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Elemental fingerprinting of tumorous and adjacent non-tumorous tissues from patients with colorectal cancer using ICP-MS, ICP-OES and chemometric analysis

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Abstract Tumorous and adjacent non-tumorous paired biopsies from 38 patients with colorectal cancer were analyzed by inductively coupled plasma mass spectrometry and inductively coupled plasma optical emission spectrometry after low-volume microwave digestion. 18 elements were investigated: Ag, Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, P, Pb, S, Se and Zn. Different chemometric tools were used for data evaluation: Wilcoxon signed rank test, Hieratical clustering analysis, principal component analysis (PCA) and linear discriminant analysis (LDA). With the exception of Al, tumours were observed to have significantly more elevated concentrations of essential elements as compared to non-tumours. On the contrary, elements considered potentially carcinogenic such as Cr, Ni, Mo or Co do not display significant differences. When PCA was applied, different components were obtained for tumorous and non-tumorous tissues. When LDA was applied for the elements studied (including essential and non-essential elements) about 90% of cases were correctly classified.

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Introduction

It has been well known for several decades that cancer is a disease in which element alterations are produced. Patients with different forms of cancer displayed significant differences in the level of some essential and non-essential elements when normal and malignant tissues are analyzed (Mulay et al. 1971; Gregoriadis et al. 1983; Rizk and Sky-Peck 1984; Drake and Sky-Peck 1989). Elemental concentration ranges in human tissues are so narrow for the correct functioning of the cells, that still today, the question of if these alterations are a consequence of the disease or can cause it is subjected to conjectures (Majewska et al. 2007).

Many studies have targeted metal-induced carcinogenicity, emphasising their role in the generation of oxygen-free radicals (Halliwell and Gutteridge 1999; Leonard et al. 2004; Valko and Morris 2005). These radicals can cause changes in DNA bases, enhanced lipid peroxidation and homeostasis. As, Cd, Co, Cr, Cu, Fe, Ni and V are the most studied metal-induced oxidative stress (Valko et al. 2006).

On the other hand, the effect of oxygen-free radicals is neutralized in the organisms by the antioxidant defense. The most efficient antioxidant

enzymes have an active metal centre; i.e. Cu, Mn or Zn is part of superoxide dismutases (SODs), the first-line antioxidant defense. Se takes part of glutathione peroxidase, i.e. the major source of protection against low levels of oxidative stress (Mates et al. 1999).

Tumours have a very high-energy demand and high rate of glucolysis. In general, essential trace elements such as Ca, Co, Cu, Fe, Mg, Mn, Mo, Ni, Se or Zn form compounds with proteins, which have important catalytic functions. In addition, the molecular mechanisms involved in carcinogenesis for some essential metals (as Ca, Fe, Mg, Ni, Zn) are likely to include binding competition among metal ions at chromatin and other regulatory molecules. This takes place in both, target cells, which give rise to tumours, and immune cells, which are responsible for controlling the tumour growth (Kasprzak 1997). Excess or deficiency of the elements may be an important factor in the development of malfunctions at cellular or subcellular level.

More difficult it is to consider this subject with regard to non-essential elements when their levels do not appear to be pathological. Few studies can be mentioned in this sense. Low non-cytotoxic concentrations of some elements such as As or Cd have been related to the inhibition of nucleotide excision repair (Hartwig 2000).

In recent years, the interest for understanding the role of alterations in element homeostasis and in the etiology of cancer has dramatically increased (Raju et al. 2006). Comparative studies carried out recently on normal and cancerous human tissues report controversial results, especially for some elements such as Cu, Fe, Ni, Se and Zn. In general, most of authors found significantly elevated concentrations or non-differences for several elements in malignant tissues (Raju et al. 2006; Ionescu et al. 2006; Ebrahim et al. 2007), however, decreases in concentration have also been reported (Zoriy et al. 2006).

The aim of this work is to investigate the elemental distribution in tumour and non-tumour adjacent colorectal tissue using several chemometric approaches. A case study with 38 patients was carried out. Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES) were applied as analytical techniques to determine 18 elements in 76 colorectal biopsies. Recently, the use of these multielemental analytical techniques has increased the possibilities of obtaining accurate information in biological tissues (Bocca et al. 2007; Alimonti et al. 2008). Ag, Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, Pb, Se and Zn were studied. S and P were also included in this study owing to their involvements in the demand of metabolic energy. Chemometric tools were applied to obtain latent information from data.

