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ORIGINAL ARTICLE

Identifying Colibactin-Producing *Escherichia coli* as Candidate Biomarker for Aggressive Colorectal Cancer

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ABSTRACT

Background*: Colorectal cancer represents the third most common cause of carcinogenesis and aggressive cancer cases have been linked to some pathogenic microorganisms, including colibactin-producing Escherichia coli. There are few biomarkers for colorectal cancer diagnosis and prognosis. However, there have been documented clinical challenges in differentiating between aggressive cancers and those that are not.* ***Objectives****: This study was designed to investigate the genetic diversity of E. coli strains of both colorectal carcinoma-associated and non-associated E. coli strains.* ***Methodology:*** *Pathogenic E. coli isolates from benign (24) and malignant (80) colorectal tissue biopsies were characterized based on bacteriological analysis. The Pks (polyketide synthase) Island, which specifically targets of E coli genes such as clbB and uidA was examined using polymerase chain reaction.* ***Results:*** *The colibactin gene (clbB) was found to be increased significantly in the malignant (62%) compared to benign (0%) colorectal tissues (p<0.001). The presence of this gene was also associated significantly with high grade (p=0.042) and tumor size (p=< 0.001) of colorectal carcinoma. In contrast, there was no significant association between uidA gene and colorectal carcinoma clinical parameters.* ***Conclusion****: The aggressiveness and development of colorectal cancer may be related to the clbB gene. Further research is required to determine the role of this gene in colorectal carcinoma and it could be used as a biomarker to identify colorectal cancer.*

INTRODUCTION

Colorectal carcinoma (CRC) is one of the most significant health issues and ranks as the fourth most prevalent cancer globally and stands as the third leading cause of cancer-related mortality [1]. It affects men and women at about the same rate [2]. In the United States, it represents the second most significant cause of cancer-related mortality, resulting in over 600,000 deaths each year [3]. In Iraq, CRC is an essential contributor to deaths from gastrointestinal malignancies and ranks as the third most prevalent cancer in Iraq, accounting for 2,328 newly diagnosed cases in 2019 [4]. About 95% of CRCs are identified with adenocarcinoma [5], while, only 4 % of tumor cases were medullary CRC [6].

The diagnosis of CRC is mostly dependent on histopathological techniques, which offer comprehensive information on tissue abnormalities that is essential for prognosis and therapy planning[7]. Hematoxylin and Eosin (H&E) staining and processing methods are used to highlight the cellular architecture and abnormalities typical of malignancy in biopsies collected by colonoscopy or surgery [8]. The grading system evaluates the degree of tumor differentiation, ranging from well-differentiated to poorly differentiated tumors, providing insight into tumor aggressiveness and prognosis [9]. In addition, the staging system, such as the TNM (Tumor, Node, Metastasis) classification, assesses the extent of tumor spread, including factors like tumor size, lymph node involvement, and presence of distant metastasis, guiding treatment decisions and predicting patient outcome [10]. Furthermore, molecular testing methods such as In Situ Hybridization (FISH) and Polymerase Chain Reaction (PCR) provide important information about chromosomal abnormalities and genetic mutations linked to colorectal cancer, allowing for the development of customized treatment plans and prognostic evaluations [11, 12]. All these methods are essential for CRC diagnosis and treatment, providing individualized patient care and better clinical results.

In the human colon, there typically exists a complex ecosystem comprising trillions of commensal bacteria. The previous finding indicates that the gut microbiota has a significant role in the formation of CRC and colonic polyps [13]. *E. coli* represent an example of these commensal bacteria frequently found in the gut microbiota of both CRC patients and healthy

individuals. However, more pathogenic strains are often identified in CRC patients compared to healthy individuals [14]. *E. coli*, a Gram-negative, facultative anaerobic, rod shape, belonging to the family Enterobacteriaceae, commonly occur in the gastrointestinal tract of several mammals, including humans [15]. *E coli* is a natural resident of the intestines in many animals, including humans. Certain strains of E. coli have the potential to cause a range of intestinal and extra-intestinal illnesses, such as diarrhea, urinary tract infections (UTI), septicemia, and neonatal meningitis [16].

