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Short Communication

A genomic explanation connecting "Mediterranean diet", olive oil and cancer: Oleic acid, the main monounsaturated Fatty acid of olive oil, induces formation of inhibitory "PEA3 transcription factor-PEA3 DNA binding site" complexes at the Her-2/*neu* (*erb*B-2) oncogene promoter in breast, ovarian and stomach cancer cells

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ABSTRACT

Olive oil is an integral ingredient of the "Mediterranean diet" and accumulating evidence suggests that it may have a potential role in lowering risk of several cancers. We recently hypothesized that the anti-cancer actions of olive oil may relate to its monounsaturated fatty acid (MUFA) oleic acid (OA; 18:1*n* − 9) content to specifically regulate oncogenes. In this study, transient transfection experiments with human Her-2/*neu* promoter-driven luciferase gene established the ability of OA to specifically repress the transcriptional activity of Her-2/*neu* gene. Gene repression was seen in tumour-derived cell lines with Her-2/*neu* gene amplification and overexpression, including SK-Br3 (⩽56% reduction), SK-OV3 (⩽75% reduction) and NCI-N87 (55% reduction) breast, ovarian and stomach cancer cell lines, respectively. Also marginal decreases in promoter activity were observed in cancer cells expressing physiological levels of Her-2/*neu* (⩽20% reduction in MCF-7 breast cancer cells). Remarkably, OA treatment in Her-2/*neu*-overexpressing cancer cells was found to induce up-regulation of the *Ets* protein polyomavirus enhancer activator 3 (PEA3), a transcriptional repressor of Her-2/*neu* promoter. Also, an intact PEA3 DNA-binding-site at endogenous Her-2/*neu* gene promoter was essential for OA-induced repression of this gene. Moreover, OA treatment failed to decrease Her-2/*neu* protein levels in MCF-7/Her2-18 transfectants, which stably express full-length human Her-2/*neu* cDNA controlled by a SV40 viral promoter. OA-induced transcriptional repression of Her-2/*neu* through the action of PEA3 protein at the promoter level may represent a novel mechanism linking "Mediterranean diet" and cancer.

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1. Introduction

The relationship between olive oil intake and cancer risk has become a controversial issue that could have very important repercussions for human health. Different studies have shown that the consumption of olive oil may have a potential role in lowering the risk of malignant neoplasms, especially breast and stomach cancer; and also in ovary, colon and endometrium cancer [1–10]. However, the mechanisms by which the effects of olive oil are mediated are not well understood. One of the remaining concerns, before going for a causal interpretation of the inverse relation between olive oil intake and cancer risk, is to establish definitely if olive oil-related anti-cancer effects can be explained by its monounsaturated fatty acid (MUFA) content (i.e., high levels of the ω-9 MUFA oleic acid) or the antioxidant components of the unsaponifiable fraction [11].

Since cancer development and progression is believed to be a multi-step process, we recently hypothesized that a novel molecular explanation for the anti-cancer actions of olive oil may relate to the ability of oleic acid (OA; 18:1$n-9$) to specifically regulate key cancer-related oncogenes [12]. Supporting our hypothesis, exogenous supplementation of cultured breast cancer cells with OA was found to significantly down-regulate the expression of Her-2/*neu* [13], a well-characterized oncogene (also called *neu* or *erb*B-2) that plays a key role in the etiology, progression and chemosensitivity of various types of human cancer [14–26]. These anti-Her-2/*neu* properties of OA offered a previously unknown molecular mechanism by which olive oil may regulate the malignant behavior of breast cancer cells. These findings generated intense public interest, since no toxicities have been reported or suspected with OA, and suggested that supplementation with OA may represent a promising dietary intervention for the prevention and/or management of Her-2/*neu*-related breast carcinomas [27,28]. However, two major questions remained to be addressed: (1) What is the molecular mechanism linking tumour cells' response to OA and Her-2/*neu* gene expression? and (2) Is the ability of OA to down-regulate Her-2/*neu* a common mechanism relevant to other types of cancer other than breast cancer?

Although overexpression of Her-2/*neu* both in tumours and in derived cell lines was originally attributed solely to amplification of the *erb*B-2 gene (usually 2- to 10-fold), an elevation in Her-2/*neu* mRNA levels per gene copy is also observed in all the cell lines examined exhibiting gene amplification [29]. This indicates that overexpression of the gene precedes and increases the likelihood of gene amplification. Indeed, an increase in transcription rate sufficient to account for the degree of overexpression has been shown in a number of Her-2/*neu*-overexpressing cancer cell lines [30]. Our current experiments sought to characterize the effects of OA treatment on the transcription rate of Her-2/*neu* gene. We have also addressed if the ability of OA to down-regulate Her-2/*neu* is a common mechanism of OA action towards tumour types reported to exhibit Her-2/*neu* overexpression, including breast, ovarian and gastric carcinomas. We report that OA promoted the up-regulation of the potent *trans*-repressor of the human Her-2/*neu* gene promoter PEA3 and could account in part the ability of OA to suppress Her-2/*neu* overexpression in cancer cells. These findings represent a novel genomic explanation linking "Mediterranean diet" and cancer, as OA-induced transcriptional repression of Her-2/*neu* gene seems to equally operate in various types of human malignancies previously shown to be influenced by olive oil consumption such as breast, ovarian and stomach carcinomas.

