Alteration of $\alpha$-tocopherol-associated protein (TAP) expression in human breast epithelial cells during breast cancer development

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Abstract

Breast cancer is the most common malignancy among women and has an age-specific incidence profile. Over the last decade, many studies have demonstrated the anticancer activity of $\alpha$-tocopherol, the main and most active form of natural vitamin E. $\alpha$-Tocopherol-associated protein (TAP) was found to be one of the major $\alpha$-tocopherol binding proteins in human serum and in liver, brain, and prostate tissues. In this study, we found that reduced TAP expression was significantly correlated with Her2/neu receptor expression, breast cancer stage and nodal stage in paired normal and cancerous breast tissue samples from 93 patients using real-time PCR analysis. A cell viability assay showed that $\alpha$-tocopheryl succinate ($\alpha$-TOS), a synthetic derivative of $\alpha$-tocopherol, enhanced the cells’ sensitivity to doxorubicin and resulted in a reduction in cell viability in breast cancers. Taken together, these data suggest that the use of vitamin E or its analogue as a dietary supplement may be beneficial for the treatment of cancer.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and is the second leading cause of death among women in the US (Hsieh, Blanca, & de Lumen, 2011; Vaz et al., 2012). Approximately 215,000 cases of invasive breast cancer and 50,000 cases of in situ ductal carcinoma were diagnosed in 2005, and 40,000 women died of invasive breast cancer (Poola et al., 2005). Survivors seek answers on how to prevent recurrence, especially women with Her2/neu receptor-negative cancers for whom preventive options are limited. A recent study reported that the use of vitamin and mineral supplements is widespread among cancer survivors and that breast cancer survivors report the highest usage (75–87%); however, the evidence that the use of such supplements is beneficial is inconclusive (Velicer & Ulrich, 2008). Thus, it is important to better understand how vitamin E compounds, which are found naturally in the diet or consumed as food additives or dietary supplements, may influence breast cancer development and recurrence.

Vitamin E is a physiological component of cellular membranes and lipoproteins. Since the discovery of vitamin E in 1922 by Evans and Bishop (Evans & Bishop, 1922), researchers have confirmed the significance of $\alpha$-tocopherol as the principal form of vitamin E in human plasma (Traber & Kayden, 1989), and it is the most active form of vitamin E (chemical structure shown in Fig. 1A). $\alpha$-Tocopherol is a well-known radical scavenger and has been widely studied as a supplemental therapy for the prevention of human cancers. Unlike the redox-active $\alpha$-tocopherol, the redox-inactive $\alpha$-tocopheryl succinate ($\alpha$-TOS) has attracted much attention over the last decade (Neuzil, 2002). $\alpha$-TOS is a naturally occurring compound that was first isolated from a green barley extract that stimulated the release of prolactin and growth hormone from pituitary cells in vitro (chemical structure is shown in Fig. 1B). Later, $\alpha$-TOS...
was reported to have various biological activities, such as the ability to inactivate the transcription factor nuclear factorκB (NF-κB) (Kanai et al., 2010; Wang et al., 2010), suppress cell growth (Huang et al., 2012), induce apoptosis (Huang et al., 2012; Kanai et al., 2010) and induce cell cycle arrest (Alleva et al., 2006; Pierpaoli et al., 2010). α-TOS has been found to induce apoptosis in a variety of cell lines, such as human breast cancer cells, neuroblastoma cells (Swettenham, Witting, Salvatore, & Neuzil, 2005), and prostate cancer cells (Basu, Grossie, Bennett, Mills, & Imrhan, 2007). Multiple signalling pathways have been suggested to function in α-TOS-induced apoptosis. These pathways may include PKC (Zhang et al., 2010), Fas (Weber et al., 2003; Zhang et al., 2011), or mitogen-activated protein kinase (MAPK) signalling (Zhao, Zhao, Yang, Neuzil, & Wu, 2007). Furthermore, it has also been reported that mitochondrial dysfunction (Stapelberg et al., 2005; Swettenham et al., 2005), lysosomal instability (Neuzil et al., 2002), and the activation of caspase cascades (Huang et al., 2012; Zhang et al., 2011) can be caused by α-TOS-induced apoptosis. Recently, α-TOS has also been used to enhance sensitivity to a clinical chemotherapeutic drug (5-fluorouracil) in the treatment of pancreatic cancer, implying that α-TOS or its analogues may be useful for future in vivo applications (Greco et al., 2010).