Phylogenetic studies have categorized *E. coli* strains into four primary groups (A, B1, B2, and D) [16, 17]. It has been observed that virulent extra-intestinal strains are predominantly associated with groups B2 and, to a lesser extent, group D, whereas most commensal strains belong to group A [16,17]. roup B2 strains are predominantly recognized for their extra intestinal pathogenicity, leading to conditions such as UTI, sepsis, and neonatal meningitis [17]. The B2 phylogenetic group is also associated with CRC due to the presence of a genomic island called polyketide synthetase (*pks*). This island contains the genetic code for the production of colibactin, a genotoxin that causes DNA damage, cell cycle disruption, mutations, and chromosomal instability in eukaryotic cells [18].

pks+ E coli infection in host cells prompt the manifestation of a senescence-associated secretory phenotype, characterized by the secretion of growth factors like hepatocyte growth factor. These factors induce proliferation in adjacent uninfected cells [19]. Consequently, a correlation has been noted between heightened E. coli colonization rates and increased prevalence of colibactin-producing strains (CoPEC) in patients diagnosed with stage III or IV tumors, suggesting a potential role in tumorigenesis [20].

The *pks* island may have a potential role to function as a catalyst for tumor growth in CRC and could serve as a reliable biomarkers for CRC diagnosis and prognosis. The aim of the present study is to exam the presence of specific colibactin genes (*clbB*) and *(uidA)* in both benign and malignant colon tissues and determine if they are associated with colorectal clinical data such as grade and stage using PCR.

METHODOLOGY

Ethics and patient samples

The study was conducted in Thi-Qar governorate, Iraq. The protocol was approved by the Al Hussein Teaching Hospital's ethical committee (No. 162 on July 8, 2023). As the study subject were patients with CRC who visited the Gastroenterology Department at Al Hussein Teaching Hospital in Al Nassiryha City and had their condition verified by histopathology reports. This study excluded patients with serious gastrointestinal infections and bleeding who were less than 20 years of age.

A total of 104 colorectal tissues samples were analyzed , including 80 CRC formalin-fixed, paraffin-embedded samples and twenty-four benign colorectal tissues. The polymerase chain reaction (PCR) assay was performed to evaluate targets genes of *E coli* such as *clbB* and *uidA* in these samples.

Tissue sample processing and DNA extraction

The samples were cut into 15 gm from formalin-fixed paraffin-embedded blocks using a microtome. These sections were collected in nuclease-free tubes and maintained at room temperature until DNA extraction and molecular were analyzed.

DNA extraction

DNA was extracted from each sample using the Promega DNA extraction kit (Genomic DNA Kit), according to the manufacturer's recommended protocol. Purified DNA was stored at −20 ◦C until further analysis.

PCR amplification:

A conventional PCR assay for the detection of *clbB* and *uidA* genes of *E. coli*. The primers used in this study are summarized in Table 1. The identification of these genes in colorectal tissue samples followed the methodology outlined by [15].

The PCR conditions used for colibactin genes amplification were as follow: an initial denaturation at 95˚C for 7 minutes, followed by 35 cycles of 30 seconds at 95 °C, annealing at 55˚C for 30 seconds, extension at 72˚C for 30 seconds, and a final extension at 72˚C for 7 minutes.Bands of the expected size from the PCR assay were excised from 1.5% agarose gels and DNA was purified through filter tips.

Table 1: The sequence of primers used in the study

Gene		Primer sequence	Tm (ºC)	Size of Product (bp)	Ref.
CIbB	*F*	GCGCATCCTCAAGAGTAAATA	55.8	283	(15)
	R	GCGCTCTATGCTCATCAACC	59.8		
uidA	*F*	TGGTAATTACCGACGAAAACGGC	61.7	147	(15)
	R	ACGCGTGGTTACAGTCTTGCG	63.7		

Statistical analysis

To calculate the data's mean, standard error, and standard deviation, Statistical analysis for Windows was used. The analysis included non-parametric, cross-table chi-square, independent sample t-test, one-way ANOVA and LSD, and Kendall's tau-b correlation. For statistical significance, a significance level of $p<0.05$ was used.

RESULTS

Clinical Data of the Study Population

The present study involved 80 CRCs (77.7%) and 24 benign colon tissues (22.3%) enrolled between September 2023 to March 2024 from the two defined hospitals in Thi-Qar, South-eastern Iraq. Of 80 CRC cases, 44 (55%) were males and 36 (45%) were females. The benign tissue were 13 (56.5%) males and 10 (43.5%) females. The age range was increased significantly in CRC patients compared to benign people (P: 0.001). Increased CRC cases were also reported in males compared to females. However, this data was not significant (P:0.776). In addition, people who reside in Urban areas have been found to have more CRC cases than those who live in rural areas (P:0.003).

