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KRAS and *MT-CO1* genes in colorectal cancer: a molecular investigation

Harmand A. Hama[1*], Bestoon S. Hasan[2], Basak Barzngy[3], Saman S. Abdulla[4], Abdulkarim Y. karim[5,6], Rozhgar A. Khailany[5], Monika Miasko[7], Janusz M. Dabrowski[8], Barbara Pucelik[9]

[1] Department of Biology, Faculty of Education, Tishk International University, Erbil, Kurdistan Region of Iraq
[2] Awat Radiation Oncology Center, Erbil, Kurdistan Region, Iraq
[3] Oncology Center, Rizgary Hospital, Erbil, Kurdistan Region, Iraq
[4] Department of Oral Diagnosis and Oral Medicine, College of Dentistry, Hawler Medical University, Erbil, Kurdistan Region, Iraq
[5] Department of Biology, College of Science, Salahaddin University-Erbil, Kurdistan region, Iraq
[6] Department of Community Health Nursing, College of Health Technology, Cihan University-Erbil, Kurdistan Region, Iraq
[7] Department of Basic Science, Faculty of Dentistry, Tishk International University, Erbil, Kurdistan Region, Iraq
[8] Faculty of Chemistry, Jagiellonian University, Krakow, Poland
[9] Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland

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ABSTRACT

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide. The tumor suppressor gene *MT-CO1,* and Kristen Rat Sarcoma Virus (*KRAS),* an oncogene are primarily responsible for controlling cell apoptosis, cell cycle arrest, and cell proliferation, and any irregularities in these genes could lead to cancer. This study aims to examine the expression of *KRAS* and *MT-CO1* in CRC biopsy specimens and investigate their relationship with one another in CRC patients residing in the Erbil city of Kurdistan Region, Iraq. The study involved categorizing 42 sets of colorectal cancer tissues and their corresponding controls based on their types and patients' clinical characteristics. The expression of *KRAS* and MT-CO1 in the samples was assessed using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), with statistical significance set at $p<0.05$. The expression of *KRAS* was found to be significantly higher in CRC compared to the control (n=42, p=0.0001). On the other hand, the expression of MT-CO1 did not exhibit significant differences compared to the control group with a p-value of 0.12. Furthermore, the Chi-square and correlation analysis results depicted that *MT-CO1* expression negatively correlates with *KRAS* expression (p= 0.0001, r= -0.047) in CRC tissues. In conclusion, the variation in the expression of *KRAS* and MT-*CO1*, and their correlations could potentially serve as a good indicator in the detection and prognosis of CRC, which might lead to better translational research on the same. However, for a better understanding of the underlying mechanisms, further analysis is required.

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Introduction

Colorectal cancer (CRC) develops in both genders, with different rates of incidence. In women, CRC is 2nd most common cancer after breast cancer, while it is the 3rd most common cancer among men with a prevalence of 10.0% globally. It is also the 3rd most common cause of death among all cancer-related mortality (1, 2). As per the 2008 statistics, the incidence and mortality due to CRC is estimated to be 50,000 of the150,000 diagnosed cases (3). Colorectal cancer is expected to increase by 2030 to reach about 2.2 million cases or even more with a mortality rate of half number of the incidents about 1.1 million (4).

Various factors have been linked with CRC incidence and prognosis. consequently, As in almost all sorts of cancer, CRC starts with alterations on the molecular level such as gene mutation or the gene expression level changes mainly in oncogenes and tumor suppressor genes (5, 6). For the purpose of expression analysis, RT-PCR is a widely used technique that allows scientists to convert RNA molecules into DNA and amplify specific genes of interest. In addition By quantifying the amount of gene expression, RT-PCR provides valuable insights into the activity and regulation of genes, contributing to our understanding of various biological processes and diseases (7, 8)

