

Parvovirus B19 is associated with benign testes as well as testicular germ cell tumours

T C Diss, L X Pan, M Q Du, H Z Peng, J R Kerr

Abstract

Aims—Parvovirus B19 has been demonstrated in testes of patients with germ cell tumours but not in controls, raising the possibility that the virus has an aetiological role in these tumours. The aims of this study were to investigate the association of the virus with germ cell tumours and to localise the virus histologically.

Methods—DNA was extracted from paraffin wax embedded sections of testes from 10 seminomas, eight teratomas, two mixed seminoma/teratomas, and 10 testes showing benign histology. Polymerase chain reaction (PCR) amplification of three regions within the NS and VP1/2 genes was carried out in duplicate on all samples. One PCR positive case (seminoma/teratoma) was examined by microdissection of histologically defined tissue components followed by PCR amplification of parvoviral sequences. Samples from PCR positive patients were immunostained using a B19 specific monoclonal antibody.

Results—Seven cases were PCR positive, these comprised two of 10 seminomas, one of two mixed tumours, none of eight teratomas, and four of 10 benign controls. PCR analysis of the material microdissected from the seminoma/teratoma showed the presence of the virus in regions of seminoma, teratoma, intratubular germ cell neoplasia, normal tubules, and connective tissue. All patient samples studied immunohistochemically were negative.

Conclusions—This confirms the presence of parvovirus B19 in a proportion of germ cell tumours; however, in one patient, the virus was widespread in the tissue components and not confined to tumour cells. In addition, the virus was present in control benign testes. These data suggest that B19 might not be of aetiological importance in germ cell tumours of testis.

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Keywords: parvovirus B19; testicular germ cell tumours; tumour aetiology

Since its discovery in 1975,¹ parvovirus B19 has been shown to cause erythema infectiosum, aplastic crisis in patients with shortened red cell survival, arthritis, and chronic bone marrow failure in immunocompromised persons.² The pathogenesis of these conditions includes both lytic infection of red blood cell progenitors and immune complex deposition. A recent study has implicated B19 in human

cancer for the first time.³ The virus was detected by polymerase chain reaction (PCR) in testes of patients with germ cell tumours but not in benign controls. This suggests that B19 might have an aetiological role in these tumours. However, it was not shown whether the virus was localised in tumour cells or in normal cells present in the tissue samples. The aims of our study were to confirm the association of the virus with germ cell tumours and to localise the virus histologically.

Methods

PATIENT SAMPLES

Paraffin wax blocks were obtained from the archives of the histopathology department at University College Medical School, London, UK. Blocks of testicular tissue were selected from 10 patients with seminomas (average age, 39 years), two with mixed germ cell tumours (seminoma/teratoma; average age, 42 years), eight with teratomas (average age, 31 years) and a control group of 10 showing benign histology (nine from patients with carcinoma of prostate; average age, 70; one with undescended testis removed at 88 years). Sections (5 µm thick) were cut from each block and placed into 0.5 ml microtubes for DNA extraction. Different regions of new microtome blades were used for each block to minimise the risk of cross contamination. Sample handling, DNA extraction, and PCR analysis were performed in laboratories that were separate from those where B19 diagnostics are carried out.

POLYMERASE CHAIN REACTION

DNA was extracted by overnight treatment at 37°C with 100–200 µl of proteinase K (200 µg/ml; Sigma, Poole, Dorset, UK) in PCR buffer (Promega, Southampton, UK), followed by heat inactivation of the enzyme.⁴ Three PCR protocols were used for the detection of parvovirus B19, targeting different regions of the viral genome (fig 1). Two 5 µl aliquots of each sample were amplified using primers directed to the parvovirus VP1/2 gene (primer sets VP1/2a (product size, 103 bp) and VP1/2b (product size, 111 bp)) and to the NS gene (product size, 103 bp). Primers were designed or adapted from published sequences³ to yield small fragments in an attempt to achieve efficient amplification from potentially highly degraded DNA samples extracted from paraffin wax embedded samples. DNA extracted from a paraffin wax block of fetal lung, which had been shown to harbour B19 by means of *in situ* hybridisation was used as a positive control, and a reaction mixture without template DNA was used as a negative control

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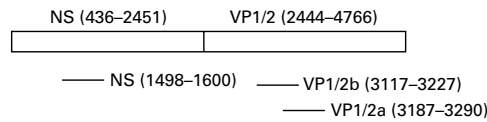


