Original Article

Immunoglobulin Receptors Expression in Indian Colon Cancer Patients and Healthy Subjects Using a Noninvasive Approach and Flowcytometry

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Submitted: 11-Sep-2019
Revised: 14-Nov-2019
Accepted: 13-Feb-2020
Published Online: 11-Jul-2020

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Access this article online

Website: www.ijabmr.org

DOI: 10.4103/ijabmr.IJABMR_191_19

Quick Response Code:

Abstract

Background: Isolation of viable colonocytes from human stool is a noninvasive and convenient approach that can be used for diagnostic, screening, management, and research on various gastrointestinal (GI) diseases including colon cancer. Limited studies are available globally and for the first time in this article, we have reported the immunoglobulin (Ig) (IgA and IgG) receptors concentration on viable colonocytes for Indian colon cancer patients using this noninvasive approach. **Materials and Methods:** Viable colonocytes from stool were isolated by the Somatic Cell Sampling and Recovery method (Noninvasive Technology, USA) and processed for the assessment of Igs (IgA and IgG) receptors expression using standard immunophenotyping and flow cytometry. **Results:** IgA and IgG receptor expression was measured and reported on these viable colonocytes. There was a significant difference in the expression of IgA and IgG receptors on viable colonocytes between colon cancer patients and healthy individuals. **Conclusion:** This noninvasive technique is a promising approach for the detection of molecular and immunological markers that will help clinicians in the diagnosis, screening, monitoring, and management of different GI diseases including colon cancer.

Keywords: *Colon cancer, colonocytes, flowcytometry, immunoglobulins, noninvasive*

Introduction

Human gastrointestinal (GI) tract is the most complicated immune organ of the body. The mucus layer, epithelial cells, and immune cells constitute the intestinal mucosal barrier which prevent the commensal microorganisms living in our intestines from reaching systemic sites and provide defense against infections from pathobionts and opportunistic and primary pathogens.[1,2] In the human gut, tissues are separated from the microbiota by a layer of epithelial cells.[3] A regulated immune balance has been shaped by the coevolution between the host and gut microbiota which is essential for the integrity of the intestine and the health of the human host. Disturbed gut immune homeostasis can lead to different GI disorders including inflammatory bowel disease (IBD) and colon cancer.[4]

The mucosal immune system is a highly integrated and finely regulated system which may have evolved as a major defense mechanism against mucosal encountered infectious agents that presents a well-tuned, two-part defense, one structured and localized and another more diffused.[5] Antigens are encountered with the primary defense line and selectively taken up into highly structured sites for the initiation of immune responses. After interaction with antigen, the effector cells such as B- and T-lymphocytes, macrophages, dendritic cells, eosinophils, basophils, and mast cells get activated and execute the appropriate action. Lymphoid tissues in the GI tract regions, which are also known as inductive sites, have evolved and facilitate antigen uptake, processing, and presentation of antigens for immune response. Gut-associated lymphoreticular tissues are major inductive sites which consist of the Peyer's patches and are units of lymphoid cells, single lymphocytes scattered in the lamina propria, and intraepithelial lymphocytes spread in the intestinal epithelia.[6]

The epithelium of the GI tract undergoes constant and rapid renewal.[7-10] Some of these cells differentiate into columnar absorptive cells and are exfoliated into the fecal stream and represent colonic epithelium and can be used for clinical

How to cite this article: Chaudhry R, Bamola VD, Samanta P, Dubey D, Bahadur T, Chandan M, *et al.* Immunoglobulin receptors expression in Indian colon cancer patients and healthy subjects using a noninvasive approach and flowcytometry. Int J App Basic Med Res 2020;10:194-9.

investigations and researches.[11-13] The life span of these differentiated cells of colonic origin is of 3–4 days and a mean generation time of about 1 day accounts for the rapid turnover of this cell population.[8,14,15] Normal colorectal epithelium renews at a high proliferative rate and must be balanced by cell loss to maintain homeostasis of the epithelial monolayer. The normal colonic epithelium contains 5×10^{10} cells and that of one-sixth to one-third of these cells are shed every 24 h, which exfoliated every day.[8,16,17] Evidences indicate that cellular efflux toward the lumen is much greater in cancers than normal epithelium and colonocytes recovery is more abundant in patients with colorectal cancer (CRC) than healthy individuals.[13]

