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ORNITHINE DECARBOXYLASE GENE EXPRESSION IN HUMAN LUNG ADENOCARCINOMA CELL (A549) TREATED WITH POMEGRANATE JUICE

Radiah Abdul Ghani*, Nik Nurasyikin Nik Abdul Malek, Najwa Farhah Md. Yusof, Elyna Fatini Jamil

Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Kuantan Campus, Jalan Istana, Bandar Indera Mahkota, 25200, Kuantan, Malaysia

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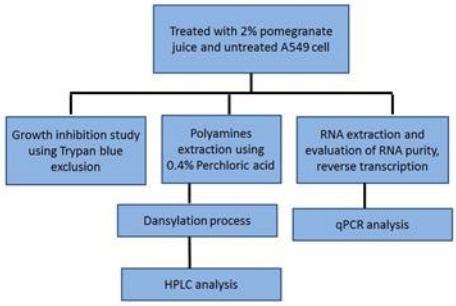
*Corresponding author
radiah@iium.edu.my

Graphical abstract



Abstract

The polyamine biosynthesis pathway plays a significant role in cell growth, both normal and malignant. As polyamines are crucial in cellular growth and differentiation, they are linked to the development of cancer, with higher polyamine level observed in cancerous cells than in healthy cells. Accordingly, suppressing the polyamine pathway has been found to disrupt tumour development. Chemoprevention is considered a more feasible option in cancer management than chemotherapy, with a focus on natural chemopreventive agent. Pomegranate is known to inhibit several progression of lung cancer, although prior studies on the chemopreventive effect of pomegranate on lung cancer did not explore into polyamine pathway. Hence, this study investigated the effect of pomegranate juice on the polyamine pathway, by focusing on the biosynthesis involving ornithine decarboxylase (ODC), the rate limiting enzyme in the pathway. Quantitative polymerase chain reaction (qPCR) was applied to quantify the changes in ODC gene expression in A549 cells treated with pomegranate. The inhibition of growth was determined using Trypan Blue exclusion and the changes in intracellular polyamine in pomegranate treated cells was observed using High Performance Liquid Chromatography (HPLC). It was found that there was inhibition of A549 cell growth and reduced in intracellular polyamine content in pomegranate treated cells. The ODC expression was significantly inhibited compared to untreated cells, with a 48-fold difference. While this finding supports the hypothesis, there is much yet to be elucidated regarding its exact mechanism.

Keywords: Polyamines, Ornithine decarboxylase (ODC), A549, chemoprevention, natural product

1.0 INTRODUCTION

Cancer is a global burden that undermines social and economic development throughout the world, with 8.2 million cancer deaths recorded in 2012 alone. While it has been the number one killer in the developed nations, more than half of the world's latest annual cancer cases occur in the developing world, making it a major public health issue (International Agency for Research on Cancer [1]. The inadequacy in controlling the mortality rates and poor prognosis for patients with common types of cancer, as well as the undesirable adverse side effects developed in response to chemotherapy,

presents the case for a prevention-based approach. One of the prevention strategies is by targeting cancer at its starting point; the cell-signalling mechanism.

Cancer research has been focusing on identifying cell-signalling pathways that are involved in the neoplastic process, and then targeting these pathways to inhibit cancer development. A particular interest lies in aiming for pathways that are activated during the stage of cell growth and perform a critical role in cancer progression. One such pathway is the polyamine pathway, recognised for its significance in the growth and development of both normal and malignant cells [2]. Targeting the polyamine biosynthesis pathway for cancer prevention offers several prospects. An established target is ornithine decarboxylase (ODC), a primary enzyme in polyamine biosynthesis [3]. Ornithine decarboxylase (ODC) is the first enzyme and the committed step in polyamine synthesis, forming putrescine by the decarboxylation of ornithine, a product from the urea cycle. The significance of elevated ODC activity as an intermediary of tumour growth has been widely investigated. ODC expression is tightly regulated by various mechanisms at transcription, post-transcriptional, and translational levels [3,4]. Inhibition of ODC expression leads to intracellular polyamine depletion, subsequently suppressing cancer progression [4].