2. Materials and methods

2.1. Materials

Phenol red-containing improved minimal essential medium (IMEM) was from Biofluids (Rockville, MD, USA). Oleic acid (18:1$n-9$) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The cultures were supplemented, where indicated, with fatty acid-free bovine serum albumin (FA-free BSA; 0.1 mg/ml) complexed with a specific concentration of OA. A BSA-OA concentrated (100×) solution was formed by mixing 1 ml BSA (10 mg/ml) with various volumes (1–10 μl) of OA (200 mg/ml) in ethanol. The concentrate was mixed for 30 min at room temperature before addition to cultures. Control cultures contained uncomplexed BSA.

The primary antibody for Her-2/*neu* immunoblotting was anti-p185$^{Her\text{-}2/neu}$ mouse monoclonal antibody from Oncogene Research Products (Clone *Ab-3*; San Diego, California, USA). Anti-PEA3 mouse monoclonal (*sc-113*) and anti-β-actin goat polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines and culture conditions

SK-Br3 (breast cancer), MDA-MB-231 (breast cancer), SK-OV3 (ovarian cancer), and NCI-N87 (gastrointestinal cancer) cell lines were obtained from the American Type Culture Collection (ATCC). MCF-7 cells stably overexpressing Her-2/*neu* oncogene (MCF-7/Her2-18) were kindly provided by Dr. Mien-Chie Hung (The University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA). Cells were routinely grown in IMEM containing 5% (v/v) heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

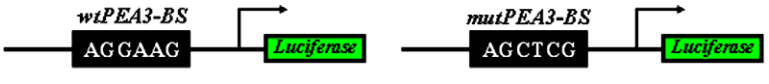
2.3. Her-2/neu promoter activity assays

Using FuGENE 6 transfection reagent (Roche Biochemicals, Indianapolis, IN) as directed by the manufacturer, overnight-serum starved cancer cells seeded into 24-well plates ($\sim 5 \times 10^4$ cells/well) were transfected in low-serum (0.1% FBS) media with 1.5 μg/well of the pGL2-luciferase (Promega, Madison, WI) construct containing a luciferase reporter gene driven by either an intact (Her-2/*neu* wild-type PEA3-binding site-luciferase) or by a mutated (Her-2/*neu* mutated PEA3-binding site-luciferase) Her-2/*neu* promoter fragment (as previously described by Xing et al. [35]) along with 150 μg/well of the internal control plasmid pRL-CMV, which was used to correct for transfection efficiency. After 18 h, the transfected cells were washed and the incubated with either ethanol (v/v) or 20 μM OA in 0.1% FBS. Approximately, 24 h after treatments, luciferase activity from cell extracts was detected with

a luciferase Assay System following manufacturer's instructions (Promega, Madison, WI, USA) using a Victor^{2TM} Multilabel Counter (Perkin–Elmer Life Sciences).

2.4. *Immunoblot analysis of Her-2/neu and PEA3 proteins*

Following treatments with OA, cells were washed twice with PBS and then lysed in buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride] for 30 min on ice. The lysates were cleared by centrifugation in an eppendorff tube (15 min at 14000*g* at 4 °C). Protein content was determined against a standardized control using the Pierce protein assay kit (Rockford, IL). Equal amounts of protein were heated in SDS sample buffer (Laemli) for 10 min at 70 °C, subjected to electrophoresis on either 3–8% NuPAGE Tris–Acetate (p185$^{Her\text{-}2/neu}$) or 10% SDS–PAGE (PEA3) and then transferred to nitrocellulose membranes. Non-specific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T [25 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20] containing 5% (w/v) non-fat dry milk. The treated filters were washed in TBS-T and then incubated with primary antibodies (1:200 for anti-p185$^{Her\text{-}2/neu}$, 1:100 for anti-PEA3 and 1:500 for anti-β-actin) for 2 h at RT in TBS-T containing 5% (w/v) non-fat dry milk. The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies in TBS-T were added for 45 min, and immunoreactive bands were detected by enhanced chemiluminiscence reagent (Pierce, Rockford, IL). Blots were re-probed with an anti-β-actin goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz,



