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Genomic Mutations of Viral Protein 1 and BK Virus Nephropathy in Kidney Transplant Recipients

Renzo Boldorini,[1]* Sara Allegrini,[1] Umberto Miglio,[1] Alessia Paganotti,[2] Claudia Veggiani,[2] Monica Mischitelli,[3] Guido Monga,[1] and Valeria Pietropaolo[3]

[1]*Faculty of Medicine, Department of Medical Sciences, University Amedeo Avogadro of East Piedmont, Novara, Italy*
[2]*Department of Pathology, Ospedale Maggiore della Carità, Novara, Italy*
[3]*Department of Public Health Sciences, "La Sapienza" University, Rome, Italy*

Genomic variability in the viral protein 1 region of BK polyomavirus (BKV) may change the ability of the virus to replicate. The significance of such changes was studied in clinical samples taken from kidney transplant patients with and without BKV nephropathy. A 94 base-pair fragment of viral protein 1 was amplified from 68 urine, 28 blood, and 12 renal biopsy samples from eight patients with BKV nephropathy, and from 100 urine samples, 17 blood and three renal biopsy samples from 41 of 218 controls. The DNA was sequenced and the amino acid changes were predicted by the Expert Protein Analysis System program (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland). Single base-pair mutations were detected more frequently in the samples from the BKV nephropathy patients than in the controls, and this was the only statistically significant finding of the study ($P < 0.05$), thus suggesting a greater genetic instability in BKV nephropathy associated strains. The amino acid changes were distributed at random in both BKV nephropathy patients and controls. However, one aspartic acid-to-asparagine substitution at residue 75 was detected in all samples of the one patient with BKV-associated nephropathy, who developed disease progression confirmed by histology, and not in any of the other patient or control samples. Whether this specific amino acid change plays a role in disease deserves further study. ***J. Med. Virol. 81:1385–1393, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: polyomavirus; BK virus nephropathy; viral capsid protein; transcriptional control region; polymerase chain reaction; sequence analysis

INTRODUCTION

The human BK polyomavirus (BKV) may cause epithelial tubular cell damage and interstitial nephritis in kidney transplant patients [Nickeleit et al., 2000]. This polyomavirus or more appropriately BKV associated with nephropathy is thought to be due to latent BKV strains reactivated by anti-rejection immunosuppressive treatment [Nickeleit et al., 2000]. However, although the frequency of BKV reactivation is very high (estimated to be up to 68%) [Hirsch et al., 2002], it has been reported that BKV-induced renal damage occurs in only 2–10% of kidney transplant patients [Vasudev et al., 2005].

As pointed out in a review [Hirsch and Steiger, 2003], many host, environmental, and viral factors may be involved in the development of BKV-associated nephropathy. A previous study [Boldorini et al., 2009] investigated the pathogenic significance of genetic variation in the non-coding transcriptional control region (TCR) of BKV strains detected in blood, urine, and renal samples from patients with and without BKV nephropathy. It was found that BKV WWT, a strain first isolated in urine samples and considered a natural variant of the archetypal BKV WW with more efficient viral replication activity [Sundsfjord et al., 1990], was more frequent in the patients with BKV nephropathy than in the controls ($P < 0.05$), but neither the frequency nor specific types of TCR rearrangements seemed to be significantly different between the two groups. In addition to the TCR, the BKV genome contains two coding regions: one codes early small t and large T (LT) antigen proteins (oncoproteins), and the other late viral capsid proteins (VP1, VP2, and VP3). VP1 has many important functions: it is responsible for DNA packaging and virus assembly, and maintaining the

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*Correspondence to: Renzo Boldorini, MD, Faculty of Medicine, Department of Medical Sciences, University Amedeo Avogadro of East Piedmont, Via Solaroli, 17, 28100 Novara, Italy.
E-mail: renzo.boldorini@med.unipmn.it

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structural integrity of the virus, and it is also essential as a receptor site for the infection of host cells [Bolen et al., 1981; Jin and Gibson, 1996].

Although the genomic structure of the early region is stable within the same and different viral strains, the structure of the late VP1 region is characterized by nucleotide polymorphisms, and a 69 base-pair region (nucleotide positions 1744–1812 of the prototype Dunlop strain) has been used to identify four main viral genotypes: group I (including the DUN, MM, and GS strains), group II (the SB strain), group III (the AS strain), and group IV (the IV and MG strains) [Jin et al., 1993]. As the VP1 region is a coding region, single base-pair mutations (substitutions, deletions, or insertions) can lead to amino acid changes affecting the ability of the virus to recognize specific receptors on the surface of target cells: for example, it has been reported that a substitution of glutamic acid with glycine at residue 92 of VP1 affects mouse polyomavirus replication and spread in laboratory animals [Freund et al., 1991].

This study investigated whether specific BKV genotypes, VP1 genomic mutations, and/or amino acid changes in the VP1 protein may cause renal disease by comparing the sequences of the 69 base-pair region in the VP1 gene detected in urine, blood, and renal biopsy samples taken from kidney transplant patients who did and did not develop nephropathy associated with BKV.

MATERIALS AND METHODS

Patients and Samples

The study included 226 renal allograft recipients who had attended the Transplant Unit of Ospedale Maggiore della Carità between 2001 and 2007, eight of whom developed BKV nephropathy (3.5%); the 218 patients (96.5%) who did not develop the disease were considered controls. The diagnosis of BKV nephropathy was made on the basis of the morphological and immunohistochemical detection of viral inclusions in tubular epithelial cells and parietal cells of Bowman's capsule in allograft biopsies [Nickeleit et al., 2000]. The epidemiological data of both groups have been published elsewhere [Boldorini et al., 2009] but, in brief, the eight patients who developed BKV nephropathy were five men and three women (mean age 51 years; range 36–65), and the 218 controls were 118 men and 100 women (mean age 51.6 years; range 22–76). As shown in Table I, the average time between organ transplantation and the development of BKV nephropathy was 37.5 months (range 12–60); the renal disease leading to chronic renal failure was: polycystic kidney disease (two cases), IgA nephropathy (two cases), arterionephrosclerosis (two cases), glomerular disease (one case), and unknown (one case). All of the patients developing BKV nephropathy had received a cadaveric organ and, during the study, none experienced graft loss due to viral infection, although one (#3) showed disease progression leading to renal fibrosis. There was no significant difference in immunosuppressive therapy between the patients with BKV nephropathy and the controls.

Post-transplant paired urine and blood samples were collected for the screening and monitoring of BKV nephropathy, as suggested by an international multidisciplinary panel [Hirsch et al., 2005]. Briefly, the samples were collected: (1) every 3 months during the first 2 years post-transplant; (2) when allograft dysfunction was noted; and (3) when allograft biopsy was performed.

