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Festen, E.A.M.; Goyette, P.; Scott, R.; Annese, V.; Zhernakova, A.; Lian, J.; Lefèbvre, C.;
Brant, S.R.; Cho, J.H.; Silverberg, M.S.; Taylor, K.D.; de Jong, D.J.; Stokkers, P.C.;
McGovern, D.; Palmieri, O.; Achkar, J.P.; Xavier, R.J.; Daly, M.J.; Duerr, R.H.; Wijmenga, C.;
Weersma, R.K.; Rioux, J.D.
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Genetic variants in the region harbouring \textit{IL2/IL21} associated with ulcerative colitis

E A M Festen,\(^1\) P Goyette,\(^2\) R Scott,\(^3\) V Annese,\(^4\) A Zhernakova,\(^5\) J Lian,\(^2\) C Lefèbre,\(^2\) S R Brant,\(^6\) J H Cho,\(^7\) M S Silverberg,\(^8\) K D Taylor,\(^9\) D J de Jong,\(^10\) P C Stokkers,\(^11\) D Mcgovern,\(^11\) O Palmieri,\(^4\) J-P Achkar,\(^12\) R J Xavier,\(^13\) M J Daly,\(^14\) R H Duerr,\(^3\) C Wijmenga,\(^2\) R K Weersma,\(^1\) J D Rioux\(^2\)

**ABSTRACT**

Objectives: Genetic susceptibility is known to play a large part in the predisposition to the inflammatory bowel diseases (IBDs) known as Crohn’s disease (CD) and ulcerative colitis (UC). The \textit{IL2/IL21} locus on \textit{4q27} is known to be a common risk locus for inflammatory disease (shown in coeliac disease, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and psoriasis), while the roles that interleukin 2 (IL2) and IL21 play in the immune response also make them attractive candidates for IBD. The objective of this study was to test for association between the \textit{IL2/IL21} locus and the IBDs.

Methods: The four single nucleotide polymorphisms (SNPs) in the \textit{IL2/IL21} locus most associated with coeliac disease were genotyped in 1590 subjects with IBD and 929 controls from The Netherlands, and then replicated in a North American cohort (2387 cases and 1266 controls) and an Italian cohort (805 cases and 421 controls), yielding a total of 4782 cases (3194 UC, 1588 CD) and 2616 controls. Allelic association testing and a pooled analysis using a Cochran–Mantel–Haenszel test were performed.

Results: All four SNPs were strongly associated with UC in all three cohorts and reached genome-wide significance in the pooled analysis (rs13151961 \(p = 1.35 \times 10^{-10}\), rs13119723 \(p = 8.60 \times 10^{-9}\), rs8640978 \(p = 3.07 \times 10^{-8}\), rs8822844 \(p = 2.77 \times 10^{-7}\)). A moderate association with CD was also found in the pooled analysis (\(p\) value range 0.0016–9.86 \(\times 10^{-4}\)).

Conclusions: Strong association for the \textit{IL2/IL21} locus with UC was found, which also confirms it as a general susceptibility locus for inflammatory disease.

Inflammatory bowel diseases (IBDs) are the most common chronic inflammatory diseases in the Western world after rheumatoid arthritis, with an incidence of about 40 per 100 000 in North America and Western Europe.\(^1\) Ulcerative colitis (UC) and Crohn’s disease (CD) are the two main types of IBD, both characterised by recurring inflammation of the digestive tract. In CD the inflammation can occur throughout the gastrointestinal tract, most commonly affecting the terminal part of the small intestine and causing weight loss and abdominal pain. In UC the disease is confined to the colon, and patients usually present with bloody diarrhoea and abdominal cramping.\(^7\)

Genetic susceptibility plays an important role in the pathogenesis of IBD. CD and UC are complex diseases with numerous genetic and environmental factors leading to disease. Epidemiological studies suggest stronger heritability in CD compared with UC.\(^1\)