	Wild-type Her-2/*neu* promoter		Mutant Her-2/*neu* promoter	
Cell line	EtOH (*v/v*)	**OA (20 μM)**	EtOH (*v/v*)	**OA (20 μM)**
SK-Br3	100	44 (95% CI = 41 to 47)	27 (95% CI = 25 to 29)	25 (95% CI = 22 to 28)
SK-OV3	100	25 (95% CI = 20 to 30)	15 (95% CI = 10 to 20)	16 (95% CI = 8 to 24)
NCI-N87	100	45 (95% CI = 41 to 46)	20 (95% CI = 14 to 26)	17 (95% CI = 10 to 24)
MCF-7	100	88 (95% CI = 80 to 96)	68 (95% CI = 49 to 81)	66 (95% CI = 60 to 72)
MCF-7/Her2-18	100	78 (95% CI = 66 to 90)	73 (95% CI = 57 to 89)	64 (95% CI = 51 to 77)

Fig. 1 – Luciferase activity in transiently transfected cells. Luciferase activity was assayed in cells that were transiently transfected with a pGL2-Luc construct containing a luciferase reporter gene under the control of a Her-2/*neu* promoter fragment containing a wild-type (*top panel, left*) or mutant (*top panel, right*) PEA3 binding site as described in Section 2. The magnitude of activation in Her-2/*neu* promoter-luciferase-transfected cells was determined after normalization of the luciferase activity obtained in cells co-transfected with equivalent amounts of the empty pGL2-luciferase vector lacking the Her-2/*neu* promoter (Ø-luciferase) and the internal control plasmid pRL-CMV. This control value was used to calculate the relative change in the transcriptional activities of Her-2/*neu*-promoter-luciferase-transfected cells in response to treatments after normalization to pRL-CMV. The activity of the wild-type promoter in untreated control cells was defined as 100%. The activity of the mutated promoter in untreated control cells was calculated relative to that found in untreated control cells transfected with the intact (Her-2/*neu* wild-type PEA3-binding site-luciferase) Her-2/*neu* promoter (=100%). Data are the mean and 95% confidence intervals (95% CI) of three experiments performed in triplicate. One-factor ANOVA was used to analyzed differences in the percentages of luciferase activity between the various treatment groups.

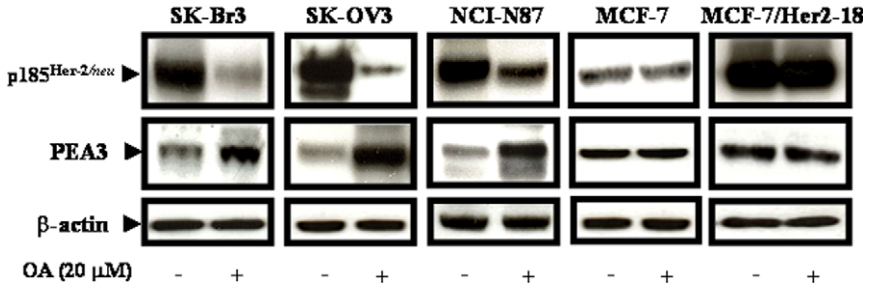


Fig. 2 – Immunoblot analysis of Her-2/*neu* and PEA3 proteins in control- and OA-treated cancer cells. SK-Br3, SK-OV3, NCI-N87, MCF-7/*neo* and MCF-7/Her2-18 cells were treated for 48 h with either ethanol (v/v) or OA (20 μM) and then subjected to immunoblot analysis of Her-2/*neu*, PEA3 and β-actin (as control) as described in Section 2. Representative blots from three independent experiments are shown.

CA) to control for protein loading and transfer. Densitometric values of protein bands were quantified using Scion Imaging Software (Scion Corp., Frederick, MD).

