TURKISH JOURNAL of ONCOLOGY

Turk J Oncol 2024;39(2):135–143
doi: 10.5505/tjo.2024.3774

ORIGINAL ARTICLE

Alterations in P53 Gene Expression in Experimental Colon Carcinoma of Wistar Rat

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OBJECTIVE

Colorectal cancer (CRC) is a major cause of mortality in the human population worldwide. After prostate and breast cancers, it is the third most prevalent cancer around the world. The tumor suppressor gene, P53, is one of the significantly mutated genes in gastrointestinal cancers, including CRC. The present study was conducted to delineate the alterations of P53 gene expression in an experimental colon carcinoma in Wistar rats.

METHODS

In this study, 20 male Wistar rats were divided into two groups, receiving phosphate buffered saline (PBS) as a control group or dimethylhydrazine (DMH), 40 mg/kg (s.c.) twice a week for 8 weeks, as the treatment group. Subsequently, biopsies were performed, and hematoxylin and eosin (H&E) staining followed by immunohistochemistry (IHC) analysis were conducted to examine changes in P53 gene expression.

RESULTS

Injection of DMH caused different types of colon tumors, including mucinous (40%), cribriform comedo-type (20%), undifferentiated (20%), signet-ring cell (10%), and serrated (10%), in which the rate of P53 gene alterations was shown to be high. According to T-test statistics, there is a statistically significant association between colonic adenocarcinoma slides and the presence of the P53 antigen-antibody complex ($P<0.05$).

CONCLUSION

In conclusion, this study confirmed the occurrence of dramatic changes in P53 gene expression in a laboratory model of colon cancer. However, more studies are required to determine the type and extent of these mutations, as well as the involved exons and introns.

Keywords: P53 gene; colon carcinoma; immunohistochemistry; Wistar rat.

INTRODUCTION

Cancers represent a pernicious class of pathologies with multiple clinical and mental consequences that can develop in different body organs, leading to millions of deaths globally.[1,2] An array of functional mechanisms is employed by normal cells, which subsequently turn them into a neoplastic growth state; these are considered cancer hallmarks, including genome instability, immortal proliferation signaling, resisting

Received: December 05, 2022
Revised: March 27, 2023
Accepted: January 13, 2024
Online: February 27, 2024

Accessible online at:
www.onkder.org

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cell death, inducing angiogenesis, evading growth suppressors, and metastasis.[3] Tumors developing in the colon and rectum, commonly referred to as colorectal cancers (CRC), are the third cause of cancer-associated mortality in both men and women, following lung, prostate, and breast cancers.[4] Reportedly, CRC initially develops as hyperplastic cells in colon epithelia, which may further advance to polyp-adenoma and carcinoma, respectively, due to several risk factors such as inflammatory bowel disease (IBD), having a history of CRC in a first-degree relative, cigarette smoking, physical inactivity, and red meat consumption.[5,6]

Most colon cancers are classified as sporadic or familial,[7] based on two distinct genomic instability pathways: i) aneuploid tumors with chromosomal instability (CIN), which dysregulate proliferation checkpoints in DNA,[8] and ii) diploid tumors with microsatellite instability (MSI) and mutating pathways.[9] The sporadic CRC of CIN-expressing phenotype is characterized by aneuploid cells, multiple chromosomal rearrangements, and the aggregation of somatic mutations in tumor suppressor genes such as adenomatous polyposis coli (APC) (80% of sporadic cases) and Kirsten rat sarcoma virus (K-ras).[10,11]

A set of potential biomarkers has been elucidated for CRC prognosis, encompassing APC, mismatch repair (MMR), K-ras, and the tumor phosphoprotein (P53) genes, as well as loss of heterozygosity in the 18q allele.[12,13] One of the major tumor suppressor genes, P53, is a 393-residue phosphoprotein containing four subunits for protein tetramerization, DNA detection, transcription, and identifying DNA damage. The protein plays a critical role in the termination of cellular growth, DNA repair process, apoptosis, oxidative stress, and cell proliferation. Studies have reported that about 60% of CRC patients demonstrate mutations in the P53 gene. Although the prognostic value of this gene is debatable, it has mostly been used as a prognostic biomarker by pathologists in both benign and malignant tumors.[14,15] There are histologic and genetic similarities between rodent and human colons;[16] hence, rodent experimental models (rats) are currently used to investigate carcinogenic mechanisms and to determine target genes and novel biomarkers of CRC. Colon carcinogenesis in laboratory rat models can be induced by dimethylhydrazine (DMH) and azoxymethane (AOM)/dextran sodium sulfate (DSS) agents.[17] The present study was performed to assess the mutation spot in exons of the P53 gene, to characterize the rate of pathologic changes, and to establish immune complexes in experimental colon carcinoma of Wistar rats.

