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Metabolic effects of cedar tar on colon cancer cell line of HCT-116: A follow-up study

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Abstract

Aim: Colon cancer is the most common cancer worldwide. Surgery and chemotherapy are widely used in treatment. Recently, the therapeutic properties of natural products, including *Cedrus libani*, have been investigated for drug development. *Cedrus libani* extract (cedar tar) has antimicrobial and anticancer properties that can mediate metabolic pathways. Metabolic changes have been identified as the hallmark of cancer, and therefore research has focused on metabolic biology and drugs in basic and clinical studies. This study aims to investigate the effects of cedar tar on the amino acid metabolism in the colon cancer cell line of HCT-116.

Materials and Methods: HCT-116 was homogenized in the phosphate buffered saline medium after being incubated with 30 µg/mL of cedar tar for 24 hours. The intracellular free amino acid profile in the samples was analyzed by liquid chromatography-mass spectrometry/mass spectrometry. Statistical data analysis was performed with SPSS V22 and MetaboAnalyst 5.0 programs.

Results: We uncovered with cedar tar treatment that apoptotic β-alanine and anserine levels increased, $p<0.05$, and pro-tumorigenic O-phosphorylethanolamine level decreased, $p<0.05$.

Conclusion: Cedar tar may be speculated to suppress the metastatic character and inhibits energy metabolism by increasing antiproliferative β-alanine and anserine amino acids and diminishing pro-tumorigenic O-phosphorylethanolamine amino acid in colon cancer cells. However, future interventional studies mediating β-alanine, anserine and O-phosphorylethanolamine amino acid levels in cancer and wild-type cells are required to conclude those as biomarkers or therapy candidates.

Introduction

According to global statistics, colon cancer (CC) is the third most common cancer worldwide, and around 2 million new patients were reported in 2018 [1]. According to statistics, approximately 2.5 million new cases will be diagnosed by 2030, and half of this number will die from CC [2]. Genetic and sporadic factors are essential triggers for the onset of CC [3]. The average life expectancy in the CC is less than five years. The CC treatment includes chemotherapy, radiotherapy, surgery, immunotherapy, and targeted therapy based on the cancer stage and patient prognosis. Oxaliplatin, 5-Fluorouracil (5-FU), capecitabine, trifluridine/tipiracil, and irinotecan are commonly used alone or in combination [4]. However, long-term use of those can reduce the patient's quality of life and cause drug resistance [5]. Over the years, many studies have been conducted to develop natural products containing multiple molecules with apoptotic effects [6]. For example, Vinca alkaloids of *Catharanthus roseus* [7], podophyllotoxin of *Podophyllum peltatum* [8], camptothecin of *Camptotheca acuminata* [9], and taxol of *Taxus brevifolia* [10] have been reviewed in primary and secondary cancer treatment research.

There are four different Cedrus species (Pinaceae) worldwide, *Cedrus libani* in Lebanon, Syria, and Turkey, *Cedrus atlantica* in Morocco and Algeria, *Cedrus brevifolia* in Cyprus, and *Cedrus deodara* in the Himalayan Mountains [11]. Cedrus species have traditionally been used in aromatherapy for clinical benefits on the musculoskele-

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tal, skin, and genitourinary [12]. Endemic in Syria and Turkey (Taurus Mountains), *Cedrus libani* tar (cedar tar) is considered to be the main forest species used for timber production; and Cedrus shavings are refined by hydro distillation to extract essential oils [13] with antimicrobial [14], anticancer [15] and analgesic effects [16]. We demonstrated the apoptotic effects of Cedar tar on colon cancer cells by molecular and biochemical examinations [15]. This follow-up study aims to enlighten cedar tar's effects on the cell amino acid profile to mechanize the published apoptotic effects of cedar tar. Cancer cells rearrange metabolic pathways to survive during the cancerous process [17, 18]. Therefore, identifying altered metabolites in these pathways is believed to promote early diagnosis research. With this ground, this study aims to create literature for future biology or treatment studies by investigating the changes in the amino acid profile. The effect of cedar tar on the amino acid profile of the HCT-116 cell line was investigated by LC-MS/MS. We explored that cedar tar extract increased apoptotic β-alanine and anserine amino acids and decreased tumorigenic O-Phosphorylethanolamine amino acids.

