A Cranberry Proanthocyanidin Inhibits Cancer-Related Processes in Human Esophageal Adenocarcinoma Cells

A Senior Honors Thesis

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by

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ABSTRACT

Esophageal cancer is the sixth most common cause of cancer death of US males today, and the rate of this cancer has increased in recent years, possibly caused by the increase in obesity, chronic acid reflux, and poor diet. Preliminary studies in our lab showed that cranberry proanthocyanidin (PAC) treatment of human esophageal adenocarcinoma inhibits multiple carcinogenic-associated processes. Thus, this study aimed to understand the mechanism by which PAC treatment affects such processes by characterizing specific markers of apoptosis and cell cycle regulation, two key pathways dysregulated during the progression to cancer. The SEG-1 human esophageal cancer cell line was utilized in this study, derived from a Barrett's-associated adenocarcinoma of the distal esophagus. The SEG-1 cells were treated with 50μg/ml PAC for 6 hrs and microarray analysis performed. WEDGE ++ was then used to remove extreme outliers, followed by EASE analysis to determine the gene ontology groups that were significantly overrepresented in the significantly up and down-regulated genes. Real-time PCR using pathway plates for apoptosis and cell cycle distribution was then used to validate the data collected from the microarray studies. Alterations in many genes were found with increased apoptosis and changes in cell cycle activity. The results support that cranberry proanthocyanidins dysregulate many processes associated with esophageal cancer development. Further studies are suggested to study PAC's affect over longer periods of time and its cancer inhibitory potential *in vivo*.

INTRODUCTION

Esophageal cancer is a rapidly increasing cause of death among males in the US, driven by an increase in chronic acid reflux (2-4) and obesity, especially male pattern obesity (5-7). Other possible causes for this increase include the presence of hiatal hernia, medication use, and various nutritional factors. Some of these nutritional factors that increase the risk of esophageal adenocarcinoma include low consumption of fruits and vegetables, high dietary cholesterol, and an elevated intake of animal protein and vitamin B12 (8-10). Once diagnosed, patients have a five year survival rate of only 14%, indicating a need for improved preventive means. Various compounds from fruit and other plant foods have shown promise in anti-cancer application; fiber, β-carotene, folate, and vitamins C and B6 all decrease the risk of developing esophageal adenocarcinoma. Research has already shown that cranberry proanthocyanidins have a role in maintaining urinary tract health (11-14). In addition, *in vitro* investigations have recently reported that cranberry extracts impact multiple cancer-associated processes in breast, colon, and prostate cells (15-19).

Proanthocyanidins, also referred to as condensed tannins, are flavanoid polymers used as determinants of flavor and astringency in wines, teas, and fruit juices. They have also been used as tanning agents for animal skins (1). Proanthocyanidins' structures vary based on the stereochemistry of the flavan-3-ol starter and extension units, the type of linkage to the 'lower' unit, the extent of polymerization, and other possible modifications. Proanthocyanidins are found in the fruits, bark, leaves, and seeds of many plants, functioning as protection against pathogens, insects, and herbivores. Different kinds of proanthocyanidin structures are found in different fruit and plant foods; in cranberries, A-type proanthocyanidins are present (1). These A-type proanthocyanidins have linkages between C_2 of the upper unit and the oxygen at C_7 of the starter

unit. The A-type structure also contains linkages between C_4 of the upper unit and positions 6 or 8 of the lower unit (1).

Earlier work in our lab showed that various carcinogenic pathways of human esophageal adenocarcinoma are inhibited with cranberry proanthocyanidin (PAC) treatment. Thus, the goal of the present study is to examine potential markers in two key pathways with the goal of understanding the mechanism by which PAC treatment affects apoptosis and cell cycle regulation.

Preliminary Studies

In a study investigating the chemopreventive potential of a cranberry proanthocyanidin (PAC) in SEG-1 human esophageal adenocarcinoma (EAC) cells, SEG-1 cells were treated with PAC at concentrations ranging from 12.5 μg/ml to 400 μg/ml and assessed over time for changes in cell viability, cell proliferation rates, cell cycle distribution, and apoptosis, compared to the vehicle-treated cells. PAC [50 μg/ml] treatment of human esophageal adenocarcinoma cancer cells was found to significantly inhibit carcinogenesis-associated processes. The administration of PAC at 50 μg/ml or greater effectively reduced cellular growth and proliferation rates, with maximum reductions at 72 to 96 hours post PAC administration. PAC-induced cell cycle arrest occurred mainly at the G1 checkpoint and decreased the fraction of cells in S-phase at 24 and 48 hours post-treatment. In addition, PAC treatment increased apoptosis 12 hours post PAC administration as determined by Annexin V staining.