A renal biopsy was performed when BKV nephropathy or transplant rejection were the suspected causes of a worsening in renal function and, in the case of persistent BKV nephropathy. The BKV nephropathy group provided 70 urine samples (a mean of 8.7 per patient; range 5–14), 63 blood samples (mean 8 per patient; range 5–14), and 17 renal biopsy samples (mean 2.1 per patient; range 1–5), and the control group provided 682 urine samples (mean 3.1 per patient;

TABLE I. Main Clinicopathologic Findings in Patients With BKV Nephropathy

Case no./ sex/age (y)	Age of transplant (mo)	Renal disease leading to chronic renal failure	Case no.	Morphological diagnosis
1/M/55	60	Polycystic kidney disease	First biopsy	Acute tubular necrosis
			Second biopsy	BKV nephropathy stage A
			Third biopsy	Cellular rejection
			Fourth biopsy	Drugs toxicity
			Fifth biopsy	Cellular rejection
2/F/32	48	IgA nephropathy	First biopsy	BKV nephropathy stage B
3/M/51	60	Polycystic kidney disease	First biopsy	BKV nephropathy stage B
			Second biopsy	BKV nephropathy stage C
			Third biopsy	BKV nephropathy stage C
4/M/55	36	Unknown	First biopsy	BKV nephropathy stage B
			Second biopsy	BKV nephropathy stage B
5/F/51	48	Arterionephrosclerosis	First biopsy	BKV nephropathy stage B
6/M/41	24	Nephroangiosclerosis	First biopsy	Polycystic kidney
			Second biopsy	BKV nephropathy stage B
7/M/65	12	Glomerular disease	First biopsy	BKV nephropathy stage A
			Second biopsy	Cellular rejection
8/F/38	12	IgA nephropathy	First biopsy	BKV nephropathy stage B

y, year; mo, month; M, male; F, female; BKV nephropathy stage according to Nickeleit et al. [2000].

range 1–11), 677 blood samples (mean 3.1 per patient; range 1–11), and 101 renal biopsy samples (taken from 68 patients: mean 1.5 per patient; range 1–5).

Urine Samples

The urine samples were concentrated by centrifugation at 700*g*/min for 10 min, and 5 ml of the urine pellets were then cytocentrifuged at 250*g*/min for 10 min onto two slides, both of which were fixed in 95% ethanol, stained with Papanicolau stain, and examined cytologically in order to identify the presence of decoy cells indicating active polyomavirus replication [Koss, 1979].

Blood Samples

The blood samples (5 ml) were collected and centrifuged immediately at 700*g*/min for 10 min, after which the serum was separated and stored in a 1.5 ml tube at −20°C.

Renal Biopsies

Three fragments of renal tissue for each sample were obtained using a 18-gauge needle, fixed in Serra fluid (ethanol, formaldehyde, and acetic acid) for 4 hr, and embedded in paraffin; 4 μm thick sections were then stained with hematoxylin and eosin, periodic acid-Schiff, silver methenamine, Masson's trichrome, and phosphotungstic acid hematoxylin. All of the renal biopsies samples contained a minimum of four glomeruli and renal medullary, and were therefore adequate to establish a reliable diagnosis.

For immunohistochemical examination, ethylendiaminetetracetic acid (EDTA) at pH 8 and microwaving at 900 W (two 3-min steps followed by two 4-min steps) were used for antigen retrieval. Immunoperoxidase staining was performed using a polyclonal antibody against VP1-3 proteins of simian virus 40 (SV40) (Lee Biomolecular Research Labs, San Diego, CA, dilution 1:20,000), which cross-reacts with human BKV and JCV [Shinohara et al., 1993]. The reactions were detected by the streptavidin-biotin method, and revealed using diaminobenzidine as chromogen.

A histological diagnosis of BKV nephropathy was made when the epithelial nuclei showed typical morphological changes [Nickeleit et al., 2000] identified by light microscopy, positively stained by immunohistochemistry with anti-SV40 antibody. The progression of renal damage induced by BKV was classified as previously proposed [Nickeleit et al., 2000]: stage A, early, with the focal involvement of tubule epithelial cells positively stained by immunohistochemistry; stage B, extensive renal involvement by cytopathic viral alterations with necrosis, diffuse chronic inflammation, and initial fibrosis; and stage C, late, with interstitial fibrosis, scarring, tubular atrophy, and with few cells with cytopathic viral alterations.

Molecular Biology

DNA extraction and PCR assay. Four 4-μm thick sections were cut from the paraffin-embedded renal tissue samples after histological work-up using a microtome blade that was cleaned with xylene between each block in order to avoid sample cross-contamination [Wright and Manos, 1990], and were then placed in 1.5 ml Eppendorf tubes. DNA was extracted using EDTA–SDS/proteinase K followed by phenol–chloroform as previously described [Ferrante et al., 1995], and resuspended with 30 μl of DEPC-treated and autocleaved pyrogen and RNase-free water.

DNA was extracted from the serum samples using commercial columns (Nucleospin virus, Macherey-Nagel, Duren, Germany) with a silica matrix with high DNA-binding capacity.

Polymerase chain reaction (PCR) of the β-actin gene was performed in all cases as a positive control of DNA extraction.

The BKV LT region was amplified in 68/70 urine samples (97.1%), 36/63 blood samples (57.1%), and 13/17 renal biopsy samples (76.5%) from all eight BKV nephropathy patients, and in 110/682 urine samples (16.1%), 20/677 blood samples (2.9%), and 3/101 renal biopsy samples (3%) from 41 patients (18.8%) in the control group.

The samples that were positive for the LT region of BKV underwent amplification of the VP1 region using VP1-7 (5′-ATC AAA GAA CTG CTC CTC AAT-3′) and VP1-2R (5′-GCA CTC CCT GCA TTT CCA AGG G-3′) as outer primers, and 327-1 (5′-CAA GTG CCA AAA CTA CTA AT-3′) and 327-2 (5′-TGC ATG AAG GTT AAG CAT GC-3′) as inner primers [Jin and Gibson, 1996]. A 327 base-pair fragment containing a region responsible for some BKV antigenic variation was obtained in all cases. Amplification was performed in a total volume of 25 μl containing 15 pmol of each primer (10 pmol for the inner PCR), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U BioTaq DNA polymerase in the presence of 1× Bioline NH_4 buffer. In the first step, 5 μl of urine or renal tissue DNA or serum DNA were added to the PCR mixture and, in the second step, 2.5 μl of template; the PCR was then performed using an Eppendorf Mastercycler gradient PCR System. The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for40 sec (30 cycles for the inner PCR), annealing at 55°C for 40 sec, and extension at 72°C for 40 sec; the cycles were terminated with a final extension at 72°C for 5 min.

All of the DNA amplification products were analyzed by 2% agarose gel electrophoresis, and visualized using ethidium bromide staining.

The sensitivity of the multiplex nested PCR was estimated by amplifying serial dilutions of a BKV-positive sample (data not shown).

Direct DNA sequencing. DNA fragments from all of the VP1-positive samples were separated by electrophoresis on 3% agarose gel, and one fragment of each sample (327 base-pair) was excised, extracted, and purified using a commercial kit (PCR clean-up gel extraction, NucleoSpin, Macherey-Nagel).

A cycle sequencing PCR reaction was performed as previously described [Boldorini et al., in press]; in order to avoid Taq polymerase errors [Jin et al., 1993],

the sequence in each sample was analyzed twice. The sequences were read manually, compared with the prototype BKV Dunlop strain, and classified into the four known genotypes [Jin et al., 1993], which are distinguished on the basis of specific polymorphisms in the portion of the VP1 region spanning nucleotides 1744–1812 (shown in Table II); the presence of any single base-pair mutations of the known sequences was also recorded. When the electropherogram showed two peaks at the same height in the same nucleotide position, these were considered to be two distinct sequences.

The changes in one or more amino acid sequences encoded by the variant nucleotide(s) were predicted by the Expert Protein Analysis System program (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland—http://www.expasy.org), and the predicted amino acid variations were compared with the prototype amino acid sequence in order to identify those that might change the chemical properties of the VP1 protein in the investigated fragment.