MATERIALS AND METHODS

Animals and General Conditions

In this study, a total of 20 Wistar rats (aged 20 weeks, weighing 300±10 grams) were purchased from the Razi Vaccine and Serum Research Institute (Karaj, Alborz) and maintained for a week to adapt in cages (5 animals per cage) at 22°C temperature, 55±10% humidity, and 12 h light/12 h darkness cycles.[18] The main experiment was conducted in the Mashhad Pathobiology Laboratory, Mashhad, Khorasan Razavi province.

Experimental Groups

The animals were randomly divided into two groups: 1) the first group (N=10) was subcutaneously (s.c.) injected with normal saline as a control group, and 2) the second group (N=10) received twice-weekly s.c. injections of DMH dissolved in EDTA (40 mg/kg) for 8 weeks to induce colon carcinoma. All procedures were conducted in accordance with the ethical guidelines of the Islamic Azad University, Sanandaj Branch.

Dissection of Rats and Colon Sampling

Eight weeks after tumor induction, sampling was conducted from the distal colon in animals of both groups for histologic evaluation and immunohistochemical analysis regarding P53 gene alterations. For this purpose, rats were anesthetized using ether, then ethically euthanized via intracardial injection of potassium chlorate. A fine incision was made in the lumbar area of the rats, and colon samples were transferred to a 10% formalin solution. A day later, the formalin solution was changed, and all specimens were stored for 2 weeks in darkness under monitored temperature and humidity.

Histologic Sections Stained with Hematoxylin and Eosin (H&E)

For the preparation of histologic sections, all specimens were initially dehydrated using a Tissue Processor device as follows: specimens were dehydrated by passing through ethanol concentrations of 50%, 70%, 80%, and 96%, as well as two absolute ethanol containers, respectively, for 2h per container. Samples were cleared using xylene impregnation twice (2h per container). Finally, impregnation was performed three times in pure paraffin (melting temperature: 58°C), for 2 hours per step. Then, specimens were placed into special blocks, filled with melted paraffin, cooled down, and ultimately subjected to a rotational microtome device to prepare histologic cuts (thickness: 4 μm). The resulting slices were placed in a 50°C water bath, then transferred onto a glass slide and subjected to a 37°C incubator for drying.

H&E staining was applied to each prepared tissue specimen following this procedure: excess paraffin was removed from each specimen using xylene impregnation twice (10 min per container). The hydration of the samples was accomplished through absolute, 95%, and 70% ethanol (1 min per container), washed with distilled water, and placed in hematoxylin stain (20 min), then rinsed. Subsequently, a 20-second impregnation was done in a combination of hydrochloric acid (1 cc) and 70% ethanol (99 cc), then washed again. Next, five-time soaking was done in lithium carbonate to achieve a light blue color, washed for 15 min, transferred to eosin for 5 min, then dehydrated using 70% and absolute ethanol (2 min per container). To clear the slices, they were also passed through two xylene wells (2 min per well). For long-term storage of prepared sections, they were covered on a glass slide with a cover glass and histologic adhesive.

