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RESEARCH ARTICLE

Analysis of Trace Elements in Formalin-Fixed Paraffin Embedded Colon Tissue Specimens Using ICP-MS

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HIGHLIGHTS

> By using FFPE tissues and preferring the ICP-MS method, the relationship between trace elements and colon cancer has been revealed more clearly.
> This study will reduce the contradictions of previous studies on the relationship between colon cancer and trace elements.

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ABSTRACT

The aim of this study was to reveal the relationship of colon cancer with some trace elements (Cr, Mn, Co, Ni, Cu, Zn, Se, Ag, Cd and Pb) and to end the contradictory results of previous studies on this subject. We wanted to eliminate the sampling and method errors shown as the cause of these contradictions. In this study, FFPE (Formalin-fixed paraffin embedded) cancer colon tissues of 12 patients with a diagnosis of colon cancer aged between 35 and 75 years and healthy colon tissues of the same people were used. The results of the analysis using ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) were evaluated statistically by using SPSS program. When cancerous and healthy tissues were compared, Pb amount was significantly higher in cancerous tissues compared to healthy tissues ($p < 0.05$). These results suggest that heavy metals accumulated in the body may be effective in the development of colon cancer.

Contents

1. Introduction

Colorectal cancer (CRC) is the third most general cancer in both women and men and is the third leading cause of cancer-related death. 60% of the CRC is observed in developed regions of the world. These differences between regions are due to various reasons. These differences are thought to be primarily related to environmental factors and

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diet [1, 2]. The role of trace elements in colon cancer falls into this category [3].

Trace elements act as enzyme components in biological systems or as catalysts in chemical reactions taking place in cells. Therefore, it is known that inadequate or excessive intake of a large number of elements causes a large number of diseases, including various types of cancer [4]. Some trace elements, such as Fe and Cu, participate in the physiological processes of the human body by activating or inhibiting enzymes [5, 6]. Zn and Cu play an important role in various processes such as RNA replication, immunity and DNA repair. However, excess Cu and Zn have shown oxidative properties by producing oxidative stress damaging different cell components including reactive oxygen species (ROS) and DNA [7]. Most trace elements affect carcinogenicity by inducing ROS production and oxidative stress [8]. Some other trace elements such as Hg, Pb and Cd can directly inhibit enzymes by binding to SH and SCH3 ligands in cysteine and methionine amino acid structures. At the same time, they can disrupt their normal function by exchanging ions of the same charge and size in the structure of metal-enzymes [9]. As a result of these, cell proliferation and disruption of genetic mutations can lead to cancer formation [10].

Many studies comparing cancer and control groups have been conducted to reveal the function of trace elements in colon cancer. In most of these studies, patient sera were analyzed. The number of studies using tissues was smaller and these studies were performed using wet tissues obtained by surgery [3, 11–13]. Yaman (2006) has shown in their study that; Conflicting results can be obtained in tissue elemental analyses for several reasons, such as tissue-based dry or wet weight, different sensitivities, the basis of analysis methods, and difficulties in sampling the sample representing the cancerous or non-cancerous area [14]. We used FFPE tissues in order to reduce these problems and to analyze many patients retrospectively. We also used the ICP-MS method, which was more sensitive than other methods and was able to measure at greater accuracy with lower concentrations.

Pathology departments and research institutes routinely use FFPE tissue blocks for histopathological examinations. The stock working solution usually consists of 4% formalin in water and this solution is diluted from 40% formalin solution and stabilized with 10% methanol. After fixation, the tissue blocks are dehydrated using dimethyl-benzene (also known as xylene) and ethanol series and embedded in paraffin. Thus, tissue samples are turned into FFPE blocks. FFPE tissues are a powerful tool to study the clinical course of diseases. The major advantage of FFPE tissues is the extensive archive of existing samples [15].

In this study, we used cancerous FFPE colon tissues of 12 colon cancer patients and healthy FFPE colon tissues of the same patients because the amount of trace elements in the human body may vary depending on the region and diet [13]. We examined the change by performing trace element analysis by ICP-MS method.

2. Materials and Method

2.1. Chemicals

All chemicals used were preferred as supra pure. Ethanol (purity >99%) and Xylene (purity >99%) were obtained from Sigma-Aldrich (St. Louis, MO). Multiple standards containing 26 elements (100 ml in 5% HNO3), 100 mL Internal Standard (100 mL in 10% HNO3), Tune Solution (500 ml in 2% HNO3), Nitric acid (purity >65%) and hydrogen peroxide (purity >31%) were purchased from Merck (Darmstadt, Germany) and were used without further purification.

2.2. FFPE Tissues

After approval of the study by the clinical research ethics committee (No: B.30.2.ATA.0.01.00/177, 29.03.2018), tissue specimens were provided from Atatürk University, Faculty of Medicine, Medical Pathology Department, Erzurum-TURKEY. In this study, 24 colon tissues, including intestinal tissue with cancer belonging to 12 patients aged between 35 and 75 years, and healthy intestinal tissue of the same patients were used. FFPE tissue samples were prepared to apply a protocol described previously [16].

