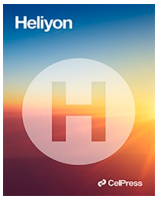
Heliyon 10 (2024) e23443

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Molecular and immunohistochemical study of *APC* exon 16 and its possible role in colorectal carcinoma development

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ARTICLE INFO

Keywords:
Carcinogenesis
Colorectal neoplasms
Exons
Human APC protein
Immunohistochemistry

ABSTRACT

Background: Colorectal cancer ranks second as a cause of cancer deaths. Mutations in the adenomatous polyposis coli (*APC*) gene, especially in exon 16, could contribute to colorectal carcinoma development. This study explored the correlations between *APC* gene exon 16 variations/expression and colorectal carcinoma progression.
Methods: In a case-control study, blood samples from 150 colorectal carcinoma patients and 50 healthy volunteers were analyzed by PCR and sequencing for *APC* exon 16 variations. The APC protein expression on tissue samples was evaluated by immunohistochemistry and statistical analyses were used to examine clinicopathological correlations.
Results: The sequencing analysis revealed a mutation in exon 16 of the *APC* gene (rs459552) in 36 % of colorectal cancer cases while absent in all non-cancer controls. Subgroup analysis by tumor grade showed higher prevalence of mutant allele in Grade II and Grade III cases, with frequencies reaching 60.0 % and 69.2 %, respectively, compared to a substantially lower prevalence of 29.4 % in Grade I patients. Immunohistochemistry showed no significant correlation between this mutation and APC expression. APC positivity proportions were 25.5 % in Grade I tumors (n = 26/102) versus 17.1 % in Grade II (n = 6/35) and 46.2 % in Grade III (n = 6/13), showing a non-significant trend of reduced positivity in higher grade tumors ($p>0.05$).
Conclusions: The frequency of *APC* exon 16 mutation (rs459552) rose significantly with increasing tumor grade. Similarly, although not statistically significant, the percentage of APC positive staining increased with poorer tumor differentiation, rather than declining. Therefore, the *APC* exon 16 mutation and expression analysis provides insights into colorectal cancer progression, with the rs459552 mutation correlating with grade and may promoting aggression.

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https://doi.org/10.1016/j.heliyon.2023.e23443
Received 7 June 2023; Received in revised form 1 December 2023; Accepted 4 December 2023
Available online 12 December 2023

1. Introduction

Colorectal cancer ranks as the second leading cause of cancer deaths. It is a highly prevalent and fatal cancer type [1]. Compared to Africa and Asia, Europe and North America exhibit higher incidence rates of colorectal cancer within gastrointestinal tract malignancies [2].

Typically, colorectal epithelial cells undergo programmed cell death (apoptosis) in a regulated fashion [3]. However, in abnormal conditions, these cells can proliferate uncontrollably and evade immune surveillance [4]. Both external influences like radiation, chemicals, smoking, diet, and lifestyle factors and internal factors such as inherited mutations, metabolic by-products, aberrant immune responses, and hormone dysfunction can trigger colorectal carcinogenesis [2,5,6]. The development may involve either solitary or multiple synergistic oncogenic hits in a temporal sequence [2,5].

Over a decade usually lapses between the initial oncogenic trigger and eventual carcinoma diagnosis. Available treatments include surgery, radiation, and chemical therapy [7]. Uncontrolled expansion of transformed cells by dysregulating cell proliferation and survival pathways facilitates tumor progression and metastasis [8,9].

Inherited and acquired genetic alterations represent principal mechanisms promoting colorectal oncogenesis [10]. The adenomatous polyposis coli (*APC*) gene on chromosome 5 (5q21.22) encodes a multi-functional tumor suppressor protein controlling Wnt signaling, cell adhesion, apoptosis, chromosome segregation, and other critical cellular processes [11]. The *APC* mutation constitutes an initiating step in the adenoma-carcinoma sequence of colorectal tumorigenesis. Both loss-of-function and gain-of-function effects may contribute to *APC* oncogenicity [11]. The former includes diminished cell-cell adhesion, dysregulated cell cycling, chromosomal instability and the latter includes augmented cell survival and motility [11–13].

Single nucleotide polymorphisms (SNPs) denote common genetic variants distributed throughout the human genome [14]. Their pathogenic contributions to cancer susceptibility are increasingly recognized and under investigation [15].

Therefore, this study aimed to explore potential links between specific exon 16 mutations in APC and the development and progression of colorectal carcinoma. Moreover, we analyzed associations between APC genetic status and certain clinicopathologic parameters.