Figure 1 Parvovirus B19 genome showing the NS and VP1/2 coding regions and approximate primer locations. Nucleotide numbers are shown in parentheses. Primer sequences were as follows: VP1/2a, CAAAAGCATGTG GAGTGAGG and CCTATAATGGTGCTCTGGG (newly designed), product size 103 bp; VP1/2b, ACCCA AGCATGACTTCAGTT³ and AGTTGGCACTAA AAGTGGCC (newly designed), product size 111 bp; NS, AATGAAAACCTTCCATTAAAT GATGTAG and CTAAAATGGCTTTGCAGCTTCTAC,⁵ product size 103 bp.

in all experiments. Reaction mixtures contained 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each dNTP, 250 ng each primer, 0.1% Triton X-100, and 0.5 U of Taq polymerase (Promega) in a total volume of 50 μl. After "hot start", 40 cycles of PCR were carried out as follows: 40 seconds at 93°C, 45 seconds at 55°C (61°C for VP1/2a primers), and 110 seconds at 72°C. Products were analysed in a separate laboratory to the one used to set up the PCR, using 10% polyacrylamide minigels that were stained with ethidium bromide and viewed under UV light. Samples that yielded products of the expected size in duplicate reactions were recorded as positive. Results were interpreted without knowledge of the diagnoses. All DNA extracts were tested for the presence of suitable DNA by amplification of a 250 bp fragment of the β-globin gene.⁵

MICRODISSECTION

Selected areas from haematoxylin and eosin stained sections from patient 11 were microdissected manually with drawn out glass pipettes using the method described by Pan *et al.*⁶ Areas of seminoma, teratoma, and intratubular germ cell neoplasia were selected, as well as areas of normal tubules and connective tissue distant from tumour foci (fig 2). Clean pipettes were used for each microdissected area to minimise cross contamination. Aliquots of ethanol solutions used to contain sections before and after microdissection were used as additional negative controls. DNA was extracted as described above, but using 25 μl of proteinase K buffer and an extended digestion step of three days. Duplicate 5 μl aliquots were analysed by PCR amplification of the parvovirus genome using VP1/2b primers. In addition, DNA was extracted from all available tissue blocks from

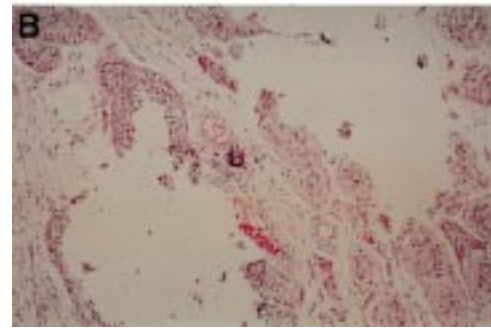
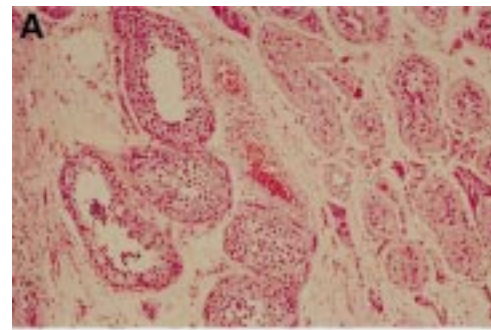


Figure 2 (A) Areas of normal tubules (left) and intratubular germ cell neoplasia (right) from patient 11 before microdissection. (B) Same areas after microdissection of each component separately. Haematoxylin and eosin stained.

patient 11 and analysed using VP1/2b primers (table 1). Microdissection of seminoma and normal components from patients 1 and 6 was also carried out, and extracts subjected to PCR using VP1/2b primers.

SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP)

VP1/2b PCR products derived from six testicular samples were analysed by SSCP using the Genephor apparatus (Pharmacia, St Albans, UK). Denatured products were run on precast 12% gels at 8 W for two and a half hours and automatically silver stained under the conditions recommended by the manufacturer.

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Immunohistochemistry was performed on the seven PCR positive cases (patients 1, 6, 11, 26, 28, 29, and 30) using a streptavidin-biotin peroxidase method with an antibody directed to the B19 VP1 and VP2 capsid proteins (Novacastra, Newcastle upon Tyne, UK).⁷

Table 1 Microdissection and polymerase chain reaction (PCR) analysis of samples from patient 11

Block	Site	Cellular composition	PCR-VP1/2b
1	Right testis, tumour	Right testis, whole section. Seminoma, teratoma, ITGCN, normal tubules	+
1	MD-A	Seminoma	+
1	MD-B	Teratoma	+
1	MD-C	Intratubular germ cell neoplasia	+
1	MD-D	Normal tubules	+
1	MD-E	Connective tissue	+
Control	Washings from microdissection experiments	No tissue	-
2	Left testis	Normal spermatogenesis. No tumour	+
3	Right testis, cord margin	Cord, vas deferens. No tumour	+
4	Right testis, lower cord	Cord, vas deferens. No tumour	+
5	Right testis, tumour	Teratoma, ITGCN, normal tubules	+
6	Right testis, distant from tumour mass	Teratoma, ITGCN, normal tubules	+

ITGCN, intratubular germ cell neoplasia; MD, microdissected area.

