Original Paper

Gene Expression Changes in Spleens and Livers of Tumour-Bearing Mice Suggest Delayed Inflammation and Attenuated Cachexia in Response to Oil Palm Phenolics

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Key Words

Cachexia · Cancer · Microarray · Oil palm phenolics · Systemic inflammation

Abstract

Background/Aim: Plant phenolics can inhibit, retard or reverse carcinogenesis, and may thus help prevent or treat cancer. Oil palm phenolics (OPP) previously showed anti-tumour activities in vivo via a cytostatic mechanism at 1,500 ppm gallic acid equivalent. Here, we report other possible molecular mechanisms by which this extract attenuates cancer, especially those concerning the immune response. **Methods:** We subcutaneously injected J558 myeloma cells in BALB/c mice and supplemented OPP orally at 1,500 ppm gallic acid equivalent. We observed the physiology parameters of these animals and harvested their spleens and livers after 18 h, 1 week and 4 weeks for microarray gene expression analysis using Illumina MouseRef-8 BeadChips. **Results:** Time course microarray analysis on spleens after injecting J558 myeloma cells in mice revealed that the immune response of tumour-bearing mice supplemented with OPP was lower compared to controls, thus suggesting delayed inflammation in response to OPP. In livers, cholesterol biosynthesis genes were upregulated while inflammatory genes were downregulated through time, further suggesting attenuation of systemic inflammation and cachexia. These effects correlated with the delayed in vivo development of syngeneic tumours in mice given OPP. **Conclusions:** This study suggests the possible utilisation of OPP as an anti-tumour and anti-cachexia agent.

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Background

Cancer is the second leading cause of death in the world [1]. In addition to DNA damage, cancer has been linked to chronic inflammation, in which transcription factors involved in inflammation have been found to play a role [2]. Experimental animal studies have shown increased cancer incidence in genetically or environmentally induced chronic inflammation [3]. Cancer is also the result of a complex interplay between the growing tumour and local or systemic responses by the host to the presence of malignancy [4]. Metastasis is the central step which makes most cancers incurable, causing 90% of human cancer deaths [5], which may occur due to specific functional organ failure, or death due to poorly defined factors with a common theme of cytokine overproduction related to cachexia [6, 7].

In addition to the current cancer treatment methods such as chemotherapy and radiotherapy, naturally occurring chemopreventive substances, such as dietary and medicinal phenolic compounds including ellagic acid [8], anthocyanidins [9], resveratrol [10], green tea extract (containing epigallocatechin gallate) [11–13] and curcumin [14–16], have also received considerable attention as cancer chemopreventive agents. Phytochemicals may work as cancer chemopreventive agents by preventing a new tumour from increasing in size or limiting the proliferation of cancer cells once limited numbers of progenitor cancer cells have evolved [17]. In addition, their anti-inflammatory properties may be beneficial, as the use of anti-inflammatory compounds, such as aspirin and non-steroidal anti-inflammatory drugs, was found to reduce cancer risk [2]. At the molecular level, dietary phenolic compounds are known to elicit vital cellular responses such as cell cycle arrest, apoptosis and differentiation by activating a cascade of molecular events [8].

Incidentally, the oil palm (*Elaeis guineensis*) contains various lipid-soluble phytochemicals which possess significant anti-oxidant and anti-cancer properties, such as carotenoids, tocopherols and tocotrienols [18–20]. Water-soluble phenolics extracted from the oil palm vegetation liquor [21], termed oil palm phenolics (OPP) [22], also showed significant biological activities against cancer cells in vitro [23] and in vivo [23, 24]. In relation to this, we previously reported a microarray analysis carried out on tumours which developed in BALB/c mice following subcutaneous injection of syngeneic J558 myeloma cells, which revealed that oral supplementation of OPP at 1,500 ppm gallic acid equivalent in these tumour-bearing animals caused a G1/S phase cell cycle arrest in the tumours [24]. This implied that OPP acted as an anti-tumour cytostatic agent at the concentration tested.

In addition to this direct anti-tumour cytostatic effect, OPP may also have other indirect effects in delaying tumour growth, such as on the immune system, which is important in removing tumour cells from the body. As the spleen and liver play important roles in the immune response, we hypothesised that certain molecular mechanisms triggered by OPP in these two organs might have important indirect anti-tumour effects. The present study reported herein is part of the same study previously published [24]. While the previous study documented only the effects of OPP on the development of tumours and their gene expression changes, the current study reports the physiology parameters of these tumour-bearing BALB/c mice and the gene expression changes caused by OPP supplementation in their spleens and livers.

Methods

OPP Samples

OPP samples were prepared according to the methods described in Sambanthamurthi et al. [21]. OPP contains numerous phenolic acids including three isomers of caffeoylshikimic acid, protocatechuic acid and *p*-hydroxybenzoic acid. The detailed composition of OPP is as described earlier [22].

Animal Feeding and Sample Collection

The protocols for animal feeding have been described previously [24]. Six-week-old male inbred BALB/c mice were purchased from the Institute of Medical Research, Kuala Lumpur, Malaysia, after ethical clearance on all experimental procedures involving animals was obtained from the Animal Care and Use Committee of the University of Malaya, Kuala Lumpur, Malaysia. The mice were injected subcutaneously at the dorsum of the neck with 200 μ l IgA-secreting J558 mouse myeloma cells (ATCC TIB-6) ($1\text{--}2 \times 10^6$ cells/200 μ l) after acclimatisation for 1 week. Immediately after the injection of the myeloma cells, the control group received distilled water while the treatment group was given OPP at 1,500 ppm gallic acid equivalent as drinking fluids ad libitum. Three harvesting periods were designated: 18 h ($n = 5$ per group), 1 week ($n = 5$ per group) and 4 weeks ($n = 15$ per group). For the last batch of animals, body weights were monitored every week, while fluid intake was monitored every day. Food intake and faecal output were monitored for 7 consecutive days in the middle of the designated feeding period (between week 2 to week 3). We found that there were no changes in these two parameters and thus did not continue with the measurements as these were not as important as the fluid intake of these mice.