Many genetic factors contributing to CD pathogenesis have been identified during the last decade. There are currently \(>30\) genes or loci associated with CD, the majority having been identified since the introduction of genome-wide association studies.\(^2\) Far fewer have been found for UC. Recently the first genome-wide association study in UC was published identifying several new loci, and another genome-wide association study will be published shortly.\(^3\)\(^4\)

Genetic studies have also shown that susceptibility genes are commonly shared between inflammatory diseases. For example, the \textit{IL2/IL21} (interleukin 2/interleukin 21) locus on chromosome \textit{4q27} has been shown to be associated with coeliac disease, type 1 diabetes, Grave’s disease, systemic lupus erythematosus, rheumatoid arthritis and psoriasis.\(^5\)\(^6\)\(^7\) Interestingly, there appear to be at least two independent association signals in this region, one conferring an increased risk of disease and the other conferring a protective effect.\(^8\)\(^9\)

There are several reasons why the \textit{IL2/IL21} locus could also represent an interesting locus for IBD. First, a number of shared autoimmune and inflammatory genes show an association with IBD: \textit{IL12B}, for example, is associated with psoriasis,\(^10\) systemic lupus erythematosus,\(^12\) asthma\(^13\) and both forms of IBD, while \textit{IL18RAP} was found to be associated with both coeliac disease\(^13\) and IBD.\(^16\) \textit{IL2/IL21} is another shared inflammatory locus, and both \textit{IL2} and \textit{IL21} are attractive functional candidate genes for association with IBD. Overexpression of \textit{IL21} in inflamed regions of the bowel of patients with IBD has been reported.\(^17\) This overexpression is most marked in CD, but a significant overexpression compared with that in diverticular disease and healthy controls is also present in UC.\(^17\) Finally, \textit{Il2\(^{-}\)}\(^2\) mice develop IBD most reminiscent of UC.\(^10\)

Given all these observations, and that IBD and coeliac disease are chronic inflammatory diseases of the gut, we were specifically interested in testing whether the \textit{IL2/IL21} region variants identified in the coeliac genome-wide association studies also have a role to play in IBD. This was achieved via a case–control association study with a three-stage design in a large cohort of patients with IBD. In addition, we performed genotype–phenotype
analysis to identify association with specific subsets of IBD. Our data unequivocally show that the IL2/IL21 locus is strongly associated with UC. We confirmed this finding in multiple IBD populations.

METHODS

Subjects

For the first phase, the cases consisted of a cohort of 1590 patients with IBD (777 CD and 813 UC) collected from the outpatient clinics of the Departments of Gastroenterology and Hepatology at the Amsterdam Medical Center (n = 752), the Radboud University Medical Centre, Nijmegen (n = 275) and the University Medical Center Groningen, The Netherlands (n = 585).19 The control cohort consisted of 929 healthy Dutch individuals who were blood donors.

To replicate the findings from the first phase, two independent cohorts were examined. The first replication cohort consists of an IBD case–control cohort (2387 cases of which 654 were CD and 1735 UC, and 1266 controls) collected through the North American NIDDK IBD Genetics Consortium (IBDGC) as described previously.20 21 Cases and geographically matched controls were ascertained through the University of Montreal, Cedars-Sinai Medical Center, Johns Hopkins University, University of Chicago, University of Pittsburgh and the University of Toronto Genetics Research Centers (GRCs). This NIDDK-IBDGC IBD cohort contained five related pairs of cases between UC and CD samples. All cases were included in the subphenotype analysis, but in the IBD analysis one member of each pair (five cases) was removed. The second replication cohort consists of an Italian IBD case–control cohort (805 cases, of which 157 were CD and 648 UC, and 421 controls) collected at the S. Giovanni Rotondo “CSS” (SGRC) Hospital in Italy. This cohort has previously been used and characterised in several association reports from our group.22 A fourth cohort consisting of 398 cases and 418 controls of Jewish descent from the USA was also included; this cohort was also collected by the NIDDK-IBDGC and has previously been characterised.20 21

