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ORIGINAL ARTICLE

Evaluation of circulating innate lymphoid cells in the early pathogenesis of mouse colorectal carcinoma

Mohsen Keykhosravi[1] · Seyed Mohammad Javadzadeh[1] · Mohsen Tehrani[1,2] · Hossein Asgarian-Omran[1,2,3] · Mohsen Rashidi[4] · Hadi Hossein-Nattaj[1] · Laleh Vahedi-Larijani[5] · Abolghasem Ajami[1,6,7]

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Abstract

Innate lymphoid cells (ILCs) have been shown to play essential roles in tumour immunity. Also, ILCs are involved in both homeostasis and inflammation. Evidence indicates that uncontrolled inflammatory response predisposes the intestine to colonic dysplasia and colorectal cancer (CRC). We investigated the frequency of three subsets of circulating ILCs in the early pathogenesis of colorectal carcinoma in the two distinct mouse models of colorectal cancer (CRC). We developed two mouse models representing the early pathogenic also reversible stages of CRC, including a chemically induced model, by administration of azoxymethane/dextran sulfate sodium (AOM/DSS), and an orthotopic mouse model, using the CT-26 cell line. Based on histopathological examinations, mice were divided into 3 groups including the dysplasia group (which consists of the chemically induced and the orthotopic induced), the chemically induced reparative change group, and a control group which was also considered in which mice were screened for stress originating from interventions and injections. Flow cytometry analysis was performed to evaluate the frequencies of ILC1, ILC2, and ILC3 in the peripheral blood of all studied mice. Altered composition of ILCs was observed in the peripheral blood of mice skewing toward ILC1s in the reparative change group compared to the control group (p value = 0.008); orthotopic-induced dysplasia and the chemically induced dysplasia groups did not differ significantly in terms of ILC subpopulations at the precancerous stages of colorectal dysplasia. A higher frequency of ILC1 in the chemically induced reparative change suggests a potentially anti-tumourigenic role participating in colorectal dysplasia.

Keywords Innate lymphoid cell · Colorectal carcinoma · Orthotopic induced · Chemically induced · Peripheral blood · Colitis-associated colorectal cancer

✉ Abolghasem Ajami
ajami36@gmail.com

Mohsen Keykhosravi
Mohsenkeykhosraviy@yahoo.com

Seyed Mohammad Javadzadeh
kayhanmj@yahoo.com

Mohsen Tehrani
drmtehrani@gmail.com

Hossein Asgarian-Omran
asgarianhossein@yahoo.com

Mohsen Rashidi
dr.mohsenrashidi@yahoo.com

Hadi Hossein-Nattaj
h.hosseinnataj@gmail.com

Laleh Vahedi-Larijani
lalevahedi@gmail.com

[1] Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[2] Gastrointestinal Cancer Research Center, Mazandaran University of Medical Sciences, Sari, Iran

[3] Immunogenetic Research Center, Mazandaran University of Medical Sciences, Sari, Iran

[4] Department of Pharmacology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[5] Department of Pathology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[6] Molecular and Cell Biology Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[7] Antimicrobial Resistance Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of death (Hull 2021). Clinical diagnosis of CRC has remained challenging due to the asymptomatic progression of CRC until the advanced stages. Therefore, a comprehensive understanding of tumor immune responses could be useful for the improvement of diagnostic and therapeutic approaches (Das et al. 2017). The innate immune system can influence carcinogenesis via direct spontaneous priming, recruitment, activation, and clonal expansion of adaptive immune cells, including B- and T-cells. On the other hand, uncontrolled long-lasting activation of the innate immune system might lead to chronic inflammation which could, in turn, facilitate tumorigenesis (Geremia and Arancibia-Cárcamo 2017; Corrales et al. 2017).

Innate lymphoid cells (ILCs) are innate counterparts of T-helper cells (Th). The term innate lymphoid cell or ILC has been traditionally used since 2010 (Spits et al. 2013); ILC subsets include that the so-called helper ILCs are characterized by a classic lymphoid morphology and divided into five main groups, based on their transcription factors and phenotypic and functional characteristics: lymphoid tissue inducer (LTi), ILC1, ILC2, ILC3, and natural killer cells (NK-cells). Helper-ILCs lack re-arranged antigen-specific receptors but produce effector cytokines (Geremia and Arancibia-Cárcamo 2017; Cording et al. 2016; Vivier et al. 2018) and exert their role in the maintaining balance of commensal containment, lymphoid organogenesis, and protection of epithelial barriers against infections and inflammation (Klose et al. 2014; Monticelli et al. 2011).