All mice were sacrificed at the designated periods via euthanasia with diethyl ether followed by exsanguination after an overnight food fast (with fluids still provided). The last batch of animals at 4 weeks was sacrificed when the presence of tumours was observed through palpation at the neck. These were the same animals of which data on their tumours had been previously published [24]. In addition to recording the weights of six major organs (spleens, livers, hearts, kidneys, lungs and brains), collection of blood samples from the last batch of animals was carried out via cardiac puncture. The spleens and livers harvested at the three designated time points were snap-frozen in liquid nitrogen and stored at -80°C until the total RNA isolation process for gene expression analysis.

Haematology Analysis

For the last batch of animals, half of the blood samples ($n = 8$) were collected in tubes containing ethylenediaminetetraacetic acid to prevent clotting. These whole blood samples were sent immediately after dissection of the animals to the Clinical Biochemistry and Haematology Laboratory, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, for haematology analysis. The analysis was carried out using the Animal Blood Counter Vet Haematology Analyser (Horiba ABX, France).

Clinical Biochemistry Analysis

In order to obtain sera, the other half of the blood samples collected from the last batch of animals ($n = 7$) were allowed to clot at room temperature for 2 h before being centrifuged at 1,000 g for 5 min, after which the supernatant layers were collected and stored at -20°C . The blood serum samples were then sent for clinical biochemistry analysis using the Roche/Hitachi 902 Chemistry Analyser (Roche/Hitachi, Switzerland) in the Clinical Biochemistry and Haematology Laboratory, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Clinical biochemistry parameters which were examined include alanine aminotransferase, aspartate aminotransferase, glucose, serum total protein, albumin, globulin, ratio of albumin to globulin, total cholesterol, triglycerides, low-density lipoproteins and high-density lipoproteins.

Microarray Gene Expression Analysis

The spleens and livers harvested at the three designated time points were used for microarray gene expression analysis. The protocols for total RNA extraction, amplification of biotinylated cRNA as well as microarray hybridisation, washing and scanning have been described previously [24]. The Illumina MouseRef-8 Version 1 Expression BeadChip Direct Hybridization Kit (Illumina, Inc., San Diego, Calif., USA) was used and the raw gene expression data obtained are available at Gene Expression Omnibus [25] (accession number: GSE36576).

Time Course Microarray Data Analysis

Quality control of the hybridisation, microarray data extraction and initial analysis were carried out using the Illumina BeadStudio software (Illumina, Inc.). Next, the GeneSpring GX software (Agilent Technologies, Santa Clara, Calif., USA), available at the Malaysia Genome Institute, Bangi, Selangor, Malaysia, was utilised for analysis of the time course dataset. The raw expression data generated using the Illumina BeadStudio software were imported into the GeneSpring GX software and normalised via data transformation by

setting measurements which were less than 0.01 to 0.01, followed by per chip normalisation to 50th percentile and per gene normalisation to median. Outlier samples were removed after hierarchical clustering analysis provided by the Illumina BeadStudio software was carried out, in addition to using the box plot, principal component analysis and hierarchical clustering tools provided by the GeneSpring GX software. Three replicates per condition (with outliers removed) were then considered for further analysis.

The data were filtered by using the Detection Level column from the Illumina data file, in which a Detection Level cut-off greater than 0.99 in at least half of the samples was applied to obtain genes significantly present. This was followed by a two-way ANOVA statistical analysis (parametric test, equal variance assumption, $p < 0.01$) to obtain three lists of genes which significantly changed with 'time', 'treatment' and 'time-treatment interaction'. Log ratios of changes in gene expression were calculated by subtracting the log value of each treatment sample from the mean log value of the 3 time-matched controls. Because there was a possibility that the 'time' gene list might have included genes whose expression levels changed with time only (changed with time similarly in both treatment and control groups), a one-way ANOVA statistical analysis (parametric test, equal variance assumption, $p < 0.01$) was performed on the log ratios of changes of the 'time' gene list in order to identify genes which changed with treatment during the time course [26].

The resultant gene list obtained was then merged with the 'treatment' and 'time-treatment interaction' gene lists identified in the two-way ANOVA test to form a final master gene list which consisted of genes significantly changed by treatment with OPP during the time course. Hierarchical clustering and principal component analysis of samples were carried out using this master list to ensure that replicates for each time point were clustered to each other. In addition, hierarchical clustering of samples was carried out on each time-treatment combination using these significant genes in order to check for similarities between these groups. We then subjected the master list to K-means clustering in order to classify the genes into two groups, upregulation or downregulation, based on the trend with time of the mean expression pattern. The genes upregulated or downregulated across time were then analysed separately in the following functional enrichment analyses.

To establish associations between treatment and the affected GenMAPPs and gene ontologies, we used the GenMAPP [27] and MAPPFinder [28] softwares (University of California at San Francisco, San Francisco, Calif., USA) for linking the gene expression data to the gene ontology (GO) hierarchy. For quantitative functional analysis, the GO-Quant software (Institute for Risk Analysis and Risk Communication, University of Washington, Seattle, Wash., USA) [29] was used. This software links results from the qualitative functional MAPPFinder analysis to the quantitative gene expression values and calculates the combined average expression values of individual significantly changed genes within each gene ontology term. To visualise the quantitative gene ontology results, hierarchical clustering was then carried out on each GO term using the euclidean distance metric and average linkage method in the TIGR MultiExperiment Viewer software (The Institute for Genomic Research, Rockville, Md., USA) [30]. Changes in regulatory networks were also analysed through the use of the Ingenuity Pathways Analysis software (Ingenuity® Systems, Redwood City, Calif., USA).

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction Validation

Two-step real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) studies were carried out on four target genes selected to represent the spleens (three time points) and livers (three time points) used in the microarray experiments, using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif., USA). The same total RNA aliquots for the microarray experiments were utilised for this analysis. The protocols for carrying out the real-time qRT-PCR experiments have been described previously [24].