Materials and methods

Apparatus and reagents

Deionised water (18.3 MΩ) from a Milli-Q purification system (Millipore, Molsheim, France) was used throughout. Ultrapure grade nitric acid (Hyperpur-Plus, Panreac, Barcelona, Spain) was used. Standard working solutions were obtained from a multielement standard stock solution ICP Multi Element Standard Certipur® VI (Merck, Darmstadt, Germany) after suitable dilution. Calibration curves were built from three replicates measurements and, in all cases, regression coefficients were higher than 0.999. Standard stock solutions (1,000 mg/L) of Ge, Rh and In (Merck) were used as internal standards.

Concentrations of minor elements (Al, Ca, Cu, Fe, K, Mg, P, S and Zn) were determined by ICP-OES using a Perkin–Elmer Optima 4300 DV spectrometer (Shelton, CT, USA), equipped with an AS-90 autosampler, axial system, a high dynamic range detector and a cross-flow type nebulizer for pneumatic nebulization. The ICP-OES measurement conditions for these elements were optimized to achieve the maximum signal-to-background ratio.

Trace element concentrations were determined by ICP-MS using a Thermo Elemental X7 Series ICP-MS equipped with an ASX-520 autosampler (Omaha, NE, USA). Two operation modes were used, standard mode with guard electrode (plasma screen) and cell collision technology (CCT). The instrument was optimized in terms of sensitivity, resolution, mass calibration and minimization of interferences (oxide ratio and polyatomic interferences).

A Reisch (Haan, Germany) mixer mill MM 2000 was used for grinding the biopsy samples. Microwave digestion of colorectal biopsies were carried out with a Multiwave 3000 oven (Anton Paar, Graz, Austria), equipped with eight PFA digestion vessels (100 ml of

capacity). The dimensions of these vessels (20 cm high, 2.5 cm of diameter) allow inserting three PFA vials (6 cm high, 1 cm diameter, Savillex, Minnetonka, USA), one over the other. In this way, 24 sample digestions were performed in one digestion run.

Different certified reference materials were used for analytical validation purposes: BCR 185R (bovine liver), NRC-CNRC TORT-2 (lobster hepatopancreas), NRC-CNRC DORM-2 (dogfish muscle) and NRC-CNRC DOLT-2 (dogfish liver).

All glassware was rinsed with acid and immersed into a 10% v/v HNO_3 solution prior to use. A class 100 laminar flow hood (Crumair, Barcelona, Spain) into a clean room was used in this work.

Analyzed samples

Paired colorectal biopsies were supplied by the anatomy pathological service of the Povisa Hospital (Vigo, Galicia, NW of Spain) from 38 patients with colorectal cancer before any chemotherapy or radiotherapy treatment. They were not occupationally exposed to heavy metals. Malignant and adjacent normal tissues (76 samples) were provided.

Characteristics of colorectal biopsies are summarized in Table 1. Classification of the tumours has been carried out from the pathological analysis of biopsies. Malignant tumour samples were adenocarcinomas, i.e. the most common type of colorectal cancer (95% of cases). The patients presented different stages or extents of disease: I (evidence of tumour growth), II (local spread), III (extensive local and regional spread) and IV (metastasis). The stage III was the most spread among patients.

Table 1 Characteristics of colorectal analyzed biopsies

Number of patients	38
Age	69 ± 11
Gender	
Female	13 (34%)
Male	25 (66%)
Tumor type	
Adenocarcinome	38
Tumor stage	
I	2 (5%)
II	9 (24%)
III	24 (63%)
IV	3 (8%)

Biopsy samples were preserved in the hospital by cryogenic freezing. Masses of biopsies were in the range 0.1–1 g wet weight. In the laboratory, samples were rinsed with deionised water and dried at 60°C to constant weight. Dry samples were ground with a mixer mill for 3 min at 60% amplitude yielding a powdered sample with a particle size <100 μm. Samples were stored in closed polyethylene vessels and kept at 4°C in a refrigerator until analysis.

Microwave sample digestions were performed in the PFA Teflon vials (6 ml) with 0.3 ml of HNO_3 following the procedure described elsewhere (Millos et al. 2008). Three subsamples of each biopsy (20–30 mg mass) were digested. The same procedure was carried out with blanks.

The accuracy of the proposed methodology was tested by analyzing four certified reference materials (BCR 185R, TORT 2, DORM 2 and DOLT 2). The results obtained were in good agreement with the certified values. For the non-certified elements, the presence of matrix effects was investigated by recovery experiments using the CRM BCR 185R. No interferences were observed for these elements. The precision of the method, expressed as relative standard deviation (RSD), was evaluated in terms of repeatability. The RSDs from five independent digestions were in the range 2–11% for all elements and the precision from three replicates of the same digestion was lower than 3%.

