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Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM–p53 cascade in colon cancer cell lines

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The Mediterranean diet is rich in extra virgin olive oil (EVOO) and associated with a lower incidence of colorectal cancer. EVOO contains phenolic extracts with potential anticarcinogenic activity. *Aim*: To assess the anticancer properties of EVOO phenolic extracts using *in vitro* models. *Methods*: Phenolic profiles of two different EVOOs (A and B) were determined. RKO and HCT116 (both p53 proficient), SW480 (p53 mutant) and HCT116$^{p53-/-}$ (p53 knocked out) cell lines were treated with EVOO extracts and assessed for cell viability. Apoptosis was determined by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay and changes in *Bax* transcript levels. Cell cycle analysis was determined by flow cytometry and western blots. To confirm the data, analysis of cell viability and cell cycle was performed with purified pinoresinol. *Results*: Chemical characterization showed that pinoresinol is the main phenol in EVOO-A, and oleocanthal predominates in EVOO-B. Only EVOO-A affected cell viability, which was significantly more pronounced in *p53*-proficient cells. At a concentration of 200 nM, *p53*-proficient cells showed increased apoptosis and G_2/M arrest. In *p53*-proficient cells, ataxia telangiectasia mutated (ATM) and its downstream-controlled proteins were upregulated after treatment, with a parallel decrease of cyclin B/cdc2. Identical results on cell viability and cell cycle were obtained with purified pinoresinol, but this required a higher concentration than in EVOO-A. *Conclusion*: Our results demonstrate that pinoresinol-rich EVOO extracts have potent chemopreventive properties and specifically upregulate the ATM–p53 cascade. This result was achieved at substantially lower concentrations in EVOO than with purified pinoresinol, indicating a possible synergic effect between the various polyphenols in olive oil.

Introduction

Colorectal cancer (CRC) is the fourth commonest cancer in Western countries, affecting over one million of new cases globally every year, with nearly 500 000 deaths (1,2). The most effective treatment for CRC is surgical resection, and early stage diagnosis is crucial for a beneficial outcome (3,4). However, primary prevention would represent the optimal strategy. At this time, the side effects of currently available chemopreventive drugs do not justify their application across the general population (2,5–7). For primary prevention purposes, candidate agents should be exceptionally safe, non-expensive and effective in suppressing the early phases of the disease. The role of diet in modulating CRC risk is a well-accepted concept. A reasonable corollary of this is that natural compounds, which are proven safe over long periods of time and easily accessible through the diet, might represent the ideal candidates as chemopreventive agents.

The Mediterranean diet is associated with beneficial health properties, including lower incidences of cardiovascular disease, age-related cognitive disease and cancer (8,9). Olive oil is a key ingredient of the Mediterranean diet, representing the main source of fat. In addition to its unsaturated fatty acids, olive oil provides a variety of minor compounds with beneficial properties (10,11). Bioactive compounds present in small quantities in food have been studied for their effects on health, and in particular phenols and polyphenols have well-established roles in disease prevention (12). Three main classes of phenolic compounds are represented in differing proportions in olive oil: simple phenols (phenolic acids and hydrolyzed secoiridoids such as hydrotyrosol and tyrosol), secoiridoids and lignans (10). Olive oils possess different phenolic profiles according to the variety of olive and technical conditions used for production (13,14).

The biological effects of polyphenols are varied and compound specific (15). For example, olive oils rich in hydroxytirosol have been demonstrated to inhibit cell proliferation and induce apoptosis in human promyelocytic leukemia and in CRC cell lines (10,16,17). The dialdehyde form of one of the secoiridoids, deacetoxy-ligstroside aglycone (also called oleocanthal), has an ability to inhibit cyclooxygenase *(COX)-1 and COX-2* similar to that seen with ibuprofen (18,19). Recently, oleuropein aglycone was found to directly regulate HER2 in breast cancer cells (20). Lignans are a major antioxidant component of olive oil and have anticancer effects in the breast, lung, skin and colon (14). Antioxidant (14) and antiviral properties (21) of lignans have been reported, and the similarities in structure between some lignans and estradiol or tamoxifen suggest possible activity against breast cancer (22).

The aim of the present study was to determine the mechanisms underlying the anticancer effects of phenolic compounds contained in two different varieties of extra virgin olive oil (EVOO) on *in vitro* models of CRC. Crude EVOO extracts were used rather than isolated phenols, testing the hypothesis that the naturally occurring family of compounds present in the oil used in the diet might have synergistic properties (10,15). We evaluated the activity of EVOO extracts on cell proliferation, apoptosis and cell cycle regulation and focused on specific pathways involved in the regulation of epithelial cell growth.

Materials and methods

Extraction and characterization of phenolic compounds from EVOO

The two EVOO preparations used in this study were obtained from olives (*Olea europaea*) of two varieties named Caiazzana and Ravece, which we called EVOO-A and EVOO-B, respectively. Both olive varieties are characteristic of the centralsouth regions of Italy. Olives were harvested in November 2005 and within 4 h, extracted in a continuous extraction plant, using a percolation–centrifugation system with hammer crushers, a centrifugal decanter and a separator. Unfiltered oils were stored in filled amber glass bottles at room temperature (between 14 and 25°C) until extraction (~2 months).

Extraction of phenols from virgin olive oil was performed according to the procedure of Vasquez-Roncero (23). Single phenolic components were identified and quantified by liquid chromatography–mass spectrometry, as described by Monti *et al.* (24), using an API-100 single-quadruple mass spectrometer (PerkinElmer Sciex Instruments, Waltham, MA) equipped with an atmospheric pressure chemical ionization ion source, and the analyses were performed using a multi-SIM detection system.

In order to express the molar concentrations of the phenolic compounds used in the mixture of EVOO as a single number, we expressed them as pinoresinol equivalents. Therefore, the sum of all the compounds present in the chromatogram was considered and quantified using a pinoresinol calibration curve.

