Short Communication

Co-enzyme Q_{10}, riboflavin and niacin supplementation on alteration of DNA repair enzyme and DNA methylation in breast cancer patients undergoing tamoxifen therapy

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In the present study, eighty-four breast cancer patients were randomized to receive a daily supplement of 100 mg co-enzyme Q_{10}, 10 mg riboflavin and 50 mg niacin (CoRN), one dosage per d along with 10 mg tamoxifen twice per d. A significant increase in poly(ADP-ribose) polymerase levels and disappearance of *RASSF1A* DNA methylation patterns were found in patients treated with supplement therapy along with tamoxifen compared to untreated breast cancer patients and tamoxifen alone-treated patients. An increase in DNA repair enzymes and disappearance of DNA methylation patterns attributes to reduction in tumour burden and may suggest good prognosis and efficacy of the treatment.

DNA repair: Methylation: Breast cancer: Tamoxifen: Co-enzyme Q_{10}: Riboflavin: Niacin

Tamoxifen (TAM) is a non-steroidal anti-oestrogen drug, which has led to an increase in both disease-free and overall survival of breast cancer patients after primary surgery[1]. A complicating factor is the relapse in breast cancer patients during tamoxifen therapy and in this subset of patients, treatment is only palliative and the recurrent breast cancer is incurable[2]. Endometrial cancer and other serious side-effects of therapy have been reported in tamoxifen-treated patients and TAM-induced DNA adducts were found in leucocyte DNA from breast cancer patients[3].

Poly(ADP-ribose) polymerase (PARP) is a highly conserved, abundant protein, with three functional domains identified within the PARP polypeptide by limited proteolysis[4]. PARP protein and associated poly(ADP-ribosyl)ation reactions are thought to play a number of roles in different biological processes such as DNA repair, recombination, apoptosis, p53 function and maintenance of genomic stability[5].

In recent years, changes in the status of DNA methylation, known as epigenetic alterations, have turned out to be one of the most common molecular alterations in human neoplasia including breast cancer[6]. Muller *et al.* [7] detected the prognostic value for *RASSF1A* methylation in pre-therapeutic sera of

patients with breast cancer. The present study has been designed to evaluate the effect of nutritional supplementation of co-enzyme Q_{10}, riboflavin and niacin (CoRN) on modulation of DNA repair enzyme PARP and *RASSF1A* DNA methylation pattern in breast cancer patients undergoing tamoxifen therapy.

Materials and methods

Study patients

Patients were recruited from the Medical Oncology Department of the Government Royapettah Hospital, Chennai, India, via their physicians according to the process approved by the Institutional Human Ethical Review Board to conduct a single blinded study. They were aged between 43 and 70 years with histopathology-confirmed breast cancer. Patients with diabetes mellitus, renal and hepatic diseases were excluded from the study.

Study design

Forty-two socio-economically and age-matched disease-free, healthy controls were recruited in group I. Two different

groups of patients were recruited for the study in groups II and III – group II: eighty-four untreated breast cancer patients; group III: eighty-four breast cancer patients treated for more than 1 year with TAM; group IV and V – group III patients followed up for 45 d (group IV) and 90 d (group V) after supplementation with co-enzyme Q_{10} (100 mg Kaneka®Q10; Kaneka Corporation, Osaka, Japan), riboflavin (10 mg; Madras Pharmaceuticals, Chennai, India) and niacin (50 mg; Madras Pharmaceuticals, Chennai, India) one dosage per d along with TAM (10 mg Nolvadex®, AstraZeneca, Bangalore, India) twice per d. Subjects were advised to maintain their usual diet during the study period and were advised not to take any other medication other than those required during the study.

Serum and DNA isolation

Serum was collected from 2 ml blood in a serum separator tube (Vacutainer; Becton Dickinson, Rutherford, NJ, USA) by centrifugation after clotting at 3000 rpm for 10 min. High molecular weight genomic DNA was isolated from whole blood by conventional phenol–chloroform and ethanol extraction[8].

