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Human endogenous retrovirus transcription profiles of the kidney and kidney-derived cell lines

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The human genome comprises approximately 8–9 % of human endogenous retroviruses (HERVs) that are transcribed with tissue specificity. However, relatively few organs have been examined in detail for individual differences in HERV transcription pattern, nor have tissue-to-cell culture comparisons been frequently performed. Using an HERV-specific DNA microarray, a core HERV transcription profile was established for the human kidney comparing 10 tissue samples. This core represents HERV groups expressed uniformly or nearly so in non-tumour kidney tissue. The profiles obtained from non-tumour tissues were compared to 10 renal tumour tissues (renal cell carcinoma, RCC) derived from the same individuals and additionally, to 22 RCC cell lines. No RCC cell line or tumour-specific differences were observed, suggesting that HERV transcription is not altered in RCC. However, when comparing tissue transcription to cell line transcription, there were consistent differences. The differences were irrespective of cancer state and included cell lines derived from non-tumour kidney tissue, suggesting that a specific alteration of HERV transcription occurs when establishing cell lines. In contrast to previous publications, all known HERV-derived tumour antigens, including those identified in RCC, were expressed both in multiple RCC cell lines and several non-tumour tissue-derived cell lines, a result that contrasts with findings from patient samples. The results establish the core kidney transcription pattern of HERVs and reveal differences between cell culture lines and tissue samples.

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INTRODUCTION

Human endogenous retroviruses (HERVs) make up approximately 8–9 % of the human genome. HERVs are footprints of ancient germ cell infections by exogenous retroviruses (Weiss, 2006). They are generally classified according to their homology to animal retroviruses. Each class has several subgroups often named based on the tRNA that binds to the retroviral primer-binding site. The class I HERV families have similarities to the mammalian γ-retroviruses that include HERV-E and HERV-H, among several other subgroups. The class II subgroups exhibit homology to mammalian β-retroviruses and include the HERV-K elements. Class III HERVs are distantly related to spumarviruses and are composed of the HERV-L and the HERV-S subgroups (Medstrand *et al.*, 2002; Griffiths, 2001). Class I and III HERVs are the oldest groups and are present throughout the primate lineage (Greenwood *et al.*, 2005; Griffiths, 2001). Among HERV sequences are more

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Supplementary material is available with the online version of this paper.

than 3000 full-length proviruses and about 8000 elements containing at least a partial *pol* gene (Jern *et al.*, 2005; Villesen *et al.*, 2004).

So far, comprehensive HERV transcription profiles have been established for only a few human tissues resulting in observation of significant interindividual variation (Frank *et al.*, 2005, 2008; Flockerzi *et al.*, 2008). The identification of a transcriptional core profile, which is characteristic for a certain tissue and detectable in all individuals, is of importance in determining the significance of transcription alterations in disease states and distinguishing disease-associated alteration from normal interindividual transcription variation. Several HERVs have been implicated in different forms of cancers, but to progress from implication to causality will require knowledge of normal interindividual HERV transcription variation (Nelson *et al.*, 2003; Ruprecht *et al.*, 2008b; Romanish *et al.*, 2010; Balada *et al.*, 2009).

HERV transcripts and proteins have been detected in many human tumours (Romanish *et al.*, 2010). For example HERV-K(HML-2) transcripts are found in testicular tumours, seminoma biopsies (Löwer *et al.*, 1993; Herbst *et al.*, 1998; Sauter *et al.*, 1995) and breast cancer tissue (Wang-Johanning *et al.*, 2003). Furthermore, HERV-encoded antigens have been detected in renal cell carcinoma (RCC) patient material [Komohara *et al.*, 2007 (Table 1); Komohara *et al.*, 2007; Takahashi *et al.*, 2008; Alves *et al.*, 2008; Wang-Johanning *et al.*, 2003, 2008; Schiavetti *et al.*, 2002;Rakoff-Nahoum *et al.*, 2006; Ishida *et al.*, 2008]. RCC is the most common neoplasia arising from the adult human kidney. It is the sixth leading cause of cancer deaths overall in the USA (Kawakami *et al.*, 2006). Several HERVs give rise to T-cell epitopes as documented by the presence of T-cells recognizing tumour cell lines harbouring respective HERV sequences (Takahashi *et al.*, 2008). We have recently identified a T-cell specificity among T-cells infiltrating the primary RCC tumour of a patient (patient 53; TCR53) that appears to recognize an antigen that is shared among many RCC tumours and appears to be absent in non-tumour kidney cells (Leisegang *et al.*, 2010). We speculate that the antigenic epitope could originate from HERV-E, a previously described HERV with RCC-associated expression (Takahashi *et al.*, 2008), or another RCC-expressed HERV sequence.

Here, we present a systematic and comprehensive analysis of the HERV transcription profiles in human non-tumour kidney, RCC tissues, RCC cell lines and cell cultures derived from non-tumour kidney tissues by using a previously established retrovirus DNA chip (Seifarth *et al.*, 2003, 2005). We utilized 10 paired tumour and adjacent non-tumour kidney tissues, 22 RCC cell lines, one non-tumour-derived epithelial kidney cell line (HK2) and four primary non-tumour kidney-derived cell cultures (NKC). The specific HERV transcript profiles of the RCC lines, HK2 and NKCs were compared to the presence or absence of the TCR53 antigen to determine if an HERV sequence correlates with the TCR recognition pattern. Furthermore, the transcription of seven known HERV tumour antigen sequences was examined in the cell lines by PCR. The data presented established the non-tumour kidney HERV transcription profile and illustrated changes in transcription between tissues versus cell culture systems. The RCC-specific changes are discussed in the context of HERV contribution to cancers.

