

No association between human parvovirus B19 and testicular germ cell cancer

T. Tolfvenstam,¹ N. Papadogiannakis,² A. Andersen³ and O. Akre⁴

^{1,2}Department of Clinical Virology¹ and Department of Pathology², Huddinge University Hospital, F68, Karolinska Institutet, SE-141 86 Stockholm, Sweden

³The Norwegian Cancer Registry, Oslo, Norway

⁴Clinical Epidemiology Unit, Department of Medicine, Karolinska Hospital, Karolinska Institutet, Stockholm, Sweden

The incidence of testicular germ cell cancer, which is the most common cancer among young male adults, is increasing. The aetiology remains unknown, although a virus has been proposed. A previous study has shown a high prevalence of human parvovirus B19 (B19) DNA in the testes of patients with testicular germ cell tumours (85%) and suggested that B19 may play a role in tumour development. To address this question of causality, seroreactivity to B19 was studied among cases ($n = 80$) and controls ($n = 241$) using serum samples drawn before the onset of disease, in addition to an elucidation of the frequency of virus DNA in a retrospectively collected 2-year testicular carcinoma series. No association was found between B19 seropositivity and the risk of testicular cancer (odds ratio = 1.03; 95% confidence interval = 0.60–1.77) nor was there any dose-response relation (P for trend = 0.53). This study did, however, confirm the observation that B19 DNA can be detected in testicular carcinoma tissue, as 4 of 24 cases were found to be positive, while no B19 DNA could be detected in the control cases. It is speculated that this finding may be due to susceptibility of the carcinoma cells to B19 virus owing to high-level expression of the viral receptor glycosphingolipid (Gb4) and possible other putative cellular factors resulting in a localized persistence initiated after the development of cancer.

Introduction

Testicular germ cell cancer is the most common cancer among young male adults. Whereas the mortality has declined owing to improved treatment, the incidence has increased by around 4% in many populations during the entire period of cancer registration (Adami *et al.*, 1994). The aetiology of the tumour remains, however, unknown. A virus aetiology has been proposed, based on epidemiological similarities to Hodgkin's disease, but at present no strong candidate agent has been suggested by previous analytical studies (Newell *et al.*, 1984).

Human parvovirus B19 (B19) is a ubiquitous pathogen that causes the childhood disease erythema infectiosum (Anderson *et al.*, 1984). It is highly prevalent in society at large, with a seroprevalence of 50–70% among adults (Cohen & Buckley,

1988). B19 is known to be a concern in pregnant women, in whom foetal infection may result in foetal death, and also among individuals with haematological disorders, in whom the infection may be fatal (Tolfvenstam *et al.*, 2001; Young, 1988). The virus mediates its pathogenicity by infecting erythroid progenitor cells by means of its cellular receptor, the blood group P antigen (Brown *et al.*, 1993). Although the virus has been shown to persist in the bone marrow of some individuals over long periods of time, it has not been previously associated with carcinogenesis (Kurtzman *et al.*, 1987; Lundqvist *et al.*, 1999). Recently, Gray *et al.* (1998) reported a high frequency of B19 DNA in testicular tissue from testicular cancer patients as compared to controls. Diss *et al.* (1999) later confirmed these findings but also concluded that B19 DNA could be found in normal testicular tissue. With the study design used by these authors, it cannot be ruled out that infection occurred subsequent to the development of cancer. To overcome this problem of reversed causality, we studied seroreactivity to B19 among cases and controls using serum samples drawn before

Author for correspondence: Thomas Tolfvenstam.

Fax +46 8 585 879 33. e-mail thomas.tolfvenstam@impi.ki.se

the onset of disease in addition to an elucidation of the frequency of viral DNAs in a retrospectively collected 2-year testicular carcinoma series in our area of referral.

Methods

■ **Study population.** We conducted a case-control study nested in a cohort of men who donated blood samples as part of the JANUS project in Norway. The JANUS project, sponsored by the Norwegian Cancer Society, was established in 1973 for the purpose of prospectively collecting serum to be used in epidemiological studies of cancer development. Blood was collected up to the end of 1991 and serum samples were stored at -25°C . A detailed description of the project and of serum collection, storage and retrieval has been published previously (Jellum *et al.*, 1995). The JANUS serum bank contains 424 938 serum samples from 293 692 individuals, none of whom had a history of cancer at the time of blood sampling, according to the Norwegian Cancer Registry.

Since 1960, all inhabitants of Norway have been assigned a unique, 11-digit identification number. All newly diagnosed cases of cancer in Norway are registered in the Cancer Registry of Norway, with compulsory reporting by both hospital departments and histopathological laboratories. Patients are entered in the Cancer Registry under their individual identification number. On a yearly basis, the identification numbers of all blood donors contained in the JANUS serum bank are linked with the Cancer Registry; as of October 1993, a total of 14 000 donors had developed some form of cancer.

