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原 著

A pilot study examining the relationship between environmental contaminants and colorectal cancer at a single medical institute in Taiwan

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Objectives: Colorectal cancer (CRC) is a major health problem with high incidence and mortality rates worldwide. The increased incidence of CRC in Taiwan may be associated with environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), nitrate, and nitrite. Accordingly, in this study, the relationship between these contaminants and CRC was investigated. **Methods:** Paired plasma and tumor tissues of CRC patients were obtained from the Tissue Bank at Kaohsiung Chang Gung Memorial Hospital. The samples were extracted and analyzed using enzyme-linked immunosorbent assay and gas chromatography-tandem mass spectrometry for detecting the concentrations of nitrate/nitrite and 16 types of PAHs, respectively, at the Super Micro Mass Research & Technology Center, Cheng Shiu University. **Results:** Nitrite and the 16 types of PAHs were undetectable in the plasma; thus, only nitrate was selected for further investigation. The data indicated that the plasma levels of nitrate were not significantly different between the CRC and control groups. Notably, the average levels of nitrate in tumor tissues were significantly higher than the average plasma levels, but the nitrate levels in paired plasma and tumor tissues did not show a significant correlation. Moreover, no significant difference in the nitrate levels of plasma and tumor tissues was found in patients with different tumor stages. **Conclusions:** In this pilot study, no significant correlation was found between the nitrate levels in plasma and tumors of CRC patients, which could be caused by the smaller study cohort. However, the result is a potentially valuable reference for further research. (*Taiwan J Public Health. 2021;**40(4)**:382-393*)

Key Words: colorectal cancer, environmental contaminants, microanalysis, enzyme-linked immunosorbent assay

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INTRODUCTIONS

Colorectal cancer (CRC) is the third-most common cancer among men and the second-most common cancer among women worldwide [1]. However, CRC is the most common malignancy among men and the second-most common malignancy among women in Taiwan [2]. The age-standardized incidence rate (ASIR) for CRC has increased over time, reaching 44.7 per 100,000 people in 2014, increasing with particular rapidity among people older than 50 years. The ASIR among persons aged 70–74 years was almost 7-fold higher than the ASIR among persons aged 45–49 years. Alongside the increase in the ASIR, the age-standardized mortality rate (ASMR) has also increased. The ASMR reported for 2006–2010 was 1.5–2-fold the ASMR value reported for 1971–1975 [3]. According to the recommendations of the American Cancer Society (ACS), persons older than 50 years should undergo colonoscopy every 10 years, a stool DNA test triennially, and a fecal occult blood test or fecal immunochemical test (FIT) annually [4]. In Taiwan, the government has promoted CRC screening since 2004, recommending that people aged 50–75 years receive a FIT biennially. If the FIT is positive, a verification examination, such as a colonoscopy, should be performed [5]. From 2015 to 2016, the screening rate was approximately 38%, and the verification rate was approximately 68%, which were slightly lower than the rates reported for western countries [2]. Thus, the development of non-invasive and tolerable diagnostic strategies represent urgent goals in the field of CRC research.

Environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), nitrate, and nitrite, have been linked to CRC tumorigenesis [6-10]. PAHs are commonly found in our living environments, and some studies have suggested that PAHs may cause indirect carcinogenic effects in many cancer types [10-13]. PAHs enter the human body through the consumption of contaminated food and water or the inhalation of cigarette smoke, automobile exhaust, and contaminated air in occupational settings [10]. In examining the potential effects of PAHs on colonic carcinogenesis, the colonic tissue of patients with ulcerative colitis, which represents a risk factor for CRC, was found to have a higher oxidation capacity for benzo(a)pyrene (a PAH) compared with the tissues of health patients, which may be due to the presence of electrophiles with higher mutagenic potential [14]. Moreover, in mice, benzo(a)pyrene was found to induce autophagy defects, which may result in oncogenic cell transformations in the colonic mucosa [15]. According to some epidemiological studies, benzo(a)pyrene intake is positively correlated with the incidence of CRC [16,17]. Nitrate and nitrite are precursors of endogenously formed N-nitroso compounds (NNO), which are carcinogens that can be inhibited by vitamin C and other antioxidants [8,18]. Endogenous nitrosation is estimated to contribute to 45%–75% of total NNO exposure, suggesting that the dietary intake of nitrate and nitrite, the precursors of endogenous nitrosation, may represent important CRC risk factors [8]. According to a cohort study and meta-analysis, nitrate and nitrate exposure are associated with the incidence of CRC [7,9], and the effects of nitrate exposure on CRC incidence depend on the exposure source (water, vegetables, and animal dietary sources) [19]. *In vivo* evidence has suggested that nitrite exposure due to processed meat consumption can promote the development of CRC [20], and nitrite intake promoted the development of CRC in the small intestine of an A/J Min/+ mouse model [21]. A multicenter study in Taiwan, however, found no significant association between nitrate in

drinking water and the risk of death from CRC [22].

Thus, we proposed this pilot study to evaluate the associations between environmental risk factors and CRC. PAHs, nitrate, and nitrite in the plasma and cancer tissue samples from CRC patients, obtained from the Tissue Bank and Biobank at Kaohsiung Chang Gung Memorial Hospital, were analyzed to elucidate the relationship between the evaluated factors and CRC.

MATERIALS AND METHODS

Clinical specimens: The G*Power analysis estimated a total sample size of 200 samples for this study. Due to limitations on specimen resources, 100 cases of fresh-frozen plasma samples (CRC, n = 50; healthy liver donor, n = 50), collected from 2011 to 2014, were obtained from the Tissue Bank at Kaohsiung Chang Gung Memorial Hospital for this pilot study. This study was approved by the Institutional Review Board (IRB) of Chang Gung Medical Foundation (IRB Number: 201700025B0). Environmental contaminants, including nitrate, nitrite, and 16 types of PAHs, were measured in the plasma samples following the standard protocols of the Super Micro Mass Research & Technology Center at Cheng Shiu University. Subsequently, 50 frozen tissue samples from patients with CRC, collected from 2011 to 2014 (36 of which were paired with plasma specimens), were obtained from the Tissue Bank at Kaohsiung Chang Gung Memorial Hospital. The tissues samples were sent to the Super Micro Mass Research & Technology Center at Cheng Shiu University for further analysis, following the approved guidelines of Chang Gung Memorial Hospital.

The determination of environmental contaminants in clinical samples: The detection methods utilized in this study included enzyme-linked immunosorbent assay (ELISA) and gas chromatography–tandem mass spectrometry (GC-MS/MS), and suitable extraction methods for the measurement of environmental contaminants were established. The limit of detection (LOD), the limit of quantitation (LOQ), and quality assurance (QA)/quality control (QC) values for each detection method are listed in Table 1. After analyzing plasma samples, nitrate levels were further examined in tissue samples obtained from patients with CRC to investigate the relationship between nitrate levels in the plasma and those in CRC tissues. The experimental design of this pilot study is illustrated in Figure 1.

Statistical analyses: All measurements of pollutant concentrations were performed in triplicate in at least three independent experiments, and the mean and standard deviation of the experimental results were calculated. Comparisons were performed using Student's *t*-test to determine significant differences between cancer and non-cancer groups. The Wilcoxon signed-rank test and Spearman's correlation analysis were used to compare values between paired plasma and cancer tissue samples. For all analyses, $p < 0.05$ was considered significant.

