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Original article

Efficacy of differential non-invasive approaches in determining the clinical course in patients with Crohn's disease

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Background: We tested the hypothesis that, elevated levels of C-reactive protein might serve as prognostic marker for the classification of Crohn's disease and its clinical course.

Objectives: The present study was undertaken to explore if serum C-reactive protein (sCRP) levels along with the conventional cytogenetic and molecular genetic approaches might serve as efficient prognostic markers with respect to disease classification and clinical course in Crohn's disease (CD) patients.

Methods: We enrolled 43 patients with Crohn's disease from Tamilnadu region, south of India. The patients were mainly classified into four groups based on disease location. A further sub-classification based on activity of disease was also undertaken in the study. Classification of type of disease activity was based on colonoscopic reports and radiological findings. An equal number of healthy normal age and sex-matched controls were also taken for the study.

Results: A positive correlation between sCRP levels, and disease location was observed. Employing a cut-off level of 10 mg/dL, the index CRP level was found to discriminate patients with different locations of the disease along with disease condition when compared to controls. Cytogenetic analysis showed sporadic changes in such patients. The widely reported NOD2 single nucleotide polymorphisms (SNPs) were absent in the patients studied.

Conclusion: Different levels of sCRP seem to correlate well with disease location and disease activity. In some of the patients, the CRP levels appeared to reflect disease activity, while in others persistently high levels seem to suggest active disease. Altogether, a positive correlation of CDAI values with CRP levels along with an increase in frequency of chromosomal aberrations was observed in CD patients.

Keywords: Chromosomal aberrations, crohn's disease, c-reactive protein, inflammation, NOD2, SNP

Crohn's disease (CD) is thought to result from an ongoing activation of the mucosal immune system leading to an inappropriate innate immune response to normal luminal factors in a genetically susceptible individual [1, 2]. In India, CD was considered rare until a decade ago, but is being increasingly recognized in recent years [3, 4]. Recent reports describe 3-10 patients per year, indicating an increase in the incidence of CD during the last decade in India [5]. Although it was earlier considered rare in Asia [6-8], a number of recent reports suggest an increase in the incidence and prevalence of IBD in the region [9-15]. The reasons for rising incidence and prevalence of Crohn's disease in India and Asia may not only be due to an increase in the number of new cases but it may partly be due to recognition of its existence and proper diagnosis of this condition [16].

The clinical expression of CD is heterogeneous with a wide spectrum of patterns and different clinical courses. Classification of CD is, therefore, difficult [17]. The introduction of biological therapies in IBD

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(Inflammatory Bowel Disease) has renewed interest in inflammatory markers (especially C-reactive protein (CRP)), given their potential to select responders to these treatments. Of all the laboratory markers, CRP is the most studied. It has been shown to have the best overall performance [18]. The production of CRP occurs almost exclusively in the liver by the hepatocytes as part of the acute phase response upon stimulation by Interleukin-6 (IL-6), Tumor necrosis factor-alpha (TNF-α) and IL-1-β originating at the site of inflammation. Its short half-life makes CRP a valuable marker to detect and follow up disease activity in CD. Moreover, it has been found to correlate with clinical parameters of disease activity in CD [19-25]. We hypothesized that serum CRP (sCRP) levels in patients with different disease locations might correlate well with location and clinical course of CD. We studied the putative association between the height of CRP levels during disease location and disease activity [26]. Thus, a new stratification of disease phenotype based on serum CRP level during active disease may affect therapeutic decision plans when considering current and novel therapies [27].

IBD is one of the precursors to colorectal cancer development. Furthermore, a wide range of chromosomal abnormalities and microsatellite instability has been reported in tumor tissue of gastrointestinal tract (GIT) cancer patients. Therefore, we planned to carry out cytogenetic genetic analyses to check the nature of such alterations. Additionally, we plan to seek association with the disease location and activity in such patients.

Since NOD2 (Nucleotide-binding Oligomerization Domain-containing protein 2) gene was found to be associated with CD in populations worldwide [28], genotyping for the three common SNPs in the gene was carried out to check the association if any in the patients. The major objective of the study was to determine the clinical course of disease in patients with CD and add an effective prognosis in a country where its diagnosis is increased in complexity due to similar inflammatory patterns observed during abdominal tuberculosis [29]. This might help in effective theranostic outcome for the patients.