All patients and controls were of European Caucasian descent. The diagnosis of IBD required (1) one or more symptoms of diarrhoea, rectal bleeding, abdominal pain, fever or complicated perianal disease, (2) occurrence of symptoms on two or more occasions separated by at least 8 weeks or ongoing symptoms of at least 6 weeks duration, and (3) objective evidence of inflammation from radiological, endoscopic and histopathological evaluation. All affected subjects fulfil clinical criteria for IBD. For patients with CD, phenotypic details were registered according to the Vienna classification. However, perianal disease was scored as an independent variable and not included in the group with penetrating disease behaviour. For patients with UC, phenotypes were described according to age of onset, maximum extent of disease (proctitis, left-sided or extensive), necessity for colectomy and the occurrence of malignancy and extraintestinal manifestations. A summary of the phenotype information available for each cohort can be found in Supplementary table 1 (CD) and Supplementary table 2 (UC).

Genotyping

We analysed the four most strongly associated single nucleotide polymorphisms (SNPs) in IL2/IL21 found by Van Heel et al: rs6822844, rs13151961, rs13119723 and rs6840978.2 Genotyping of the Dutch cohort was performed using TaqMan technology, while SNP genotyping assays for PCR were supplied by Applied Biosystems (Foster City, California, USA), as described.6 The patient and control DNA samples were processed in 384-well plates and each plate also contained 16 genotyping controls (4 duplicates of 4 Centre d’Étude du Polymorphisme Humain (CEPH) DNA).

Genotyping of 1577 samples from the North American IBD cohort was performed using primer extension chemistry and mass spectrometric analysis (iPLEX assay, Sequenom, San Diego, California, USA) on the Sequenom MassArray. This was performed at the Laboratory for Genetics and Genomic Medicine of Inflammation (www.inflammagen.org) of the Université de Montréal and at The University of Pittsburgh. Data from an additional set of 2917 North American IBD samples were also obtained from genotyping on Illumina HumanHap300 or HumanHap550 Genotyping BeadChips (Illumina, San Diego, California, USA) as was previously reported in the IBDGC’s CD and UC genome-wide association studies.23 24

Genotyping for the Italian cohort was also performed at the Laboratory for Genetics and Genomic Medicine of Inflammation, using primer extension chemistry and mass spectrometric analysis on the Sequenom MassArray. The patient and control DNA samples were again processed in 384-well plates and each plate also contained 16 genotyping controls (4 duplicates of 4 CEPH DNA). All SNPs were validated, and we obtained >99.9% concordance between our genotype data and the CEU data available from HapMap.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was tested by comparing the expected and observed genotypes in a 2 × 3 χ² table. Controls did not show deviation from HWE (p value (HWE) >0.001). Differences in allele and genotype distribution in the cases and controls of the individual cohorts were tested for significance by the χ² test. Analyses for association between genotype and subphenotypes were also performed with the χ² test. A significant threshold for p values was determined at <0.05. Odds ratios (ORs) were calculated and the CIs were approximated using Woolf’s method with Haldane’s correction. Power calculations were performed using the online Genetic Power Calculator by Shaun Purcell (http://pngu.mgh.harvard.edu/~purcell/gps/).24

Combined analysis of the different cohorts was performed by Cochran–Mantel–Haenszel meta-analysis.

RESULTS

Initially the rs13151961, rs13119723, rs6840978 and rs6822844 SNPs were tested in 1590 Dutch patients (777 patients with CD and 813 patients with UC) and 929 healthy controls. The minor alleles of all four SNPs tested were associated with IBD with a p value range between 0.00095 and 0.00039 and an OR between 0.76 and 0.78. This association was even stronger in the UC subgroup (p value range 0.00085–0.00001 and OR range 0.71–0.67). In the CD subgroup, the rs15119723 SNP was borderline significant with a p value of 0.0527, while only a trend towards association was observed for the other SNPs. This indicated that the association of the IL2/IL21 locus with IBD was coming predominantly from the UC subgroup. The results are shown in table 1.