Chemometric analysis

Chemometric data analysis was performed with SPSS for Windows version 14.0 (SPSS Inc., Chicago, IL). Wilcoxon signed rank test, i.e. a non-parametric statistical paired sample test, was used for assessing the differences between the two populations (tumorous and non-tumorous biopsies). Multivariate methods such as Hieratical clustering analysis (HCA), principal component analysis (PCA) and linear discriminant analysis (LDA) were used as tools to investigate the relationships between variables and samples.

Multivariate analysis of colon tissue data was performed on both raw and log-transformed data. No substantial differences were observed and the results were used directly (concentration in μg/g).

Results and discussion

Elemental concentrations in paired colorectal biopsies

Seventy-six colorectal biopsies (tumour and non tumour) were analyzed in order to obtain the elemental fingerprinting of these samples. Analytical results, from three sample preparations and three replicates of each one, are summarized in Table 2 as mean, median, minimum and maximum levels for each element. Elemental concentrations found in both tumorous and non-tumorous biopsies were in the following ranges: Ag, Co, Pb (<0.1 μg/g), Mo, Cd, Ni, Mn, Se, Cr (0.1–1 μg/g); Cu, Al, Zn, Fe (10–200 μg/g); Mg, Ca, K (300–1,500 μg/g).

A different variability of elemental concentrations in tumorous and adjacent non-tumorous samples was observed. The most abundant metals, i.e. Mg, K and to a lesser extent Ca, displayed a similar variability in tumorous and non-tumorous tissues (Mg 55 and 51%; K 88 and 84%; Ca 40 and 80% for non-tumorous and tumorous biopsies, respectively). A similar finding occurs for the essential elements Zn, Cu and Se (CV: Zn 33 and 31%; Cu 41 and 43%; Se 48 and 46% for non-tumorous and tumorous biopsies, respectively). For the remaining metals, variability is higher in the group of tumorous biopsies than in the non-tumorous ones and the largest variability is found for the non-essential elements. Thus, for Co, CVs are 50 and 140% and for a non-essential element as Pb these values are much higher, i.e. 140 and 248% in non-tumorous and tumourous biopsies, respectively.

As can be observed in Fig. 1, tumorous tissues of colon show higher element concentration than non-tumorous tissues for the same patient. In this figure, the average ratio of element concentration in tumorous and healthy tissues is shown. Mn and Ca content in tumours can exceed that of non-tumorous tissues by a factor higher than 2; for Se, Mg, K and Al this factor is between 2 and 1.5; for Cu, Cr, Fe, Mo, Ni, Pb and Zn between 1.5 and 1; for Ag and Co is 1; and only for Cd is <1.

Table 2 Elemental concentrations in tumorous and non-tumorous tissues

Element	Non-tumorous tissue (μg/g), $n = 38$			Tumorous tissue (μg/g), $n = 38$			δ Parameter
	Mean	Median	Min–max	Mean	Median	Min–max	
Ag	0.02	0.010	0.002–0.16	0.02	0.01	0.002–0.09	0.819
Co	0.05	0.04	0.02–0.14	0.06	0.04	0.01–0.53	0.488
Pb	0.13	0.06	0.002–2.1	0.22	0.07	0.002–4.9	0.724
Mo	0.20	0.12	0.03–1.1	0.35	0.14	0.03–5.8	0.373
Cd	0.23	0.19	0.005–0.65	0.14	0.12	0.03–5.8	0.010*
Ni	0.54	0.35	0.02–5.6	1.27	0.40	0.02–27	0.509
Mn	1.1	0.6	0.23–7.7	2.3	1.5	0.66–20	0.001*
Se	0.9	0.7	0.10–1.9	1.6	1.3	0.75–3.8	0.000*
Cr	1.5	0.8	0.16–9.5	2.7	1.1	0.11–42	0.415
Cu	6.2	6.0	0.12–13.2	8.5	8.5	0.23–17.4	0.000*
Al	28	17	4.0–90	44	28	6.1–238	0.008*
Zn	79	79	29–131	89	90	31–150	0.100
Fe	125	106	18–313	194	154	20–500	0.003*
Mg	323	296	26–896	664	573	189–1,977	0.000*
Ca	459	416	81–955	1,462	978	372–7,217	0.000*
K	1,219	819	107–3,693	2,241	1,464	195–6,833	0.000*
P	2,130	1,954	248–5,918	5,134	5,107	1,447–10,994	0.000*
S	4,118	3,979	377–7,127	5,887	5,756	2,950–9,109	0.000*

Wilcoxon signed rank test (paired samples)

* Significant difference ($P < 0.05$ and 0.01)