Materials and methods

All patients and controls who agreed to participate in the study were required to sign informed consent forms. All the procedures were carried out in accordance with ethical standards laid down in the 1964 Declaration of Helsinki. Patients with CD were recruited in and around Coimbatore city, South India, during their first visit to the various Gastroenterology Institutes, during their routine checkup on remission in Gastroenterology departments and local health clinics and when hospitalized during an exacerbation of the disease. We divided the patients with CD into four categories based on disease location namely Group I with Ileocolon location, Group II with ileal location, Group III with colonic location, and Group IV with perianal location. An additional three categories based on disease activity as new, relapse, and remission was included in the study. For relapse, the highest CRP level during the exacerbation, within the first four weeks after the onset of symptoms, was taken as an index CRP level [26].

Of the patients, thirty-seven were males, and six were females, between 21 and 55 years of age (mean of 34.48±9.25). An equal number of age and sex matched controls was also taken for the study. Excluded were any individuals with other inflammatory disease conditions (arthritis, IBS, etc.) and infections. Thirty-three patients of our study were under different medications. Of them 11 were taking steroids (systemic and topical), 14 were taking 5-ASA+Probiotics, four were taking 6-MP/azathioprine, and four were taking antibiotics like metronidazole/ciprofloxacin.

Comparison of subgroups

The three subgroups namely new patients with active disease, patients on remission, and patients with relapse were also compared in addition to the main categorization.

Measurement of CRP in serum

Venous blood was drawn from patients and collected in oxalate-coated tubes for serum separation and collection. The quantitative concentration of sCRP was determined by the immunoturbidimetric assay using kits with the help of UV-Vis spectrophotometer. The lowest serum-CRP concentration with this method is 0.3 mg/dL. The maximum detection limit was 10 mg/dL.

Chromosomal aberration assay

The blood sampling and cell culture procedures were essentially the same as those used for normal control individuals. Briefly, 0.5 ml blood was added to 4.5 ml RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1%

streptomycin-penicillin, 0.2 ml reagent grade phytohemagglutinin, and was incubated at 37°C. After 50 hours, cultures were treated with 0.1 g/ml colcemid to block cells in mitosis. Lymphocytes were harvested after 52 hours by centrifuging cells to remove culture medium (800-1000r/min), added with hypotonic solution (KCl, 0.075 mol/L) at 37°C for 20 minutes to swell the cells, and treated twice with Carnoy's fixative (3:1 (v/v) ratio of methanol : acetic acid). Slides were carefully dried on a hot plate (56°C, two minutes), and then stained using the Giemsa-banding (G-banding) technique and 100 well spread metaphases per slide were screened [30].

Molecular genetic analysis

Genotyping of the three Single Nucleotide Polymorphisms (SNPs) within the NOD2 gene, denoted Arg702Trp, Gly908Arg, and 980fs981 (GenBank accession numbers G67950, G67951, G67955, respectively) was performed using PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism). PCR-RFLP analyses for Arg702Trp and Gly908Arg were carried out according to the method described by Mendoza et al. [31] and genotyping of 980fs981 was carried out [32].

Statistical Analysis

All continuous variable data were summarized and are displayed as mean±SD for each patient group. All the categorical data were summarized and displayed as the number and percentage of participants in each group. A paired Student *t* test was used to compare between CRP levels of patients and controls in different groups. In order to evaluate differences between groups and sub-groups, one-way ANOVA followed by Tukey's HSD (honestly significant differences) post hoc test was performed among them.

CDAI

CDAI scores were evaluated as described by Best et al. [33]. The CDAI scores were also compared with sCRP levels to check the correlation between the two variables. Pearson correlations for confounding variables were used to evaluate the association between the sCRP and the scores of disease activity in CD patients. p value <.05 was considered as statistically significant.

Results

Forty-three patients with CD and equal number of age and sex matched controls was included for the study. The controls were mostly recruited as subjects who underwent routine master health check-ups in hospitals and local health clinics. The mean±SD of CRP in various groups of IBD is reported in **Table 1**.