Immunohistochemical Analysis

A new section (3 μm thick) was prepared from paraffin-embedded blocks of those slides positive for colon cancer in histologic evaluation. Immunohistochemical staining for P53 gene alterations was performed using the Benchmark XT automatic slide preparation system (Ventana Company, Arizona, USA), as follows: slices were initially placed in a water bath (56–60°C, 5 min), then transferred onto positively charged Poly-L-lysine coated glass slides and soaked in three xylene wells (5 min each) to remove paraffin. The sample hydration was done by placing them in absolute, 95%, 75%, and 50% ethanol (10 min each) and twice washing with distilled water. The antigen retrieval step was done using heat induction, so that slides were incubated in Tris-EDTA at 98 °C for 20 min, then cooled down to ambient temperature. Triple washing was done with distilled water for 5 min, and slides were incubated in 3% hydrogen peroxide for 10 min at room temperature, to terminate the endogenous peroxidase activity that may cause false-positive results. Twice washing (5 min) was again performed and slides were incubated in tris-buffered saline (TBS) for 5 min. A hydrophobic barrier pen was utilized to delineate the sample section and a UV inhibitor blocker agent was applied to each slide for an hour at room temperature. This blocker agent was further eliminated by TBS washing, specific anti-P53 antibodies (Ventana Company, Arizona, USA) were applied to the slides for 30 min, washed with TBS, and secondary antibodies were used for 20 min. The slides were triple washed with TBS (5 min each), diaminobenzidine (DAB) substrate and H_2O_2 were added to form a brown color, the washing step was repeated, then a UV copper solution was added (5 min) for better coloring. After TBS washing, hematoxylin stain was applied for 3 min, rinsed with tap water (3 min) and hydrated using 50%, 75%, 95% and absolute ethanol (10 min each), placed three times in xylene, then finally sealed with proper adhesive and alterations in the P53 gene were evaluated using the standard protocol of the World Association of Pathologists.[19]

Table 1 Classification of the colon cancers induced by DMH in Wistar rats

Classification	Properties	n	%
Staging	Stage 1	–	–
	Stage 2	2	20
	Stage 3	5	50
	Stage 4	3	30
Lymphatic metastasis	Negative	2	20
	Positive	8	80
Vascular invasion	Negative	2	20
	Positive	8	80
Tumor size (mm)	Negative	1	10
	Positive	9	90

DMH: Dimethylhydrazine

Statistical Analysis

All data were analyzed using SPSS version 22 for Windows. The statistical analysis was performed using the T-test with a 5% level of significance.

RESULTS

Histopathology Results

Based on H&E staining of paraffin-embedded colon specimens, the normal intestinal structure was seen in Wistar rats of the control group, whereas aberrant crypt foci (ACF) induced by DMH injection along with hyperplastic epithelium and infiltration of mononuclear cells were observed in the cancer group. It is noteworthy that ACF is a possible predisposing factor to form adenoma and adenocarcinoma; hence, they can assist us in the early detection of cancers. Moreover, 5 types of CRC were recognized in experimental animals, including mucinous (40%), cribriform comedo-type (20%), undifferentiated mucinous signet-ring cell (20%), signet-ring cell (10%), and serrated (10%), based on the WHO classification (Table 1).

Immunohistochemistry (IHC) Analysis

Significant variations in P53 protein level were observed in histologic colon sections of DMH-treated rats, in comparison with the control group. Those

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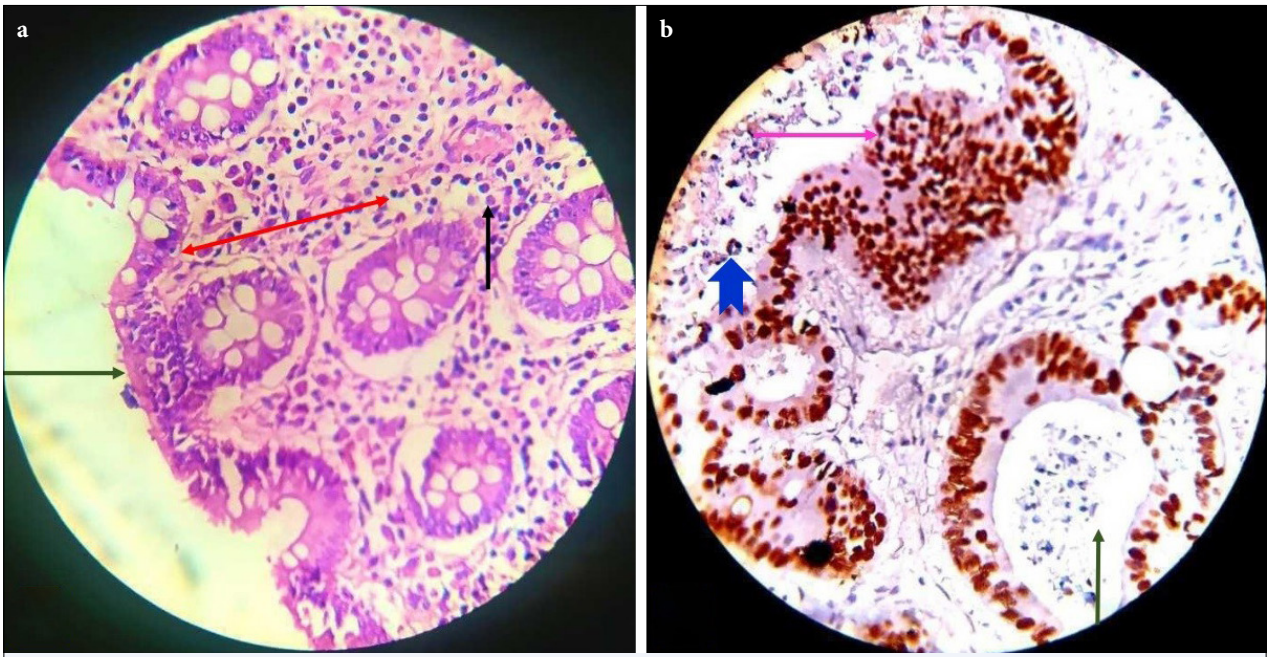