Individual ILC subsets emerge from the common lymphoid progenitors (CLP) after a series of differentiation stages that include the bipotent $CXCR6^+$ (chemokine CXC motif receptor 6) ILC/NK progenitors (αLP) and the common "helper-like" ILC precursors (CHILP), which become common ILC progenitors (ILCP). Different sets of transcription factors control how ILCs are generated. The thymocyte selection-associated high-mobility group box protein (TOX), GATA3, the zinc finger and BTB domain-containing protein 16 (PLZF) (Constantinides et al. 2015), and inhibitor of DNA-binding 2 (ID2) are transcription factors that control the development of the ILCP. ILC1 requires T-bet; development of ILC2 is controlled by GATA3, Notch, ROR, transcription factor 1 (TCF1), and growth factor-independent 1 (GFI1) (Klose et al. 2014; Hoyler et al. 2012). ILC3 differentiation depends on RORγt, Notch, TCF1, RUNX1, and aryl hydrocarbon receptor (AHR). Differentiating of NK cells is regulated by Eomesodermin (Eomes), TOX, v-Ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1), T-bet, and Runt-related transcription factor 3 (RUNX3) (Tang et al. 2012; Montaldo et al. 2014).

In humans, ILC1s produce IFN-γ (interferon gamma) in response to IL-12 (interleukin 12) and IL-15, group 1 ILCs (ILC1) could be divided into two subgroups based on expression of Cluster of Differentiation-127; $CD127^-$ ILC1s are responsive to IL-12 and IL-15 (Fuchs et al. 2013). $CD127^+$ ILC1s which are mainly located in the lamina propria and can be derived from ILC3 under the influence of IL-12 (Krämer et al. 2017; Bernink et al. 2015); $CD127^+$ ILC1s react to IL-12 and IL-18 by producing IFN-γ. Group 2 ILCs (ILC2s) represent a major subset in the mouse intestine, but are infrequent in the adult human gut; majority of human ILC2s are positive for prostaglandin D2 (PGD2) receptor or CRTH2; furthermore, they are responsive to alarmin molecules: IL-33, IL-25, TSLP, and PGD2 by producing type 2 cytokines mainly IL-4, IL-5, and IL-13 in a transcription factor GATA3-dependent manner (Loyon et al. 2019; Mjösberg et al. 2011). Several subsets comprise the ILC3 compartment; functional ILC3s are divided into two subgroups based on expression of the natural cytotoxicity receptor NKp44 (or NKp46 in mice), $NKp44^+$ ILC3s that secrete IL-22 and $NKp44^-$ ILC3s that produce IL-17F (Klose et al. 2014; Nussbaum et al. 2017; Irshad et al. 2017). In vitro cultures of both mice and humans, ILC3s show that the predominant ILC3 population in the human gut produces IL-22 (Coorens et al. 2019), which promotes intestinal epithelial integrity. However, the production of GM-CSF and IL-17 by ILC3 in mice and humans has been reported, which has been demonstrated to promote inflammation in a mouse model of colitis (Hazenberg and Spits 2014; Pearson et al. 2016; Satoh-Takayama et al. 2008). Additionally, findings now show that LTi cells and their closely related group, ILC3, play a central role in maintaining long-term memory $CD4^+$ T cells in mammals (Shikhagaie et al. 2017).

Although several roles of ILCs in various types of cancers have been unveiled in some studies (Eisenring et al. 2010; Gronke et al. 2019; Huber et al. 2012; Kirchberger et al. 2013), little is known about ILC frequency and composition in mouse peripheral blood during early pathogenesis of colorectal-carcinoma progression. In this experimental study, two distinct mouse models of CRC were used, to evaluate ILC frequency in peripheral blood of mice during early CRC pathologic changes to illustrate whether circulating ILCs participate in mouse early-stage CRC development.