Abbreviations: ATM, ataxia telangiectasia mutated; CRC, colorectal cancer; EVOO, extra virgin olive oil; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction.

Thus, the concentrations of the phenolic compounds were expressed in pinoresinol equivalents.

Cell culture and treatment

The human CRC cell lines RKO, SW480 and HCT116 were obtained from the American Type Culture Collection, Manassas, VA. HCT116$^{p53-/-}$ (*p53* knockout) cells were kindly obtained from Bert Vogelstein. RKO and HCT116 cells have microsatellite instability as a consequence of promoter methylation and homozygous mutation in *hMLH1*, respectively. Both cell lines have wild-type *p53*. SW480 is a microsatellite stable cell line with inactivating mutations in the *p53* gene (25). The cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (Life technologies, Grand Island, NY) and 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco, Invitrogen Corporation, Carlsbad, CA). The cultures were maintained at 37°C in 5% CO_2. The treatment with EVOO phenolic extracts was performed based on the Mean Inhibition concentration (IC50) results obtained, while changing the conditioned media every 48 h. Pinoresinol was purchased from PhytoLab GmbH and Co. KG (Vestenbergsgreuth, Germany), resuspended in methanol, aliquoted and stored at −80°C. As reported by the company, the Pinoresinol was obtained from plants and was >95% pure.

Cell viability (MTT assay)

Cells were seeded at a density of 3000 cells per well in a 96-well plate. The next day, cells were treated with concentrations ranging from 0 to 20 μM of EVOO phenolic extracts dissolved in methanol. The cells were treated with concentrations of purified pinoresinol ranging from 0 to 70 μM.

An appropriate amount of methanol was also added to the control wells. After 96 h of treatment, the cells were incubated with a solution of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St Louis, MO) at a concentration of 0.5 μg/μl for 3 h at 37°C. The cells were lysed in a buffer containing 10% sodium dodecyl sulfate and 0.01 N HCl and analyzed after 12 h of incubation at 37°C. Colored formazan converted from MTT by viable cells was measured at 570 nm by a microplate reader. Experiments were performed in triplicate.

Determination of the induction of apoptosis

Late apoptotic events were analyzed by TUNEL assay using the *In Situ* Cell Death Detection Kit (Roche, Branchburg, NJ). Briefly, the cells were plated on glass coverslips in 24-well plates at a concentration of 3000 cells per well followed the next day by 96 h of treatment at the final concentration of 200 nM EVOO phenolic extracts, changing the conditioned media every 48 h. An equal amount of methanol was used in the untreated, control cells. The TUNEL assay was performed according to the manufacturer's protocol. Pretreatment with DNase I (3000 U/ml in 50 mM Tris–HCl, 1 mg/ml bovine serum albumin) was used for the positive controls. Apoptotic cells were visualized under an AxioSkop2 multichannel epi-fluorescent microscope and processed by AxioVision software (Carl Zeiss, Thornwood, NY). Each experiment was repeated three independent times.

Real-time polymerase chain reaction for measurement of Bax expression

Real-time polymerase chain reaction (PCR) was performed to evaluate changes in the transcript levels of the proapoptotic gene *Bax*. After treatment, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. TaqMan One-Step RT–PCR Master Mix (Roche) and the TaqMan Gene Expression Assay for *Bax* (Hs 00180269; Applied Biosystems, Foster City, CA) were used. One microgram of RNA from each sample was used as the template. Glyceraldehyde-3-Phosphate Dehydrogenase was used as an endogenous control. The ABI Prism 7000 Sequence Detection System (ABI 7000 SDS) was used for real-time PCR analysis. Thermal cycling conditions were designed as follows: RNA retro-transcription at 48°C for 30 min followed by an initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantification of gene expression was performed using the comparative C_T method (ΔΔCt). Each evaluation was performed in triplicate in three independent experiments.

Cell cycle analysis

The effects of EVOO phenolic extracts on cell cycle profiles were evaluated by flow cytometry. Cell cycle distribution was based on an evaluation of the amount of DNA stained with propidium iodide. Cells were plated at a density of 5×10^5 cells per plate in 100 mm dishes, synchronized by serum deprivation for 48 h and finally treated with 200 nM of EVOO-A and 10 μM of EVOO-B, with the conditioned media changed every other day, for a total duration of 96 h. We followed the same protocol for the treatment with purified pinoresinol, with a final concentration of 700 nM. The cells were harvested, resuspended at a density of 5×10^6 cells/ml in cold phosphate-buffered saline and fixed with 80% ethanol overnight at −20°C. The next day, the cells were washed, resuspended in 300 μl of phosphate-buffered saline, incubated with 160 μg/ml of boiled and renatured ribonuclease A for 15 min at 37°C and stained with 80 μg/ml of propidium iodide for 30 min. DNA content was evaluated by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Cell cycle distribution was determined using the ModFit DNA Analysis Software (Verity Software House, Topsham, ME). Each experiment was performed three independent times.

To evaluate the involvement of the *ATM* axis, cells were also subjected to pretreatment with caffeine (Sigma-Aldrich Chemical Company, St Louis, MO) at a concentration of 5 mM, 3 h prior to treatment with EVOO.