Ornithine decarboxylase (ODC) is the first enzyme and the committed step in polyamine synthesis, forming putrescine by the decarboxylation of ornithine, a product from the urea cycle. The significance of elevated ODC activity as an intermediary of tumour growth has been widely investigated. ODC expression is tightly regulated by various mechanisms at transcription, post-transcriptional, and translational levels (Pegg, 2006; Perez-Leal & Merali, 2012). The extensive regulatory networks controlling ODC emphasises its importance as a critical enzyme vital for normal cell growth and development. The importance of ODC is further proven *in vivo* by data showing that the homozygous deletion of ODC in mice caused death after 3.5 days post-fertilization (Pendeville *et al.*, 2001).

A highlight in the field of chemoprevention is the extensive range of naturally occurring food compounds that have been found to prevent cancer progression [5].These natural chemopreventive agents, consumed through dietary means, are an attractive strategy in today's fight against cancer. One such naturally occurring chemopreventive agent is *Punica granatum* (pomegranate). The medicinal properties of pomegranate have been highly regarded throughout the millennia. To modern researchers, pomegranate's allure lies not only in its biochemistry, but also in its esteemed status stretching back to the ancient cultures of China, Indus Valley, and Greece, where it is featured in the works of Homer, Babylonian texts, the Quran, and Egyptian art and mythology [6].In Islam, pomegranate is upheld as one of the *sunnah* (prophetic) foods—foods that are established as customary for Muslims on the basis of Prophet Muhammad's teachings and practices.

One potential use of pomegranate as a chemopreventive agent is to inhibit prosurvival pathways in human lung carcinoma A549 cells. According to [7], pomegranate impedes lung tumour development by MAPK, PI3K/Akt, and NF-κB signalling interference; all of which are important to promote cell survival or cell proliferation. Nevertheless, the need for a pathway that is not only involved in all types of cells, but is also specifically targeting cancer cells is highly needed [2]. One possible pathway that has not yet been evaluated with pomegranate is the polyamine pathway.

Therefore, this study aimed to investigate the effect of pomegranate juice on the polyamine metabolism, by focusing on the biosynthesis pathway involving ODC enzyme. It attempted to test the hypothesis that pomegranate juice inhibits polyamine biosynthesis via polyamine enzyme ODC, leading to a decrease in ODC gene expression.

2.0 EXPERIMENTAL

2.1 Cell Culture Methods

The A549 cells, a human lung adenocarcinoma cell lines, were grown in Dulbecco's Modified Eagle Medium (DMEM) with a high glucose and L-glutamine, 10% (v/v) Fetal Bovine Serum, and 1% (v/v) Penicillin-Streptomycin. The cells were grown at 37°C in a humidified atmosphere flushed with 5% CO_2. Cells were routinely subcultured every 2-3 days, and were seeded in six-well plate at 3.2 x10^3 and grown for 48 h prior treatment.

2.2 Sample Preparation for qPCR

A549 cells treated with pomegrenate (2% (v/v) [17] at several of time exposure (0-48 h) were washed twice with PBS followed by the addition of TRIzol reagent for the extraction of total RNA following the manufacturer's (TRIzol) instructions. The yield and quality of total RNA was measured by absorbance at 260/280 nm. One µg of total RNA and 0.5 µg of the random primers were used for reverse transcription following the manufacturer's instructions. The resulting cDNA was diluted to 100 µl with Diethylpyrocarbonate treated water and used as a template for real-time PCR. Briefly, PCR primers were designed with a melting temperature (T_m) of 65-95 °C. Amplicon size was 50–150 bases. Forward and reverse primers spanning exon-exon junctions were selected to avoid amplification of genome sequences (for OCD, 5'-AAAACATGGGCGCTTACACT (forward primer) and

TGGAATTGCTGCATGAGTTG-3′ (reverse primer); for actin, 5′- AGTCCTGTGGCATCCACGAAA (forward primer) and GTCATACTCCTGCTTGCTGA -3′ (reverse primer); for GAPDH, 5′- TCCCTGAGCTGAACGGGA AG (forward primer) and GGA GGA GTG GGT GTC GCT GT-3′ (reverse primer).

2.3 Polymerase Chain Reaction (PCR) Analysis

Quantitative values were obtained from the threshold cycle value (*Ct*), which is the point where a significant increase of fluorescence is first detected. The transcript number of rat β-actin was quantified as an internal RNA control, and each sample was normalized on the basis of its β-actin content. The relative gene expression level of each sample was then normalized to the control. Final results, expressed as *n*-fold difference in gene expression relative to β-actin and and calibrator were determined by subtracting the average *Ct* value of a target gene from the corresponding *Ct* value of the β-actin gene.

