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ORIGINAL ARTICLE

Occupational Exposure to Heavy Metal Initiate Carcinogenesis Though BRAF/KRAS Over Expression and DNA Methylation

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KEYWORDS

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ABSTRACT: Colorectal carcinoma (CRC) is a leading cause of death especially in industries worker. The aim of this study is to identify the role of heavy elements exposure on CRC DNA methylation. The study was conducted on 25 CRC patients. Biopsies were taken by colonoscopy from malignant tissue and adjacent normal tissues for comparative assessment of BRAF/KRAS, methylated MLH1 and MGMT between the normal and malignant tissues by using real time PCR. In an attempt to identify wither heavy metal like Lead, Aluminum and Mn have a role in cancer development or not, we compared their levels in the serum of 25 CRC patients and 25 normal volunteers by using atomic absorption. The expressions of BRAF/KRAS, methylated MLH1 and MGMT were significantly higher in malignant tissues compared to normal tissues (p value<0.001). Additionally, the levels of lead and aluminum but not Mn were statistically significantly increased in CRC patients compared to normal controls (p value<0.001). Lead and Aluminum were positively correlated with all studied parameters. Heavy metals act as starting signals for carcinogenesis through DNA methylation.

INTRODUCTION

Colorectal cancer can be considered as of the most common causes of cancer deaths worldwide. The risk factors of CRC are smoking, polyps and intestinal inflammatory disease [1]. cancer occur as a result of interaction between multiple epigenetic changes that leads to inactivation of tumor suppressor genes, DNA repair enzymes and conversion of proto-oncogenes to oncogenes and DNA methylation changes [2].

BRAF is a proto-oncogene, it codes for BRAF protein which is a serine/threonine-protein kinase. The B-RAF protein is involved in signals that promote cell growth [3]. More over KRAS is a proto-oncogene, it controls cell proliferation. KRAS mutation disrupt the negative signaling, therefore cells continuously proliferate and develop cancer [4].

The mitogen activated protein kinase (*MAPK)*

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pathway plays an important role in tumor cell proliferation and survival. RAS is a component of MAPK signaling pathway that is activated by a ligand binding to a receptor tyrosine kinase (RTK) such as the epidermal growth factor receptor (EGFR). Activated KRAS leads to downstream activation of the effector BRAF [5, 6].

DNA methylation is one of the epigenetic modifications. It means the addition of a methyl group to a cytosine residue. Methylation of tumor suppressor genes result in transcriptional inhibition and carcinogenesis. MLH1 and MGMT genes codes for DNA repair enzymes and are considered as a suppressor genes. CRC is much frequently occurring with their promoter methylation [7] [8].

The exposure to heavy metals increases the cancer risk, not only due to DNA damage as a result of induction of reactive oxygen species, but also due to affection of DNA repair mechanisms [9]. We investigated the role of heavy metals exposure in pathogenesis of cancer colon development.

MATERIALS AND METHODS

Twenty-five patients' industries worker (11females and 14 males and with age range 24-75 years) with biopsy proven colorectal adenocarcinoma were enrolled in this study. The patients were recruited. Tissue collection was approved by all patients after written informed consent. During colonoscopy, biopsies (2mm size each) were taken from malignant lesions and adjacent apparently normal mucosa. The specimens were sent for histopathological evaluation.

Quantitative RT-PCR of BRAF and KRAS genes expression

The malignant and normal tissues were homogenized for total RNA isolation with GeneJET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). for reverse transcription, 5µl the total RNA from each sample were used with subsequent amplification with Bioline, Amedian life science company, U.K. (SensiFAST ™ SYBR Hi-ROX) One-step Kit (catalog number PI-50217 V) in a 48-well plate using the Step-one instrument (Applied Biosystems, U.S.A.). The expressions of BRAF and KRAS genes were calculated relative to the mean critical threshold (CT) values of housekeeping gene ;GAPDH by the Ct method. Thermal profile was 10 minutes at 95ºC for enzyme activation followed by 40 cycles of 15 seconds at 95ºC, 20 seconds at 55ºC and 30 second at 72ºC for the amplification step. Primers' sequences for Braf were; forward: 5´-CTTCATGAAGACCTCACAGT-3´ and reverse: 5´-CATCCACAAAATGGATCCAG-3´. Kras primers were, forward: 5´-AAAATGACTGAATATAAACTTGTGG-3´, and reverse: 5´-TCTATTGTTGGATCATATTCGTC-3´. GAPDH primers were forward: 5´-GTGCGTGCCCAGTTGAACCA-3´, and reverse: 5´-CGGCTGACTGTCGAACAGGA-3´.