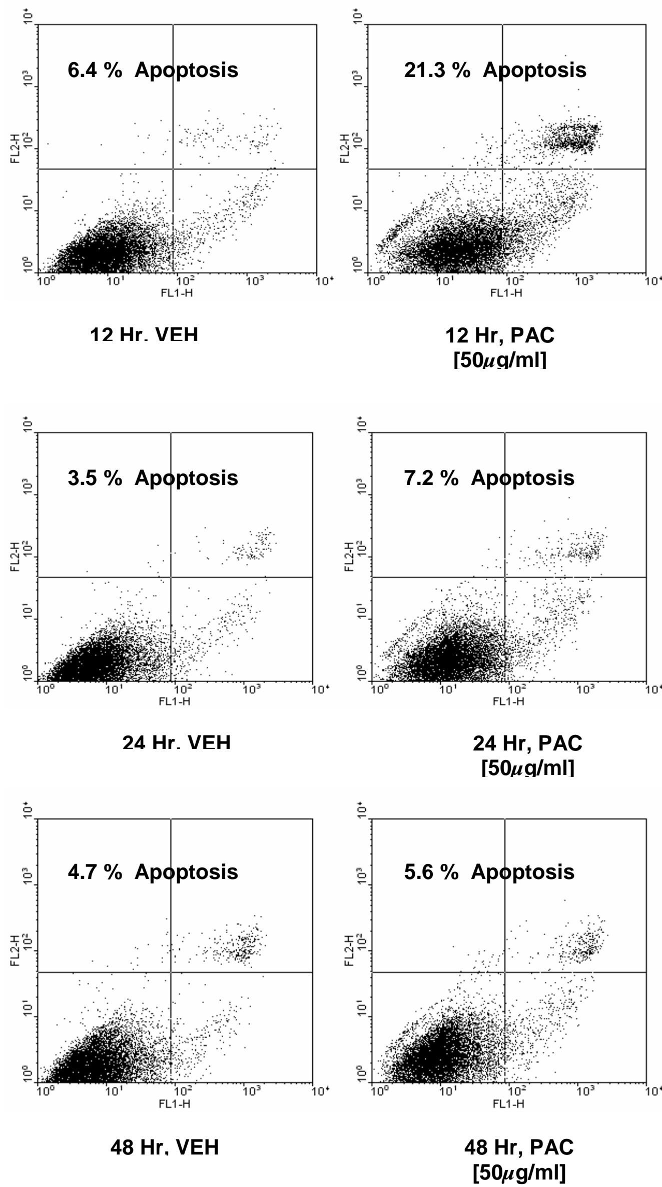

Fig. 1 Induction of Apoptosis following Treatment with a Cranberry Proanthocyanidins

PAC treatment of SEG-1 cells significantly increased apoptosis 12 and 24 hours post-treatment and maximum apoptosis induction occurred 12 hours post PAC treatment.

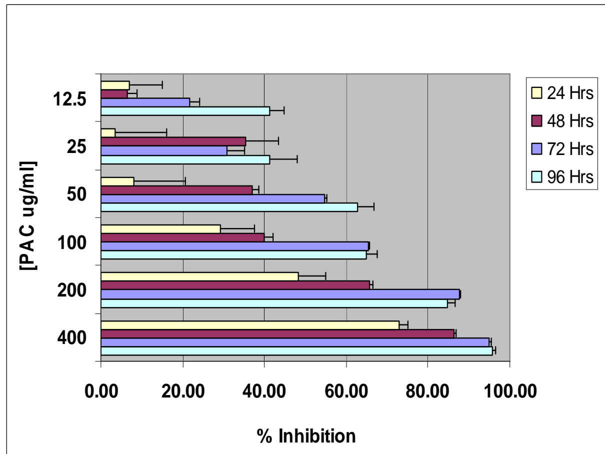

Fig. 2 Inhibition of Cell Viability by PAC

PAC treatment of SEG-1 cells decreased cell viability in a time and concentration dependent manner.