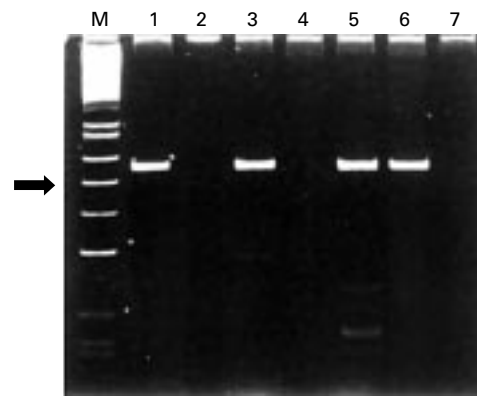


Figure 3 Polyacrylamide gel of VP1/2b PCR products. Lane M, ϕ X-HinI molecular weight markers (the 100 bp fragment is indicated by an arrow); lane 1, positive control showing 111 bp fragment; lane 2, negative control; lane 3, patient 26; lane 4, patient 27; lane 5, patient 28; lane 6, patient 29; lane 7, patient 30.

Results

POLYMERASE CHAIN REACTION

Seven of the 30 patients analysed were positive. Three benign controls and one mixed seminoma/teratoma were positive with all three sets of primers. Two seminomas were positive with the NS and VP1/2b sets and a single benign control was positive with the NS primers alone (fig 3; table 2). Three patients yielded PCR products with a single primer set in one of two initial duplicate reactions. Two further repeats were negative in each patient, and these samples were recorded as negative to exclude sporadic false positives. Positive and negative controls gave the appropriate results in each experiment. β -Globin PCR confirmed the

Table 2 Patient samples and results of polymerase chain reaction (PCR) and immunohistochemical (IHC) analyses

Patient	Age (years)	Diagnosis	PCR-VP1/2A	PCR-VP1/2B	PCR-NS	IHC
1	54	SEM	-	+	+	-
2	43	SEM	-	-	-	ND
3	41	SEM	-	-	-	ND
4	39	SEM	-	-	-	ND
5	38	SEM	-	-	-	ND
6	35	SEM	-	+	+	-
7	33	SEM	-	-	-	ND
8	33	SEM	-	-	-	ND
9	29	SEM	-	-	-	ND
10	45	SEM	-	-	-	ND
11	30	S + T	+	+	+	-
12	25	S + T	-	-	-	ND
13	35	TER	-	-	-	ND
14	30	TER	-	-	-	ND
15	26	TER	-	-	-	ND
16	29	TER	-	-	-	ND
17	26	TER	-	-	-	ND
18	20	TER	-	-	-	ND
19	51	TER	-	-	-	ND
20	31	TER	-	-	-	ND
21	80	CA-P	-	-	-	ND
22	74	CA-P	-	-	-	ND
23	70	CA-P	-	-	-	ND
24	69	CA-P	-	-	-	ND
25	76	CA-P	-	-	-	ND
26	77	CA-P	+	+	+	-
27	72	CA-P	-	-	-	ND
28	62	CA-P	+	+	+	-
29	47	CA-P	+	+	+	-
30	88	UND	-	-	+	-
Total			4/30	6/30	7/30	0/7

SEM, seminoma; S + T, seminoma/teratoma; TER, teratoma; CA-P, uninvolved testis from patient with carcinoma of the prostate; UND, undescended testes; ND, not done.

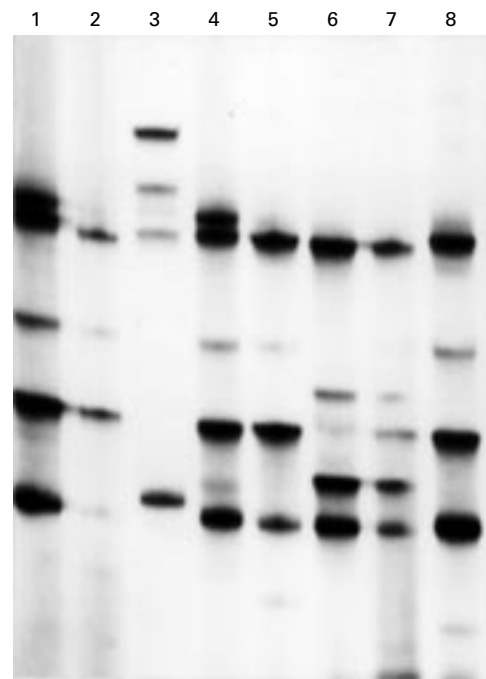


Figure 4 Single strand conformational polymorphism gel showing VP1/2b PCR products from six different patients (two different products from patients 1 and 26 have been run). Lane 1, patient 1; lane 2, patient 26; lane 3, patient 6; lane 4, patient 1; lane 5, patient 26; lane 6, patient 28; lane 7, patient 29; lane 8, patient 11.

presence of amplifiable DNA in all extracts from complete sections.

