High prevalence of enteroviral genomic sequences in myocardium from cases of endemic cardiomyopathy (Keshan disease) in China

Y Li, T Peng, Y Yang, C Niu, L C Archard, H Zhang

Abstract

Objective—To verify the aetiological involvement of enterovirus and identify the viral genomic sequences in Keshan disease.

Design—Formalin fixed, paraffin embedded myocardial necropsy tissue samples were collected in Keshan disease endemic regions. Fourteen cases with a histologically confirmed diagnosis of subacute or chronic Keshan disease were studied. Control tissue included 10 samples of myocardium from cases of cerebral trauma and one from accidental acid intoxication. One sample from a case of enteroviral myocarditis was used as a positive control. The presence of viral genomic RNA was investigated using an established reverse transcription nested polymerase chain reaction (PCR) coupled with direct nucleotide sequencing. Further investigations of PCR positive samples included in situ antigen detection or hybridisation to confirm positive results.

Results—Nine of 14 myocardial samples from Keshan disease cases and the positive control were positive for the enteroviral RNA. All the controls were negative. Six of the PCR positive samples were investigated further by in situ enteroviral antigen or RNA detection and all were positive. DNA sequencing of six representative PCR products confirmed that they were homologous to the 5’ non-translated region of enteroviral genomic RNA. Five had highest homology to coxsackievirus B genotypes and one was identical to poliovirus type 3.

Conclusions—These results support an aetiological role for enteroviral infection in Keshan disease. Nucleotide sequence data suggest that coxsackievirus B or coxsackie B like viruses are often involved in Keshan disease.

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Keywords: enterovirus; coxsackievirus; cardiomyopathy; Keshan disease
Methods

PATIENTS AND MYOCARDIAL TISSUE SAMPLES
All patients lived in Keshan disease endemic rural areas in the Chuxiong region of Yunnan Province in southwest China. Their hygienic and socioeconomic status was considered low. Fourteen cases of Keshan disease (age range 0.5 to 25 years) occurring between 1983 and 1996 were investigated (table 1). Ventricular tissue was obtained from each patient within 12 hours of death, fixed in 10% buffered formalin, paraffin embedded, and stored in sealed plastic bags. Histopathological examinations of sections from each sample by Professor B Gu confirmed the clinical diagnosis of subacute Keshan disease in seven cases and chronic Keshan disease in the remainder. All subacute cases died within a week of onset. Control samples were obtained from 11 cases of accidental death occurring between 1985 and 1995, including 10 cases of cerebral trauma and one of acid intoxication (table 1). Among these, eight were from Keshan disease areas and the remaining three were from non-endemic areas. Ventricular tissue was obtained within 12 hours of death. Histologically these hearts were normal. The positive control in this study was explanted myocardium from a patient with chronic myocarditis from a non-endemic area (enteroviral genomic RNA and capsid antigens had been detected in this tissue).

PREPARATION OF TOTAL RNA
Paraffin embedded tissue samples were de-waxed in xylene and washed with ethanol. RNA was recovered by the tri-reagent method according to the supplier’s instructions (Sigma, Poole, UK).

REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION
Established reverse transcription nested polymerase chain reaction (RT-NPCR) methods were used to detect enteroviral RNA. Details of PCR and second stage nested PCR primers are given in table 2. Primers for enterovirus sequence amplification are targeted to conserved motifs within the 5’NTR or cap region of enterovirus genome in a mouse model of viral myocarditis. To establish the aetiological role of enteroviruses in Keshan disease and to characterise the genomic sequence of the viruses involved, we conducted a multicentre study using an established RT-PCR technique coupled with direct nucleotide sequencing. The reliability of these techniques has been evaluated in an experimental model of coxsackievirus B3 myocarditis and used successfully in our laboratory to identify enterovirus involvement in human heart muscle disease.

Table 2 PCR primers used in the study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interviruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F252</td>
<td>GGGCCCTGATGGCGGCTAA</td>
<td>452–470</td>
</tr>
<tr>
<td>R68</td>
<td>GGGACCTTCACACCAACCNC</td>
<td>1177–1196</td>
</tr>
<tr>
<td>F24</td>
<td>CTACCTTGGGTGTCCG</td>
<td>544–559</td>
</tr>
<tr>
<td>R253</td>
<td>GATACT(7)GAGGNCACCAT</td>
<td>742–758</td>
</tr>
<tr>
<td>F16</td>
<td>TTAAACACCCTGGGTGTTG</td>
<td>1–20</td>
</tr>
<tr>
<td>R5</td>
<td>TCACGCGATGGCCAACTCCCA</td>
<td>625–644</td>
</tr>
<tr>
<td>F3</td>
<td>CTGTATACAGGTTACCTTTG</td>
<td>54–73</td>
</tr>
<tr>
<td>R8</td>
<td>AAAAAACGGAACCACAGGTA</td>
<td>545–564</td>
</tr>
<tr>
<td>P2*</td>
<td>CCCCCGACTGATGTACATA</td>
<td>180–199</td>
</tr>
</tbody>
</table>