CRC is one of the most common malignancies and the third leading cause of cancer-related mortality worldwide.[18] The incidence was higher in developed countries but is rapidly increasing in historically low-risk areas including Asia.[19] Colonoscopy, endoscopy, sigmoidoscopy, barium enemas, and biopsy are the standard procedures for the diagnosis of GI disorders including IBD and CRC. However, these procedures are highly invasive, expensive, and inconvenient for most of the patients, especially for pediatric patients.[20] Assay of molecular and immunological markers in stool represents a promising noninvasive approach to screen CRC and IBD.[13] Stool testing has several advantages over other available screening approaches because stool testing is noninvasive and not requires unpleasant cathartic preparation, formal health care visit, or time away from routine activities.[13] A study has suggested that the use of stool colonocytes can be a valuable noninvasive approach for studying gut pathophysiology in the neonatal period, as studies of GI patho-physiology are not feasible by biopsies in human neonates and pediatric population. In this study, researchers have examined the utility of live colonocytes in the stool to study cellular markers during early neonatal life and reported the expression of IgA, IgG, cluster of differentiation-45 cells, and toll-like receptors-2 and 4.[10]

In the present study, we have assessed Ig (IgA and IgG) receptors expression on viable colonocytes recovered by noninvasive technique in patients of Indian colon cancer and healthy individuals, using specific florochrome-conjugated antibodies and flow cytometry. For the first time, we report the concentration of Ig (IgA and IgG) receptors on viable colonocytes in the patients of colon carcinoma and its comparison with healthy population. In our previous study,[9] we have reported the normal range of IgA and IgG receptors on viable colonocytes in healthy adults and children from India. This noninvasive technique can be useful for the detection of molecular and immunological markers using a stool that can be used for the assessment of gut immunity, screening, and management of different GI diseases including colon cancer even in small clinical, diagnostic, and health-care setups.

Materials and Methods

Collection of sample

After obtaining ethical approval from the "Institute Ethics Committee" of All India Institute of Medical Sciences, New Delhi, India, individuals were recruited for the study. The study was conducted at All India Institute of Medical Sciences, New Delhi, India. Before enrollment the detail participant information sheet of the study was provided to the participants and objectives, expected outcomes of the study and associated risks were explained to all the participants. After screening of the individuals in the hospital ward as per the inclusion and exclusion criteria, only those individuals were enrolled who were willing to participate in the study and ready to give written participant informed consent. After screening of 300 patients and 100 healthy individuals, we have enrolled 24 participants including 12 healthy individuals and 12 patients of colon cancer as per the exclusion and inclusion criteria of the study. The participants were asked to collect stool samples for the study. For the collection of a fresh stool sample, a sterile stool collection vial was provided and each participant was explained and instructed for proper collection of the sample to maintain maximum sterile condition. It was ensured that the collected stool sample should be transported to the laboratory in the minimum possible duration. After receiving in the laboratory, the samples were processed immediately to isolate viable colonocytes and immunophenotyping.

Isolation of viable colonocytes

A noninvasive approach was applied to isolate viable colonocytes from the stool. These viable colonocytes were isolated by Somatic Cell Sampling and Recovery (SCSR) method (Noninvasive Technology, USA) with some in-house modifications.[9] In brief, stool samples (0.5–1.0 g) were collected in a specific collection vial provided and adequately mixed. After mixing, the samples were filtered by a 330 µm nylon mesh to remove large particles. The first filtrate was again passed through a second filter (40 µm, BD-Becton, Dickinson and Company, USA) to remove undesired large particles. The final filtrate was collected in a fresh falcon tube (50 ml). The final suspension underlaid with 10 ml of cushion solution provided in the kit. After adding cushion medium, the sample was centrifuged at 200 × g for 10 min in a refrigerated centrifuge. After centrifugation, the exfoliated cells or colonocytes were aspirated and recovered from the interface. The collected fraction containing colonocytes was washed three times with phosphate-buffered saline (PBS). After adequate washing, cell pellets were suspended in 200 µl PBS.[9]