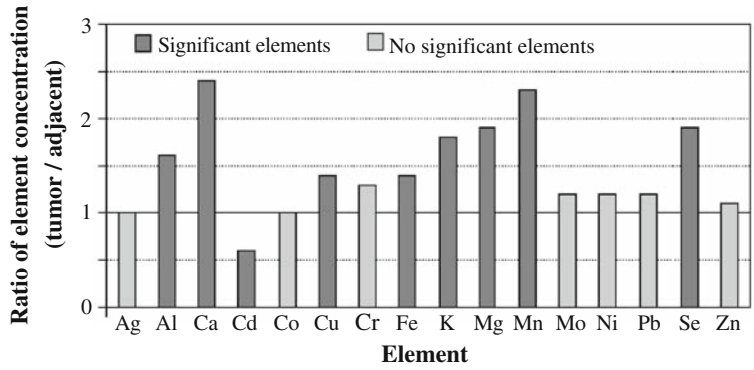

Fig. 1 Elemental concentration ratios of tumorous-to-adjacent non-tumorous tissues

Elemental concentrations reported in the literature for colon tissues display a large variability (Table 3), which can be attributed to different factors such as diet, environment, age, etc.

Statistical comparison of data

Wilcoxon signed rank test was applied to establish whether there were significant differences between average elemental concentrations in non-tumorous and tumorous tissues The values for the statistical parameter (δ) corresponding to the above test are shown in Table 2. Tumorous tissues were observed to have significantly more elevated contents of Mn, Se, Cu, Al, Fe, Mg, Ca, K, P and S. In contrast, a significantly lower level of Cd was found in tumour tissues. No significant differences were found for Ag, Co, Pb, Mo, Ni, Zn and Cr. Although non-significant differences were found for Zn, significant differences exist ($P < 0.05$) for the Cu/Zn concentration ratio when comparing the non-tumorous and tumorous tissues ($\delta = 0.037$). It has been suggested that the ratio Cu/Zn is a good indicator of the extent and prognosis in carcinomas (Gupta et al. 2005).

We found that with the exception of Al, all the elements that are significantly accumulated in tumorous tissues correspond to the essential group. P and S accumulation in tumorous tissues allows confirming a major demand of metabolic energy. Phosphorylation and de-phosphorylation of proteins are essential processes for the growth of tumour cells. The total S/P ratio in cell cultures has been showed a potential tool to distinguish malignant cell lines. This ratio between colorectal human tumorous and normal colon tissue has been found to be in the range 1.57–1.93 (Bandura et al. 2004). In our work, an average ratio of 1.69 has been found.

Elements such as Se, Cu, Zn and Mn are essential trace elements involved in some enzymes that protect the cells against the free radicals. Se is a cofactor of glutathione peroxidase and Cu/Zn and Mn are cofactors of SODs. Colorectal carcinogenesis has been associated with serious oxidative stress. A statistically significant increase in the level of Cu/Zn-SOD, glutathione peroxidase (Se) and glutathione reductase as well as lipid peroxidation products, has been observed in tumorous tissues in comparison with normal colon tissues (Skrzydlewska et al. 2005).

Selenium has been suggested that the intake of Se reduces risk of colorectal cancer, but epidemiological studies have not been able to show a consistent protective action (Jacobs et al. 2004). Gastrointestinal glutathione peroxidase (GI-GPx) and selenoprotein P (SePP) are considered to provide protection against reactive oxygen species, thereby reducing DNA damage and preventing development of colon cancer. Reduced SePP expression and increased GI-GPx expression in colorectal cancers has been observed. In general, the balance implies an increase in selenoprotein expression (Al-Taie et al. 2004).

Enhanced levels and activity of enzyme Mn-SOD has been well established in colorectal cancer (Janssen et al. 1999). It is known that this enzyme suppresses cell growth in different tumour cell lines. It has been demonstrated that Mn-SOD induces p53-dependent senescence in colorectal cancer cells (Behrend et al. 2005). Immunohistochemical expression of Mn-SOD has been proposed as a marker of malignant potential in colorectal carcinoma (Nozoe et al. 2003).