Statistical Analysis

Statistical analysis was carried out by using the two-tailed unpaired Student t test available in the Microsoft Excel software (Microsoft Corporation, Redmond, Wash., USA) unless otherwise stated. Differences with p values of less than 0.05 were considered statistically significant.

Results

Physiology Parameters of Tumour-Bearing Mice

We found that OPP supplementation did not affect the well-being of the tumour-bearing mice in this study, as no significant changes were observed in the body weights (fig. 1a), organ weights (fig. 1b), fluid intake (fig. 1c), food intake (fig. 1d) and faecal output (fig. 1e) of the

Fig. 1. Physiology parameters of tumour-bearing mice were not changed by OPP. **a** Body weights; $n = 15$. **b** Organ weights; $n = 15$. **c** Timeline of fluid intake; $n = 3$ cages (3–4 mice per cage). **d** Timeline of food intake; $n = 3$ cages (3–4 mice per cage). **e** Timeline of faecal output; $n = 3$ cages (3–4 mice per cage). * $p < 0.05$ vs. control. Error bars indicate SEM.

last batch of animals in the treatment group compared to those in the control group. An increase in the fluid intake of mice in the treatment group on the first 2–3 days might have been caused by the initial attraction of the mice to the smell of OPP. This fluid intake was not significantly different thereafter throughout the course of the study.

Unexpectedly, however, haematology analysis on whole blood samples obtained from the last batch of mice in the treatment group showed a significant reduction in the levels of lymphocytes and monocytes (table 1), and thus implied a lower immune response in these animals compared to those in the control group. While a lower immune response might at first suggest weak immunity against tumours, we found that this was not the case as mice in

Table 1. Haematology and clinical biochemistry parameters of mouse blood samples

Test	Control	Treatment
Haematology		
Red blood cells, $\times 10^{12}/l$	8.77 $\pm$ 0.21 ^a	8.82 $\pm$ 0.16 ^a
Haemoglobin, g/l	134 $\pm$ 3 ^a	135 $\pm$ 2 ^a
Haematocrit/packed cell volume, l/l	0.36 $\pm$ 0.01 ^a	0.36 $\pm$ 0.01 ^a
Mean corpuscular volume, fl	41 $\pm$ 1 ^a	41 $\pm$ 1 ^a
Mean corpuscular haemoglobin concentration, g/l	375 $\pm$ 5 ^a	373 $\pm$ 7 ^a
White blood cells, $\times 10^9/l$	1.5 $\pm$ 0.3 ^a	0.8 $\pm$ 0.1 ^a
Band neutrophils, $\times 10^9/l$	0.02 $\pm$ 0.00 ^a	0.01 $\pm$ 0.00 ^a
Segmented neutrophils, $\times 10^9/l$	0.72 $\pm$ 0.21 ^a	0.41 $\pm$ 0.05 ^a
Lymphocytes, $\times 10^9/l$	0.71 $\pm$ 0.15 ^a	0.34 $\pm$ 0.02 ^b
Monocytes, $\times 10^9/l$	0.03 $\pm$ 0.01 ^a	0.01 $\pm$ 0.00 ^b
Eosinophils, $\times 10^9/l$	0.01 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a
Basophils, $\times 10^9/l$	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a
Thrombocytes, $\times 10^9/l$	570 $\pm$ 45 ^a	608 $\pm$ 25 ^a
Prothrombin, g/l	73 $\pm$ 1 ^a	77 $\pm$ 1 ^a
Clinical biochemistry		
Alanine aminotransferase, U/l	22.2 $\pm$ 2.0 ^a	27.6 $\pm$ 2.9 ^a
Aspartate aminotransferase, U/l	159.4 $\pm$ 18.4 ^a	172.5 $\pm$ 31.6 ^a
Glucose, mmol/l	5.2 $\pm$ 0.2 ^a	6.2 $\pm$ 0.3 ^b
Serum total protein, g/l	54.6 $\pm$ 1.5 ^a	50.9 $\pm$ 1.3 ^a
Albumin, g/l	33.6 $\pm$ 1.0 ^a	31.2 $\pm$ 1.3 ^a
Globulin, g/l	21.0 $\pm$ 0.8 ^a	19.7 $\pm$ 0.4 ^a
Albumin:globulin	1.6 $\pm$ 0.1 ^a	1.6 $\pm$ 0.1 ^a
Total cholesterol, mmol/l	3.72 $\pm$ 0.17 ^a	3.41 $\pm$ 0.27 ^a
Triglycerides, mmol/l	0.95 $\pm$ 0.04 ^a	1.14 $\pm$ 0.08 ^a
Low-density lipoproteins, mmol/l	0.30 $\pm$ 0.03 ^a	0.28 $\pm$ 0.03 ^a
High-density lipoproteins, mmol/l	3.19 $\pm$ 0.15 ^a	2.90 $\pm$ 0.22 ^a

All data are expressed as means $\pm$ SEM. Means with different superscript letters are significantly different ($p < 0.05$). OPP decreased the lymphocyte and monocyte levels while it increased the glucose levels of tumour-bearing mice when compared to the control group. Other parameters remained unchanged. Group size: for the haematology analysis, $n = 8$; for the clinical biochemistry analysis, $n = 7$.

the treatment group developed tumours which were smaller compared to mice in the control group. The results for the measurements of these tumours have previously been reported by us [24]. Clinical biochemistry analysis on the blood serum samples obtained (table 1) showed that OPP supplementation caused no significant changes in the parameters measured, except for slightly raising glucose levels, possibly due to the fruit sugar content of the extract.

Gene Expression Changes in the Spleen and Liver

We harvested the spleens and livers of the tumour-bearing mice at three different time points (18 h, 1 week and 4 weeks) after injection with the J558 myeloma cells, and carried out a time course microarray analysis on these two organs. For the spleen, we found that 2,213 genes were upregulated across time, while 1,308 genes were downregulated across time. For the liver, we found that 571 genes were upregulated across time, while 249 genes were downregulated across time. These genes, together with their fold changes for each time period, are given in online supplementary material 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000357948).