2.3. Tissue Preparation and ICP-MS Analysis

FFPE blocks cut in a microtome thickness of 4 μm were placed on glass coverslips, folded and placed in centrifuge tubes. It was stored in a refrigerator at 4 ° C in the dark. Trace element analysis in FFPE tissues of colorectal cancer patients was started by removing paraffin from the tissue surface. The tissues were placed in different eppendorf tubes (2 mL) and heated to 60 ° C. 1 ml of xylene was added to the tissues, stirred for 15 min at 300 rpm and centrifuged at 15000 rpm + 4 ° C for 10 min. The xylene phase was discarded with a pipette and the samples were dried in the oven to completely remove the solvent. This procedure was repeated three times. Then 1 mL of 100% ethanol was added to the samples for rehydration, mixed with vortex until a suspension was obtained (about 1 min) and centrifuged again at 15000 rpm. The ethanol phase was removed by pipette over the sample and the ethanol completely evaporated in the oven. The same process was repeated with 95% ethanol, 70% ethanol and distilled water.

The standard solutions of the elements (Cr, Mn, Co, Ni, Cu, Zn, Se, Ag, Cd and Pb) to be analyzed were prepared using 2% nitric acid solution at increasing concentrations. These were then introduced to the instrument ready for analysis and the calibration curves plotted. During the analysis, in order to correct the deviations occurring in the calibration curve, it was given to the internal standard device including Indium, Scandium, Germanium and Bismuth which represent the periodic table (Table 1). The samples were analyzed by the determined method and the results were obtained in ppb. The results were then converted to μg gr-1 tissue and statistical analyses were performed. To analysis trace elements by ICP-MS, a previously described method was followed with minor modifications [17].

Table 1 ICP-MS operating conditions

Parameters	
Radio frequency power	1550 W
Sample uptake rate	0.40 mL/min.
Ar Plasma gas flow rate	15 L/min.
Ar auxiliary gas flow rate	1 L/min.

Carrier gas flow rate	0.99 L/min.
He flow rate	4.3 mL/min.

Before analysis, 100 µL from each samples were digested in 2 mL pure mixture acid (1/5, HClO4/HNO3) at 120 0C for 120 minutes via microwave oven (Ethos Easy, Milestone). The elements (Cr, Mn, Co, Ni, Cu, Zn, Se, Ag, Cd and Pb) were assessed in the samples using inductively coupled plasma- mass spectrometry (ICP-MS, Agilent 7700). For measurements, 20× dilutions of samples were prepared in 1 % HNO3 in acid-treated 14 mL conical tubes with an 8 mm sampling depth. Data were quantified with external standards for elements in 1 % HNO3. The internal standard consisting of 45Sc, 209Bi and 72Ge was used. For each sample, data were acquired in triple and averaged.

2.4. Statistical Analysis

In the study, IBM SPSS 20.0 package program were used for statistical analysis. Independent samples t-test were used to compare trace element concentrations in cancer and healthy tissues. $p < 0.05$ was considered significant.

3. Results and Discussion

Trace elements are basic micronutrients involved in many important biological processes, such as the activity of antioxidant enzymes, cell division and acting as cofactors for differentiation. Imbalances in trace element levels may adversely affect human health. Epidemiological evidence suggests that altered concentrations are associated with the onset of fatal diseases such as cancer [18]. Some trace elements can change biological processes and are considered carcinogenic substances. These genotoxic carcinogens are altering the genetic make-up of cells causing multiple mutations in the critical genes in the human body which may lead to irreversible permanent DNA damage and cancer development. Accumulation or deficiency of these elements may lead to the development of CRC [19].

Various factors such as habit, genetics, nutrition, behavioral and occupational status have been identified as cancer risk factors [20]. The identification of these risk factors is important both in the prevention and treatment of cancer [21]. Epidemiological studies have shown a higher incidence of cancer in areas with environmental pollution by industrial and agricultural wastes and trace elements and radioactive materials [22, 23]. Although many studies have been carried out on this subject, the role of trace elements in cancer is not fully understood [7].

In our study, we wanted to reveal the relationship between colon cancer and trace elements and it was observed that Mn, Ag and Cd concentrations decreased in tumor tissues compared to healthy tissues. Concentrations of Cr, Co, Ni, Cu, Zn, Se and Pb showed an increase in tumor tissues compared to healthy tissues. However, only Pb element change was statistically significant ($p < 0.05$) (Table 2).