Table 3 Some published data of elemental concentrations in colorectal biopsies

Element	Gregoriadis et al. (1983)		Drake and Sky-Peck (1989)		Majewska et al. (2007)		Bocca et al. (2007)	Alimonti et al. (2008)		
	Adjacent ($n = 33$)	Tumour ($n = 18$)	Adjacent ($n = 15$)	Tumour ($n = 15$)	Benign colon polyps ($n = 42$)	Tumour ($n = 73$)	Healthy ($n = 10$)	Healthy ($n = 17$)	Adjacent nonpolypotic ($n = 15$)	Polypotic ($n = 15$)
Ag							16.1 ± 13.1[a]			
Co							35.5 ± 28.1[a]	29.8 ± 15.9[a]	39.8 ± 25.4[a]	19 ± 16[a]
Pb	107 ± 115[a]	136 ± 154[a]	1.36 ± 0.71[b]	1.03 ± 0.59[b]			137 ± 50.0[a]	194 ± 145[a]	156 ± 109[a]	155 ± 145[a]
Mo			2.40 ± 0.58[b]	2.19 ± 0.23[b]			150 ± 55.0[a]			
Cd							199 ± 112[a]	222 ± 97[a]	298 ± 120[a]	97 ± 66[a]
Ni	70 ± 61[a]	110 ± 76[a]	0.94 ± 0.54[b]	0.88 ± 0.32[b]			123 ± 40.0[a]			
Mn	70 ± 60[a]	78 ± 63[a]	2.92 ± 1.19[b]	1.68 ± 0.32[b]			3,343 ± 1,258[a]	3,724 ± 1,938[a]	4,950 ± 2,500[a]	3,558 ± 1,091[a]
Se			1.53 ± 0.33[b]	1.26 ± 0.28[b]	0.545 ± 0.132[b]	0.816 ± 0.577[b]	737 ± 359[a]	611 ± 351[a]	949 ± 377[a]	1,559 ± 527[a]
Cr			1.31 ± 0.65[b]	1.21 ± 0.31[b]			445 ± 342[a]	365 ± 196[a]	509 ± 284[a]	261 ± 252[a]
Cu	1.27 ± 0.33[b]	1.76 ± 0.58[b]	14.1 ± 4.4[b]	8.9 ± 3.4[b]	3.73 ± 1.53[b]	3.55 ± 2.36[b]	26.0 ± 10.0[b]	27 ± 12[b]	29 ± 11[b]	20 ± 5[b]
Al							2,746 ± 1,449[a]	3,726 ± 2,099[a]	3,254 ± 1,425[a]	2,817 ± 3,201[a]
Zn	14.4 ± 2.9[b]	14.3 ± 2.8[b]	64.1 ± 20.5[b]	98.2 ± 18.7[b]	9.65 ± 4.50[b]	14.8 ± 9.63[b]	89.5 ± 19.4[b]	94 ± 23[b]	100 ± 22[b]	93 ± 26[b]
Fe			189 ± 50.5[b]	129 ± 41.2[b]	43.1 ± 8.33[b]	45.0 ± 33.4[b]	121 ± 75.0[b]	117 ± 66[b]	130 ± 48[b]	246 ± 150[b]
Mg							1,094 ± 239[b]	1,079 ± 244[b]	1,105 ± 209[b]	1,263 ± 318[b]
Ca			591 ± 253[b]	393 ± 195[b]			819 ± 304[b]	855 ± 246[b]	732 ± 271[b]	754 ± 252[b]
K	1,405 ± 495[b]	2,149 ± 686[b]								

[a] Results are expressed as ng/g

[b] Results are expressed as μg/g

Elevated Cu levels have been found in a wide spectrum of tumours of cancer patients. This has been mostly related to angiogenesis, i.e. development of new blood vessels. Cancer cells synthesize their own angiogenic stimulators or recruit endothelial cells to synthesize them (Gupte and Mumper 2009). Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis. Copper ions have been identificated as an important endogeneous angiogenesis stimulators (Nasulewicz et al. 2004). Copper stimulates the proliferation and migration of endothelial cells and is required for the secretion of several angiogenic factors by tumour cells (Lowndes and Harris 2005). Cu deficiency has been proposed as an anti-cancer strategy. Copper chelators reduce tumour growth and microvascular density in animal models. (Goodman et al. 2004). An unifying mechanism of action by which copper chelation inhibits endothelial cell proliferation and tumour secretion of angiogenic factors remains to be elucidated, but possible targets include copper-dependent enzymes, chaperones, and transporters (Lowndes and Harris 2005).

Although, Zn is essential for cell growth as well as in the control of gene transcription and differentiation (Vallee and Auld 1990), accumulation of this element in tumorous tissues was not observed in this work. Zn homeostasis is coordinated through Zn transporter proteins (ZnT), that include ZIP group increases the intracellular level and cation diffusion facilitator (CDF), and metallothioneines. Gurusamy et al. (2008) explain the low levels of Zn found in liver metastases in comparison with normal adjacent tissues as a variation in the levels of Zn transporters and a possible increase in the utilization of Zn by the fast growing tumour tissues. An irregular distribution of Zn in tumours might explain these results. Low Zn levels in tumours and high Zn levels in marginal areas of tumours have been observed (Ujiie et al. 1995). Thus, these results can be compatible with Zn requirements of the tumorous cells for proliferation. Zn causes growth arrest in colon cancer cells (Jaiswal and Narayan 2004).

