

Perinuclear Neutrophil Antibodies Are Not Markers for Genetic Susceptibility or Indicators of Genetic Heterogeneity in Familial Ulcerative Colitis

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OBJECTIVES: Autoantibodies that bind to the perinuclear region of neutrophils have been suggested to represent subclinical markers of genetic susceptibility and indicators of genetic heterogeneity in ulcerative colitis (UC). However, results from more recent studies have contradicted this statement. To address this discrepancy, we assayed perinuclear neutrophil antibodies in the UC-affected members of 53 families with at least one relative pair affected with UC.

METHODS: Perinuclear neutrophil antibodies were detected using an indirect immunofluorescence assay.

RESULTS: Perinuclear neutrophil antibodies were present in 53% of the study subjects with ulcerative colitis (UC). In 15 families (28%), all members with UC were positive for perinuclear neutrophil antibodies, whereas in 12 families (23%), none of the UC-affected members were positive. In the remaining 26 families (49%), the results for the UC-affected relatives were discordant. Similar results were found when only sibling pairs were evaluated. Statistical analysis revealed that for all affected relative pairings, observed concordance did not significantly exceed expected concordance. Logistic regression analyses demonstrated that the degree of relationship was not enough to predict concordance for perinuclear neutrophil antibodies, and that these antibodies are not markers of genetic heterogeneity.

CONCLUSIONS: Perinuclear neutrophil antibodies are not markers for genetic susceptibility or indicators of genetic heterogeneity in UC. (*Am J Gastroenterol* 2002;97:2343–2349. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

Mechanisms that lead to the development of inflammatory bowel disease (IBD) are not well understood, but genetic factors clearly play an important role (1). Although recent studies have shown evidence for linkage between IBD and loci on specific chromosomes (2–12), genetic heterogeneity within IBD is a challenge that must be faced as the genome

is searched for IBD susceptibility genes. In addition, the genetics of IBD are complex because of the likely involvement of more than one susceptibility gene, lack of simple Mendelian inheritance, and incomplete penetrance (1). The identification of markers that define genetically more homogeneous subgroups of IBD would be of great help in the search for susceptibility genes (1).

Autoantibodies that bind to the perinuclear region of neutrophils are highly specific and moderately sensitive markers for ulcerative colitis (UC) (13–15). They are found in 44–86% of subjects with UC, 0–6% of subjects with non-IBD GI disorders (irritable bowel syndrome, bacterial colitis, and celiac sprue), and 0–6% of healthy controls with no family history of IBD (13–24). Two studies showed that unaffected relatives of patients with UC had a significantly higher frequency (16–30%) of perinuclear neutrophil antibodies compared to healthy controls (0–3%) (16, 17). One of these studies also showed that the frequency of perinuclear neutrophil antibodies was increased in relatives of antibody-positive probands compared to relatives of antibody-negative probands (16). Together, these data have been used to suggest that perinuclear neutrophil antibodies are markers of genetic susceptibility and indicators of genetic heterogeneity in UC (16, 17). However, subsequent studies have contradicted these results and suggested that perinuclear neutrophil antibodies are neither markers of genetic susceptibility nor indicators of genetic heterogeneity in UC (18–25). The reasons for these discrepancies are unclear, but possible explanations have included differences in techniques for antibody detection as well as distinct populations and ethnic groups in each study (18–23).

Although IBD genetics are complex, genetic susceptibility factors for affected members within the same family should be similar. Therefore, if perinuclear neutrophil antibodies are truly markers of genetic susceptibility or indicators of genetic heterogeneity in UC, then multiple UC-affected relatives within single families should have a high degree of concordance for these antibodies. To test this hypothesis, we evaluated the presence of perinuclear neu-

trophil antibodies in UC-affected members of 53 families with at least one UC-affected relative pair.