RESULTS

The human kidney core HERV transcription profile

Using RNA from non-tumour healthy kidney tissues from 10 different patients, we established the core kidney HERV transcription profile (Table 1). Fourteen HERV subgroups, HERV-E elements (E4-1 and Seq32), HERV-W, ERV-9 elements (Seq63, ERV9 and Seq59), HERV-K (HML-2) elements (HERV-K10, HERV-K2.HOM, HERV-KHP1 and HERV-KD1.2), HML-3 elements (Seq26 and HML-3), HML-6 and HML-8 were transcribed in eight or more individuals for tissue samples, all primary NKC cultures and non-tumour HK2 kidney cell line (Table 1). The cut-off value of 13 positives of 15 samples is similar to that used by Frank *et al.* (2005, 2008). Because samples 4807N and 4757N gave weaker overall signals for class I retrovirus-like elements (see Supplementary Fig. S2, available in JGV Online), a slightly less stringent cut-off for the core profile was used (87 % in this study versus 94 % in Frank *et al.*, 2005) to account for potential underscoring of very frequently active HERVs in kidney tissues. Microarray images of a core profile versus a non-core profile HERV transcription are shown in Fig. 1. It should be noted that each positive spot on the microarray can represent multiple HERV loci as most HERVs are multicopy elements with sufficient sequence homology that they cannot be distinguished on an individual basis. Thus, HML-3 spots, for example, are representative for HML-3 subgroup elements, not single locus expression.

HERV transcription in RCC tumours closely reflects that of the non-tumour kidney tissue

Comparison of HERV transcription in the non-tumour tissue to the patient-matched RCC tissue revealed striking similarity with the kidney 'core' HERV profile (Table 1). No RCC-specific up- or downregulation of HERVs was observed. This suggests that cancer manifestation does not grossly alter the HERV transcription.

Cell culture-induced changes in HERV transcription

Next, we analysed the transcription pattern in short-term cultures (passages 2–4) of primary non-tumour kidney cell cultures of four patients and found complete overlap when compared to the tissue core kidney profile. However, the cell cultures expressed additional HERV subgroups that

Table 1. Number of samples positive for HERV transcription for each sample type

Bold indicates HERVs belonging to the kidney core profile.

HERV family	Subgroup*	Primary cultures of NKC and HK2 cells in HK2 media (n=5)	RCC cell lines (n=19)†	Non-tumour kidney tissue (tumour adjacent) (n=10) showing percentages of tissues with transcription of respective HERV	Tumour tissue (n=10)	Core profile non-tumour kidney tissue‡	Transcribed with different frequency among cell lines and tissues	Infrequently transcribed or random among sample types
Gammavirus-like								
HERV-S	HERV-S	60 %	21 %	30 %	20 %			X
	Seq77	0	5 %	10 %	0			X
HERV-I	HERV-I	0	21 %	50 %	60 %		X	
	HERV-IP-T47D	40 %	42 %	30 %	50 %			X
	Seq65	40 %	26 %	60 %	60 %		X	
HERV-T	S71pCRTK6	100 %	84 %	20 %	30 %		X	
	S71pCRTK1	20 %	32 %	30 %	40 %			X
HERV-FRD	HERV-FRD	100 %	90 %	50 %	40 %		X	
	HS49C23	0	0	50 %	50 %		X	
	HERV-Z	0	0	0	20 %			X
HERV-E	E4-1	100 %	100 %	100 %	100 %	**X**		
	Seq32	100 %	84 %	80 %	80 %	**X**		
HERV-H	RGH2	100 %	100 %	30 %	30 %		X	
	HERV-H	0	0	30 %	40 %		X	
	Seq66	80 %	68 %	40 %	30 %		X	
HERV-ADP	HERV-ADP	0	0	0	0			X
HERV-W	HERV-W	100 %	100 %	80 %	70 %	**X**		
ERV-9	Seq64	80 %	90 %	40 %	30 %			X
	Seq63	100 %	100 %	80 %	70 %	**X**		
	ERV9	100 %	100 %	80 %	80 %	**X**		
	Seq59	100 %	100 %	80 %	80 %	**X**		
	Seq60	100 %	95 %	30 %	10 %		X	
HERV-F	HERV-F2	20 %	16 %	10 %	10 %			X
	HERV-F	0	5 %	40 %	50 %		X	
	HERV-Fb	100 %	100 %	50 %	50 %		X	
HERV-R	HERV-Rb	0	0	60 %	70 %		X	
	ERV3	40 %	26 %	60 %	70 %		X	
Betavirus-like								
HML-1	HML-1	20 %	16 %	0	0		X	
	Seq29	20 %	16 %	0	0		X	
HML-2	HERV-K10	100 %	90 %	90 %	80 %	**X**		
	HERV-K2.HOM	100 %	90 %	90 %	90 %	**X**		
	HERV-KHP1	100 %	95 %	90 %	80 %	**X**		
	HERV-KD1.2	100 %	95 %	100 %	80 %	**X**		
HML-3	Seq26	100 %	100 %	80 %	70 %	**X**		
	Seq34	100 %	100 %	50 %	30 %		X	
	HML-3	100 %	100 %	90 %	80 %	**X**		
	HERV1	100 %	100 %	40 %	30 %		X	
	Seq43	100 %	100 %	30 %	30 %		X	
HML-4	Seq10	100 %	100 %	30 %	20 %		X	
	HERV-K-T47D	100 %	100 %	20 %	30 %		X	
HML-5	HML-5	80 %	79 %	20 %	10 %		X	
HML-6	HML-6	100 %	100 %	80 %	90 %	**X**		

Table 1. cont.