The *KRAS* gene is an oncogene located on chromosome 12p12.1 which encodes the KRAS protein (9). The KRAS protein is a crucial protein that regulates the RAS/MAPK pathway. The proliferation signals transmit via the KRAS protein to activate proliferation-related genes in the nucleus (10). Therefore, mutations in the *KRAS* gene that lead to a constitutively active protein or up and down-regulation of its mRNA might result in cellular abnormalities and diseases including cancer. Mutations in *KRAS* are very common among all cancer types. Clinically, they account for 20–30% in lung cancer, 30–50% in colon and 70–90% in pancreatic carcinomas (11). Generally, mutated *KRAS* has a higher expression rate than their wild-type counterparts (12). Overexpression of mutated KRAS leads to an increase in cell proliferation in CRC also (13). Moreover, Zaanan, Okamoto (14) reported that mutated KRAS increases the BCL-2 expression, an antiapoptotic

* Corresponding author. Email: harmand.ali@tiu.edu.iq

protein thereby rate of apoptosis.

The *MT-CO1* gene also called Cytochrome *c* oxidase subunit 1 (MT-CO1) or COI is one of 37 genes on the mitochondrial chromosome between 5,904-7,445 (15). The CO1 is a part of the Cytochrome oxidase complex (COX) that takes the main role in reducing the molecular oxygen to water and oxidizes the reduced cytochrome c (16). At the same time, it has a role in apoptosis regulation, homeostasis of calcium, ATP synthesis and ROS production (16). CO1 gene doesn't have an intron region, and its expression results in CytcO subunit I which is a part of CytcO subunits, mutations or alterations in expression level cause various health problems including cancer. Mutations in these genes can lead to the apoptosis process mainly intrinsic apoptosis pathway since cytochrome c along with Apaf1 are responsible for proteasome production (17).

Our aim in this study is to investigate the expression level of *KRAS* and *CO1* gene among CRC patients from the Kurdistan region, Iraq. Moreover, to assess their expression rate correlation with CRC occurrence. Additionally, hence these genes have an impact on apoptosis to examine their expression correlation with each other in tissues of CRC patients.

Materials and Methods

Patients and samples

Overall, 84 tissue samples (42 tumor tissue and 42 control) from CRC patients were collected from Rizgary Hospital and Zhin International Hospital in Erbil, Kurdistan Region of Iraq between October 2018 and April 2019. The samples were preserved in RNALater at -20 ℃ until RNA analysis. The study was approved by the Human Research Ethics Committee of Salahaddin University-Erbil and followed ethical standards for human experimentation, including obtaining written consent from all participants. The study protocol was registered under "Trial registration: 4S/55 on April 4, 2022".

RNA extraction

To extract the RNA from the colorectal tissue samples the *ExiPrep*™ Tissue total RNA kit was used based on the manufacture instruction (Bioneer, Korea). To determine the quantity and quality of total extracted RNA the Biophotometer (Eppendorf, Germany, model: Biophotometer Plus 6132) was used.

Complementary DNA synthesis

The extracted RNA of *KRAS* and *MT-CO1* genes reverse-transcribed into cDNA according to the instructions included with the cDNA synthesis Ipsogen RT Kit (Qiagen, GmbH, Hilden, Germany), thus gene expression level can be measured. To regulate the required condition for the cDNA, thermal cycling processes Master-cycler pro-PCR System (Eppendorf, German) was used. Then, the cDNA was amplified using RT-PCR employing the expression primers described in Table 1.

Expression analysis of the targeted genes:

For *KRAS* and *CO1* expression detection, the RT-PCR method was utilized using Master-cycler pro-PCR System (Eppendorf, German) with RT^2 SYBR Green ROX FAST Mastermix (Qiagen GmbH, Hilden, Germany). Moreover, like internal control, Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was used as a housekeeping gene (20). The cDNA for *KRAS* and *CO1* were subjected to RT-PCR using a set of primers designed based on exon-exon junction using the online tool primer-BLAST. GAPDH primers were used as previously described by (20) and indicated in Table 1.

