Adenovirus 12 E1A gene detection by polymerase chain reaction in both the normal and coeliac duodenum

M Lawler, P Humphries, C O'Farrelly, H Hoey, O Sheils, M Jeffers, D S O'Brain, D Kelleher

Abstract
A 12 amino acid sequence from the adenovirus 12 E1B protein is homologous at the protein level with a similar 12-mer derived from the wheat protein α-gliadin. It has been suggested that exposure to Ad 12 could sensitize individuals to gliadins with resultant gluten sensitive enteropathy. In this study, the polymerase chain reaction (PCR) was used to analyse duodenal biopsy tissue from patients with coeliac disease for the presence of Ad 12. The sensitivity of the assay system was at least 1 in 10^5 cells and specificity was confirmed both by probing with an internal oligonucleotide and by direct sequencing. Ad 12 sequences were detected in three of 17 patients with adult coeliac disease and in five of 16 adult controls with normal duodenal biopsies. Since exposure to the virus would be predicted to occur in infancy we also studied patients with childhood coeliac disease diagnosed at less than 1 year of age. Ad 12 was positive in three of 10 childhood coeliac patients and one of seven controls. In addition, we studied a cohort of patients who presented with a diarrhoeal illness and associated anti α-gliadin antibodies in 1983. These patients had duodenal biopsies performed at this time. One of three patients with abnormal histology had detectable Ad 12 while two of 14 with normal findings were positive for Ad 12. Finally, the potential oncogenic nature of Ad 12 prompted examination of a group of patients with intestinal tumours. Ad 12 DNA was, however, in only two of 19 tumour samples tested. These data indicate that Ad 12 can be successfully detected using PCR on paraffin embedded tissue. Furthermore, Ad 12 was detected at a relatively high level in normal duodenum. The results do not, however, support the hypothesis that prior exposure to Ad 12 is implicated in the pathogenesis of coeliac disease.

TABLE 1 Results of polymerase chain reaction (PCR) amplification of adenovirus 12 (Ad 12) DNA from a cohort of children aged 1–4 years presenting with a diarrhoeal illness and elevated antibodies to α-gliadin in 1983. Anti-α-gliadin antibody levels are expressed as ELISA index (EI). A normal level of <1.5 was established from the mean ± 2 SD of indices of a cohort of 25 normal age matched children.

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Biopsy findings</th>
<th>Anti-α gliadin (EI)</th>
<th>PCR for Ad 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flat mucosa</td>
<td>8-7</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>2-8</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>2-6</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Flat mucosa</td>
<td>12-3</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>3-2</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>6-9</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>2-5</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>9-6</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>3-2</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>2-4</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>Increased IEL</td>
<td>2-7</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>Normal</td>
<td>1-8</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
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<td>3-2</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>Normal</td>
<td>1-8</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>Normal</td>
<td>1-9</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
<td>Normal</td>
<td>2-0</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>Normal</td>
<td>2-0</td>
<td>No</td>
</tr>
</tbody>
</table>
Adenovirus 12 E1A gene detection by PCR

<table>
<thead>
<tr>
<th>A1-1/2-1 OUTER</th>
<th>CTTGAGACTCGGAGA</th>
<th>CAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATTTACGAGCCAGTGAACCTGAGCCTACGGTGGT</td>
<td>346</td>
<td></td>
</tr>
<tr>
<td>A1-1 NESTED</td>
<td>TCGATACTGACCTGGAGA</td>
<td>Alu 1 Sau 11a</td>
</tr>
<tr>
<td>AGCTATGACCTGGAGA</td>
<td>TATTTACGAGGGCAGAGTGAACTCTGAGCCTCTACGTGTGGGTT</td>
<td></td>
</tr>
<tr>
<td>TTCCGTCTCTTTTACGAACTGTATGATCTTGATGTGGAGTCTGCCGG</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>AAGAGACGGTCG</td>
<td>AAGAGACGGTCG</td>
<td></td>
</tr>
<tr>
<td>1:</td>
<td>436</td>
<td></td>
</tr>
<tr>
<td>Figure 1: Sequence of adenovirus 12 E1a region showing the primers used in this study.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The whole region can be amplified using primers A1-1 (outer) and A2-2. However, for amplification from paraffin embedded tissue this region is too large (579 bp) and so A1-1 (outer) and A2-2 (outer) are used to give a fragment size of 286 bp. Use of the nested inner primers (A1-1 nested and A1-2 nested) then gives an internal fragment of 149 bp which can be cut by the enzyme Alu I (AAG CTG) to give diagnostic fragment sizes of 67 bp and 82 bp. The sites for Pvu II (CAO GTG) and Sau 11a (GATC) are also shown.

