

## Short reports

# Absence of HHV-8 DNA sequences in malignant mesothelioma

V Ascoli, F Nardi, C Carnovale Scalzo, S Signoretti, A Pistilli, F Lo Coco

### Abstract

**Human herpesvirus 8 (HHV-8) associated primary effusion lymphomas arise and grow in the body cavities as effusions, but it is not known whether the lining of body cavities and mesothelium derived malignancies are potential targets of HHV-8 infection. We examined a series of 13 diffuse malignant mesotheliomas and four mesothelial cell rich effusion samples of non-neoplastic aetiology from non-immunodepressed patients using the polymerase chain reaction to detect HHV-8 specific sequences. HHV-8 amplification products were absent in diffuse malignant mesotheliomas and in non-neoplastic effusions samples. These results suggest that HHV-8 has a selective tropism among body cavity based tumours, being confined to primary effusion lymphomas.**

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Keywords: mesothelioma; primary effusion lymphoma; human herpesvirus 8

Human herpesvirus 8 (HHV-8) DNA sequences have been isolated from Kaposi's sarcoma lesions and some lymphoproliferative disorders, including primary effusion lymphoma, a rare high grade B cell lymphoma arising in the body cavities.<sup>1-3</sup> Recent studies suggest that HHV-8 might be more widespread than described originally, whereas other groups argue against this suggestion.<sup>2,3</sup> Thus, there is much interest about HHV-8 distribution in normal tissues, inflammatory conditions, and tumours. The restriction of HHV-8 to specific cell types and/or pathological entities suggests a role for this agent in the pathogenesis of HHV-8 associated diseases.

Regarding body cavity based tumours other than primary effusion lymphomas, those of submesothelial origin do not harbour HHV-8 sequences.<sup>4,5</sup> To investigate whether HHV-8 infection is associated with mesothelial derived tumours, we tested a series of diffuse malignant mesotheliomas using the polymerase chain reaction (PCR) to amplify specific viral sequences. We also analysed DNA in mesothelial cell rich effusion samples of non-neoplastic aetiology from non-immunodepressed patients.

### Methods

A total of 20 samples was investigated for the presence of HHV-8 including: (1) serous effusions (n = 3) and tissue samples (n = 10) from 13 patients affected by histologically and immunophenotypically proved diffuse malignant mesothelioma; (2) serous effusions (n = 4) obtained from individuals with non-neoplastic underlying diseases (cirrhosis and inflammatory conditions); and (3) skin biopsies (n = 3) from three non-human immunodeficiency virus (HIV) infected Italian patients with classic Kaposi's sarcoma (nodular lesions), as positive controls.

High molecular weight DNA was extracted from OCT frozen tissue samples (n = 10), DMSO frozen cell suspensions (n = 7), and paraffin wax embedded biopsies (n = 3) collected at diagnosis. DNA was obtained by cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. The primers and PCR conditions used to amplify HHV-8 sequences have been reported elsewhere.<sup>1</sup> In all experiments, a positive control using DNA derived from one of the above mentioned Kaposi's sarcoma biopsies and a negative control (all reagents plus water and no DNA) were included. To verify the presence of amplifiable DNA, a 294 base pair fragment of the Bcl-6 first intron gene was also amplified in all cases. To determine the sensitivity of our assay, the DNA obtained from a Kaposi's sarcoma biopsy was diluted serially by mixing with DNA from non-HHV-8 infected normal lymphocytes. A specific amplification band could be visualised on the ethidium bromide stained agarose gel only at a 1/10 dilution of Kaposi's sarcoma DNA; no fragments were visible when 1/100 and 1/1000 dilutions were amplified.

### Results

Among the patients with diffuse malignant mesothelioma, four were women and nine were men, and the median age at the time of diagnosis was 60 years (range, 31-78). Diffuse malignant mesotheliomas arose from pleura (n = 9) and peritoneum (n = 4), including eight epithelial, three sarcomatous, and two biphasic variants. Patients with non-neoplastic disorders included three women and one man with a median age of 40 years (range, 20-64). None of

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the patients had any clinical signs of immunodepression.