RESULTS

Establishment of detection methods for environmental contaminants

The methods developed in this study enabled the detection of environmental contaminants in human clinical plasma and tissue samples. Methods for the analysis of nitrate, nitrite, and 16 different PAHs were successfully established using ELISA and GC-MS/MS techniques (Table 2). In this pilot study, only nitrate was detectable in CRC plasma samples using ELISA. The linear calibration curve for nitrate was 12.5–175 μM, with a

Table 1. The limit of detection (LOD), limit of quantitation (LOQ) and quality assurance (QA)/quality control (QC) for the detection of nitrate, nitrite and 16 PHAs

Items	Methods	LOD	LOQ	QA/QC
Nitrate	Cayman Nitrate/Nitrite Colorimetric Assay Kit (Item No. 780001)	-	1.5 μM	1. The calibration curve was re-established for each batch experiment. 2. The standard and blank were duplicated in each batch experiment. 3. Actual standard or nitrate/nitrite concentration in each sample were obtained after the deletion of blank.
Nitrite	Cayman Nitrate/Nitrite Colorimetric Assay Kit (Item No. 780001)	-	4 μM	Same as above.
16 PAHs	GC-MS/MS	0.05~2 ng/ml	0.1~5 ng/ml	1. QC check sample, standard and specimen to be tested were pre-processing at the same time. 2. The calibration curve was re-established for each batch study. 3. After every 20 samples are analyzed, the laboratory control sample was used to check the applicability of calibration curve.

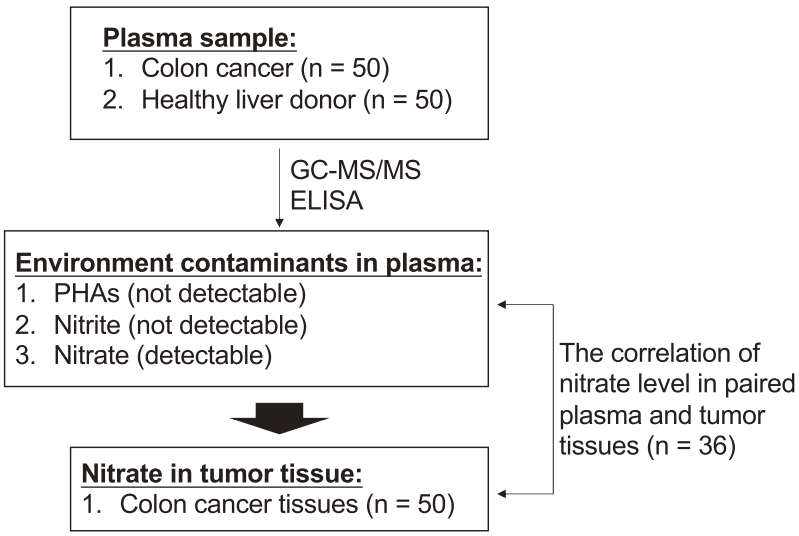

Figure 1. The experimental design in this pilot study

correlation coefficient of 0.9996. Based on these results, only the nitrate level was further analyzed in frozen colonic tumor tissues in this pilot study.

No significant difference in plasma nitrate levels exists between CRC patients and healthy liver donors

The clinical characteristics and nitrate concentrations in the plasma samples

Table 2. The established detection methods of environmental contaminants

Contaminants	Methods	Units	Range	R^2
Nitrate	ELISA	μM	12.5-175	0.9996
Nitrite	ELISA	μM	4-70	0.9998
Naphthalene	GC-MS/MS	ng/mL	5-50	0.990
Acenaphthylene	GC-MS/MS	ng/mL	0.25-10	0.996
Acenaphthene	GC-MS/MS	ng/mL	0.25-10	0.997
Fluorene	GC-MS/MS	ng/mL	0.25-25	0.994
Phenanthrene	GC-MS/MS	ng/mL	0.25-50	0.999
Anthracene	GC-MS/MS	ng/mL	0.1-10	0.999
Fluoranthene	GC-MS/MS	ng/mL	0.1-50	0.998
Pyrene	GC-MS/MS	ng/mL	0.1-50	0.998
Benzo[a]anthracene	GC-MS/MS	ng/mL	0.50-25	0.999
Chrysene	GC-MS/MS	ng/mL	0.10-25	0.997
Benzo[b]fluorarthene	GC-MS/MS	ng/mL	0.1-50	0.998
Benzo[k]fluoranthene	GC-MS/MS	ng/mL	0.1-25	0.998
Benzo[a]pyrene	GC-MS/MS	ng/mL	0.1-50	0.999
Indeno(123-cd)pyrene	GC-MS/MS	ng/mL	0.1-50	0.996
Dibenz[a,h]anthracene	GC-MS/MS	ng/mL	0.1-50	0.995
Benzo[ghi]perylene	GC-MS/MS	ng/mL	0.1-50	0.996