In this study, we measured the expression of TAP in paired clinical breast cancer and normal tissues from 93 patients by real-time PCR and in 12 breast cancer cell lines by western blot analysis. The data show that TAP is preferentially expressed in normal breast cells relative to tumour lesions, with an average of 1.4-fold higher expression data were normalised to the level of -glucuronidase expression in normal breast epithelial cells. These data imply that the use of -tocopherol or its analogues as supplements may be beneficial during chemotherapy for the treatment of human breast cancers.

2.2. Cell proliferation and viability assays

Cell growth, proliferation and viability were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Chou et al., 2007). Stock solutions of 10× α-TOS and DOX (Sigma–Aldrich Corporation, St. Louis, MO) were prepared in dimethylsulphoxide (DMSO). This assay was repeated three times with duplicate samples.

2.3. Protein extraction, immunoblotting, and antibodies

To determine the level of TAP protein expression, the various normal and cancerous breast cells were washed once with ice-cold phosphate-buffered saline and lysed on ice in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM sodium fluoride and 200 μM sodium orthovanadate) containing protease inhibitors, as previously described (Ho et al., 2005). Total protein from each sample (50 μg) was resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and analysed by western blotting. Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody and rabbit polyclonal anti-TAP antibody were purchased from Abcam Inc. (Cambridge, MA, USA). Alkaline phosphatase-coupled anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GAPDH and anti-TAP primary antibodies were used at 1:2,000 and 1:8000 dilutions, respectively. After incubation with primary antibodies for 2 h, the membranes were incubated with secondary antibodies at a 1:4,000 dilution for 1 h. The specific protein complexes were identified by incubation with chromogenic substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate; KPL, Inc., Gaithersburg, MD, USA). The assay was repeated twice with duplicate samples.

2.4. Real-time quantitative PCR

Total RNA was isolated from human cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. TAP-specific primers (forward: 5′-TGGGACGGGATTTGGTT-TT-3′ and reverse: 5′-TTGAGATGAACTTGTAGG-3′) were synthesised by MB Mission BioTech (Taipei, Taiwan). Our TAP expression data were normalised to the level of β-glucuronidase (GUS) (forward: 5′-agttggctgctagtattgg-3′ and reverse: 5′-aagcggcgttaagg-3′), which has been reported to be an ideal control gene because of its consistent expression level (Aerts, Gonzales, & Topalian, 2004). A LightCycler thermocycler (Roche
Molecular Biochemicals, Mannheim, Germany) was used for real-time PCR analysis. The TAP mRNA fluorescence intensity was measured and normalised to the GUS expression using the built-in Roche LightCycler Software, Version 4.

2.5. Immunohistochemistry staining

To determine whether TAP could be detected in human breast cancer cell lines, immunohistochemistry assays were performed using stage III breast cancer tissue slides. Paraffin-embedded breast tumour tissues that had been excised either from patients or from xenografted-tumours were cut into 8 μM sections. The sections were preincubated in 3% H2O2 and 0.3% Triton X-100 before microwaving for antigen retrieval. For TAP immunostaining, sections were microwaved in Citrate Buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 10 min. Following this step, sections were blocked in 5% horse serum (Chemicon, Temecula, CA, USA) for 30 min and subsequently incubated with 1:400 diluted TAP antibody for 2 h at room temperature. Following incubation with the primary antibodies, the stains were developed according to the streptavidin–biotin–peroxidase method using an LSAB 2 kit purchased from DAKO (Carpinteria, CA, USA). Briefly, sections were washed in phosphate-buffered saline and incubated with a biotinylated anti-rabbit secondary antibody. They were then washed again in the same buffer and incubated with streptavidin–biotin–peroxidase complex. Staining was completed after incubation with a substrate–chromogen solution. The appropriate length of incubation in the DAB solution was determined by viewing the samples under a low-power microscope. The slides were then dehydrated and cover slipped using DPX (Sigma–Aldrich, St. Louis, MO, USA).

Both adjacent sections and same slides were counterstained with haematoxylin for general histological orientation.