The majority of CRC patients were in grade II (75%) compared to those with grade 1 (7.5%) and grade III (17.5) (P:<0.001). Furthermore, stage III was detected in almost 40% of CRC patients. In contrast, stages I and II were observed in 15% and 11% of CRC cases, respectively, and the stage was unknowable in the remaining cancer cases (p: < 0.001). The majority of CRC cases were in tumor size T3-4 compared to T1-2 (P:<0.001). In contrast, 18 cases of CRC were in the N0 stage compared to 8 cases with N1 (P:<0.001) Table 2.

Table 2: The distribution of malignant and benign patients according to age, sex

The clinical data			Number	%	P value
Number of samples	Benign		23	22.3	0.001
	Malignant		80	77.7	
Age range	23-32		4	5	0.001
	33-42		16	20	
	43-52		22	27.5	
	53-62		18	22.5	
	63-73		14	17.5	
	<73		6	7.5	
Sex	CRC	Male	44	55	0.776
		Female	36	45	
	Benign	Male	13	56.5	
		Female	10	43.5	
Residency	CRC	City	58	72.5	0.003
		Rural	22	27.5	
	Benign	City	12	52.2	
		Rural	11	47.8	
Grade	Grade 1		6	7.5	<0.001
	Grade2		60	75	
	Grade 3		14	17.5	
Stage	Stage 1		8	15.38	0.001
	Stage II		6	11.54	
	Stage III		20	38.46	
	N/A		18	34.62	
T category	T1-T2		7	8.75	<0.001
	T3-T4		20	25	
	N/A		53	66.25	
N category	N0		18	22.5	<0.001
	N1		8	10	
	N/A		54	67.5	
M category	M0		0	0	n/a
	M1		0	0	
	N/A		80	100	

Prevalence of *E. coli* genes in malignant and benign tissues

The study found both genes, *clbB, and uidA*, in benign and malignant colorectal tissues. Statistically, the *clbB* gene was found to be increased significantly in malignant (62%) compared with benign (0%) colorectal tissues (P:<0.001). In contrast, there was no significant association between *uidA* gene and CRC (Figure 1 &Table 3).