Immunoglobulin receptor measurement immunophenotyping and flow cytometry

A wet preparation of the final cell suspension was viewed under a phase-contrast microscope to check the numbers

of cells. Cells were stained with trypan blue (0.4%) to distinguish viable cells from necrotic cells and observed in phase-contrast microscope (PH 2, Nikon, Japan). Cells were also counted in a Coulter Counter (Z2 Coulter Particle Count and Size Analyzer, Beckman Coulter) to obtain counts and size distribution. This SCSR method normally gives the yield of 20–40 million cells per gram of stool and size distribution histograms show the existence of 2 distinct populations, one between 2–5 μ and another between 5–8 μ.[8-10,21] In our experiment in all assays, a total count of 3×10^3 cells per tube were processed for further staining with fluorochrome-conjugated antibody. To measure the IgA and IgG receptor concentration on viable colonocytes, the cells were incubated with specific antibodies using a standard protocol. In this study, we used IgA-fluorescein isothiocyanate (FITC)-conjugated and IgG-Phycoerythrin (PE)-conjugated antibodies (Sigma) [Figure 1].

Results

In this study, we have recruited a total of 24 individuals including 12 healthy individuals and 12 patients of colon cancer as per the exclusion and inclusion criteria of the study. The mean age of healthy individuals was 34 ± 11 years with a median of 30.5 years (range from 18 to 58 years). The mean age of colon cancer patients recruited in the study was 48.6 ± 12.9 years with a median of 47 years (range from 25 to 60 years). All the statistical analyses were carried out using Microsoft Excel and GraphPad Prism software USA.

The range of IgA receptors on viable colonocytes isolated from the stool of healthy individuals was varied from 50.8% to 71.2% with a mean concentration of 66.2% ± 5.4% and median value of 67.1%. The range of IgG receptors on viable colonocytes isolated from stool of healthy individuals was varied from 45.5% to 70.5% with a mean concentration of 64.3% ± 7.5% and median value of 66.7% [results are summarized in Table 1 and Figure 2]. The range of IgA receptor on viable colonocytes isolated from the stool of patients of colon carcinoma was varied from 47.6% to 65.3% with a mean concentration of 53.5% ± 5% and median value of 51.2%. The range of IgG receptor on viable colonocytes isolated from stool of patients of colon carcinoma was varied from 48.4% to 63.8% with a mean concentration of 55.4% ± 5.7% and median value of 56.4%. The differences in the mean concentrations of IgA and IgG receptors were statistically significant when compared with the mean concentrations of IgA and IgG receptors in healthy individuals (for the mean IgA, $P = 0.0027$ and for the mean IgG, $P = 0.0045$).

Discussion

In this study, we have measured Ig (IgA and IgG) receptors expression on viable colonocytes in the patients of Indian colon cancer and healthy individuals recovered by noninvasive technique. Most of the diagnosis and researches which require colonic epithelial cells are highly invasive and involve endoscopy and biopsy. Among available screening approaches, stool testing is noninvasive which saves the patient from unpleasant cathartic preparation, frequent health-care setup visit, and time from routine procedure preparations.[13] Stool is a heterogeneous mixture consists of undigested food residues, microflora, endogenous secretions, and exfoliated epithelial cells from the walls of GI tract.[12] These exfoliated epithelial cells are exclusively of colonic origin and representative of the entire colon. Therefore, isolation of theses exfoliated viable colonocytes from human stool is a noninvasive as well as a highly convenient approach that can be used for diagnostic