Fig. 2. Venn diagram showing the number of genes significantly changed by time, treatment as well as time-treatment interaction and hierarchical clustering of time-treatment combinations using significant genes. **a** Venn diagram for spleens. **b** Venn diagram for livers. Genes were selected using a two-way ANOVA (parametric test, equal variance assumption, $p < 0.01$), with time as the first factor and treatment as the second factor. **c** Hierarchical clustering groups for spleens. **d** Hierarchical clustering groups for livers. The focus of this hierarchical clustering was to find similarities among different time-treatment combinations. Groups which were more similar to each other clustered closer together.

The Venn diagrams generated from the microarray data are shown in figure 2a and b, for the spleen and liver, respectively. It should be noted that the number of genes changed in the ‘time only’ (coloured red) and the ‘treatment only’ (coloured green) categories was the highest in the spleen, indicating that the injection of J558 myeloma cells in mice resulted in large changes in this organ as time went by, and that the supplementation of mice with OPP also had a large effect in this organ. However, the number of genes changed in the ‘interaction only’ category (coloured blue) was the lowest and might thus reflect a low interaction effect between time and treatment, further suggesting that these two factors acted mostly independently of each other. In other words, the administration of OPP did not interfere with the functions of the spleen as time went by.

Figure 2c and d shows the hierarchical clustering analysis carried out on each time-treatment combination, using genes selected as significant from the spleen and liver, respectively. For the spleen, control and treatment groups at 4 weeks were clustered together, while the control group at 1 week was closer to groups at 4 weeks, and the treatment group at 1 week was closer to groups at 18 h. This suggests that by supplementing the tumour-bearing mice with OPP, the occurrence of tumours, and thus the immune response, was delayed. For the liver, the control and treatment groups at 1 week and 4 weeks were separated according to treatment. This shows that OPP supplementation caused major gene expression changes in the liver as well.

Genes which were upregulated or downregulated across time were further analysed separately in a functional enrichment analysis. GenMAPPs and gene ontologies that significantly changed across time in spleens and livers of mice supplemented with OPP are listed in online supplementary material 2.

Quantitative functional analysis of significant gene ontologies in the spleen (online suppl. material 3) showed the upregulation of catabolic metabolism, energy production, transcription, translation, proteasome degradation, cholesterol biosynthesis and oxidative stress response across time. Gene ontologies which were downregulated across time involved the immune response, such as antigen processing and presentation, signalling of transcription factors involved in immunity, mitogen-activated protein kinase (MAPK) signalling, interleukin signalling and cell adhesion.

As it was surprising to find that the immune response was downregulated across time, we carried out further analyses specifically on immune-related genes, which showed that the immune response was being triggered in the treatment group, albeit slightly delayed when compared to the control group (fig. 3). Further hierarchical clustering of the groups based on these genes (inset in fig. 3) showed that while the control and treatment groups were similar at 18 h, the control group at 4 weeks and the treatment group at 1 week were clustered separately from the other groups. Furthermore, the treatment group at 4 weeks clustered together with the control group at 1 week, indicating that the immune response being triggered in the treatment group was delayed compared to the control group.

Concordantly, genes involved in proteasome degradation associated with antigen processing and presentation (fig. 4a) and genes involved in the electron transport chain associated with energy production (fig. 4b) were upregulated across time. This indicates that the immune response was being triggered later in the treatment group compared to the control group, possibly due to the delayed growth of tumours. On the other hand, genes regulated by transforming growth factor- β (*Tgfb1*) (fig. 5a) and nuclear factor- κ B (*Nfkb2*) (fig. 5b) were downregulated, thus indicating delayed inflammation.

Quantitative functional analysis of significant gene ontologies in the liver was also carried out (online suppl. material 4). Livers of tumour-bearing mice fed OPP showed an upregulation of cholesterol biosynthesis across time (fig. 6a). Other functions upregulated across time include hormone metabolism, proteasome degradation and nitric oxide biosynthesis.

As we previously showed that liver cholesterol biosynthesis genes were downregulated by OPP supplementation in BALB/c mice on a normal diet [31], it is thus surprising to find the same pathway upregulated in the treatment group in the present study. Thus, we further analysed the expression changes of hepatic cholesterol biosynthesis genes across time in the treatment group as compared to the control group based on their expression levels in different time-treatment combinations. We found that the control group showed a decrease in the expression of liver cholesterol biosynthesis genes across time, while the treatment group showed an increase in the expression of these genes across time (fig. 6b). Hierarchical clustering of the groups based on these genes revealed that while the control and treatment groups were similar at 18 h, the control groups at 1 week and 4 weeks were clustered separately from the treatment groups at 1 week and 4 weeks (inset in fig. 6b). In fact, the treatment

Fig. 3. Spleen immune-related genes classified as downregulated across time in the treatment group as compared to the control group shown based on their expression levels in different time-treatment combinations. While both control and treatment groups showed an increase in the expression of spleen immunity genes across time, those in the treatment group showed lower expression levels. The control group at 4 weeks showed higher expression levels of immunity genes while the treatment group at 1 week showed lower expression levels when compared to the rest of the groups. Further hierarchical clustering of the groups based on these genes showed that while the control and treatment groups were similar at 18 h, the control group at 4 weeks and the treatment group at 1 week were clustered separately from the other groups (**inset**). Furthermore, the treatment group at 4 weeks was clustered together with the control group at 1 week, thus indicating that the immune response being triggered in mice given OPP was delayed compared to the controls.

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groups at 1 week and 4 weeks were clustered nearer to the control and treatment groups at 18 h. This indicates that OPP supplementation to these tumour-bearing mice prevented the downregulation of cholesterol biosynthesis genes observed in the control groups, possibly as an effect of reduced tumour growth.

On the other hand, genes involved in an inflammatory network were downregulated across time (fig. 6c). Other functions downregulated across time were adipogenesis, insulin signalling, apoptosis and nuclear receptors.