In all cases, informed consent was obtained using protocols approved by the local institutional review board in all
Table 1  Summary of the association results in our screening (Dutch) and replication (North American and Italian) cohorts, as well as the combined results following the Cochran–Mantel–Haenszel meta-analysis

<table>
<thead>
<tr>
<th>SNP</th>
<th>A1</th>
<th>A2</th>
<th>MAF controls</th>
<th>MAF cases</th>
<th>p Value</th>
<th>OR (95% CI)</th>
<th>MAF controls</th>
<th>MAF cases</th>
<th>p Value</th>
<th>OR (95% CI)</th>
<th>MAF controls</th>
<th>MAF cases</th>
<th>p Value</th>
<th>OR (95% CI)</th>
<th>Combined p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13151961</td>
<td>G</td>
<td>A</td>
<td>0.19</td>
<td>0.15</td>
<td>0.00039</td>
<td>0.85 (0.71 to 1.02)</td>
<td>0.17</td>
<td>0.14</td>
<td>0.0002</td>
<td>0.78 (0.68 to 0.89)</td>
<td>0.16</td>
<td>0.12</td>
<td>0.0007</td>
<td>0.66 (0.52 to 0.84)</td>
<td>1.41 x 10^-8</td>
</tr>
<tr>
<td>rs13119723</td>
<td>G</td>
<td>A</td>
<td>0.16</td>
<td>0.13</td>
<td>0.00093</td>
<td>0.76 (0.65 to 0.89)</td>
<td>0.16</td>
<td>0.13</td>
<td>0.0005</td>
<td>0.78 (0.68 to 0.89)</td>
<td>0.17</td>
<td>0.12</td>
<td>0.0028</td>
<td>0.70 (0.55 to 0.89)</td>
<td>1.32 x 10^-8</td>
</tr>
<tr>
<td>rs6840978</td>
<td>T</td>
<td>C</td>
<td>0.22</td>
<td>0.18</td>
<td>0.00067</td>
<td>0.78 (0.67 to 0.90)</td>
<td>0.21</td>
<td>0.17</td>
<td>0.0006</td>
<td>0.81 (0.71 to 0.91)</td>
<td>0.20</td>
<td>0.16</td>
<td>0.0160</td>
<td>0.77 (0.62 to 0.95)</td>
<td>6.17 x 10^-8</td>
</tr>
<tr>
<td>rs6822844</td>
<td>T</td>
<td>G</td>
<td>0.19</td>
<td>0.15</td>
<td>0.00070</td>
<td>0.77 (0.66 to 0.89)</td>
<td>0.17</td>
<td>0.14</td>
<td>0.0005</td>
<td>0.79 (0.69 to 0.89)</td>
<td>0.16</td>
<td>0.11</td>
<td>0.0005</td>
<td>0.65 (0.51 to 0.83)</td>
<td>7.45 x 10^-8</td>
</tr>
</tbody>
</table>

Original Dutch cohort (1590 IBD (777 CD, 813 UC), 929 controls), replication cohorts: North American (2387 IBD (553 CD, 1733 UC), 1266 controls); Italian (805 IBD (157 CD, 648 UC), 421 controls). All p values are two-tailed.

CD, Crohn’s disease; IBD, inflammatory bowel disease; MAF, minor allele frequency; SNP, single nucleotide polymorphism; UC, ulcerative colitis.
Inflammatory bowel disease

participating institutions. All DNA samples and data in this study were denominated.

To replicate these findings, we studied two independent cohorts. In the North American cohort (2387 IBD cases (654 CD and 1733 UC) and 1266 controls), we observed association with the same alleles of all SNPs in IBD (p value range 0.0011–0.0003 and OR range 0.77–0.81). As in the original cohort, this effect was strongest in the UC subgroup of the cohort (p value range 0.0046–0.0004 and OR range 0.77–0.81). In the CD subgroup of the North American cohort, a moderate association with the same alleles was also observed (p value range 0.0123–0.0011). Testing of all four SNPs in the Italian cohort (805 IBD cases (157 CD, 648 UC) and 421 controls) showed the same strong association of the minor alleles in UC as seen in the original cohort, with a p value range between 0.0123 and 0.0002 and an OR range between 0.75 and 0.62. The CD subgroup of the Italian cohort showed only a trend towards association with the same alleles, which was not significant, with a p value range between 0.3495 and 0.0873. The results are shown in table 1.