Materials and Methods

Cell preparation for cell culture and amino acid profile

HCT-116 cells, obtained from the American Type Culture Collection (ATCC) and stocked under appropriate conditions, were used in the study. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM-F12) (Sigma-Aldrich Cat No: D9785, USA) media, 10% Fetal bovine serum (FBS) (Sigma-Aldrich Cat No: F7524, USA) and 1% L-glutamine (Sigma-Aldrich Cat No: 59202C, USA), 1% penicillin/streptomycin (Sigma-Aldrich Cat No: P4333, USA) were used for growing the cells. Cells were taken into 25 cm^2 flasks and incubated at 37 C, 5% CO_2, and 95% humidity, and after filling 80% of the flask, the cells were seeded into experimental dishes of 6 cm^2 plates. Then, we administrated a 30 g/mL dose of cedar tar and examined the intracellular free amino acid profile after incubating for 24 hours. The administrated dose here was calculated in our previous study [15]. After the incubation, the cells were washed two times with cold 1x phosphate buffered saline (PBS) (Sigma-Aldrich Cat No: D8537, USA) to remove the remaining medium. Next, the cells were scraped off the plates to prepare a cell suspension in 1 ml of PBS. The cell suspension was centrifuged at 1200 rpm for 5 minutes, and the supernatant was removed. After washing the pellet with PBS, we lysed it with a homogenizer (Qiagen, TissueLyser LT, Germany) at +4 °C for 10 minutes. The lysate was centrifuged at 14,000 rpm, and the supernatant was used for amino acid profiling in LC-MS/MS.

Investigation of free amino acid profile by LC-MS/MS

Intracellular free amino acid analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Shimadzu-8045) according to the kit protocol (Bome Trivitron, Trimaris-BR130030, Turkey). We used a derivative method in the analysis based on the kit protocol. 50 µL of sample is mixed with an internal standard solution consisting of 20 amino acids with C13 and N15 labeled atoms prepared in 0.1 M HCl. Essential organic buffer components were added for pH balance. A chloroform/isooctane mixture containing 5% alkyl chloroformate as an active ingredient was added to the sample mixture and incubated at room temperature for 3 minutes. Derived amino acids were removed by centrifugation from the supernatant containing organic solvents. The signal of the MS device is increased in parallel with the increased molecular weight of esterified amino acids. Trimaris Amino Acid LC-MS/MS column (250mm x 2mm, 3µM) with C18 phase filler was used for the chromatographic separation, and multiple reaction monitoring (MRM) mode was preferred to analyze.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences V22.0 (IBM SPSS Inc., Chicago, IL, USA). The results are given as mean ± standard deviation (Sd). Shapiro-Wilk test was used to assess whether there is a difference between data and Gaussian distributions. The means of two independent groups were compared with Student's t-test. MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to compare the amino acid levels. Clustering was determined by Principal Component Analysis (PCA). The amino acids that contribute most to the clustering were defined by variable importance in projection (VIP) analysis. *$p<0.05$ was considered significant.

Results

Effects of cedar tar on the free amino acid profile

The results of the amino acid profile (41 amino acids) are summarized in Table 1. Accordingly, cedar tar increased apoptotic β-alanine and anserine levels and decreased tumorigenic O-Phosphorylethanolamine levels in colon cancer cells (*$p<0.05$).