Real-Time qRT-PCR Validation

To confirm the microarray results, the expression levels of four target genes, i.e. *Sirt1* (spleen), *Tgfb1* (spleen), *Rxra* (liver) and *Tiam1* (liver) (online suppl. material 5A), were measured using real-time qRT-PCR. The expression levels of these target genes were normalised to the geometric mean of the three most stable housekeeping genes, chosen from the four tested, namely *Rasa1*, *Sema4a*, *Tpst2* and *18SrRNA*. As shown in online supplementary material 5B, correlation of fold changes obtained by the two gene expression profiling techniques was high ($R^2 = 0.9444$), thus validating the microarray data obtained.

Discussion

OPP isolated from the oil palm vegetation liquor has various biological activities [21–24, 31–33]. We previously found that OPP may function as an anti-proliferative agent against cancer development [23]. We also discovered that OPP exerted in vivo anti-tumour effects via a cytostatic mechanism by inducing a G1/S phase cell cycle arrest at 1,500 ppm gallic acid equivalent [24]. In addition to this direct anti-tumour cytostatic effect, OPP may also have other indirect effects in delaying tumour growth, such as by acting on the immune system to eliminate tumour cells from the body. The present study thus extends the findings of our previously published study [24], by reporting the physiology parameters of the tumour-bearing BALB/c mice and the gene expression changes caused by OPP supplementation in their spleens and livers.

Mice on OPP Showed Normal Physiology albeit Having Smaller Tumours

Despite the fact that mice given OPP developed significantly smaller tumours, their well-being was not in any case different from the tumour-bearing controls, as shown by the physiology studies carried out. While the lower amounts of lymphocytes and monocytes in the blood samples of mice in the treatment group at first seemed to suggest lower immunity against tumour development, it was found that this was not true as animals in the treatment group developed smaller tumours when compared to the tumour-bearing controls [24].

Fig. 5. Genes downregulated across time in the spleen. **a** Genes downregulated in the *Tgfb1* network. *Tgfb1* is most frequently upregulated in tumours and is associated with enhanced tumour angiogenesis and poor prognosis in several cancers. In addition to the downregulation of *Tgfb1* in spleens of mice belonging to the treatment group, we also found a network of genes which were under the direct regulation of this gene, which suggests a delayed immune response in these mice. **b** Genes downregulated in the *Nfkb2* network. *Nfkb* is a widely known pro-inflammatory transcription factor which is constitutively activated in tumours. It has been linked to cellular transformation, proliferation, apoptosis suppression, invasion, angiogenesis and metastasis. Besides *Nfkb2*, genes involved in MAPK signalling, tumour necrosis factor receptors and chemokines were downregulated as well. This indicates a downregulation of inflammation and immune activation in spleens of mice treated with OPP, possibly due to suppressed tumour growth.

Fig. 6. Genes regulated across time in the liver. **a** Genes upregulated across time in the cholesterol biosynthesis pathway. Among the genes upregulated across time in the liver by OPP are *Hmgcs1* (3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1), *Pmkv* (phosphomevalonate kinase), *Idi1* (isopentenyl-diphosphate delta isomerase), *Fdps* (farnesyl diphosphate synthetase), *Sqle* (squalene epoxidase), *Cyp51* (cytochrome P450 51), *Nsdhl* [NAD(P) dependent steroid dehydrogenase-like] and *Dhcr7* (7-dehydrocholesterol reductase). **b** Liver cholesterol biosynthesis-related genes classified as upregulated across time in the treatment group as compared to the control group shown based on their expression levels in different time-treatment combinations. While the control group showed a decrease in the expression of liver cholesterol biosynthesis genes across time, the treatment group showed an increase in the expression of these genes across time. Further hierarchical clustering of the groups based on these genes showed that while the control and treatment groups were similar at 18 h, the control groups at 1 week and 4 weeks were clustered separately from the treatment groups at 1 week and 4 weeks (inset). In fact, the treatment groups at 1 week and 4 weeks were clustered nearer to the control and treatment groups at 18 h. This indicates that OPP supplementation to these tumour-bearing mice prevented the downregulation of cholesterol biosynthesis genes observed in the control groups, possibly as an effect of reduced tumour growth. **c** Genes downregulated across time in the inflammation network. Genes involved in adipogenesis and inflammation were downregulated across time in the livers of mice given OPP. Examples of these genes include *Cebpb* (C/EBP- β), *Foxo1* (forkhead box O1), *Egr1* (early growth response-1), *Gab1* (growth factor receptor bound protein 2-associated protein-1), *Tiam1* (T cell lymphoma invasion and metastasis-1) and *Pbaf1* (pre-B cell colony-enhancing factor-1).

Interestingly, it was found in a previous study that the amounts of immune cells were higher at peak tumour size [34]. Thus, the lower immune response of mice in the treatment group suggests that OPP delays tumour growth by affecting the micro-environment of the tumour instead of activating the immune response. By directly affecting the tumour cell cycle, the immune response of mice treated with OPP was thus not triggered as fast compared to that of the tumour-bearing controls.

Delayed Activation of the Immune Response in the Spleens of Mice Given OPP Implies Delayed Tumour Growth and Delayed Inflammation

The immune system has three primary roles in the prevention of tumours through immune surveillance. First, it protects the host against virus-induced tumours by eliminating viral infections. Second, timely elimination of pathogens and resolution of inflammation can prevent the establishment of a tumour-promoting environment. Third, the immune system recognises pre-cancerous or cancerous cells and eliminates them before they can cause harm [35]. However, it has also been recognised that the immune system may have an immunostimulatory role as well, in which immune infiltrates in tumour cells facilitate tumour progression by secreting growth factors, inflammatory mediators and free radicals [36], thereby playing paradoxical roles during cancer development [37]. The duality of the immune system in cancer arises in part because of the decentralised nature of the immune system and the bilateral nature of the interaction between the tumour and the immune system of the host [36]. Thus, the concept of immuno-editing has recently been introduced to explain this dual role of the immune system in tumour development [38]. Immuno-editing is divided into three phases: elimination in which the immune system detects and eliminates tumour cells, equilibrium between the immune system and the developing tumour, and escape in which the immune system is no longer able to contain tumour growth, resulting in a progressively growing tumour unrestrained by immune pressure [39].