DNA extraction

Total DNA was isolated from ultrasonic homogenized tumor and normal adjacent tissues biopsies using DNA extraction kit (Qiagene, USA) according to instructions of manufacture.

Bisulfite-PCR methylation analysis

For MLH1 and MGMT epigenetic study, methylation specific PCR (MSP) was used, which was designed to identify promoter region hypermethylation changes .MSP entails initial modifications of the DNA by sodium bisulfite that converts all unmethylated cytosine to uracil and subsequent amplifications with primers specific for methylated DNA. Methylated cytosine is protected from the effect of sodium bisulfite. Bisulfite treatment was performed according to kit instruction (Qiagene, USA).

Quantitative Real-Time PCR to Measure DNA Methylation

Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment. primer sequences were chosen for regions containing frequent cytosines (Cs) and CpG islands near the 3´end of the primers. MLH1 primer was forward: 5´AGGAAGAGCGGATAGCGATTT-3´, reverse: 5´-TCTTCGTCCCTCCCTAAAACG-3´ and probe sequence (5´–3´) 6FAM-CCCGCTACCTAAAAAAATATACGCTTACGCG-BHQ-1. MGMT primer was forward: 5´-GCGTTTCGACGTTCGTAGGT-3´, reverse primer, 5´-CACTCTTCCGAAAACGAAACG-3´and probe sequence (5´–3´) 6FAM-CGCAAACGATACGCACCGCGA-BHQ-1. After Sodium bisulfite conversion of genomic DNA and primers and probes preparation, the bisulfate modified DNA was amplified with the Taqman Master Kit (Qiagene USA) in a 48-well plate using the StepOne™ (Applied Biosystems, Foster City, CA, USA) for quantitative real-time PCR. Changes in the expression of target genes MGMT and MLH1 were measured relative to the mean critical threshold (CT) values of universal human control DNA methylated gene, by the ΔΔCt method.

Assessment of serum levels of Lead, Aluminum and Mn

The levels of Lead, Aluminum and Mn were detected in serum samples. Instrumental conditions for metals are based on Analytical Methods of Atomic Absorption Spectrometry (ASS) (Perkin Elmer). For each element calibration curve equation was linear and passing through point zero during measurement.

Statistical methods

Data was coded as mean and standard deviation using the statistical package SPSS version 22. Comparisons between the normal and malignant tissues were done using paired t test, while comparison between CRC patients and control were done using paired t test. Correlations between quantitative variables were done using Pearson correlation coefficient. Regression analysis was used to detect the risk factors for CRC. p value < 0.05 is considered statistically significant.

RESULTS

Histopathological examination of malignant tissues revealed adenocarcinoma; 28% (n=7) were grade I, 32% (n=8) were grade IIA, 32% (n=8) were grade IIB, 8% (n=2) were grade IIC and none was grade III.

BRAF, KRAS, methylated MLH1 and methylated MGMT highly expressed in malignant tissue

Statistically significant increase in BRAF and KRAS gene expression in malignant tissues relative to normal tissues (p value<0.001) (Figure 1A,1B).

Statistically significant increase in methylated MLH1 and methylated MGMT expression in malignant tissues relative to normal tissues (p value<0.001) (Figure 1C, 1D).

BRAF is significantly correlated with methylated MLH1 (p value<0.001 & r = 0.845) (Figure 2A) also significantly correlated with methylated MGMT (p value<0.001 & r = 0.832) (Figure 2B).