The mesothelial origin of tumour cells in diffuse malignant mesothelioma samples was determined by immunoperoxidase staining with monoclonal antibodies against cytokeratin, vimentin, Leu-M1 (CD15), Ber-EP4, carcinoembryonic antigen, epithelial membrane antigen, and mesothelial cells (antibody HBME-1). Reactive and mesotheliomatous effusions contained a variable number of CD5 positive lymphocytes (T cells) and CD68 positive cells (monocytes/macrophages), as well as cytokeratin/HBME-1 positive mesothelial cells.

PCR studies showed an absence of HHV-8 DNA sequences in all diffuse malignant mesothelioma samples and in all the non-neoplastic effusions analysed, whereas HHV-8 DNA was amplified in the DNAs obtained from the three Kaposi's sarcoma biopsies. The HHV-8 amplification signals visualised after PCR in Kaposi's sarcoma DNAs were weak, in keeping with the finding that a low number of viral copies is usually detected in such samples.<sup>6</sup>

### Discussion

Several studies investigating the presence of HHV-8 in tissues and diseases other than Kaposi's sarcoma, multicentric Castlemann's disease, and primary effusion lymphoma have reported conflicting observations.<sup>2,3</sup> HHV-8 DNA has been found in non-Kaposi's sarcoma skin lesions developing in post-transplant patients and non-Kaposi's sarcoma malignant vascular tumours. Other results argue against the presence of HHV-8 in endothelial Kaposi's sarcoma simulators or vascular lesions other than Kaposi's sarcoma and skin cancers of immunosuppressed patients; sporadic non-primary effusion lymphomas were found to harbour HHV-8 DNA. More recent studies have shown that HHV-8 may also infect a proportion of healthy adults.<sup>3,7</sup>

This study indicates that HHV-8 is not present in diffuse malignant mesotheliomas and non-neoplastic effusions containing mesothelial cells. Our PCR sensitivity allowed us to detect a 1/10 dilution of HHV-8 infected Kaposi's sarcoma DNA, a tumour in which low viral copy numbers are usually detected.<sup>6</sup> Therefore, our findings should also rule out the possibility of HHV-8 infection in diffuse malignant mesotheliomas with very low viral load. As to the suggestion that polymorphisms within the Kaposi's sarcoma Bam 330 region of the HHV-8 genome could contribute to false negative results, we believe that this is unlikely, although it cannot be formally ruled out. Although HHV-8 DNA sequences can be detected in some normal tissues and circulating lymphocytes,<sup>2,3</sup> the linings of body cavities are not targets for HHV-8 infection. Regarding body cavity based tumours, only primary effusion lymphomas are found consistently to contain HHV-8 sequences. Two recent studies have reported the absence of detectable HHV-8 in primary vascular tumours of subme-

sothelial origin.<sup>4,5</sup> Our findings extend these observations by showing that HHV-8 is absent in diffuse malignant mesothelioma, the only mesothelium derived malignancy related to body cavities. Thus, our study supports the view that the virus is distributed in a non-ubiquitous manner, and is characteristically restricted to specific pathological entities. Except for primary effusion lymphomas, HHV-8 does not seem to play an important pathogenetic role in any other tumour related to body cavities.

Molecular mimicry of human cytokines is a newly recognised characteristic of HHV-8,<sup>8</sup> which encodes homologues to multiple host cell genes including interleukin 6 (vIL-6). A common feature of some HHV-8 associated diseases is the upregulation of IL-6.<sup>3,8,9</sup> Interestingly, this characteristic is shared with diffuse malignant mesothelioma.<sup>10</sup> IL-6 is a growth factor for HHV-8 associated lesions, whereas its biological significance in diffuse malignant mesothelioma is unclear. It is not related to growth control mechanisms but is linked to systemic paraneoplastic events (fever, acute-phase reaction, cachexia, and thrombocytosis) associated with the disease.<sup>10</sup> Our study suggests indirectly that within the spectrum of IL-6 producing proliferative disorders, diffuse malignant mesotheliomas belong to a group lacking HHV-8 sequences and expressing human IL-6 (huIL-6). In contrast, other entities, such as primary effusion lymphomas, contain HHV-8 DNA and express vIL-6.<sup>8</sup>

The primary localisation of primary effusion lymphomas in the serosal cavity might be because of the favourable conditions for the growth of HHV-8 infected circulating lymphocytes in this location, through huIL-6-mediated paracrine stimulation by mesothelial cells and vIL-6 mediated autocrine loops.