Total Fe in the body and high dietary Fe intake are considered risk factors for colorectal cancer. It is due to its redox characteristics and the ability to generate reactive oxygen species (ROS) (Toyokuni 1996). Significant associations between this kind of cancer and iron intake, transferrin iron saturation and serum iron has been found (Weinberg 1994; Richardson et al. 2008). Colorectal cancer progression has been recently associated with increased expression in Fe import proteins and a block in Fe export. This results in an increase of intracellular Fe, which may induce proliferation and repress cell adhesion (Brookes et al. 2006). It has been well-established that tumorous cells generally have higher levels of transferrin receptor 1 (TfR1), a cell membrane-associated glycoprotein involved in iron homeostasis and cell growth, than their normal adjacent cells (Larrick and Cresswell 1979). More recently, transferrin receptor 2 (TfR2) has been also found to be expressed in a wide range of neoplastic cells and tumours (Kawabata et al. 2000; Nakamaki et al. 2004). Excess risk of colorectal cancer has been observed by Knekt et al. (1994) in subjects with transferrin saturation level exceeding 60% (in adult humans ~25–30% of Fe in the body is bound to ferritin (Eisenstein 2000). Fe is crucial for cellular growth and then, for continuous proliferation of the tumorous cells, it is necessary an iron supplement. This made tumorous cells express more transferrin receptors to produce transferrin or transferrin-like proteins and to obtain iron. Colon environment favors this process because important quantities of unabsorbed iron are found in the lumen of the colon (Weinberg 1994). In addition, it is probable that Fe plays an important role in angiogenesis (Richardson et al. 2008).

Ca is considered a chemopreventive agent for colorectal cancer. This element can reduce the risk of colorectal tumours by binding bile and fatty acids (potentially carcinogenic compounds) in the bowel or by the action of the calcium-sensing-receptors (CaSRs) on colonic epithelium (Peters et al. 2004). Colon tumours often start with the losses of functional APC (adenomatous polyposis coli) followed by losses of a top–down cryptal Ca gradient and functional cellular CaSRs (Whitfield 2009). Low dietary calcium may be related to inhibition of apoptosis and possibly to an increase in cell proliferation (Owen 2001) and the differentiation of colon cancer cell lines has been associated with changes in Ca homeostasis (Aung et al. 2007). In addition, Ca-ATPases are important in many cell functions involved in intracellular signal transduction, control of proliferation, programmed cell death or synthesis of mature proteins (Chung et al. 2006).

Magnesium plays an important role in cell proliferation according to membrane magnesium mitosis

model cell growth (Rubin 2007). It has been clearly established that proliferating cells contain more magnesium than quiescent cells and a decrease in magnesium availability influences the cell proliferation rate. Scarce information has been published about magnesium and tumour growth, but in contrast with normal cells, these only ceased their growing when extracellular magnesium decreased drastically. The avidity of tumours towards magnesium explains hypomagnesaemia in cancer and the chemotherapy for its normalization (Wolf et al. 2009). Mg deficiency modulates tumour expression of genes involved in the control of cell cycle, stress response, proteolysis and adhesion cells (Maier et al. 2007; Dai et al. 2007). Mg has been also related to angiogenesis through the endothelial cell migration (Lapidos et al. 2001) and the VEGF (Maier et al. 2007). Recently, it has been suggested that a high Mg intake may reduce the occurrence of colorectal cancer (Larsson et al. 2005; Folsom and Hong 2006; van den Brandt et al. 2007).

K is required by glycolytic enzymes, this has much higher activity in carcinoma of rectum and colon than in adjacent non tumour tissues. High activity of pyruvate kinase in colon and rectum tumorous cells has been observed, what would explain the accumulation of this element in tumorous tissues (Reddy et al. 2003).

Al is the most abundant metal in the earth′s crust, however, is not an essential element for life. The exposure to this element has been related to neurodegenerative diseases and more recently, to breast cancer with aluminium based antiperspirants (Darbre 2005; Exley et al. 2007). It has been demonstrated also that the administration of Al to rat is involved in oxidative stress (González et al. 2007). In spite of the interest of this element, there are few studies in which Al determination has been carried out in tumour tissues. Al is omnipresent in everyday life in the developed countries and this can result in an increase in the body burden. Ng et al. (1997) found significantly higher contents of this element in breast cancer biopsies but no differences between healthy and tumorous tissues were found in brain tissues (Andrasi et al. 1995).

