area; and rate of linear growth (V/tumor age), Vage.

Statistical analysis

Statistical analyses were performed using SPSS software (Version 15.0). The non-parametric Mann–Whitney’s U-test was used for quantitative data and the chi-squared test for qualitative data. The non-parametric correlations were carried out through Kendall’s tau-b and Spearman’s rho tests. The level of significance was established at P < 0.05. Only mammary adenocarcinomas with noticeable levels of a particular protein studied were considered.

Results

Expression and activity of the ErbB receptors

Immunoblot analyses revealed no differences in the tumor expression of the membrane-bound full-length proteins ErbB1, ErbB2, ErbB3 and ErbB4 due to the dietary lipids (Figure 1). Regarding ErbB4, the levels of the 80 kDa form, identified as a membrane-bound and/or soluble (m80/s80) cleavage product by comparison
with a positive control cell line, were significantly lower in the HFOO group, as compared with the HFCO group ($P < 0.013$). The levels of the active phospho-ErbB receptors (p-ErbB1 Tyr1068, p-ErbB2 Tyr1248, p-ErbB3 Tyr1289 and p-ErbB4 Tyr 1056) were not significantly changed by dietary lipids either. It is noteworthy that the ErbB1 receptor was found not to be phosphorylated on tyrosine 1173, and the only phospho-ErbB4 was that corresponding to the 80 kDa form.

**p21Ras expression and activity**

The p21Ras levels were significantly higher in total ($P = 0.019$) and membrane ($P = 0.030$) extracts from the HFOO group, as compared with the control, and also to the HFCO group ($P = 0.037$ and $P = 0.032$, respectively) (Figure 2). No differences were observed in the p21Ras levels in the cytosolic fractions, which were lower than those of membranes. The active GTP-bound p21Ras levels were significantly diminished in the HFOO group both in total ($P = 0.046$) and membrane fraction ($P = 0.014$) extracts, as compared with the control, and the HFCO group ($P = 0.01$ and $P = 0.012$, respectively). No significant amounts of p21Ras-GTP were found in the cytosol. No changes in p21Ras and p21Ras-GTP levels were observed between the HFCO and the control.

**c-Ha-ras1 mutation status**

To determine whether the high EVOO diet modified Ras activity by changing the mutation frequency of c-Ha-ras1, the occurrence of the...
activity levels found in the HFOO group were related to a specific modulation of the mevalonate pathway. The mRNA expression of HMG-CoA reductase and SQS was analyzed by real-time PCR. The median values for the former were 1.83 (LF, n = 17), 1.66 (HFCO, n = 12) and 1.80 (HFOO, n = 12). The medians for SQS were 1.11 (LF, n = 17), 1.00 (HFCO, n = 12) and 1.23 (HFOO, n = 12). In both cases, differences were not statistically significant.

Ras effector pathways status
The Raf-1/ERK1/2, PI3K/Akt and RalGDS/RalA/B pathways were evaluated through the analysis of the expression and activity of ERK1/2, Akt and RalA/B proteins, respectively. As shown in Figure 3A, the protein levels of ERK1/2 were significantly reduced in tumors from the HFCO (P = 0.022) and HFOO groups (P = 0.019), in comparison with the control. As for ERK1/2 activity, a non-significant increase of >2-fold in the medians of the phosphorylated proteins due to the high-fat diets was found. Whereas almost all tumors (92.9–100%) expressed phospho-ERK1, the percentage of positive phospho-ERK2 tumors was LF = 50% (7/14), HFCO = 84.2% (16/19) and HFOO = 56.3% (9/16), these differences being statistically significant (P = 0.037) for the HFCO group, when compared with the control. As regards the PI3K/Akt pathway (Figure 3B), a significant decrease in the Akt expression levels in the HFCO (P = 0.01) and HFOO groups (P = 0.03), as compared with the control, was found. In contrast, the phospho-Akt levels were reduced by ~2-fold in the HFOO group, in comparison with the other groups, which displayed similar levels. The HFCO group showed the highest phospho-Akt:total Akt ratio (median values: LF = 0.024; HFCO = 0.08 and HFOO = 0.026). Finally, as regards the RalGDS/RalA/B pathway (Figure 3C and D), neither expression nor activity levels of RalA/B proteins were different among experimental groups.