A Cochran–Mantel–Haenszel meta-analysis of the results from all three cohorts showed a very convincing association of all IL2/IL21 SNPs in IBD (p value range 7.45×10⁻⁸–1.41×10⁻⁷). In UC, this effect also reached genome-wide significance, with a p value of 5.07×10⁻⁶ for rs6840978 and a p value of 1.35×10⁻¹⁰ for rs13151961. The meta-analysis showed a moderate association with CD for all four SNPs with the same alleles (p value range 0.0016–9.86×10⁻¹⁰).

The fourth cohort consisting of patients with a Jewish background was analysed separately; these results are depicted in table 2. We did not find a significant association between any of the SNPs and CD in this cohort. We were reluctant to add this cohort to the meta-analysis for all patients with CD because of the large discrepancy in minor allele frequency (MAF) between Jewish controls and controls from the other cohorts: the MAF for SNP rs13119723 in Jewish controls was 0.06, while the MAF in the other cohorts was between 0.16 and 0.17. We performed a meta-analysis of all CD cohorts including the Jewish cohort (data not shown), which yielded a p value of 1.4×10⁻³ for SNP rs13151961, a p value of 1.0×10⁻⁴ for SNP rs15119723, a p value of 4.1×10⁻⁴ for SNP rs6822844 and a p value of 1.4×10⁻³ for SNP rs6840978.

Because the association of the IL2/IL21 locus with CD is much more moderate than that with UC it might be that the association is mainly with colonic disease. If this were the case, then we would predict that the association signal from CD comes exclusively from disease localised in the colon. To test this hypothesis, we performed a within-cases analysis for the association in colonic and non-colonic CD. However, this did not yield any significant results. Further genotype-phenotype analysis for disease localisation or extent, disease behaviour, necessity for operation, the occurrence of malignancy and extraintestinal manifestations did not yield any phenotype-specific associations (data not shown). Although phenotype data were available for a large proportion of cases (80% for both CD and UC) this might still be due to a lack of power in each specific subgroup to detect true genotype-phenotype associations.

Another possible explanation for the comparatively modest association with CD is the relatively low total number of patients with CD: 1588 patients with CD compared with 3194 patients with UC. This, however, does not appear likely as the power calculations showed that with the 1588 patients with CD we have in our study there is 95% power to detect an effect with an OR of 0.85, which is similar to that observed in UC.

DISCUSSION

In the current study we have identified and replicated a novel association between genetic variants in the IL2/IL21 locus and IBD (OR 0.66; p value 1.4×10⁻³), with the strongest evidence of association in UC (OR 0.62; p value 1.35×10⁻³). This association is consistent with the recent findings of a common protective allele in coeliac disease, rheumatoid arthritis, psoriasis and type 1 diabetes, and thus confirms this locus as a general risk locus for inflammatory disease.Δ6–10

This locus on chromosome 4q27 comprises a region of 480 kb of extensive linkage disequilibrium (LD) that harbours the testis nuclear RNA-binding protein (TENR) gene, a gene encoding a protein of unknown function (KIAA1109), and genes encoding the IL2 and IL21 cytokines. TENR is expressed primarily in testis, and KIAA1109 transcripts are ubiquitous, hence their roles in inflammatory diseases are not particularly compelling, which leaves IL2 and IL21 as the most likely candidates for disease association in the region.Δ6 As previously reported in other immune diseases, the four SNPs tested and found to be associated with IBD in this study are correlated to each other (with r² correlation coefficients ranging from 0.5 to 0.97) and are all located in non-coding regions within this 480 kb LD block. Two SNPs, rs13151961 and rs13119723, are situated in intronic regions of the KIAA1109 gene. SNP rs6822844 is located in the intergenic region between IL2 and IL21, and SNP rs6840978 is located downstream of IL21. These SNPs are not known to have an effect on expression of the genes in the IL2–IL21 region.Δ21