HERV family	Subgroup*	Primary cultures of NKC and HK2 cells in HK2 media (n=5)	RCC cell lines (n=19)†	Non-tumour kidney tissue (tumour adjacent) (n=10) showing percentages of tissues with transcription of respective HERV	Tumour tissue (n=10)	Core profile non-tumour kidney tissue‡	Transcribed with different frequency among cell lines and tissues	Infrequently transcribed or random among sample types
	Seq38	100 %	100 %	70 %	60 %			X
	Seq56	80 %	100 %	10 %	20 %		X	
HML-7	NMWV7	20 %	53 %	100 %	80 %		X	
HML-8	NMWV3	100 %	74 %	90 %	80 %	X		
HML-9	NMWV9	80 %	100 %	80 %	70 %		X	
HML-10	HERV-KC4	100 %	84 %	30 %	50 %		X	
	Seq31	60 %	47 %	60 %	20 %			X
Spumavirus-like								
HERV-L	HERV-L	100 %	47 %	60 %	40 %			X
	Seq39	100 %	47 %	50 %	30 %			X
	Seq45	60 %	37 %	30 %	30 %			X
	Seq51	100 %	84 %	60 %	50 %			X
	Seq58	60 %	26 %	20 %	20 %			X

*HERV names from Seifarth *et al.* (2003).

†The three variants of the 786-0 cell line were analysed separately from the other 19 RCC cell lines.

‡Core profile of transcribed HERVs in non-tumour kidney tissues, NKC and HK2 cells in HK2 media. HERV transcription was defined as belonging to the core if transcribed in 80 % of tissues and 100 % of cell cultures.

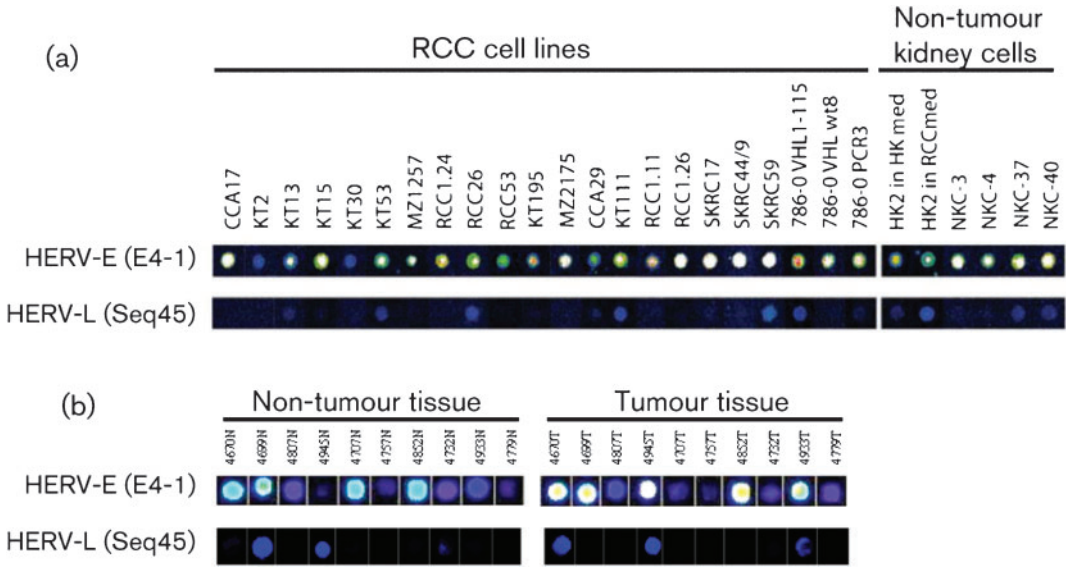

Fig. 1. HERV microarray example images of cell culture and tissues. HERV-E (E4-1) belongs to the core profile and is contrasted with a non-core profile HERV (HERV-L Seq45). (a) Enhanced contrast and brightness false-colour microarray images of the 22 RCC and four non-tumour kidney cultures and one non-tumour kidney cell line HK2 grown in different media. (b) False-colour images of non-tumour and tumour kidney tissues.

were infrequently transcribed in the tissues (Table 1 and Supplementary Fig. S1, available in JGV Online). These included HERV-T (S71pCRTK1 and S71pCRTK6), HERV-FRD (FRD and HS49C23), HERV-H (RGH2, HERV-H and Seq66), ERV-9 (Seq60 and Seq64), HERV-F (-F and Fb), HERV-R (HERV-Rb), ERV3, HML-1 (Seq29), HML-3 (Seq34, HERV1 and Seq43), HML-4 (Seq10 and HERV-KT47D), HML-5, HML-6 (Seq56), HML-7, HML-9, HML-10 (HERV-KC4 and Seq31) and HERV-L elements (Seq39 and Seq51). Interestingly, all the HERV sequences that were induced in the primary non-tumour kidney cells by short-term culture were also found to be expressed in RCC cell lines (Table 1). Given that these same HERVs were uniformly or frequently expressed in the RCC cell lines but were consistently infrequent in the tumour tissues, these differences probably represent changes in HERV transcription that occurred in response to cell culture establishment and thus do not reflect pathology. The uniformity with which the culture-induced differences occurred suggests that the cell culture conditions drastically alter HERV transcription by a common mechanism, which influences the same HERV LTRs (Table 1).