2.6. Statistical methods

According to the REMARK criteria for tumour marker studies developed in a previous report (McShane et al., 2005), all data were expressed as mean ± SD and univariate analysis was used to compare the TAP mRNA expression ratios for the paired tumour/normal samples. The TAP mRNA ratios were compared for samples grouped by age, ER status, PR status, Her2/neu status, tumour size, nodal status and disease stage. All statistical comparisons were performed using the SigmaPlot graphing software (San Jose, CA, USA) and the Statistical Package for the Social Sciences v.13 (SPSS, Chicago, IL, USA). A P-value <0.05 was considered statistically significant and all statistical tests were two-sided.

3. Results

3.1. TAP expression in normal and malignant human breast cell lines

Many studies have reported that TAP is strongly expressed in many malignant human cancers, including prostate, brain and breast cancers. TAP was previously found to be down regulated in prostate cancer cell lines and human prostate carcinoma tissue samples. To determine whether high TAP expression can also be observed in normal human breast epithelial cells, we examined the TAP expression in various cell lines derived from benign and malignant breast tissue for which the cancer malignancy status was known. As shown in Fig. 2, basal breast cancer cells had the highest level of malignancy (MB-231, MB-435 and HS-578T), followed by luminal B cells positive for Her2/neu (MB-453 and SKBR-3); the least malignant breast cancer cells were luminal A and ER- or PR-positive cells (BT-474, BT-483, MCF-7, T-47D and ZR-75). Of the various cell lines shown in Fig. 2, the normal breast epithelial cells (MCF-10A) had the highest levels of TAP expression. These data support the mRNA analysis results of a previous study that showed that TAP was more highly expressed in MCF-10A cells than in MB-231 and MCF-7 cells, implying that γ-tocopherol (vitamin E) may play a role in normal breast function.

3.2. TAP expression in normal and tumourous human breast tissues

As described above, TAP may be an important protein in the response to vitamin E in normal breast epithelial cells. We next examined the TAP mRNA levels in 93 pairs of tumour and normal tissue samples using real-time PCR analysis. The real-time PCR results were calculated and are presented as the TAP mRNA expression patterns in the tumour and surrounding normal tissues. The average TAP expression levels in the normal and tumour tissues were equal, with approximately 5.2 × 103 copies of TAP RNA per μg of total RNA (Fig. 3A). However, when comparing the TAP expression level in the paired normal and tumour samples, the TAP expression in normal cells was 1.4-fold greater than that in tumour cells. These data suggest that, in general, TAP may not be expressed at significantly different levels in normal and tumour lesions; however, the relative TAP expression levels in individual normal and tumour tissue pairs may change during breast tumourigenesis.

3.3. Expression of TAP in advanced stage breast tumour tissues

We next determined whether the relative TAP expression level is correlated with clinical pathology factors. The results of the statistical analysis of TAP expression in samples grouped based on clinical factors and biological markers are shown in Table 1. The average age of the study population was 54 years (range, 24 to 90). The majority (97.6%) had been diagnosed with stage I, II, or III breast cancer. We compared the TAP expression levels in the normal and tumour tissues from the 93 patients and found significant differences in nodal status (P = .018), the breast cancer stage (P = .02), and the Her2/neu (P = .016) status, whereas no differences were found for age at the time of surgery, tumour size, ER status and PR status.

First, the data demonstrate that the level of TAP mRNA significantly decreased as breast cancer stage increased; the more advanced stages of breast cancer had lower TAP expression levels in the tumour tissue relative to the levels in the normal tissue. The difference between the normal and tumour tissues was approximately 1.8-fold for stage 2 (1-fold, P = 0.043) and stage 3 (1-fold, P = 0.048) breast cancers. In the clinical breast cancer staging system, tumour size, distant metastases and lymph node infiltration are the three main factors that define the cancer status. We found that the relative TAP expression level in normal samples vs. tumour samples increased significantly from stage 0 nodal status, which had a relative TAP expression level of 1.7-fold, to stage 1...
(1-fold; \( P = 0.052 \)) and stage 2, with a 0.6-fold relative TAP expression ratio \( (P = 0.039) \). This clinical information confirms that normal breast cells have higher TAP expression levels than tumour cells in vivo. Moreover, TAP expression may decrease significantly during breast cancer development, which includes the development of characteristics such as acquired migration ability.