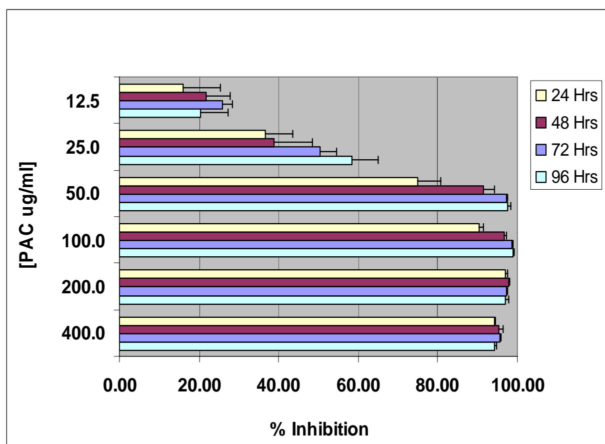

Fig. 3 Inhibition of Cell Proliferation by PAC

PAC treatment of SEG-1 cells decreased cell proliferation in a time and concentration dependent manner up to 50μg/ml.

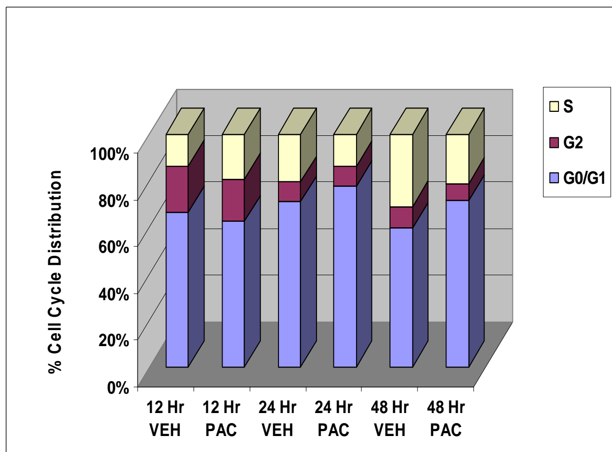

Fig. 4 Cell Cycle Distribution of SEG-1 EAC Cells following PAC Treatment [50 μg/ml]

PAC treatment of SEG-1 cells decreased cells in S-Phase (24, 48 hrs) and increased cells in G1.

METHODS

Cell Culture

The SEG-1 human esophageal cancer cell line was utilized in this series of experiments. The SEG-1 cell lines was derived from a Barrett's-associated adenocarcinoma of the distal esophagus and was a kind gift from Dr. David Beer of the University of Michigan, Ann Arbor, MI. The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) with 0-10% FBS depending on the experiment. The cells were maintained as monolayers at 37°C in 5% CO_2 throughout all of the studies.

Preparation of the PAC Compound

The cranberry PAC compound used in this study was obtained from Dr. Amy Howell of Rutgers University, Chatsworth, NJ. Using bioassay-directed fractionation, the cranberry proanthocyanidin extract was isolated. The cranberry fruit was homogenized with acetone and filtered, discarding the pulp. The collected proanthocyanidin extract was concentrated under reduced pressure to remove the acetone, and then diluted with water. Its aqueous phase was further extracted with EtOAc and the resultant residue was freeze-dried, dissolved in EtOH, and loaded onto a Sephadex column. The adsorbed proanthocyanidins were eluted from the column with 60% aqueous acetone, and the acetone was rotary evaporated off and the resulting residue freeze-dried.

Microarray Studies

The SEG-1 esophageal adenocarcinoma cells were plated in T75 flasks at $3x10^6$ cells, treated with 50 μg/ml PAC for 6 hrs, rinsed and the media replaced. Cells at six hours post PAC

exposure were harvested and their RNA isolated for expression analysis. A minimum of 10 μg of RNA per experimental group was reverse transcribed, and labeled by incorporating biotinylated nucleotides during in vitro transcription. Then, the labeled cRNA was hybridized to the human genome gene chip U133 2.0 plus (Affymetrix, Santa Clara, CA) following fragmentation. Specific transcripts were bound to the corresponding oligonucleotide probes on the chip and the biotinylated cRNA bound fragments were detected via a streptavidin-antibody-phycoerthin conjugate. Data was then compared by relative fluorescence intensity values; the OSU Microarray Core performed the genome-wide expression determination. The results used WEDGE ++ to remove extreme outliers, followed by EASE analysis to determine the gene ontology groups that were significantly overrepresented in the significantly up and down-regulated genes.