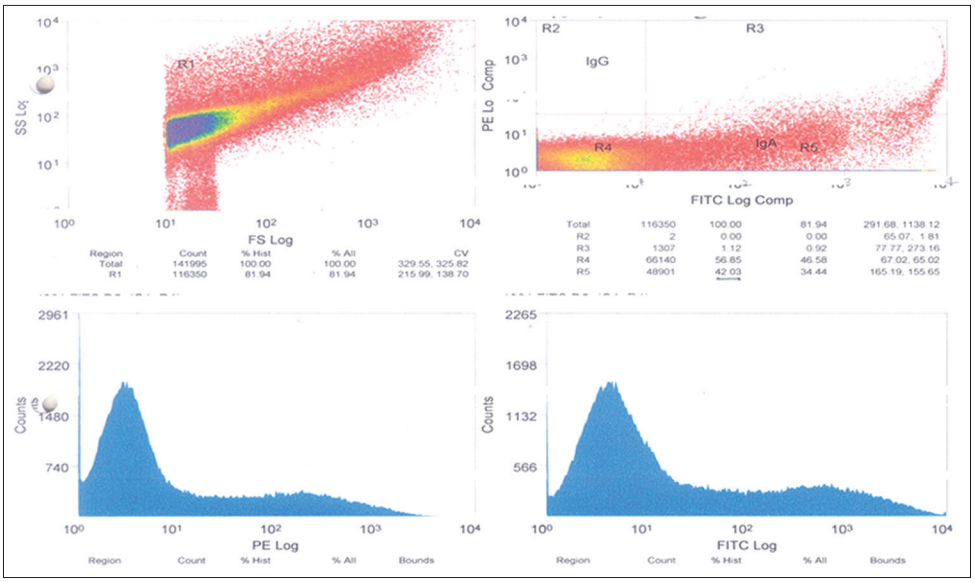

Figure 1: Immunoglobulin A and immunoglobulin G receptor expression on viable colonocytes assessed by different fluorochrome (immunoglobulin A-fluorescein isothiocyanate and immunoglobulinG-Phycoerythrin (PE)) using standard flowcytometry

Table 1: Immunoglobulin A and immunoglobulin G receptor concentration on viable colonocytes isolated from stool samples of the subjects of both the study groups

	Colon cancer group		Healthy group		*P* (between both groups) Colon cancer versus healthy	
	IgA (%)	IgG (%)	IgA (%)	IgG (%)	IgA (%)	IgG (%)
Mean±SD	53.5±5.0	55.4±5.7	66.2±5.7	64.3±7.5	0.0027	0.0045
Range (minimum-maximum)	47.6-65.3	48.4-63.8	50.8-71.2	45.5-70.5		
Median	51.2	56.4	67.1	66.7		

IgA: Immunoglobulin A, IgG: Immunoglobulin G, SD: Standard deviation

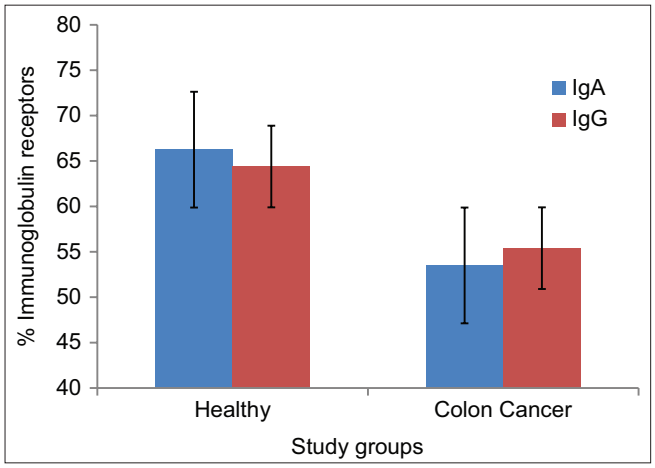

Figure 2: Immunoglobulin A and immunoglobulin G receptor concentration on viable colonocytes isolated from stool samples of the subjects of different study groups

and research purposes. Life span of these exfoliated colonocytes is of 3–4 days.[8,14,15] It is estimated that the normal colonic epithelium contains 5×10^{10} cells and that one-sixth to one-third of these cells are shed every 24 h, which exfoliated every day.[8,16,17] Our study highlights this noninvasive approach for isolation of viable colonocytes from stool which can be used for the detection of the Ig receptors and other surface antigens. This noninvasive approach can be useful for the research and diagnosis of various clinical conditions.