Western blotting analysis

Protein extraction was performed using Ripa Buffer (Santa Cruz Biotechnology, Santa Cruz, CA) combined with 10 μl/ml of phenylmethylsulfonyl fluoride solution, 10 μl/ml of sodium orthovanadate solution and 10 μl/ml of protease inhibitor cocktail. The appropriate amount of lysis buffer was added to each sample and the pellets were sheared with a syringe. The cell lysates were incubated for 1 h on ice and centrifuged for 10 min to obtain clear supernatants. The protein concentration was measured using the bicinchoninic acid assay protein assay kit (Pierce, Rockford, IL) as indicated by the manufacturer. Aliquots of 40 μg of protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Transferred proteins were stained with Ponceau red to confirm successful transfer, followed by blocking with 5% non-fat milk or 5% bovine serum albumin in Tris-buffered Saline Tween-20 (50 mM Tris, pH 7.6, 150 mM NaCl and 0.1% Tween 20). Membranes were then probed with the specific primary antibody followed by incubation with the appropriate secondary antibody, including goat anti-mouse IgG–horseradish peroxidase (Santa Cruz Biotechnology), donkey anti-goat IgG–horseradish peroxidase (Santa Cruz Biotechnology) and anti-rabbit IgG–horseradish peroxidase (GE Healthcare Life Science Corp., Piscataway, NJ). The membranes were visualized using the ECL Plus chemiluminescence system and scanned with a Storm 840 PhosphorImager (Amersham Biosciences Arlington Heights, IL). Quantification of the bands was performed using ImageQuant 5.2 spot densitometric software (Molecular Dynamics, Sunnyvale, CA). The expression levels of the proteins were normalized to the expression of the housekeeping protein β-actin. The primary antibodies, including anti-cyclin D1 (clone A-12), anti-cyclin E (clone HE 12), anti-cyclin B1 (clone D11), anti-p53 (clone DO-1), anti-p21 (clone F5), anti-GADD45 (C4), anti-14-3-3σ (N14), Chk1 (G4) and Chk2 (B4) were obtained from Santa Cruz Biotechnology and incubated for 3 h at room temperature. The primary antibodies anti-phospho-p53 (Ser15) and anti-cdc2 were purchased from Cell Signaling Technology (Danvers, MA), and the anti-ATM (clone 3E8) was purchased from GeneTex (San Antonio, TX) and incubated overnight at 4°C. All antibodies were used at a working concentration of 2 μg/ml, and all experiments were performed at least twice.

Statistical analysis

A two-way analysis of variance model, including the effects for cell line, dose and interaction between cell line and dose, was used to evaluate the relationship between cell viability and those effects. The relative amount of *Bax* gene expression was quantified using the comparative C_T method ($\Delta\Delta C_T$). A one-way analysis of variance approach was used to compare the fold changes among the cell lines, and the Tukey–Kramer method for pair-wise comparisons. The chi-square test was performed to assess the differences in the cell cycle distributions before and after treatment in each cell line, and then a two-way analysis of variance including the effects for cell line and treatment were used to assess the treatment effect on G_2/M cell cycle checkpoint arrest. A *t*-test was used to test the treatment effect on gene expression in *p53*-proficient and *p53*-deficient cell lines. Significance was assumed for a value of $P < 0.05$.

Results

Identification and concentration of EVOO compounds in two different types of olive oil

The phenolic compound compositions in the two EVOO samples obtained from the two different olive varieties (EVOO-A and EVOO-B) are shown in Table I. The main phenolic compound in EVOO-A was pinoresinol (62%), which made up <20% of EVOO-B. EVOO-B contained more of the ortho dihydroxy derivatives, thus having higher antioxidant activity than EVOO-A (data not shown). Interestingly, EVOO-B contained a relatively large amount of p-HPEA-EDA (oleocanthal, the dialdehyde form of decarboxymethyl ligstroside aglycone) that is considered to be the anti-inflammatory moiety, while in EVOO-A, oleocanthal represented only 6% of the total phenolic extract.

Table I. Composition of the two EVOO phenolic extracts

Main phenolic compounds in EVOO extracts (%)		
	EVOO-A	EVOO-B
3,4-DHPEA-EDA	0	22.4
p-HPEA-EDA (oleocanthal)	6	35
3,4-DHPEA-EA	16.4	16.5
p-HPEA-EA	15.6	7.5
Pinoresinol	62	18.6

3,4-DHPEA-EDA, dialdehyde of decarboxymethyl oleuropein aglycone; p-HPEA-EDA (oleocanthal), dialdehyde of decarboxymethyl ligstroside aglycone; 3,4-DHPEA-EA, oleuropein aglycone; p-HPEA-EA, ligstroside aglycone.

Pinoresinol-rich EVOO extract (EVOO-A) decreases cell viability in p53-proficient cell lines

Cell viability was evaluated by the MTT assay. First, we compared the effects of the two EVOO extracts (EVOO-A and EVOO-B) using two models: RKO and SW480. Cells were treated with EVOO-A and EVOO-B at concentrations ranging from 20 nM to 20 μM for 4 days. Only the pinoresinol-rich EVOO-A inhibited cellular viability in a concentration-dependent manner (Figure 1A). Furthermore, among the EVOO-A-treated samples, the inhibition of viability in RKO (*p53* proficient) was significantly more pronounced compared with SW480 (*p53* mutant), starting at 200 nM ($P < 0.0001$). Thus, the pinoresinol-rich EVOO-A extract was selected for subsequent studies.

In order to clarify whether the differences in viability after EVOO-A treatment were explained by the different genetic profiles of the models used, we evaluated the effects of EVOO-A on cell viability in a larger panel of cell lines, including the syngeneic pair, HCT116 (*p53* proficient) and HCT116$^{p53-/-}$ (*p53* knocked out). Our results show that the effects on the cell viability were significantly more pronounced in the *p53*-proficient cell lines ($P < 0.0001$) (Figure 1B), with a decrease in viability of 50% (IC50) in the *p53*-proficient cells, versus 19% ± 1.7% in SW480 and 13% ± 1.5% in HCT116$^{p53-/-}$.

Based on these data, in the next series of experiments the treatment was performed at the concentration of 200 nM EVOO-A for 4 days, changing the conditioned media every 48 h.