Next, TAP protein localisation was determined by immunohistochemical (IHC) staining of frozen tumour sections, which revealed an increase in TAP protein expression in normal tissues (Fig. 4, brown stain in the left panel, indicated by the green arrows).

By contrast, advanced-stage tumour tissues (migration cells) diagnosed as invasive ductal and lobular carcinomas did not express substantial levels of TAP (Fig. 4, brown stain in the right panel, indicated by the red arrows). HE staining is shown to indicate the cell position and cell type for IHC staining.

3.4. \( \alpha \)-Tocopheryl succinate enhances the chemotherapeutic sensitivity of Her2/neu-negative breast cancer cells

\( \alpha \)-TOS, a derivative of natural \( \alpha \)-tocopherol (vitamin E), has been demonstrated to inhibit the proliferation of various types of cancer cells in vitro and in vivo. Cells sensitive to the anti-tumour effects of \( \alpha \)-TOS include human neuroblastoma cells (Swettenham et al., 2005), prostate carcinoma cells (Basu et al., 2007), promyelocytic cells and breast cancer cells (Liang et al., 2012; Wang et al., 2010). Adriamycin (also called doxorubicin) is a type of anthracycline antibiotic that is also used as a chemotherapeutic agent for a variety of cancers, including breast cancer. The aim of this experiment was to evaluate whether the sensitivity of human breast cancer cells to doxorubicin (DOX) is enhanced by \( \alpha \)-TOS. Here, we used \( \alpha \)-TOS, DOX and the combination of 100 nM DOX with different concentrations of \( \alpha \)-TOS to measure the effects on the drug sensitivities of MDA-MB-231, SKBR-3 and MCF-10A cells, as shown in Fig. 5. The data showed that \( \alpha \)-TOS alone has minor cell toxicity at a concentration of 50 \( \mu \)M in normal and cancerous breast cells, resulting in approximately 90% cell viability after 48 h of treatment. DOX alone had strong anti-cancer activities on the SKBR-3 and MDA-MB-231 cell lines, resulting in 60 and 42% cell viability after treatment with 100 nM DOX, as shown in Fig. 5A and B, respectively. However, the cytotoxicity of DOX was limited to breast cancer cells; the treatment of normal breast cells (MCF-10A) with DOX had little effect on cell viability, as shown in Fig. 5C. We found that the combination of a low concentration of DOX (100 nM) and \( \alpha \)-TOS significantly reduced cell toxicity in an \( \alpha \)-TOS dose-dependent manner (from 70% with 100 nM DOX alone to 55% with 100 nM DOX plus 10 \( \mu \)M \( \alpha \)-TOS) in MDA-MB-231 cells, whereas SKBR-3 and MCF-10A cells remained unaffected by the combination treatment. These results demonstrate that the addition of a very small amount of \( \alpha \)-TOS significantly enhanced the anti-cancer response to DOX in MDA-MB-231 breast cancer cells but not in normal breast cells from the MDA-MB-231 cell line.

4. Discussion

HER2-positive breast cancers account for 20 to 30 percent of all breast cancers and are identified by the over expression of HER2 in tumour tissues. HER2-positive breast cancers are more aggressive...
than other breast cancers and have an increased probability of metastasis. Despite recent advances in treatment, HER2/neu-positive and HER2/neu-negative breast cancers continue to be a major cause of death in women. Trastuzumab (Herceptin®), a humanised antibody that targets the extracellular domain of HER2/neu, has become the standard of care for treating early stage and metastatic HER2/neu-over expressing breast cancers. Many studies have shown that α-tocopherol and other forms of vitamin E have anti-tumour activity against several breast cancers, both in vitro and in vivo (Comitato et al., 2010; Wang et al., 2010; Yu et al., 2008). Several studies have also shown that combining α-tocopheroxyacetic acid (α-TOS, an analogue of α-tocopherol) with a HER2/neu-specific antibody (Herceptin) results in an enhanced cytotoxic effect against HER2/neu-positive cell lines but not against HER2/neu-negative cell lines. This result suggests that low doses of α-tocopherol may be therapeutic in combination with a HER2/neu-specific antibody. We found that TAP was specifically expressed in HER2/neu-negative breast tissues in 93 pairs of normal and tumour samples in vivo and that a combination of expressed in HER2/neu-negative breast tissues in 93 pairs of normal and tumour samples in vivo and that a combination of 

**Table 1**

Univariate analysis of prognostic factors and TAP expression in normal and tumour tissues.