KRAS is significantly correlated with methylated MLH1 (p value<0.001 & r = 0.914) (Figure 2C) also significantly correlated with methylated MGMT (p value<0.001 & r = 0.92) (Figure 2D).

Lead and Aluminum elevated in CRC

The serum levels of lead and aluminum are significantly increased in CRC patients than the normal control (p value <0.001) (Figure 3A, 3B). While no significant difference In Mn level between CRC and normal control (p value=0.38) (Figure 3C).

Lead is significantly correlated to (methylated MLH1, methylated MGMT, BRAF, KRAS (P value <0.001& r= 0.496, 0.414, 0.602, 0.621 respectively) (Figure 4 A, B, C, D)

Aluminum is significantly correlated to (methylated MLH1, methylated MGMT, BRAF, KRAS (P value 0.003,0.009, 0.002, 0.001 & r= 0.438,0.392 0.457, 0.45 respectively) (Figure 4,E,F,G,H).

No significant correlation between Mn and (methylated MLH1, methylated MGMT, BRAF, KRAS (P value >0.05).

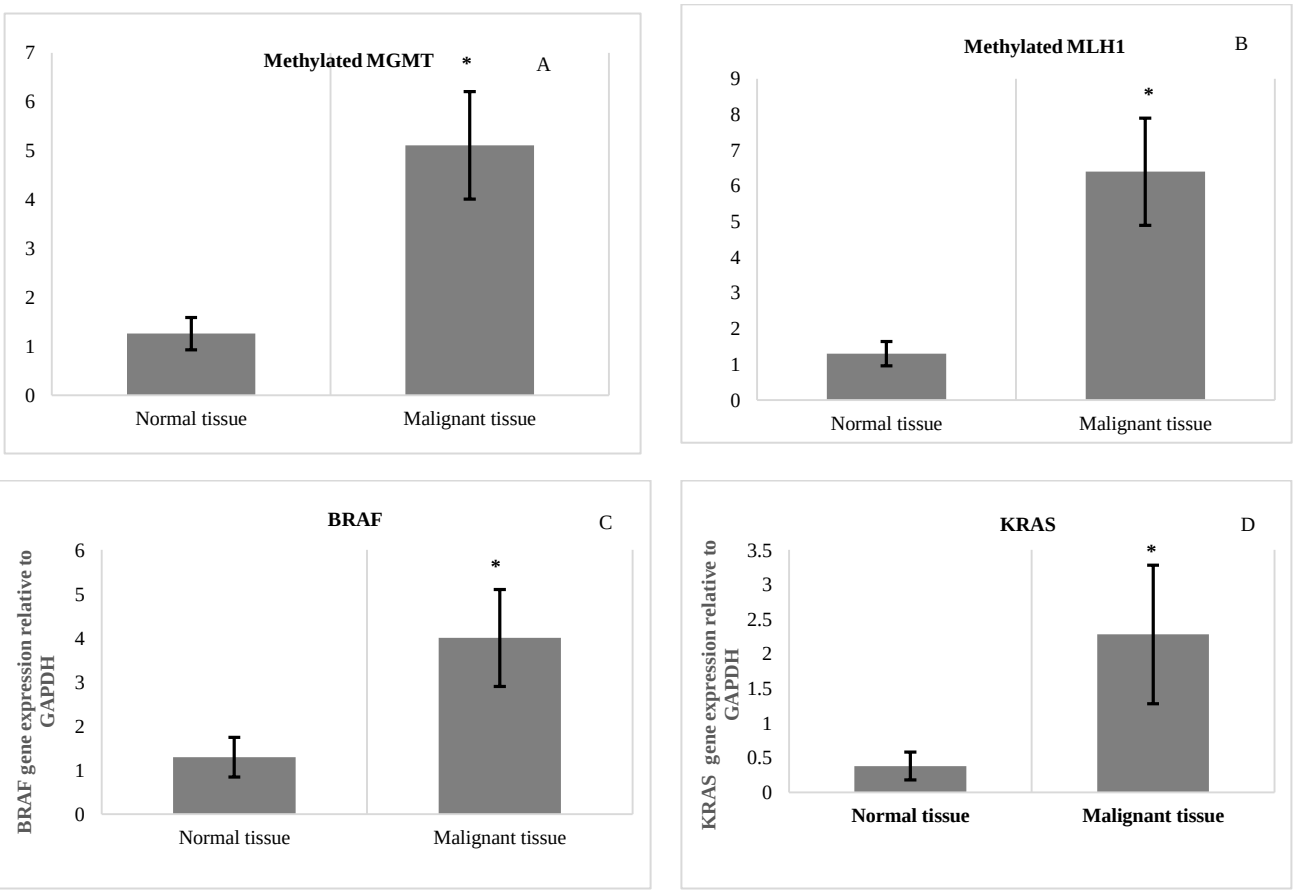

Figure 1. Expression of BRAF, KRAS and methylated MLH1& MGMT in normal versus malignant tissues. (*) Statistically significant compared to normal tissues.