were compared between the cancer and control groups (Table 3). Age and sex were significantly different between CRC patients and healthy liver donors. The mean age in the cancer group was older than that in the control group, and up to 70% of CRC patients were men. The nitrate levels in plasma samples did not differ significantly between the CRC and control groups (Figure 2). To adjust for the effects of age and sex on these outcomes, binary logistic regression was used, which showed no significant difference in the plasma nitrate content between groups (p = 0.766).

No significant correlation exists between nitrate levels in paired tumor tissues and plasma from CRC patients.

Among the patients associated with cancer tissue samples, approximately 70% were men, 98% of patients had low-grade tumors, and only 2% of patients had high-grade tumors (Table 4). In the 36 paired samples (both plasma and tissue samples collected from the same patient), the nitrate levels in the tissue samples were significantly higher than those in the plasma ($p < 0.001$, Wilcoxon signed-rank test; Table 5). However, the nitrate levels in the paired plasma and cancer tissue samples did not show a significant correlation (r = 0.139, $p = 0.419$, Spearman's correlation test; Figure 3), and no significant differences in nitrate levels in either the plasma or tissue samples were observed between early (stage 0, I, and II) and advanced (stage III and IV) stages of CRC (Table 6). No significant differences in nitrate levels were observed for either plasma or tumor tissues among stages 0, I, II, III, and IV (Figure 4). Notably, tumor nitrate levels were significantly higher than plasma nitrate levels in stages I–IV CRC (Figure 5). Taken together, these findings showed that nitrate levels in tumor tissues were more abundant than those in plasma samples; however, plasma nitrate levels do not appear to be a suitable non-invasive diagnostic or prognostic marker for CRC.

Table 3. Nitrate concentration in the plasma samples

	Group 1 (Cancer) (N = 50)	Group 2 (Control) (N = 50)	p-value
Nitrate[a] (μM)	19.38 (14.55, 26.69)	20.96 (14.55, 37.12)	0.510
Age[b]	64.86 ± 12.21	26.58 ± 6.42	< 0.001
Sex (Male)	35 (70%)	12 (24%)	< 0.001

1. Data are expressed as [a]median (interquartile range) and [b]mean ± SD (standard deviation)
2. The limitation for detection is 12.5 μM.

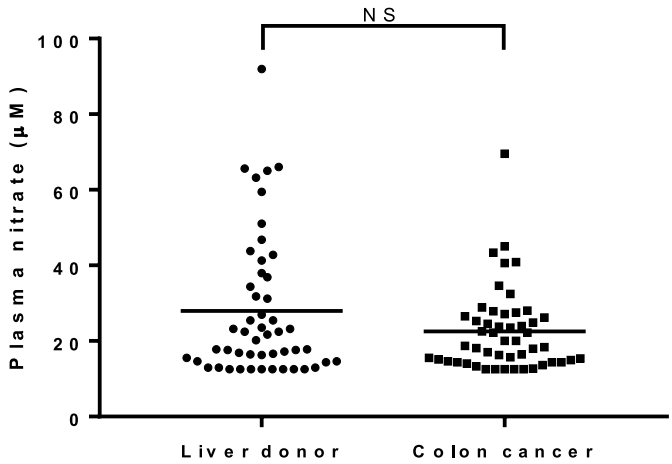

Figure 2. Plasma nitrate levels between liver and colorectal cancer groups

The ELISA analysis for nitrate in plasma samples of liver donors and colorectal cancer patients. NS = not significant.