Human β actin
| HBAF1 | ATCTGGACACACACCTTCTACATTGAGGCTGC | 263–294 |
| HBAR1 | CCGTATACCTCGTCTGGATCATCCACATGC | 1069–1100 |
| HBAR2 | TACATACCTGCTGGGCTAAGG | 750–769 |
| HBAR2 | CACTGTTGCGGCTACAGG | 885–904 |

*Hybridisation probe
denaturation for one minute at 94°C, primer annealing for one minute at 55°C, and template extension for three minutes at 72°C for 30 cycles. Second stage nested PCR was performed on 5 µl of the initial PCR reaction products as described above except for the addition of the nested primers F24 and R253 or F3 and R8 (0.1 nmol each) and reduction of the template extension time to two minutes.

The RT-NPCR for β actin was employed to verify the quality of total RNA extracted from tissue samples and was performed similarly, except for the anealing temperature of 65°C for first stage PCR and 55°C for NPCR.

Precautions taken to avoid false positive results caused by contamination during RT-NPCR included the use of filter tips and of separate rooms, containment cabinets, and pipettes for RNA extraction, assembly of reaction mixes, and enzymatic amplification.

ANALYSIS OF SEQUENCE DATA

The forward and reverse sequence data from each PCR product were aligned using the SeqEd program (PE-Biosystems, Warrington, UK). The consensus sequence was compared with other amplicons and to the DNA sequence database (GenBank) using the program AssemblyLIGN (Oxford Molecular Group, Oxford, UK) or the GCG group of programs at SERC Sequenot facility, Daresbury, UK.

IN SITU DETECTION OF VIRAL ANTIGENS AND GENOMIC RNA

Paraffin sections were dewaxed and rehydrated by standard histological methods. An enterovirus group specific monoclonal antibody (Mab) 5-D8/1 (DAKO, Cambridge, UK) was used to detect the viral capsid protein VP1. Immunostaining was carried out by an indirect immunohistochemical assay after heat treatment of sections for antigen retrieval and the EnVision™ detection system (Dako, UK) was employed to visualise antibody-antigen complex. For in situ hybridisation, either a mixture of PCR primers (F252, F24, R253, R68, P2; table 2) or a single primer (P2) was labelled with digoxigenin for use as probe. Hybridisation was carried out according to the manufacturer’s instructions (Boehringer Mannheim, Bracknell, Berkshire, UK). It was anticipated that multiple primer probes would enhance the hybridisation signal.

Results

ENTEROVIRAL RNA IN MYOCARDIAL TISSUE FROM KESHAN DISEASE

Total RNA preparations from myocardial tissue samples were amplified by RT-NPCR, initially with primer pairs F252/R68 for the first round PCR and F24/R253 for the nested PCR. Nine of 14 Keshan disease samples and the myocarditis control were positive for enteroviral RNA. As anticipated, the amplified NPCR products were approximately 215 base pairs (bp) (fig 1A). The reverse transcription products (cDNA) of positive samples were resuspended with different primer pairs—F16/R5 for the first round and F3/R8 for the nested PCR. The expected 511 bp product was amplified in all samples tested (fig 1A). These results were reproducible in repeated experiments by different workers. In contrast, all 11 normal heart tissue samples were negative.

RT-NPCR for the housekeeping gene, β actin, was positive in all samples (fig 1B), excluding false negative results. All reagent controls lacking RNA were negative. PCR contamination from the environment was assessed by DNase or RNase (Promega) treatment of the RNA preparations from patients 3, 8, and 9. The PCR positivity was not affected by DNase digestion, but was abolished by RNase treatment. PCR without reverse transcription failed to yield a product (fig 2). These results confirm that the RT-NPCR products seen were not false positives owing to DNA contamination or to cross reactivity with human DNA in the samples.
PCR products were sequenced to confirm and characterise enterovirus in the detected clinical samples. Each purified NPCR product was sequenced in both directions. Consensus sequences without ambiguity were obtained from six NPCR products amplified with primer pairs F3/R8 or F24/R253, or both, and compared with the sequence database in GenBank. Five of the six sequences showed greatest homology with the 5'NTR of coxsackievirus B3, and the remaining one was identical to the 5'NTR of wild type poliovirus type 3 (strain P3/119).26 Among the coxsackievirus-like sequences, two were identical with greatest homology to coxsackievirus B3 (88.6%) or to those in the coxsackievirus-like phylogenetic cluster (GenBank accession Nos AF198383 and AF198384).27 Three cases (3, 8, and 9) were identical to coxsackievirus B3 strain Nancy.28 DNA sequences of the NPCR product (primers F24/R253) from the positive myocarditis control showed 97–99% similarity with coxsackievirus B3. Its GenBank accession No is AF197926 (table 3).