Fig. 1. Tubular adenoma comprising sample dysplastic crypt-like glands. (a) red arrow: sections of ABC hyperplastic crypts, green arrow: hyperplastic proliferation, black arrow: mononuclear infiltrate around crypts; H&E stain (b) specific detection of P53 protein (color tonality: 4), green arrow: dirty necrosis within gland lumen, pink arrow: granulomatous status (++), blue arrow: macrophage infiltration (+); IHC.
ABC: The avidin-biotin-peroxidase complex; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry.

slides identified as adenocarcinoma were sorted based on Duke's staging system (A-D), so that Duke-0 is the first stage, at which CRC is still within the inner layer of the colon or rectum, so-called as carcinoma. Other stages include the outgrowth of the tumor in the submucosal layer (A), the intestinal muscular layer (B), involving draining lymph nodes (C) and metastasis to other body parts such as liver, lungs, and ovaries (D). This staging was validated using TNM criteria.

In the following, slides were evaluated regarding the formation of a color complex for P53. Based on Figure 1, tubular adenoma was formed, consisting of a sample, dysplastic and crypt-like glands; ACF and mononuclear cell infiltration around crypts as well as detected P53 protein using specific antibodies were shown. In Figure 2, signet-ring cell carcinoma, as a rare CRC, was a pronounced finding, characterized by mucinous, foamy intracytoplasmic vacuoles that push aside the nucleus. Another major finding was a moderately differentiated adenocarcinoma with complex glandular structures in a desmoplastic stroma (Fig. 3) as well as traditional serrated adenoma with low-grade cytologic dysplasia and eosinophilia (Fig. 4). Furthermore, mucinous adenocarcinoma was observed, showing abundant extracellular mucin (Fig. 5). Based on the tumor staging system, most tumors belonged to stage 3 with a 50% expression level, followed by stage 4 (30%) and stage 2 (20%). Most tumors (80%) had lymphatic metastasis, vascular invasion (80%) with a tumor size over 3 millimeters (90%). The highest color tonality in association with P53 protein expression in different types of cancer belonged to slides number 1, 2, and 8 as well as signet-ring cell carcinoma, moderately differentiated adenocarcinoma, and tubular adenoma. Based on T-test statistical results, there was a remarkable significant association between colon adenocarcinoma slides and the level of P53-specific antigen-antibody complexes ($p<0.05$).

DISCUSSION

Colon cancer is one of the principal causes of mortality due to gastrointestinal cancers worldwide. Still, there is a paucity of data on the apoptotic and anti-apoptotic genes involved in CRC. Apoptosis is a physiological response to stress, such as tumors, so that tumor cells can evade apoptotic pathways and remain proliferative and invasive.[20] The P53 gene, usually recognized as tumor protein 53 (TP53), is located on chromosome number 17 and acts as a tumor-suppressor gene, playing a critical role in cell cycle inhibition, apoptosis, genetic