MATERIALS AND METHODS

Study Subjects

As part of ongoing institutional review board-approved studies of the genetic basis of IBD at the University of Pittsburgh and the Cleveland Clinic Foundation, blood samples were collected from families with two or more IBD-affected members. The geographic distribution of these families spanned the entire United States. A total of 53 families with two or more UC-affected members constituted the study group. In 40 families, all affected members had UC, whereas in the other 13 families, at least two members had UC but another member had either Crohn's disease or indeterminate colitis. Only subjects with UC were analyzed. Diagnoses of IBD were confirmed by review of medical records.

Assay for Neutrophil Antibodies

An indirect immunofluorescence assay was used for the detection of neutrophil antibodies. Serum samples were diluted 1:20 in phosphate-buffered saline (PBS) and 35 μ l of each diluted sample were then placed in wells containing ethanol-fixed human neutrophils (INOVA Diagnostics, San Diego, CA). After incubation for 30 min in a moist chamber at room temperature, the serum was washed off using PBS, and the slides were then placed in a Coplin jar containing PBS for 5 min. Antihuman IgG (goat) conjugated to fluorescein (INOVA Diagnostics) was added to each well, followed by incubation in a moist chamber for 30 min. The fluorescein conjugate was then washed off with PBS, and the slides were placed in a Coplin jar containing PBS for 5 min. Coverslips were applied to the slides using a mounting medium (INOVA Diagnostics). A positive control was run with the test samples.

Slides were viewed using an epifluorescence-equipped microscope. Each slide was read by two independent observers who were blinded to the relationships of the study subjects. If there was a discrepancy between the two readings or if the staining pattern was difficult to interpret, the above-described procedure was repeated and the slides were again read in a blinded fashion. Only a perinuclear staining pattern was considered positive.

Statistical Analysis

Data were analyzed by ranking affected relative pairs according to their concordance or discordance for perinuclear neutrophil antibodies. *Observed* concordance ratios within affected pairs were analyzed by examining 1) relatives with either both having or both lacking perinuclear neutrophil antibodies, and 2) only relatives both having positive perinuclear neutrophil antibodies. *Expected* concordance ratios were calculated as the probability of sharing one allele identical by descent or twice the kinship coefficient. Ob-

served and expected concordance ratios were compared using a test of binomial proportions based on the hypothesis that if perinuclear neutrophil antibodies are markers of genetic susceptibility, then observed ratios should be identical to or greater than the expected degree of sharing. This method is analogous to a standard nonparametric linkage analysis, in that we are looking for increased sharing of perinuclear neutrophil antibodies (increased concordance in antibody status) in relative pairs affected with UC. The advantage of using this method is that it is completely model independent, which allows us *not* to make any assumptions regarding modes of inheritance. This is important because the genetics of IBD are complex and do not follow simple Mendelian modes of inheritance.

To determine whether the degree of relationship between individuals had an influence on concordance for perinuclear neutrophil antibodies, we performed a logistic regression using the kinship coefficient as a measure of degree of relationship between individuals within a pair. The kinship coefficient of two individuals (I, J) is the probability that a randomly chosen allele from one is identical by descent with a randomly chosen allele from the other (26). In general, if there are m meioses from I to the nearest common ancestor and then back to J, then the kinship coefficient (ϕ) is calculated as:

$$\phi = \sum_i \left(\frac{1}{2}\right)^{m_i + 1}$$

where i is the number of genetic paths connecting I and J and some common ancestor (26).

For logistic regression, we modeled the kinship coefficient as the independent variable and the probability of concordance for perinuclear neutrophil antibodies as the dependent variable.

RESULTS

Of the 53 study families, there were 43 families with two UC-affected members, seven with three UC-affected members, one with four UC-affected members, and two with five UC-affected members. Therefore, a total of 121 subjects (58 male and 63 female) were analyzed for the presence of perinuclear neutrophil antibodies. The distribution of affected relative pair relationships is shown in Table 1.