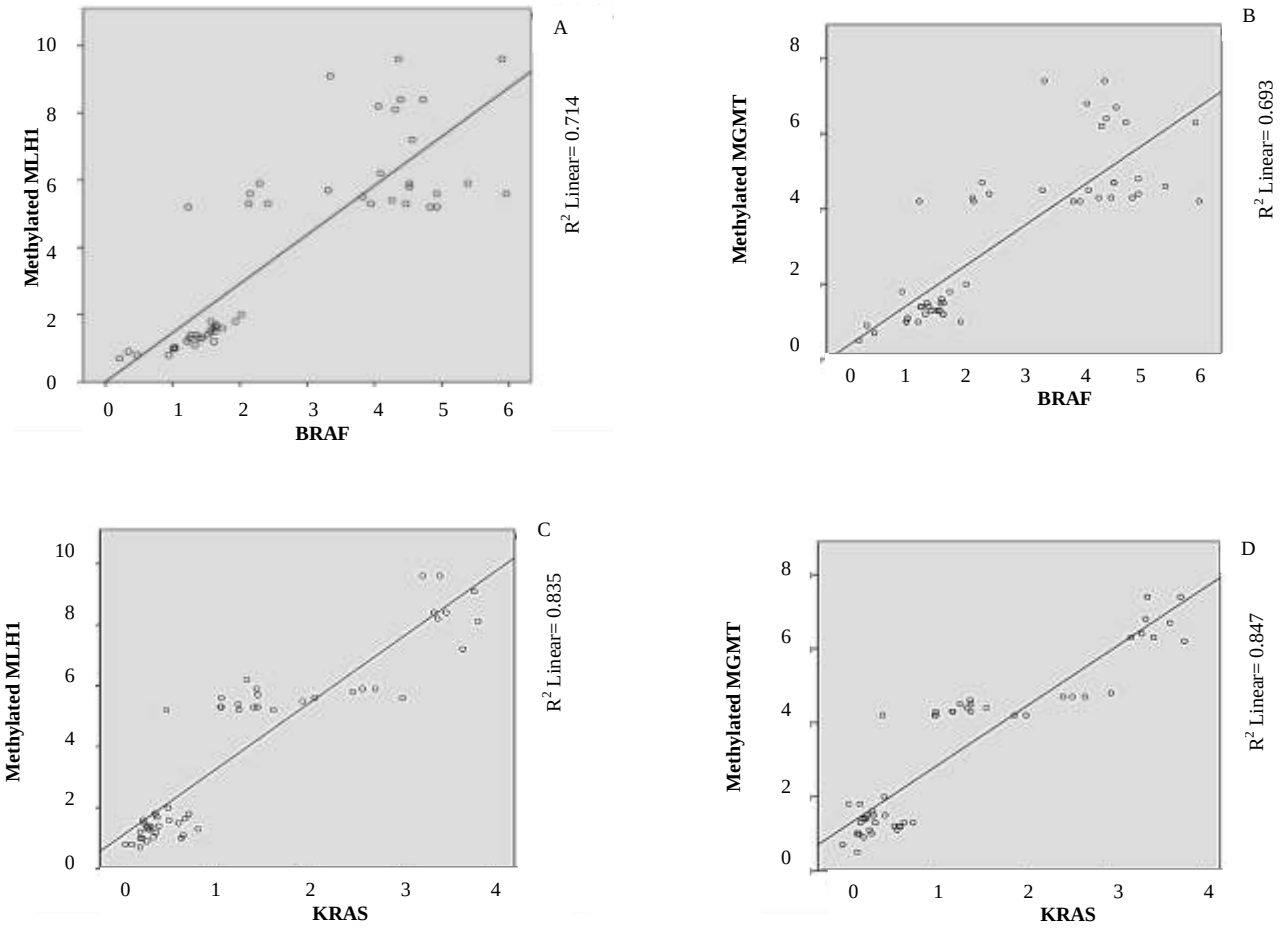

Figure 2. Correlation between BRAF, KRAS and methylated MLH1& MGMT.

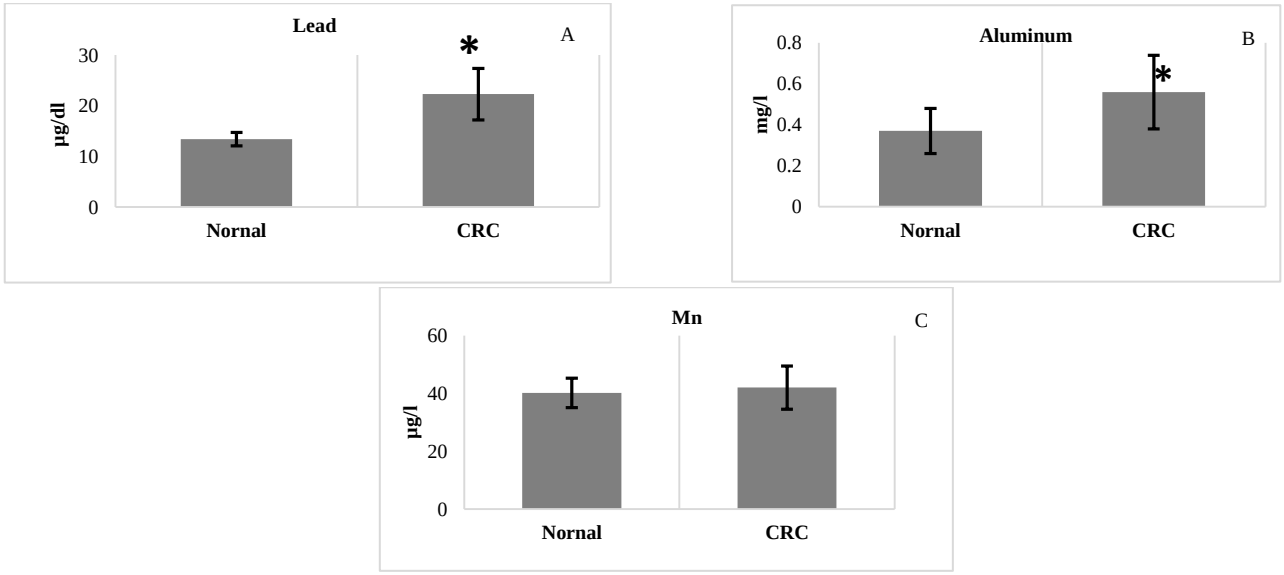

Figure 3. The levels of Lead, Aluminum and Mn in CRC patients versus normal controls (*) statistically significant compared to normal controls.