MICRODISSECTION

B19 PCR products of the expected size were amplified from all microdissected tissue components (seminoma, teratoma, intratubular germ cell neoplasia, normal tubules, and connective tissue) from patient 11 (table 1). The same results were seen from at least two separate microdissections and PCR amplifications of each component. All tissue blocks from this patient were also shown to contain B19 viral DNA (table 1). β -Globin PCR products were amplified from all DNA samples from patient 11. No parvovirus PCR products were amplified from microdissected areas from patients 1 and 6, and β -globin products were amplified from less than 50% of the samples from these patients, suggesting that insufficient suitable DNA had been extracted.

SINGLE STRAND CONFORMATIONAL

POLYMORPHISM

VP1/2b PCR products from six patients showed four different patterns on the SSCP gel (fig 4). These patterns were reproducible on repeated analysis and all results from a given patient were consistent.

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No positive signal was seen in any sample studied, although the B19 control gave the expected pattern of staining.

Discussion

Gray *et al* report the presence of B19 DNA in 33 of 39 frozen tissue samples from patients with testicular germ cell tumours.³ In our

study, we were able to confirm the presence of the virus in germ cell tumours, although in only three of 20 patients. The previous study reported no B19 DNA in 12 benign controls, whereas we found evidence of the virus in four of our 10 benign samples.

Microdissection of histologically defined tissue components in a single germ cell tumour suggested that the virus was widespread throughout the section, including benign tissue as well as areas of seminoma and teratoma. It is possible that rare B19 positive tumour cells contaminated our microdissected benign tissue samples, although whole sections of cord and vas deferens and a left testicular biopsy showing no evidence of tumour cells were also positive. At present we can only speculate as to which cells carry the virus, because our attempts to localise the virus using *in situ* hybridisation have not been successful to date, probably because of low viral copy numbers (not shown). We cannot definitely rule out cross contamination of microdissected samples with B19 DNA, although this is unlikely in view of the reproducibility of our results and the precautions taken.

The reasons for our much lower detection rates in tumour samples could be related to our use of paraffin wax embedded samples as opposed to frozen tissue samples, or to the use of different primers. Paraffin wax embedded samples yield relatively degraded DNA, which might have resulted in a lower sensitivity in our study. However, our design of primers that amplified short fragments, the use of primers that targeted multiple sites, the positivity of extracts using control primers, and our increased detection rate in benign testes argue against this. Nevertheless, it is possible that samples with low cellular viral infection rates and low copy number were falsely negative using paraffin wax embedded tissue samples. Our increased detection rate in benign controls might be related to the age of the patients studied or to the selection of cases, although the total numbers studied to date are low. The incidence of parvovirus infection might also have been underestimated as a result of variability of the B19 genome, which is known to occur during persistent infection.⁸ SSCP analysis of B19 PCR products confirmed variability^{3, 8, 9} within the viral genome and suggested that our positive signals were not the result of cross contamination of samples. The use of multiple primers for parvovirus detection is therefore necessary, although four of our seven positive cases were amplified with all three sets of primers.

Our results indicate that B19 DNA might be present in non-tumour cells in orchidectomies performed for the treatment of germ cell tumours, and that the virus is present in benign testes. Therefore, the virus might be a passenger in germ cell tumours. Whether infection of germ cells by B19 occurs, and whether it confers an increased risk of germ cell tumours, and at what stage infection occurs, remain to be elucidated. Lack of expression of viral capsid genes in our patients supports the hypothesis that the virus is inactive. The means of B19 persistence is unclear at present. B19 DNA has also been detected in the synovium of patients with osteoarthritis and rheumatoid arthritis in the absence of expression of the gene encoding the B19 capsid protein.¹⁰ The cell type that harbours the virus and the mechanism of persistence in these conditions are unknown.

In conclusion, we have confirmed the presence of parvovirus B19 in a proportion of patients with testicular germ cell tumours and shown that benign testes might also be infected. Our findings suggest that the virus, even if present in tumours, is not restricted to tumour cells, and they indicate that the virus might not be involved directly in the evolution of germ cell tumours. Further work is required to understand the site of viral incorporation, the importance of that event, and to determine the range of cells and tissues in which it is located.

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