Table 2 Trace element levels in cancerous and healthy colon tissues (ppm)

Trace Element	Healthy tissue mean ± SD	Tumor tissue mean ± SD	P value
Cr	1.014±0.329	1.334±0.423	0.137
Mn	1.971±1.234	1.560±0.776	0.489
Co	0.024±0.014	0.038±0.027	0.219
Ni	0.474±0.276	0.682±0.496	0.336
Cu	22.787±9.052	39.491±25.585	0.110
Zn	53.518±15.538	69.440±23.509	0.152
Se	1.388±0.421	1.686±0.634	0.307
Ag	0.076±0.096	0.041±0.043	0.431
Cd	0.165±0.091	0.149±0.071	0.731
Pb*	0.347±0.138	0.847±0.487	0.016

*: Statistically significant difference ($p < 0.05$)

Lead is a heavy metal toxic and carcinogenic to the organism. It shows the effect of lead as a heavy metal by accumulating in blood and various tissues. Radicals formed by the effect of lead are described as a strong lipid peroxidation initiator that plays a role as the primary mechanism in the formation of cell damage by disrupting the cell membrane structure [24]. It is known that lead is a human toxin that can affect many organs and functions, including the bone marrow and the nervous system, and that the lead is not genotoxic in vitro, but possibly enhances the mutagenicity of other mutagens acting through inhibition of DNA repair [25, 26]. This information explains the significant difference in the amount of lead in our study. This information confirms the results of our study.

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality worldwide [18]. Many studies have been conducted to compare the concentrations of trace elements in normal and cancerous tissues, and to investigate the role of trace elements in colon cancer, and different results have been obtained (Table 3).

Table 3 Changes in trace element concentrations in colon tissues in the literature

		Cr		**Mn**		**Co**		**Ni**		**Cu**	
Study, Year	Method	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor
Gupta and Shukla, 1993	AAS	NA		NA			↑	NA		NA	
Milde et al., 2001	AAS	NA		NA		NA		NA		↑	
Magalhães et al., 2010	TXRF	NA		NA		NA		NA			↑
Rinaldia et al., 2015	ICP-AES		↑	NA		NA		NA		NA	
Sohrabi et al., 2018	FAAS		↑		↑	NA		NA		unchanged	
Carvalho et al., 2007	TXRF	NA		NA		NA		NA			↑
Majewska et al., 2007	TXRF	NA		NA		NA		NA			↑
Our study	ICP-MS		↑	↑			↑		↑		↑

		Zn		**Se**		**Ag**		**Cd**		**Pb**	
Study(year)	Method	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor	Healthy	Tumor
Gupta and Shukla, 1993	AAS	↑		NA		NA		NA		NA	
Milde et al., 2001	AAS	↑			↑	NA		NA		NA	
Magalhãeset al., 2010	TXRF		↑	↑		NA		NA		NA	
Rinaldia et al., 2015	ICP-AES		↑	NA		NA		NA		NA	
Sohrabi et al., 2018	FAAS		↑	NA		NA		NA			↑
Carvalho et al., 2007	TXRF	unchanged		NA		NA		NA		NA	
Majewskaet al., 2007	TXRF		↑		↑	NA		NA		NA	
Our study	ICP-MS		↑		↑	↑		↑			↑

NA: not available, ↑: increased, AAS: Atomic absorption spectrometry, TXRF: Total reflection x-ray fluorescence, ICP-AES: Inductively coupled plasma atomic emission spectrometry, FAAS: Flame atomic absorption spectroscopy, ICP-MS: Inductively coupled plasma- mass spectrometry.

When Table 3 is examined, it is seen that the previous studies are full of contradictions. For example, although Zn amount was higher in healthy tissues in two of the studies [27, 28], Zn amount was higher in tumor tissues compared to healthy tissues in four other studies [3, 29–31]. Although Milde and co-workers have concluded that the amount of Cu higher in healthy tissues than in cancerous tissues, Magalhães et al. (2010), Carvalho et al. (2007) [32] and Majewska et al. (2007) have concluded that the amount of Cu is higher in cancerous tissues. In addition, when the results of selenium analysis are examined in Table 3, there are other contradictions. Milde et al. (2001) and Majewska et al. (2007) have shown that selenium is higher in cancerous tissues than in healthy tissues, while Magalhães et al. (2010) say the opposite.

Two factors stand out as the cause of the differences in the studies on trace element analysis in cancerous and healthy tissues. The first is considered to be difficulties in sampling, while the other is related to the selected analysis method [15]. We used tissues with FFPE in the sample collection, as the sample representing the cancerous or non-cancerous area could not be extracted accurately during surgery. Because FFPE tissues can be examined under a microscope to distinguish between cancerous and non-cancerous tissue. In addition, the use of tissue with FFPE eliminates any errors that may arise from tissue-based dry or wet weight. We used ICP-MS method in order to eliminate the negativity that may arise from the analysis method. Because ICP-MS method is more advantageous compared to methods used for trace element analysis such as AAS, ICP-OES. Detection limits provided by ICP-MS cannot be achieved by other methods and isotope analysis cannot be performed. ICP-MS can be used with a wide range of linearity (ppt-ppm). It also provides a significant advantage in terms of analysis time compared to other methods [33, 34].

4. Conclusions

In our study, we found that Pb was significantly higher in cancerous tissues than healthy tissues ($p < 0.05$). These results will lead to studies on diagnosis and treatment of cancer patients. In our study, using FFPE tissues and preferring ICP-MS method, we think that the relationship between trace elements and cancer will be more clearly revealed and the contradictions of studies on colon cancer trace element analysis will be reduced. We also believe that this study will shed light on the researches on the trace element levels of cancerous and healthy tissues.

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Conflict of Interest

Authors declare that they have no conflict of interest with any person, institution, or company.

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