Table 4. Demographic characteristics of cancer tissue samples (n = 50)

Variable	
Nitrate[a] (μM)	56.68 (45.38, 100.46)
Age[b]	63.95 ± 13.50
Sex (Male)	26 (70.3%)
Histologic grade	
Low-Grade (Well-Differentiated to Moderately Differentiated)	49 (98%)
High-Grade (Poorly Differentiated to Undifferentiated)	1 (2%)
T, N, M stage	
0	1 (2%)
I	16 (32%)
II	11 (22%)
III	16 (32%)
IV	6 (12%)
Recurrence/metastasis	
Yes	22 (44%)
No	28 (56%)

1. Data are expressed as [a]median (interquartile range) and [b]mean ± SD (standard deviation)
2. The limitation for detection is 25 μM.

Table 5. Nitrate concentration in paired plasma and tumor tissue samples of colorectal cancer group

Nitrate in plasma (μM)	Nitrate in tumor (μM)	p-value
18.07 (14.37, 26.23)	56.68 (45.38, 100.46)	< 0.001

1. Data are expressed as median (interquartile range), N = 36.

Table 6. Difference of nitrate concentrations in both plasma and tissue samples between early stages and advanced stages of colorectal cancer

Variable	Early stage (0, I, II)	Advanced stage (III, IV)	p-value
Nitrate in plasma (μM)	18.07 (14.37, 24.30)	23.85 (15.30, 27.59)	0.273
Nitrate in cancer tissue (μM)	55.75 (45.11, 106.47)	60.46 (45.38, 106.35)	0.764

1. Data are expressed as median (interquartile range).

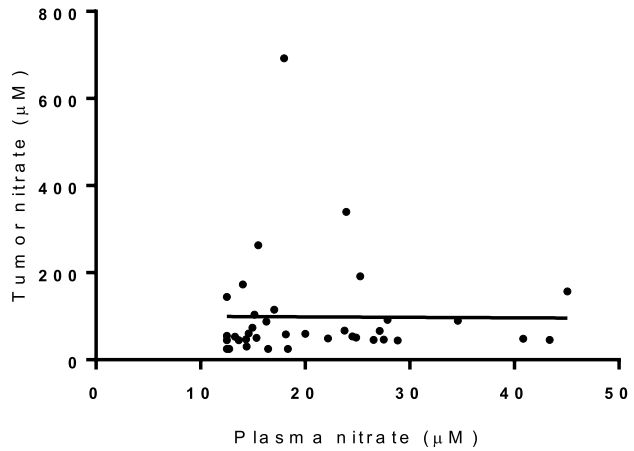

Figure 3. The correlation of plasma nitrate levels between tumor tissues and plasma samples in colorectal cancer group

The ELISA analysis for nitrate in tumor tissues and plasma samples of colorectal cancer patients.

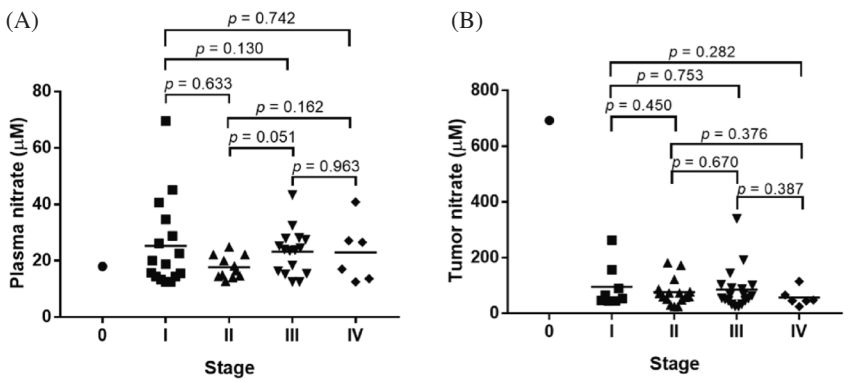

Figure 4. The plasma and tumor nitrate levels in colorectal cancer patients with different stages