Methods

PATIENTS GROUP, CHOICE OF SECTION, AND HISTOLOGICAL CONFIRMATION

Duodenal biopsy specims from patients with coeliac disease were obtained from archival material at St James’s Hospital. Diagnoses were based on histological criteria and response to gluten free diet in all cases. Diagnoses were confirmed histologically beforehand in all cases. Seventeen adult coeliac patients were entered in the study and duodenal and jejunal samples were analysed from these patients and from 16 age matched controls. Ten childhood coeliac patients diagnosed within the first year of life were studied with seven age matched controls. We also analysed a cohort of children who presented to a pediatric unit with acute diarrhoeal illnesses. This cohort was selected on the basis of the finding of circulating antibodies to α gliadin (Table I). Finally, material from a group of 17 patients with a variety of intestinal tumours (nine colorectal tumours, four ampullary tumours, and five duodenal tumours) were analysed for the presence of Ad 12 DNA sequences.

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**REAGENTS**

The BMK line containing integrated E1A sequences was obtained from Dr Alex van der Eb (University of Leiden, The Netherlands). Oligomers to be used as primers for PCR were constructed from published sequences of E1A (Fig 1) and synthesised on the
round amplification. This tissue, fixed in formalin, was dewaxed, then dewaxed using xylenes and DNA extraction was performed. Cells in Ad 12 virus were prepared for PCR as described in the Methods section. They were amplified by first round PCR (A, lanes 1–4) or nested PCR (B, lanes 5–8). Amplification of primers from the rhodopsin gene (C) served as a positive control (lanes 2–7).

Lane 1: undiluted sample; lane 2: 10^{-1} dilution; lane 3: 10^{-2} dilution; lane 4: 10^{-3} dilution; lane 5: 10^{-4} dilution (nested PCR); lane 6: 10^{-3} dilution (nested PCR); lane 7: 10^{-4} dilution (nested PCR); lane 8 undiluted sample (nested PCR).

Model 391 Oligonucleotide Synthesizer (Applied Biosystems, Foster City, CA, USA). Thermus aquaticus DNA polymerase (Taq polymerase) was obtained from Perkin Elmer Cetus (Emeryville, CA, USA). Alu I, Pvu II and Sau 3a restriction enzymes and deoxynucleoside triphosphates (dNTPs) were obtained from Bethesda Research Laboratories (Bethesda, MD, USA).

PCR ON PARAFFIN FIXED SPECIMENS

Design of primers
The entire sequence could be amplified using primers A1-1 and A2-2 defining outer sequences of the region (Fig 1). This sequence was too large for amplification from paraffin fixed tissue, however, and hence primers A1-1 and A1-2 were used to give a fragment size of 286 base pairs (bp). Use of the nested primers (A1-1 nested and A1-2 nested) then gives an internal fragment of 149 base pairs (bp). Primers from the rhodopsin and anti-thrombin III loci were used to amplify these human sequences in the same tube as the Ad 12 amplification. This served as a positive control to ensure that reaction conditions would promote amplification in all cases.

PCR
All amplifications were performed in a 50 µl reaction volume using conditions already described. For nested PCR, excess primers and dNTPs were removed from first round PCR by centrifugation through spin columns. Some 2 µl of the initial amplification product were then subjected to second round amplification using internal primers (Fig 1). In order to examine the sensitivity of the assay system, serial dilution of BMK cells in Ad 12 negative human neutrophils was performed. Cells were then pelleted, formalin fixed, and paraffin embedded. Sections through these cell blocks were then dewaxed, DNA extracted, and subjected to 30 cycles of amplification. For all amplifications, the procedures recommended by Kwok and Higuchi were followed to avoid contamination.