Apoptosis analysis
In view of the EVOO-induced changes in ERK1/2 and Akt activities, it was investigated whether dietary lipids may modify the apoptosis rate. The analysis of Caspase-3 through immunoblotting showed that EVOO significantly increased the levels of 17 kDa active Caspase-3, as compared with the control. As regards the PI3K/Akt pathway (Figure 3B), a significant decrease in the Akt expression levels in the HFCO (P = 0.01) and HFOO groups (P = 0.03), as compared with the control, was found. In contrast, the phospho-Akt levels were reduced by ~2-fold in the HFOO group, in comparison with the other groups, which displayed similar levels. The HFCO group showed the highest phospho-Akt:total Akt ratio (median values: LF = 0.024; HFCO = 0.08 and HFOO = 0.026). Finally, as regards the RalGDS/RalA/B pathway (Figure 3C and D), neither expression nor activity levels of RalA/B proteins were different among experimental groups.

Cellular proliferation
Tumor cell proliferation was firstly evaluated by measuring mitotic activity in hematoxylin and eosin-stained paraffin-embedded tumor sections. Adenocarcinomas from the HFCO group displayed a higher mitotic index than the other groups (P = 0.033), with a similar level in the HFOO group (P = 0.017). In the HFCO group the Ubi-PCNA levels were higher than in the control (P = 0.033) and the HFOO groups (P = 0.047). The analysis of Caspase-3 through immunoblotting showed that EVOO significantly increased the levels of 17 kDa active Caspase-3, as compared with the control. As regards the PI3K/Akt pathway (Figure 3B), a significant decrease in the Akt expression levels in the HFCO (P = 0.01) and HFOO groups (P = 0.03), as compared with the control, was found. In contrast, the phospho-Akt levels were reduced by ~2-fold in the HFOO group, in comparison with the other groups, which displayed similar levels. The HFCO group showed the highest phospho-Akt:total Akt ratio (median values: LF = 0.024; HFCO = 0.08 and HFOO = 0.026). Finally, as regards the RalGDS/RalA/B pathway (Figure 3C and D), neither expression nor activity levels of RalA/B proteins were different among experimental groups.

8-oxo-dG assay
In view of the changes in the Ubi-PCNA levels, we asked ourselves whether mammary adenocarcinomas displayed different DNA damage levels due to dietary lipids. The levels of the pre-mutagenic 8-oxo-dG base were measured in the genomic DNA isolated from tumors of the distinct dietary groups. The median values obtained were

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**Fig. 2.** Total and activated p21Ras levels in the experimental mammary adenocarcinomas from rats fed a low-fat, high-fat corn oil or high-fat olive oil diet (LF, HFCO and HFOO groups, respectively). (A) Representative immunoblots of 21 kDa total Ras and GTP-bound Ras (activated) in the different protein extracts (total, membrane and cytosol) from each dietary group. No p21Ras-GTP band was detected in the cytosol fraction. Median values for total p21Ras (B) and p21Ras-GTP (C) are shown. A p21 Ras (Gly12) Western Blot Standard (Calbiochem) was used for quantitative purposes. The number of samples with detectable levels of protein/the total number of samples analyzed, and the percentage that those expressing samples represent, is indicated under each histogram. *P < 0.05, non-parametric Mann–Whitney’s U-test.
as follows (8-oxo-dG/10^6dG): LF = 3.03 (n = 21); HFCO = 2.22 (n = 25) and HFOO = 2.97 (n = 25). The differences were not statistically significant.

Tumor phenotype

Here, it was verified that the group of tumors analyzed in this work for each dietary treatment displayed a different phenotype (Table I). Thus, adenocarcinomas from the HFCO group showed a higher morphological malignancy, based on the greater degree of the Scarff-Bloom-Richardson scheme and the higher mitotic activity (P = 0.033 and P = 0.013, respectively, versus the control group), and a more aggressive growth as indicated by the higher values of the tumor volume parameters (Vs, P = 0.05; Area, P = 0.029 and V/age, P = 0.018; versus the HFOO group). In contrast, adenocarcinomas from the HFOO group showed morphological and clinical features consistent with a lower degree of malignancy, similar to control tumors.