Statistical Analysis

The data were analyzed statistically using Fisher's exact test, with P values of <0.05 being considered significant (95% confidence interval).

RESULTS

The BKV VP1 region was amplifiable in all 68 urine samples (100%), 28/36 blood samples (77.7%), and 12/13 renal biopsy samples (92.3%) from all of the BKV nephropathy patients who were positive for the LT region. One of the 13 renal biopsies with histologically proven BKV nephropathy was negative for VP1 amplification, probably because of the small quantity of renal tissue available for molecular analysis after histological and immunohistochemical processing. The VP1 region was amplifiable in 100/110 urine samples (91%), 17/20 blood samples (85%), and three renal biopsy samples (100%) from the 41 control patients.

Table III shows the distribution of BKV genotypes and subtypes in the two groups. The subtypes MM, GS, IV, and MG were distributed at random in the BKV nephropathy group (25% each), whereas SB and AS were never detected. Conversely, all of the BKV subtypes were represented in the control group, with MM, being the prevailing subtype (51.2%; $P < 0.05$) within this group and in comparison with the BKV nephropathy group, although this latter was not statistically significant (25%; $P = 0.25$).

Sequence analyses of the polymorphic VP1 gene sites showed single base-pair mutations in 75% of the BKV nephropathy patients and only 17% of the controls ($P < 0.05$); furthermore, they were only identified in the urine samples of the control group, but in samples of all types in BKV nephropathy group. Figure 1 shows the frequency of the genomic mutations in the VP1 region of

TABLE II. Nucleotide Sequences of VP1 Positions 1744–1812 Used to Assign BKV Genotypes and Subtypes [Jin et al., 1993] (A), and Related Amino Acids (B)

	1744–1746	1747–1749	1750–1752	1753–1755	1756–1758	1759–1761	1762–1764	1765–1767	1768–1770	1771–1773	1774–1776	1777–1779	1780–1782	1783–1785	1786–1788	1789–1791	1792–1794	1795–1797	1798–1800	1801–1803	1804–1806	1807–1809	1810–1812
A																							
I (DUN)	GAA	AAC	CTT	AGG	GGC	TTT	AGT	CTA	AAG	CTA	AGT	GCT	GAA	AAT	GAC	TTT	AGC	AGT	GAT	AGC	CCA	GAG	AGA
I (PT)																				AGT			
I (MM)																						GAA	
I (GS)																						GAC	
II (SB)	GAT					TAT					ACT				GCC		GAC					GAC	AAA
III (AS)	GAT					TAT		CAG	CAC						GCC		GAG					GAC	
IV (IV)	AAT	GAC				TAT			AGA		ACT			ACT	GCC		GAG					GAC	
IV (MG)	AAT	GAC				TAT			AGA		ACT			ACT	GCC		GAC					GAC	
	1744–1746,	1747–1749,	1750–1752,	1753–1755,	1756–1758,	1759–1761,	1762–1764,	1765–1767,	1768–1770,	1771–1773,	1774–1776,	1777–1779,	1780–1782,	1783–1785,	1786–1788,	1789–1791,	1792–1794,	1795–1797,	1798–1800,	1801–1803,	1804–1806,	1807–1809,	1810–1812,
B																							
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
I (DUN-PT-MM)	Glu	Asn	Leu	Arg	Gly	Phe	Ser	Leu	Lys	Leu	Ser	Ala	Glu	Asn	Asp	Phe	Ser	Ser	Asp	Ser	Pro	Glu	Arg
I (GS)																						Asp	
II (SB)	Asp					Tyr					Thr				Ala		Asp					Asp	Lys
III (AS)	Asp					Tyr		Gln	His						Ala		Glu					Asp	
IV (IV)	Asn	Asp				Tyr			Arg		Thr			Thr	Ala		Glu					Asp	
IV (MG)	Asn	Asp				Tyr			Arg		Thr			Thr	Ala		Asp					Asp	

TABLE III. BKV Genotype and Subtype Distributions in Patients From BKV Nephropathy and Control Group

Genotype[a]	Subtype[a]	No. of patients: BKV nephropathy group	No. of patients: Control group
I	MM	2/8 (25%)	21/41 (51.2%)
	GS	2/8 (25%)	4/41 (9.8%)
II	SB	0/8	4/41 (9.8%)
III	AS	0/8	1/41 (2.4%)
IV	IV	2/8 (25%)	4/41 (9.8%)
	MG	2/8 (25%)	7/41 (17%)
	Total number	8	41

[a]BKV genotypes and subtypes according to Jin et al. [1993] and Randhawa et al. [2002].

all of the amplified fragments obtained from the samples of both the BKV nephropathy and control groups. As can be seen, genomic rearrangements of blocks of sequences in the VP1 region were never detected in the amplified fragments, but single base-pair substitutions were frequent in the urine samples of the BKV nephropathy patients (52% vs. 16.4%; $P < 0.05$), and were also detected in 21.2% of the blood and 29.4% of the renal biopsy samples.

Table IV shows the distribution and type of amino acid changes generated by the single base-pair mutations in the amplified fragments. The changes were distributed at random in both groups as only two patients in each group showed the same change: aspartic acid to asparagine at residue 62 (#2 and #4) and glutamic acid to alanine at residue 73 (#6 and #8) in the BKV nephropathy group, and arginine to lysine at residue 69 (#6 and #7) and glutamic acid to glutamine at residue 73 (#6 and #7) in the controls. It is worth noting that patient #3, who developed renal fibrosis due to BKV infection (BKV nephropathy stage C), showed an aspartic acid to asparagine substitution at residue 75 in all of the amplified fragments that was not identified in any of the samples from either group. Finally, it is also worth noting that the amino acid changes were stable in all of the patients from both groups.