Separation of groups by principal component analysis

Principal component analysis (PCA) is a technique that reduces the dimensions of a dataset to increase interpretability and minimizes information loss. In addition, this technique creates new uncorrelated variables to maximize variance. Finding such new variables solves the eigenvalue/eigenvector problem in the data, which makes PCA an adaptive data analysis technique [19]. We performed

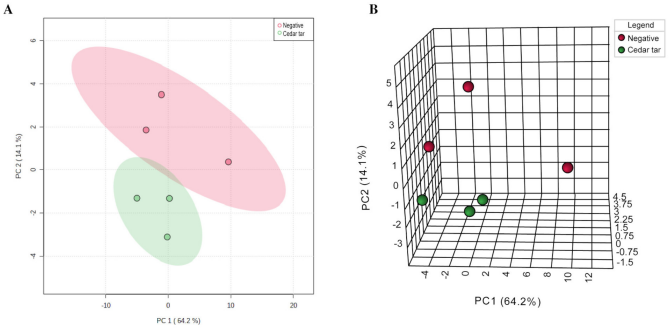

Figure 1. A: Amino acid profile of HCT-116 cells and control groups. B: Principal component analysis graphs.

Table 1. Quantitative analysis of intracellular amino acids between groups.

Amino acid (µmol/L)	Abbreviations	Negative	Cedar tar (30 g/mL)	p value
1-Methylhistidine	1-MHis	4.41±1.71	7.77±3.60	0.219
2-Aminoadipic Acid	2-Aaa	2.00±0.96	1.44±1.05	0.532
3-Aminoisobutyric Acid	3-Aiba	6.22±1.96	6.97±1.99	0.664
3-Methylhistidine	3-MHis	1.87±0.59	1.95±0.45	0.871
4-Hydroxyproline	4-Hyp	10.37±3.70	12.95±5.65	0.545
5-Hydroxylysine	5-Hyl	0.45±0.21	0.37±0.05	0.546
Alanine	Ala	417.28±282.38	185.56±15.81	0.291
Alloisoleucine	Allo-Ile	117.54±73.89	89.84±33.05	0.585
Anserine	Ans	0.05±0.04	0.20±0.06	0.028
Arginine	Arg	739.52±366.33	529.95±175.66	0.422
Argininosuccinic Acid	ASA	30.19±22.28	23.49±5.81	0.641
Asparagine	Asn	139.02±50.70	88.01±38.56	0.238
β-Alanine	β-Ala	6.14±2.58	17.61±1.43	0.003
Carnosine	Car	5.50±3.70	5.19±2.14	0.998
Citrulline	Cit	16.56±10.52	12.85±5.71	0.620
Cystine	Cys	42.08±13.54	35.67±3.38	0.471
Cystathionine	Cyt	1.00±0.81	1.06±0.50	0.918
Ethanolamine	Eta	1.26±0.46	1.12±0.53	0.319
Gamma-Aminobutyric Acid	GABA	6.77±1.84	7.43±0.15	0.569
Glutamine	Gln	1824.62±807.05	1643.82±349.12	0.740
Glutamic Acid	Glu	383.03±74.73	351.60±1.80	0.507
Histidine	His	122.14±31.76	123.51±45.47	0.968
Homocitrulline	HCit	4.87±2.25	2.62±1.06	0.192
Isoleucine	Ile	542.99±331.04	418.34±157.37	0.587
Leucine	Leu	666.36±360.36	472.47±157.45	0.441
Lysine	Lys	620.03±361.47	419.97±139.24	0.422
Methionine	Met	129.64±66.51	96.81±28.18	0.475
Norvaline	Nva	13.69±6.74	8.99±2.98	0.331
O-Phosphorylethanolamine	O-PEa	50.05±11.28	22.67±5.21	0.019
O-Phosphoserine	O-PSer	0.67±0.16	1.10±0.77	0.398
Ornithine	Orn	87.81±34.67	71.22±15.21	0.490
Phenylanalanine	Phe	313.13±154.95	214.61±66.71	0.369
Proline	Pro	276.85±147.59	196.50±136.88	0.527
Sarcosine	Sar	230.67±111.93	106.67±45.33	0.150
Serine	Ser	381.72±168.69	305.32±73.03	0.511
Taurine	Tau	110.36±85.11	67.90±53.27	0.504
Threonine	Thr	972.35±659.54	685.38±281.80	0.526
Trans-4-Hydroxyproline	T-4-Hyp	25.26±13.50	25.04±8.75	0.982
Tryptophan	Trp	48.30±26.04	28.92±8.74	0.289
Tyrosine	Tyr	319.76±164.96	202.17±49.66	0.303
Valine	Val	630.91±383.65	438.23±141.27	0.460