To confirm that the biologic effects were due to the activity of pinoresinol, we performed MTT assays with purified pinoresinol. We observed a reduction in cell viability in a concentration-dependent manner only in the p53-proficient cells ($P = 0.0002$) (supplementary Figure 1, available at *Carcinogenesis* Online). Interestingly, this effect required a higher concentration of pinoresinol (IC50 of 700 nM) compared with what is present in EVOO-A (124 nM), sugggesting a synergistic effect in the EVOO-A mixture.

Pinoresinol-rich EVOO-A extract induces apoptosis in p53-proficient cell lines

Apoptosis was determined either by TUNEL assay, as a measure of DNA fragmentation, and by real-time PCR for *Bax*, a proapoptotic gene in the *p53*-downstream pathway. As shown in Figure 2A, apoptotic cells were visible in the preparations obtained after treatment in both the *p53*-proficient cell lines (RKO and HCT116), whereas no staining was detectable in SW480 and HCT116$^{p53-/-}$. The induction of apoptosis was evaluated further by measuring transcript levels of *Bax*. A significant increase in *Bax* messenger RNA was observed in RKO and HCT116 cells after treatment, whereas no changes were detected in the *p53*-deficient cell lines SW480 and HCT116$^{p53-/-}$ ($P < 0.0001$, Figure 2B, left panel). Furthermore, the *Bax* transcript levels in *p53*-proficient and *p53*-deficient cell groups were significantly different after treatment ($P < 0.004$), suggesting that changes in p53 activity directly modified the expression of *Bax* (Figure 2B, right panel).

To test the hypothesis that p53-mediated apoptosis was a specific effect of pinoresinol-rich olive oil, we evaluated the expression of *Bax* after treating the cells with EVOO-B. Based on the MTT results, no significant effects on cell viability were detected at concentrations ranging from 200 nM to 20 μM. Moreover, when the cells were treated with EVOO-B at concentrations ranging from 200 nM to 20 μM, no changes in *Bax* transcripts were detected (data not shown).

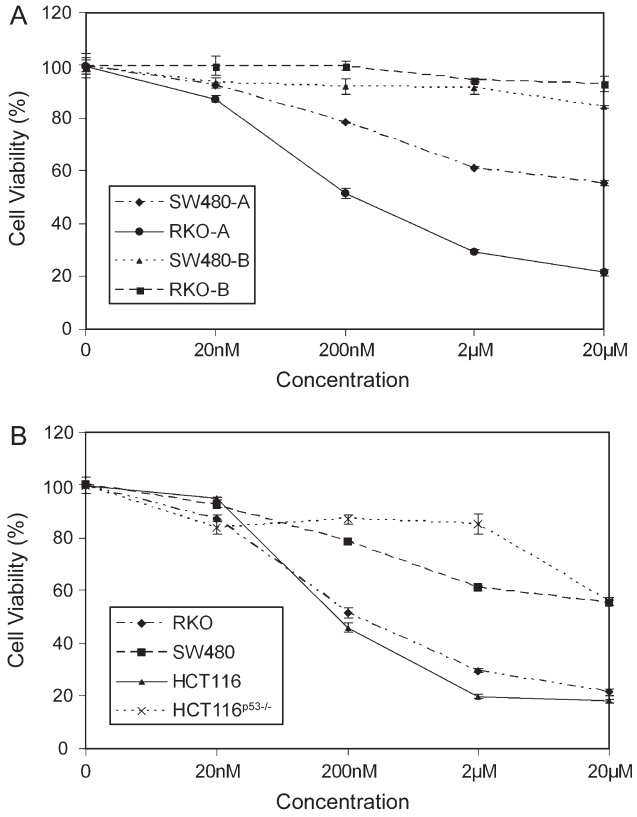

Fig. 1. Cell viability. (**A**) Cell viability (MTT assay) in RKO and SW480 cell lines after 96 h of EVOO-B (upper curves) and EVOO-A (lower curves). Cell viability is expressed as a ratio of the absorbance between treated cells and untreated controls. Each point is a mean ± SE of three independent experiments. Only the pinoresinol-rich EVOO-A (the two lower curves) inhibited cellular proliferation in a concentration-dependent manner. The *P* value for overall comparison between EVOO-A and EVOO-B is <0.0001, and a significant difference was seen starting at the second dose. Furthermore, among the EVOO-A-treated samples, the inhibition of proliferation in RKO (wild-type *p53*) was significantly greater than with EVOO-A-treated SW480 (inactive *p53*), starting at 200 nM ($P < 0.0001$). (**B**) Cell viability after 96 h of EVOO-A treatment in HCT116$^{p53-/-}$ and SW480 (inactive *p53*, upper curves) and RKO and HCT116 (wild-type *p53*, lower curves). A significant difference between RKO/HCT116 and SW480/HCT116$^{p53-/-}$ was found starting at the 200 nM dose ($P < 0.0001$).

Pinoresinol-rich EVOO-A extract induces G_2/M cell cycle arrest selectively in p53-proficient cell lines

The effects of EVOO-A treatment on cell cycle progression were studied by flow cytometry. First, the cells were synchronized by serum deprivation and subsequently treated every 48 h, for 96 h, with 200 nM of EVOO-A. Each experiment was repeated three times. As shown in Figure 3A, in the *p53*-proficient cell lines, EVOO-A treatment induced a significant arrest in G_2/M, with an increase in the G_2 population in RKO and HCT116 from 0.96% ± 0.056% and 2.08% ± 0.3% to 45% ± 9.1% and 48% ± 2.6%, respectively ($P < 0.0001$). Corresponding decreases of G_1 and S phase cells were observed. In the *p53*-deficient cell lines, a slight, but not significant, increase in the S phase was observed.