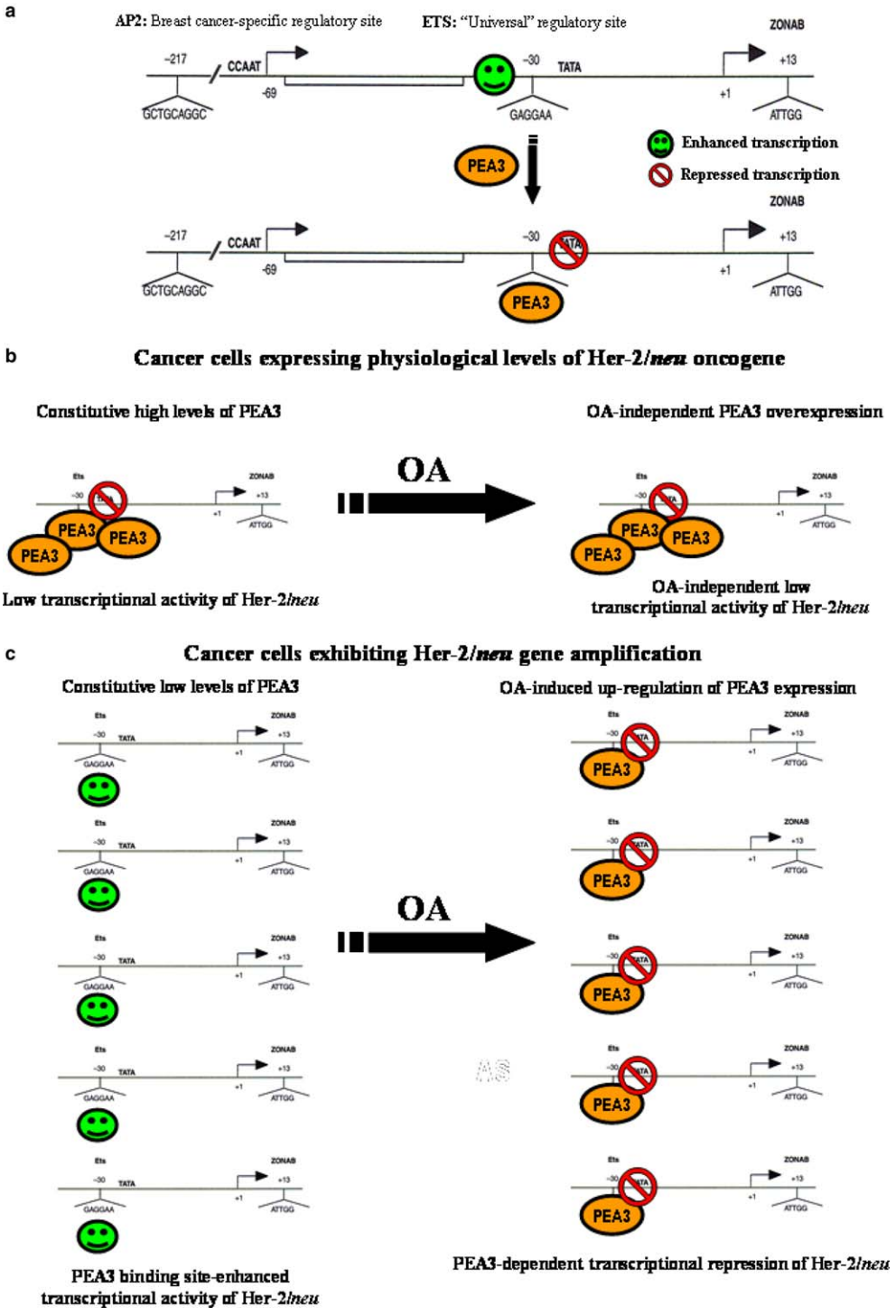
2.5. Statistical analysis

Data are the mean and 95% confidence intervals (95% CI) of three independent experiments. A two-way ANOVA was used to analyze differences in the percentage of luciferase activity between the treatment and the control groups.

3. Results

3.1. Exogenous supplementation with OA inhibits Her-2/neu gene promoter activity in breast, ovarian and stomach cancer cells

We used reporter gene expression analysis to characterize the effects of OA on Her-2/*neu* oncogene transcription. We performed transient transfection experiments with a luciferase reporter gene driven by the wild-type Her-2/*neu* promoter



(p *Nulit*; Fig. 1, top panel). Exogenous supplementation with OA (20 μM; 24 h) was found to profoundly repress the activity of Her-2/*neu* gene promoter in SK-Br3 breast cancer cells (up to 56% inhibition; Fig. 1; bottom panel). Accordingly, a significant reduction on the expression levels of Her-2/*neu* transcripts (Her-2/*neu* mRNA) was observed in semi-quantitative RT-PCR analyses of RNA isolated from OA-treated SK-Br3 cells (data not shown). Similar to SK-Br3 breast cancer cells, Her-2/*neu*-overexpressing SK-OV3 ovarian and NCI-N87 stomach tumour-derived cell lines demonstrated a dramatic reduction (up to 75% and 55% inhibition, respectively) on Her-2/*neu* gene promoter activity upon OA treatment (Fig. 1; bottom panel).

Although the precise molecular mechanisms governing Her-2/*neu* promoter activity in Her-2/*neu*-overexpressing cancer cells are far from being totally defined, two main groups of transcription factors, namely the AP-2 and the *Ets* families of transcription factors, have been shown to be required for both maximal promoter activity and associated with overexpression of the gene in cancer [31]. Recent studies showed that AP-2-regulated Her-2/*neu* promoter regions have different roles in breast and non-breast cancer cells [31–34], while the *Ets* sites appear to be non-tissue specific ("universal") regulators of Her-2/*neu* promoter activity [31,35,36]. In the absence of exogenous supplementation with OA, Her-2/*neu*-overexpressing SK-Br3, SK-OV3 and NCI-N87 cancer cells did express low to undetectable levels of the DNA-binding protein PEA3, a member of the *Ets* transcription factor family. PEA3 specifically targets a DNA sequence on the Her-2/*neu* promoter that suppresses Her-2/*neu* overexpression and inhibits Her-2/*neu*-dependent tumourigenesis [35,36]. Interestingly, a significant up-regulation of PEA3 protein expression, concomitantly with down-regulation of Her-2/*neu* protein expression, occurred following OA treatment in SK-Br3, SK-OV3 and NCI-N87 cancer cells (Fig. 2). Exogenous supplementation with OA failed to modulate AP-2 expression levels in Her-2/*neu*-overexpressing cancer cells (data not shown).