A significant increase in the sCRP concentration was seen in individuals with CD as opposed to the controls. The mean±SD of sCRP for patients of different groups in which aberrations were scored was

Table 1. Differences of serum CRP between patients and controls in various groups

Particulars	Number of subjects examined	Serum CRP values (mg/dL) mean SD	Serum CRP values (mg/dL) paired T test values	Significance p<.05
Group I				
Controls	19	0.3474±0.069	4.333	S*
Experimentals	19	2.989±2.680		S
Group II				
Controls	11	0.363±0.1026	5.419	S
Experimentals	11	3.927±2.156		S
Group III				
Controls	8	0.337±0.106	3.721	S
Experimentals	8	3.062±2.112		S
Group IV				
Controls	5	0.360±0.0547	3.500	S
Experimentals	5	2.560±1.4028		S

Group I: Patients with disease present in ileum and colon; **Group II:** Patients with disease confined to small intestine; **Group III:** Patients with disease confined to colon; **Group IV:** Patients with disease in perianal region. S* - refers to significant

5.2±7.38. Due to small sample size, the p values were not significant when comparison between groups was considered (**Table 2**), though the values were significant for comparison between new and patients with remission as compared to patients with relapse (**Table 3**). In addition, mean sCRP levels for new patients was higher in the three sub-categories of disease activity patients studied (**Table 4**).

In the present study, chromosomal aberrations were present in chromosomes 1, 3, 5, 6, 9, 12, X, and 22. An increase in the frequency of aberrations with an increase in CRP concentration was found in the sub-group of patients categorized on basis of disease activity in the present study (**Table 5**). Two cases (CDP017 and CDP015) had a family history of the disease (4.6%) and the rest were all sporadic cases (95.4%) of CD. Two of the 43 control subjects studied (4.65%) showed minor chromosomal aberrations.

In our study, none of the patients and controls was found to have the three SNP variants of the NOD2 gene. The CDAI scores in the patients ranged from 80-290 and disease was found to be of mild-moderate type in the patients studied. Pearson's correlation between sCRP levels and CDAI scores yielded a value of 0.833, which was found to be significant at p <.001 as shown in **Figure 1**.

Therapy

As in patients in other countries, a high dependence on steroid therapy and salicylates was found in patients.

Table 2. Statistical data pertaining to Crohn's disease patients with different disease (Groups I, II, III, and IV). One way ANOVA followed by Tukey's comparison between four groups of patients with p<0.01

Sum of Squares	df	M Square	Fisher F value	Sig (p)
Between groups: 8 .819	3	2.940	0.497	0.687
Within groups: 230.788	39	5.918		
Total: 239.607	42			

Table 3. One-way ANOVA followed by Tukey's comparison between three sub-groups of patients (new, remissions, and relapse)

Sum of Squares	df	M Square	Fisher F value	Sig (p)
Between groups: 97.3579	2	48.679	13.5028	<0.0001
Within groups: 155.0214	43	3.6051		
Total: 252.793	45			

HSD [.05] =1.77; HSD [.01] =2.25. M1 vs. M2 p<.01; M1=mean of Sample 1, M2=mean of sample 2

Table 4. Mean CRP level in the three sub-categories of disease activity with levels in corresponding control subjects

Disease activity	CD	Mean±SD
Active/New	20 (46.5%)	4.66±2.20
Controls		0.37±0.10
Remission	15 (34.8%)	1.25±0.71
Controls		0.34±0.06
Relapse	8 (18.6%)	3.15±1.55
Controls		0.32±0.04

Table 5. Frequency of aberrations (per 100 metaphases scored) in CD patients of the present study

Group	CRP (mean SD)	Number of cells scored	Frequency of aberrations
Group I	2.989±2.68	1900	7.3%
Group II	3.927±2.156	1100	3.3%
Group III	3.062±2.112	800	0.5%
Group IV	2.560±1.402	500	0%
New	4.66%±2.20	2000	7.9%
Remission	1.25±0.71	1500	0.9%
Relapse	3.15±1.55	800	2.3%