Persons eligible as cases for this nested case-control analysis were male blood donors listed in the Cancer Registry through 1993 who had been diagnosed with invasive testicular cancer. There were 81 eligible cases but one case was excluded due to a lack of sample material. Three blood donors, matched with each index case by birth date (within 1 year), were randomly selected from the JANUS serum bank as potential controls. To verify that a potential control was alive and had not been diagnosed with testicular cancer at the time of diagnosis of the corresponding case, we examined the records, linked through the individual identification number, in the Death Registry and the Cancer Registry of Norway. One control was excluded on this basis and one case was excluded due to a lack of sample material, leaving 241 to be included in the serological analysis.

To confirm the overrepresentation of B19 DNA in germ cell cancer reported in the previous studies in a Scandinavian setting, testicular tissue samples were collected retrospectively from all cases of testicular carcinoma referred during the years 1998–1999 ($n = 24$) to the Department of Pathology at Huddinge University Hospital (Stockholm, Sweden), which serves all three hospitals in the southern part of the greater Stockholm area. Histopathologically, 21 cases represented seminomas, two cases were mixed testicular carcinomas and one case was an embryonic carcinoma. Controls were included retrospectively and consisted of all cases of orchidectomy referred to the same unit during the years 1996–1999, in which the diagnosis was not testicular carcinoma and the tissue was histopathologically normal ($n = 11$). In these cases, orchidectomy was performed owing to suspected malignancy (five cases), prostatic cancer (four cases) and complicated hydrocele (two cases).

■ **Ethical approval.** This study was approved by the Ethics Committee of Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden.

■ **Serum analyses.** B19-specific IgG class antibodies were measured by a sandwich ELISA (Biotrin). Antibody levels were expressed as IU/ml and determined by five-point co-titration of the WHO B19 standard in

each analysis. Information on seroreactivity to cytomegalovirus, Epstein–Barr virus, *Chlamydia trachomatis*, human papillomavirus types 16 and 18, herpes simplex virus types 1 and 2 and human herpesvirus type 8 was used in the multivariate analysis to control for potential confounding. The methods used for detecting seroreactivity to these agents are described elsewhere (Akre *et al.*, 1999).

■ **Tissue analysis.** Tissue sections of 25 mg were cut from the paraffin-embedded material representing the tumour, as indicated by the corresponding haematoxylin and eosin (H&E)-stained slide. Xenol and ethanol washings subsequently removed the paraffin and DNA was extracted using QiaAmp DNA Mini kit (Qiagen), according to the manufacturer's instructions. The extraction procedure was verified by amplification of a conserved region in the chromosomal major histocompatibility complex class II gene (Ehrlich & Bugawan, 1989). B19-specific DNA was amplified by nested PCR, as described previously, with the exception of the use of a modified outer forward primer (5' GGCAGCATGTGTAAAGTGG 3') (Broliden *et al.*, 1998). Amplification resulted in a 284 bp fragment originating from the B19 non-structural protein (NS1). Immunohistochemistry was performed on formalin-fixed microtome-cut tissue sections using a monoclonal antibody directed to the B19 structural protein 2 (VP2), visualized by a peroxidase system. The slides were reviewed, together with routinely stained H&E tissue sections, by a senior pathologist experienced in B19-induced histopathology.

■ **Statistical analysis.** The prevalence of seropositivity, as well as mean titres, were determined. In addition to analyses of the risk of being seropositive, dose-response relationships were evaluated by dichotomizing seropositivity into high (above median) and low (below median) positive titres based on the distribution in the seropositive population. Data were modelled by means of conditional logistic regression using the SAS statistical package. The parameters and standard errors in the models were converted to odds ratios (ORs) with 95% confidence intervals (CIs). All reported *P* values are two-tailed. Separate analyses for the two main histological subgroups of testicular cancer, seminomas and non-seminomas, were performed to evaluate potential aetiological differences.

Results

The mean age at the time of blood sample collection was 35.7 years. The mean follow-up between sample collection and diagnosis of testicular cancer among cases was 7.0 years (range 0.4–19.1 years; median 5.5 years). Among cases, 54 (68%) were of the seminoma type, 21 (26%) were of the non-seminoma type and five (6%) were of unknown histology. There was no difference in the seroprevalence of B19 between cases and controls (64 and 63%, respectively). Furthermore, the difference in mean absorbance was negligible, being 0.85 among cases and 0.79 among controls. Excluding three cases and three controls showing equivocal serological results, no association was found between B19 seropositivity and the risk of testicular cancer (OR = 1.03; 95% CI = 0.60–1.77) (Table 1) nor was there any dose-response relation when seropositivity was divided into high and low absorbances (*P* for trend = 0.53). When testicular cancer was differentiated into histological subgroups, the lack of association remained for seminoma type. For non-seminoma types, the risk was elevated among the seropositive men (Table 2). This finding was not

Table 1. Association between testicular cancer and seroreactivity to parvovirus B19

Cases showing equivocal serological result were not included in the analysis (P for trend = 0.53).