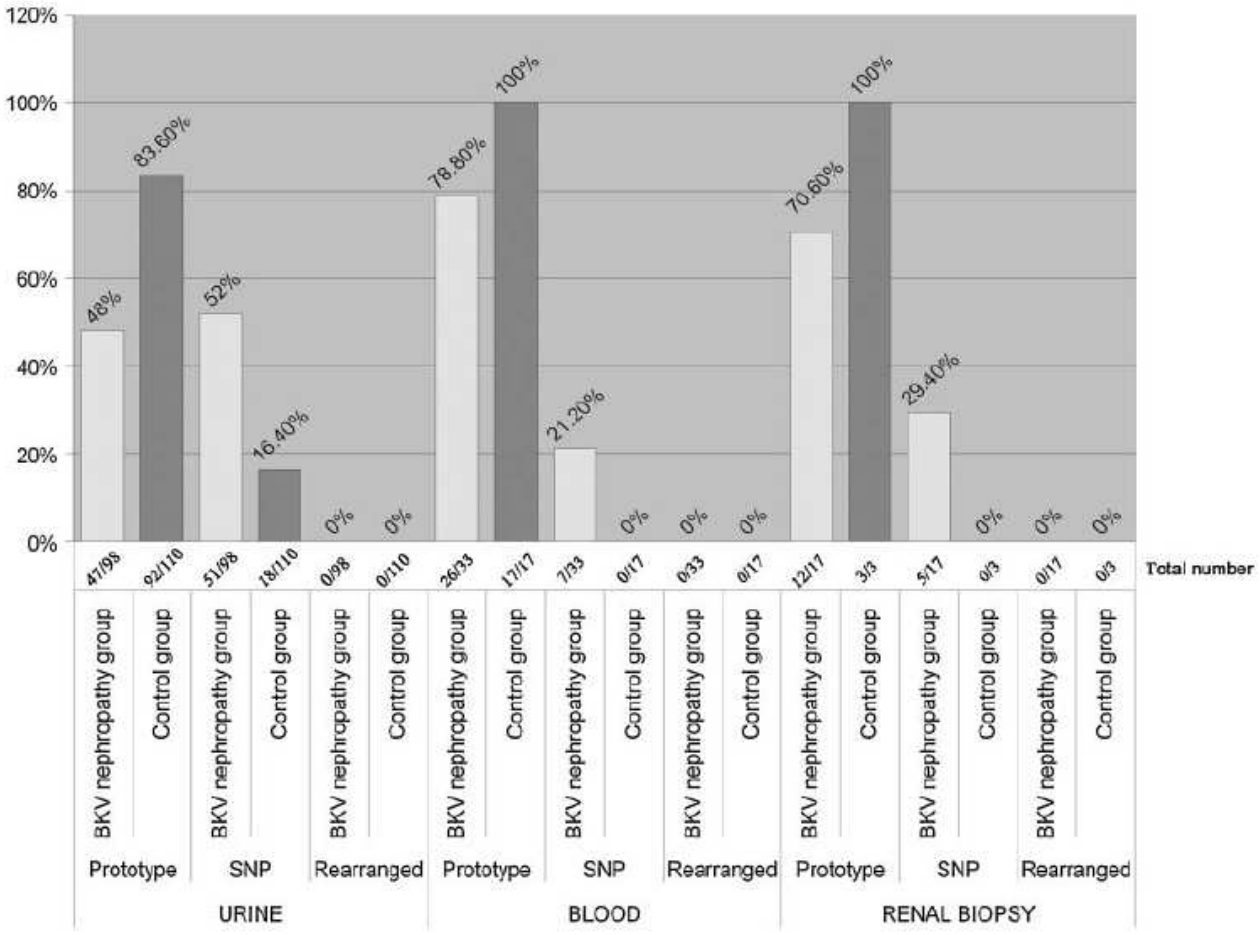

Fig. 1. Diagrammatic representation of BKV genomic mutations in Viral Protein 1 region detected in urine, blood, and renal biopsy samples from BK Virus nephropathy and control group. First line SNP, single nucleotide polymorphism.

TABLE IV. Viral Protein 1 Amino Acid Changes in Each Amplified Fragment From Patients of the BK Nephropathy Group (A) and Controls (B)

A

		Amino acid positions														
Patient	Sample	Asn → Lys 62	Asp → Asn 62	Leu → Pro 63	Lys → Arg 69	Glu → Thr 73	Glu → Ala 73	Glu → Gln 73	Glu → Lys 73	Thr → Ser 74	Asp → Asn 75	Glu → Gln 77	Ser → Arg 77	Ser → Lys 77	Asp → His 77	Asp → His 82
BKV nephropathy group																
#2	Urine		7					7				11				
	Blood		1					1				1				
	Renal biopsy							1				1				
#3	Urine					3					17					
	Blood										3					
	Renal biopsy										3					
#4	Urine		7	1					3	1					1	
#5	Urine	6											9	1		4
	Blood	1											1			1
#6	Urine				4		3									
	Blood				1		1									
	Renal biopsy				1											
#8	Blood						1									

B

		Amino acid positions																
Patient	Sample	Asn → Lys 61	Asp → Asn 61	Asp → Asn 62	Leu → Val 68	Arg → Lys 69	Glu → Lys 73	Glu → Gln 73	Asn → Lys 74	Δ Ala 75	Phe → Leu 76	Asp → Glu 77	Asp → Asn 77	Ser → Ile 78	Ser → Arg 80	Asp → Glu 82	Glu → Gln 82	Asp → Asn 82
Controls																		
#1	Urine		1									1				2		
#2	Urine										1							
#3	Urine						3											
#4	Urine				1									2	1		1	
#5	Urine								1									
#6	Urine					3		3					4					1
#7	Urine	2		3		3		3		3								

, urine; , blood; , renal biopsy; Δ, deletion.

Table V shows the BKV genotypes and nucleotide changes in sequential samples taken before and after the diagnosis of BKV nephropathy from each patient. The BKV genotypes, subtypes, and single base-pair mutations were stable in all cases, regardless of the sample type or time of sample collection.

The BKV genotypes and subtypes in the control group were also stable in the urine samples taken at different times (data not shown).

DISCUSSION

The VP1 region of BKV encodes the major viral capsid protein, which plays a structural role in viral architecture and bears important antigen epitopes that are crucial for viral attachment to target cells [Jin and Gibson, 1996].

Nucleotide polymorphisms in the VP1 genomic sequence 1744–1812 are used to identify BKV genotypes

TABLE V. BKV Genotypes and Nucleotide Changes in Sequential Samples Before and After the Diagnosis of Nephropathy in BKV Nephropathy Patients

			Number of samples and nucleotide position of SNPs		
Case no.	Genotype	BKV nephropathy stage	Urine	Blood	Renal biopsy
#1	Genotype I (MM)	Before BKV nephropathy	1 prototype	N/A	1 negative
		BKV nephropathy	1 prototype	1 negative	1 prototype
		After BKV nephropathy	2 negative 4 prototype	6 negative	2 negative
#2	Genotype IV (IV)	Before BKV nephropathy	1 SNP 1792	1 prototype	
		BKV nephropathy	1 prototype 1 SNP 1747; 1780; 1792	1 prototype 1 SNP 1747; 1780; 1792	1 prototype 1 SNP 1780; 1792
		After BKV nephropathy	7 prototype 3 SNP 1792 6 SNP 1747; 1780; 1792	2 prototype 1 negative	N/A
#3	Genotype I (GS)	Before BKV nephropathy	1 SNP 1786	N/A	N/A
		BKV nephropathy	1 SNP 1786	1 SNP 1786	1 prototype 1 SNP 1786
		After BKV nephropathy	1 prototype 12 SNP 1786 3 SNP 1780; 1781; 1786	2 prototype 2 SNP 1786 7 negative	2 prototype 2 SNP 1786
#4	Genotype IV (MG)	Before BKV nephropathy	1 prototype 1 SNP 1747; 1751	1 prototype	N/A
		BKV nephropathy	1 prototype 1 SNP 1747; 1780	1 prototype	1 prototype
		After BKV nephropathy	6 prototype 1 SNP 1747; 1780; 1792 1 SNP 1747 2 SNP 1747; 1780 1 SNP 1747; 1784	4 prototype 2 negative	1 prototype
#5	Genotype I (GS)	Before BKV nephropathy	2 prototype	2 prototype	N/A
		BKV nephropathy	1 prototype 1 SNP 1749; 1794; 1807	N/A	1 prototype
		After BKV nephropathy	4 prototype 3 SNP 1749; 1794; 1807 2 SNP 1749 5 SNP 1794 1 SNP 1793; 1794	2 prototype 1 SNP 1794; 1807 6 negative	N/A
#6	Genotype I (MM)	Before BKV nephropathy	2 prototype 1 SNP 1769	2 prototype 1 SNP 1769; 1781	1 prototype 1 negative
		BKV nephropathy	1 prototype 1 SNP 1769; 1781	1 negative	1 prototype 1 SNP 1769
		After BKV nephropathy	4 prototype 2 SNP 1769; 1781	2 prototype 1 negative	N/A
#7	Genotype IV (IV)	Before BKV nephropathy	1 prototype	1 prototype	N/A
		BKV nephropathy	1 prototype	N/A	1 prototype
		After BKV nephropathy	3 prototype	2 negative	N/A
#8	Genotype IV (MG)	Before BKV nephropathy	2 prototype	2 prototype 1 SNP 1781	N/A
		BKV nephropathy	1 prototype	1 prototype	1 prototype
		After BKV nephropathy	2 prototype	2 prototype	N/A

SNP, BKV single nucleotide polymorphism according to Jin et al. [1993].
N/A, not available; negative, BKV genome not found.