Moreover, it was found that different culture media could influence HERV transcription. This was seen with the established non-tumour proximal tubular kidney epithelial cell line HK2, which was cultured under two different conditions (see Methods). Altered HERV transcription in response to the media included HERV-I, HERV-H (subgroup HERV-H), and HERV-F (subgroup HERV-F) (Supplementary Table S1, available in JGV Online). Notably, the affected HERVs did not belong to the core profile.

Additionally, three RCC cell lines all derived from the same parental cell line but transfected with either the wild-type von Hippel Lindau (VHL) gene (786-0 VHLwt8), a truncated VHL gene (786-0 VHL1-115) or the empty vector (786-0 PCR3) (see Methods), also demonstrated differences in HERV expression. HERV-I (HERV-I and HERV-IP-T47D), HERV-T (S71pCRTK6 and S71pCRTK1), HERV-H (subgroup HERV-H), ERV9 (Seq64), HERV-R (ERV3), HML-1 (HML-1 and Seq29), HML-7, and HERV-L (Seq39, Seq45 and Seq58) were transcribed differently among the three RCC cell lines with different VHL transcription (Supplementary Table S1). Again, none of the altered HERVs were part of the core profile.

HERV sequences as potential targets for RCC-reactive TCR53

The HERV transcription profiles of the RCC lines (n=22), primary non-tumour kidney cultures (NKC, n=4) and the established HK2 non-tumour kidney epithelial cell line was compared to the presence or absence of the TCR53 epitope. Fifteen of the 22 RCC cell lines expressed the TCR53 epitope, but none of the four NKC cultures (Leisegang *et al.*, 2010). Thus, while the antigen recognized by TCR53 appears to be restricted to RCC cell lines, it is not present in all RCC cell lines.

Positive (n=15) and negative (n=11) cell lines or cultures did not reveal any HERV subgroup that was present or respectively absent in the majority of cells of one or the other group (Supplementary Table S2, available in JGV Online). Thus, the HERV transcription pattern does not provide evidence that an HERV sequence is the antigen recognized by the RCC-reactive TCR53. However, it cannot be excluded that a single HERV locus among a subgroup of multicopy HERV elements may be differentially expressed.

Quantitative analysis of HERV transcription differences between tumour and non-tumour tissue samples

Although there was no difference in terms of HERV presence or absence in tumour-derived versus non-tumour-derived RNA (Table 1), the intensity of the microarray signals differed among samples (Fig. 1). To examine whether the differences in HERV transcription levels between RCC and non-tumour tissue RNA that were statistically significant, the microarray data were analysed densitometrically and each HERV normalized to the housekeeping gene HPRT, which was consistently expressed among samples. No statistically significant difference was observed, reinforcing that HERV transcription is unaltered in RCC (not shown).

The results of the microarray analysis were confirmed by quantitative real-time RT-PCR (qRT-PCR) for HERV-K groups HML-3 and HML-5 performed on selected samples (five and three RCC tissues, respectively) (Supplementary Fig. S3, available in JGV Online). The qRT-PCR results were generally consistent with the observed microarray analysis and did not support RCC-specific increase or decrease of transcription of the HERVs tested.

Transcription of known HERV-encoded tumour antigens in RCC cell lines, the cell line HK2 and primary NKC cultures

A number of tumour-specific antigens derived from various HERV elements have been identified in several human tumours such as breast carcinoma, colorectal carcinoma, melanoma, prostate carcinoma, RCC and seminoma (Shastri, 1996; Rakoff-Nahoum *et al.*, 2006; Ishida *et al.*, 2008; Chen *et al.*, 2004; Schiavetti *et al.*, 2002; Alves *et al.*, 2008; Takahashi *et al.*, 2008; Wang-Johanning *et al.*, 2008) (Table 2). Primers specifically amplifying transcripts of these HERV antigens were adapted from the literature and used to screen the RCC and HK2 cell lines as well as the primary NKC cultures (Fig. 2). HERVs HERV-E-RCC8, HERV-E-RCC9, HERV-K-102 and HERV-K-M1976 were expressed in all analysed cell cultures, including those from non-tumour kidney, thus exhibiting no cancer-associated expression pattern. HERV-H-Xp22 was absent in some RCC lines (10 of 20) and also absent in the immortalized non-tumour kidney cell line HK2 and one (of four) primary

Table 2. HERV-derived antigens

HERV	GenBank accession no.	Cancer type	Location	Antigen detection	Chromosome	PCR primers (5′–3′)	T_m (°C)	Reference
HERV-E RCC8	AL133408	Kidney	HERV-E LTR/pro/pol	T-cell response against antigen (delete)	6q	F, GAACACCGGGAAGGAATCG; R, TCTGCGGCTTGCTGCAT	59	Takahashi *et al.* (2008)
HERV-E RCC9	AL133408	Kidney	HERV-E LTR/pro/pol	T-cell response	6q	F, TGGAACATAGCCCCTTTGTG; R, GGATCCAGGCCGGAATTC	59	Takahashi *et al.* (2008)
HERV-H Xp22	EF194101	Colorectal	HERV-H gag	Bioinformatical construction	Xp22	F, CTTCCCTCCGTGTCTTTACG; R, AAGATTAGACACACTCAGCAACG	59	Alves *et al.* (2008)
HERV-K-102	AF164610.1	Breast	HERV-K (HML-2) env	T-cell response	Unknown	F, ATTGGCAACACCGTATTCTGCT; R, CAGTCAAAATATGGACGGATGGT	59	Wang-Johanning *et al.* (2008)
HERV-K-MEL	AC092357.2	Melanoma	HERV-K (HML-2) env	T-cell response	16	F, TGCAGAGGATATAAGGAGAT; R, GGATCAAACTGCAAGGCA	61	Schiavetti *et al.* (2002)
HERV-K M1976	M14123	Seminoma	HERV-K (HML-2) gag	T-cell response	Unknown	F, CCATTGCTTATGGACATAGACTC A; R, GAGGATTGGCAGCCCTATTT	59	Rakoff-Nahoum *et al.* (2006)
HERV-K-NGO-Pr-54	AP000346	Prostate	HERV-K (HML-2) gag	Antibody reactivity	22q11	F, CAGCGATGGCGTCTAATTCA; R, AGTGGGCGGCTGAGGATAC	63	Ishida *et al.* (2008)