2. Materials and methods

2.1. Study design, participants and ethics

This study utilized both molecular biology and immunohistochemistry techniques in a case-control design. It was conducted from January 2022 to February 2023 after approval by the Institutional Review Boards of Kufa University, College of Science (Kufa, Iraq) and Alsadr Medical City (Najaf, Iraq) with approval number of T/3206 (dated in 06-Dec-2021). All participants provided written informed consent prior to enrollment.

The study population consisted of 200 Iraqi adults who underwent colonoscopy or abdominal CT scanning between December 2021 and February 2023, followed by tumor resection surgery. They were categorized into a colorectal carcinoma (n = 150) with histologically confirmed adenocarcinoma or a non-cancer control group (n = 50) with colitis. The controls were age- and sex-matched to the patients.

Five milliliters of peripheral whole blood samples were collected preoperatively on vacutainer tubes containing EDTA as anticoagulant (Becton Dickinson, USA). These samples were maintained at 4 °C until receipt of corresponding formalin-fixed paraffin-embedded tissue specimens after surgical resection. Only samples with pathology-confirmed malignant transformation (adenocarcinoma) were selected for further analysis. The blood samples were labelled with the same unique numeric identifiers as the matched tissues.

Genomic DNA isolation and downstream molecular analyses were performed on the blood samples. Sections from the tumor tissues and margins were used for immunohistochemical assays.

2.2. Molecular studies

2.2.1. The primers

The polymerase chain reaction (PCR) primers used to amplify exon 16 of the APC gene were commercially synthesized by Macrogen Inc. (Seoul, Korea). The forward primer sequence was 5′-ACCCAACAAAAATCAGTTAGATG-3′ and the reverse primer was 5′-GTGGCTGGTAACTTTAGCCTC-3′, yielding a 410 base pair amplicon [16]. Lyophilized primer aliquots received at varying concentrations were first centrifuged at 13,000 rpm for 3 min. Then, each tube was reconstituted to 100 pmol/μl stock solutions using 300 μl nuclease-free water as instructed by the manufacturer. For preparing 10 pmol/μl working solutions, 10 μl of the primer stock solution (stored at −20 °C) was diluted with 90 μl nuclease-free water.

2.2.2. Genomic DNA isolation

Genomic DNA was extracted from peripheral blood samples using the ReliaPrep™ Blood gDNA Miniprep System (Promega Co., USA) per the manufacturer's guidelines [17].

2.2.3. DNA quantification

The extracted DNA concentration and purity were evaluated by measuring the absorbance at 260 nm and ratios of 260/280 nm

using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The quantification was performed as described previously [18].

2.2.4. PCR optimization

To identify the optimal annealing temperature for the *APC* exon 16 primers, DNA templates were amplified using the same primer pair at various temperatures: 55 °C, 58 °C, 60 °C, 63 °C, and 65 °C. The PCR was performed in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) using 20 μl reactions containing 10 μl GoTaq Green Master Mix (2X, Promega, USA), 1 μl of each primer (10 pmol), 6 μl of nuclease-free water, and 2 μl of template DNA. The cycling conditions were: initial denaturation at 94 °C × 4 min; 30 cycles of denaturation at 94 °C × 30 s, annealing at 55 °C, 58 °C, 60 °C, 63 °C, or 65 °C × 30 s, extension at 72 °C × 30 s; and final extension at 72 °C × 7 min, followed by a cooling step at 4 °C for 10 min.

2.2.5. PCR amplification

PCR was performed using the following thermo-cycling conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s; final extension at 72 °C for 7 min.

2.2.6. Agarose gel electrophoresis

Agarose gels were prepared and electrophoresed as described previously [19]. PCR products (5 μl) were loaded directly into the wells alongside 100bp DNA ladder before applying 70 V, 60 mA constant current for 60 min. Gels were stained with ethidium bromide and DNA migration visualized under UV light using a BioRad Gel Doc XR + system.

2.2.7. Standard sequencing

Sanger sequencing was performed by Macrogen Co. (Seoul, Korea)using an ABI 3730XL automated capillary DNA analyzer. Chromatograms received by email were analyzed with Geneious Prime software.

2.3. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were assayed for APC protein expression using immunohistochemistry protocols described previously [20–22]. Breast carcinoma and colon colitis tissues were used as positive and negative controls respectively. Primary antibody exclusion controlled for non-specific binding.