2.4 Growth Inhibition

A549 cell growth was determined using the Trypan Blue exclusion assay. To determine cell number, 100 μl of cell suspension was mixed with 900 μl of Trypan Blue and cells were counted using an improved Neubauer haemocytometer. This method also allowed viability to be determined, since Trypan Blue penetrates non- viable cells, staining them blue. A modification of the method described by Lowry *et al*. [15] was used to determine total cellular protein. Samples were quantified against standards, prepared from a stock solution of 500 μg/ml BSA, to provide a range of standards from 0 to 250 μg/ml BSA.

2.5 Intracellular Polyamine Content

After the appropriate time interval, HL-60 cells were harvested by the removal of cells and medium to a sterile 15 ml centrifuge tube. To ensure that all cells had been removed from the plate, the plate was rinsed with an equal volume of RFC10 which was added to the tube. Next, the cell suspension was centrifuged at 2800 *g*av for 5 min. The supernatant was discarded and the pellet was washed twice in 1 ml of complete PBS before being transferred to a clean microtube. The cell suspension was centrifuged again at 7500 *g*av for 5 min and the supernatant discarded before resuspending the pellet in 300 μl of 0.2 M PCA. This was placed on ice for 30 min in order to extract the acid-soluble fraction. After this time each tube was centrifuged at 7500 ***g***av for 5 min and the supernatant was removed to a clean Eppendorf tube. This was stored at −20◦C until analysis by HPLC. The method of HPLC used was that of pre-column dansylation. Samples were dansylated at 25◦C overnight, extracted in toluene, blown to dryness in a nitrogen stream, and then reconstituted in 200 μl of acetonitrile. Samples were analysed by reverse-phase HPLC on a Hichrom RPB 5 μm column using a gradient of 100% acetonitrile to 40:60 (v/v) acetonitrile/water.

3.0 RESULTS AND DISCUSSION

The expression level of (ODC) is assayed for up- or down-regulation using relative quantification analysis method. The dosage used as previous findings [17]. Figure 1 shows the relative normalized expression for each treatment group over time. From this figure, the relative expression of ODC was reduced in treated samples when compared to untreated samples. It was shown to be significantly lower than untreated cells at 48 hours of treatment, with a 7-fold decrease ($p<0.05$). The result illustrated the inhibitory effect of pomegranate on ODC expression in A549 cells.

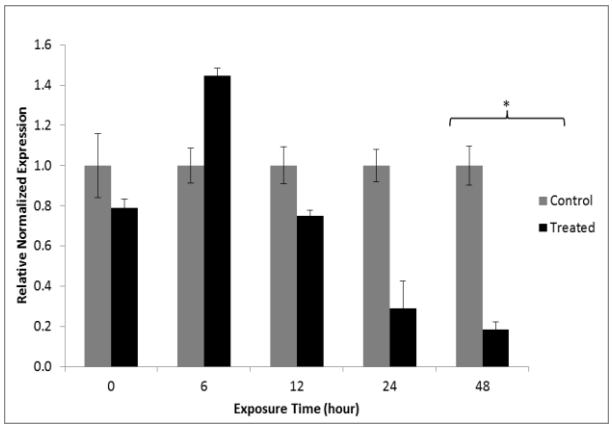


Figure 1 The Ornithine Decarboxylase Gene Expression

Ornithine decarboxylase (ODC) is an important enzyme for the regulation of polyamine metabolic pathway. It is the rate-limiting enzyme in the synthesis of polyamines. Polyamines contribute to various cellular functions such as cell division and cell growth, and polyamine concentration increases primarily through an upregulation of ODC. Studies have demonstrated the important roles of ODC in tumour development and metastasis. This upregulation of ODC is correlated with increased cell proliferation as well as tumourigenesis [8]. Figure 2 showed that the A549 cells's growth was inhibited after the addition of 2% (v/v) pomegranate juice. This inhibition was concurrent with the reduction in the number of total polyamines in treated cells (Table 1).

The downregulation of ODC observed in Figure 1 could reflect a decrease in polyamine synthesis and thus inhibition of tumourigenesis (Table 1). This finding indicates that pomegranate juice exerts its antitumourigenesis effect by altering the polyamine

metabolism. This is supported by previous findings in which cells and tissues derived from transgenic mice with reduced ODC gene copy number exhibited reduction in ODC activity, a reduction in polyamine content, and a strongly retarded tumourigenesis [9, 10]. It was shown that modest reductions in ODC activity could lead to marked resistance to tumour development.