### Table 3 DNA sequence analysis of nested polymerase chain reaction products

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sequenced products</th>
<th>Sequence identity*</th>
<th>Virus</th>
<th>Accession number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>F3/R8</td>
<td>372/420 (88.6%)</td>
<td>CVB3</td>
<td>M88483</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>366/420 (87.1%)</td>
<td>Echo 7</td>
<td>L76401</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>365/420 (86.9%)</td>
<td>CVB3</td>
<td>U57056</td>
<td>30</td>
</tr>
<tr>
<td>3, 8, 9</td>
<td>F3/R8</td>
<td>420/420 (100%)</td>
<td>CVB3</td>
<td>M33854</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>417/420 (99.3%)</td>
<td>CVB3</td>
<td>M88483</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>405/420 (96.4%)</td>
<td>CVB3</td>
<td>U57056</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>F3/R8</td>
<td>460/460 (100%)</td>
<td>Polio 3</td>
<td>X01076</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>F3/R8</td>
<td>459/460 (99.8%)</td>
<td>Sabin 3</td>
<td>X00925</td>
<td>31</td>
</tr>
<tr>
<td>+</td>
<td>F24/R253</td>
<td>174/175 (99.4%)</td>
<td>CVB3</td>
<td>M33854</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>F24/R253</td>
<td>172/175 (98.3%)</td>
<td>CVB3</td>
<td>M74567</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>F24/R253</td>
<td>171/175 (97.7%)</td>
<td>CVB3</td>
<td>M88483</td>
<td>29</td>
</tr>
</tbody>
</table>

*Consensus sequence derived from both forward and reverse sequencing of each NPCR product was used for sequence alignment. The first 20–45 nucleotides in an original sequence data from either direction were not included in consensus sequences because they usually contain ambiguous sequences.

+ positive control.

ENTEROVIRAL CHARACTERISATION BY DIRECT DNA SEQUENCING OF PCR PRODUCTS

PCR products were sequenced to confirm and characterise enterovirus in the detected clinical samples. Each purified NPCR product was sequenced in both directions. Consensus sequences without ambiguity were obtained from six NPCR products amplified with primer pairs F3/R8 or F24/R253, or both, and compared with the sequence database in GenBank. Five of the six sequences showed greatest homology with the 5'NTR of coxsackievirus B3, and the remaining one was identical to the 5'NTR of wild type poliovirus type 3 (strain P3/119).26 Among the coxsackievirus-like sequences, two were identical with greatest homology to coxsackievirus B3 (88.6%) or to those in the coxsackievirus-like phylogenetic cluster (GenBank accession Nos AF198383 and AF198384).27 Three cases (3, 8, and 9) were identical to coxsackievirus B3 strain Nancy.28 DNA sequences of the NPCR product (primers F24/R253) from the positive myocarditis control showed 97–99% similarity with coxsackievirus B3. Its GenBank accession No is AF197926 (table 3).

ENTEROVIRAL CAPSID PROTEINS AND GENOMIC RNA IN SITU IN KESHAN DISEASE HEARTS

Having observed sequences identical to coxsackievirus B3 in samples from three Keshan disease patients, further experiments were performed to exclude the possibility of contamination from environmental or incidental sources by coxsackievirus B3 or its genomic RNA or cDNA, in which case viral antigens or genomic RNA would not be localised in the cytoplasm of myofibres. Tissue sections from patients 3, 5, 6, 8, 9, and 10 were examined using in situ antigen detection or in situ hybridisation techniques. Further tissue sections were not available from patients 1, 2, and 4 for in situ detection. Either viral capsid protein VP1 or viral genomic RNA was detected in these six samples by immunohistochemistry using Mab 5-D8/1, or by in situ hybridisation using the digoxigenin labelled enterovirus specific probes. Signals were localised in the cytoplasm of affected myofibres (fig 3), confirming the involvement of enterovirus in Keshan disease and the positive results obtained by RT-NPCR. Hybridisation signals were stronger when using multiple oligoprobes than when using a single oligoprobe, as expected. Viral antigen was also detected in sections from the positive control.

