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Gene expression changes in colon tissues from colorectal cancer patients following the intake of an ellagitannin-containing pomegranate extract: a randomized clinical trial

María A. Nuñez-Sánchez[a], Antonio González-Sarrías[a], Rocío García-Villalba[a], Tamara Monedero-Saiz[a,b], Noelia V. García-Talavera[b], María B. Gómez-Sánchez[b], Carmen Sánchez-Álvarez[b], Ana M. García-Albert[c], Francisco J. Rodríguez-Gil[c], Miguel Ruiz-Marín[d], Francisco A. Pastor-Quirante[e], Francisco Martínez-Díaz[e], Francisco A. Tomás-Barberán[a], Juan Carlos Espín[a,*], María-Teresa García-Conesa[a,*]

[a]*Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, 30100 Campus de Espinardo, Murcia, Spain*
[b]*Nutrition, Hospital Reina Sofía, Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain*
[c]*Digestive, Hospital Reina Sofía, Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain*
[d]*Surgery, Hospital Reina Sofía, Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain*
[e]*Anatomical Pathology Services, Hospital Reina Sofía, Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain*

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Abstract

The clinical evidence of dietary polyphenols as colorectal cancer (CRC) chemopreventive compounds is very weak. Verification in humans of tissue-specific molecular regulation by the intake of polyphenols requires complex clinical trials that allow for the procurement of sufficient pre- and postsupplementation tissue samples. Ellagitannins (ETs), ellagic acid (EA) and their gut microbiota-derived metabolites, the urolithins, modify gene expression in colon normal and cancer cultured cells. We conducted here the first clinical trial with 35 CRC patients daily supplemented with 900 mg of an ET-containing pomegranate extract (PE) and evaluated the expression of various CRC-related genes in normal and cancerous colon tissues before (biopsies) and after (surgical specimens) 5–35 days of supplementation. Tissues were also obtained from 10 control patients (no supplementation) that confirmed a large, gene- and tissue-specific interindividual variability and impact of the experimental protocol on gene expression, with some genes induced (*MYC, CD44, CDKN1A, CTNNB1*), some repressed (*CASP3*) and others not affected (*KRAS*). Despite these issues, the consumption of the PE was significantly associated with a counterbalance effect in the expression of *CD44*, *CTNNB1*, *CDKN1A*, *EGFR* and *TYMs*, suggesting that the intake of this PE modulated the impact of the protocol on gene expression in a gene- and tissue-specific manner. These effects were not associated with the individuals' capacity to produce specific urolithins (*i.e.*, metabotypes) or the levels of urolithins and EA in the colon tissues and did not reproduce *in vitro* effects evidencing the difficulty of demonstrating *in vivo* the *in vitro* results.

Keywords: Clinical trial; Gene expression; Colon tissues; Ellagitannins; Interindividual variability; Urolithins; Pomegranate extract

1. Introduction

Numerous preclinical *in vitro* and animal studies have given evidence of the regulation of cell processes by polyphenols *via* (epi)genetic changes as a general mechanism of action underlying the health benefits attributed to these compounds [1]. Demonstration in humans of these changes remains essential to better comprehend the link between polyphenols intake and health effects, but, in this

Abbreviations: ABCG2, ATP-binding cassette subfamily G member 2 gene; *APC*, adenomatous polyposis coli gene; *BIRC5*, baculoviral inhibitor of apoptosis repeat-containing 5 gene; *CASP3*, apoptosis-related cysteine peptidase (caspase 3) gene; *CDKN1A*, cyclin-dependent kinase inhibitor 1A gene (p21, Cip1); CRC, colorectal cancer; *CD44*, cluster of differentiation 44 gene (CD44 molecule, Indian blood group); *CTNNB1*, β-catenin gene; EA, ellagic acid; *EGFR*, epidermal growth factor receptor gene; ETs, ellagitannins; FC, fold-change; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase gene; *GUSB*, β-glucuronidase gene; *HPRT1*, hypoxanthine phosphoribosyltransferase 1 gene; IsoUro-A, isourolithin A; *KRAS*, GTPase V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *M*, gene stability measure; Mb, malignant biopsies; MiR, microRNA; Ms, malignant surgery specimens; *MYC*, V-myc avian myelocytomatosis viral oncogene homolog; Nb, normal biopsies; Ns, normal surgery specimens; PE, pomegranate extract; RT-PCR, real-time polymerase chain reaction; *TP53*, tumor protein p53 gene; *TYMS*, thymidylate synthase gene; Uro-A, -B, -C, -D, -M6, urolithins A, B, C, D and M6, respectively; *18S*, eukaryotic 18S rRNA gene

* Corresponding authors at: Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, 30100 Campus de Espinardo, Murcia, Spain. Tel.: +34 968 396276; fax: +34 968 396213.
E-mail addresses: jcespin@cebas.csic.es (J.C. Espín), mtconesa@cebas.csic.es (M.-T. García-Conesa).