Traditionally, cancer therapy has focused on cytotoxic agents rather than on therapies that ameliorate the by-product effects of cancer cells or the pro-inflammatory host response to their presence. However, pro-inflammatory cytokines, such as tumour necrosis factor- α , interleukin-1, interleukin-6, interleukin-11 and transforming growth factor- β , have been shown to induce DNA damage. They also inhibit repair, inhibit apoptosis, facilitate tumour growth, invasion and metastasis, induce angiogenesis, as well as contribute to maintaining a chronic state of inflammation by way of a self-activating feedback loop, causing morbidity and mortality in cancerous patients through activation of multiple signalling pathways [7].

In the present study, when the time course gene expression profiles of spleens in tumour-bearing mice were investigated, it was found that a majority of the genes which had a lower expression in the treatment group compared to the control group was involved in the immune response. These immune-related functions include antigen processing and presentation, MAPK signalling, interleukin signalling and cell adhesion. These results were at first surprising, as it was hypothesised that the immune response in the treatment group would be upregulated due to possible immunostimulatory effects of OPP supplementation in preventing cancer, as shown by a previous study carried out on tumour-bearing mice using curcumin [40]. Although this was not the case, the findings obtained in the present study were consistent with the observations made in several other previous studies, in which curcumin suppressed the immune response and lymphocyte proliferation, mainly through the inhibition of NF- κ B [41–43]. In these previous studies, curcumin was found to suppress the mitogen-, interleukin-2- or allo-antigen-induced proliferation of splenic lymphocytes and the development of cytotoxic T lymphocytes [41], although the generation of lymphokine-activated killer cells was less influenced [42]. Curcumin also inhibited immunostimulatory functions of dendritic cells [43]. In addition, the cancer chemopreventive agent dithiolethione was found to mediate its anti-cancer effects via downregulation of antigen presentation genes [44]. Hence, OPP may help delay inflammation in the prevention or treatment of cancer.

It is also tempting to speculate that perhaps the higher immune response triggered in the control mice in the present study was due to tumour metastasis into the spleens. However, J558 cells are not known to be metastatic in vivo in immunocompetent mice and do not

migrate into local lymph nodes and other lymphoid tissues [45]. Thus, the lower expression levels of genes involved in the immune response might then seem to suggest a possible immunosuppressive or immunotoxic effect of OPP supplementation, as shown by a synthetic flavonoid called flavopiridol, which is being tested as a cancer chemotherapeutic agent [46]. Flavopiridol is a potent growth inhibitory flavone that can selectively block cell cycle progression [47]. It is known to induce apoptosis of normal lymphoid cells and cause immunosuppression. This property has been suggested as a possible mechanism for its anti-tumour activity in vivo against human leukaemia and lymphoma xenografts [46]. Cells from haematopoietic neoplasms appear to be very susceptible to flavopiridol-induced apoptosis [48]. Hence, these effects of flavopiridol seem to suggest that certain phenolics such as flavonoids may cause immunosuppression or immunotoxicity. However, OPP does not contain significant amounts of flavonoids but comprises mostly phenolic acids [22].

In the present study, although immune-related genes were found to have lower expression levels in the treatment group compared to the control group, further analysis of the genes revealed that the spleens of mice in the treatment group actually showed a delayed immune response, as the expression levels of these immunity genes did increase across time, albeit slightly delayed. Some of these immune-related genes are those encoding cathepsins, cluster of differentiation antigenic markers, chemokines, colony-stimulating factors, complement components, major histocompatibility complexes, antibodies, interferons, interleukins, MAPKs, nuclear factors and receptors, phospholipases, tyrosine phosphatases and kinases, *Ras/Rho/Rac* oncogenes, signal transducer and activator of transcription genes, Son cell proliferation proteins, thromboxane receptors and tumour necrosis factor receptors. Hierarchical clustering of the groups based on these immune-related genes showed that while the control and treatment groups were similar at 18 h, the control group at 4 weeks and the treatment group at 1 week were clustered separately from the other groups. Furthermore, the treatment group at 4 weeks was clustered together with the control group at 1 week, thus indicating that the immune response being triggered in the treatment group was delayed when compared to the control group. Therefore, while OPP supplementation did not impair the immune system as shown by flavopiridol, this extract did not trigger the immune system (especially the adaptive immune response) against the tumour cells either.

A role for the innate arm of the immune response which includes the dual role of the complement system in the immune surveillance of cancer [49, 50], however, cannot be discounted yet. This is because the regulation of complement genes was not very clear in this study. The complement system acts as an adjuvant for the antibody-mediated killing of cancer cells. However, Markiewski et al. [51] previously suggested that contrary to this, complement inhibition may also have a therapeutic role in treating cancers with an inflammatory component. This is because the deficiency of certain complement proteins such as C5a retards tumour growth. Interestingly, the complement protein C5a acts as an anaphylatoxin which releases inflammatory mediators and increases the production of reactive oxygen as well as nitrogen species by myeloid-derived suppressor cells recruited by tumours. In fact, the complement system has also been linked to other inflammatory diseases besides cancer [52]. Thus, further studies on the complement system would be useful, given its dual role in immune surveillance and inflammation.

Perhaps the two most interesting transcription factors which were found significantly downregulated through time in the spleen by OPP supplementation are *Tgfb1* and *Nfkb2*. *Tgfb1* is most frequently upregulated in tumours, and high tumour burden as well as circulating plasma levels of *Tgfb1* are associated with enhanced tumour angiogenesis and poor prognosis in several cancers [53–58]. In addition, *Tgfb1* may also suppress natural killer cell activity and is known to incur tamoxifen resistance in breast cancer cells [59]. *Nfkb* is a well-

known pro-inflammatory transcription factor which is constitutively activated in tumours, and it has been linked to carcinogenesis, promotion of cell proliferation, suppression of cell death, angiogenesis and metastasis [60]. In line with this, *Sirt1*, which is known to block the expression of *Nfkb*, thus preventing pro-inflammatory responses in cancer [61], was also upregulated through time by OPP supplementation in the spleens of mice in the treatment group, and this suggests a delayed inflammatory response. At 4 weeks, the upregulation of genes involved in proteasome degradation in the spleens of mice by OPP supplementation may indicate the beginning of the antigen processing and presentation process [62], thus initiating the adaptive immune response. The upregulation of genes involved in the electron transport chain may indicate an increase in energy production required for immune-related processes as well.