The ELISA analysis for nitrate in (**A**) plasma samples and (**B**) tumor tissues of colorectal cancer patients with different stages

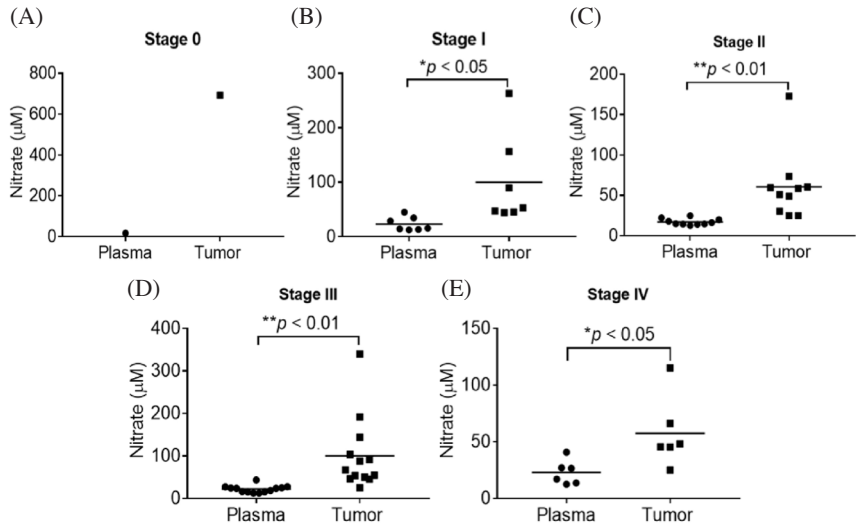

Figure 5. The comparison of plasma and tumor nitrate levels in different stages

The ELISA analysis for nitrate levels of tumor tissues and plasma samples of colorectal cancer patients in stage (A) 0, (B) I, (C) II, (D) III and (E) IV. ($^{*}p < 0.05$; $^{**}p < 0.01$).

DISCUSSIONS

In this pilot study, we analyzed the levels of environmental contaminants, including 16 different PAHs, nitrate, and nitrite, in plasma samples collected from CRC patients and controls, followed by the analysis of nitrate levels in 50 CRC tissue samples. A major limitation of this study was that the age of the CRC group was significantly older than that of the liver donor group, and male patients were more prominently represented in the cancer group compared with the control group due to limits on sample availability. The risk of CRC development has been reported to increase with age [23]. Moreover, in our study, 70% of the CRC group were men, and the overall incidence of CRC was reported as higher among men in a study performed in the UK, which might be the result of exogenous or endogenous factors pre-diagnosis associated with a higher incidence rate [24]. According to a large-scale cohort study performed at multiple liver transplant centers, the age of liver donors averaged 35 years [25]. In this pilot study, these factors likely contributed to the significant differences in age and sex observed between the CRC and liver donor groups. Although binary logistic regression was used to adjust for the effects of age and sex on the statistical results, the differences in age and sex between the CRC and liver donor groups may have interfered with the analytical results obtained from this pilot study. A modified study condition or a larger-scale cohort study should be considered in further research.

Nitrate was the only detectable environmental contaminant among nitrate, nitrite, and 16 types of PAHs in the plasma samples examined in this pilot study. Patients with CRC and active inflammatory bowel disease were found to have significantly higher plasma nitrate/nitrite levels [26]. Another study, however, indicated no significant difference in plasma nitrate/nitrite levels between CRC patients and healthy controls [27]. In this

pilot study, the plasma nitrate levels did not differ significantly between the cancer and control groups. Differences in the racial and ethnic backgrounds among different study cohorts may contribute to discrepancies in the experimental results. Moreover, different extraction and detection methods could also explain these differences.