IL2 is secreted in an autocrine fashion by antigen-stimulated T cells, and stimulates T cell activation and proliferation. In these T cells, IL2 stimulates the production of the proinflammatory cytokines interferon γ and IL4. Furthermore, IL2 has an important role in regulating the adaptive immune response by stimulating T regulatory (CD4⁺ CD25⁺) cells and by its ability to stimulate activation-induced cell death in antigen-activated T cells.Δ23 IL21 is also a T cell-derived cytokine; it stimulates class switching to immunoglobulin G (IgG) in B cells and regulates natural killer cell proliferation and differentiation. IL21 augments proliferation in cells of the monocyte–macrophage lineage and induces an immunosuppressive phenotype by stimulating the formation of immature monocytes that inhibit antigen-specific T cell proliferation. During inflammatory processes, the receptor for IL21, IL21R, can be found on non-immune cells, such as colon epithelial cells or fibroblasts. When stimulated by IL21, these cells secrete proteins that mobilise T cells to areas of immune challenge.Δ27

The overexpression of IL21 in patients with IBD compared with healthy controls and patients with diverticulitis shows the importance of this interleukin in the inflammatory process of

Table 2 Association of the IL2/IL21 SNPs in a Jewish cohort

<table>
<thead>
<tr>
<th>SNP</th>
<th>A1</th>
<th>A2</th>
<th>MAF controls</th>
<th>MAF cases</th>
<th>p Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13151961</td>
<td>G</td>
<td>A</td>
<td>0.07</td>
<td>0.06</td>
<td>0.5691</td>
<td>0.89 (0.60 to 1.30)</td>
</tr>
<tr>
<td>rs13119723</td>
<td>G</td>
<td>A</td>
<td>0.06</td>
<td>0.05</td>
<td>0.6388</td>
<td>0.89 (0.58 to 1.37)</td>
</tr>
<tr>
<td>rs6840978</td>
<td>T</td>
<td>C</td>
<td>0.07</td>
<td>0.06</td>
<td>0.3790</td>
<td>0.83 (0.56 to 1.23)</td>
</tr>
<tr>
<td>rs6822844</td>
<td>T</td>
<td>G</td>
<td>0.14</td>
<td>0.14</td>
<td>0.7468</td>
<td>0.96 (0.72 to 1.27)</td>
</tr>
</tbody>
</table>

*Jewish CD cohort (398 cases, 418 controls). All p values are two-tailed. CD, Crohn’s disease; IL2, interleukin 2; IL21, interleukin 21; MAF, minor allele frequency; SNP, single nucleotide polymorphism.*
both CD and UC.\(^\text{17}\) Interestingly, Monteleone et al observed the increase in IL21 expression level predominantly in the CD subgroup of patients with IBD, whereas we here observed a stronger association of the IL2/IL21 locus with UC rather than CD. Although speculative, this prioritises the IL2 gene as the gene more likely to be involved. IL2 is an attractive functional candidate gene for UC pathogenesis, as the IL2/\(\gamma\)C mouse develops a disease similar to UC, supporting an association between IL2 and UC.\(^\text{18}\) The fact that calcineurin inhibitors, which mainly suppress the expression of IL2, are effective in treatment-resistant UC, but not in CD, might also point to a key role for this interleukin in UC.\(^\text{19}\) Further support for the importance of IL2 in UC comes from the fact that a pilot trial with antibodies against the IL2 receptor in treatment-resistant UC was successful.\(^\text{20}\) The fact that both a lack of IL2 and an excess of IL2 predispose to colitis is however puzzling. Further functional studies on these genetic variants are needed to define the specific role for the IL2/IL21 locus in the pathogenesis of IBDs.