Parvovirus B19 seroreactivity	Number of cases ($n = 77$)	Number of controls ($n = 238$)	OR (95% CI) adjusted for age
Seronegative	28	87	1 (referent)
Seropositive	49	151	1.03 (0.60–1.77)
Seropositive, below median	20	77	0.81 (0.40–1.61)
Seropositive, above median	29	74	1.23 (0.67–2.26)

Table 2. Association between seroreactivity to parvovirus B19 and risk of testicular cancer, divided into histological subgroups

Cases showing equivocal serological result were not included in the analysis.

Parvovirus B19 seroreactivity	Age-adjusted OR (95% CI)	
	Seminoma ($n = 54/161$)	Non-seminoma ($n = 19/62$)
Seronegative	1 (referent)	1 (referent)
Seropositive	0.90 (0.48–1.67)	2.64 (0.66–10.57)
Seropositive, below median	0.57 (0.24–1.32)	3.26 (0.66–16.02)
Seropositive, above median	1.21 (0.60–2.42)	2.36 (0.55–10.19)

statistically significant, however, and the analysis was based on only 21 non-seminoma cases. Adjustment in the multivariate analysis for seroreactivity to cytomegalovirus, Epstein–Barr virus, *C. trachomatis*, human papillomavirus types 16 and 18, herpes simplex virus types 1 and 2 and human herpesvirus type 8 revealed no confounding of the association between B19 and the risk of testicular cancer.

Four of 21 cases (19%) of seminoma were found to be positive by direct B19 DNA amplification. None of the other types of testicular carcinoma (mixed tumour and embryonal carcinoma) and none of the control cases were found to be positive ($P = 0.272$, Fisher's exact test). No typical histopathological signs of B19 infection or positive immunohistochemical staining could be detected in any case (Morey *et al.*, 1992).

Discussion

In the present study, we have prospectively elucidated the association between testicular cancer and B19 infection by serology to minimize the risk of false-positive results generated by reverse causality. In addition, we investigated the prevalence of B19 DNA in a testicular tumour tissue series to assess the previously reported observation of a considerably higher frequency of B19 DNA in the testes of testicular carcinoma patients as compared to controls in a Scandinavian setting.

Serologically, we found no support for an association between B19 infection and the risk of testicular cancer, as cases and controls did not differ in terms of seropositivity or of quantitative antibody levels. This finding is consistent with the fact that B19 is believed to be a cytolytic virus, exerting its pathological effect by a rapid replication and subsequent lysis of the cell, whereupon the virus is cleared from the body by the host immune responses. Although there is evidence of a capacity of the virus to persist at low replication rates in the bone marrow, indicated by prolonged PCR positivity confined to haematogenerative sites, there is no indication of integration of the viral genome, establishment of latency, teratogenic effect during pregnancy or immunocompromizing effects of the infection, or other viral factors associated with oncogenic potential. Although the increased frequency of B19 DNA in tumours from individuals with testicular cancer, now demonstrated in three reports, is unlikely to reflect a causal relationship between the virus and the disease, it is interesting to speculate about this observation. Several authors have described an increased expression of glycolipid (Gb4), a synonym of the P antigen, in testicular carcinoma cells on studying the composition of these cells, suggesting that the overexpression of Gb4 is of potential diagnostic value (Ohyama *et al.*, 1990; Olie *et al.*, 1996; Wenk *et al.*, 1994). As the P antigen is known to be required for B19 infection of a cell, this could render these tumour cells susceptible to B19 infection

(Weigel-Kelley *et al.*, 2001). However, other unknown factors, co-receptors or intracellular factors are also necessary for infection, as demonstrated by the narrow tropism of B19 to certain stages of erythroid progenitor cells, in spite of low-level expression of the P antigen on a variety of organ-specific cells, such as endothelial cells, trophoblasts and myocardial cells, where no replication of B19 has been demonstrated (Cooling *et al.*, 1995). Infection of testicular carcinoma cells specifically would, therefore, also require this unknown factor to be present in erythroid progenitor cells. The negative immunohistochemical staining in the B19 DNA-positive specimens could be explained by a limited production of capsid proteins; such a phenomenon has been described previously by Pallier and colleagues when B19 infection in mainly non-permissive cells has been shown to express NS1 *in vitro* but some degree of DNA replication still persisted over long periods of time without cytolysis (Pallier *et al.*, 1997). However, among cases of intrauterine foetal death in which B19 was the only plausible causative agent, only two of nine DNA-positive cases scored positive in immunohistochemistry, which indicates that the assay has low sensitivity (Tolfvenstam *et al.*, 2001).

In summary, in this study we found no indication of an aetiological role of B19 in the development of testicular carcinoma but confirmed the observation of an over-representation of B19 DNA-positivity in testicular tumours compared to controls. We speculate that this finding may be due to susceptibility of the carcinoma cells to B19 infection owing to high-level expression of the viral receptor and possibly other putative cellular factors. This infection would have occurred subsequent to the development of cancer and would have a defect, low-grade replication, possibly protected by the blood–testis barrier.

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