NKC culture. Transcripts of the HERV-K-MEL gene were detected in only one of the primary NKC cultures. The antigen HERV-K-NGO-P-54 was transcribed in 22 of the 28 analysed samples, irrespective of whether or not they are non-tumour or cancer in origin. There was no correlation between HERV transcription and the presence or absence of the TCR53 epitope. Moreover, transcription of each HERV described to be a tumour-associated antigen was not restricted to cancer cell lines but was also found in non-tumour kidney tissues.

DISCUSSION

Alterations in HERV transcriptional activity are observed in many human cancers (for reviews see (Romanish *et al.*, 2010; Ruprecht *et al.*, 2008a). Some HERV sequences have also been found in RCC where it was shown that they give rise to cancer-associated T-cell reactivity (Takahashi *et al.*, 2008). In order to better understand potential disease-associated changes in HERV transcription in the kidney, we established the core kidney HERV transcription profile using retrovirus-specific DNA microarrays and compared it to the transcription profile of kidney cancer tissues. It should be noted that the microarray is based on the detection of the *pol* gene (Seifarth *et al.*, 2005). The advantage is that HERV group-specific *pol* sequences flanked by conserved motifs make the gene amenable to microarray discrimination. This means, however, that it cannot be excluded that other HERV genes such as *env*-derived sequences exhibit different expression patterns. Previous studies of brain tissue (Frank *et al.*, 2005) and mammary gland tissue (Frank *et al.*, 2008) observed interindividual variation in HERV transcription with some HERV groups expressed in all individuals and some in one or few. The HERV transcription profile in kidney tissue contrasts with the profile observed in cultured cells. While there is a core of commonly expressed HERVs, cell cultures were consistently found to express additional HERVs. The expression of the additional HERV sequences was found to be independent of whether the cell culture was from non-tumour kidney tissue or from RCC tumour. Different microarray production does not explain this result as the extra HERVs were transcribed in some of the tissue samples as well but at much lower frequency among individuals. Given the consistency of this difference among all cell cultures tested, the data suggest that HERV transcription in kidney tissue and kidney cell cultures is not entirely comparable. They additionally suggest that there is a common mechanism involved in the alteration of HERV transcription that occurs when cells are propagated under culture conditions. The induction of HERV transcription occurs quickly as had already been seen in primary non-tumour kidney cultures within only two passages. Within the organ, kidney and RCC cells are mostly quiescent with very few cells positive for the proliferation marker Ki67 (Nakano *et al.*, 2001). Thus, HERV induction could be related to the cell proliferation prompted by the cell culture conditions.

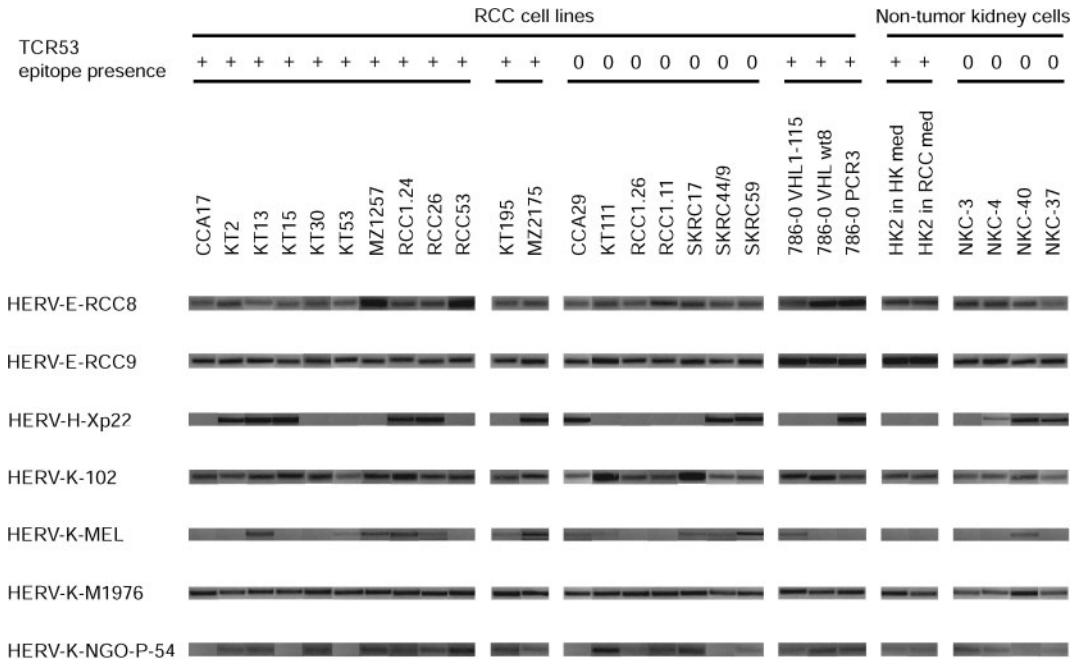

Fig. 2. Seven HERV sequences, which are described as encoding antigens in various cancer types, were analysed by PCR to determine their transcription in RCC cell lines and non-tumour kidney cell line (HK2) and primary NKC cultures. None of the investigated HERVs were restricted to cancer cell lines; rather the transcription was also found in the immortalized non-tumour kidney cell line HK2 and the primary NKC cultures. The presence or absence of the TCR53 epitope was determined previously (Leisegang *et al.* 2010) and is indicated by + or 0.