An equivalent protective association signal of the IL2/IL21 locus with coeliac disease, rheumatoid arthritis, type 1 diabetes and psoriasis has previously been reported. This shows that this locus plays an important role in inflammatory diseases. Previously MAGIC, PARD3, MYO1D and IL1R8P were reported to be associated with both coeliac disease and UC.\(^\text{21}\) The IL2–IL21 locus is now the fifth locus to be associated with both diseases, further supporting a model where a common set of biological pathways lead to coeliac disease and UC. Interestingly, multiple SNPs in this same region, that are independent of the SNPs studied herein, have recently been reported to confer risk of type 1 diabetes and potentially of coeliac disease.\(^\text{22}\) Although these SNPs conferring risk were not tested in the current study a published study in CD (rs17388568, \(p = 1.7 \times 10^{-4}\); rs716501, \(p = 3.8 \times 10^{-4}\)) potentially supports the presence of alleles conferring increased risk for disease.\(^\text{23}\) Further examination of these risk-conferring alleles are warranted in CD and UC.

Extensive sequencing in coeliac cases and matched controls, as well as functional studies, will be needed to find the true causal variant in the IL2/IL21 locus and determine the molecular mechanisms by which this locus influences an individual’s risk of multiple immune-mediated diseases.

**Author affiliations:**
1. Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;
2. Laboratory in Genetics and Genomic Medicine of Inflammation, Montreal Heart Institute, Université de Montréal, Montreal, Canada;
3. Division of Gastroenterology, Hepatology and Nutrition, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA;
4. UU.OO. Gastroenterology ed Endocrinologia Digestiva, Ospedale “Casal Sofia della Sofferenza”, RDCS, San Giovanni Rotondo, Italy;
5. Complex Genetics Section, Department of Medical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands;
6. Harvey M and Lyn P Meyerhoff Inflammatory Bowel Disease Center, Department of Medicine, Johns Hopkins University, Baltimore, Maryland, USA;
7. Departments of Medicine and Genetics, Division of Gastroenterology, Inflammatory Bowel Disease (IBD) Center, Yale University, New Haven, Connecticut, USA;
8. Mount Sinai Hospital IBD Centre, University of Toronto, Toronto, Ontario, Canada;
9. Medical Genetics Institute and Inflammatory Bowel Disease (IBD) Center, Cedars-Sinai Medical Center, Los Angeles, California, USA;
10. Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;
11. Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands;
12. Center for Inflammatory Bowel Disease, Department of Gastroenterology & Hepatology, Cleveland Clinic, Cleveland, Ohio, USA;
13. Center for Computational and Integrative Biology and Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Massachusetts, USA;
14. Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

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**Competing interests:** None.

**Patient consent:** Informed consent was obtained using protocols approved by the local institutional review board in all participating institutions.

**REFERENCES**

ANSWER

From the question on page 744

The CT scan (fig 1) revealed a thickened terminal ileum. The endoscopic biopsy revealed inflammation only. She received right hemicolectomy and partial ileum resection for persistent ileal obstruction. During the operation, segmental stiffness of the terminal ileum was noted (fig 2). Pathological examination (fig 3) showed metastatic lobular carcinoma, which is compatible with the previous histological finding of breast cancer. The cancer cells involved submucosa and muscularis propria but sparing the mucosa, which explained the inconclusive finding by endoscopic biopsy.

A thickened terminal ileum should remind clinicians of Crohn’s disease, tuberculosis, ischaemia, adenocarcinoma, lymphoma and rarely metastatic cancer. Metastatic breast cancer is the leading cause of small intestinal obstruction resulting from metastatic cancers, with an incidence of up to 16%. The interval between the primary tumour and gastrointestinal tract metastasis may span >10 years. Most of these cases were disseminated and lobular in type. Metastatic cancer should be considered in such patients with a history of lobular carcinoma of the breast.

REFERENCES


Figure 1 Abdominal CT revealed a diffuse dilated small bowel with a transitional zone at the terminal ileum; the terminal ileum wall was very enhanced and thickened (arrow).

Figure 2 Operation specimens reveal segmental stiffness with a nodular surface of the terminal ileum (arrows).

Figure 3 Pathology revealed (A) uniform-sized discohesive cells with round cell morphology and concentric vesicular nuclei, which had characteristics of lobular breast carcinoma. (B) Diffuse expression of oestrogen receptor protein.