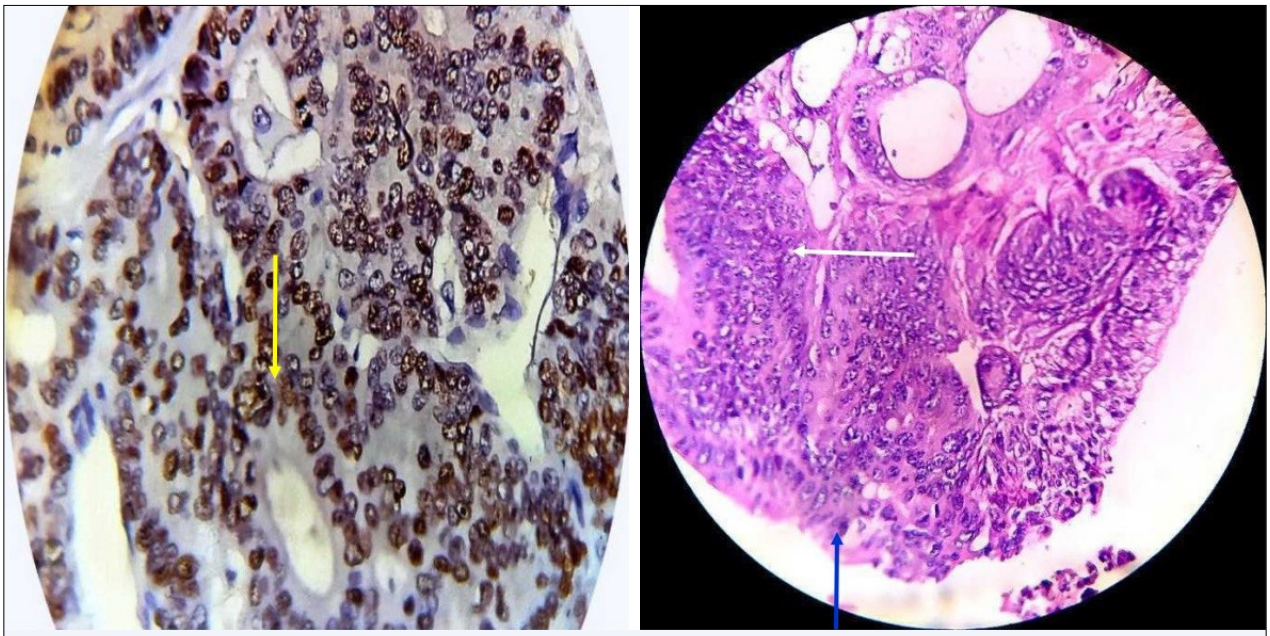

Fig. 2. Signet-ring cell carcinoma. Yellow arrow: mucinous, foamy intracytoplasmic vacuoles which push aside the nucleus. specific detection of P53 protein (color tonality: 4), white arrow: granulomatous status (++), blue arrow: macrophage infiltration (+), IHC.