We then tested the hypothesis that the same effects on the cell cycle dynamics would be obtained after treating the cells with EVOO-B. No changes in the cell cycle profiles were demonstrated after EVOO-B

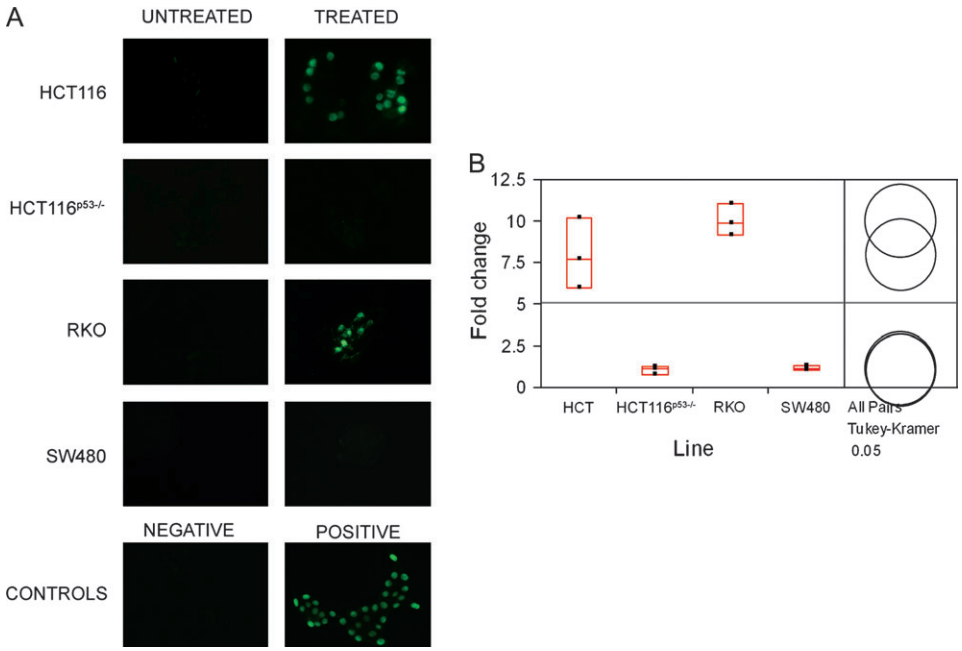

Fig. 2. Apoptotic response to EVOO-A treatment. (**A**) Apoptosis was evaluated by TUNEL assay as a measure of DNA fragmentation. The cells were treated for 96 h with EVOO-A at a concentration of 200 nM. TUNEL-positive cells were observed in RKO and HCT116 (*p53* proficient) after treatment. No staining was found after treating SW480 and HCT116$^{p53-/-}$. Pretreatment with DNase I was used as a positive control, whereas incubation without terminal deoxynucleotidyltransferase was performed as the negative control. Reproducible results were obtained in three independent experiments. (**B**) Changes in the expression of *Bax* were evaluated by real-time PCR. Each point on the left represents an independent experiment performed in triplicate. The relative amounts of *Bax* transcripts were quantified using the comparative C_T method ($\Delta\Delta C_T$). HCT116 and RKO expressed significantly more *Bax* than HCT116$^{p53-/-}$ and SW480 ($P < 0.0001$). On the right side, using a pair-wise Tukey–Kramer test, *p53*-proficient and -deficient cell lines were distributed into two statistically different groups: HCT116 and RKO (upper circles) versus HCT116$^{p53-/-}$ and SW480 (lower circles), $P < 0.004$.

treatment in the two models of CRC (data not shown). Taken together, these results suggest that only pinoresinol-rich olive oil affects apoptosis and cell cycle arrest, and that these effects are exclusively found in the *p53*-proficient cells. Additionally, we replicated the effects of EVOO-A on cell cycle using purified pinoresinol (supplementary Figure 2, available at *Carcinogenesis* Online). Pinoresinol induced a G_2/M arrest with an increase in the G_2 population only in the *p53*-competent RKO and HCT116 cells from 4.5 ± 0.1 to 31.3 ± 1.1 and from 2.35 ± 0.6 to 33.3 ± 1.1, respectively ($P < 0.001$).

Pinoresinol-induced G_2/M arrest is mediated by the ATM cascade in p53-proficient cell lines

Based on the observation of G_2/M cell cycle checkpoint arrest only in the *p53*-proficient cell lines after pinoresinol-rich olive oil treatment, we tested the hypothesis that the status of the p53 cascade mediated these changes. The activation of the cyclin-dependent kinase cdc2 and the cdc2–cyclin B complex are crucial for entry into mitosis. *p53* activation depends on ATM status, and three *p53*-downstream pathways can inhibit the cdc2–cyclin B complex. These involve p21$^{cip/waf}$, 14-3-3σ and GADD45, respectively. Furthermore, two kinases, Chk1 and Chk2, can mediate G_2 arrest by stabilizing p53 through phosphorylation at its N-terminus (26). As shown in Figure 4, increases of p53 ($P = 0.035$) and its activated form, phospho-p53 (Ser15) ($P = 0.029$), were observed after treatment specifically in the *p53*-proficient cell lines, followed by increases in the downstream targets p21$^{cip/waf}$ ($P = 0.002$), GADD45 ($P = 0.005$) and 14-3-3σ ($P = 0.004$). In RKO and HCT116, the activation of these regulatory pathways resulted in a corresponding decrease in cdc2 protein levels ($P = 0.002$, Figure 5). A significant decrease in cyclin E ($P = 0.009$), controlled by cdc2, was also observed, whereas no changes were observed in cyclin B and D levels.