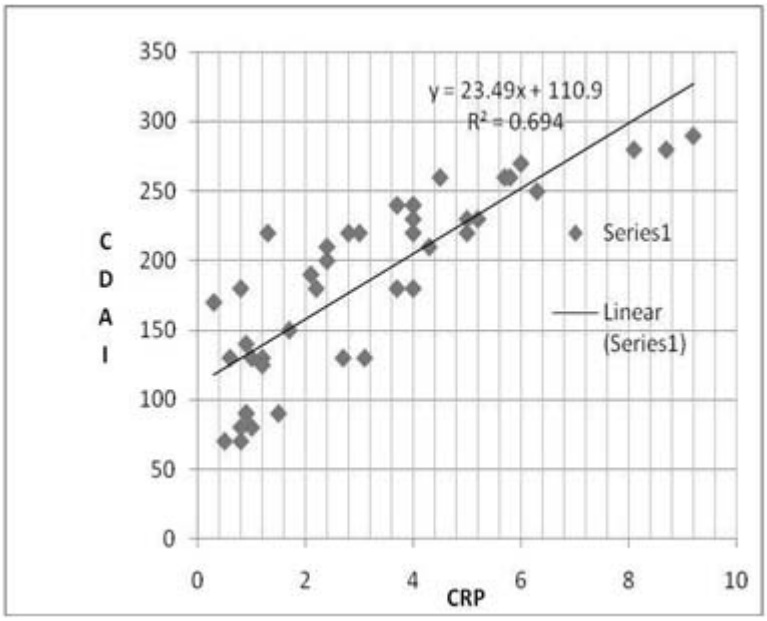

Figure 1. Linear correlation between CRP concentration and CDAI scores

Discussion

In the present study, more males were found to be affected as compared to females (37 and 6 respectively) as has been the case in an earlier study from the country [29]. Concerning Asian population, in the Chinese and Japanese races, there are some similarities in the pattern of CD, including a predominance of ileocolonic involvement and male gender predominance (70-77% in the Japanese and 71% in the Chinese) [34, 35]. In our study, a clear increase in CRP levels as compared to controls was observed. In addition, within groups, values were found to be significant between new patients and patients on remission.

The resurgence of interest in C-reactive protein, a major liver-derived acute phase protein, has provided a better definition of disease flare-up [4] when abscess formation or infections are ruled out. The level of serum CRP acts as a robust marker of intestinal inflammation and as an excellent biological marker of response for patients with elevated CRP and active luminal disease. The therapeutic action of some of the newer biologics is indeed more striking on stratification of treatment response according to serum CRP level [36, 37].

It has been shown that quantitative CRP measurements help determine the degree of disease activity in patients with IBD. The introduction of sensitive CRP assays to clinical practice enabled

evaluation of CRP concentrations that were previously thought to be within normal limits when studied with less sensitive assays. We therefore wished to determine the correlation between sCRP and IBD disease activity to determine whether there is any correlation between sCRP concentrations and clinical course of the disease

Chromosomal anomalies

The present study has revealed chromosomal instability on specific chromosomes in lymphocytes of sporadic CD patients and a couple of hereditary patients compared to healthy unrelated controls. A high frequency of aberrations involving specific chromosomes harboring particular genes involved, directly or indirectly, in tumorigenesis, indicates a constitutional chromosomal instability. Cytogenetic analysis of lymphocytes as an adjunct to other tests has the potential to be used for surveillance of the subjects at risk for carcinogenesis [38].

In our study, deletions were observed in long arms of chromosomes 1, 3, 5, 9, 12, and X. Recurrent losses, including 1p, 14q, 10q, 13q, 15q, 18q, and 22q, and gain of 5p, 12q, 17q, and 20q have been reported as genetic markers with prognostic potential in gastrointestinal stromal tumors [39]. Though a database is required to characterize aberrations as a marker of cancer risk [40], there is evidence that increased frequency of chromosomal aberrations in blood lymphocytes is predictor of cancer [41-43]. Structural and numerical aberrations in chromosome 1, 2, 5, 7, 12, 14, 17, 18, and 21 have been reported earlier also in lymphocytes of GIT cancer patients [44-46]. It has been reported that chromosome segments frequently lost are regions of candidate loci for tumor suppressor genes and those frequently gained are candidate loci of dominantly acting growth regulator genes [47].