Perinuclear neutrophil antibodies were present in 64 of the 121 study subjects (53%). Figure 1 demonstrates the antibody status within each of the families. In 15 families (28%), all UC-affected members were positive for perinuclear neutrophil antibodies, whereas in 12 families (23%), all UC-affected members were negative. In the remaining 26 families (49%), the UC-affected members were discordant for perinuclear neutrophil antibodies. Of the 10 families with three or more UC-affected members, none had complete concordance for presence of perinuclear antibodies, whereas in one family, all three UC-affected members were

Table 1. Data for Affected Relative Pairs

Type of Affected Pair	Kinship Coefficient	Total Number of Pairs*	Concordant Pairs†		Discordant Pairs	Concordance Ratios			
			Positive	Negative		Observed‡	Expected	Difference§	95% CI
Siblings	0.250	42	11	12	19	0.37	0.50	NS	0.22–0.55
Parent-offspring	0.250	11	5	4	2	0.71	1.00	<0.05	0.36–0.92
Grandparent-grandchild	0.125	2	0	1	1	0.00	0.25	NS	
Avuncular	0.125	19	4	5	10	0.29	0.25	NS	0.11–0.52
First cousins	0.062	8	3	0	5	0.38	0.12	NS	0.14–0.70
First cousins once removed	0.031	8	1	2	5	0.17	0.06	NS	0.03–0.57

* These pairings represent all possible pairings within families. Of the 53 families in the study, there were 43 with two affected relatives (43 paired relationships), seven with three affected relatives (21 possible paired relationships), one with four affected relatives (six possible paired relationships), and two with five affected relatives (20 possible paired relationships). This leads to a total of 90 possible paired relationships.

† Positive and negative pairs are those pairs that are concordant for (respectively) either presence or absence of antibody.

‡ Observed concordance ratios were based only on the affected relative pairs who were concordant or discordant for presence of perinuclear neutrophil antibodies.

§ Difference is calculated as the comparison of the observed concordance ratio vs expected ratio.

negative for perinuclear antibodies (10% concordance ratio).

The data were then analyzed for sibling pairs only (Fig. 2). There were 29 families with two affected siblings, one family with three affected siblings, and one family with five affected siblings. Of 66 subjects, 36 (55%) were perinuclear neutrophil antibody positive. In 10 families (32%), both siblings were positive for perinuclear neutrophil antibodies, whereas in six (19%), both siblings were negative. In the remaining 15 families (48%), including the two families with more than two affected siblings, there was discordance for perinuclear neutrophil antibodies.

Statistical analysis was performed based on concordance or discordance for perinuclear neutrophil antibodies by affected relative pair relationships (Table 1). As can be seen, the observed concordance ratios (based only on the affected relative pairs who were discordant or concordant for positive antibody titer) did not differ significantly from the expected concordance ratios except for parent-offspring pairs, in whom a significantly less than expected difference

was noted. This finding in parent-offspring pairs would suggest that perinuclear neutrophil antibodies are not markers of genetic susceptibility to UC. However, the difference was not large and could be due to recombination between putative genetic determinants for UC and perinuclear neutrophil antibodies. The observed concordance ratio for siblings (0.37), although not low enough to be incompatible with the expected concordance ratio (0.50), still approached a significantly lower than expected difference as well. Had our sample size been somewhat larger (*e.g.*, twice as large), then the observed concordance ratio would have been significantly lower than 50%, again suggesting that perinuclear neutrophil antibodies are not markers of genetic susceptibility to UC. Similarly, for the other affected relative pairings, the observed and expected concordance ratios were not significantly different from each other, a finding that is probably due to small sample sizes.