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area, clinical trials are limited and show weak evidence of the effects of these compounds on molecular expression in human tissues. Various reasons are responsible for this: (a) the high interindividual variability in the human capacity to metabolize and absorb the compounds as well as in the ability to respond to these compounds and (or) their metabolites [2] and (b) difficulties in obtaining sufficient number of participants and high-quality samples.

Ellagitannins (ETs) and ellagic acid (EA) are phenolic compounds abundant in foods such as pomegranate, walnuts or berries. The metabolic conversion of these compounds by the gut microbiota to form some intermediate urolithins (Uro) (Uro-M5, Uro-M6,Uro-M7, Uro-D, Uro-C) and the main final metabolites Uro-A, IsoUro-A and Uro-B in animals and humans has been thoroughly reported [3–6]. Considerably high concentrations of these urolithins can be found in the feces (mM range) and in the colon tissues (μM range) of volunteers that consumed pomegranate extracts (PEs) and/or walnuts [7,8]. It is also known now that the capacity of producing urolithins varies between individuals depending on their microbiota composition, and thus, humans can be classified into different metabolizing phenotypes (metabotypes): (a) metabotype A that produces Uro-A, (b) metabotype B that produces IsoUro-A and/or Uro-B in addition to Uro-A and (c) metabotype 0 or nonproducers [7,9]. A recent and interesting appreciation is that the distribution of these metabotypes appears to be related to the health status. Along these lines, the urolithin metabotype B has been reported to be more abundant in persons with gut dysbiosis such as obese subjects, individuals with metabolic syndrome or colorectal cancer (CRC) patients [8,9].

Urolithins, the main colon microbial metabolites derived from the polyphenols ETs, have gained a lot of interest for their potential health benefits. Among these, the potentiality of these molecules to reduce or prevent CRC development has been repeatedly evidenced in cultured colon cancer cells [10]. Each of the main urolithins, EA and mixtures of these metabolites (reproducing the concentrations found in the colon tissues from individuals with metabotypes A or B) is able to inhibit the growth of these cells and interfere with cell cycle and apoptotic death mechanisms. These phenotypic responses are accompanied by substantial molecular changes in several key cancer deregulated genes (*e.g.*, *CDKN1A*, *MYC*, *TP53*) and microRNAs (miRs; miR-224, miR-215) [11,12]. Also, there were marked differences in the response between normal and cancerous cells, changes commonly observed in all the cells investigated (*e.g.*, induction of *CDKN1A*) with independence of the metabolite tested, and metabolite- and cell-specific changes [12]. These *in vitro* results need to be verified in humans, and thus, clinical studies with appropriate designs to investigate tissue molecular changes must be implemented [13]. We conducted the first clinical trial in CRC patients where we provided evidence of the *in vivo* availability and concentrations of urolithins in the colon following a short-term supplementation with ET-containing PEs [8] and also reported some specific and significant changes in various miRs in the human colon normal and malignant tissues after the intake of the PE [14].

Using the same colon samples obtained from those CRC patients, the aim of the present research was to test whether it was also possible to detect significant differential expression for various specific genes in colon human tissues in response to the supplementation with a PE. The selection of these genes was based on: (a) genes that are key hallmarks for cancer development (*e.g.*, *APC*, *CTNNB1*, *KRAS*, *EGFR*, *TP53*, *MYC*, *BIRC5*) [15] and (b) previous *in vitro* studies have shown changes in these genes in colon normal and cancer cultured cells exposed to the urolithins and EA (*e.g.*, *CDKN1A*, *ABCG2*, *CASP3*) [12]. The partial objectives of this research were (a) to examine the interindividual variability in the expression levels of the selected molecular markers in the human normal and cancerous colon tissues, (b) to evaluate the impact of the experimental protocol on the expression levels of these markers and (c) to try to discriminate specific changes in gene expression in the human colon tissues that might be attributed to the consumption of the PE and (or) the presence of urolithins in the colon tissues.

2. Materials and methods

2.1. Pomegranate extracts

PEs (PE-1 and PE-2) were kindly supplied by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). The composition of PE-1 and PE-2 has been previously reported in detail [8] and differs primarily in their ET (punicalagin)/EA ratio: (a) PE-1 (low ET/EA ratio) contains 72 mg/g punicalagin, 2 mg/g punicalin and 294 mg/g EA derivatives, and (b) PE-2 (high ET/EA ratio) contains 155 mg/g punicalagin, 5.4 mg/g punicalin and 28 mg/g EA derivatives.