Restriction enzyme digestion
The Alu I enzyme is predicted to cut the amplified sequence in two fragments of known molecular weight, 67 bp and 82 bp and allow confirmation of the identity of the amplified product. In addition, we also performed restriction digests with Pvu II (which overlaps the Alu I site) and Sau 11Ia, which yields fragments of 71 and 78 bp. All

Figure 2: Sensitivity of the assay system.

A. Negative human neutrophils and prepared for PCR as described in the Methods section. They were amplified by first round PCR (A, lanes 1–4) or nested PCR (B, lanes 5–8). Amplification of primers from the rhodopsin gene (C) served as a positive control (lanes 2–7).

Lane 1: undiluted sample; lane 2: 10^{-1} dilution; lane 3: 10^{-2} dilution; lane 4: 10^{-3} dilution; lane 5: 10^{-4} dilution (nested PCR); lane 6: 10^{-3} dilution (nested PCR); lane 7: 10^{-4} dilution (nested PCR); lane 8 undiluted sample (nested PCR).

Figure 3: Restriction enzyme digestion of amplified DNA.

Lane 1 marker DNA; lane 2 uncleaved DNA; lane 3 Alu 1 cut DNA; lane 4 uncleaved DNA; lane 5 Sau 3a cut DNA; lane 6 marker DNA.

Each positive sample was tested with Alu 1, Sau 3a and Pvu 2 (not shown) to confirm the identity of the amplified product.

Preparation of genetic material
A number of sections (3 mm) were cut from each block using a new knife for each block to avoid cross contamination. Samples were dewaxed using mixed xylenes and DNA was extracted using a modified proteinase K/SDS method as previously described. Samples were prepared in a separate area from DNA amplification as a precaution against contamination.
Adenovirus 12 E1A gene detection by PCR

**Figure 4**: (A) Subset of coeliac patient samples amplified to detect adenovirus 12 (Ad 12) DNA sequences.

Lanes 1, 14: control Ad 12 cell line (1:10 000 dilution) amplified with outer primers; lanes 2, 13: nested PCR of control dilution; lanes 3, 12: negative control; lanes 4-11: coeliac patients coamplified with Ad 12 (nested) and rhodopsin primers. Lanes 7, 9, and 10 show a positive signal which was confirmed by oligonucleotide hybridisation and restriction enzyme digestion.

(B) Subset of control duodenum samples amplified to detect Ad 12 E1a DNA sequences.

Lane 1: control Ad 12 E1a cell line DNA cell line DNA (1:10 000 dilution) amplified with outer primers; lane 2: nested PCR of 1:10 000 control dilution; lane 7: negative control; lanes 3-6, 8 control duodenum amplified with Ad 12 (nested) and rhodopsin primers. Lanes 3, 6, and 8 showed a positive signal which was confirmed by oligonucleotide hybridisation and restriction enzyme digestion.

Restriction digests were performed using the manufacturers' instructions.

**Oligonucleotide hybridisation**

An internal oligonucleotide was designed of the sequence 5' CGC GTT TAT TGT TCT GTC AGC TGA 3' (position 391-415) to be used as a probe. This oligonucleotide was labelled at the 5' end with a radioactive deoxyctosine triphosphate using polynucleotide kinase. Amplified material was denatured and transferred to nylon membranes using a Hybridot manifold (BRL).

**Table II. Adenovirus 12 (Ad 12) positivity as judged by polymerase chain reaction in different patient groups**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No studied</th>
<th>Ad 12+ve</th>
<th>NR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult coeliac disease</td>
<td>17</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Normal duodenum</td>
<td>16</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Childhood coeliac disease</td>
<td>10</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Normal duodenum (&lt;1 y)</td>
<td>7</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

NR* = No results were achieved in these samples as all had been fixed in Bouin's medium which we found refractory to DNA analysis.