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# Evidence for a common mutation in hereditary pancreatitis

S M Bell, C Bennett, A F Markham, N J Lench

## Abstract

**Hereditary pancreatitis is an autosomal dominant disorder with incomplete penetrance. It is characterised by recurring episodes of severe abdominal pain and often presents in childhood. Recently, a mutation in the cationic trypsinogen gene was identified in this disease. Previously, only one mutation at residue 117 of the trypsinogen gene has been found in five separate hereditary pancreatitis families, four from the USA and one from Italy. Alteration of the Arg117 site is believed to disrupt a fail-safe mechanism for the inactivation of trypsin, leading to autodigestion of the pancreas under certain conditions. Molecular analysis of the trypsinogen gene was carried out on a hereditary pancreatitis family from the UK. The same G to A mutation at residue 117 was identified in this family, suggesting that this is a common mutation in hereditary pancreatitis.**

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Keywords: hereditary pancreatitis; cationic trypsinogen gene mutation; polymerase chain reaction

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Hereditary pancreatitis is a rare autosomal dominant disorder with variable expression. Hereditary pancreatitis is characterised by pan-

creatitis with early onset, usually before the age of 10. In general, patients seem to improve later in life. In most cases, the attacks are of nuisance value only, often precipitated by emotional upset, alcohol, or high fat intake. Only in a small number of cases is this disease life threatening. Recently, a linkage was identified between hereditary pancreatitis and the markers D7S661 and D7S676 at chromosome 7q35, a region containing the cationic trypsinogen gene.<sup>1,2</sup> Mutational analysis of this gene in affected individuals identified a G to A transition resulting in an Arg to His amino acid substitution at residue 117.<sup>3</sup> It has been postulated that pancreatitis is caused by the inappropriate activation of pancreatic proenzymes. Trypsinogen is an inactive proenzyme for trypsin that is activated by the removal of the eight amino terminal amino acids. Normally, a small amount of active trypsin is present within the pancreas and, in general, it is inactivated rapidly by the pancreatic secretory trypsin inhibitor. Occasionally, when trypsin activity exceeds that of the pancreatic secretory trypsin inhibitor, a number of proenzymes are activated, such as mesotrypsin and enzyme Y. These enzymes are thought to be part of a feedback loop that inactivates trypsinogen and trypsin. *x* Ray crystal structure analysis, molecular modelling, and protein digestion data indicate that residue Arg 117 is a trypsin sensitive site, which can be cleaved by mesotrypsin and enzyme Y. The replacement of Arg 117 with His removes this putative fail-safe mechanism, leading to autodigestion of the pancreas and pancreatitis.<sup>3</sup> Here, we present the genetic analysis of a white hereditary pancreatitis family from the UK.

## Methods

### PATIENTS

Patients were from a five generation hereditary pancreatitis family that had 10 affected members, demonstrated autosomal dominant inheritance, and originated from northern England (fig 1).

### ISOLATION OF GENOMIC DNA

Blood samples were obtained from five affected members of the family, individuals II:1, III:2, III:5, IV:2, and IV:6 (fig 1), representing three generations of the family. Peripheral blood mononuclear cells were separated from 3 ml of whole blood and genomic DNA was extracted using the Gentra Systems Puregene DNA purification protocol (Gentra Systems, Minneapolis, USA), which is based on the salt precipitation method.