![Figure 3](http://heart.bmj.com/)
Enteroviral capsid protein VP1 and genomic RNA were not found in tissue sections of control samples.

**Discussion**

Since an outbreak of Keshan disease in 1935, speculation on the aetiology of the disease has been focused on selenium deficiency and virus infection. Selenium is a component of glutathione peroxidase, and deficiency of this antioxidant is linked to cardiovascular diseases, including Keshan disease and cardiomyopathy. In the present study, exact selenium status of these Keshan disease patients and controls before their death was not known, but Chuxiong Region is noted for selenium deficiency in the environment and in its inhabitants, and is one of the high risk Keshan disease endemic regions. Selenium supplementation has been instituted in China in Keshan disease endemic areas, but despite restoring the plasma selenium and the decrease in incidence of Keshan disease in last two decades, the programme has not eliminated Keshan disease. In addition, it is known that Keshan disease has annual and seasonal fluctuation of incidence.

These observations have led to a hypothesis that selenium deficiency (with other nutritional factors, for example vitamin E) is an important environmental and nutritional predisposing factor or one of the key interacting factors, but not the only factor in the development of Keshan disease. One mechanism by which selenium deficiency may act in the pathogenesis of Keshan disease is to increase cardiovirulence of enteroviruses by changes at the viral nucleotide sequence level. This is supported by a mouse model where the gene for the selenium dependent enzyme glutathione peroxidase 1 (GPX-1) was disrupted (Gpx1−/−). Damage to the heart of Gpx1−/− mice after infection with an avirulent coxsackievirus was caused by mutations in the viral genome, which generated a cardiovirulent strain.

Enteroviruses were implicated initially in Keshan disease by virus isolation, serology, and recently the detection of enteroviral genomic RNA in the myocardium from patients at different stages of the disease. In one study, 18 of 21 formalin fixed, paraffin embedded necropsy heart tissue samples were positive by RT-NPCR specific for the 5'NTR of enteroviral RNA, but all 10 normal heart tissues were negative. Some of the same subacute Keshan disease samples had been investigated earlier by an independent laboratory using in situ hybridisation, and 18 of 20 samples were positive for enteroviral RNA. In this study a cDNA clone of 5'NTR of coxsackievirus B3 was employed as probe. Similar observations were made in acute or chronic Keshan disease. Immunohistochemistry results demonstrated extensive viral antigen distribution in these samples—that is, a large area of myocardium had been infected by enterovirus (fig 3B and our unpublished data).

Sequence analysis of PCR products shows that most enteroviral sequences (five of six) detected in the myocardium from cases of Keshan disease resemble those of coxsackie B-like viruses, in accordance with recent studies on patients with myocarditis and dilated cardiomyopathy. However, the 5'NTR sequences of enteroviruses do not necessarily correlate with serotypes, although several phylogenetic clusters could be distinguished on this basis. Identification of serotype related nucleotide sequences requires analysis of regions coding for viral capsid proteins, for example VP1.

A question that immediately arises is whether the three sequences (in patients 3, 8, and 9) were caused by PCR contamination, and how and when that could occur. Could contamination be excluded? Further experiments suggest that the presence of coxsackievirus B3 sequences identical to the 5'NTR of the Nancy strain in these samples is unlikely to reflect incidental cDNA or virus contamination because first, the positive RT-NPCR result was not abolished by DNase treatment of the RNA preparation; second, PCR amplification of the RNA extract without reverse transcription failed to yield products; and third, viral capsid protein VP1 and genomic RNA were detected in tissue sections and localised in cytoplasm of myofibres (fig 3).

Enterovirus sequences from one positive sample matched poliovirus type 3. The significance of this finding is not clear, but myocarditis was observed at necropsy examination in a proportion of patients who died of acute poliomyelitis before the poliovirus immunisation programme. In further studies, poliovirus was isolated from the hearts of three of five persons who died of the disease. These isolates caused not only the characteristic lesions in the anterior horn of monkeys but also myocardial lesions resembling those observed in the human disease. Another possibility is that the child had a recent dose of live polio vaccine. As no official medical records are available, this speculation cannot be confirmed. However, live polio vaccine boosters had been offered to children within China in the WHO campaign for eradication of poliomyelitis. The enterovirus group specific RT-NPCR would be able to detect poliovirus in blood in heart tissue; as no more tissue from this case is available for in situ...
Enterovirus and Keshan disease

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