Methylation-specific PCR

RASSF1A DNA methylation: bisulphite modification. Bisulfite modification was done by following the method of Herman et al. [9]. Briefly, 1 μg DNA was denatured by incubating with 5·5 μl NaOH (2 M) for 10 min at 37°C. Subsequently, 30 μl hydroquinone (10 mM; Sigma) and 520 μl sodium bisulphite (3 M; Sigma) at pH 5, freshly prepared, were added and mixed. The DNA was overlaid with four drops of mineral oil and the sample was incubated at 50°C for 16 h. Bisulphite-modified DNA was purified using a Wizard DNA CleanUp System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Methylation-specific PCR. CpG islands in *RASSF1A* genes were detected by methylation-specific PCR following the method of Fackler et al. [10]. Forward and reverse primers were synthesized which corresponds to the predicted sequence of methylated or unmethylated genomic DNA after sodium bisulphite treatment. For the reaction, 1 μl sodium bisulphite-treated DNA was added to 24 μl reaction buffer (1·25 mM-dNTP, 16·6 mM-$(NH_4)_2SO_4$, 67 mM-2-amino-2-hydroxymethyl-1,3-propanediol (Tris), pH 8·8, 6·7 mM-$MgCl_2$, 10 mM-β-mercaptoethanol, 0·1 % DMSO and 1·25 U RedTaq; Sigma, St. Louis, MO, USA) containing 100 ng each of forward and reverse primers specific to the unmethylated and methylated DNA sequences. Conditions were 94°C for 5 min, followed by thirty-five cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, with a final extension cycle of 72°C for 5 min. The PCR products were resolved by electrophoresis in a 2 % agarose gel and the ethidium bromide-stained PCR products were imaged with the Eagle Eye II Video System (Stratagene, La Jolla, CA, USA). *RASSF1A* unmethylated DNA (180 bp): forward: 5′-GGT TGT ATT TGG TTG GAG TG; reverse: 5′-CTA CAA ACC TTT ACA CAC AAC A. *RASSF1A* methylated DNA (160 bp): forward: 5′-GTT GGT ATT CGT TGG GCG C; reverse: 5′-GCA CCA CGT ATA CGT AAC G.

Analysis of poly(ADP-ribose) polymerase by immunoblotting

Serum protein concentration was estimated by the method of Lowry et al. [11]. Protein (50 mg) was boiled with sample solubilizing buffer for 5 min and separated on 10 % SDS–PAGE by the method of Laemmli[12]. The gel was transferred using blotting buffer (25 mM-Tris, 192 mM-glycine and 10 % methanol) on to a nitrocellulose membrane (Hybond C + ; Amersham Life Sciences) at 30 V for 3 h. The membrane was incubated with 1:1000 anti-PARP primary antibody (Calbiochem International, CA, USA) for 3 h. After extensive washing the membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bangalore GeneI, Chennai, India; 1:500 dilution). The bands were detected using the DAB/hydrogen peroxide chromogen system (6 mg 3,3′-diaminobenzidine dihydrochloride and 30 mg nickel chloride in 10 ml 50 mM-Tris–HCl, pH 7·5 containing 10 μl H_2O_2).

Statistical analysis

Statistical analysis was performed with one-way ANOVA followed by least significant difference test using the Statistical Package for Social Science version 10.0 (SPSS, Chicago, IL, USA). Values are expressed as means and standard deviations.

Results

Protein expression of PARP (116 kDa) protein was determined by immunoblot using anti-PARP antibody. Fig. 1 shows the protein expression pattern in different study groups. In group IV and V patients there was a significant increase ($P < 0.01$) in PARP concentration compared to groups I, II and III. In group I controls no *RASSF1A* DNA methylation pattern was found, but all the eighty-four patients in group II had methylated *RASSF1A* DNA pattern. In group III patients *RASSF1A* DNA methylation was found in thirty out of eighty-four patients. Methylation pattern changed in twenty of these patients in group IV and group V leading to disappearance of *RASSF1A* DNA methylation. Fig. 2 shows a representative methylation pattern of *RASSF1A* in breast cancer patients. Lane 5 shows the methylated *RASSF1A* DNA and the disappearance of the methylated pattern in lanes 6 and 7 after CoRN supplementation.

Discussion

Cancer patients are usually exposed to high levels of DNA-damaging agents. In addition, they may have compromised nutritional status due to the disease process itself (cachexia) or due to the side-effects of TAM and chemotherapy (nausea, vomiting, impaired gastrointestinal function)[3]. The purpose of the present study was to determine whether CoRN supplementation alters the extent of DNA damage and alters *RASSF1A* methylation.