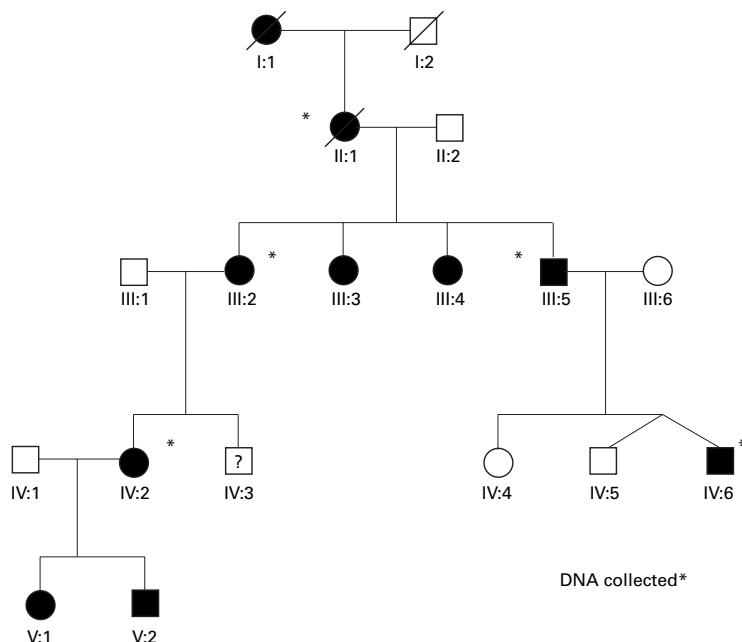


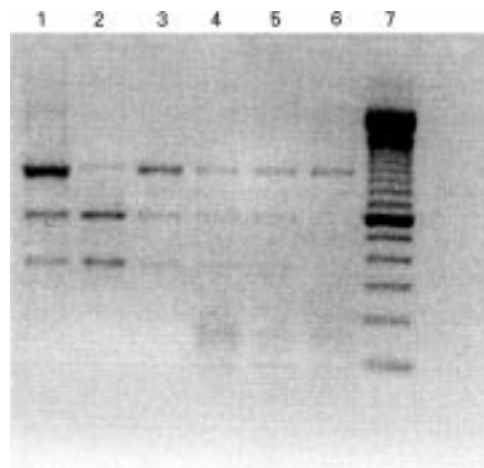
Figure 1 Hereditary pancreatitis pedigree. Closed symbols, affected individuals; circles, women; squares, men; diagonal lines, dead.

## POLYMERASE CHAIN REACTION (PCR)

A 911 base pair (bp) fragment containing exon 3 of the trypsinogen gene was amplified using PCR conditions described previously.<sup>3</sup> Briefly, PCR was carried out in a 25 µl reaction volume containing: 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100), 2.5 mM MgCl<sub>2</sub>, 200 µM dATP, dCTP, dGTP, dTTP, 10 pmols of the forward primer: dGGTCCTGGGTCTCATACCTT and 10 pmols of the reverse primer: dGGGTAG-GAGGCTTCACACTT, 1 unit of Taq DNA polymerase (Promega, Southampton, UK), and 10 ng genomic DNA. The PCR amplification was carried out under the following conditions: 95°C for five minutes, then 40 cycles of 95°C for one minute, 64°C for one minute, and 72°C for two minutes, followed by a final extension step at 72°C for 10 minutes. The PCR products (10 µl aliquots) were analysed on a 1.5% agarose gel stained with ethidium bromide.

## RESTRICTION ENZYME DIGESTION

The G to A mutation at residue 117 of the trypsinogen gene creates a restriction site recognised by the enzyme AflIII. The amplified PCR products were digested overnight at 37°C with AflIII according to the manufacturer's instructions (New England Biolabs, Hitchin, Hertfordshire, UK). The restriction digestion products (10 µl aliquots) were analysed on a 1.5% agarose gel stained with ethidium bromide.



**Figure 2** Restriction enzyme analysis of the cationic trypsinogen gene. The cationic trypsin gene was amplified by the polymerase chain reaction (PCR). The PCR products were digested with the restriction enzyme AflIII and analysed on a 1.5% agarose gel stained with ethidium bromide. The figure represents the reverse image of the agarose gel. Lanes 1 to 5 contain affected family members II:1, III:2, III:5, IV:2, and IV:6, respectively. Three bands are visible in each lane: a 911 base pair (bp) band (normal allele) and two smaller bands 565 bp and 346 bp in size (mutant allele). Lane 6 contains an unaffected control showing the large 911 bp band only, which represents the normal allele. Lane 7 contains a 100 bp molecular weight size marker. This figure illustrates that the five affected individuals are heterozygous for the AflIII restriction enzyme site that recognises the hereditary pancreatitis G to A mutation.

## SEQUENCE ANALYSIS OF PCR PRODUCTS

The PCR product was desalted and separated from unincorporated nucleotides and primers using a Microcon 100 column (Amicon, Beverly, USA). The concentrated template (1 µl) was sequenced with an internal primer,<sup>3</sup> dTGACCCACATCCCTCTG, using a PRISM ready reaction terminator cycle sequencing kit (Applied Biosystems, Warrington, Cheshire, UK) on an automated fluorescence DNA sequencer (Applied Biosystems 377).