To perform PCR, 50µL of the reaction mixture was made in PCR tubes. This mixture contained 2.5 µL of cDNA template, OnePCR^{TM} master mix (GeneDirex, Korea), 1 µL of forward primer, 1 µL of reverse primer, and 20.5 µL of ddH2O. The cycling conditions were set as follows: initial denaturation at 95℃ for 10 minutes, 35 cycles of denaturation at 95 ℃ for 45 seconds, annealing at temperatures specified in Table 1 for 30 seconds, extension at 72℃ for 45 seconds, and final extension at 72℃ for 4 minutes.

Statistical analysis

The study utilized GraphPad Prism 7 to analyze data obtained from relative quantification RT-PCR performed in triplicate. The threshold cycle (Ct) values for *KRAS* and *CO1* were obtained and normalized using the ct-DNA of the housekeeping gene (GAPDH). The ΔCt method was used to calculate relative changes in gene expression for both tumor and control samples separately. Student's t-test was used to compare mRNA expression in colon and rectum cancerous tissues with their respective controls, with a significance level set at ≤0.05. Descriptive statistical methods such as mean, standard deviation, median, minimum, maximum rate, and frequency were used where appropriate.

Results

In this study, the expression profiles of *KRAS* and *MT-CO1* for 42 pair of samples of CRC patients at the Rzgary Hospital and Zheen International Hospital were studied.

It was found that *KRAS* expression was higher (over-expressed) in 38 tumor tissue samples as compared to their respective controls "Figure 1". The results revealed that the mRNA expression rate from tumor samples was signi-

Table 1. Primer sequences, PCR product size of the targeted region of KRAS, CO1, and GAPDH genes, and optimal annealing temperature.

Primer	Sequence 5'- 3'	PCR product Size	Optimum Annealing temperature	References
KRAS gene	F-TCTTGCCTCCCTACCTTCCACAT R- CTGTCAGATTCTCTTGAGCCCTG	211	64.3°C	(18)
CO1 gene	F- ATCCAGCAGCTGGAGGGTCT R- TGAATCTTGGGGGTTCGGCG	222	59.1°C	(19)
GAPDH gene	F- GGGTGATGCTGGTGCTGAGTATGT R- AAGAATGGGAGTTGCTGTTGAAGTC	700	68.1°C	(20)

ficantly higher as in controls (P value=0.0001), as calculated by student t-test; $p \leq 0.05$, "Figure 2". Besides in regards to *MT-CO1* expression, it was found to be higher in 20 and lower in 16 tumor tissues compared to their normal adjacent tissues (control) as indicated in Figure 3. However, statistically no significant difference has been found ($p = 0.1201$, T-test; $p > 0,05$) "Figure 4".

Following the relative expression of the *KRAS* and *CO1* gene, the link between *KRAS* and *CO1* expression in CRC was investigated. The statistical results of Chi-square and Pearson Rho tests for the association between *KRAS* and *CO1* expression showed a significant relationship with a value of $p = 0.0001$, and a negative correlation (Rho -0.047) as shown in Figure 5.

Discussion

The current study aimed to explore the profiles of *KRAS* and *MT-CO1* gene expressions in colorectal cancer (CRC) patients, and to study the potential link between the expression of these genes. This study is the first expression-based study of *KRAS* and *MT-CO1* among Iraqi CRC patients and their expression association has not been investigated previously. Consequently, the findings revealed interesting results regarding the expression patterns and potential association between the targeted genes.

Our results show that the gene expression of *KRAS* in tumor samples was significantly higher than in the non-cancerous ones "Figure 2". This suggests that the *KRAS* gene may play a crucial role in CRC, as its over-expression has been associated with various aspects of cancer development and progression. Similar results were also reported by other groups (12, 13, 21). Furthermore, the elevation of *KRAS* expression was detected in lung adenocarcinoma, pancreatic ductal adenocarcinoma, and colon adenocarcinoma samples (12). However, Nakayama, Funakoshi-Tago (22) has reported *KRAS* downregulation among colorectal cancer cell lines after treatment with less than 5% concentration of coffee.