The nitrate level was significantly higher in CRC tissues than in paired plasma samples. Both food intake and dietary style may affect the nitrate levels in blood and organs [28,29]. Inducible nitric oxide synthase (iNOS) is upregulated in CRC tissues compared with non-tumor regions [30,31], and iNOS overexpression in cancer can promote nitric oxide (NO) production, which affects the biological functions of tumor cells, such as tumor angiogenesis and DNA repair [32]. Total nitrate/nitrite levels have been suggested to serve as an indicator of NO production or iNOS activity [33-35], which would explain the observed increase in local nitrate levels in the primary tumor relative to the plasma sample. We also studied the correlation between nitrate levels in paired plasma and CRC tissue samples in this pilot study. We identified 36 paired samples among the 50 plasma and 50 tumor tissues. In this pilot study, no significant correlation was identified for the nitrate levels between paired plasma and tumor tissues; however, this study was limited by a relatively small sample size with insufficient statistical power. iNOS activity has previously been found to be significantly and positively related to tumor stage in CRC [30]. In this pilot study, however, no significant differences were observed for the levels of nitrate in either the plasma or the tissue samples according to tumor stage. Dietary changes made by the patients after being diagnosed as CRC may modify colonic microbiota compositions and proinflammatory protein levels (such as iNOS), which can affect nitrate levels in plasma and tissues [36].

In summary, we successfully established analytic strategies for measuring PAHs, nitrite, and nitrate using GC-MS/MS and ELISA assays in this pilot study and found that the nitrate level was significantly higher in local tumor tissues than in plasma samples. No significant difference was identified in plasma nitrate levels between the control and CRC groups, and the nitrate contents in the plasma and tumor tissues of CRC patients were not significantly correlated. This pilot study was performed as a cross-sectional study with some limitations. The incubation period for CRC can be as long as 10 years or longer; therefore, correlations between environmental contaminants detected in the plasma of patients with CRC and their dietary styles may be worth further investigation in a future study. The result of this pilot study could potentially serve as an important reference for further related research.

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環境汙染物與大腸直腸癌關聯性之台灣單一醫學中心前驅性研究

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目標：大腸直腸癌（CRC）在惡性腫瘤中具高發生率與死亡率，台灣CRC病患急劇增加可能與環境汙染物（多環芳香烴碳氫類化合物、硝酸鹽和亞硝酸鹽類）有關。因此，本篇研究探討此類汙染物與台灣CRC的相關性。**方法：**配對的CRC患者血漿與腫瘤組織檢體取自高雄長庚紀念醫院組織銀行，由正修科技大學超微量研究科技中心萃取後，以酵素免疫分析法測定其中之硝酸鹽及亞硝酸鹽濃度，另以氣相層析儀串聯質譜儀進行16種多環芳香烴偵測。**結果：**因血漿中所含之亞硝酸鹽及多環芳香烴均未檢出，後續實驗針對硝酸鹽進一步分析。實驗結果顯示CRC病患血漿的硝酸鹽濃度與對照組無顯著差別，但腫瘤組織中的硝酸鹽含量顯著高於配對的血漿。但是，硝酸鹽濃度在配對的CRC血漿與腫瘤組織中不具顯著關聯，且不同期別CRC的血漿與腫瘤組織硝酸鹽含量也無顯著差異。**結論：**此較小樣本數之前驅性研究顯示血液與腫瘤中硝酸鹽含量與CRC的相關性並不顯著，但是可提供後續進一步研究的重要潛在參考依據。（台灣衛誌 2021；**40(4)**：382-393）

關鍵詞：大腸癌、環境汙染物、微量分析、酵素免疫分析法

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