Notably, there were no differences between non-tumour kidney and tumour kidney tissue or between non-tumour kidney cultures or RCC cell lines. When analysed quantitatively, there were no statistically significant differences in the levels of transcription. Thus, pattern and transcription levels remained unaffected by tumour status. Some differences were observed that were related to the expression of VHL protein variants in a given cell line (786-0 cell line). However, none of the HERVs that varied among the VHL-altered cell line belonged to the core profile and varied in transcription in other cell lines.

The results are somewhat surprising in light of the description of HERV-derived RCC-specific antigens (Takahashi *et al.*, 2008). Based on this publication we had speculated that the RCC-associated T-cell reactivity (TCR53), which we had identified (Leisegang *et al.*, 2010), could be related to an RCC-expressed HERV sequence. However, the transcription pattern of HERVs in the RCC lines and normal kidney cultures did not match the pattern of TCR53 epitope presence, thus not supporting the hypothesis that an HERV *gag* sequence is involved in TCR53 specificity. However, it is possible that differential expression of a single HERV locus among a subgroup of closely related HERV elements may not be detected by microarray analysis (Frank *et al.*, 2008). We therefore analysed the two published RCC-specific HERV sequences and several other HERV sequences that are thought to be tumour antigens, but observed that all were expressed in RCC cell lines and non-tumour kidney cultures. Unfortunately, insufficient human kidney tissue material was available for a similar analysis. The lack of tumour specificity of antigen expression could be due to cell line alterations in expression. Alternatively or additionally, it could mean that the reported tumour antigens are not truly tumour-specific and are more generally expressed. Further work on tissue samples will be needed to clarify this point.

In conclusion, we have established a core HERV transcription profile for the human non-tumour kidney and we describe, for the first time, the dramatic impact that cell culture has on the transcription of HERV sequences. This indicates that data obtained with cell cultures cannot be used to deduce the 'native' organ-associated HERV profile. Moreover, we report that the HERV profile of RCC tissues very closely follows that of the non-tumour kidney, suggesting that RCC development and manifestation do not impact HERV transcription. However, other kidney diseases may alter HERV transcription by either qualitatively or quantitatively changing the core HERV profile.

METHODS

Tissue collection. Human kidney samples were obtained from the Biorepository of the Eastern Virginia Medical School (Eastern Virginia Medical School, Norfolk, VA, USA IRB # 07-02-EX-0031)

Table 3. Kidney tissue samples and pathology information from Eastern Virginia Medical School

CA#, Cancer sample number.

CA#	Tissue type	Stage/Fuhrman nuclear grade*
4670	Clear cell	pT3bN0Mx/ 3
4699	Clear cell	pT3aNxMx/ 2
4807	Clear cell	pT3aNxMx/ 2-3
4945	Clear cell and granular cell	pT3N3Mx/ 2-3
4707	Papillary renal cell	pT1bNxMx/ 2
4757	Clear cell	pT2NxMx/ 3
4852	Clear cell with sarcomatoid differentiation	pT3N0Mx/ 4
4732	Unspecified renal cell	pT1bNxMx/ 2
4933	Clear cell	pT3bNxMx/ 2
4779	Clear cell	pT1bNxMx/ 4

*Fuhrman *et al.* (1982).

(Table 3). Whole kidney sections were stored at −80 °C in plastic bags with no preservatives. Tumours and adjacent normal tissue were harvested at Sentara Norfolk General Hospital, at the Urology Department, at the time of total or partial nephrectomy from RCC patients (Fuhrman *et al.*, 1982). All samples were obtained only from subjects who consented to the Eastern Virginia Medical School RCC biomarker discovery research protocol. All samples were carefully analysed by one of three genito-urinary specialized pathologists. The sampling strategy involved obtaining equivalent size sections from non-tumour and cancerous tissues, minimum of 1 cm apart, from the same individual. Tumours and non-tumour kidney areas were defined by a pathologist (RL). Tissue samples were taken using a 6 mm^3 biopsy punch and were added directly to a minimum of 180 µl buffer RNA Later (Qiagen) solution containing 0.1 % 2-mercaptoethanol.