The IARC (International Agency for Research on Cancer) considers Cd as a human carcinogen (IARC 1993). Cd is involved in genotoxic mechanisms as the induction of single-strand DNA breaks, the inhibition of DNA repair, the activation of protoncogenes and the inhibition of apoptosis (Navarro Silvera and Rohan 2007). Inconsistent results on Cd in tumorous tissues have been published. Higher contents in breast (Ionescu et al. 2006) and lung (Martin Mateo et al. 1990) tumorous tissues have been found in comparison to non tumorous tissues. No differences have been found in liver (Mai et al. 2006), lung (Kuo et al. 2006) or colon (Martin Mateo et al. 1990). A decrease in Cd levels in kidney (Feustel et al. 1986) and liver (Tashiro et al. 2003) tumours has also been published. In adenomatous polyps, associated with a greater risk of cancer, significantly lower levels have been found (Alimonti et al. 2008). Metallothioneins take part of Cd detoxication and they also protect the tissues from the effects of free radicals; these processes can be related to a decrease in cadmium levels in tumorous tissues (Reddy et al. 2003).

For the other elements considered in this study (i.e. Ag, Co, Cr, Mo, Ni and Pb), no differences were found between tumorous and non-tumorous tissues. Co, Cr, Mo and Ni have been related to induction of oxidative stress produced in metal carcinogenesis (Valko et al. 2006; Richardson-Boedler 2007).

Multivariate data analysis

For an insight into the similarity of samples, hierarchical clustering analysis was applied. Figure 2 shows a dendogram for the 76 samples. Two possible cluster solutions may be discussed. Cluster solution 2 is a homogeneous grouping, where all samples corresponded to tumorous tissues. Cluster 1 is an inhomogeneous cluster where the adjacent tissues and an important number of tumorous tissues are grouped resulting in inconclusive results. When cluster analysis is applied to 18 features (i.e. elements), solutions can be explained as a function of their concentration.

PCA was applied to two different subsets of data (tumorous and non-tumorous biopsies). By applying PCA with varimax rotation to the matrix formed by 18 variables (elements) and 38 non-tumorous samples, five principal components (PCs) were extracted that accounted for 77.6% of the total variance. When PCA was applied to tumorous samples (varimax rotation, 18 variables and 38 samples), 5 PCs described 79.0% of the total variance. Table 4 shows the elements that load the different PCs and the common variance for both tumorous tissues and non-

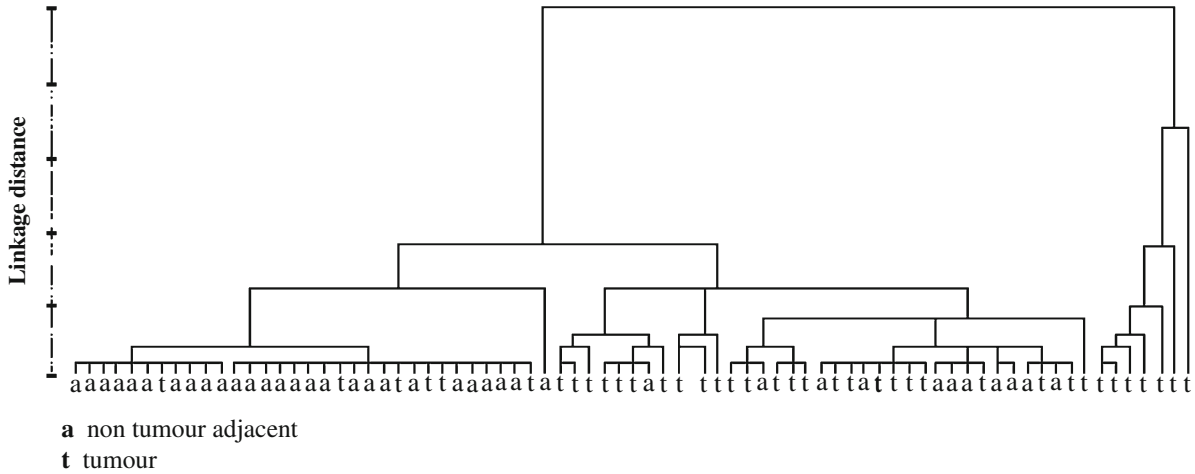

Fig. 2 Dendogram for the 76 samples analyzed

Table 4 Principal component analysis with varimax rotation for adjacent non-tumorous and tumorous tissues (18 features and 38 samples)

Component	Non-tumorous tissues		Tumorous tissues	
	Elements	Common variance (%)	Elements	Common variance (%)
1	Cu, Se, Ca, Fe, K, Mg, P, S	32.2	Mo, Co, Ni, Cr	25.6
2	Mo, Co, Ni, Cr	19.2	Cu, Fe, K, P, S	23.6
3	Pb, Mn	12.7	Se, Ca, Mg	14.3
4	Ag, Al	7.0	Cd, Pb, Mn, Zn	9.8
5	Cd, Zn	6.5	Al	5.7
	Accumulated variance	77.6		79.0

tumorous ones. In non-tumorous tissues, the first PC is loaded by essential elements related to the normal metabolism and the antioxidant defense system. On the contrary, these elements load different PCs in tumorous tissues. Mo, Co, Ni and Cr load the first PC in the tumorous tissues, whereas all these elements load PC 2 in the non-tumorous tissues. In the tumorous biopsies, PC 2 is loaded by essential elements and displays a similar variance as compared to PC 1. PCA confirms the need to consider groups of elements in order to understand the variation of the elemental concentrations in tissues.