(I–IV) and subtypes (DUN, PT, MM, GS, SB, AS, IV, and MG) [Jin et al., 1993]. Genotype I is the most frequent in the human population, followed by genotypes II, IV, and III. There are significant geographical differences, as well as differences between bone marrow and kidney transplant patients, although not enough evidence to relate the different BKV genotypes and/or subtypes to reactivation, particular diseases or particular groups of immunosuppressed patients [Jin et al., 1993].

In this study the frequency of BKV genotypes and subtypes in eight patients with BKV nephropathy was examined and compared with those observed in 41 kidney transplant patients without BKV nephropathy. A different distribution was found between the two groups of patients, with the absence of subtypes SB and AS (genotypes II and III) in the BKV nephropathy group and the prevalence of subtype MM, (genotype I) in the controls. However, as both genotypes and subtypes were stable in sequential samples from the same patient, and there were only a few BKV nephropathy patients, these differences may simply reflect individual or geographical variability in the BKVs circulating in the population as reported in epidemiological studies [Knowels, 2001].

VP1 gene sequence analyses showed that single base-pair mutations were significantly more frequent in the patients with BKV nephropathy than in the controls and this was the only statistically significant finding of the study. Furthermore, they were identified in amplified fragments from urine, blood, and renal biopsy samples taken from the BKV nephropathy patients, but only in the urine samples of the controls. The biological and clinical significance of this genetic variability is unknown but, in line with a previous study [Randhawa et al., 2002], it can be explained tentatively by hypothesizing that more genomically unstable viral strains may have a particular tendency to cause clinical disease. Alternatively, it is possible that the heterogeneity of BKV strains circulating in the human population is greater than previously thought.

The amino acid changes generated by base-pair mutations were distributed at random in the two groups; interestingly, a mutation converting aspartic acid to asparagine at residue 75 was detected in all of the samples taken at different times from one patient with BKV nephropathy, but never in any of the other samples from either group. Three sequential renal biopsies of this patient showed disease progression leading to renal fibrosis, thus suggesting that this specific amino acidic change may have played a role in the worsening of the disease. As there are no other published studies comparing BKV VP1 sequences in urine, blood, and renal biopsy samples taken at different time from kidney transplant patients with and without BKV nephropathy, the results of the present study are not readily comparable with those of other authors. One study of the significance of VP1 mutations in JC polyomavirus in patients with progressive multifocal leukoencephalopathy found that mutations were more frequent in those who survived longer, thus suggesting that they may be associated with disease progression [Zheng et al., 2005]. Previous studies [Dubensky et al., 1991; Freund et al., 1991] have found that amino acid changes occurring in specific positions of the VP1 gene of mouse polyomaviruses generated from PTA, RA, and A2 strains lead to in vitro plaque size and viral hemoagglutination behaviors that affect their ability to replicate and spread in mice. However, these results were obtained in experimental animals using a polyomavirus species that is different from BKV and may not be comparable with the results obtained in the present study.

Finally, a study that sequenced the DNA of the VP1 region of BKVs isolated from 49 sequential renal biopsies taken from 24 BKV nephropathy patients at different times during the course of disease (no data concerning urine or blood samples were reported) found frequent base-pair mutations and amino acid changes [Randhawa et al., 2002]; furthermore, unlike the results of the present study in which the genomic structure of the isolated BKVs were stable in sequential samples, examination of the data at different times indicated that the genetic structure of the virus may have undergone continuous evolution related to the progression of the disease. The authors hypothesized that VP1 nucleotide and amino acid variation may have implications for escaping host immunity and the development of antiviral drug resistance but, as their study was retrospective, and the interval between successive samples was up to 6 years, it is possible that the variability in genomic structure was related to the long intervals between the renal biopsies.

In conclusion, clinical sample data collected during this study seem to indicate greater genomic instability in the structure of the BKV VP1 region in patients who developed renal disease than in the controls but, as the single base-pair mutations and amino acid changes were distributed at random, their clinical significance is unknown. Finally, the mutation converting aspartic acid to asparagine at residue 75, which was detected in all of samples of the only BKV nephropathy patient in whom the disease progressed is of potential interest, but further studies of a larger number of BKV nephropathy patients are needed to understand the real significance of this change.

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REFERENCES

Boldorini R, Allegrini S, Miglio U, Paganotti A, Veggiani C. 2009. Detection, distribution and pathological significance of BK virus strains isolated from kidney transplant patients with and without polyomavirus-associated nephropathy. Arch Pathol Lab Med 133: 766–774.

Bolen JB, Anders DG, Trempy J, Consigli RA. 1981. Differences in the subpopulations of the structural proteins of polyoma virions and capsids: Biological functions of the multiple VP1 species. J Virol 37:80–91.

Dubensky TW, Freund R, Dawe CJ, Benjamin TL. 1991. Polyomavirus replication in mice: Influences of VP1 type and route of inoculation. J Virol 65:342–349.

Ferrante P, Caldarelli-Stefano R, Omodeo-Zorini E, Vago L, Boldorini R, Costanzi G. 1995. PCR detection of JC virus DNA in brain tissue

from patients with and without progressive multifocal leukoencephalopathy. J Med Virol 47:219–225.

Freund R, Garcea RL, Sahli R, Benjamin TL. 1991. A single-amino-acid substitution in polyoma virus VP1 correlates with plaque size and hemagglutination behaviour. J Virol 65:350–355.

Hirsch HH, Steiger J. 2003. Polyomavirus BK. Lancet Infect Dis 3:611–623.

Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Steiger J. 2002. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipient. N Engl J Med 347:488–496.

Hirsch HH, Brennan DC, Drachenberg CB, Ginevri F, Gordon J, Limaye AP, Mihatsch MJ, Nickeleit V, Ramos E, Randhawa P, Shapiro R, Steiger J, Suthanthiran M, Trofe J. 2005. Polyomavirus-associated nephropathy in renal transplantation: Interdisciplinary analyses and recommendations. Transplantation 79:1277–1286.

Jin L, Gibson PE. 1996. Genomic function and variation of human polyomavirus BK (BKV). Rev Med Virol 6:201–214.

Jin L, Gibson PE, Booth JC, Clewley JP. 1993. Genomic typing of BK virus in clinical specimens by direct sequencing of polymerase chain reaction products. J Med Virol 41:11–17.

Knowels WA. 2001. The epidemiology of BK virus and the occurrence of antigenic and genomic subtypes. In: Khalili K, Stoner GL, editors. Human polyomaviruses—Molecular and clinical perspectives. New York: Wiley-Liss, Inc. pp 527–559.

Koss L. 1979. The urinary tract in absence of cancer. In: Koss L, editor. Diagnostic cytology and its histopathologic bases. 3rd edition. Philadelphia: Lippincott JB, Inc. pp 711–748.