3.2. *OA-induced transcriptional repression of Her-2/neu gene requires an intact PEA3 binding site at the endogenous Her-2/neu promoter*

To examine whether the increased PEA3 levels might mediate the inhibition of Her-2/*neu* transcription in OA-treated Her-2/*neu*-overexpressing cancer cells, we examined the effects of OA in transcription from a promoter bearing a mutated PEA3 binding site (at −33 to −28) that is known to abolish PEA3 binding [35]. For this analysis we used the same luciferase reporter gene construct described above but containing a Her-2/*neu* promoter mutation at the PEA3 binding site (5′-GAGGAA-3′ to 5′-GAGCTC-3′) (Fig. 1, top panel, right). When the levels of wild-type and mutant promoter activities were compared in the absence of OA treatment, the mutant promoter was drastically less active than the wild-type promoter in SK-Br3, SK-OV3 and NCI-N86 cells (up to 73%, 85% and 80% reduction, respectively). These results support the notion that a PEA3 binding site on the Her-2/*neu* promoter acts as a positive regulatory element necessary for elevated expression of Her-2/*neu* oncogene in cancer cells [31,35,36]. Remarkably, the luciferase reporter gene driven by the PEA3 site-mutated sequence was not subject to negative regulation in OA-supplemented SK-Br3, SK-OV3 and NCI-N87 cells (Fig. 1, bottom panel).

The above findings strongly suggested that the formation of inhibitory complexes "PEA3 protein-PEA3 DNA binding site" at the endogenous Her-2/*neu* promoter could be required for OA-induced transcriptional repression of Her-2/*neu* gene in Her-2/*neu*-overexpressing cancer cells. This suggestion was supported further when the effects of OA treatment on Her-2/*neu* protein expression, Her-2/*neu* promoter activity and PEA3 accumulation were characterized in MCF-7 breast cancer cells, with naturally expressing physiological levels of Her-2/*neu*; and MCF-7 cells engineered to overexpress Her-2/*neu* under the transcriptional control of a different promoter (i.e., MCF-7/Her2-18 stable transfectants expressing full-length human Her-2/*neu* cDNA under SV40 promoter

Fig. 3 – Working model for OA-induced transcriptional repression of Her-2/*neu* oncogene in cancer cells. (a) Features of the Her-2/*neu* promoter. The Her-2/*neu* promoter from −75 to +15 is represented, with an additional area illustrating sequences upstream of −200. The major (+1 bp) and minor (−69 bp) transcription start sites are indicated with arrows and the positions of the TATA (−22 to −26 bp) and CCAAT (−71 to −75 bp) boxes are marked. The relative positions of the main transcription factor binding sites AP-2, *Ets* and ZONAB are indicated, with the sequences below each giving the core binding site defined for each factor (modified from [31]). Mutation of the *Ets* binding site (EBS; GAGGAA), at −33 to −28, impairs reporter activity [35], while it has also been reported that binding of *Ets* factors to the EBS induces a severe bend in the DNA [48]. At least 10 different *Ets* proteins have been found in cancer cells at varying levels but, of those, only PEA3 has so far been shown to correlate in distribution with Her-2/*neu* overexpression. It is likely that if the EBS is occupied by PEA3 then the TATA-binding protein will not be able to access the closely associated TATA box, thus repressing the Her-2/*neu* promoter. (b) Cancer cells expressing physiological levels of Her-2/*neu* naturally exhibit high levels of the *trans*-repressor PEA3 and constitutive low transcriptional activity of the Her-2/*neu* gene promoter. In this scenario, exogenous supplementation with OA does not modulate PEA3 expression and, therefore, Her-2/*neu* gene promoter activity continues to be inhibited by PEA3. (c) Cancer cells bearing Her-2/*neu* gene amplification naturally express low to undetectable levels of the trans-repressor PEA3 and, therefore, a PEA3 binding site-enhanced transcriptional activity of the Her-2/*neu* gene promoter. Exogenous supplementation with OA promotes accumulation of the *trans*-repressor PEA3 and, hence, occupation of the PEA3 binding site. OA-induced formation of inhibitory "PEA3-PEA3 DNA binding site" complexes at the Her-2/*neu* gene promoter in Her-2/*neu* gene-amplified cancer cells may represent a novel genomic explanation linking "Mediterranean diet" and cancer as it seems to equally operate in various types of human malignancies in which olive oil has previously been shown to exert protective effects.