2.2. Study design and intervention

This randomized controlled trial (Clinicaltrials.gov, NCT01916239, 30 July 2013) was designed to investigate the potential effects of a supplementation with ET-containing PE in the expression of several genes in the colon tissues obtained from CRC patients. The study was approved by the Spanish National Research Council's Bioethics Committee (Madrid, Spain) and by the Clinical Ethics Committee at Reina Sofia University Hospital (Murcia, Spain) (reference 03/2011) and conducted in accordance with the guidelines in the Declaration of Helsinki and its amendments. Details of the trial can be found in previous reports [8,14]. Briefly, eligible patients with programmed colonoscopy (n=2501) gave their written informed consent prior to participating. Inclusion criteria were as follows: age over 18 years and confirmed CRC diagnosis with resectable tumor by programmed surgery, World Health Organization performance status between 0 and 3, alanine aminotransferase >2.5-fold, hemoglobin >10 g/dl, creatinine <140 mM and serum bilirubin >1.5-fold above the normal values range. Exclusion criteria were as follows: patients that did not satisfy the inclusion criteria and (or), treatment with chemotherapy or radiotherapy 1 month prior to recruitment, alcoholism, active peptic ulcer, pregnancy/breastfeeding, habitual intake of food supplements and treatment with steroids or other anti-inflammatory drugs 1 week prior to recruitment.

Fifty-seven patients were recruited and several initial (baseline) endoscopic colon biopsies taken: two samples from normal tissue (normal biopsy, Nb) and four samples from malignant tissues (malignant biopsy, Mb). The samples were rapidly stabilized in RNAlater reagent (Qiagen, Madrid, Spain) and stored at −80°C until use. Patients were then randomly allocated into three groups: a control group (n=14) that did not consume any PE and two groups that consumed two capsules (2×450=900 mg) daily of the PE-1 (n=22) or the PE-2 (n=21). The supplementation period lasted from the initial colonoscopy until the programmed surgery and was on average 13.6±7.5 days (range: 5–35 days). The last dose was consumed approximately 10–12 h before surgery. It is important to note that, during the supplementation period, none of the participants was taking any anticancer drug or treatment. At surgery, normal (Ns, ~10 cm adjacent to the tumor) and malignant (Ms) tissues were taken approximately 2 h after initiating the surgical resection. These colon tissue specimens were rapidly taken to the Anatomical Pathology Service. Portions of both types of tissue were cut out, immersed in RNAlater stabilization reagent and stored at −80°C until use. A total of 45 patients completed the trial: control group (n=10), PE-1 group (n=19) and PE-2 (n=16).

2.3. RNA extraction and cDNA preparation

All colon tissue samples (0.02 g obtained from a pool of different pieces of each sample) were homogenized with lysis buffer (Qiagen) using an IKA T10 Ultra-Turrax equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24,000 rpm at 4 C for 1 minute. Total RNA extraction and quality assessment were performed as reported elsewhere [14]. Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 and RNA Integrity Number values above 6.0 that indicate acceptable RNA integrity for reverse transcriptase polymerase chain reaction (RT-PCR) assays [16] were used for gene expression analysis. cDNA was obtained from RNA following reverse transcription according to the manufacturer's instructions (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, CA, USA) and quantified using the Nanodrop spectrophotometer system (ND-1000 3.3 Nanodrop Technologies, USA).

2.4. Gene expression analyses: RT-PCR arrays

Gene expression levels were evaluated using TaqMan Array 96-Well Plates (Applied Biosystems, ABI, CA, USA) designed to analyze 12 genes. A total quantity of 100 ng of cDNA obtained from each treatment was added to each well of the TaqMan Array Plate together with the standard master mix (Applied Biosystems, CA, USA) to a final volume of 20 μl, and the quantitative PCRs were run on the 7500 RT-PCR System (Applied Biosystems) following manufacturer's conditions. These plates contained gene-specific primers (ABI) for four generally accepted reference genes: *18S* (Eukaryotic 18S rRNA, Hs99999901_s1), *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase, Hs99999905_m1), *HPRT1* (Hypoxanthine phosphoribosyltransferase 1, Hs99999909_m1) and *GUSB* (Glucuronidase, beta, Hs99999908_m1). *HPRT1*, *GUSB* and *GAPDH* were considered suitable reference genes for gene expression normalization in tumor and normal tissue from colon cancer patients based on geNorm and/or