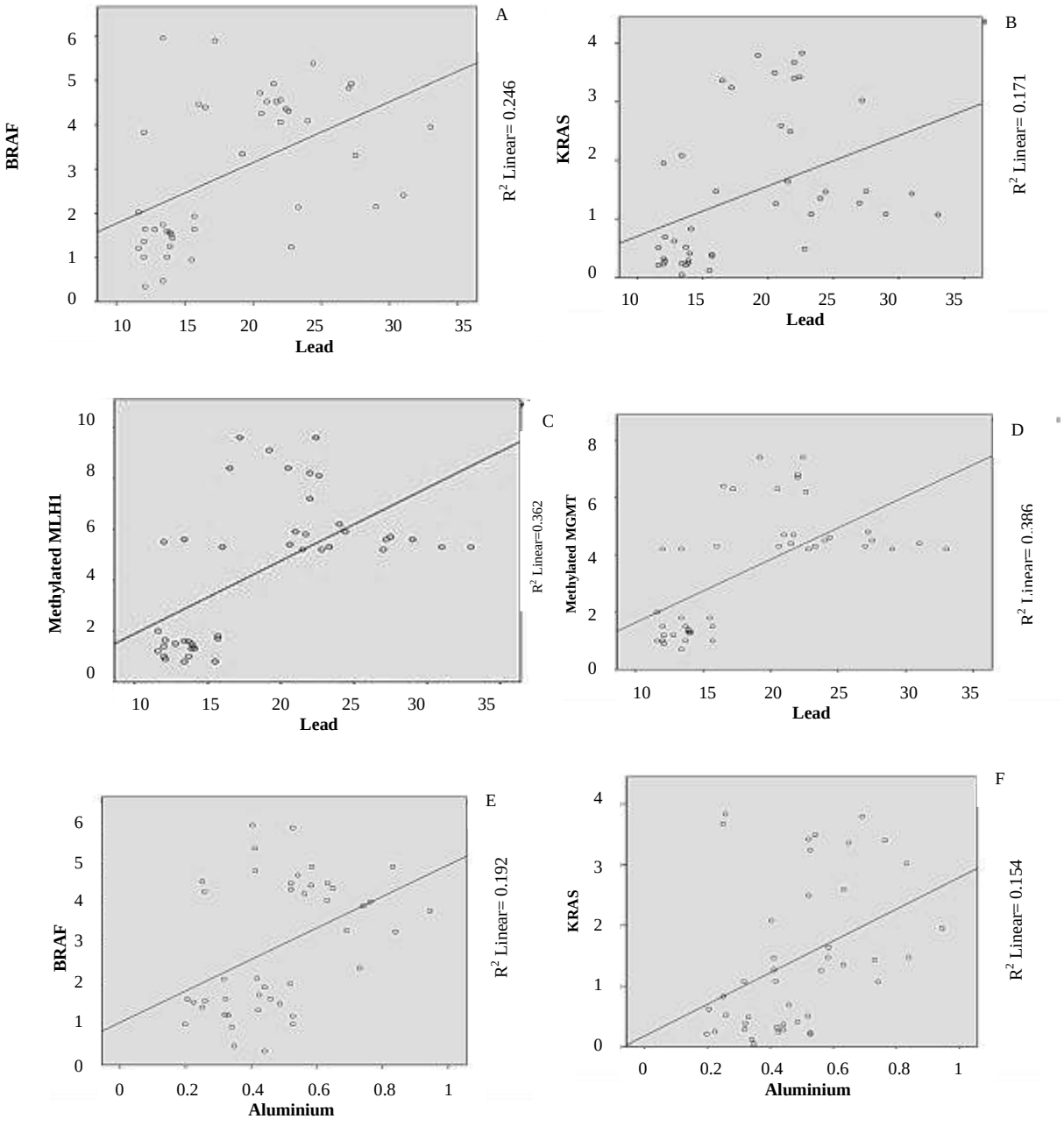

Figure 4. Correlation between Lead, Aluminum and BRAF, KRAS, methylated MLH1& MGMT