Materials and methods

Mice

Forty-eight male BALB/c mice aged 6–8 weeks, weight range between 18 and 25 g, were used in this interventional case–control study (January 2019–June 2020). Mice

were maintained at the Laboratory Animal Care Institute at Mazandaran University of Medical Sciences (MAZUMS), according to the animal care guidelines. All of the maintenance and laboratory test processes were approved by the ethics committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1397.2674). All animals were housed in plastic cages (5 mice/cage) with free access to drinking water and basal diet pellet under controlled conditions of humidity, light/dark cycle, and temperature (23 ± 2 °C). They were assigned into 3 experimental groups chemically induced ($n = 18$), orthotopic injected ($n = 12$), and the control group ($n = 18$).

The chemically induced mouse model of CRC

Azoxymethane (AOM), as a genotoxic carcinogen, was purchased from Santa Cruz Chemical (Dallas, TX, US), and dextran sulfate sodium (DSS), as a non-genotoxic carcinogen, was purchased from Sigma-Aldrich (Aurora, OH, US). The method described in the Suzuki et al. work with a slight modification was implemented in this study (Suzuki et al. 2005); eighteen mice were treated with an intraperitoneal administration (15 mg/kg body weight) of AOM followed by 7 days of recovery. On day 7 after the AOM injection, mice were orally administered DSS (2.5% in drinking water) for a week. On day 21, mice were injected again with a single dose of AOM (7.5 mg/kg) and then DSS (2.5% in drinking water) for a week (from day 28 to day 35). The control group was injected with a single dose of normal saline (15 mg/kg) on day 1 and another dose of phosphate buffer saline (PBS) (7.5 mg/kg) on day 21; then, on days 7 to 14 and 28 to 35, they received 2.5% normal saline in drinking water. There was no further intervention performed up to the 80th day.

Orthotopic mouse model of CRC

Initially, fourteen mice were injected with CT-26, mouse adenocarcinoma cell line. Optimized 2×10^6 numbers of cells were suspended in 1 mL PBS. The mice were anaesthetized, shaved, and prepped with povidone-iodine. Laparotomy was performed to expose the caecum, and then, 50 µL of the cell suspension (1×10^6 cells per mouse) was injected into the caecum wall according to the methodology adjusted from Miller et al. study (Miller et al. 2016). Finally, the caecum was returned to the abdominal cavity, and the incision was sutured. Two mice out of fourteen died during the surgical process; the 12 remaining mice underwent the recovery process without receiving antibiotic treatment and continued to bear growing lesions until their sample collection and sacrifice for pathological diagnosis on days 25 ($n = 6$) and day 40 ($n = 6$) after cell injection. The dysplastic lesion was detected in 8 mice (66% evidence rate) of this group; 4 mice were lesion-free and thus excluded from the study.

Antibodies and preparations

The following anti-mouse lineage cocktail antibodies: FITC anti-mCD3/FITC/anti-mGr-1/FITC anti-mCD11b/FITC anti-mCD45R (B220)/FITC anti-Ter-119; in addition, APC anti-mouse IL-33Rα (ST2), PE/Cy7 anti-mouse CD45, and PE anti-mouse CD117 (c-Kit) were used (all purchased from Biolegend). Corresponding isotype control antibodies were used to ensure specific antibody binding to the target rather than nonspecific bindings or artifacts. Considering the utility of a 4-color staining panel in the following experiment, before evaluation of ILCs in our collected samples, FM2 control and one 4-color control negative panel sample tubes were used to obtain a valid compensation matrix and determine appropriate quadrant marker placement for the panel; also, PMT voltages were adjusted and optimized for evaluation of target cells.

Sample collection

Twelve mice were selected for each time sampling on days 80, 105, and 120 (6 mice in the control group and 6 from the chemically induced group). In line with chemically induced mice, all orthotopically injected mice were anaesthetized with intraperitoneal administration of xylazine (16 mg/kg) and ketamine (120 mg/kg) on days 25 and 40 after cell line injection. Whole blood was collected with the cardiac puncture method, and blood samples were transferred to ethylene diamine tetra-acetic acid (EDTA) 10% containing tubes and mixed well; then, 100 µL of blood were aliquoted into 2-mL microtubes and labeled with appropriate amounts of antibodies for 40 min in the dark at 4 °C. Then, samples underwent red blood cell lysis with cold ammonium chloride lysing solution. Cells were then washed and suspended in FACS buffer (1X PBS, 50 µM EDTA, 0.2% BSA) twice and then suspended in fixation buffer (PBS + 2% paraformaldehyde) until sample evaluation on a BD FACS CALIBUR flow cytometer (Fig. 1).