The *KRAS* gene mutations happen in different cancer cases they result in expression rate alteration. Stephens, Yi (12) has confirmed that mutated *KRAS* gene generally has a higher expression rate than their wild-type KRAS counterparts, but not in all tumor types. The expression level of the *KRAS* gene can also impact the expression of Epidermal Growth Factor Receptor (EGFR) expression

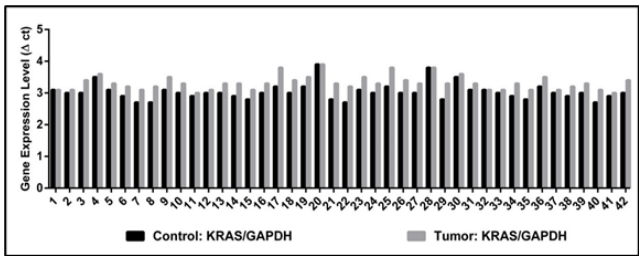

Figure 1. The mRNA expression levels of normal controls and tumors, according to Δct of KRAS/GAPDH. Among all samples, it was observed that the mRNA expression levels of 38 cancerous tissues were higher than those of normal control tissues.

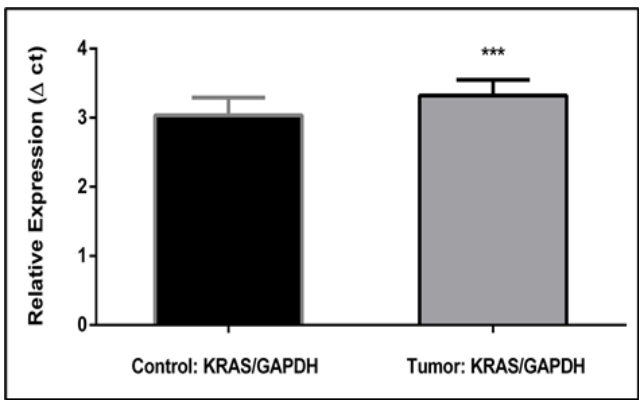

Figure 2. The statistical results for mRNA expression of *KRAS/GAPDH* gene in both normal controls and tumor samples among CRC patients (p=0,0001). The average mean of samples was (control = 3.035 and tumor = 3.321).

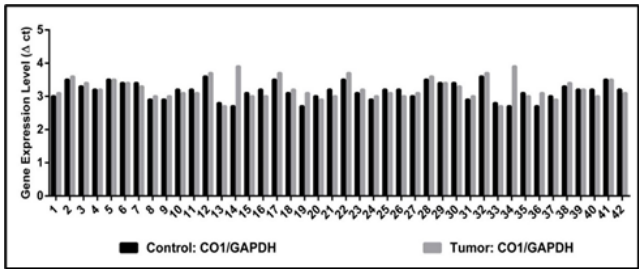

Figure 3. The mRNA expression level of each normal controls and tumors according to Δct of *CO1/GAPDH*. The mRNA expression level of 20 cancerous tissue were increased and 16 were decreased compared to normal control tissues.

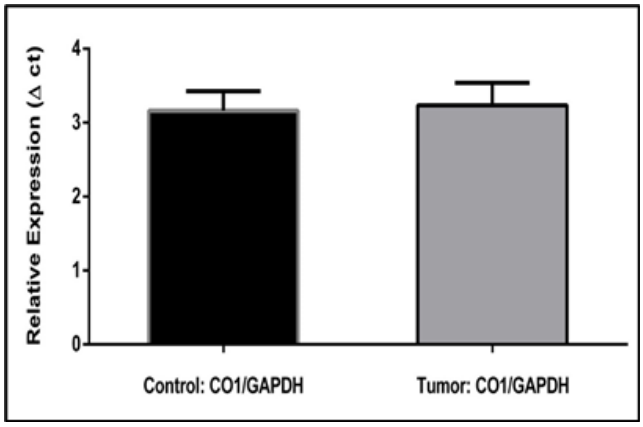

Figure 4. The statistical results of mRNA expression of the CO1/GAPDH gene in both normal controls and tumor samples among CRC patients (p=0,1201). The average mean of control samples = 3.161 and tumor = 3.233.