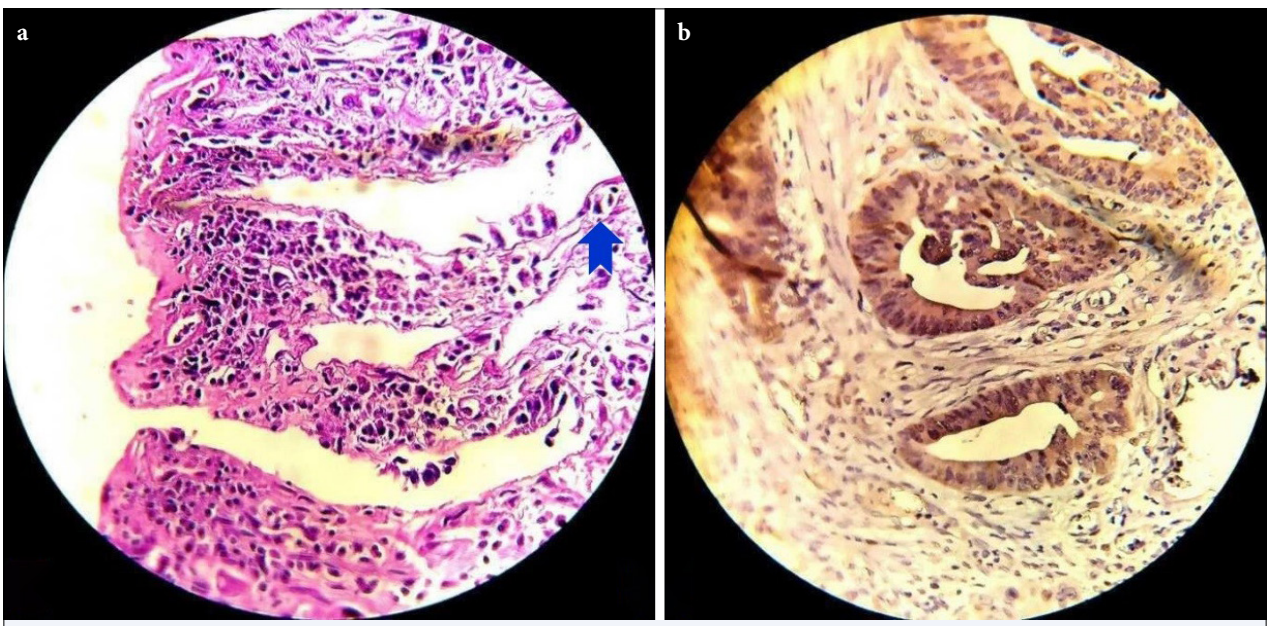

Fig. 3. Moderately-differentiated adenocarcinoma. (a) complex glandular structures are observed in a desmoplastic stroma, blue arrow: macrophage infiltration (+) (b) specific detection of P53 protein (color tonality: 3), granulomatous status (-); IHC.

stability, and blocking angiogenesis. In other words, this gene is actually a guardian of the genome which prevents instability throughout the genetic material. Any mutation or deficiency in the P53 gene leads to immortal cell proliferation and the subsequent chance of cancer development. In normal conditions, only low levels of P53 protein are expressed, but it can dramatically increase in the case of any cellular stress or even DNA damage, in order to repair genetic dysfunctions and terminate the cell cycle through apoptosis.[21,22]

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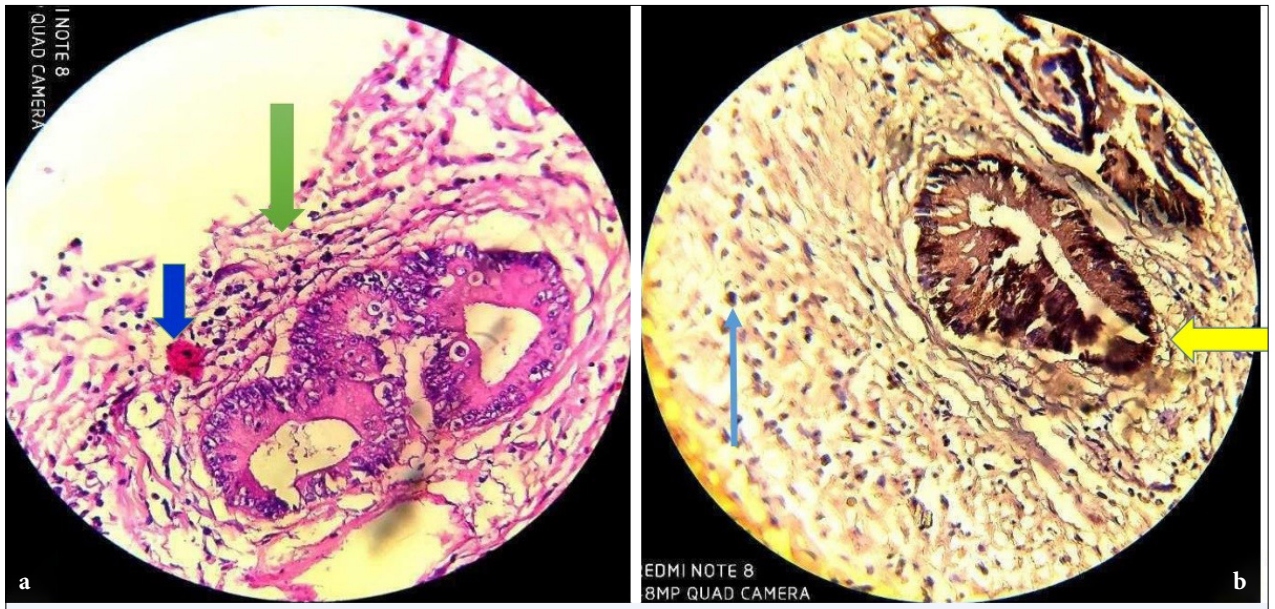

Fig. 4. Traditional serrated adenoma. (a) yellow arrow: lumen entry, green arrow: low-grade cytological dysplasia, blue arrow: cytological eosinophilia. (b) specific detection of P53 protein (color tonality: 3), pink arrow: granulomatous status (-), blue arrow: macrophage infiltration (+); IHC.

In this study, we investigated the P53 protein expression changes in experimental colon carcinoma of Wistar rats, through histopathologic and IHC analyses.