Real Time Polymerase Chain Reaction (PCR)

In this study, real time PCR was used to validate the data from the microarray studies in the previously mentioned study. Real time PCR is used to continuously collect fluorescent signals from polymerase chain reactions throughout the cycles. Pathway plates for apoptosis and cell cycle distribution were used in this study. These were obtained from SuperArray, Frederick, MD. The Bio-Rad Icycler machine was used in this study, using a 96 well plate format. Two experimental cocktails were mixed in RNAse and DNAse-free tubes. In the first was added 1225µl of the SuperArray Master Mix for ICycler, 98µl of the diluted first strand of cDNA, and 1127µl of ddH_2O. To the second was added 100µl of Master Mix and 100µl of ddH_2O. A 1:160 dilution of the original input RNA plus ddH_2O was made as a no-reverse-transcription (NRT) control. Five 10-fold serial dilutions of the first experimental cocktail were mixed with 3µl of the

first cocktail with 27µl of the second. Four more 10-fold serial dilutions of this mixture were also mixed in the same fashion. The 96 well plate was loaded with 25µl of mixture into each well as follows: the first experimental cocktail was aliquoted into wells A1 through H5. Each serial dilution was aliquoted into the appropriate well H6-H10, respectively. The second experimental cocktail was loaded into well H12 for the no template (NCT) control. Into well H11, 24µl of the second experimental cocktail was aliquoted as the NRT control. Lastly, 1µl of the 1:160 diluted RNA was added into well H11 as the NRT control. Optical thin-wall 8-cap strips were attached to the wells after plate loading was completed. The plate was then placed in the centrifuge plate carrier and spun for 3 minutes at 4°C at 1,000 RPM's to ensure proper mixing of reagents. After centrifuging, the plate was placed on the ICycler machine. The ICycler program designed for SuperArray's optimized primer sets was run for 40 cycles and the data was obtained using the ICycler program. The samples were then incubated at 2°C and the plates then saved in the 4°C fridge for possible future agarose gel runs of the samples.

RESULTS

Table 1. Real-Time PCR PAC-Induced Changes in Gene Expression in SEG-1 EAC Cells for Apoptosis Pathway Plate

**** Denotes unvalidated regulation data from microarray data**

Genes Up-Regulated (>2 fold)	*Fold-Change*
AKT1	1.23
BAK1	1.87
BCL2L1	2.14
BID	3.25
DFFA	2.00
MCL1	2.00
TNFRSF10A	3.03
TNFRSF25	14.93
TNFSF7	1.32
TP73	22.63
TRADD	12.13
CASP10**	2.46
AVEN**	
CIDEB**	
NOL3**	
Genes Down-Regulated (<2 fold)	*Fold-Change*
APAF1	1.41
BAG4	2.46
BIRC4	6.96
BNIP2	6.50
BNIP3L	3.25
CARD8	3.73
CASP3	8.00
CASP4	13.00
TNFRSF11B	8.57
TRAF2	1.07
BIRC6**	
FAS**	

Table 2. Real-Time PCR PAC-Induced Changes in Gene Expression in SEG-1 EAC Cells for Cell Cycle Pathway Plate

***All have been validated with microarray data**

Genes Up-Regulated (>2 fold)	Fold-Change
ANAPC2	2.38
DIRAS3	2.07
CDC34	1.11
DNM2	1.57
E2F4	1.11
Genes Down-Regulated (<2 fold)	***Fold-Change***
ANAPC4	4.76
ATM	2.73
BCCIP	15.45
BRCA2	1.11
CCNC	4.76
CCND1	2.55
CCNT2	1.93
CDC16	3.14
CDC2	5.10
CDC20	7.21
CDK8	19.03
CDKN3	2.55
CHEK1	23.43
CUL1	8.28
GTF2H1	2.93
KNTC1	2.55
MAD2L1	4.14
MKI67	57.68
MNAT1	7.21
MRE11A	6.73
RBBP8	8.88
RBL1	1.19
TFDP2	4.76
PCNA	3.61

Table 3. PAC Induced Gene Expression in SEG-1 EAC Cells by Gene Ontology Groups

****From KK EASE data**

Groups Up-Regulated	*Groups Down-Regulated*
cell growth and/or maintenance	response to DNA damage stimulus
biosynthesis	mitotic cell cycle
vesicle-mediated transport	response to endogenous stimulus
	DNA metabolism
	cell cycle
	DNA repair
	M phase
	nuclear division
	nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism
	DNA replication and chromosome cycle
	M phase of mitotic cell cycle
	Mitosis
	cell proliferation
	regulation of mitosis
	RNA splicing
	nuclear mRNA splicing\, via spliceosome
	RNA splicing\, via transesterification reactions with bulged adenosine as nucleophile
	RNA splicing\, via transesterification reactions
	DNA recombination
	RNA processing
	mitotic checkpoint
	G1/S transition of mitotic cell cycle
	RNA metabolism
	mRNA metabolism
	cell cycle checkpoint
	chromosome segregation
	mRNA processing

DISCUSSION

In this study, potential markers in apoptosis and cell cycle regulation pathways were studied to understand how PAC treatment affects these two key pathways. Through validation with real-time PCR, several gene markers were found to have an impact on inducing important gene expression changes in the SEG-1 EAC cells for both the apoptosis and cell cycle pathway plates.