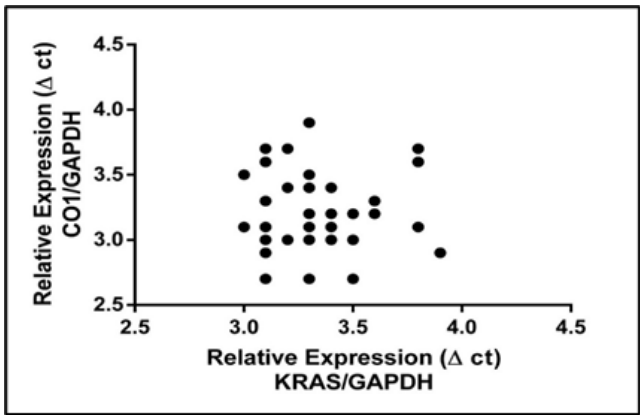

Figure 5. Correlation analysis of the expression of *CO1* and *KRAS* gene in colorectal cancer patients. The correlation coefficient (r= -0.04).

rate, miRNA and changes in those proteins which are responsible for the regulation of KRAS activities affect its expression level (12, 23). The mutated KRAS that expresses in higher amounts according to wild-type KRAS leads to increased cell proliferation in colorectal cancer (13, 24). The idea of KRAS decreasing apoptosis in CRC and other cancer types due to mutation is still debatable. Nevertheless, Okamoto, Zaanan (25) have reported that mutated KRAS increases the BCL-2 expression, an antiapoptotic protein thereby decreasing the rate of apoptosis. Moreover, declining in apoptosis as a result of KRAS mutation and overexpression was reported by Ward, Todd (26). The Liu, Jakubowski (13) group has concluded that KRAS mutation in a few cases increases spontaneous apoptosis. Furthermore, Maciag, Sithanandam (27) also reported that mutated KRAS in lung cancer resulted in COX-2 peroxidase activation and DNA damage that led to intrinsic apoptosis pathway activation. Although there have been contradicting results on the impact of KRAS on apoptosis, loss of apoptosis is the major hallmark of cancer (28, 29). We believe that *KRAS* mutation might be one of the reasons for preventing apoptosis mainly in CRC. It has also been suggested that the G12C KRAS and EGFR mutation should be concomitantly inhibited to overcome resistance to KRAS G12C blockade in CRC (30).

On the other hand, the results revealed that among 42 pairs of samples, the expression rates of mRNA for *CO1* in tumor samples were increased in 20 patients and decreased in 16 patients compared to the expression level of normal (control) samples. However, the statistical analysis did not reveal significant different as indicated in Figure 4. This suggests that *MT-CO1* may not be a key player in CRC, at least based on the expression level alterations. Despite of our findings, the alteration of *CO1* expression has been detected in some other cancer cases. Takesue, Kawakubo (31) has reported that *MT-CO1* expression has dramatically decreased in esophageal squamous cell carcinoma, thus resulting in losing the ability to activate caspase-3 in triggering apoptosis. Furthermore, alternation in the *MT-CO1* gene along with another gene that encodes for CytcO subunits II and III and their expression level changes has been found in prostate cancer (32). Similarly, Reznik, Wang (33) have reported that *MT-CO1* expression was downregulated in several cancer types including esophageal, head and neck squamous, breast, kidney clear cell, liver and colorectal cancers. However, they also mentioned that their expression differs from other mitochondrial genes the downregulation of complex IV (*MT-CO1, MT-CO2, MT-CO3)* genes and occurs in only those cancer types in which mtDNA and mtRNA are strongly depleted. The expression of *MT-CO1* in highly differentiated colon carcinoma was higher (overexpressed) compared to moderately or poorly differentiated colon carcinoma cell lines (34). Additionally, the expression of *CO1* directly affects Reactive Oxygen Species (ROS) production by mitochondria, which acts as a key effector mediating differential expression of apoptosis and DNA damage pathway-related genes (35). Wallace et al showed that *MT-COI* was overexpressed in late CRC among all their studied transcripts, suggesting that increased expressions in certain mt genes and elevated levels of ROS may potentially play a critical role in the colorectal tumors evolving from adenopolyps to malignant lesions (36)