control). MCF-7/Her2-18 cells are known to express 45-times the level of Her-2/*neu* than parental MCF-7 cells or the MCF-7/*neo* control sub-line expressing a neomycin phosphotransferase gene [37]. Her-2/*neu* and PEA3 protein levels in MCF-7/*neo* cells were not significantly affected by exogenous supplementation with OA, while the luciferase reporter activity of the wild-type Her-2/*neu* promoter was slightly reduced by either OA treatment (up to 12% reduction; Fig. 1, bottom panel) or mutation of the PEA3 binding sequence (up to 32% reduction; Fig. 1, bottom panel). Equivalent results were found in wild-type MCF-7 cells (data not shown). Importantly, there were no important effects of OA supplementation on Her-2/*neu* gene promoter activity (up to 22% reduction; Fig. 1, bottom panel) and Her-2/*neu*-PEA3 protein levels in MCF-7/Her2-18 transfectants (Fig. 2).

4. Discussion

The observations reported in this study demonstrate that: (i) the PEA3 binding motif on the Her-2/*neu* promoter functions as a positive regulatory element for Her-2/*neu* gene transcription solely in cancer cells naturally exhibiting both Her-2/*neu* gene amplification and Her-2/*neu* protein overexpression (Fig. 3*a*); (ii) There is an inverse correlation between PEA3 and Her-2/*neu* expression, with low PEA3 expression occurring in Her-2/*neu*-overexpressing cancer cells and high PEA3 expression occurring in low-Her-2/*neu*-expressors, and (iii) the ability of OA to down-regulate Her-2/*neu* promoter activity and to suppress Her-2/*neu* protein overexpression, while concomitantly up-regulating PEA3 expression, is restricted to cancer cells naturally exhibiting Her-2/*neu* gene amplification, as OA exposure does not modulate Her-2/*neu* protein levels when Her-2/*neu* gene is overexpressed under the control of a viral promoter. Therefore, it is reasonable to suggest that PEA3-induced down-regulation of Her-2/*neu* promoter activity is a major molecular mechanism underlying the anti-Her-2/*neu* effects observed upon exogenous supplementation with OA of Her-2/*neu* gene-amplified cancer cells (Fig. 3(b) and (c)).

Overexpression of the Her-2/*neu* oncogene is a frequent molecular event in multiple human cancers. Her-2/*neu* codes for a transmembrane tyrosine kinase orphan receptor $p185^{Her\text{-}2/neu}$ that regulates biological functions as diverse as cellular proliferation, differentiation, motility and apoptosis. Therefore, modulation of Her-2/*neu* levels must be tightly regulated for normal cellular function. Accordingly, in vitro and animal studies clearly demonstrate that deregulated Her-2/*neu* expression plays a pivotal role in malignant transformation, tumourigenesis and metastasis. Patients with Her-2/*neu*-overexpressing cancer are associated with unfavorable prognosis, shorter relapse time, and low survival rate [14–26].

Treatments that target Her-2/*neu* in cancer cells have been shown to be useful strategies to significantly reverse the malignancy induced by Her-2/*neu* overexpression. The humanized anti-Her-2/*neu* antibody trastuzumab (Herceptin™) has proven to be effective in clinical trials in patients with metastatic breast cancer [38–42]. In addition, tyrosine kinase inhibitors can also target the Her-2/*neu* oncogenic activity [43,44]. Alternatively, Her-2/*neu* overexpression can be repressed by attenuating the promoter activity of the Her-2/*neu* gene [31,36,45]. The rationale is that it will be more efficient to reduce Her-2/*neu* levels by preventing the transcription of 2–10 gene copies than trying to neutralize up to 10^6 receptor molecules commonly found in overexpressing cells [36]. Although this approach has mainly been used in vitro, one strategy, based on the observation that overexpression of the *Ets* factor PEA3 reduced Her-2/*neu* expression, has also been examined in preclinical trials. Breast and ovarian tumour-derived cell lines with either low expression or overexpression of Her-2/*neu* growing as xenografts in nude mice were treated with daily injections of liposome-conjugated PEA3 expression plasmid. Significant reductions in tumour growth were observed in the Her-2/*neu*-overexpressing groups, with some mice surviving over a year, whereas growth of the tumours with low expression was unaffected [35].