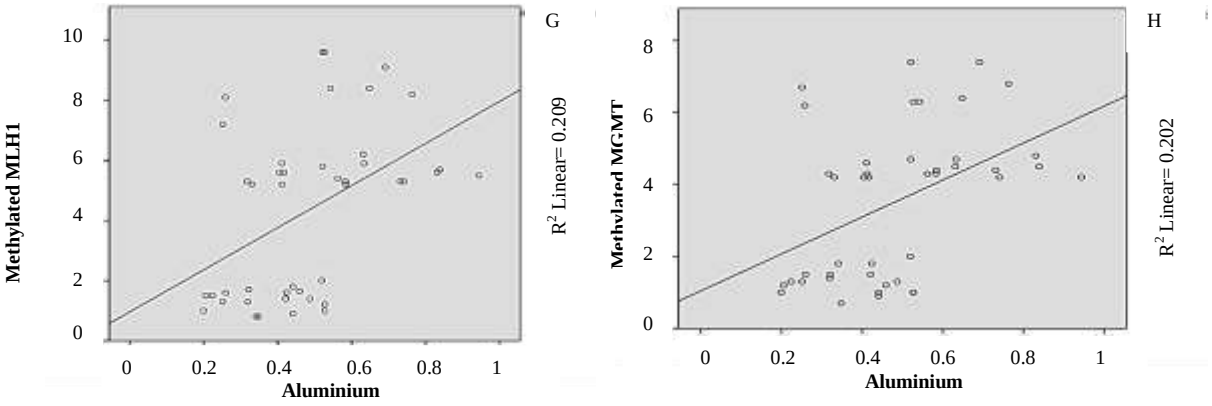

Figure 4. Continued.

BRAF, KRAS, methylated MLH1, methylated MGMT, Lead, Aluminum increase the risk of CRC

Regression analysis for studied parameter revealed that methylated MLH1, methylated MGMT, BRAF, KRAS, lead, aluminum significantly increase the risk of CRC (p value <0.05). (Table1)

BRAF, KRAS, methylated MLH1, methylated MGMT increased in advanced tumor stage

Methylated MLH1, methylated MGMT, BRAF, KRAS, expression significantly increased with more progression in tumor stage (p value <0.05), while no significant difference in lead, aluminum and Mn levels in different tumor stages (p value>0.05) (Table 2).

Table 1. Assessment of relative risk of studied parameters to develop CRC.

	P value	OR	95% CI
BRAF	0.012	160.22	3.07-8397
KRAS	0.006	180	13.7-9450
Methylated MLH1	0.02	0.16	0.145-0.184
Methylated MGMT	0.013	0.22	0.195-0.248
Lead	0.004	2.212	1.28-3.821
Aluminum	0.003	353	15.76-790
Mn	0.376	1.045	0.949-1.15

Table 2. Levels of all studied parameters in different tumor stages.

Tumor stage/variables	0	I	IIA	IIB	IIC	P Value
Braf	1.2±0.01	3.2±1.04	4.4±1.01	4.05±0.02	6.7±0.13	0.007
Kras	0.49±0.02	1.33±0.3	2.08±0.64	3.5±0.21	3.8±0.12	<0.001
Methylated MLH1	5.2±0.03	5.4±0.18	5.6±0.27	8.5±0.81	9.3±0.22	<0.001
Methylated MGMT	4.2±0.05	4.3±0.11	4.5±0.22	6.8±0.48	7.2±0.13	<0.001
Lead	22.8±8.5	26.11±6.9	20.7±4.3	20.3±2.4	24±0.24	0.19
Mn	55±12.3	41.8±5.6	43.37±7.7	39.5±8.6	40±7.2	0.41
Aluminum	0.33±0.04	0.62±0.24	0.56±0.13	0.52±0.16	0.63±0.02	0.6

DISCUSSION

CRC is a leading cause of death especially in industries worker besides lung cancer. It might be caused by exposure to heavy metal as lead and aluminum. We found that their serum levels are significantly higher than the normal control. This agreed with previous study that revealed significant difference in zinc, copper, aluminum and lead between cancerous and healthy tissues, which indicates the effect of environmental pollution in CRC development [10]. Another study reported that higher levels of Cu, Mg, Pb, Cr, Zn and Cd were significantly detected in patients with metastatic colon cancer compared to healthy subjects [11].