Linear discriminant analysis (LDA) was used in the classification and identification of the two groups of samples (tumorous and non-tumorous biopsies) on the basis of the values of the predictor variables (elemental concentrations). The squared Mahalanobis distance was used as a measurement of the differences between the examined populations. When all elements were used, one canonical discriminant function explained the 100% of the variance (Wilks′ Lambda was 0.436, $P = 0.00$). This discriminant function classified correctly the 90.8% of the cases (Table 5). In order to reduce the number of elements necessary for classification, the backward stepwise inclusion method was

Table 5 Classification results for adjacent non-tumorous and tumorous tissues discriminations

Group	Number of cases	Predicted group	
		Non-tumorous	Tumorous
All elements			
Adjacent	38	35 (92.1%)	3 (7.9%)
Tumour	38	4 (10.5%)	34 (89.5%)
Total	90.8% of the cases classified correctly		
Without Ag and Pb			
Adjacent	38	36 (94.7%)	2 (5.3%)
Tumour	38	4 (10.5%)	34 (89.5%)
Total	92.1% of the cases classified correctly		

Table 6 Classification results for stages

Group/stages	Number of cases	Predicted group				
		0	I	II	III	IV
0	38	34 (89.5%)	0 (0%)	2 (5.3%)	2 (5.3%)	0 (0%)
I	2	0 (0%)	1 (50%)	0 (0%)	1 (50%)	0 (0%)
II	8	1 (12.5%)	0 (0%)	6 (75%)	1 (12.5%)	0 (0%)
III	25	3 (12%)	1 (4%)	2 (8%)	17 (68%)	2 (8%)
IV	3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (100%)
		80.3% of the cases classified correctly				

used. Three elements were used in this model (P, Se and S), however, only 85.5% of samples were correctly classified. Then, others alternatives were used for element reduction. If Ag and Pb are excluded (elements with δ parameter >0.7 in Wilcoxon signed rank test) the 92.1% of the cases can be correctly classified (Table 5). If we exclude those elements for which non-significant differences were found between tumorous and non-tumorous, such as Ag, Co, Pb, Mo, Ni and Cr, this percentage decreased up to 84.2%. When the different components obtained in the PCA were used as elements for classification, the different LDAs did not improve the classification accuracy. Results indicate that between 92 and 95% of the non-tumorous biopsies and 90% of the tumorous biopsies are correctly classified only when an elevated number of elements is used (16 or 18), including essential and non-essential elements.

Moreover, LDA was also applied for classification of the different biopsies according to the tumour stage. The obtained results are shown in Table 6. The 80.3% of cases were correctly classified with four canonical functions (function 1 explained the 57.1% of total variance; function 2 the 23.4%, function 3 the 12.6% and function 4 the 7.0%). About 90% of non-tumorous biopsies, 75% of stage II biopsies, 70% of stage of III, 50% of stage I (only with two cases) and 100% of tissues with metastasis (stage IV) were correctly classified when all elements are used for this purpose.

Conclusions

In this work, consistent results regarding trace elements in the colorectal cancer are presented. The use of the same patient for evaluating the concentration of different elements in cancerous and non-cancerous tissues offers a more reliable comparison because genetic, environmental or dietary differences are eliminated. 38 patients and 76 analyzed samples are an important pool for this kind of studies.

The differences found in this work in the elemental content for tumorous and adjacent non-tumorous tissues were clear. In general, with the exception of aluminium, we can conclude that tumours accumulate essential trace and minor elements. Higher levels found in tumorous tissues could be a consequence of disease progression.

The PCA study suggests that non-accumulated elements such as Co, Cr, Mo and Ni in tumours loaded the first factor when PCA is applied to tumorous samples and the second one when PCA is applied to non-tumorous samples. This finding could point out a different behaviour in tumorous and non-tumorous tissues.

LDA confirms the need to include these elements in order to differentiate between tumorous and non-tumorous tissues. In this work, an important classification percentage was achieved when elemental concentrations were used as an indicator of cancer and, to a lesser extent, of the disease stage. This finding is promising, but further efforts need to be made so as to unambiguously select which elements must be considered. In this respect, the use of multielemental sensitive techniques such as ICP-MS and ICP-OES facilitate enormously this task.

The behaviour of the Al in regard to the other elements suggests that the accumulation of Al in colorectal tumours must be studied in depth.

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