Upregulation of Hepatic Cholesterol Biosynthesis Genes and Downregulation of Genes Involved in Inflammation by OPP Supplementation through Time Suggest Attenuation of Systemic Inflammation and Cachexia

Cancer patients normally suffer from cachexia, due to the activation of catabolism pathways which degrade skeletal muscle (proteolysis-inducing factors) and adipose tissue (lipid-mobilising factors), thus resulting in loss of lean and fat mass [63]. In recent years, cachexia has been understood as a result of major metabolic abnormalities due to a combination of host cytokine release and tumour products rather than a simple increase in energy consumption by the tumour and subsequent starvation of the cancer patient [6]. Patients with cancer cachexia frequently show an elevated resting energy expenditure, as a result of an increase in the Cori cycle, glucose and triglyceride-fatty acid recycling as well as gluconeogenesis [64]. These patients also have increased hepatic acute-phase reactants, increased protein turnover, increased urinary nitrogen loss, hyperlipidaemia, hyperinsulinaemia, insulin resistance and glucose intolerance [65]. As the liver is involved in many of these altered processes in cachexia, the effects of administering OPP on the livers of the tumour-bearing mice in the present study were investigated next.

It was surprising to find that among the functions significantly upregulated across time by OPP supplementation in the liver, cholesterol biosynthesis appeared the most prominent. When analysed based on their expression levels in different time-treatment combinations, OPP supplementation to the tumour-bearing mice was found to prevent the downregulation of cholesterol biosynthesis genes observed in the control groups, possibly as an effect of reduced tumour growth. In BALB/c mice on a normal diet, it was previously reported that OPP supplementation was able to downregulate hepatic cholesterol biosynthesis genes and may thus be able to prevent atherosclerosis and cardiovascular disease [31]. Therefore, it was unusual to find that the same pathway was upregulated in the treatment group in the present study. This, however, shows that OPP behaves differently in different biological conditions, and further suggests that it has pleiotropic effects in preserving the in vivo pro-oxidant/anti-oxidant balance in wellness maintenance and chronic disease prevention.

Furthermore, although hypercholesterolaemia is widely known to be linked to cardiovascular disease, hypocholesterolaemia, on the other hand, is linked to increased cancer mortality risks and has been attributed in part to the effect of pre-existing cancer [66, 67]. Many compounds and hormones derived from the cholesterol metabolic pathway could be potentially affected by the rate of cholesterol biosynthesis, and they have an impact on cancer incidence [66]. In addition, hypocholesterolaemia has been identified as a predictor of nosocomial infection, death and length of stay in hospitalised patients [68], a predictor of in-hospital mortality in the elderly [69] and a predictor of death in chronic haemodialysis patients [70]. Decreased serum cholesterol may also indicate a poor prognosis in liver cancer patients [71]. The paradox of the protective effect of total cholesterol levels on cancer risk, however, does

not represent an etiological link (low cholesterol as a cause of cancer), but may reflect the advance of the clinical course of cancer (low cholesterol as an effect of cancer) [72]. In fact, total cholesterol synthesis was found to be lower in Yoshida AH130 ascites hepatoma tumour-bearing mice compared to controls [73]. Thus, it is reasoned that by supplementing tumour-bearing mice with OPP, the incidence and progression of cancer were suppressed, and as an effect, the downregulation of cholesterol biosynthesis and thus cancer mortality was reduced in these mice.

In investigating the effects of phenolics, much interest has been placed on transcription factors which regulate the expression of pro-inflammatory mediators such as CCAAT/enhancer-binding proteins (C/EBPs), activator protein-1 and NF- κ B [74]. In line with this, the downregulation of genes involved in adipogenesis and inflammation across time in the livers of mice given OPP was observed. Examples of these genes include *Cebpb* (C/EBP- β), *Foxo1* (forkhead box O1), *Egr1* (early growth response-1), *Gab1* (growth factor receptor bound protein 2-associated protein-1), *Tiam1* (T cell lymphoma invasion and metastasis-1) and *Pbaf1* (pre-B cell colony-enhancing factor-1).

Cebpb has recently been shown to play a role in the oncogenic gene regulation activities of cyclin D1 [75]. It is known to be strongly upregulated at the transcriptional level by inflammatory stimuli [76] such as turpentine oil and bacterial lipopolysaccharide, and by recombinant cytokines such as tumour necrosis factor- α , interleukin-1 and interleukin-6 [77]. *Cebpb* is also involved in lipid accumulation in diet-induced non-alcoholic steatohepatitis [78], hepatic steatosis (fatty liver), gluconeogenesis and diabetes [79]. The downregulation of this gene in the livers through time thus suggests a reduction of inflammation in the tumour-bearing mice given OPP.

Foxo1 is a master regulator of tumour necrosis factor-induced fibroblast apoptosis [80]. The decrease in nuclear *Foxo1* decreases gluconeogenesis and reduces fasting blood glucose [81]. Reduction of *Foxo1* gene expression using antisense oligonucleotide was found to improve hepatic and peripheral insulin action, thus improving insulin resistance [82]. *Foxo1* also has significant roles in extrahepatic tissues, and the overexpression of *Foxo1* in myocytes leads to muscle atrophy and possibly sarcopaenia [83]. Thus, the downregulation of *Foxo1* in the present study through time suggests a possible reduction in insulin resistance and inflammation, which are linked to cancer cachexia.