Cell exfoliation from colonic epithelium appears to be a relatively increases in neoplasia when cell removal by apoptosis does not function properly. Studies have reported that colonocyte exfoliation in the human colon constitutes a unique mechanism that can undergo significant changes under different physiological and pathological conditions, and therefore, exfoliated colonocyte analysis may open new approaches to CRC screening and early diagnosis.[22] In the current study, this noninvasive approach used to assess the mucosal immune system in the patients of Indian colon cancer and healthy individuals. We recovered a significant number of viable colonocytes from fresh stool samples and measured IgA and IgG receptor expression on these viable colonocytes. We have reported the significant difference in the expression of IgA and IgG receptors on viable colonocytes, which are supported by other studies. Although our study is a pioneer in its coverage, studies also reported that cellular efflux toward the lumen is greater from cancers than normal epithelium and colonocytes recovery is more abundant from patients with CRC than healthy individuals.[13] Osborn and Ahlquist have suggested that neoplasms exfoliate abundantly into the lumen and that DNA recovered from stool can be assayed with sensitive techniques; there is a strong biologic rationale to pursue this emerging technology in the marker discovery and technologic refinements.[13]

In our previous study, we have reported the use of this noninvasive technique to recover viable colonocytes from the stool samples of healthy adults and children to assess IgA and IgG receptors expression.[9] Now, we have applied this technique to assess gut immunity in colon cancer patients and compare that with healthy individuals. Other studies also suggested that the use of stool colonocytes is a valuable noninvasive approach for studying gut pathophysiology in the neonates as GI studies are not feasible by biopsies in pediatrics population and reported the expression of IgA, IgG, a cluster of differentiation-45 cells, and toll-like receptors.[10] Another study also reported the use of this noninvasive technique to isolate viable colonocytes from stools in IBD individuals and normal controls and showed altered levels of inflammatory markers and cytokine expression in the IBD individuals compared to controls.[23]

A study reported the use of this technique to assess the associations between markers of CRC stem cells and adenomas among different ethnic groups. Matsushita *et al. (2005) have isolated colonocytes* from patients with CRC and healthy volunteers to assess cytological examination and DNA analysis for mutations of the APC, K-ras, and p53 genes. Wu *et al*. also demonstrated that the detection of molecular markers in stool samples is a potential strategy for CRC screening by evaluating the feasibility of detecting miR-21 and miR-92a in stool samples of patients with CRC or polyps.[20] They have detected MiRNA levels in CRC tissues and stool samples by real-time quantitative reverse transcription PCR.

Mucosal IgA serves a variety of functions including the first line of immune defense at mucosal surfaces and thought to protect intestinal mucosal surfaces against colonization and invasion by pathogenic microorganisms.[24,25] Evidence indicates that IgA responses are highly dependent on intestinal colonization by commensal microorganisms that

neutralize microbial toxins and pathogens. IgG provides essential host defense and immunoregulatory functions at the mucosal surfaces. Studies reported the detection of IgA and IgG in fecal samples from humans with intestinal infections.[26,27] In this study, we have reported the significant difference in the mean concentrations of IgA and IgG between colon cancer patients and healthy individuals. We observed and believe that this noninvasive technique is a promising approach for the detection of molecular and immunological markers of gut health during screening and management of different GI diseases.

Conclusion

Due to the scarcity of noninvasive, easy and affordable techniques for investigating immune response and mechanisms during the treatment and management of the disease, especially in small health-care settings, is still a challenge. Standard procedures for the diagnosis and monitoring of GI disease including colon cancer are endoscopy, colonoscopy, sigmoidoscopy, and biopsy, but these procedures are highly invasive, expensive, and inconvenient for most of the patient, especially for pediatric patients (Wu *et al*. 2011). To understand the immune response and mechanism in the human intestines during the development and course of disease, genetic and microbial markers should be assessed at more frequent intervals during the disease development and treatment regime, but the enrollment of volunteers in such studies is difficult due to the involvement of invasive procedures for required sampling; therefore, a noninvasive technique is prerequisite to answer the physiological questions that remain unanswered. Promising noninvasive approaches may help to explore new targets in pathogens, boost host mucosal immunity, and modulate the host microbiota to enhance colonization resistance because the more we learn about the players and their interactions, the more we can develop to combat with enteric pathogens. In view of this study and our previous experience, we are convinced that this noninvasive technique is a promising approach for the detection of molecular and immunological markers during screening and management of different GI diseases including colon cancer, although large-scale intensive studies are required to establish novel diagnostic markers. This study will encourage the use of this noninvasive technique for various GI disorders even in the rural, remote, and small health-care settings that will also help the clinician in the diagnosis, monitoring, and management of different GI diseases including colon cancer.

Acknowledgment

The corresponding author acknowledges the Department of Biotechnology, Government of India, for supporting the study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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