Finally, we investigated the upstream regulator of the p53 axis, ATM and the ATM-controlled Chk1 and Chk2 proteins. EVOO-A treatment induced a significant increase of ATM protein levels exclusively in *p53*-proficient cell lines (Figure 5A). Significant changes in Chk1 ($P = 0.015$) and Chk2 ($P = 0.02$) protein expression were also detected in RKO and HCT116-treated samples. In the *p53*-deficient cell lines SW480 and HCT116$^{p53-/-}$, the system was not affected by the treatment (Figure 5). Finally, to demonstrate the involvement of the ATM axis, RKO and HCT116 were pretreated with caffeine 3 h before treatment with EVOO-A. Pretreatment with caffeine resulted in no changes in cell cycles compared with untreated controls (Figure 5B) ($P > 0.05$). Additionally, pretreatment with caffeine resulted in the same suppression of G_2/M arrest induced by pinoresinol in HCT116 and RKO, indicating that the biological effects on the ATM–p53 axis were due to pinoresinol activity (no difference when compared with untreated controls; $P = 0.73$) (supplementary Figure 2, available at *Carcinogenesis* Online).

Taken together, these findings suggest that the EVOO-A induced G_2/M arrest found in RKO and HCT116 was dependent on both the ATM–p53 as well as the ATM-Chk1/Chk2 cascades. The latter system may directly mediate ATM–p53 G_2/M arrest, and may contribute to the cell cycle arrest by enhancing p53 stabilization.

Discussion

In this study, we have demonstrated that pinoresinol-rich EVOO (EVOO-A) is able to decrease the cell viability, to induce apoptosis and modulate cell cycle dynamics in CRC cell lines. Our results provide evidence for the specific involvement of the ATM–p53 axis in the anticancer effects of pinoresinol.

The Mediterranean diet is associated with lower incidences of many diseases, including CRC (8,9). This diet is characterized by the consumption of unsaturated fat, and olive oil is its principal source. There is evidence that the quality rather than the quantity of fat may be important in modulating many disease processes, and

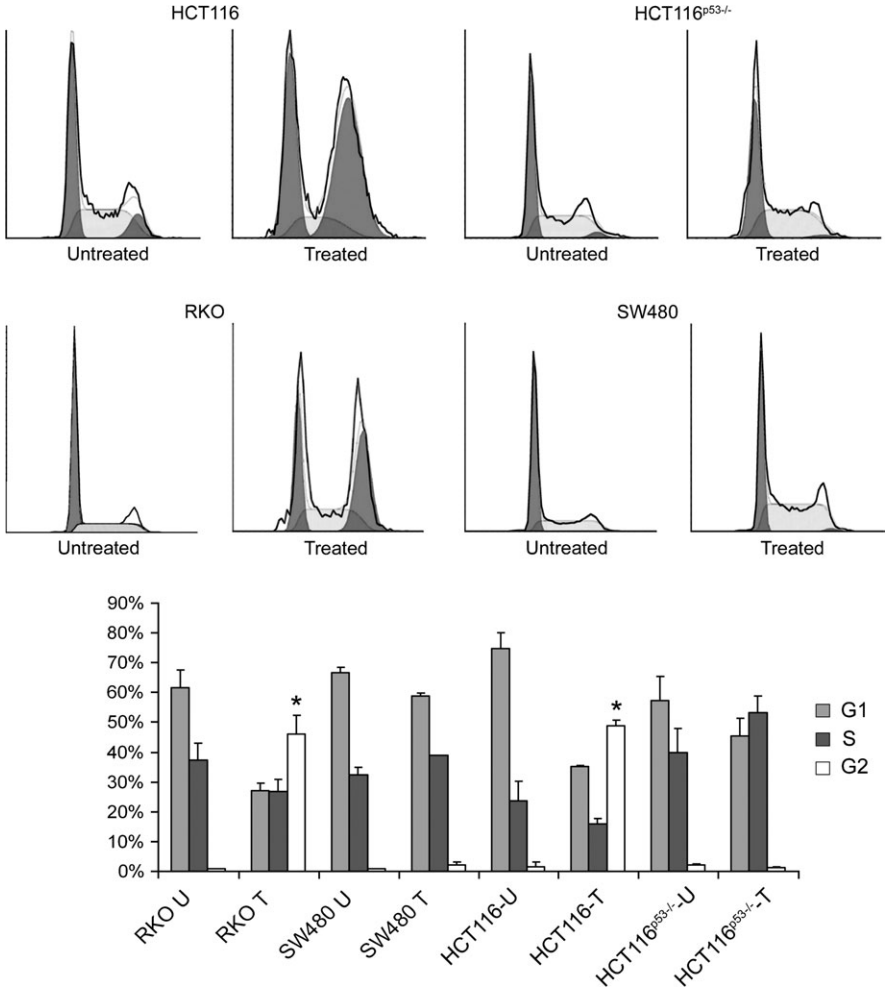

Fig. 3. Cell cycle analysis in EVOO-A-treated samples and untreated controls. Representative flow cytometry cell cycle profiles for each cell line are shown in the upper panels. Each experiment was repeated three times and the distribution of the mean among the different experiments is shown in the histograms in the lower half of the figure. *A significant arrest in the G_2/M phase was found in RKO and HCT116 cells after treatment ($P < 0.0001$). No significant changes in the cell cycle profiles were demonstrated in *p53*-deficient cell lines in response to the EVOO-A treatment.