Histopathological examinations

The chemically induced group of mice was sacrificed for macroscopic evaluation of the colon during 3 series of sample collection on days 80, 105, and 120; also, the orthotopic-induced group of mice was sacrificed at 25 and 40 days after cell line injection (6 mice per time). Colon and caecum were dissected and fixed in 10% buffered formalin for at least 24 h and prepared in paraffin-embedded sections after hematoxylin and eosin (H&E) staining to proceed with histopathological examinations.

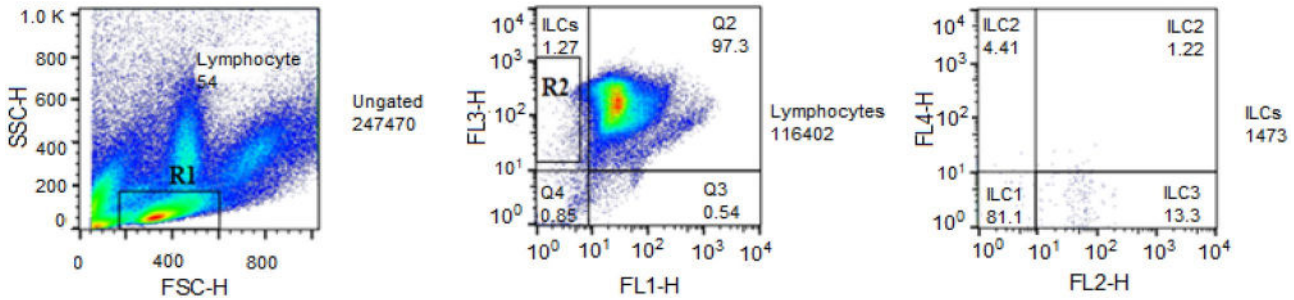

Fig. 1 Samples were analyzed using a BD FACS CALIBUR flow cytometry device. The lymphocyte populations were gated in agreement with the conventional identification, Lin $^{-}$ CD45^{+} events showing total ILCs; aim to analyze the ILC subpopulations, daughter dot plot was derived from Lin^{-} CD45^{+}, and events were further gated into ILC1 (Lin^{-} CD45^{+} CD117^{-} ST-2^{-}), ILC2 (Lin^{-} CD45^{+} CD117$^{\pm}$ ST-2^{+}), and ILC3 (Lin- CD45^{+} CD117^{+} ST-2^{-})

Statistical analysis

Numerical data were analyzed and defined as the median with a min–max range. Regarding sample size and normality test analysis, the Kruskal–Wallis test was used for statistical analysis followed by Dunn's multiple comparison test. The whole statistical process was evaluated with GraphPad Prism v 9.4 (p values less than 0.05 were considered significant with $*p < 0.05$).

Results

Mice colonic tissues were examined by a pathologist to detect mucosal ulcerations, dysplasia, and lesions. Mice were assigned mice into 4 groups, based on their pathological status, including the control group ($n = 18$), the chemically induced reparative change ($n = 10$), and the chemically induced dysplasia ($n = 8$) group, as well as the orthotopic dysplasia group ($n = 8$); as expected, mice in the control group showed histo-pathologically normal appearance with no inflammation. Mild infiltrate of immune cells into the lamina propria with obvious inflammation, often including crypt abscess formation, was described as chemically induced reparative change ($n = 10$), and the dysplasia group which involved colonic mucosal dysplasia diagnosed mice according to all characteristics of dysplasia, such as hyperchromatic nuclei, increased nuclei, and mitotic cells consisting of either low or high grades ($n = 16$) (Fig. 2).

ILC frequency

To investigate whether conversions of peripheral blood ILCs are involved in the CRC development process, we compared the frequency of ILC1s, ILC2s, and ILC3s among all study groups. The frequency of total ILCs in the chemically induced reparative change group was slightly higher in comparison to other groups, but it was not statistically significant (data not shown) (Fig. 3).