<table>
<thead>
<tr>
<th>Factors</th>
<th>No. of patients</th>
<th>TAP N/T ratio</th>
<th>P-value</th>
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<tr>
<td></td>
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<td>Mean ± se</td>
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<td>Age</td>
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<td>&lt;50yr</td>
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<td>≥50yr</td>
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<td>Size of tumor</td>
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<tr>
<td>T1</td>
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<td>1.66 ± 0.34</td>
<td>.464</td>
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<tr>
<td>T2</td>
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<td>T3</td>
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<td>T4</td>
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<td>Nodal status</td>
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<td>N0</td>
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<td>N2</td>
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<tr>
<td>N3</td>
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Data were analyzed using univariate analyses. A P-value <0.05 was considered as statistically significant. All P-values are two-sided.

* Fold ratios of TAP mRNA expression were determined in normal/tumor paired samples.
  * Average fold ratio of TAP mRNA expression in each group.

**Fig. 4.** Immunolocalisation of the TAP protein in human invasive ductal and lobular carcinoma breast tumour tissues. The tumour tissue samples were cut into 8 μm serial sections and stained with antibodies specific to human TAP. I.H.C., immunohistochemistry stain; H.E., hematoxylin and eosin stain. The normal breast cells are indicated by green arrows; the malignant or metastatic breast cells are indicated by red arrows. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 5.** Cell viability in response to α-tocopheroyacetic acid (α-TOS) alone, DOX alone and the combination of 100 nM DOX and various concentrations of α-TOS after 48 h in MDA-MB-231, SKBR-3 and MCF-10A cells. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, in which an increase in cell number is reflected by an increased OD540nm at the indicated time points. The experiment was repeated three times with replicate samples. The data points represent the means, and the error bars indicate the 95% confidence intervals.
Vitamin E has been widely studied as a supplement for preventing human cancers, especially prostate and breast cancers. However, the results have been not consistent, in part due to the limitations of epidemiological and experimental studies. Depending upon the number and position of methyl groups on the chromanol ring, Vitamin E can be defined in α-, β-, γ-, or δ-tocopherols (Yang & Suh, 2012). Professor Chung S. Yang and his colleagues in Rogus University showed several pieces of evidence that γ-, or δ-tocopherols, due to the unmethylated carbons at the 5-position at the chromanol ring, are more effective than α-tocopherols in trapping reactive nitrogen species (RNS) or in other words, enhancing cancer preventive functions (Li et al., 2011; Smolarek & Suh, 2011; Yang & Suh, 2012). Despite the success of the above experiments, disappointing results were found from several recent large-scale intervention human trials with α-tocopherol alone. A proposal was made to use a combination therapy with Vitamin E due to its less toxic, anti-oxidative activities (Hazewindus, Haenen, Welsey, & Bast, 2012; Jaswir, Kobayashi, Koyama, Eichii, & Nagao, 2012), when used in combination with target or hormone thera-pies. Furthermore, TAP expression is another important factor for responding to different types of tocopherols in cells. In our studies, the preferential expression of TAP, the α-tocopherol-binding protein, in normal/benign breast cells indicates that there may be a preferential accumulation of α-tocopherol, the principal and most active isoform of vitamin E, in normal/benign luminal epithelial cells, and the down regulation of TAP in both in situ and invasive carcinomas suggests a correlation between the reduction of tocopherol levels and the initiation of carcinogenesis. We interpret this result as strong, albeit indirect, in vivo evidence in support of the use of vitamin E as a supplement to prevent breast cancer.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Chia-Hwa Lee and Ka-Wai Tam performed the experimental work, design, and data analysis. Chih-Hsiung Wu, Ka-Wai Tam, Ching-Shyang Chen, Ching-Shui Huang and Shih-Hsin Tu kindly provided the human breast specimens. Chi-Tang Ho provided use-

References