The protein expression of PARP in group IV and V patients was found to be increased compared to the other groups, which proves the up-regulation of PARP repair enzyme by CoRN supplementation. Niacin is the dietary precursor for the synthesis of NAD^+, which is the sole substrate for the nuclear enzyme PARP. PARP binds to, and is specifically acti-
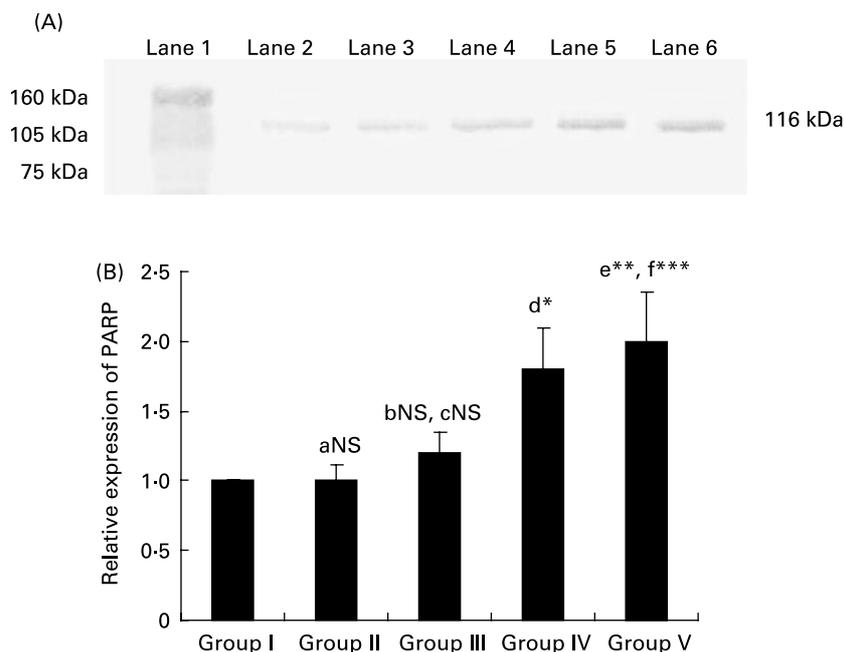
Fig. 1. Poly(ADP-ribose) polymerase (PARP) expression levels in different study subjects. (A) Lane 1, molecular-weight marker; lane 2, control subjects (group I); lane 3, untreated breast cancer patients (group II); lane 4, breast cancer patients treated with tamoxifen (group III); lane 5, breast cancer patients treated with tamoxifen + co-enzyme Q_{10}, riboflavin and niacin (CoRN) (45 d) (group IV); lane 6, breast cancer patients treated with tamoxifen + CoRN (90 d) (group IV). (B) Values are means with their standard deviations depicted by vertical bars. Comparisons were made between: a, group I and group II; b, group I and III; c, group II and III; d, group III and IV; e, group III and V; f, group I and V. Statistical significance is expressed as: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

vated by, DNA single- and double-strand breaks, representing one of the earliest responses to DNA damage in the cell. Upon activation, PARP synthesizes poly(ADP-ribose) (pADPr) from NAD^+, on itself and on a number of acceptor proteins involved in the maintenance of chromatin architecture and DNA metabolism[13]. Riboflavin captures reactive metabolites like TAM and carcinogens to form a complex and thereby prevents formation of DNA adducts, prevents DNA methylation and maintains genomic stability[14,15]. Earlier reports have shown that riboflavin deficiency enhances the induction of the three repair-associated enzymes by carcinogens, and that riboflavin supplementation suppresses this phenomenon[16]. This is possibly because of the protection riboflavin provides against DNA damage, by increasing the levels of PARP. Co-enzyme Q_{10} has been found to increase the DNA repair rate by protecting the cells against further oxidative DNA damage[17,18] and has been found to enhance the DNA repair enzyme PARP activity[18].

Earlier studies in our laboratory have proved the beneficial effect of CoRN supplementation; CoRN has been found to enhance the expression of the tumour suppressor gene MnSOD. It has been found to restore lipid peroxide levels and activities of the enzymic and non-enzymic antioxidants to normal levels[19]. Cofactors of CoRN have been found to inhibit host energy loss by increasing the gluconeogenesis pathway, thereby preventing cancer cachexia[14]. CoRN supplementation to breast cancer patients has been found to counteract the TAM-induced hyperlipidaemia to normal levels[20], reduce the tumour marker levels of CEA and CA 15-3[21] and decrease the levels of serum cytokines IL-1β, IL-6, IL-8, TNF-α and vascular endothelial growth factor[22]. In the present study, we found disappearance of *RASSF1A* DNA methylation in the serum of ten patients treated with CoRN. Serum *RASSF1A* DNA methylation is an easy means of detecting the treatment prognosis for patients undergoing adjuvant TAM treatment as it could help in early detection of recurrence in these patients[23]. Disappearance of *RASSF1A* methylation during supplement therapy with CoRN in these patients may help in preventing the occurrence of recurrence.
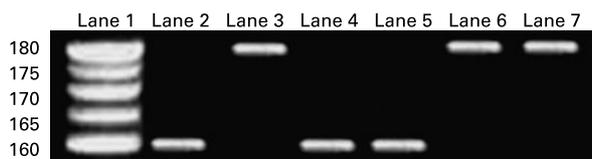

Fig. 2. Methylation-specific PCR of *RASSF1A* pattern in the study subjects. Lane 1, DNA marker; lane 2, breast cancer cell line MCF-7 used as a positive control showing methylated DNA; lane 3, normal lymphocyte DNA used as positive control for unmethylated DNA; lane 4, untreated breast cancer patients; lane 5, breast cancer patients treated with tamoxifen; lane 6, breast cancer patients treated with tamoxifen + co-enzyme Q_{10}, riboflavin and niacin (CoRN) (45 d); lane 7, breast cancer patients treated with tamoxifen + CoRN (90 d). 160 bp, Methylated DNA; 180 bp, unmethylated DNA.

Acknowledgements

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