Discussion

The present study shows that the EVOO diet, which negatively modulates the experimental mammary carcinogenesis, decreases
Ras activity, without the involvement of ErbB receptors, and generates a particular cell signaling scenario resulting in a proliferation–apoptosis balance tilted in favor of apoptosis. In contrast, the HCO diet stimulates mammary cancer through a combination of Ras-independent cell signaling pathways, which deflects this balance in favor of the survival of transformed mammary cells. Moreover, the

![Fig. 4](image-url)

**Fig. 4.** Apoptotic cell death in the experimental mammary adenocarcinomas from rats fed a low-fat, high-fat corn oil or high-fat olive oil diet (LF, HFCO and HFOO groups, respectively). (A) Western blot was used to evaluate the expression levels of the 17 kDa fragment resulting from the cleavage of Caspase-3 upon activation. The median values for each group are represented. The number of samples with detectable Caspase-3/the total number of samples analyzed, and the percentage that those expressing samples represent, is indicated under each histogram. *P < 0.05, non-parametric Mann–Whitney’s U-test. (B) Tumor histological sections from each group were subjected to TUNEL assay to detect apoptotic nuclei (brown) and counterstained with 0.5% methyl green to detect intact nuclei (green). Apoptotic cells were identified as cells with nuclear brown staining and morphological characteristics of apoptosis such as nuclear condensation, a perinuclear halo and apoptotic bodies. Slides from rat mammary gland in regression after weaning were used as positive controls, whereas DNase I-treated sections without terminal deoxynucleotidyl transferase enzyme were performed as negative controls. Low-power pictures at ×200 original magnification, high-power pictures (insets) at ×400 (LF and HFCO groups) or ×600 (HFOO) original magnification.

![Fig. 5](image-url)

**Fig. 5.** Cell proliferation in the experimental mammary adenocarcinomas from rats fed a low-fat, high-fat corn oil or high-fat olive oil diet (LF, HFCO and HFOO groups, respectively). (A) Tumor mitotic activity was evaluated by histopathological analysis of hematoxylin and eosin-stained sections of tumors. Original magnification ×400. (B) Two forms of the PCNA protein were detected by western blot analysis, a 36 kDa band corresponding to the unmodified protein (lower panel), and a 46 kDa band resulting from PCNA mono-ubiquitylation (upper panel). Median values from each form in the experimental groups are shown. One-hundred percent of the tumors analyzed expressed both PCNA forms, as is indicated under each histogram. *P < 0.05, non-parametric Mann–Whitney’s U-test.
### Table I. Characterization of the tumor phenotype

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Tumor volume was calculated as $V = \frac{4}{3} \pi (d_1/2) (d_2/2)^2$, throughout the study and as $V = \frac{4}{3} \pi (d_1/2) (d_2/2) (d_3/2)$, at sacrifice, where $d_1$, $d_2$ and $d_3$ are the three tumor diameters. LF, low-fat diet group; HFCO, high-fat corn oil diet group; HFOO, high-fat olive oil diet group; n, number of tumors analyzed; M, mean; m, median; SD, standard deviation.

aScore in the Scarff-Bloom-Richardson (SBR) grading method. 1 = Grade I or well-differentiated adenocarcinomas; 2 = Grade II or moderately differentiated adenocarcinomas; 3 = Grade III or poorly differentiated adenocarcinomas.
bScore in the tumor mitotic activity. 1 = ≤3 mitoses in 10 high-power fields at $\times$400 magnification; 2 = 4–6 mitoses; 3 = 7–9 mitoses; 4 = 10–19 mitoses and 5 = ≥20 mitoses.
cTumor volume at sacrifice.
dArea below the tumor growth curve.
eRate of linear growth.

EVOO diet was indirectly associated with lower levels of tumor DNA damage. All of these data confirm and significantly extend our previous works showing the more benign tumor phenotype of mammary cancer under the influence of EVOO.