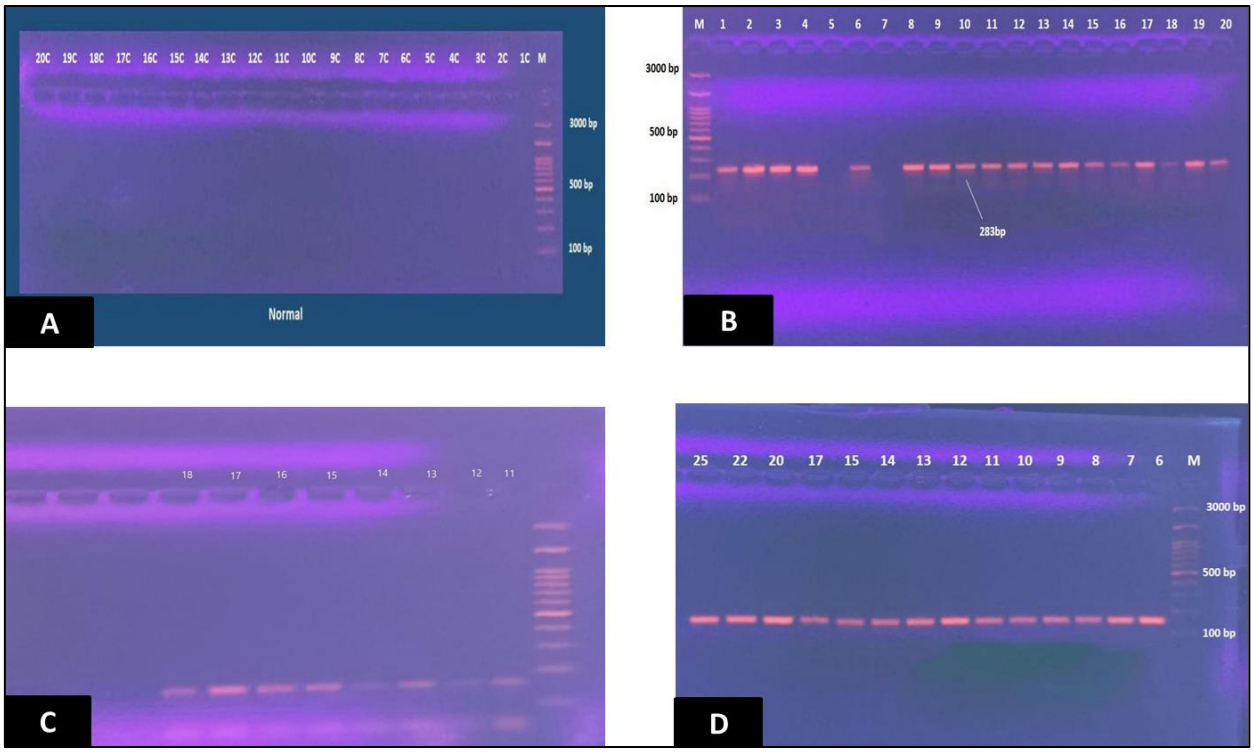

Fig. 1: Shows the presence of *E. coli* genes, *cIbB and uidA*, in malignant and benign tissues of colon. A) The benign colon tissue was free from *cIbB* gene. B) The CRC tissue showed the presence of *cIbB gene. C) uidA* gene was found in benign colon tissue. D) *uidA* gene was found in CRC tissue. The size of the PCR product is (283,147bp) respectively. The gel was 1.5% at 80 volt / 80 min. DNA ladder (100-3000).

Association between *E. coli* genes (*cIbB* and *uidA*) and CRC Parameters

The *cIbB* was increased significantly in CRC patients with grade III compared to those with grade I or II (P:0.042). This gene was also increased significantly in patients with grade III compared to those with grade 1 & II (P:0.042). In addition, a significant association was also seen between the presence of *cIbB* gene and tumor size (P:<0.001), but not with lymph node involvement.

The *uidA* gene results showed that there was no significant difference in the presence of this gene when comparing CRC tissues to benign tissues (p:0.72). In addition, there was no association between *uidA* gene and CRC parameters, including grade and stage (Table 3).

Table 3: Association between *E coli* genes (*cIbB* and *uidA*) with CRC clinical parameters

Tissue Gene	Clinical Parameter	Positive		Negative		Total		p. value
		No.	%	No.	%	No.	%	
cIbB	CRC	50	62.5	30	37.5	80	77.7	< 0.001
	Benign	0	0	23	%100	23	23.3	
	Grade I	2	2.5	4	5.0	6	7.5	0.042
	Grade II	42	52.5	18	22.5	60	75	
	Grade III	6	7.5	8	10.0	14	17.5	
	T1-2	6	23.08	0	0.00	6	23.08	< 0.001
	T3-4	12	46.15	8	30.77	20	76.92	
	N0	12	46.15	6	23.08	18	69.23	0.452
	N1	6	23.08	2	7.69	8	30.77	
uidA	CRC	76	95	4	5	80	100	0.72
	Benign	23	100	0	0	23	100	
	Grade I	6	7.5	0	0	6	7.5	0.091
	Grade II	58	72.2	2	2.5	60	75	
	Grade III	12	10	2	2.5	14	12.5	
	T1-2	6	23.08	0	0.00	6	23.08	-----
	T3-3	20	76.92	0	0.00	20	76.92	
	N0	18	69.23	0	0.00	18	69.23	.....
	N1	8	30.77	0	0.00	8	30.77	

DISCUSSION

The present study examined the presence of *pks* genes such as *cIbB and uid A* using PCR as a possible biomarker for CRC diagnosis and prognosis. Our study demonstrated a high incidence of CRC in males compared to females. This data agreed with the previous finding of Siegel et. al. [21]. This might be explained by men's tendency to consume a diet characterized by high levels of red meat, alcohol, and tobacco. Men are more likely than women to accumulate visceral fat, which is closely linked to an increased risk of CRC [21]. This data was disagreed with a study reported that males exhibit an elevated predisposition to CRC compared with females, predominantly attributed to hormonal differentials and other associated risk factors [22] .This data was contradicted by other studies which reported that the incidence of CRC was equivalent between men and women [13,23].