2.4. Statistical analyses

GraphPad Prism v8 (GraphPad Software Inc., USA) was used for statistical analyses. Continuous variables were presented as mean ± standard deviation and inter-group comparisons performed by two-tailed unpaired Student's t-tests (and Mann-Whitney's *U* test). Frequency and percentage (%) were used to present nominal variables, and the chi-square test was used to compare these variables between the studied groups. Associations were evaluated using chi-square analysis and the strength of associations were assessed by odds ratios with 95 % confidence intervals and *p*-values. The p value of ≤ 0.05 defined the statistical significance.

3. Results

The study included a total of 200 adults, with 150 diagnosed colorectal carcinoma cases and 50 non-cancer controls (Table 1). Among the patients, there were 77 males with a mean age of 69.74 ± 6.74 years and 73 females aged 65.88 ± 9.30 years.

Based on tumor grade, there were 102, 35, and 13 cases categorized as Grade I, II, and III respectively. Among males, 40 (51.9 %),

Table 1
General characteristics of the participants.

	Patients (n = 150)	Control (n = 50)	P
Age (years)	67.73 ± 8.32	65.9 ± 9.12	0.306
Sex (M/F)	77/73	30/20	0.287
Tumor grade, n(%)			
Grade I	102 (68 %)	–	–
Grade II	35 (23.3 %)	–	
Grade III	13 (8.7 %)	–	
Tumor site, n(%)			
Right colon	59 (39.3 %)	–	–
Left colon	59 (39.3 %)	–	
Rectum	32 (21.3 %)	–	
Histological type			
Conventional AC	82 (54.6 %)	–	–
Signet ring AC	49 (32.7 %)	–	
Mucinous AC	19 (12.7 %)	–	

Data is presented as means and standard deviation for age, frequencies for sex and as frequencies and percentages for the disease stage. AC = adenocarcinoma.

24 (31.1 %), and 13 (16.8 %) cases were Grade I, II, and III tumors, compared to 62 (84.9 %), 11 (15.1 %), and 0 (0 %) in females, representing a significant difference in grade distribution ($p < 0.05$, Table 1).

With regards to tumor locations, 59 cases each occurred in the right and left colon versus 32 were diagnosed in the rectum, without significant gender variations ($p > 0.05$). Specifically, 30 (38.9 %) cases were diagnosed in each right and left colon, and 17 (22 %) cases were diagnosed in the rectum in males. In females, 29 (39.7 %) cases were diagnosed in each right and left colon, and 5 (20.5 %) were diagnosed in the rectum.

Histopathological analysis identified 82 cases of conventional adenocarcinoma (AC), 49 mucin-producing signet ring AC, and 19 mucinous AC, without significant differences by gender ($p > 0.05$). In males, frequencies were 44 (57.1 %), 23 (23.8 %), and 10 (12.9 %) for conventional, signet ring, and mucinous subtypes, respectively. Corresponding values in females were 38 (52 %), 26 (35.6 %), and 9 (12.3 %).

The target 410bp region of APC exon 16 was successfully amplified, by PCR, from human genomic DNA samples. Agarose gel electrophoresis revealed specific bands in lanes 1–15 corresponding to the anticipated amplicon size, alongside a 100bp DNA ladder (Fig. 1).

Sanger sequencing chromatograms were analyzed for *APC* exon 16 amplicons from patients and controls (Tables 2 and 3, Supplementary Figs. 1 and 2). The rs459552 SNP resulting in a heterozygous A > T substitution was observed in 54 (36 %) patient cases compared to 0 controls (Table 2). The distribution of AA and AT genotypes significantly differed across tumor grades ($p = 0.001$, Table 3). The AA genotype, encoding the wildtype APC protein, predominated in 70.6 % of Grade I tumors but only 40.0 % and 30.8 % of Grade II and III cases, respectively. On the other hand, the mutant AT genotype was detected in 29.4 % of Grade I tumors while reaching frequencies of 60.0 % in Grade II and 69.2 % in Grade III. There was a trend toward enrichment of the mutant genotype with increasing de-differentiation status.

Further analysis indicated APC-negative immunohistochemistry staining in 71 SNP-negative colorectal cancer cases versus 25 SNP-negative cases with positive staining (Table 4). Among 54 SNP-positive cancer cases, 41 and 13 were biomarker-negative and –positive respectively. No significant associations found between SNP status and APC expression ($p > 0.05$) or colorectal carcinoma risk (odds ratio 1.11, 95 % CI 0.52–2.38, $p > 0.05$).