## Results

The trypsinogen gene was amplified from five affected family members and an unaffected control. Restriction enzyme digestion of the PCR products using the restriction enzyme AflIII found that all five affected family members were heterozygous for the presence of the restriction enzyme site (fig 2). The enzyme AflIII recognises the restriction enzyme site 5'A▼CGTGT 3', which is created by a G to A base pair mutation in the trypsinogen gene. Three bands were seen in the affected individuals: a large band (911 bp) and two smaller bands (565 bp and 346 bp); only the large 911 bp band was seen in the unaffected control. The large band represents the normal allele and the two smaller bands represent the mutant allele, indicating that the G to A mutation is present in the five affected individuals examined, but not the normal control. The PCR amplifications and restriction enzyme digestions were repeated to confirm the results obtained. DNA sequencing of the PCR product from the common ancestor (II:2) confirmed the presence of the G to A mutation. This G to A transition results in an amino acid substitution of arginine (CGC) for histidine (CAC) at residue 117 of the trypsinogen gene.

## Discussion

This study describes the identification of a G to A mutation at residue 117 of the cationic trypsinogen gene in a UK hereditary pancreatitis family from Yorkshire. Our study is the first to confirm the involvement of the cationic trypsinogen gene in hereditary pancreatitis. One hundred hereditary pancreatitis kindreds have been reported worldwide; most of the families are white, although other ethnic groups have been identified.<sup>4</sup> Previously, this G to A mutation has been identified in five hereditary pancreatitis kindreds, four from the USA and one from Italy. One hundred and forty unaffected individuals were also screened for the AflIII restriction enzyme site to determine that this was not a polymorphism. Initially, the presence of the same mutation in five different families was thought to be the result of a common ancestor. Haplotype analysis of the USA families did identify a common 4cM region associated with the hereditary pancreatitis phenotype, indicating that these families are distantly related. Our family from northern England could be distantly related to the USA families, which have English ancestry. However, the Italian family from Naples had a unique haplotype, suggesting that the same mutation has oc-

curred on two separate occasions.<sup>3</sup> Our study identifying the same mutation in a UK family provides further evidence that this is a common mutational event involved in hereditary pancreatitis.

Hereditary pancreatitis is one of the most common causes of chronic or recurrent pancreatitis in childhood. The identification of trypsinogen as the hereditary pancreatitis gene means that individuals carrying the gene mutation can be identified before the onset of pancreatitis, thus facilitating the future treatment of the disease. The genetic confirmation of the clinical diagnosis of hereditary pancreatitis in the family under study means that counselling and improved clinical care can be provided for family members. The study of other hereditary pancreatitis kindreds will determine the frequency of this G to A mutation in the trypsinogen gene, whether mutations exist elsewhere in the gene, and whether hereditary pancreatitis is genetically heterogeneous (that is, whether other hereditary pancreatitis loci exist). The clinical presentation of hereditary pancreatitis is the same as that for pancreatitis caused by other factors, such as alcohol abuse or gallstones, apart from the occurrence of childhood attacks. The identification of the type of mutations present in hereditary pancreatitis kindreds might indicate whether the screening of sporadic pancreatitis cases is warranted, because some members of the general population may be more susceptible than others to developing pancreatitis in adulthood when exposed to factors such as high alcohol intake.

Normally, the amount of active trypsin in the pancreas is controlled by pancreatic secretory trypsin inhibitor. However, occasionally—for example, after large meals or excessive alcohol, the inhibitory effect of pancreatic secretory trypsin inhibitor is overwhelmed and proteolytic enzymes that digest trypsin are activated as a fail-safe mechanism to prevent autodiges-

tion and pancreatitis.  $\alpha$  Ray crystallography of trypsinogen, to determine its three dimensional structure, has shown that the Arg 117 site is in a critical position in the peptide chain, connecting the two globular domains of the trypsin molecule.<sup>3–5</sup> Molecular modelling indicates that this Arg 117 residue is at a trypsin-like cleavage site<sup>6</sup>; this has been confirmed by in vitro protein digestion studies, which have shown that this is the primary site for proteolysis of trypsin.<sup>7</sup> While cleavage at this site does not inactivate trypsin, it is believed to expose other proteolytic sites, leading to rapid digestion of the protein.<sup>7</sup> The G to A mutation found in hereditary pancreatitis results in an Arg to His substitution at residue 117, consequently making trypsinogen resistant to these proteolytic enzymes. An understanding of the mechanisms involved in hereditary pancreatitis will facilitate the development of treatments for both the disease itself and sporadic pancreatitis caused by other factors.

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