Our results indicated the presence of five types of CRC, comprising mucinous (40%), cribriform comedotype (20%), undifferentiated mucinous signet-ring cell (20%), signet-ring cell (10%), and serrated (10%). We also showed a substantial statistically significant association between colon adenocarcinoma slides and the level of P53-specific antigen-antibody complexes (P<0.05). In Bhattacharya et al.[23]'s study (2019), the expression of beta-catenin in colorectal neoplasms and its role as an additional prognostic marker in colorectal adenocarcinoma was evaluated using the IHC method; they demonstrated a statistically significant (P=0.004) association in beta-catenin expression in benign, pre-cancer, and malignant neoplasms, showing a gradual transition from membranous to nuclear positivity. Also, the nuclear score of this gene was correlated with colorectal adenocarcinoma, according to the American Joint Committee on Cancer (AJCC). The pathologic findings of this study were in line with ours regarding the expression level of the target gene, while we did not assess the type of cancer. Elsabah and Adel (2013) performed a study to detect K-ras expression in 26 metastatic colorectal cancer patients using IHC and Rap1A polyclonal antibody. In about 42.3% of cases, cytoplasmic positivity of K-ras was observed, which was shown to be moderate or strong in 15.4% and 26.9% of examined patients, respectively. About 50% of adenocarcinoma variant cases were positive for K-ras expression, whereas it was negative in all signet-ring and mucinous types. The K-ras expression positivity was noticeable in 50% and 38.9% of moderately-differentiated GII colorectal and poorly-differentiated GIII carcinomas, respectively. Also, in 40% and 45.5% of lymph node metastatic and non-metastatic tumors, a positive color for K-ras expression was found, respectively. Of note, no statistically significant correlation was observed between clinicopathological parameters and K-ras positivity, in contrast with our study. However, the pathological finding of this study was consistent with ours.[24]

Another study in 2000 compared IHC using the DO-7 antibody for detection of P53 gene accumulation and molecular techniques for P53 gene mutation in CRC. The accumulation of P53 protein was found in 20 out of 38 patients (53%), whereas its mutation was detected in 21 of 38 individuals (55%). Among these, 15 patients (39%) showed overexpression in exons 5–8.[25] The rate of P53 gene mutation in this study was partly consistent with our result, while the cancer type was not determined in our study. In Prieto et al.[26]'s study (2017), the expression of P53 and beta-catenin in topographic compartments of CRC and its prognostic value following surgery were assessed. The immunoexpression of beta-catenin showed an increasing gradient