Nickeleit V, Hirsch HH, Zeiler M, Gudat F, Prince O, Thiel G, Mihatsch MJ. 2000. BK-virus nephropathy in renal transplants-tubular necrosis, MHC-class II expression and rejection in a puzzling game. Nephrol Dial Transplant 15:324–332.

Randhawa P, Khaleel-Ur-Rehman K, Swalsky PA, Vats A, Scantlebury V, Shapiro R, Finkelstein S. 2002. DNA sequencing of viral capsid protein VP-1 region in patients with BK virus interstitial nephritis. Transplantation 73:1090–1094.

Shinohara T, Matsuda M, Cheng SH, Marshall J, Fujita M, Nagashima K. 1993. BK virus infection of the human urinary tract. J Med Virol 41:301–305.

Sundsfjord A, Johansen T, Flaegstad T, Moens U, Villand P, Subramani S, Traavik T. 1990. At least two types of control regions can be found among naturally occurring BK virus strains. J Virol 64:3864–3871.

Vasudev B, Hariharan S, Hussain SA, Zhu YR, Bresnahan BA, Cohen EP. 2005. BK virus nephritis: Risk factors, timing, and outcome in renal transplant recipients. Kidney Int 68:1834–1839.

Wright DK, Manos MM. 1990. Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Svinskyy JJ, White TJ, editors. PCR protocols. San Diego: Academic Press, Inc. pp 153–158.

Zheng HY, Ikegaya H, Takasaka T, Matsushima-Ohno T, Sakurai M, Kanazawa I, Kishida S, Nagashima K, Kitamura T, Yogo Y. 2005. Characterization of the VP1 loop mutations widespread among JC Polyomavirus isolates associated with progressive multifocal leukoencephalopathy. Biochem Biophys Res Commun 333:996–1002.

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TEACHING CASES

Primary vulvar Ewing's sarcoma/primitive neuroectodermal tumor in a post-menopausal woman: A case report

Renzo Boldorini [a], Francesca Riboni [b,*], Silvia Cristina [c], Sara Allegrini [a], Stefano Valentini [c], Maurizio Muscarà [c], Giovanni Ruspa [d]

[a] Department of Pathology, Maggiore Hospital, University School of Medicine "Amedeo Avogadro, Novara, Italy
[b] Department of Obstetrics and Gynecology, Maggiore Hospital, University School of Medicine "Amedeo Avogadro", via solaroli 17, 28100 Novara, Italy
[c] Unit of Pathology, SS. Trinità Hospital, Borgomanero, Italy
[d] Department of Obstetrics and Gynecology, SS. Trinità Hospital, Borgomanero, Italy

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ABSTRACT

Ewing's sarcomas/peripheral primitive neuroectodermal tumors (ES/pPNETs) are high-grade malignant neoplasms rarely found outside the skeletal system. Only 12 cases of vulvar ES/pPNET have so far been reported, all involving children or women of child-bearing age. We describe the case of a 52-year-old woman who was admitted to our hospital for the local excision of a 4 cm vulvar mass, originally thought to be a Bartholin's gland cyst. It was subsequently found to consist of small round cells positive for anti-CD99 antibody, thus suggesting a diagnosis of ES/pPNET. The demonstration of EWSR1 gene translocations by means of fluorescent *in situ* hybridization excluded small-cell carcinoma, squamous cell carcinoma of the small type, Merkel cell carcinoma, and lymphoblastic lymphoma. After surgery, the patient received six cycles of polychemotherapy and radiotherapy; she is still alive and well after 1 year of follow-up. Our findings underline the crucial role of molecular biology techniques in the differential diagnosis of small round cell tumors in these unusual locations.

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Introduction

Ewing sarcomas/peripheral primitive neuroectodermal tumors (ES/pPNETs) are high-grade malignant neoplasms that typically arise within the skeletal system [1,2], although extra-skeletal sites [3,4] such as the chest wall, lower extremities, and the paravertebral region have been observed in about 15% of the cases [4]. Female genital tract involvement is rare, but ovarian, uterine cervix and corpus, and vaginal localizations have been reported in individual cases or small patient series [4–19].

A primary ES/pPNET of the vulva was first described by Scherr et al. [9], and only about 12 cases have been reported so far, all involving children or adult women of child-bearing age (Table 1). We describe and discuss the differential diagnosis of the first case of an ES/pPNET primarily arising in the vulva of a post-menopausal woman, whose diagnosis was confirmed by fluorescence *in situ* hybridization (FISH). As ES/pPNETs are histologically indistinguishable from other small cell tumors potentially affecting the vulva, the crucial role of diagnostic techniques, such as FISH and molecular biology, is underlined.

A 52-year-old woman, para 0, was admitted to the hospital of Borgomanero, Italy, with a vulvar mass. Her past medical history was unremarkable. A physical examination revealed a palpable, painless, and fixed nodule (about 4 cm in diameter) located in the lower third of the right labium major, and extending into the vestibule. Pelvic examination of the uterus and adnexae was negative. Given the suspicion of a Bartholin gland cyst, the patient underwent local excision of the mass with negative macroscopic margins. Her post-operative course was regular: computed tomography of the abdomen and pelvis, chest radiography, and a bone scan showed no evidence of metastases, and the results of all routine clinical laboratory tests remained within the normal range.

After surgery, the patient received six cycles of first-line polychemotherapy with vincristine, adriamycin, and ifosfamide, alternated with ifosfamide and etoposide, and then a total of 44 Gy radiotherapy to the pelvis and vulva, with a 16 Gy boost to the vulva. Six months later, a physical examination detected nothing of note, and chest radiograph and pelvic and abdominal contrast computed tomography showed the absence of any mass or adenopathy.

Twelve months after diagnosis, she is alive and well without any clinical signs of a local recurrence or metastasis.

Materials and methods

The tumor specimen was sampled, fixed in 10% neutral formalin, and embedded in paraffin, after which 4-μm thick

* Corresponding author. Tel.: +39 0321 3733780.
E-mail address: frriboni@tin.it (F. Riboni).

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Table 1
Clinical and pathological features of the primary EWS/pPNETs of the vulva reported in the literature.

Authors (Ref.)	Age (years)	Site	Size (cm)	Therapy	Molecular confirmation	Follow-up (months)
Scherr et al. [9]	10	Left labium major	6.5	Surgical excision	None	NA
Vang et al. [12]	28	Right labium minor	0.9	Surgical excision+CT	RT-PCR	AW, 18
McCluggage et al. [17]	40	Right labium minor	3	Surgical excision+CT	FISH	AW, 12
McCluggage et al. [17]	20	Right labium	6.5	NA	FISH	Died, pulmonary metastasis
McCluggage et al. [17]	19	Vulva, NOS	4	Surgical excision+CT	RT-PCR+FISH	NA
Takeshima et al. [13]	45	Right labium minor	3	Surgical excision	None	AW 36
Moodley and Jordaan [16]	26	Right labium major	5	Surgical excision+RT/CT	None	NA
Nirenberg et al. [10]	20	Right labium major	12	Surgical excision+ RT+CT	None	Died 18
Lazure et al. [14]	35	Vulva, NOS	20	Surgical excision+CT	RT-PCR	AW 7
Paredes et al. [11]	29	Left vulva	5	Surgical excision+RT/CT	None	AW 8
Habib et al. [8]	23	Vulva, NOS	Not reported	Surgical excision	None	NA
Fong et al. [18]	17	Vulva, left	Two fragments 0.7 and 2.1	Surgical excision+CT	RT-PCR	AW 12
Boldorini et al. (this study)	52	Right labium major	4	Surgical excision+CT/RT	FISH	AW 12

CT: chemotherapy; NA: not available; AW: alive and well; RT-PCR: reverse-transcription polymerase chain reaction; FISH: fluorescent *in situ* hybridization; RT: radiotherapy.

sections were stained in hematoxylin–eosin and periodic-acid Schiff (PAS) with and without diastase digestion.