Several lines of evidence indicate that *Egr1* is also an important regulator of inflammation. For example, *Egr1* response elements are present in the promoters of several pro-inflammatory genes, including *Icam1* (intercellular adhesion molecule-1) and *Tnfa* [84, 85], and they directly regulate the expression of these latter genes. *Egr1* is also involved in mediating cholestatic liver injury [86]. Overexpression of *Gab1* was found to significantly upregulate NF- κ B transcription and DNA-binding activities, which are involved in the inflammatory process in glioblastoma cells [87]. The downregulation of these two genes, *Egr1* and *Gab1*, through time by OPP supplementation thus also suggests a reduction in inflammation.

The expression of *Tiam1* is highly related to the metastatic potential of colorectal carcinoma cells [88] and the knockout of this gene reduces the growth potential and invasion of these cells [89]. *Pbaf1* is upregulated in neutrophils and monocytes by interleukin-1 β , and it functions as a novel inhibitor of apoptosis in response to a variety of inflammatory stimuli during experimental inflammation and clinical sepsis [90]. Its expression is also induced in mammals by lipopolysaccharide and pro-inflammatory cytokines during the infection of human foetal membranes [91]. Therefore, the downregulation of *Tiam1* and *Pbaf1* through time also suggests an attenuation of the inflammatory response by OPP supplementation in the livers of mice. Along these lines, it should also be noted here that two alternative splice transcripts of *Il6ra* (interleukin-6 receptor- α) were also expressed at lower levels in the livers of mice given OPP when compared to the controls.

In addition, two cytochrome P450 isozymes (*Cyp2a4*, *Cyp2a5*), which are overexpressed in a variety of human tumours and generate procarcinogens [92], were downregulated across time in the livers of mice given OPP. The inhibition of these genes may thus have implications for the prevention and treatment of cancer. In addition, two genes which have been reported to be downregulated by hepatotoxicants, such as acetaminophen, 1-naphthylisothiocyanate, carbon tetrachloride, chloroform, erythromycin estolate and tetracycline [93], were upregulated by OPP supplementation in the livers through time as well. The two genes are *Abcb11* (ATP-binding cassette subfamily B member 11) and *Pygl* (liver glycogen phosphorylase). These results indicate that OPP supplementation may prevent the toxic effects of tumour growth in these mice.

Additionally, a gene which is downregulated by acute-phase response mediators, *Rxra* (retinoid X receptor- α) [94], was upregulated through time by OPP supplementation. Ligands of retinoid X receptors such as the retinoids are known to suppress tumourigenesis, and the downregulation of the *Rxra* gene has been implicated in the early stages of human prostate cancer development [95]. The upregulation of this gene by OPP supplementation further supports the anti-tumour effects of this extract in the livers of the mice.

Limitations of the Study

It is important to point out here that the data presented in the current work were obtained from only a single experimental model using J558 IgA-secreting myeloma cells. Thus, the extrapolation of these results to other experimental and clinical situations has to be made carefully due to the enormous diversity of neoplastic diseases. In addition, other cell lines and animal models would also need to be experimented in the future to confirm the anti-cancer effects of OPP in vivo. With this being said, however, we have previously shown that OPP exhibited anti-proliferative effects on various tumour cells in vitro but conversely enhances the growth of normal cells [23]. Among the cell lines tested were mouse IgA-secreting myeloma (J558), oestrogen-receptor-positive human breast adenocarcinoma (MCF7), human lung carcinoma (A549), Madin Darby canine kidney (MDCK), *Aedes albopictus* larvae (C6/36) and Syrian baby hamster kidney (BHK) cells. The reason we chose to work with the J558 cell line in vivo in the present and past cancer studies [23, 24] was because the cell line was syngeneic with the mouse model (BALB/c) that we had been using for our previous mouse experiments [31–33], and it was thus easier for us to work with this cell line. Other cell lines would require the use of athymic nude mice or severe combined immunodeficient mice, with suitable facilities to work with these animals needed as well.

We also acknowledge that to a great extent, most of the results obtained in this study are inferring a link between OPP and its physiological outcomes based on gene expression profiles. Thus, further supporting evidence of altered biochemistry in these mice as suggested by their gene expression analysis would be necessary to pinpoint a precise cause-and-effect relationship and the related biochemical sequences of events involved. As mentioned previously, although the regulation of complement genes was not very clear in the present study, this system may have a role to play in the anti-tumour effects of OPP. Therefore, additional targeted experiments would have to be carried out in the future to further identify the mechanism of tumour growth inhibition by OPP, especially on the complement system. These include carrying out complement haemolysis to demonstrate and quantify the activity of serum complement proteins [96] after OPP supplementation in certain cancer models, as well as immunofluorescence or fluorescence-activated cell sorting flow cytometry analysis to identify whether certain immune cell subpopulations or complement proteins have been altered [51].

Conclusions

The gene expression changes observed in the spleens and livers of tumour-bearing mice given OPP in this study suggest that OPP supplementation did not induce pro-inflammatory cytotoxic effects. On the contrary, OPP was shown to delay inflammation, in addition to its *in vivo* cytostatic effects previously reported, which may be useful in the prevention and treatment of cancer and its related cachexia. One way of doing this would be to administer OPP with standard anti-cancer medical procedures such as radiotherapy and chemotherapy for reducing the effects of systemic inflammation and cachexia caused by cancer.

The findings about systemic inflammation and cachexia in this study are also interesting given that this wasting condition is so prevalent not only in certain late-stage cancers, but also in other conditions including acquired immunodeficiency syndrome (AIDS), multiple sclerosis, tuberculosis and some hormone deficiencies. Thus, OPP may also find possible uses in patients or animal models with some of these other disease states. However, this would involve a new series of experiments, and maybe more interesting results could be obtained with one or more of these diseases rather than with cancer alone. In fact, cytokine overproduction has also been found in a huge range of other diseases besides cancer, ranging from arthritis to influenza. This has been described in terms of a ‘cytokine storm’, as recently reviewed by Tisoncik et al. [97]. Thus, the non-cancer roles of cytokines in these other diseases would also be interesting to study in the future, in addition to their roles in cancer.

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