RCC cell lines, immortalized kidney cell line HK2, primary non-tumour kidney cell cultures and TCR53 epitope presence. The RCC cell lines used in this study are described in detail in Leisegang *et al.* (2010). They are derived from primary human RCC tumours by spontaneous outgrowth after placing tumour suspensions in culture. No means of cell transformation were applied. Three variants of the RCC cell line 786-0 were used. These variants are the 786-0 VHLwt8 cell line, transfected with the wild-type von Hippel Lindau gene (VHLwt8), the 786-0 VHL1-115 cell line, transfected with a truncated VHL gene encoding aa 1–115, and the 786-0 PCR3 cell line, transfected with the empty vector (Iliopoulos *et al.*, 1995). HK2 was purchased from ATCC (ATCC CRL-2190). It is an immortalized human proximal epithelial kidney cell line, which was established by transformation with E6/E7 genetic elements of the human papilloma virus HPV16. All cell lines were examined regularly regarding their growth behaviour and morphological characteristics. All cell lines were grown in VLE-RPMI 1640 (FG1415) medium (Biochrom) with very low endotoxin. RPMI was further supplemented with FCS (12 %) and penicillin/streptomycin (1 %), except for HK2, which was grown either in HK2 medium (DMEM F-12 HAM (Sigma) supplemented with L-glutamine, 1 % Insulin-Transferrin-Selenium-X (Sigma), 0.1 µM hydrocortisone (Sigma), T3 (3,3′,-triodo-L-thyrosine; 10 µg ml^{-1}) (Sigma) and EGF (0.01 µg ml^{-1}) (Sigma) or RCC medium (RPMI 1640 supplemented with 12 % FCS, 1 % L-glutamine, 1 % non-essential amino acids (Biochrom), 1 % sodium pyruvate and 1 % penicillin/streptomycin; Invitrogen).

NKC are described elsewhere (Leisegang *et al.*, 2010). Briefly, they are short-term cultures (passage 2– 4) of cells from non-tumour kidney cortices obtained from RCC patients undergoing complete nephrectomy at the Urological Department of the University Hospital Grosshadern at the Ludwig-Maximilians-University Munich. They were maintained in RPMI 1640 medium supplemented with 10 % FCS, 100 U penicillin–streptomycin ml^{-1}, 1 mM L-glutamine, 1 mM sodium pyruvate, 1 % non-essential amino acids and 1 % Insulin-Transferrin-Selenium-X as described previously (Leisegang *et al.*, 2010). Determination of TCR53 epitope presence in the cell lines and NKCs is described in Leisegang *et al.* (2010).

RNA extraction from human tissues. Disposable probes attached to a TissueRuptor were used to disrupt the samples at full speed until no debris remained (Qiagen). The lysate was then transferred to a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at maximum speed. The homogenized lysate was transferred to a genomic DNA (gDNA) Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 s at 8000 ***g***. One volume of 70 % ethanol (350 µl or 600 µl) was added to the flow-through. The samples were then processed according to the manufacturer's directions.

A DNase digestion step on the RNA was performed to ensure no residual DNA was in the sample prior to a second RNA clean-up procedure. The eluted RNA was mixed with 10 µl Buffer RDD and 2.5 µl DNase stock I solution followed by incubation at room temperature for 10 min. The above procedure was repeated beginning with the transfer of the mixture to the gDNA Eliminator spin column. A total of 100 µl was added to the membrane for the final elution step.

Cell lines were harvested, pelleted and immediately placed on ice and processed for RNA extraction using an RNeasy kit (Qiagen) according to manufacturer's instructions. DNase treatment was performed using RQ1 RNase-free DNase (Qiagen) according to the supplier's instructions.

To ensure that all gDNA was removed by DNase treatment PCR with HERV-L LTR-specific primer pair was performed for the cell lines. This primer pair amplifies a great number of HERV-loci. Alternatively, the mixed oligonucleotides for pre-amplification before microarray hybridization were used and checked by gel electrophoresis. The same strategy was used for the human tissue sample RNA preparations. Further experiments were only conducted on PCR-negative RNA.

Reverse transcription and PCR. Reverse transcription of 500 ng to 1 µg human tissue RNA was performed using Q-script (Quanta BioSciences) after the RNA tested free of contamination. A second PCR was then performed using the same protocol as above to amplify the cDNA. For cell lines, cDNA synthesis was performed using the Superscript First-Strand Synthesis System for RT-PCR according to manufacturer's instructions.

Two different mixed oligonucleotide primer (MOP) sets were used for multiplex-PCR. The primer sequences were derived from two highly conserved amino acid motives (VLPQG and YM/VDDI/LL) present in all retroviral RT-proteins and flank an approximately 90 bp sequence. This ensures the amplification of various RT-associated elements and the specificity of the PCR (Seifarth *et al.*, 2003). The antisense- ('reverse'-) primers were modified with the fluorochrome Cy3 at their 5′ end, which allows the labelling of all synthesized PCR products (Seifarth *et al.*, 2003). In addition, 6 bp long 'clamp'-sequences were implemented at the 5′ end of each primer, which stabilize the binding of the primer during the PCR. The MOP-C-

primer set is based on degenerate primers described by Shih *et al.* (1989) and permits the amplification of human and vertebrate γ-retroviral RT-sequences and various exogenous virus sequences. The other primer set (MOP-A) enables the amplification of human β-retroviral RT-sequences. The PCR for each primer mixture was performed separately to allow the optimal amplification of the retrovirus-related elements. Oligonucleotides specific for human housekeeping genes (MOP-HKG) served as internal control for the quality of the RNA and the reproducibility of the microarray data (Seifarth *et al.*, 2003). For the human tissues, the PCR mixture contained 2.5 µl cDNA, 0.5 µl HKG primer mixture, 2.0 µl MOP-A primer mixture or 2.0 µl MOP-C primer mixture and 45 µl Platinum *Taq* polymerase (Invitrogen). All PCR programs were performed on the Bio-Rad MyCycler thermocycler (Hercules) using the HKG with either the MOP-A or MOP-C oligonucleotide primers (100 nM final concentration) in 1 mM phosphate buffer (1.5 mM $MgCl_2$), with Platinum *Taq* polymerase and standard buffers (Applied Biosystems). Negative-amplification controls were included with each sample to monitor for contamination. RNA extraction and pre-amplification steps for PCR were carried out in separate UV-exposed hoods to prevent DNA contamination with separate pipettes, disposable sterile tubes, filter tips, sterile reagents and solutions were used throughout the procedures. All reagents and tubes were irradiated with UV light to minimize the potential contamination. The cycling parameters included an initial denaturation of 9.5 min at 95 °C, followed by 30 s at 45 °C; annealing for 30 s at 60 °C in the initial three cycles then stepping down to 50 °C annealing for the remaining 42 cycles, 72 °C for 30 s and a final extension of 2–7 min at 72 °C.