Immunohistochemistry identified APC protein expression in 38 colorectal carcinoma cases, including 26/102 (25.5 %) Grade I cases, 6/35 (17.1 %) Grade II cases, and 6/13 (46.2 %) Grade III cases (Table 5). The remaining APC-negative cases showed a distribution of 78 (69.6 %), 28 (25 %), and 6 (5.3 %) for Grades I, II, and III respectively. A non-significant declining trend occurred in positivity rates with increasing tumor grade ($p > 0.05$). As shown in Table 5, 26/102 (25.5 %) Grade I cases were APC-positive compared to 6/35 (17.1 %) Grade II and 6/13 (46.2 %) Grade III cases. Representative micrographs demonstrate heterogeneous APC immunostaining tensities in colon adenocarcinoma sections (Figs. 2–6).

4. Discussion

Colorectal or large bowel carcinoma arises from the uncontrolled proliferation of cells that line the colon and rectum. Normally, these cells undergo programmed cell death [23]. Various external (e.g. radiation, chemicals) and internal (e.g. inherited mutations) factors can prompt gene alterations that dysregulate cell cycle progression and apoptosis, ultimately enabling tumor formation [2,5,6]. While genetic mutations frequently occur, those presenting a selective advantage persist. Ultimately cells accumulate sufficient mutations to escape growth control [24]. Whereas normal cells respond to signals regulating processes like proliferation and survival, transformed cells lose this coordinated control [25]. Reflecting underlying genomic instability, accumulated mutations override internal fail-safes, permitting autonomous growth [26]. Though initial genetic hits occur years earlier, carcinoma diagnosis usually follows years afterwards, given the prolonged subclinical course. Available treatments aim to surgically excise the tumor, chemically

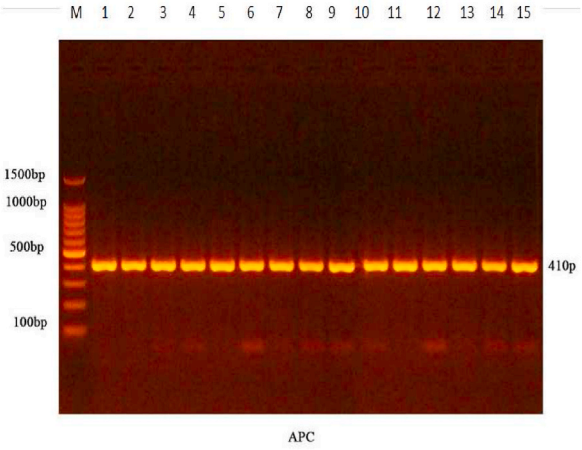

Fig. 1. Agarose gel electrophoresis of *APC* exon 16 PCR products.

Table 2
Occurrence of SNP rs459552 genotypes in study groups.

	Genotype	Patients group (n = 150)	Control group (n = 50)	*P*-value
		No. (%)	No. (%)	
APC gene (ID: 100992823)	AA	96 (64)	50 (100)	<0.001
	AT	54 (36)	0 (0)	

Table 3
Association between tumor grade and *APC* rs459552 genotype.

Tumor grade	AA, n(%)	AT, n(%)	Total	*P*-value
Grade I	72 (70.6)	30 (29.4)	87	0.012
Grade II	20 (57.1)	15 (42.9)	35	
Grade III	4 (30.8)	9 (69.2)	13	

Table 4
Association between *APC* SNP (rs459552) and protein expression.

	Expression		Odds ratio		
Genotype	+ve, n(%)	-ve, n(%)	OR	95 % Cl	*P*-value
AA	25 (26)	71 (74)	1.11	0.52–2.38	0.847
AT	13 (24)	41 (76)			

Table 5
APC immunohistochemical staining frequency by tumor grade.

	APC expression		Total	*P*-value
	+ve, n(%)	-ve, n(%)		
Tumor grade				
Grade I	26 (26.0)	76 (74.5)	102	0.121
Grade II	6 (17.1)	29 (82.9)	35	
Grade III	6 (46.2)	7 (53.8)	13	

Data is presented as frequencies and percentages for the disease stage.