We observed a significantly higher percentage of ILC1 in the chemically induced reparative change group compared to the control group (p value = 0.0262) followed by the chemically induced and the orthopic induced dysplasia groups. Surprisingly, there were no significant differences in terms of ILC subpopulations between the orthotopic-induced dysplasia and the chemically induced dysplasia groups in comparison with the control group during the early stage of CRC pathogenesis.

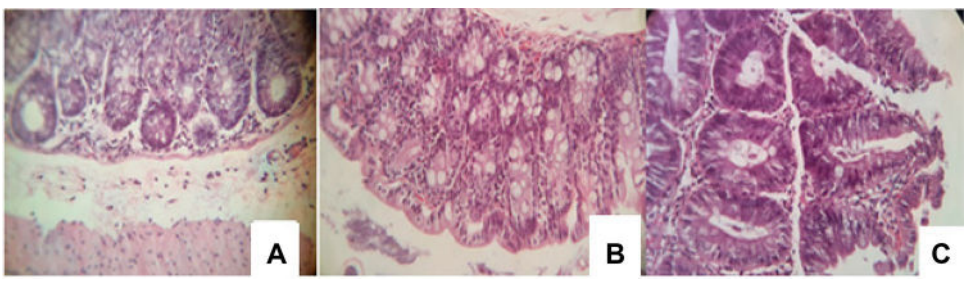

Fig. 2 Histological microscopic examination. Hematoxylin & Eosin (H&E)-stained cross sections of mice colonic tissues (magnification × 40), showing the normal appearance of the control group (**A**). Reparative change is depicted with the hemorrhagic and inflamed scheme (**B**). The slide represents a microscopic observation of the dysplastic alteration in the mouse colon (**C**)

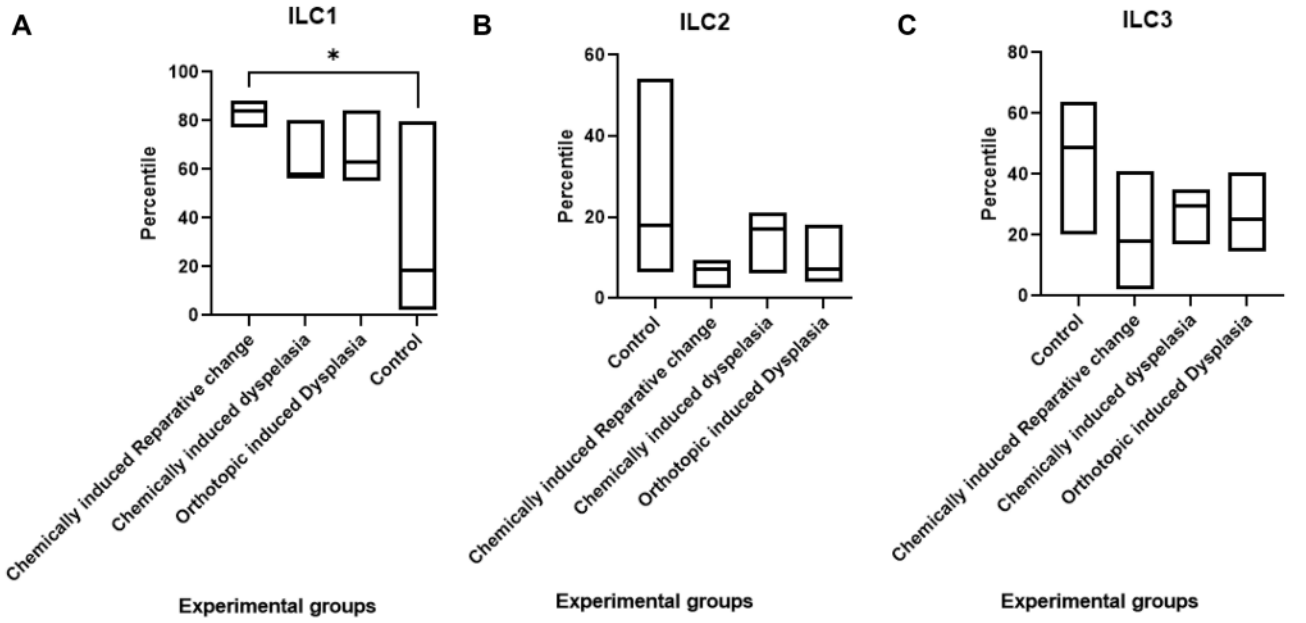

Fig. 3 ILC composition in peripheral blood of experimental groups. Representative columns showing ILC1 percentage among pathological groups (**A**). Representative columns of ILC2 percentages among experimental groups (**B**) and ILC3 percentages in several experimental groups have been shown (**C**). The bars show the median with a min–max range, $*p < 0.05$