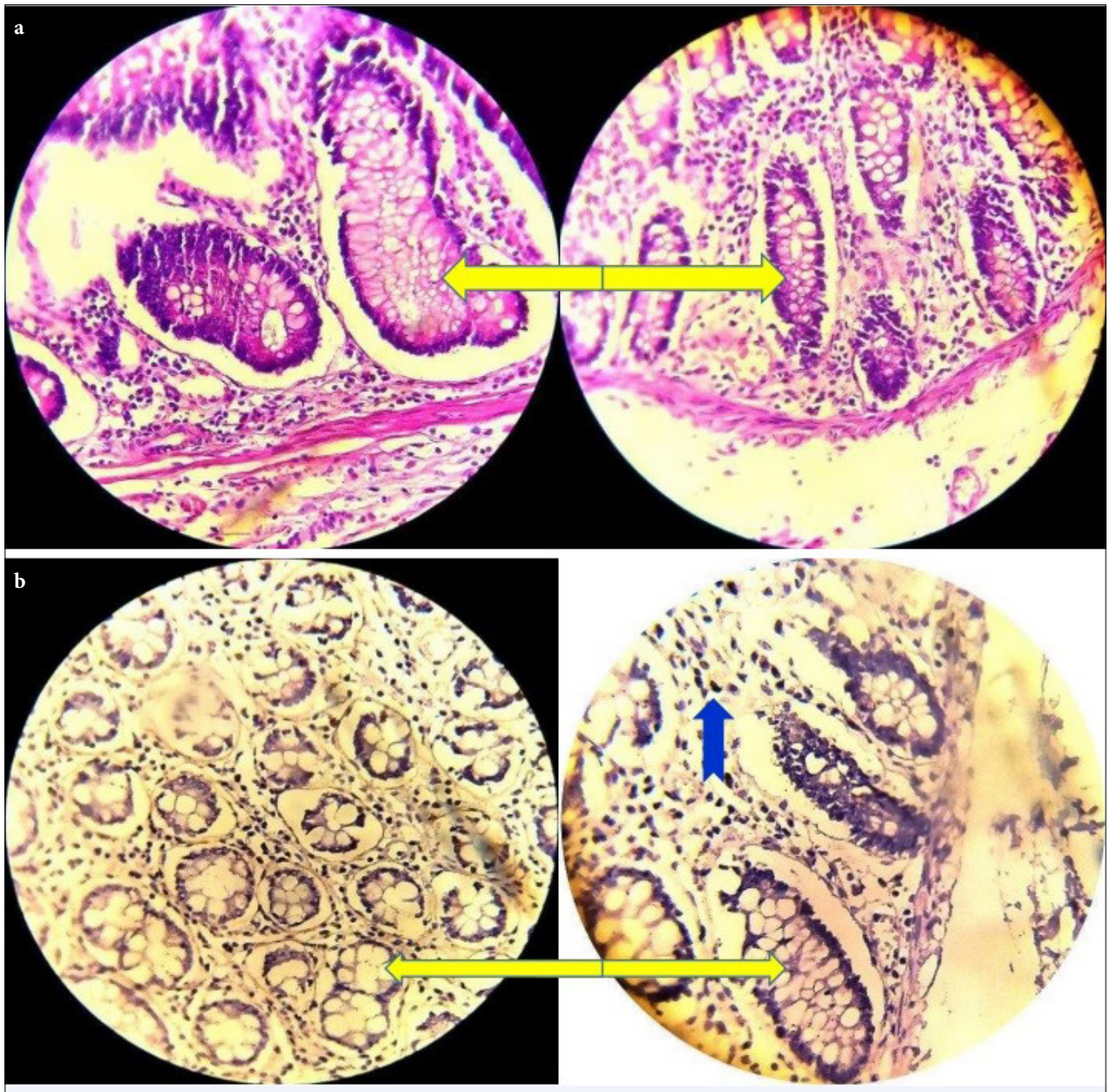

Fig. 5. Mucinous adenocarcinoma. (A) Abundant extracellular mucin. (B) specific detection of P53 protein (color tonality: 1), pink arrow: granulomatous status (-), blue arrow: macrophage infiltration (++); IHC.

associated with the depth of the tumor, and this nuclear immunoexpression was much higher in the deep compartment. Notably, P53 expression without beta-catenin co-expression demonstrated a higher rate of survival in patients than the simultaneous expression of both genes. Nakayama and Oshima (2019) showed a missense-type mutation, P53 R270H, causing submucosal invasion and promoting metastasis, whereas the loss of wild-type P53 has a limited impact on invasion. Altogether, it was found that missense-type P53 mutations along with the loss of P53 wild type catalyze the activation of both oncogenic and inflammatory pathways, leading to late-stage CRC.[27] In a recent study, Cho et al.[28] (2020) evaluated the overexpression and mutation of P53 exons 4–8 in canine intestinal adenocarcinoma; they showed that P53 overexpression could be a prognostic factor of the presence of neoplasia in the dog intestine, while this overexpression process may occur through other distinct mechanisms rather than P53 mutations in exons 4–8.

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CONCLUSION

As a final word, the current study detected considerable variations in P53 gene expression in colon cancer of Wistar rats; however, more in-depth studies are highly demanded to identify the type and rate of mutations in the P53 gene and possible involved exons and introns. It is also suggested to simultaneously trace the alterations in P53 and K-ras genes' expression.

Ethics Committee Approval: The study was approved by the Islamic Azad University, Sanandaj Branch Research Ethics Committee (no: IR.IAU.SDJ.REC.1402.118, date: 10/03/2022).

Authorship contributions: Concept – P.M.; Design – P.M.; Supervision – P.M.; Funding – B.N.G.; Materials – P.M., B.N.G.; Data collection and/or processing – P.M., B.N.G., S.G.; Data analysis and/or interpretation – S.G.; Literature search – B.N.G.; Writing – P.M.; Critical review – P.M.

Conflict of Interest: All authors declared no conflict of interest.

Use of AI for Writing Assistance: Not declared.

Financial Support: This work was supported by the Islamic Azad University, Sanandaj Branch, Sanandaj, Iran.

Peer-review: Externally peer-reviewed.

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