BCL2-antagonist/killer 1 (BAK1) is expressed in many tissues, but is mainly found in its highest levels in heart and skeletal muscle. With the presence of a proper stimulus, BAK1 binds to the repressor BCL2 gene. BCL2 is an apoptosis regulator, and when BAK1 binds to it, it is antagonized. Programmed cell death is thus accelerated (20). Through its up-regulated nature induced by the PAC compound, BAK1's activity is enhanced and apoptosis occurs more frequently.

BH3 interacting domain death agonist (BID) is involved in inducing apoptotic activity and countering the anti-apoptotic effects of the BCL-2 family. While isoform 3 does not induce apoptosis, isoforms 1, 2, and 4, have all been found to promote apoptosis. Isoforms 2 and 3 are found to be expressed in the spleen, bone marrow, and the cerebral and cerebellar cortex. Isoform 2 is expressed in the spleen, pancreas, and placenta, and isoform 4 is expressed in the lung and pancreas. Expression of isoform 3 occurs in the lung, pancreas, and spleen (20). This study showed that PAC treatment up-regulated the activity of BID, thus increasing apoptosis.

Member 10A of the tumor necrosis factor receptor superfamily (TNFRSF10A) plays an important role in transducing the cell death signal, and thus inducing apoptosis. This gene is found to be highly expressed in the spleen, blood leukocytes, small intestine, thymus, and breast carcinoma cells. Studies suggest, however, that FADD, a death domain containing adaptor protein, may be needed to mediate apoptosis with the protein encoded by TNFRSF10A (20). PAC treatment of SEG-1 EAC cells was shown to induce up-regulation of TNFRSF10A, which subsequently increases apoptosis activation.

Tumor protein P73 (TP73) is found in a chromosome region that is frequently mutated in many cell lines of human cancers, but studies show that contrary to its mapping region, it is not mutated often. When TP73 is overproduced, it induces apoptosis. Some studies show that TP73

may also be a tumor suppressor protein. It is expressed in a wide variety of tissues, such as the brain, kidneys, placenta, colon, heart, liver, spleen, skeletal muscle, prostate, thymus, and pancreas (20). Up-regulation of TP73 was found to be induced by PAC treatment in this study, increasing the induction of apoptosis.

The protein encoded by the NFRSF1A-associated via death domain (TRADD) gene is a death domain that contains an adaptor molecule that interacts with TNFRSF1A/TNFR1, which mediates cell death signaling. TRADD provides an important shuttling system between the nucleus and the cytoplasm. TRADD binds to TRAF2, an apoptosis inhibitor, and suppresses the anti-apoptotic activity of TRAF2. TRADD induces apoptosis through many different mechanisms, from both the nucleus and the cytoplasm. It is found to be expressed in all examined tissues (20). PAC treatment was found to induce up-regulation of TRADD, increasing the amount of apoptosis.

Caspase 3 (CASP3), an apoptosis-related cysteine protease, is involved in the activation cascade of caspases involved in apoptosis, and this sequential activation of caspases has a central role in executing cell apoptosis. It is found in its highest expression levels in cells of the immune system, but also in high levels in the lung, spleen, heart, liver, and kidney. Moderate expression levels are found in the brain and skeletal muscle, and its lowest expression levels are found in the testis. It is also found to be expressed in many cell lines (20). Because of its central role in apoptosis execution, it would be beneficial for CASP3 activity to be upregulated; however, it was found that PAC induced down-regulation of this gene and other members of its family.

Member 8 of the caspase recruitment domain family (CARD8) and the rest of its family of proteins are found to be involved in pathways that activate caspases. The activation of caspases plays an important role in executing cell apoptosis. CARD8 is found to be highly

expressed in the lung, ovary, testis, and placenta. It has lower expression levels in the heart, kidney, and liver, and it is also expressed in the spleen, lymph nodes, and bone marrow (20). CARD8, another important player in activating apoptosis, was found to be down-regulated by the PAC treatment.