To examine further the relationship between kinship coefficient and probability of concordance, two logistic regression analyses were performed. In the first analysis, all con-

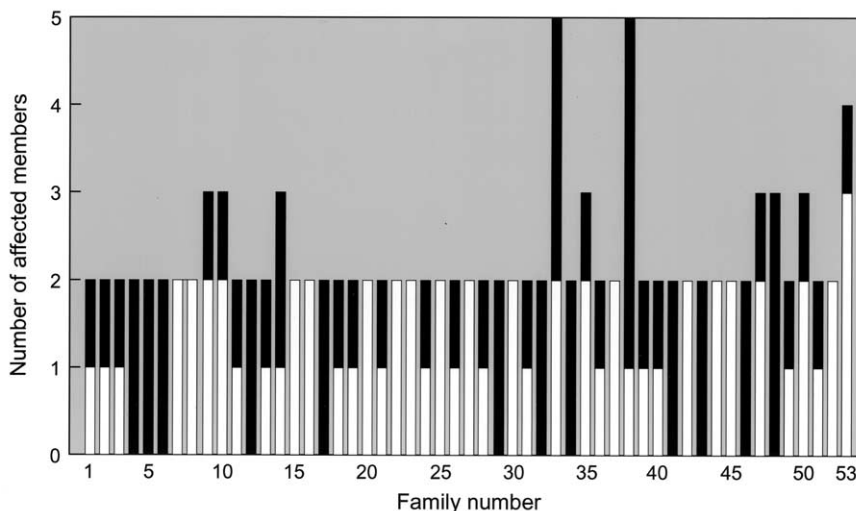


Figure 1. Perinuclear neutrophil antibody distribution within each family member for the entire study group (□ = perinuclear neutrophil antibody positive; ■ = perinuclear neutrophil antibody negative).

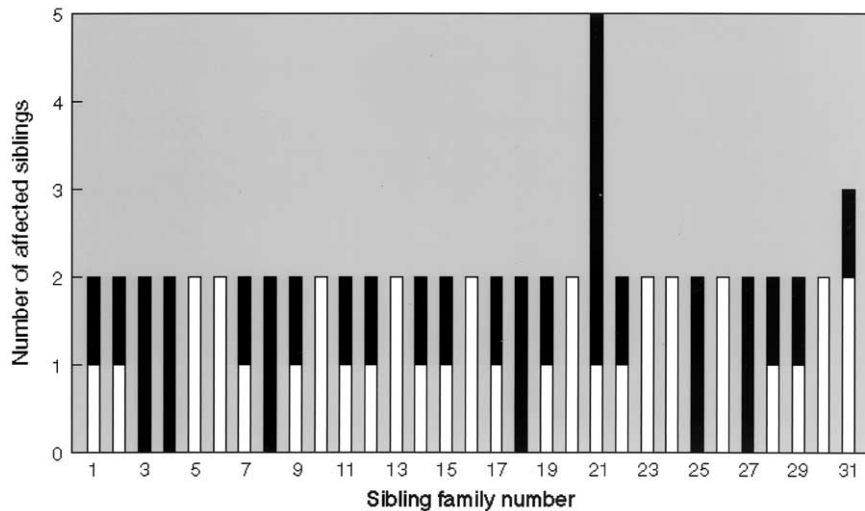


Figure 2. Perinuclear neutrophil antibody distribution within each family for sibling pairs only (□ = perinuclear neutrophil antibody positive; ■ = perinuclear antibody negative).

cordant (both antibody-positive and antibody-negative concordance) and discordant pairs that were formed from nonoverlapping individuals within the pedigrees were used. This analysis examines the influence of kinship coefficient on the probability of antibody concordance in a pair, and directly addresses the coheritability of UC and perinuclear neutrophil antibodies. In the second analysis, only discordant pairs and antibody-positive concordant pairs were considered. This analysis addresses the issue of perinuclear neutrophil antibodies as a marker of genetic heterogeneity. If the presence of perinuclear neutrophil antibodies is a marker for a unique subset of UC patients, then there should be an observable cosegregation of UC and these antibodies within that subset, with a significant effect for regression against the kinship coefficients. As can be seen from the coefficient tables (Tables 2 and 3), the p value attributed to kinship coefficient as a predictor was not significant for either analysis ($p = 0.59$ when all concordant pairs were considered; $p = 0.86$ when only antibody-positive concordant pairs were considered). These results are not surprising when one considers only the sibling-sibling and parent-offspring pairs. Genetically, these two pairs seem similar because parent-offspring pairs always share exactly one allele identical by descent and siblings share on average one allele identical by descent. Despite this genetic similarity, the concordance ratios for each pair were very different in this study (Table 1). Indeed, for the 11 parent-offspring pairs, nine pairs were concordant for the presence ($N = 5$) or absence ($N = 4$) of perinuclear neutrophil antibodies, whereas for the 42 sibling-sibling pairs, there was an almost