This report shows a proof of concept in which OA-promoted accumulation of the potent *trans*-repressor of the human Her-2/*neu* promoter PEA3 is a key molecular feature that may account, at least in part, for the down-regulatory effects of OA on the expression of Her-2/*neu* oncogene in cancer cells. These findings do not prove, however, that exogenous supplementation with OA exclusively suppresses Her-2/*neu* overexpression via PEA3. It could be argue that other Her-2/*neu* promoter interacting factors such as AP-2, a family of highly homologous proteins all of which can activate the Her-2/*neu* promoter [31–34], may explain the blockade of Her-2/*neu* promoter activity observed upon OA exposure. While recent studies suggest that AP-2 is not a major player in the increased levels of Her-2/*neu* transcripts in colon and ovary cancer cells, thus suggesting that the promoter regions leading to Her-2/*neu* overexpression are different in breast and non-breast cancer cells [34], we failed to demonstrate any effects of OA on AP-2 levels in Her-2/*neu*-overexpressing cancer cells (data not shown). Considering that OA exposure similarly impaired Her-2/*neu* promoter activity and concomitantly up-regulated PEA3 expression in all the Her-2/*neu*-overexpressing cell models evaluated, our results favor the hypothesis that PEA3 and its *Ets* binding site at the Her-2/*neu* promoter are the main down-stream effectors explaining OA-induced repression of Her-2/*neu* oncogene in breast, ovarian and stomach cancer cells (Fig. 3).

Our earlier results established that the combined treatment with OA and trastuzumab (Herceptin™), a monoclonal antibody that targets the ECD of Her-2/*neu*, synergistically increased the extent of apoptotic cell death in Her-2/*neu* overexpressors and strongly impaired the ability of Her-2/*neu*-overexpressing cancer cells to grow under anchorage-independent conditions [13]. Considering that OA appears to mitigate Her-2/*neu* overexpression *via* PEA3 binding to the Her-2/*neu* promoter, this mechanism of action should not be affected by the mechanisms of resistance described for trastuzumab-based anti-Her-2/*neu* immunotherapy [46,47]. Taking into account that systemic drugs or gene therapy vectors to specifically repress Her-2/*neu* promoter activity are far to be developed in the short term, it is reasonable to suggest that OA-based dietary interventions might become a novel strategy to improve our current management of Her-2/*neu*-overexpressing tumours with poor prognosis. Nevertheless, the ability of OA to promote the formation of inhibitory "*Ets* transcription factor PEA3-PEA3 DNA binding site" complexes

at the Her-2/*neu* gene promoter may represent a novel genomic explanation linking "Mediterranean diet" and cancer. The ability of OA to transcriptionally repress Her-2/*neu* overexpression in a PEA3-dependent manner seems to equally operate in various types of human malignancies previously shown to be influenced by olive oil consumption including breast, ovarian and stomach carcinomas.

Conflict of interest statement

None declared.