Our initial expectation was that the modulatory effects of dietary lipids on mammary cancer would involve changes in ErbB signaling since this receptor family plays important roles in mammary development and human breast cancer (14,27). However, the expression and activity of ErbB1, ErbB2 and ErbB3 were not altered due to the diet, suggesting that these receptors are not required for the lipid effects on mammary carcinogenesis. We previously reported that the HCO diet increased mRNA levels of the ErbB1/EGFR truncated receptor, which seems to interfere in the appropriate ErbB signaling (28). Unfortunately, it was not possible to confirm this possibility because of the lack of suitable antibodies. No effect on EGFR expression and ErbB2 suppression due to the OA or phenols have been reported in ErbB2-overexpressing breast cancer cells (29). We found no changes either in the expression of the full-length ErbB4, but levels of the 80 kDa band significantly decreased in the HFOO group, as compared with the HFCO group. This band may correspond both to the transmembrane peptide (m80) and the soluble intracellular domain (s80), also called 4ICD. 4ICD may be generated sequentially by the ligand-binding and kinase activity-dependent proteolytic processing of ErbB4 and appears to mediate most of ErbB4 activities in the breast, although its biological significance in breast cancer is poorly known (30). Since the mammary gland exclusively expresses a cleavable ErbB4 isoform (31), the m80/s80 band detectable in ErbB4 and phospho-ErbB4 immunoblots would correspond to the activated receptor, but differences were only statistically significant in the first case. Further investigation will be necessary to establish the potential role of ErbB4 in EVOO effects.

The possible involvement of p21Ras in the dietary fat-breast cancer link was next investigated. The EVOO diet significantly decreased p21Ras activity, but it significantly increased protein levels. In contrast, no changes were observed in p21Ras due to the HCO diet. These findings suggest that p21Ras has a role in the negative modulatory effect of EVOO on breast cancer, whereas it is not involved in the stimulatory effect of the corn oil. As far as we know, this is the first report to show that EVOO influences in the p21Ras status in cancer. In particular, our data indicated that EVOO interferes somehow in its activation, resulting in an increase of the relative levels of the non-activated protein. The possible mechanisms by which EVOO underregulates p21Ras activity could include either (i) decreasing the ras mutation rate or (ii) modifying the regulation of the mevalonate pathway, which is the source of the prenyl groups needed for the posttranslational modification of p21Ras and its subsequent activation (32). The first possibility can be ruled out because the frequency of the activating c-Ha-ras1 mutation was not changed due to the diet. Interestingly, the median age of the mutation-bearing tumors was higher than that of the wild-type tumors, irrespective of the diet, which would be consistent with a selective advantage of growth conferred by ras mutation to the mammary tumors (33). To our knowledge, there are no published data about the influence of olive oil on tumor ras mutation. A decreased percentage of tumors harboring ras mutations due to HCO diets has been described in other mammary cancer models (34). To address the second possibility, we analyzed the mRNA expression of HMG-CoA reductase and SQS. Results discarded this possibility, at least at the enzyme expression level. They did not support the idea either that the chemopreventive effect of squalene on cancer, a minor component of EVOO, is due to the HMG-CoA reductase inhibition (35). It is probably that squalene should be at very high doses (1%) to exert this effect. We cannot rule out, however, that EVOO may modulate the enzyme activity or other critical steps of the p21Ras processing.

Long-chain n-3 PUFA has also been described to decrease Ras levels and/or activity in rat colon (23,36). It has been proposed that this may be the result of changes in membrane lipid composition, specifically the lipid raft and caveolae, thus altering the membrane structure and function and, in this way, influencing the intracellular trafficking and subcellular location of lipidated proteins such as p21Ras (37). The effects observed in p21Ras due to the EVOO diet could also be a consequence of an OA enrichment of cell membranes. Future studies will address this possibility.

When Ras effector pathways were examined, increased ERK1/2 activity and reduced ERK1/2 expression were found owing to the high-fat diets, the differences in the expression being statistically significant. These results suggest an unspecific effect of these diets on the Ras effector pathways. Further investigation will address this possibility.