**Blots were probed with the 24 mer internal oligonucleotide, washed under high stringency and subjected to autoradiography for eight to 24 hours.**

**ANTI-GLIADIN ANTIBODY DETECTION**

Antibodies to α gliadin were measured using an ELISA method as previously described.13

**Results**

**SENSITIVITY OF ASSAY**

PCR using synthetic oligonucleotides specific for Ad 12 E1A sequences was capable of detecting the presence of E1A sequences in paraffin embedded sections of BMK cells containing the integrated E1 region. No other adenovirus sequences were identified in this cell line. The sensitivity of identification was such that 1:10⁶ cells could be identified by PCR using this technique with nested primers (Fig 2). The specificity of the amplification was confirmed by cleavage of the amplified fragment using the restriction enzyme *Alu I* into two fragments of 67 bp and 82 bp, as predicted from the location of the *Alu I* site in the nucleotide sequence of the published Ad 12 sequence (Figs 1 and 3). In addition, the restriction enzymes *Sau II* (Fig 3) and *Pvu II* (data not shown) gave diagnostic DNA fragments when the amplification product was digested with either of them. Thus, this technique was sensitive and specific for Ad 12 amplification in formalin fixed, paraffin embedded tissue.

**AD 12 DETECTION IN ADULT COEILIAC PATIENTS**

Adenovirus sequences were detected by PCR in duodenal or jejunal sections of three of 17 patients with adult coeliac disease (Table II). In all these cases these sequences were detected using Ad 12 specific primers. An example of the results achieved is shown in Figure 4(A). However, Ad 12 sequences were also detected in five of 16 patients with normal duodenal biopsy tissues (Table II, Fig 4(B)). These sequences were confirmed as Ad 12 both by restriction enzyme digestion as above and also by direct sequencing in one case (data not shown). No other adenoviral sequences were detected. The DNA sequence obtained correlated directly with the published Ad 12 sequence. In addition, we examined biopsy material from other sites, mainly from the...
AD 12 DETECTION IN PATIENTS WITH INTESTINAL ADENOCARCINOMAS

Since Ad 12 has been reported to be oncogenic, we also examined material from a range of intestinal adenocarcinomas for Ad 12 DNA. Briefly, Ad 12 DNA was detected in none of nine colorectal carcinoma samples, none of four ampullary carcinoma samples and in two of five duodenal carcinoma samples tested. Thus, in total two of 19 tumour samples tested were positive for Ad 12 DNA.

DNA extraction and PCR amplification was achieved from all samples in the study with the exception of those that had been fixed with Bouin’s fluid. Extension of proteinase k digestion times and a variety of modifications in the extraction protocol did not yield material of a sufficient quality for routine PCR.

Discussion

Coeliac disease is an HLA linked disease which is tightly linked to HLA DQ2 and, possibly, independently linked to HLA DP sequences. Up to 20% of the normal population carry these HLA types. Furthermore, concordance in identical twins is only approximately 75%. These data have led to the suggestion that either a second gene or alternatively an environmental factor may be responsible for the full expression of the disease. Further support for the concept of an additional environmental factor comes from data indicating a reduction of incidence of coeliac disease in childhood in regions of high prevalence and it has been suggested that an infective agent such as a virus with changing virulence may be implicated. Sequence analysis of the A-gliadin molecule and of the Ad 12 E1B sequence have shown that there is significant sequence homology between a 12 amino acid sequence on Ad 12 and the A-gliadin molecule. This has led to the suggestion that Adenovirus 12 may have a role in the pathogenesis of coeliac disease by molecular mimicry. The region of homology is 12 amino acids long, approximately the same size as epitopes recognised by T cells. Furthermore, antibodies to the A-gliadin sequence have been shown to cross react with viral antigens.

The suggestion that Ad 12 might be implicated in the pathogenesis of coeliac disease was supported by a study in which a cohort of coeliac individuals had significantly increased titres of antibody reactive against the Ad 12 E1A protein relative to controls. Subsequent studies failed to show antibodies to E1B in patients with coeliac disease. Furthermore, a study using the PCR failed to show Ad 12 E1B sequences in coeliac patients. In the current study, we have performed amplification using the PCR and E1A primers to detect Ad 12 sequences in two of these children with normal biopsies and one of three with abnormal biopsies (Table I). There was no correlation between the anti-α gliadin antibody titres and the presence or absence of Ad 12 DNA.