Immunohistochemistry was performed using an automated staining machine (LabVision Corporation, Bio-Optica); the slides were incubated for 1 h with the following primary antibodies: CD99 (clone HO36-1.1, dilution 1:50, Cell Marque), vimentin (clone AB2, dilution 1:600, Neomarkers), CK20 (clone Ks20.8, dilution 1:200, Biocare), CK7 (clone OB-TL12/30, dilution 1:150, Biogenex), cytokeratin (clone AE1/AE3, dilution 1:200, Neomarkers), epithelial membrane antigen (Clone AB3, dilution 1:200, Dako), CD45 (clone LCA88, dilution 1:80, Biogenex), desmin (clone D33, dilution 1:50, Neomarkers), synaptophysin (clone SNP 88, dilution 1:80, Biogenex), and chromogranin (clone LK2H10+PHE5, dilution 1:150, Biocare). The subsequent reactions were performed using the Novolink Polymer Detecting System kit (Novocastra, Menarini) and incubation with universal secondary antibody, and the signal was visualized using 3,3′-diaminobenzidine hydrochloride.

FISH analysis was performed using the commercial Vysis® LSI® EWSR1 (22q12) Dual Color Break Apart Rearrangement Probe (Abbott Molecular), which consists of two FISH DNA probes: the first is labeled in SpectrumOrange, flanks the 5′ side of the EWSR1 gene, and extends inward into intron 4; the second is labeled in SpectrumGreen and flanks the 3′ side of the EWSR1 gene. The most common chromosomal translocation, t(11;22) (q24;q12), and variants t(21;22) (q22;q12), t(7;22) (p22;q12), and t(2;22) (q33;q12) are all identified by the same signal pattern, which consists of one signal fusion and two separate green and orange signals within the nuclei of neoplastic cells. The cells were detected using a fluorescent microscope (Axioplan 2 Imaging, Zeiss). About 80 neoplastic cells were selected, and the images were captured by means of dedicated software (Axiovision 4, Zeiss).

Results

Macroscopic examination showed a non-encapsulated brownish mass with a smooth surface and a maximum diameter of 4 cm; it was fleshy on the cut surface, and free of necrosis.

Microscopically, the tumor seemed to be located just beneath the squamous epithelium of the vulva, and consisted of relatively small round cells with ill-defined borders, scanty cytoplasm, and hyperchromatic nuclei with small nucleoli (Fig. 1). The mitotic count was 6 × 10 high power fields, and necrosis was frequent. No Homer–Wright rosettes were identified.

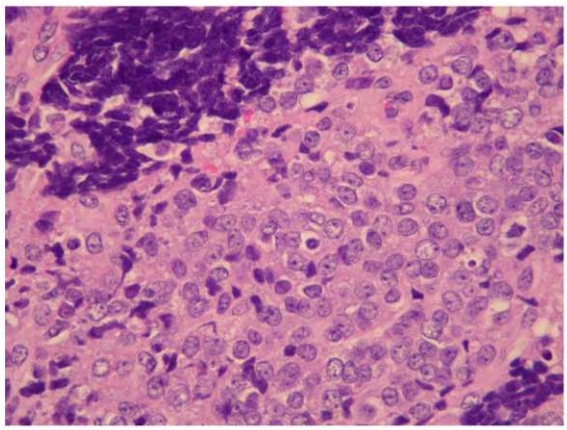

Fig. 1. The micrograph shows the classical characteristics of a small round cell tumor: perfectly round nuclei, with small and inconspicous nucleoli and scanty cytoplasm. Hematoxylin–eosin; original magnification 250 ×.

PAS staining showed cytoplasmic granules in a small percentage of the neoplastic cells, which were diffusely immunoreactive for CD99 (Fig. 2) and cytokeratin AE1/AE3, and focally for vimentin, epithelial membrane antigen, and neurofilaments; CK20, CK7, desmin, synaptophysin, and chromogranin were negative.

FISH analysis identified the typical pattern of chromosomal translocation involving the EWSR1 gene in about 70% of neoplastic cells (Fig. 3).

A diagnosis of primary vulvar ES/pPNET was made on the basis of the microscopic, immunohistochemical, and FISH data.

Discussion

Two main categories of PNETs are currently recognized on the basis of their cell origin and location: central PNETs arise from the neural tube and involve the brain and spinal cord, whereas peripheral PNETs arise from the neural crest and involve soft tissue and bone. ES, pPNETs, and small-cell tumors of the thoracopulmonary region (Askin tumors) are all members of the Ewing family of tumors (EFT), and characterized by similar phenotypical and molecular features: round small-cell morphology, immunohistochemical positivity to anti-CD99 antibody, and

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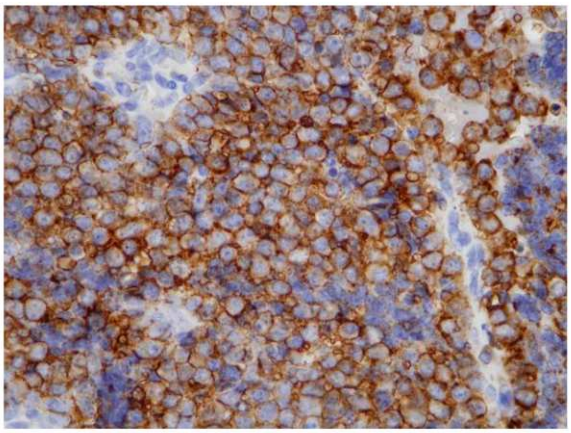

Fig. 2. The neoplastic cells showed strong and diffuse anti-CD99 antibody immunoreactivity along the cytoplasmic membranes. Anti-CD 99 antibody, slight hematoxylin counterstain; original magnification 250 × .

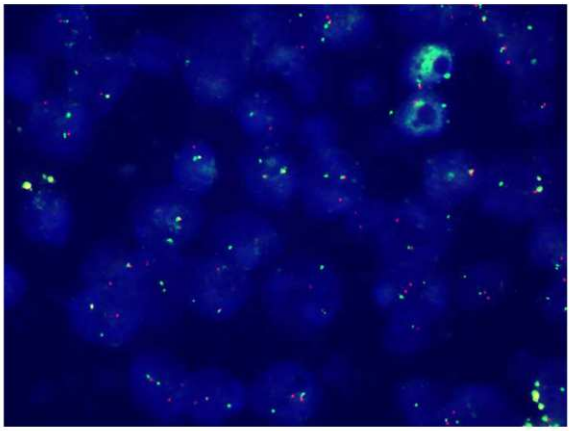

Fig. 3. Fluorescent *in situ* hybridization showing the typical pattern of a chromosomal translocation involving the EWSR1 gene: one signal fusion and two separate green and orange signals can be seen in most of the nuclei of the neoplastic cells. Dual Color Break Apart Rearrangement Probe, DAPI stain; original magnification 1000 × .

the expression of EWS/ETS gene fusion, which has been identified in virtually all cases.