the PCA to visualize the separation of amino acid clusters in the cedar tar-treated HCT-116 cells, illustrated in Figure 1A-B. Principal component 1 (PC1) and principal component 2 (PC2) accounted for 64.2% and 14.1% of the variance in the data, respectively. PCA analysis showed significant clustering and separation in amino acid profile between HCT-116 cells treated with cedar tar and control groups.

Screening of metabolites

Variable importance in projection (VIP) analysis was performed to determine which amino acids changed significantly in HCT-116 and control cells (Figure 2). The concept in this analysis is that the higher the VIP score (>1), the higher the probability of leaving. We determined that the first three amino acids with the highest VIP score were β-alanine, anserine, and O-Phosphorylethanolamine. The increases in these three amino acid scores indicate that the contribution of those to the separation between groups is worth considering.

Discussion

Many studies have aimed to elucidate the mechanisms by which natural products affect cancer cells and to determine the changing parameters, and therefore those have focused on both elucidating the effects of natural products and identifying biomarkers that may be used in early diagnosis. Amino acids facilitate the survival and proliferation of cancer cells under genotoxic, oxidative, and nutritional stress. Therefore, targeting amino acid metabolism seems to be a

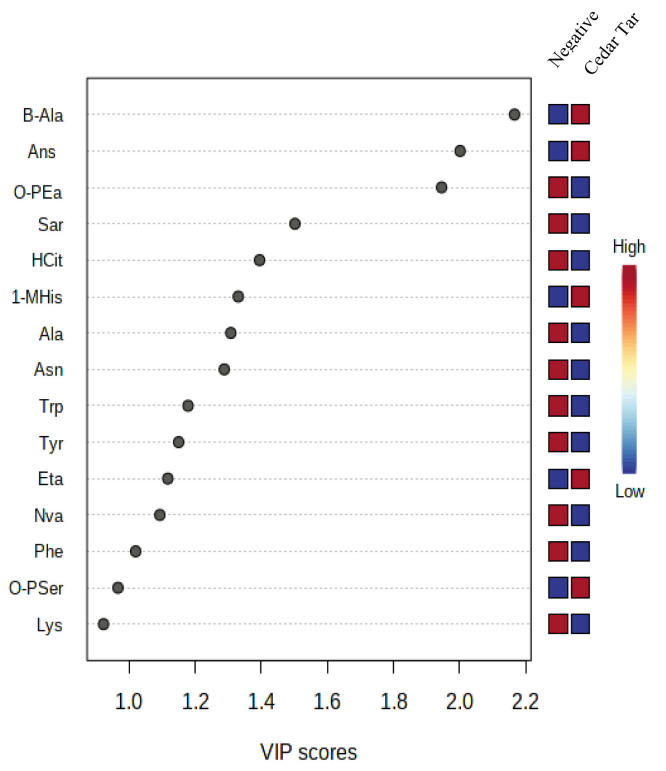

Figure 2. VIP chart listing amino acids based on their importance (Red: High level; Blue: Low level).