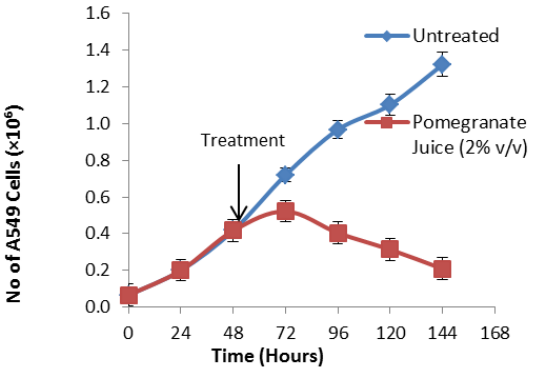


Figure 2 The effect of pomegranate juice on the A549 cell growth

Table 1 The Total Polyamines in A549

Time after exposure (h)	Untreated (mg/mol)	Treated with Pomegranate (mg/mol)
0	5.75	5.75
24	6.10	4.35
48	7.21	3.11
72	7.98	2.65
96	9.11	1.18

Agents that block induction of ODC can therefore prevent tumour formation. Hence, ODC inhibition is shown to be a promising tool to screen for inhibitors of tumourigenesis. In the present study, treatment with pomegranate juice resulted in down-regulation of ODC expression. This indicates the chemopreventive mechanism of pomegranate could be due to its inhibitory effect on ODC expression. The finding of this study is in agreement with a study on pomegranate inhibition of tumourigenisis in mice, in which found the pomegranate was found to inhibit the ODC expression and impeded hyperplasia [11].

The recognition that polyamines are required for cell growth and that their metabolic pathway is frequently dysregulated in cancers led to the development of inhibitors for each step of the polyamine biosynthetic pathway, including for ODC. A well-known ODC inhibitor is 2-difluoromethylornithine (DFMO). Inhibition of ODC by DFMO produced a near complete depletion of polyamines putrescine and spermidine, which was accompanied by a substantial decrease in cellular growth rate [12].Thus, there is a convincing case that ODC is a viable target for chemoprevention.

Other studies also provided insights into the underlying molecular mechanisms of natural chemopreventive agents by focusing on the key role of the polyamine metabolism in cancer cells. It has been revealed previously that the effects of resveratrol, a chemopreventive agent found in grape skins, peanuts, and red wine, on the growth and polyamine metabolism of human colon cancer cells [13, 14]. Treatment of the cells with resveratrol resulted in a decrease in ODC activity, followed by tumour growth inhibition. Similarly *in vivo*, a study on tumourigenesis in rats with induced cancers and treated with ellagic acid and *B. purpurea* respectively, and it was observed that the reduced ODC expression was in tandem with lowered tumour incidences. These studies substantiated the potentiality of natural chemopreventive agents in suppressing tumour progression via ODC and the polyamine pathway [15, 16].

In essence, the finding of this study supported the hypothesis that pomegranate juice, as a chemopreventive agent, inhibits polyamine biosynthesis in A549 human lung carcinoma cells via a downregulation of ODC. It suggested the importance of polyamines in tumour progression and underscored the rationale of targeting polyamine metabolism as a potential cancer intervention.

4.0 CONCLUSION

Despite advances in lung cancer chemotherapy, there is a pressing need for effective lung cancer chemoprevention beyond smoking cessation. After the discovery that dysregulated polyamine levels were a hallmark for numerous tumour types, anti-tumourigenic therapy began targeting the polyamine biosynthetic pathway. Pomegranate, a natural chemopreventive agent with known cancer prosurvival pathways inhibitive properties, was studied for its effect on the polyamine biosynthesis pathway. Ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis pathway, is the subject of intense study among researchers, including as a target for chemopreventive agents. This study was aimed to investigate the effect of pomegranate on ODC gene expression in human lung carcinoma A549 cells. The expression of ODC in treated and untreated cells were compared, and it was found that ODC expression was significantly lower in treated cells than that of untreated cells after 6 hours of treatment with pomegranate, by a 48-fold difference. The preliminary finding supports the hypothesis that pomegranate inhibits the polyamine pathway via polyamine biosynthesis enzyme ODC.

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