Extra-skeletal ES/pPNETs have been reported in various sites, the most frequent being the chest wall, lower extremities, and the paravertebral region. However, they have only rarely been found in the female genital tract.

As shown in Table 1, the mean age at the time of the initial diagnosis of previous cases is 26 years (median 23, range 10–45), but our patient was older and post-menopausal. Therefore, excluding small round blue-cell tumors of children, such as rhabdomyosarcoma and neuroblastoma, the main differential diagnoses that were taken in account were primary or metastatic small-cell carcinoma, poorly differentiated squamous cell carcinoma of the small type, non-Hodgkin lymphoblastic lymphoma, malignant melanoma, and Merkel cell carcinoma, all of which share a similar morphology of round neoplastic cells with a high mitotic index. In addition, as a number of them (small-cell carcinomas and lymphomas) may stain positively for anti-CD99 antibody, a diagnosis of extra-skeletal ES/pPNETs has to be supported by the RT-PCR or FISH demonstration of the presence of the EWSR1 fusion transcript and/or gene translocations. These techniques can be regarded as "gold standards", which raises the question as to whether such tumors can be diagnosed only on the basis of their classic morphology and CD99 positivity.

Like McCluggage et al. [17], we believe that molecular confirmation is necessary when the tumor has unusual morphological features, is located in an unusual site, or (as in our case) develops in a patient falling outside the usual age group, but it is worth noting that only six of the published cases of vulvar ES/pPNET were molecularly confirmed (Table 1).

As only a few cases have been described, it is difficult to predict whether ES/pPNETs of the vulva behave differently from those arising in skeletal or more frequent extra-skeletal sites, and establish the best way of treating them. Table 1 shows that they have so far been treated by means of surgical excision alone (three cases), surgery associated with chemotherapy (five cases), or chemo- and radiotherapy (three cases); the treatment of one case was not described. Follow-up information has been provided in eight cases: two patients died because of the disease; the other six were alive and free of disease 7–36 months after diagnosis.

Our patient underwent surgery, and then received six cycles of first-line polychemotherapy followed by radiotherapy; 12 months after diagnosis, she is alive and well, and shows no signs of a local recurrence or metastasis. These albeit limited data suggest a possibly more favorable prognosis than that reported for more common ES/pPNETs, probably because of the superficial location, early detection, and complete removal of the neoplasm.

In conclusion, our case of an ES/pPNET primarily arising in the vulva of a post-menopausal woman was successfully treated by means of local excision, chemotherapy, and radiotherapy. FISH-based differential diagnosis excluded more frequent age-related tumors of the vulva, such as primary or metastatic small-cell carcinomas, poorly differentiated squamous cell carcinomas of the small type, and Merkel cell carcinomas. We suggest that molecular biology techniques are useful in the differential diagnosis of small round-cell tumors, especially unusual locations.

References

[1] M. Teft, G.F. Vawter, A. Mitus, Paravertebral "round cell" tumors in children, Radiology 92 (1969) 1501–1509.
[2] P. Gaona-Luviano, E. Unda-Franco, L. Gonzalez-Jara, P. Romero, H. Medina-Franco, Primitive neuroectodermal tumor of the vagina, Gynecol. Oncol. 91 (2003) 456–458.
[3] H. Grier, The Ewing family of tumors, Ewing sarcoma and primitive neuroectodermal tumors, Pediatr. Clin. North Am. 44 (1997) 991–1003.
[4] G. Cenacchi, G. Pasquinelli, L. Montanaro, et al., Primary endocervical extraosseous Ewing sarcoma/PNET, Int. J. Gynecol. Pathol. 17 (1998) 63–68.
[5] D. Daya, H. Lukka, P. Clement, Primitive neuroectodermal tumors of the uterus: a report of four cases, Hum. Pathol. 23 (1992) 1120–1129.
[6] J.Y. Park, S. Lee, H. Jin Kang, H.S. Kim, S.Y. Park, Case report. Primary Ewing's sarcoma-primitive neuroectodermal tumor of the uterus: a case report and literature review, Gynecol. Oncol. 106 (2007) 427–432.
[7] J. Farley, J.D. O'Boyle, J. Heaton, S. Remmenga, Extraosseous Ewing sarcoma of the vagina, Obstet. Gynecol. 96 (2009) 832–834.
[8] K. Habib, J.F. Finet, F. Plantier, et al., A rare lesion of the vulva, Arch. Anat. Cytol. Pathol. 40 (1992) 158–159.
[9] G.R. Scherr, G. d'Ablaing, J.G. Ouzounian, Peripheral primitive neuroectodermal tumor of the vulva, Gynecol. Oncol. 54 (1994) 254–258.
[10] A. Nirenberg, A.G. Ostör, J. Slavin, C.B. Riley, R.M. Rome, Primary vulvar sarcomas, Int. J. Gynecol. Pathol. 14 (1995) 55–62.
[11] E. Paredes, A. Duarte, A. Couceiro, et al., A peripheral neuroectodermal tumor of the vulva, Acta Med. Port. 8 (1995) 161–163.
[12] R. Vang, J.K. Taubenberger, C.M. Mannion, K. Bijwaard, A. Malpica, N.G. Ordonez, F.A. Tavassoli, S.A. Silver, Primary vulvar and vaginal extraosseous Ewing's sarcoma/peripheral neuroectodermal tumor: diagnostic confirmation with CD99 immunostaining and reverse transcriptase–polymerase chain reaction, Int. J. Gynecol. Pathol. 19 (2000) 103–109.
[13] N. Takeshima, T. Tabata, H. Nishida, et al., Peripheral primitive neuroectodermal tumor of the vulva: report of a case with imprint cytology, Acta Cytol. 45 (2001) 1049–1052.
[14] T. Lazure, I.A. Alsamad, S. Meuric, D. Orbach, M. Fabre, Primary uterine and vulvar Ewing's sarcoma/peripheral neuroectodermal tumors in children: two unusual locations, Ann. Pathol. 21 (2001) 263–266.

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[15] X. Liao, X. Xin, X. Lu, Case report. Primary Ewing's sarcoma-primitive neuroectodermal tumor of the vagina, Gynecol. Oncol. 92 (2004) 684–688.
[16] M. Moodley, A. Jordaan, Ewing's sarcoma of the vulva – a case report, Int. J. Gynecol. Cancer 15 (2005) 1177–1178.
[17] W.G. McCluggage, V.P. Sumathi, M.R. Nucci, M. Hirsch, P. Dal Cin, M. Wells, M. Adrienne, A.M. Flanagan, C. Cyril Fisher, Ewing family of tumours involving the vulva and vagina: report of a series of four cases, J. Clin. Pathol. 60 (2007) 674–680.
[18] Y.E. Fong, D. Dolores López-Terrada, Q. Zhai, Case study. Primary Ewing sarcoma/peripheral primitive neuroectodermal tumor of the vulva, Hum. Pathol. 39 (2008) 1535–1539.
[19] S. Mittal, G. Sumana, M. Gupta, B. Gupta, Primitive neuroectodermal tumor of the uterus: a case report, Int. J. Gynecol. Cancer 17 (2007) 524–527.

Please cite this article as: R. Boldorini, et al., Primary vulvar Ewing's sarcoma/primitive neuroectodermal tumor in a post-menopausal woman: A case..., Pathol. Res. Practice (2009), doi:10.1016/j.prp.2009.07.006