potential therapeutic strategy for cancer patients [20]. In addition, amino acids have been suggested as markers for early cancer diagnosis [21]. An ideal cancer biomarker is characterized by specificity, sensitivity, and reliability [21, 22]. Amino acids are essential molecules mediating epigenetic regulation, post-transcriptional modification, redox balance, intracellular energy regulation, biosynthetic support, and homeostatic balance [23]. Increased biosynthetic activities are characteristic of metabolic reprogramming [20, 24]. However, some amino acid metabolites may contribute to tumorigenic and anti-tumorigenic activities. A product of arginine metabolism, nitric oxide (NO) promotes, for example, angiogenesis and tumorigenesis and acts as a tumor suppressor by upregulating tumor protein (TP53) [23]. Various amino acid functions have provided researchers the multiple targets for developing drugs [25, 26]. Determining amino acids and metabolite changes by natural products paved the way for researchers to design drugs [27]. Our previous study showed cedar tar's molecular and biochemical effects on colon cancer cells [15]. In addition to testing the relationship between cell death pathways and cedar tar, we also investigated, in this follow-up study, cedar tar extract' impact on the amino acids profile to mechanize the observed apoptotic findings on colon cancer cells.

In this context, the effect of cedar tar on the amino acid profile was investigated by LC-MS/MS, and the recorded apoptotic effects of the extract [15] were tested to have therapeutic value in terms of amino acid profile by principal component analysis. Forty-one amino acids were screened, and potential biomarkers and drug targets were identified. Fluctuated amino acids were summarized in detail by comparing their cellular functions with the literature. Accordingly, β-alanine plays a role in the metabolic pathways of cell migration and proliferation [28, 29]. A study has evidenced that β-alanine regulates cytoplasmic acidity [30] and has antitumor effects, including suppressing cell migration and triggering apoptotic effect [31, 32]. Abatement of β-alanine amino acid causes an increase in metastatic behavior in gastric cancer; on the contrary, depressing it prevents recurrence and increases the overall survival time [33, 34]. Our study found that the β-alanine amino acid level increased in colon cancer cells treated with cedar tar compared to untreated cells (*$p<0.05$), which is consistent with our previous study [15].

Metabolic reprogramming of energy metabolisms by cancer cells is essential for continuous proliferation and increased survival. Therefore, the cancerous cells adapt to rearrange the energy metabolism when exposed to nutrient starvation and hypoxia. Glutamine is firstly used as the glucose precursor in glucose deprivation, and in this way, the cells ensure energy production under catastrophic conditions. Studies revealed that pro-tumorigenic metabolite O-Phosphorylethanolamine accumulation diminished proliferation, development, and survival in cancer cells [35-37]; however, we found that O-Phosphorylethanolamine amino acid level decreased with cedar tar administration (*$p<0.05$). The anserine amino acid mediates H+ ion buffering, antioxidation, muscle contractility modulation, and metabolism regulation [38]. In vivo neurodegenerative models have shown that anserine benefits neurodegeneration and disease-related behaviors such as memory loss [39]. In addition, an in vitro study suggested that anserine amino acid inhibits tumor formation and promotes apoptosis of tumor cells [40], which is consistent with our study presenting that the anserine amino acid level increased in colon cancer cells after being treated with cedar tar. Cedar tar extract may be suggested as an anti-tumorigenic candidate for the HCT-116 line. However, commercial cell lines' phenotype and genotype are often different from those of primary cells [41-43]. Therefore, inferencing based on the commercial cell line data may be open to speculation. Also, this study provides the first line of evidence; in other words, we present observational data which needs to be corroborated with loss-of-function approaches using manipulation strategies such as miRNA to regulate levels of amino acids.

Conclusion

Ethics approval

This study did not use any data related to human or animal subjects. All studies were conducted using cell culture samples.

Author contributions

Concept (ET., KE., IK., OY., YK., MT., SA., ME., YA.); Design (ET., KE., IK.,OY., YK., MT., SA., ME., YA.); Supervision (ET., IK.); Fundings (ET., IK.); Materials (ET., KE., IK., YK.); Data Collection and Processing (ET., KE., IK., OY., MT., SA., ME., YA.); Analysis and Interpretation (ET., KE., IK., OY., MT., SA., ME., YA.); Literature Review (ET., IK.); Writing (ET., IK., YA.); Critical Review (ET.).

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