The study also investigated the potential link between *KRAS* and *CO1* expression in colorectal cancer. The statistical analysis demonstrated a significant relationship ($p = 0.0001$) which implies that there is a correlation between the expression of these two genes in CRC. The negative correlation (Rho -0.047) as shown in Figure 5 suggests that higher *KRAS* expression tends to be associated with lower MT-CO1 expression in CRC patients. This correlation is close to zero, indicating a weak correlation, but it may imply a regulatory relationship between *KRAS* and *MT-CO1* or indicate shared pathways or mechanisms involved in CRC pathogenesis. However, we couldn't find previous studies that explain the correlation of *KRAS* with *CO1* expression. Nevertheless, Venesio, Balsamo (37) reported that *KRAS* mutations have a significant association with *MT-CO1* mutations in CRC. Consequently, the underlying molecular mechanisms and functional implications of this correlation remain to be elucidated, requiring further investigation.

Overall, these findings provide valuable insights into the molecular characteristics of CRC. The over-expression of KRAS in tumor tissues supports its potential role as a therapeutic target or a biomarker for CRC. Additionally, the observed correlation between KRAS and MT-CO1 expression suggests a potential interplay between these genes in CRC pathogenesis, although further investigation is warranted to understand the underlying mechanisms.

It is important to acknowledge the study was limited by less sample size, also conducting in only two specific hospitals may limit the generalizability of the finding. Moreover, gene expression analysis in our study focused only on mRNA levels, and further studies examining protein expression and functional assays are needed to validate and explore the functional significance of these gene expressions in CRC. Extensive research on a higher sample may be useful in delineating the mechanism and the trends of *KRAS* mutations in the Kurdistan population of Iraq and thereby devising targeted therapy in the future.

Conclusion

This study supports our understanding of the expression profiles of *KRAS* and *MT-CO1* genes in CRC patients. It provides evidence of increased *KRAS* expression among CRC patients. This suggests that over-expression of the *KRAS* gene could be a risk factor for CRC development, and it can be considered as a potential diagnostic biomarker for CRC. However, there was no significant difference in the expression of *CO1*, especially among CRC patients of this geographical region, indicating that *CO1* might not have a direct impact on CRC occurrence. Moreover, the noticed significant association with a negative correlation between *KRAS* and *CO1* suggests that there is a tendency for higher *KRAS* expression to be associated with lower *MT-CO1* expression in CRC patients. These findings demand further investigation with larger sample sizes and functional assays to validate the molecular interactions and functional implications of *KRAS* and *MT-CO1* in CRC.

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Conflict of interest

The authors declare no conflict of interest.

Author's contribution

All authors have contributed to preparing the current manuscript as the following; Harmand A. Hama; Conceptualization, methodology, formal analysis, data curation, visualization and writing—original draft preparation. Rozhgar A. Khailany; sample processing, investigation, methodology. Bestoon S. Hasan, Saman S. Abdulla, and Basak Barzngy; sample collection and resources, Monika H Miasko, Zanko H Jawhar; validation, review and editing, Abdulkarim Y Karim; editing, reviewing and supervision. Janusz M. Dabrowski and Barbara Pucelik; project administration, Review & Editing, and funding acquisition.

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