For the cell lines, the KAPA 2G Robust DNA-Polymerase system was used containing 19.8 µl, PCR-grade water, 10 µl 5× KAPA 2G Buffer A, 2 µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM), 2 µl primer mixture MOP-A/MOP-C, 1 µl MOP-HKG, 10 µl 5× Enhancer 1, 2 µl 25× Enhancer 2, 0.2 µl KAPA 2G Robust DNA-Polymerase and 2 µl template cDNA for a total volume of 50 µl. Cycling conditions were an initial denaturation at 95 °C for 1 min, three cycles of 94 °C 30 s, 45 °C 3 min, 72 °C 2 min followed by 30 cycles of 94 °C 30 s, 50 °C 2 min, 72 °C 2 min and a final 72 °C extension for 7 min. PCR products using RNA or cDNA as template were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining. PCR-amplified cDNA was purified using a MinElute PCR Purification kit (Qiagen), according to manufacturer's directions for tissues.

Microarrays. Purified PCR-amplified cDNA was annealed to microarray slides (Epoxy2 Barcoded Slides; VWR) containing the complementary sequence to known HERV oligonucleotides. Microarray slides were spotted (SpotArray24; Packard BioScience) in triplicate at Eastern Virginia Medical School (Norfolk, VA) in separate UV-exposed hoods to prevent DNA contamination. Separate pipettes, disposable sterile tubes, filter tips, sterile reagents and solutions were used throughout procedures. All reagents and tubes were irradiated with UV light to minimize contamination. Microarray slides were blocked for 2–24 h, using 20 µl ArrayIt BlockIt (ArrayIt Corporation) buffer in microarray hybridization chambers at 60 °C. Slides were then washed with ArrayIt wash buffers: 2 min in 1× Wash Buffer A, 2 min in 2× SSC, 1 min in double-deionized water. Purified PCR products (10 µl) were mixed with 18.4 µl 1.25× Hybridization buffer and 2 µl nuclease-free water and incubated at 42 °C for 5 min.

The mixture was then added to the microarray and covered with a coverslip. The microarrays were placed in a Hybex hybridization chamber overnight at 60 °C. Double-deionized water (1 ml) was added to the top of the chamber to keep the microarrays hydrated. A second wash cycle was performed after DNA annealing to remove un-annealed DNA: 5 min in 1× Wash Buffer A; 5 min in 1× Wash Buffer B; and 1 s in 1× Wash Buffer C. Several preliminary studies were conducted to determine autofluorescence of the slides. Water control slides were scanned prior to sample slides in order to observe autofluorescence levels. The scanner was reduced to 60 % Photo-multiplier tube to remove the observed autofluorescence. Microarray slides were scanned using a microarray scanner (ScanArray Express; Packard BioScience). False-colour spot images were used to determined low or high expression levels. The microarray dataset images are shown in the Supplementary material (available in JGV Online).

For cell lines, similar arrays were produced using a GMS arrayer (three hits per dot) using oligonucleotides synthesized by MWG Biotech AG. Slides were incubated 1–4 h in a humid chamber and then dried 24 h in the dark. The slides were shaken vigorously for 2 min in 0.2 % SDS and two times for 1 min in ddH_2O. They were placed for 5 min in a $NaBH_4$-solution, washed for 1 min in 0.2 % SDS and then twice for 2 min in ddH_2O. The slides were placed in 50 ml Falcon tubes and centrifuged for 2 min at low speed and stored at room temperature until use. Hybridization then proceeded as described in Seifarth *et al.* (2003). The microarrays were scanned with an Affymetrix GMS 418 array scanner, further processing of the pictures were done with ImaGene Software tool package (resulting pictures were visualized in false-colours), the pictures were saved as tagged image file (tif) and bitmap file (bmp) and the alignments were created with the support of Adobe Photoshop CS3 Extended and Adobe Illustrator CS3 (Supplementary material). Densitometric analysis was performed with the program Image J 1.37v. Data analysis was performed using GraphPad Prism 5.0. Significance was calculated using unpaired Student's *t*-test and using a cut-off of $P<0.05$ for significance. The microarray dataset images are shown in the Supplementary material.

Detection of HERV antigen transcripts. Primers used to detect seven known HERV-derived tumour antigens were developed and tested for amplification of the correct sequences by sequencing each product from a test sample. Primers that amplified the correct sequences were used on the cell line-derived RNA. The primer sequences are shown in Table 1. Amplification conditions were performed in ddH2O (39.5 µl), 5 µl Expand High Fidelity buffer with 5 µl 10× $MgCl_2$, 1 µl dNTPs (10 mM), 1 µl Primer fwd (10 µM), 1 µl Primer rev (10 µM), 0.5 µl Expand High Fidelity Polymerase, and 2 µl template cDNA for a total of 50 µl. Cycling parameters included an initial denaturation at 95 °C for 2 min followed by 30 cycles at 95 °C for 30 s, 59–61 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 5 min. Products were visualized on ethidium stained agarose gels.

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