We found no statistical significant difference in Mn levels between CRC patient and normal participants., this coinceds with a study on metal distribution in colorectal biopsies that also revelead no significant difference between malignant and normal tissues [12] in contast to another study which reported that th level of Mn is significantly lower in malignant tissues than normal tissues [10].

Metals cause the overproduction of reactive oxygen species that induce DNA damage, lipid peroxidation [13].In addition, Metals can induce modifications on DNA repair proteins including, catalytic activities, compartmentalization and DNA binding, stability. Moreover, the positively charged metals can bind directly to DNA making it un assessable to the scanning of repair enzyme [14].

We found statistical significant increase in methylated MLH1 and MGMT in malignant tissues compared to normal tissues. Both lead and aluminum but not Mn were positively correlated with methylated MLH1 and methylated MGMT. This is agreed with previous study that revealed that metals induce MLH1 promoter silencing which can initiate carcinogenesis [15], and agreed also with previous study reported that methylated MLH1 and MGMT were elevated in the peripheral blood and malignant tissues of CRC patients [16]. Another study reviewed that metals alter the DNA methylation by both hyper- and hypo methylation modification. Metal can induce carcinogenesis by hyper methylation of DNA repair enzymes like MLH1 producing their silencing, and hypo methylation of proto-oncogenes producing their activation [17]. More study reported that metals target histone methyltransferases and demethylases, which affect histone methylation of gene promoter, leading to the silencing of specific tumor suppressor genes such as MLH1 [18].

Previous studies showed that lead exposure may modulate gene expression by affecting the epigenetic status. In addition, lead exposure induces alterations on DNA methylation in exposed workers with subsequent disturbances in the regulation of gene expression [19]. Another study for the effect of metals on genome reported that aluminum induces DNA damage and promotes methylation of DNA repair enzymes [20].

BRAF and *KRAS* are important members of RAS/RAF/MAPK signaling pathway, which regulates cell growth, differentiation, proliferation, and apoptosis in malignant and nonmalignant cells [21]. We found statistically significant increase in BRAF and KRAS gene expression in malignant tissue compared to normal tissues, together with their positive correlation with methylated MLH1 and MGMT repair enzymes on one side and lead, aluminum metal on other side. More over the expressions of BRAF and KRAS are significantly higher with advanced tumor stage. This agreed with a previous study reported that BRAF and KRAS are highly expressed in CRC [22] and MLH1 promoter methylation has been associated with *BRAF* and *KRAS* mutation in CRC [23]. More over MLH1 promoter methylation and *BRAF* and *KRAS* mutation have been correlated with tumor staging and tumor differentiation [24]. Other studies reported that KRAS and *BRAF* mutations are coexisted in CRC and are implicated in disease progression and therapeutic

responses [25], and mKRAS and mBRAF are prognostic factors in CRC patients [26]. Furthermore, another study revealed that, MGMT hypermethylation is associated with KRAS mutations in CRC [27], and there is Synergistic interrelationship between KRAS genes and MGMT gene promoter hypermethylation in CRC and these markers can be used as diagnostic/prognostic markers for CRC [28].

In conclusion, the industries workers are more liable to develop CRC due to metal exposure. Metal can initiate carcinogens by ROS production and DNA damage with subsequent mutation such as conversion of the proto-oncogenes BRAF/KRAS to oncogenes. Metals also can affect DNA repair mechanism by silencing methylation of repair enzyme like MLH1 and MGMT. The mutant BRAF/KRAS and methylated MLH1 and MGMT act synergistically for cancer progression and advancement. Thus, heavy metal exposure like lead, aluminum acts as start signal for carcinogenesis but not related to cancer progression. We recommended an experimental adenocarcinoma model for studying BRAF/KRAS inhibitors.

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethical approval

This article has Beni Suef University human consent and ethical approval.

Author contributions

Ahmed Talaat Abd Elaziz (Corresponding author): Practical research work and data analysis. Sahar Hassan Ahmed: Research design, data interpretation and analysis. Hanaa M Elzahed: Research design and data interpretation and analysis. Wael Fathi: clinical samples and results interpretation. All authors shared in writing and revision of manuscript.

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