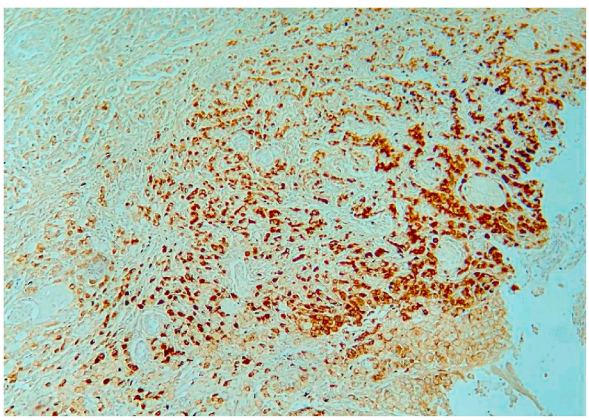

Fig. 2. Photomicrograph shows invasive adenocarcinoma with cells demonstrating strong, diffuse cytoplasmic and membranous staining for APC protein (immunoperoxidase, original magnification 100X).

or physically destroy cancer cells, or modulate biological pathways [7].

Tumor grading represents a microscopic assessment of how closely cancer cells and tissue architecture resemble their non-neoplastic counterparts [27]. Grade I means well-differentiated morphology with minimal deviations; Grade II is moderately

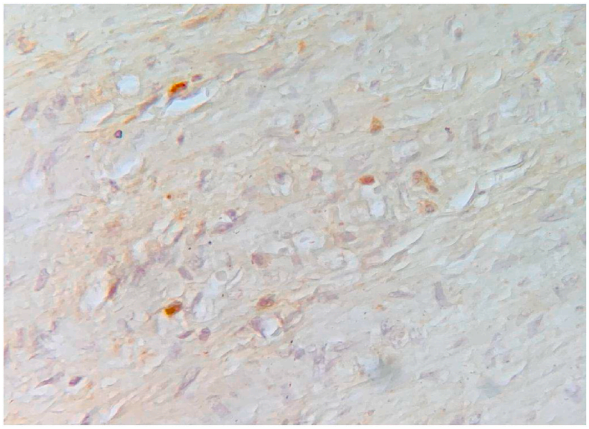

Fig. 3. Poorly differentiated colon adenocarcinoma section with rare tumor cells displaying moderate intensity APC immunoreactivity (immunoperoxidase, original magnification 400X).

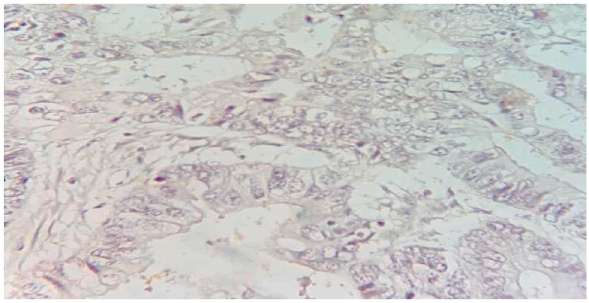

Fig. 4. Well-formed colon adenocarcinoma gland lacking discernible APC staining (immunoperoxidase, 400X).

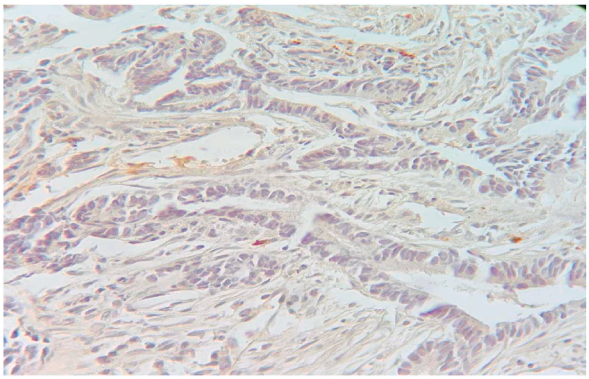

Fig. 5. Section of well-differentiated colonic adenocarcinoma without detectable APC immunohistochemical labeling (immunoperoxidase, 400X).

differentiated, Grade III exhibits poorly differentiated features portending aggressive clinical behavior [27]. Among the current 150 colorectal carcinoma patients, 102 (68 %) were classified as Grade I, 35 (23 %) as Grade II and 13 (9 %) as Grade III. Prior studies noted between 7.5 and 23 % Grade III incidence proportions [28–30]. Besides, Grade I representation ranged from 4.4 to 28 % across these studies. Reasons underlying variability in subgroup frequencies warrants further investigation but could signify population-specific factors influencing tumor differentiation pathways during colorectal oncogenesis.