AD 12 DETECTION IN CHILDHOOD COELIAC PATIENTS

Ad 12 was detected in duodenal biopsy specimens from three of 10 coeliac patients diagnosed within the first year of life. Ad 12 was also detected in one of seven normal duodenal biopsies performed during the first year of life (Table II).

AD 12 DETECTION IN CHILDREN WITH ANTIBODIES TO α GLIADIN

All of these children with antibodies to α gliadin (Table I) were subsequently followed up and had at least one duodenal biopsy. Two patients were found to have villous atrophy consistent with coeliac disease and a further case had a significant increase in the intraepithelial lymphocyte count. PCR amplification detected Ad 12 sequences...
Sequences in archival material. The E1A sequence was chosen since this sequence may integrate into the host genome and might potentially serve as a marker not only for current infection but also for prior infection. We have shown that the assay is sensitive and specific using paraffin fixed material. Furthermore, Ad 12 E1A sequences are detectable in approximately 25% of adult biopsy specimens. Our data are in broad agreement with those reported in previous studies using fresh biopsy tissues. However, we did not see any specific association of the detection of E1A sequences with coeliac disease. Furthermore, there did not seem to be an individual clinical syndrome associated with the detection of Ad 12 sequences.

Since the concept of molecular mimicry could also include the possibility that infection during childhood with subsequent eradication of viral antigen might permit recognition of the gliadin peptide in later life, we also examined the duodenal biopsy specimens of patients with coeliac disease detected during the first year of life, and frequently during the first 6 months. In these cases, the incidence of Ad 12 detection did not differ significantly from the adult incidence of Ad 12. Nor did Ad 12 positivity correlate significantly with anti-α gliadin antibody detection in infants with diarrhoeal illness. These data suggest that Ad 12 is not associated with the development of coeliac disease. It is still possible that infection in very early infancy could result in disease. However, given the very high rate of detection of this sequence, it suggests a relatively high prevalence of the virus. The prevalence seems to be similar in both adults and children suggesting that infection occurs at a very young age.

In view of the documented high prevalence of E1A sequences, we were concerned that this oncogenic virus might be associated with tumour development in the gastrointestinal tract. Patients with coeliac disease have an increased frequency of adenocarcinoma of the gastrointestinal tract. E1A sequences are known to affect cellular proliferation by a number of means including interaction with tumour suppressor genes and interactions with cyclin.19 20 While E1A sequences are known to transform cells in vitro,21 and Ad 12 E1A sequences are associated with the development of gastrointestinal carcinoma in transgenic mice,9 there is as yet no evidence of adenovirus involvement in the development of human cancers. In this study we have not observed any association between the presence of Ad 12 E1A DNA and the development of carcinoma of the gastrointestinal tract in humans.

PCR of paraffin embedded material is a robust technique and has been useful in the assessment of archival material.12 22 The lack of amplification seen in this study from any material that had been previously fixed with Bouin’s fluid makes us recommend that this fixative is not used in material that may subsequently be needed for DNA analysis. A recent study has identified Ad 12 in both adult and childhood duodenal biopsy tissues.23 The current study differs from both that study and the other study previously described8 in that E1A DNA sequences were studied. Secondly, the assay system was adapted for high sensitivity with paraffin embedded tissue. Furthermore, we have confirmed: (i) that Ad 12 is not present in newly diagnosed coeliac patients less than 1 year of age and (ii) that anti-gliadin antibodies may be detected in infantile diarrhoeal illnesses which are not associated with adenovirus 12. These data indicate that cross reactivity to Ad 12 is not implicated in anti-gliadin antibody generation in children and secondly that Ad 12 is not implicated in the continuing pathogenesis of infantile coeliac disease. One cannot, however, entirely exclude the possibility that brief infection of the neonate without integration might serve as an initiation event for coeliac disease. Since Ad 12 positivity is not uncommon in our patient population, in agreement with previous serological and molecular studies,6 8 23 this makes it unlikely that Ad 12 is implicated in the pathogenesis of coeliac disease.


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