The current study also reported an increase in the CRC risk factor in elderly people compared to other age groups. This was consistence with the recent findings that the prevalence of CRC exhibits a notable increase in individuals aged 50 and above, with the propensity for disease manifestation escalating with each successive decade following the age of 40[1,24]. Malignancies are infrequent among individuals below the age of 40, except in cases where a substantial familial predisposition to the ailment exists [24]. The reason behind this observation lies in the age-related decline in immune functionality among the elderly populace compared to their younger people, consequently amplifying the susceptibility to CRC development Moreover, the prevalence of CRC was reported to be increased in urban area compared to the rural area. This data comes in agreement with the previous CRC report [25.] However, it disagreed with previous findings [26.] The reason for these differences may be that living in the city exposes people to many risk factors such as pollutants, industrial emissions, and vehicular exhaust as well as the type of food which is usually a home grow fresh food in rural areas.

Two genes linked to virulence factors were examined in this study using the PCR method. The current data showed no significant difference between the presence of uidA gene when comparing CRC to the benign tissues and, the difference in CRC grades and stages. This data agreed with previous findings [15, 27]. In contrast, it disagreed with other previous findings [28]. Moreover, this study aimed to assess whether uidA gene and CRC grade were linked. Our data indicated a negative correlation between uidA and CRC grade which agreed with findings of the previous data [15,19]. but It is in disagreement with another study [27]. The findings of the present study showed no significant difference between *uidA* gene and the stage of the disease. This was agreed with previous reports [15,19] but was disagreement with the study of Villariba-Tolentino *et.al.* [27]. This difference may be a result of the existence of the uidA gene in phylogroups A and B1are typically not

disease-causing and also present in Phylogroups B2 and D are associated with both intestinal and extra-intestinal disorders[29]. Taken together, these findings suggest that this gene was not associated with CRR development and prognosis.

The second gene linked to virulence factors was CIbB. Our results showed that the presence of this gene was higher in CRC tissue compared to benign tissues. This agree with previous findings [17, 30]. In contrast, the current result disagreed with the study of Villariba-Tolentino *et.al.* [27]. The observed variation could have emerged from the utilization of different tissue samples for molecular analysis, potentially exerting an influence on the resultant outcomes. In addition, There was a significant association between the presence of this gene and CRC grade. These results agreed with the findings of the previous data [27] but disagreed with another finding [19]. This difference may be a result of the size and type of samples.

The present indicated a significant difference between *CIbB* and the clinical stage. *CIbB* was observed to be higher in patients with tumor size T3-4 compared to those with T1-2. This result was consistent with a previous study [20,27] but it is inconsistent with previous reports of de Oliveira Alves *et al.* [31].The discrepancy may be because *pks+ E. coli* might impact carcinogenesis at various stages of cancer [30].

The present data showed no significant association between the presence of *cIbB* gene and the clinical stage N. This agreed with previous findings [15,19,32]. In contrast, it was disagreed with the study of Bonnet *et. al.* [20]. These differences may arise due to the involvement of regional lymph nodes in stages III and IV. During these stages, affected lymph nodes undergo alterations that can lead to lymphangiogenesis, and the formation of lymphatic vessels. This process may help in the elimination of harmful bacteria, such as *pks+ E. coli*, in the CRC advanced stages [30]. Certain strains of *E. coli* containing a gene cluster known as the *pks* island have been implicated in the development of CRC. Taken together, These data suggest that the presence of *cIbB* gene may increase the risk of CRC and is potentially associated with Clinical grade and stage of malignancy. This study's primary restriction is the absence of clinical information on metastasis because the tissue samples were obtained via endoscopy.

CONCLUSION

The presence of *pks+* bacteria appears to have a significant role in the progression of CRC, particularly concerning the colibactin gene (*cIbB*), which is associated with higher grades and advanced stages (T3-4). In contrast, *uidA* gene may have no role in CRC development and progression. Furthermore, determining the functional role of these genes in colon cell lines through tissue culture will be highly beneficial.

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Author contributions:

Oula and Dhafer conceptualized the project, conducted the experimental procedures, drafted the initial articles, and conducted the statistical analysis. Dhafer and Rash managed the data collection. Ola and Dhafer collaborated on writing, reviewing, and editing the material. The authors have reviewed and approved the final manuscript.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

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