A mosaic was found in one of the patients CDP034 (45XO/ 46, XX, del(6p-). A mosaic karyotype 46, XX / 45, XO was observed in an earlier study conducted by our lab on gastrointestinal lesions [48] suggesting the possible role of the missing chromosome in the suppression of dysplasia in such subjects. Although in Turner's syndrome patients, there have been rare reports of a variety of symptomatic vascular malformations of the gastrointestinal tract, more common are instances of IBD [49]. The association of CD with Turner's syndrome might reflect X chromosome involvement in disease pathogenesis.

In our study, translocations involving chromosomes one and three [46, XY, t (1p-:3q+)] were observed in a couple of patients and chromosomes six and 16 [(46, XY, t (6q-:16q+)] were found in a single patient. According to Richard et al. [50], despite considerable inter individual variations; increased chromosome breakage and rearrangement may be the signs of chromosome instability in the predisposition to colorectal cancer. In addition to the translocations, we observed few more structural aberrations viz; 46, XY del(5q-), 46, XY del(3q-), 46, XY del(6p-), 46, XY del(9q-), and 46, XY del(12q-).

In addition, a higher sCRP level was found to be associated with more or less higher frequency of chromosomal aberrations detected in such patients (mean SD of 5.2±7.38). An increased sample size along with other molecular cytogenetic approaches may enable establishment of a pattern for correlating increased sCRP levels with aberrations in different regions. It may also help to narrow down the critical regions in each identified chromosome and to characterize the putative genes involved in IBD. Additionally, gene for CRP is present in chromosome 1q, which might get affected in IBD as in one of the patients (CDP033) of present investigation showed, thus accounting for increase in such patients.

The aberrations may be suggestive of regions containing mutational or polymorphic changes in genes thus contributing to certain drug tolerance/resistance pattern that can be used for a pharmacogenomic advantage in such patients due to adverse effects of systemic steroid and antipurine analog based therapy. For example, steroid naive, dependent and refractory types may be decided beforehand and appropriate alternate safe regimen prescribed.

Our study showed two of the patients had a family history of the disease and one of them was found to have a chromosomal aberration [46, XX, del(3q-)]. The greatest risk factor for the development of IBD is having an affected family member with this disease. First-degree relatives, especially siblings, are at greatest risk, but the risk also extends to distant relatives [51].

In our study, none of the patients and controls harbored the NOD2 SNPs reported elsewhere. Three apparently common mutations in the NOD2 gene have been reported in up to 30% of sporadic patients with Crohn's disease in western countries, which when analyzed in patients of the Indian subcontinent, were found to be uncommon [52].

Altogether, the study reiterates that the clinical phenotypes of Asian IBD resemble the white population in general, but with some differences, including higher prevalence of males, and higher prevalence of ileocolonic involvement among Asian Crohn's disease patients [42]. A family history for CD is uncommon and may indicate a lesser genetic predisposition to the disease than in Western populations. However, time trend studies of the prevalence of IBD are necessary to investigate this further [53]. Unfortunately, data on the epidemiology and clinical characteristics of IBD in many Asian countries, and for that matter in most developing countries, are scarce [13]. Investigating the early stages of IBD as it emerges in new populations may provide a new opportunity to study its pathophysiology [54].

The above-mentioned data might hint for use of simple conventional tools for tracking changes in IBD patients. The present findings might have special relevance for therapeutic interventions if carried over a longer range and time span. They also show that for a given change in sCRP concentration, there is a similar change in disease activity score for patients with CD. A limitation of our analysis is the small number of patients. Another limitation of the study is that several patients (on remission and relapse) were under various medications with anti-inflammatory activity though a standard criterion for the investigations has been followed and this could not be used against selection as the disease is on the rise in the subcontinent. Obviously one cannot exclude the possibility that this could have an effect on the results of the sCRP concentrations. Until more markers become available, the use of CRP should be seen as an additive, non-invasive tool for clinical observation and physical examination in a disease of unknown etiology as Crohn's disease. In addition, cytogenetic analysis should be used as an additional tool for detecting gross chromosomal changes that might lead to molecular clues in unraveling the mediators of this disease condition. Hence, in the absence of specific markers for diagnosis, a combination of tools may prove to be effective in determining the clinical course in a disease as complex as Crohn's thus aiding in surveillance.

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