The sequencing analysis in the current study identified the *APC* gene SNP rs459552 (A > T substitution) in *APC* exon 16 among 54/150 (36 %) colorectal cancer patients versus 0/50 (0 %) non-cancer controls. Moreover, it also showed that the AT genotype frequency increased significantly along with advanced tumor grades. Immunohistochemistry revealed no significant correlation between this mutation and APC protein expression. Among mutant cases, 41 had negative and 13 had positive APC staining. These results indicate the rs459552 polymorphism without diagnostic or prognostic utility in colorectal cancer. Our findings differ from some other studies

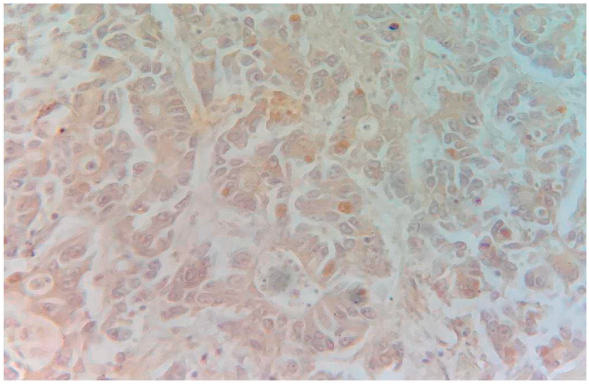

Fig. 6. Focal moderate APC immunopositivity observed in a subset of colonic adenocarcinoma cells (immunoperoxidase, 400X).

examining rs459552 in colorectal cancer [16,31,32]. Feng et al. detected this mutation in 24/196 (12 %) patients compared to 53/279 (19 %) controls, with no significant differences ($p = 0.062$) [16]. Collectively, these data argue against a pathogenic role of this specific *APC* mutation in colonic tumorigenesis among the studied population. The conflicting findings between studies on the rs459552 variant may be due to differences in patient populations or the limited sample sizes. Further mechanistic work is still needed to definitively rule out possible impacts of this variant on *APC* tumor suppressor function that may translate to subtle or context-dependent colon cancer risk. In the meantime, analyses incorporating environmental factors or other colon cancer risk SNPs may uncover contributions of the rs459552 variant through gene-gene/gene-environment interactions.

Immunohistochemistry data showed a trend towards decreasing APC positivity with advancing tumor grade, categorized as 26 % APC-positive among Grade I cases compared to 17.1 % in Grade II and 46.2 % in Grade III. At first glance, the Grade III frequency appears higher than Grade I. However, the small Grade III subgroup size (n = 13) limits the statistical power. In contrast, among rs459552 SNP-positive cases, mutation frequency increased significantly with grade, from 29.4 % in Grade I up to 69.2 % in Grade III. While overall APC loss loosely correlates with poorer differentiation, rs459552 variant enrichment associates specifically with higher grade lesions in the studied cohort.

This distinct pattern suggests unique influences of rs459552 versus APC loss-of-function. The D1822V substitution occurs within a mutation cluster region rather than causing truncation [16,33,34]. Structural impacts on the APC protein likely differ from truncating mutations associated with total loss of expression [35]. Additional mechanistic work should investigate rs459552 effects on beta-catenin regulation and other signaling involved in tissue architecture and aggression.

The limitations of the current study include the small subgroup sample sizes, particularly for higher grade tumors, restricting statistical power for some analyses, as well as the lack of mechanistic functional investigation into the specific impacts of the studied *APC* exon 16 mutation on pathways involved in cancer progression.

5. Conclusions

To conclude, this study found the frequency of the *APC* exon 16 mutation rs459552 rose significantly with increasing tumor grade. Moreover, the percentage of APC-positive immunohistochemical staining trended higher in less differentiated Grade III tumors. However, small subgroup sizes limited statistical power for differentiation analyses. While the mutation and expression data here provide initial clues into factors enabling tumor aggression, further research on larger cohorts should explore relationships between specific *APC* variants, maintenance of tissue homeostasis, and the acquisition of invasion capabilities during malignant transformation. Elucidating the context-dependent functional effects of particular exon 16 substitutions would offer enhanced understanding of signaling pathways controlling colorectal cancer advancement.

Funding resources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author (HKA: hamid.altameme@yahoo.com).

CRediT authorship contribution statement

Conceptualization: HKA, RMA, RHA. Data curation: HKA. Formal analysis: HKA. Funding acquisition: N/A. Investigation: HKA. Methodology: HKA, HAA. Project administration: HKA, RMA. Resources: HKA, HRA, DAA, MTA. Supervision: RMA, RHA. Validation:

HKA, MTA. Visualization: HKA. Writing – original draft: HKA, DAA. Writing – review & editing: RMA, RHA, HAA.

Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23443

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