even split between discordant ($N = 19$) and concordant pairs ($N = 23$). These results indicate that degree of relationship alone is not enough to predict discordance or concordance for perinuclear neutrophil antibodies.

DISCUSSION

The results of this study suggest that perinuclear neutrophil antibodies are not markers of genetic susceptibility or of genetic heterogeneity in UC. Of 53 families with two or more UC-affected members, complete concordance for the presence of perinuclear neutrophil antibodies in the UC-affected members was demonstrated in only 15 families (28%), and discordance for perinuclear neutrophil antibodies among UC-affected relatives was demonstrated in 49% of families. This pattern was consistent when only sibling pairs, in whom a greater degree of genetic sharing would be expected, were analyzed. Furthermore, analysis of concordance ratios revealed significantly reduced differences between observed and expected concordance ratios for parent-offspring pairs. In addition, the comparison of concordance ratios for sibling pairs would have been significant at the same proportions for a larger collection, leading us to suspect reduced concordance in these pairs as well. The finding of an observed concordance ratio that was less than the expected ratio suggests that perinuclear neutrophil antibodies are not markers of genetic susceptibility to UC. This statement is supported by the logistic regression analysis, which demonstrated that degree of relationship alone was not enough to predict concordance for perinuclear neutro-

Table 2. Logistic Model Coefficients Table for All Concordance

	Coefficient	SE	Coefficient/SE	χ^2	p Value
Constant (α)	0.14	0.73	0.19	0.04	0.85
Kinship coefficient (β)	1.91	3.52	0.54	0.29	0.59

Table 3. Logistic Model Coefficients Table for Positive-Antibody Concordance

	Coefficient	SE	Coefficient/SE	χ^2	<i>p</i> Value
Constant (α)	-0.18	0.78	-0.23	0.05	0.82
Kinship coefficient (β)	0.68	3.78	0.18	0.03	0.86

phil antibodies. In addition, our data suggest that perinuclear neutrophil antibodies are not markers of genetic heterogeneity within the UC population because logistic regression modeling in the perinuclear neutrophil antibody-positive UC subpopulation failed to detect heritability of concordance for the antibody.

Our results contradict those of prior studies from North America (16) and Germany (17), which suggested that perinuclear neutrophil antibodies might represent markers of genetic susceptibility for UC. In these studies, unaffected relatives of UC subjects had statistically significantly increased frequencies of perinuclear neutrophil antibodies compared to healthy controls. The North American study (16) also suggested that there was evidence for genetic heterogeneity in UC because perinuclear neutrophil antibody-positive probands were more likely to have an antibody-positive unaffected relative than probands who were antibody negative. A similar trend was seen in the German study when only antibody titers of $\geq 1:100$ were considered positive, but this trend did not reach statistical significance. Our findings, including discordant perinuclear neutrophil antibody status among UC-affected relatives in 49% of families, would argue that these antibodies are not markers for genetic susceptibility or indicators of genetic heterogeneity in UC.

Our results are in agreement with those of seven other published studies (18–24) and the abstract of one study (25) that did not find evidence that perinuclear neutrophil antibodies are markers for genetic susceptibility to UC. Six of these studies (18–23) evaluated nonfamilial cases of UC and found low rates of positive perinuclear neutrophil antibodies (range of 0–7%) in unaffected relatives of UC patients. These frequencies were much lower than those in the North American and German studies in which 16–30% of unaffected relatives were perinuclear neutrophil antibody-positive. In addition, the low frequencies of positive perinuclear neutrophil antibodies were similar to those in a wide spectrum of control groups that included healthy subjects, unaffected spouses of UC subjects, unaffected relatives of patients with Crohn's disease, patients with the irritable bowel syndrome, and patients with celiac disease (range of 0–6%).