V-akt murine thymoma viral oncogene homolog 1 (AKT1) is a general protein kinase that can phosphorylate many various proteins. It has a wide variety of roles, from mediating glucose transport and antiapoptotic effects, to promoting glycogen synthesis. It has been found in all human cell types that have been thus far analyzed, and is thus not tissue specific. By activating the serine/threonine kinase in AKT1, apoptosis can be suppressed through the phosphorylation and subsequent inactivation of apoptotic pathway components (20). AKT1 was found to be up-regulated through PAC treatment.

Anaphase promoting complex subunit 2 (ANAPC2) controls the progression of the cell cycle pathway through mitosis and the G1 phase. It promotes the metaphase to anaphase transition during the process of mitosis (20). Its activity was found to be up-regulated through PAC treatment.

Cell division cycle 34 (CDC34) is important in catalyzing the covalent attachment of ubiquitin to other proteins. The protein encoded by this gene is part of a large multiprotein complex that is used in the degredation of cell cycle G1 regulators. It is also used in the initiation of DNA replication (20). CDC34 expression was up-regulated with PAC treatment.

Dynamin 2 (DNM2) encodes a protein that is involved in making microtubule bundles, and it is capable of binding and hydrolyzing GTP. The family of dynamin proteins is associated with microtubles and is involved in vesicular transporting processes such as endocytosis and cell

motility (20). In this study, PAC treatment up-regulated the expression of DNM2 in the cell cycle pathway plate.

E2F transcription factor 4 (E2F4) is present in the growth-arrested state of the cell cycle. The E2F family of transcription factors has an important role in controlling the cell cycle pathway and in controlling the activity of tumor suppressor proteins. It has also been found to assist in the suppression of proliferation-associated genes. The complex it forms with DRTF1 has been found to control the cell-cycle progression from the G1 to S phase of mitosis. Studies show that increased expression of members of this family may be linked with human cancer. It is found in a wide variety of tissues, such as the heart, brain, placenta, lungs, liver, skeletal muscle, kidneys, and the pancreas (20). The expression of E2F4 was up-regulated through PAC treatment.

Anaphase promoting complex subunit 4 (ANAPC4) is a large protein complex that supports the metaphase-anaphase transition during mitosis. The precise function of the gene product of ANAPC4 is not yet known; however, it is known that is needed for ubiquination conjugation (20). PAC treatment induced down-regulation of ANAPC4.

Ataxia telangiectasia mutated (ATM) plays a role in signal transduction, cell cycle control, and DNA repair. It may also play a role in tumor suppression. Along with similar kinase ATR, ATM functions as an important cell cycle checkpoint in the signaling pathways for a cell's response to DNA damage (20). Through PAC treatment, the expression of ATM was down-regulated.

CDK inhibitor p21 binding protein (BCCIP) is known to interact with BRCA2 and p21 proteins. It may play an important role as a cofactor for BRCA2 in tumor suppression. Over-

expression of BCCIP has been found to delay the progression of G1 to S phase in mitosis, and it also elevates p21 expression (20). BCCIP's activity was down-regulated with PAC treatment.

Cyclin C (CCNC) is a member of the cyclin family and is found in its highest expression levels in the pancreas. It is also expressed at high levels in the heart, liver, skeletal muscle, and the kidney, and at low levels in the brain. CCNC may be an important factor in regulating transcription, as it binds to and activates kinase CDK8, which phosphorylates the large subunit of RNA polymerase II. The highest levels of mRNAs for this gene are found in the G1 phase of the cell cycle pathway (20). The expression of CCNIC was down regulated with PAC treatment.

Cyclin D1 (CCND1) plays an important role in controlling the cell cycle at the G1/S (start) transition. It also interacts with the tumor suppression protein Rb. In many different kinds of tumors, it has been found that mutations, amplification, or over expression of CCND1 are present. These aberrations have a strong impact in altering cell cycle progression (20). CCND1 expression was down regulated through PAC treatment.

CHK1 checkpoint homolog (s. pombe) (CHEK1) has an important role in the checkpoint mediated cell cycle arrest after the presence of DNA damage or unreplicated DNA is found. It also may negatively regulate cell cycle progression during undisturbed cell cycles. CHEK1 is found in its highest expression levels in the thymus, testis, small intestine, and the colon (20). PAC treatment induced down regulation of this gene.

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