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REFERENCES

1. Martin-Moreno JM, Willett P, Gorgojo L, et al. Dietary fat, olive oil intake and breast cancer risk. *Int J Cancer* 1994;**58**:774–80.
2. Landa MC, Frago N, Tres A. Diet and risk of breast cancer in Spain. *Eur J Cancer Prev* 1994;**3**:313–20.
3. Trichopoulou A, Katsouyanni K, Stuver S, et al. Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. *J Natl Cancer Inst* 1995;**87**:110–6.
4. La Vecchia C, Negri E, Franceschi S, et al. Olive oil, other dietary fats, and the risk of breast cancer. *Cancer Causes Control* 1995;**6**:545–50.
5. Simonsen NR, Fernandez-Crehuet NJ, Martin-Moreno JM, et al. Tissue stores of individual monounsaturated fatty acids and breast cancer: the EURAMIC study. European Community Multicenter Study on antioxidants, myocardial infarction, and breast cancer. *Am J Clin Nutr* 1998;**68**:134–41.
6. Stoneham M, Goldacre M, Seagroatt V, et al. Olive oil, diet and colorectal cancer: an ecological study and a hypothesis. *J Epidemiol Community Health* 2000;**54**:756–60.
7. Braga C, La Vecchia C, Franceschi S, et al. Olive oil, other seasoning fats, and the risk of colorectal carcinoma. *Cancer* 1998;**82**:448–53.
8. Tzonou A, Hsieh C, Polychronopoulou A, et al. Diet and ovarian cancer: a case-control study in Greece. *Int J Cancer* 1993;**55**:411–4.
9. Tzonou A, Lipworth L, Kalandidi A, et al. Dietary factors and the risk of endometrial cancer: a case-control study in Greece. *Br J Cancer* 1996;**73**:1284–90.
10. Zamora-Ardoy MA, Banez Sanchez F, Banez Sanchez C, et al. Olive oil: influence and benefits on some pathologies. *An Med Interna* 2004;**21**:138–42.
11. Martin-Moreno JM. The role of olive oil in lowering cancer risk: is the real gold or simply pinchbeck? *J Epidemiol Community Health* 2000;**54**:726–7.
12. Menendez JA, Ropero S, Lupu R, et al. Dietary fatty acids regulate the activation status of Her-2/neu (c-erbB-2) oncogene in breast cancer cells. *Ann Oncol* 2004;**15**:1719–21.
13. Menendez JA, Vellon L, Colomer R, et al. Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu gene amplification. *Ann Oncol* 2005;**16**:359–71.
14. Yamamoto T, Ikawa S, Akiyama T, et al. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* 1986;**319**:230–4.
15. Bargmann CI, Hung M-C, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 1986;**319**:226–30.
16. Daly RJ. Take your partners, please–signal diversification by the erbB family of receptor tyrosine kinases. *Growth Factors* 1999;**16**:255–63.
17. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–37.
18. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;**235**:177–82.
19. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;**244**:707–12.
20. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Stem Cells* 1998;**16**:413–28.
21. Di Fiore PP, Pierce JH, Kraus MH, et al. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987;**237**:178–82.
22. Hudziak RM, Schlessinger J, Ullrich A. Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumourigenesis of NIH 3T3 cells. *Proc Natl Acad Sci USA* 1987;**84**:7159–63.
23. Tan M, Yao J, Yu D. Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. *Cancer Res* 1997;**57**:1199–205.
24. Eccles SA. The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. *J Mammary Gland Biol Neoplasia* 2001;**6**:393–406.
25. Yu D, Hung M-C. Role of erbB2 in breast cancer chemosensitivity. *Bioessays* 2000;**22**:673–80.
26. Dowsett M. Overexpression of HER-2 as a resistance mechanism to hormonal therapy for breast cancer. *Endocrinol Relat Cancer* 2001;**8**:191–5.
27. Marchetti P. Natural medicine: a 'new frontier' in oncology? *Ann Oncol* 2005;**16**:339–40.
28. Nelson R. Oleic acid suppresses overexpression of ERBB2 oncogene. *Lancet Oncol* 2005;**6**:69.
29. Kraus MH, Popescu NC, Amsbaugh SC, et al. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumour cell lines by different molecular mechanisms. *EMBO J* 1987;**6**:605–10.
30. Bates NP, Hurst HC. Transcriptional regulation of type I receptor tyrosine kinases in the mammary gland. *J Mammary Gland Biol Neoplasia* 1997;**2**:153–63.
31. Hurst HC. Update on HER-2 as a target for cancer therapy. The ERBB2 promoter and its exploitation for cancer treatment. *Breast Cancer Res* 2001;**3**:395–8.
32. Bosher JM, Williams T, Hurst HC. The developmentally regulated transcription factor AP-2 is involved in c-erbB-2

overexpression in human mammary carcinoma. *Proc Natl Acad Sci USA* 1995;**92**:744–7.
33. Bosher JM, Totty NF, Hsuan JJ, et al. A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* 1996;**13**:1701–7.
34. Vernimmen D, Gueders M, Pisvin S, et al. Different mechanisms are implicated in ERBB2 gene overexpression in breast and in other cancers. *Br J Cancer* 2003;**89**:899–906.
35. Xing X, Wang SC, Xia W, et al. The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumourigenesis. *Nat Med* 2000;**6**:189–95.
36. Wang SC, Hung M-C. Transcriptional targeting of the HER-2/neu oncogene. *Drugs Today (Barc)* 2000;**36**:835–43.
37. Benz CC, Scott GK, Sarup JC, et al. Estrogen-dependent, tamoxifen-resistant tumourigenic growth of MCF-7 cells transfected with Her-2/neu. *Breast Cancer Res Treat* 1993;**24**:85–95.
38. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992;**89**:4285–9.
39. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996;**14**:737–44.
40. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999;**17**:2639–48.
41. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 2002;**20**:719–26.
42. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;**344**:783–92.
43. Allen LF, Eiseman IA, Fry DW, et al. CI-1033, an irreversible pan-erbB receptor inhibitor and its potential for the treatment of breast cancer. *Semin Oncol* 2003;**30**(5 Suppl. 16):65–78.
44. Burris III HA. Dual kinase inhibition in the treatment of breast cancer: initial experience with the EGFR/ErbB-2 inhibitor lapatinib. *Oncologist* 2004;**9**(Suppl. 3):10–5.
45. Wang SC, Zhang L, Hortobagyi GN, et al. Targeting HER2: recent developments and future directions for breast cancer patients. *Semin Oncol* 2001;**28**(6 Suppl 18):21–9.
46. Lu Y, Zi X, Zhao Y, et al. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* 2001;**93**:1852–7.
47. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumour inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 2004;**6**:117–27.
48. Scott GK, Chang CH, Erny KM, et al. Ets regulation of the erbB2 promoter. *Oncogene* 2000;**19**(2):153–63.