If perinuclear neutrophil antibodies are truly markers of genetic susceptibility to UC, then a high degree of concordance for these antibodies should be observed among UC-affected relatives and especially among monozygotic twins with at least one UC-affected twin. A group of investigators from England demonstrated that the frequency of perinuclear and cytoplasmic neutrophil antibodies was no different for familial UC (44%) than that for nonfamilial UC (46%)

(19). These investigators also noted that among families with two or more UC subjects, all affected members were positive for neutrophil antibodies in only 14% of the families. This finding is similar to our finding that concordance for the presence of perinuclear neutrophil antibodies among all UC-affected relatives was observed in only 28% of families. Another study evaluated perinuclear neutrophil antibody status in 12 pairs of monozygotic twins with at least one UC-affected twin (24). There was no difference in the frequency of perinuclear neutrophil antibodies when the affected twins were compared to cases of nonfamilial UC. In addition, of the 10 healthy twins, only two (20%) were positive for perinuclear neutrophil antibodies. Similarly, a study of neutrophil antibodies in monozygotic twins discordant for UC recently presented in abstract form demonstrated that only two of 17 (12%) monozygotic unaffected twins were positive for neutrophil antibodies by an indirect immunofluorescence assay compared with five of 17 (29%) of the UC-affected twins (25). The results from the studies with monozygotic twins are of particular interest because they suggest that even when there is complete genetic sharing, perinuclear neutrophil antibody status is no different than that for comparable groups of nonfamilial UC patients or healthy controls. Our study further supports the results from these trials and argues against a genetic association between perinuclear neutrophil antibodies and UC.

The reasons for discrepancies in results between the initial North American and German studies and those of several other studies are unclear. A possible explanation could relate to differences in the technique of neutrophil antibody detection. Indirect immunofluorescence, either alone or in conjunction with an ELISA, was used in all of the studies discussed. However, a recent study demonstrated that the detection of perinuclear neutrophil antibodies from the same cohort of patients varied greatly among six different laboratories (27). Because the results of that study are available only in abstract form, the methods used for antibody detection in each laboratory are not known but presumably involved enzyme-linked immunosorbent assays and/or indirect immunofluorescence. Another possible reason for the discrepant results is the fact that two studies considered either perinuclear or cytoplasmic neutrophil staining as positive (19, 21). However, both of these studies found low rates of neutrophil antibodies in unaffected relatives and, thus, the detection of cytoplasmic antibodies would not seem to have affected the results or conclusions of these trials. Another possibility for different study results could relate to changes in perinuclear neutrophil antibody status over time or with UC disease activity. One study showed that changes in perinuclear neutrophil antibody status (both

positive to negative and *vice versa*) occurred in 25% of patients during a mean follow-up of 51 months (28). Another study found low rates of antibody positivity in patients with quiescent UC (29), but other studies have demonstrated persistence of perinuclear neutrophil antibodies more than 5 yr after colectomy (13, 30). Finally, the discrepancy in results could be related to the influence of distinct populations and ethnic backgrounds on genetic factors. Before our study, the North American study (16) was the only one from this continent, whereas all the other trials came from Europe. Given the results of our study which included an ethnically heterogeneous population from across the United States as well as similar results from several single country trials in Europe that may have involved more homogeneous populations, it seems unlikely that specific populations or ethnicity had significant effects on perinuclear neutrophil antibody expression.

In conclusion, the results of our study in conjunction with those from several other trials suggest that neutrophil antibodies with perinuclear immunofluorescence staining are not markers for genetic susceptibility to UC. These antibodies also do not seem to be indicators of genetic heterogeneity in UC.

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