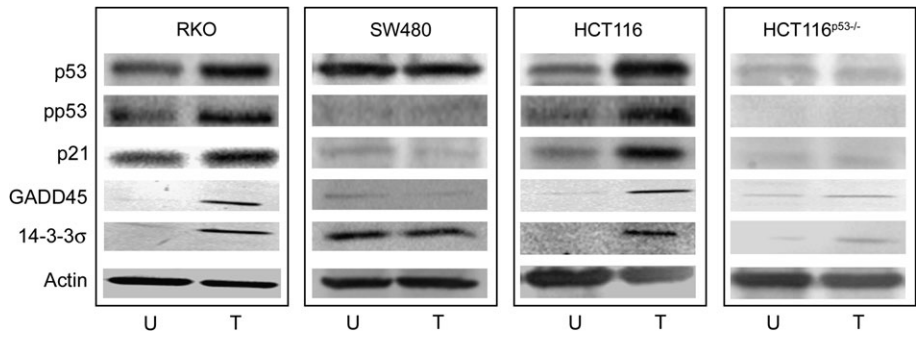

Fig. 4. Western blot analysis of p53 and p53-regulated proteins before and after EVOO-A. The treatment of RKO and HCT116 with EVOO-A led to a significant activation and phosphorylation of p53 and the downstream proteins $p21^{cip1/waf1}$, GADD45 and 14-3-3σ. No change in the expression of the p53-regulated axis was observed in SW480 or HCT116$^{p53-/-}$ after treatment. U (untreated), control, T (treated).

polyunsaturated fat diets are associated with lower incidences of cardiovascular diseases and cancers (10,11,14).

However, in addition to its unsaturated fatty acids, olive oil is the source of several micronutrient components with beneficial properties, such as α-tocopherol, carotenoids, sterols and phenolic compounds (11). Certain seed oils that are not associated with health-promoting properties are richer in polyunsaturated fat and vitamin E/tocopherol than olive oil, but do not contain the phenolic compounds (14). Thus, the nutritional issues relevant to a healthful diet may be subtle and complex.

Olive oils are relatively constant in terms of lipid composition, but the micronutrient contents vary based upon the location of cultivation, climate, olive variety and oil production techniques (14). Since the biological effects of dietary phenols are compound specific (15), we

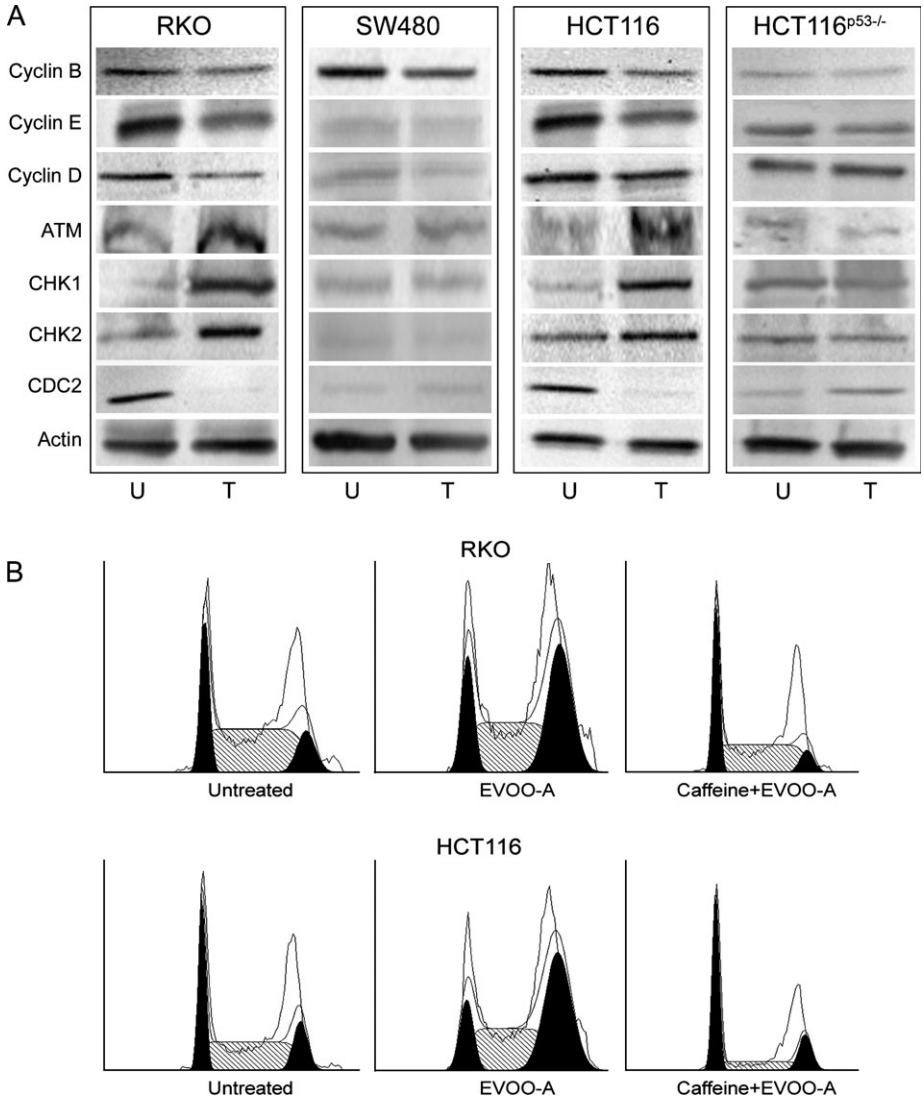

Fig. 5. Evaluation of the involvement of ATM axis in the EVOO-A effects on p53-proficient cells. (**A**) Significant changes were observed after treatment of RKO and HCT116 for ATM, Chk1, Chk2 and cdc2 (increased expression) and cyclin E (decreased expression). A slight decrease in cyclin B and no change in cyclin D expression was evident in the *p53*-proficient cell lines. No changes in protein expression were detected after treatment of the *p53*-deficient cell lines. U (untreated), control, T (treated). (**B**) Flow cytometry cell cycle profiles in RKO and HCT116 cells with and without pretreatment with caffeine. Pretreatment with caffeine was able to abrogate the G_2/M arrest determined by EVOO-A treatment, confirming the involvement of the ATM axis in EVOO-A effects (no differences compared with untreated controls; $P > 0.05$).

wished to characterize and examine different olive oils independently to understand the possible chemopreventive mechanisms involved.