Discussion

This work examined how ILCs alter in terms of subset composition during the early pathogenesis of CRC development using two established mouse models of CRC, including adenocarcinoma cell line injected mouse model (Miller et al. 2016) and chemical component (AOM/DSS)-treated model with taking benefit of a well-known inflammation-related CAC (Miller et al. 2016). We find that the circulating ILC compartment in the chemically induced mice with reparative change demonstrated the shift, skewing toward elevated numbers of ILC1, while ILC2 and ILC3 remained unchanged. However, the nature of this alteration was not investigated in this study; other researchers have proposed that the ILC alterations in the composition of subpopulations might be related to the ILC expression pattern of homing/trafficking receptors (Kim et al. 2015; Satoh-Takayama et al. 2014; Mackley et al. 2015). Furthermore, ILCs can regulate their balance and dynamically replenish their population by employing self-renewing cells that seed non-lymphoid organs during ontogeny. Also, ILC repertoire is continuously replaced by precursors in the lymphoid organs (Klose et al. 2014; Montaldo et al. 2014; Bando et al. 2015; Chen et al. 2016; Chea et al. 2015), or via intragroup plasticity due to epigenetic rearrangement in a reversible process (Bernink et al. 2015; Lim et al. 2016; Parker et al. 2020; Viant et al. 2016). Notably, cytokines seem to be the common critical factor of the all-aforementioned mechanisms and significantly influence the onset, progression, and outcome of many chronic diseases, including cancer; however, they might not always exert their role directly on epithelial and pre-malignant cells. The IL-12 cytokine family has been discovered by other workers to significantly influence inflammatory bowel disease (IBD) in humans and several CAC mice models (Kullberg et al. 2006; Cox et al. 2012). Additionally, previous researches have shown that IL-12 is responsible for ILC3 conversion into $CD127^{+}$ILC1 in mucosal tissues (Bernink et al. 2015) and derives ILC2-to-ILC1 plasticity in ILC2s obtained from blood (Lim et al. 2016); those findings raised the possibility of an involvement of type 1 polarizing cytokines specially the IL-12 cytokine family cytokines in the induced phenotypic conversion of ILC3 and ILC2 into ILC1; we suggest a key role mediated by type 1 cytokines as a possible explanation for the rise of the ILC1 in the chemically induced reparative change group mice.

However, our research was limited by challenges such as the assessment of the extremely small population of innate lymphoid cells in peripheral blood, the unavailability of transgenic mouse models, and the limited access to high-throughput techniques. There have been an increasing number of researches that have shed light on innate immune cell-mediated therapies in recent decades (Chen et al. 2016; Lanuza et al. 2022; Shields et al. 2020; Xiao et al. 2019); thus, deciphering the highly plastic innate lymphoid cells, which are remarkably evolved to exert their decisive roles by either producing cytokines or direct cytotoxicity, could be a promising and futuristic candidate for orchestrating immune

responses and taking steps toward treating colorectal cancer and other chronic diseases, as a result of our deep understanding of innate lymphoid cells and their various aspects.

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Author contribution All authors contributed to the study. Abolghasem Ajami, Mohsen Tehrani, and Hossein Asgarian-Omran designed and conducted the research. Mohsen Rashidi managed the induction of a cancer model in mice. Laleh Vahedi-Larijani assessed the pathological status of samples. Seyed Mohammad Javadzadeh, Hadi Hossein-Nattaj, and Mohsen Keykhosravi carried out the assays, contributed to data collection and data analysis, and prepared the manuscript. All co-authors have read, informed, and approved the paper for publication in the journal of clinical comparative pathology.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were under the ethical standards of the institution or practice at which the studies were conducted. All of the processes of maintenance and laboratory tests were approved by the ethic committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1397.2674).

Informed consent For this type of study, informed consent is not required.

Consent for publication For this type of study, consent for publication is not required.

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