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The EVOO diet also decreased both Akt expression and activation, whereas the HCO diet only decreased its expression. Given that Akt contributes significantly to survival and proliferation in cancer, and reduced phospho-Akt levels have been associated with apoptosis induction (41), our results suggest that EVOO effects may be linked to a downregulation of the pro-survival p21Ras/Akt pathway. In
contrast, tumors from the HFCO group displayed the highest phospho-Akt:total Akt ratio, consistent with the stimulating effect of this diet on tumor growth. Currently, we have no explanation for the decreased Akt expression in both high-fat diet groups. In fact, its transcriptional regulation remains largely unknown. In human breast cancer cells, OA both increased (42) and decreased (29) active Akt, and in colon cancer, hydroxytyrosol, an antioxidant from EVOO, abolished Akt phosphorylation and induced a transient ERK1/2 phosphorylation–dephosphorylation (43).

We also investigated the Rap1GDS–RapA/B pathway. Rap proteins regulate vesicle trafficking, actin cytoskeletal dynamics and transcription in response to mitogenic cascades (15). They seem to have a pivotal contribution to the regulatory framework supporting tumorigenesis (18). Dietary lipids did not modify the expression or activity of RapA/B proteins, suggesting a Rap-independent modulation of mammary carcinogenesis as well as a specificity of Ras signaling in the case of the EVOO diet.

The Akt downregulation due to the EVOO diet may seem contradictory to its upregulatory effect on ERK1/2 activity since the latter mainly provides cell survival and proliferation signals. However, a pro-apoptotic role of ERK1/2 depending on the kinetics and duration of its activation has been suggested (43). Thus, under the influence of the EVOO diet, the relative balance between ERK1/2 and Akt pathways would result in a pro-apoptotic cellular scenario. Interestingly, a variety of anticancer drugs that stimulate apoptosis have been reported to downregulate the Akt pathway and upregulate the ERK one (44,45). Consistent with this hypothesis, the activated Caspase-3 levels increased in the HFOO group. Caspase-3 is considered the main executor of the caspase cascade involved in both extrinsic and intrinsic apoptosis (46). TUNEL staining of mammary tumor sections confirmed increased apoptosis due to the EVOO diet. At present, there is growing evidence of apoptosis modulation by conjugated LA (47), long-chain n-3 PUFA (48) and OA and olive oil (42,49,50).

Our results also showed that the high EVOO diet did not exert any significant effect on tumor cell proliferation, as the mitotic index and PCNA levels indicated. Thus, a proliferation–apoptosis balance tilted toward apoptosis might contribute to the ability of this diet to negatively regulate tumor growth. On the other hand, the HCO diet significantly increased tumor mitotic activity. The fact that the PCNA levels did not reflect this effect may be due to the loss of the simple relation between PCNA expression and cell proliferation that has been described in some cancers (51). The higher proliferation rate associated with the HCO diet is consistent with our results supporting its role in transducing survival/proliferation signals and, therefore, with its stimulatory effect on cancer.

Interestingly, Ub-PCNA levels were significantly decreased in the HFOO group and non-significantly increased in the HFCO group. PCNA ubiquitination specifically occurs when DNA is damaged or replication forks stalled. PCNA mono-ubiquitination mediates translesion synthesis and poly-ubiquitination promotes error-free DNA damage tolerance pathways (52). Our data suggest less DNA damage owing to the EVOO diet and higher genotoxic stress due to the corn oil diet. Nevertheless, no significant changes were observed for the pre-mutagenic 8-oxo-dG base, which is one of the most common biomarkers of oxidative DNA damage (53). It is probably that the 8-oxo-dG levels did not reflect the total DNA adduct levels and that dietary lipids may have an effect on other DNA lesions. High levels of malondialdehyde-derived and etheno-DNA adducts have been described due to n-6 PUFA (54), whereas sunflower and olive oils did not increase levels of hepatic 1,2N2-propanodeoxyguanosine and 8-oxo-dG (55). The effect of EVOO on the oxidative DNA damage and the relationship with its pro-apoptotic effect must be further investigated.

In summary, even though caution must be exercised when applying experimental data to human breast cancer, our findings emphasize the importance that certain dietary habits may have on cancer promotion. Through a different modulation of cell signaling pathways, which modifies the cell proliferation–apoptosis balance, and probably a different level of DNA damage, the high EVOO and corn oil diets exert differential effects on this cancer. These results would help in the design of future strategies of secondary, and even primary, prevention of breast cancer.

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