On the basis of these considerations, we focused on the phenolic fraction of two different varieties of olive grown in southcentral Italy. As expected, the two varieties had significantly different phenolic compositions, in which pinoresinol was the major phenolic fraction in EVOO-A, and oleocanthal was the main component of EVOO-B. When compared with EVOO-A, our data showed that the oleocanthal-rich variety had a relatively weak ability to alter cell viability in two different types of CRC cells. On the other hand, pinoresinol-rich EVOO-A had a potent effect in decreasing cell viability at concentrations as low as 200 nM, a concentration that corresponds to 124 nM pinoresinol.

Our first results demonstrated different responses to treatment between the cell lines, being more pronounced in RKO than in SW480. We therefore tested the effects of EVOO-A on a broader panel of cell lines that included HCT116 and its syngeneic partner lacking *p53*, HCT116$^{p53-/-}$. EVOO-A treatment caused a decrease of cell viability in RKO and HCT116 cells, together with an increase of apoptosis and prominent G_2/M arrest, whereas no significant changes were demonstrable in SW480 and HCT116$^{p53-/-}$. Importantly, we obtained the same effects on cell viability after treating the cells with higher concentrations (700 nM) of purified pinoresinol, confirming that the major biological effects were due to the activity of pinoresinol, and indicating that a synergistic effect was obtained in the EVOO-A mixture. The principal difference between these cells is the presence of a functional p53 axis in RKO and HCT116, which is crucial for the regulation of both apoptosis and cell cycle arrest. Cellular stresses and DNA damage typically trigger the *p53* tumor suppressor gene to mediate a series of antiproliferative strategies that preserve genomic fidelity by inducing both cell cycle arrest and apoptosis (27,28). One important link between p53 and apoptosis is based on the transcriptional control of proapoptotic members of the *Bcl-2* family, such as *Bax* (27). Our results showed that the pinoresinol-rich oil was able to increase apoptosis, as demonstrated by DNA fragmentation and increased *Bax* transcription, but only in cells with an intact p53 axis. In terms of cell cycle regulation, p53 can mediate both G_1 and G_2 phase arrest. For control of the G_2/M checkpoint, *p53* is directly controlled

by the *ATM–ATR* genes (29–31). Pinoresinol-rich olive oil induced G_2 cell cycle arrest in *p53*-proficient cell lines. Inhibitory phosphorylation of cdc2/cyclin B is essential for G_2 arrest, and this is regulated by p53-dependent and -independent pathways, both of which are downstream of *ATM* (30,31). Our results clearly demonstrated that G_2 arrest by pinoresinol-rich olive oil takes place through the upregulation of ATM–p53 and their downstream pathways ($p21^{cip/waf}$, GADD45 and 14-3-3σ) with a reciprocal decrease in cdc2. In addition, our results suggest that activation of the Chk1 and Chk2 kinases contributes to the G_2/M arrest. Chk1 and Chk2 mediate the stabilization of p53 by the phosphorylation of serine 20 in the N-terminal region (26,32,33), which might explain why the G_2/M arrest was found exclusively in the *p53*-proficient cells. We demonstrated that the effect on the cell cycle was due to the activity of pinoresinol by treating the cells with the purified compound. Interestingly, pretreatment of the cells with caffeine prior to pinoresinol repressed the effects on G_2/M arrest obtained by pinoresinol alone, indicating that pinoresinol is an activator of the ATM/p53 axis.

Loss of functional *p53* occurs frequently, but it typically characterizes the late phases of tumor evolution (34). Sporadic CRC is the end result of a slowly evolving multistep process, in which the histopathological changes are driven by specific genetic mutations. Biallelic inactivation of the *APC* gene mediates the transition to adenoma formation as a very early event in most colorectal neoplasms, and *K-RAS* mutations are associated with additional growth in about half of colorectal neoplasms. Biallelic loss of *p53* is specifically associated with the adenoma-to-carcinoma transition, and is usually a late event (35).

Any chemopreventive strategy must be effective in the early phases of the evolution of cancer. Thus, a strategy that requires an intact p53 axis would be suitable for the prevention of colorectal neoplasia. One would not anticipate therapeutic strategies that requires intact p53 activity would be effective in killing cancer cells, but this is precisely the type of mechanism one might expect to protect non-neoplastic colonic epithelium from entering into the neoplastic pathway. Based on our results, it is reasonable to speculate that the action of pinoresinol is to enhance the p53 cascade and to prevent the expansion of mutated epithelial clones.

To date, colon cancer prevention guidelines have been completely focused on screening and monitoring high-risk patients, and chemopreventive strategies have never been formally introduced, largely because of the absence of safe agents with proven efficacy (2,6,7,36). Ultimately, effective preventive programs should begin with data-based primary prevention. The Mediterranean diet, encouraging a balanced intake of macronutrients and rich with health-promoting micronutrients, might represent a potential source of nutrients for primary prevention. It is rational to identify novel chemopreventive candidates from the diets of geographical locations that enjoy specific health benefits. Other polyphenols from this diet, such as gallic acid and resveratrol, have been demonstrated to modulate ATM and to induce changes in cell cycle dynamics via Chk1 and Chk2 activation (37,38). It is helpful, and perhaps necessary, to understand the molecular mechanisms by which these nutriceuticals work. Furthermore, it may require a combination of compounds that work through complementary pathways to develop a cancer-preventing diet or intervention, and we propose that using a mechanistic approach to the identification of chemopreventive agents is a useful strategy.

In summary, this study demonstrates that pinoresinol-rich EVOO induces cell cycle arrest and apoptosis in CRC cells by inducing the ATM–p53 axis. In particular, our study indicates that pinoresinol-rich EVOO might be an effective agent in the chemoprevention of CRC. Further *in vivo* studies are warranted to understand the chemopreventive properties of these extracts.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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