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Detection of Human Endogenous Retrovirus Type K-Specific Transcripts in Testicular Parenchyma and Testicular Germ Cell Tumors of Adolescents and Adults

Clinical and Biological Implications

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Testicular germ cell tumors (TGCTs) of adolescents and adults have been shown to contain proteins of the human endogenous retrovirus type K family. In a recent study, expression of these retroviral sequences was confirmed using *in situ* hybridization, which also showed expression in carcinoma *in situ*, the precursor of all TGCTs. Because of the clinical significance of a test for early diagnosis of TGCTs, we studied whether expression of human endogenous retrovirus type K genes could be an informative parameter. Therefore, we investigated TGCTs of various histologies and testicular parenchyma with and without carcinoma *in situ* using reverse transcription-polymerase chain reaction for expression of the *gag*, *env*, and *prt* genes. The *gag* and *prt* genes were expressed in all samples tested. The *env* transcripts were not found in TGCTs showing somatic differentiation only but could be detected in most normal testicular parenchyma samples. Therefore, detection of human endogenous retrovirus type K transcripts cannot be used for early diagnosis of TGCTs. Simultaneous expression of multiple *gag* sequences was found both in normal parenchyma and TGCTs, and we demonstrated that expression of *gag* sequences with an extra G, necessary to generate a functional protein, was not limited to TGCTs. *(Am J Pathol 1998, 153:1277–1282)*

Testicular germ cell tumors (TGCTs) of adolescents and adults are divided into two clinically and histologically distinct entities: the seminomas and the nonseminomatous TGCTs.[1–3] The seminomas are composed of neoplastic primitive germ cells. Nonseminomatous TGCTs are neoplastic caricatures of early development and may be composed of a stem cell population of embryonal carcinoma, giving rise to teratoma (somatic tissue) and yolk sac tumor and choriocarcinoma (extraembryonic tissues).[4] Seminomas and nonseminomatous TGCTs originate from a common precursor known as carcinoma *in situ* (CIS)[5] or intratubular germ cell neoplasia.[6] Epidemiological data and histological findings indicate that CIS is formed during intrauterine development[7,8] and will always progress to an invasive cancer.[9]

TGCTs are the most common cancer in Caucasian males between 15 and 45 years of age. For most European countries, as well as the USA, an increasing incidence has been reported.[10–12] Several well defined risk factors, among them a familial history, have been identified for this cancer.[13–16] Up to about 90% of the invasive TGCTs can be cured using orchidectomy alone or in combination with irradiation and/or chemotherapy.[2] CIS, however, can be effectively treated by low-dose irradiation with minimal side effects.[17] This treatment prevents the development of an invasive TGCT, underscoring the clinical relevance of a sensitive and specific marker for CIS.

The presence of human endogenous retrovirus type K (HERV-K) proteins in TGCTs has been recognized for several years.[18] Patients with a TGCT frequently show a specific immune response to these proteins.[18,19] Using *in situ* hybridization, Herbst et al[20] showed that the *gag* and *env* genes of HERV-K are expressed in all histological elements of TGCTs, except teratoma. Expression of HERV-K genes was also detected in CIS, whereas normal cells of the testis were negative. These data suggest that transcripts of HERV-K-specific genes, such as *gag* and *env*, could be used as a marker for TGCTs, in particular

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for diagnosis of CIS. To test this hypothesis, we developed a reverse transcription polymerase chain reaction (RT-PCR) for *gag*, *env*, and *prt* expression. A series of invasive TGCTs with different histologies, testicular parenchyma with varying amounts of CIS, and normal testicular parenchyma were studied. In addition, we show that multiple *gag* sequences are simultaneously expressed in TGCTs and in normal testicular parenchyma. At variance with previous findings, we show that expression of a specific *gag* variant, containing an extra G, is not unique for TGCTs. We conclude that detection of HERV-K transcripts cannot be used for early diagnosis of TGCTs.

Materials and Methods

Tissue Samples

Tumor and parenchyma was sampled from orchidectomy specimens with TGCTs and nonneoplastic conditions collected at the pathology departments of collaborating hospitals. Representative samples were divided into two parts; one was immediately snap frozen in liquid nitrogen, and the other was formalin-fixed and paraffin-embedded. Samples of normal testicular parenchyma obtained from autopsies of men who died from causes other than testicular cancer were processed in the same way. TGCTs were classified on the basis of morphology and immunohistochemistry according to the World Health Organization classification.[3] The presence of CIS was visualized on acetone-fixed, frozen tissue sections using a direct enzyme-histochemical staining method, as previously described.[21] The percentage of seminiferous tubules containing CIS was scored.

RNA Isolation and RT-PCR

Total RNA was isolated from 10 to 15 frozen tissue sections of 20-μm thickness using RNAzol (Tel-Test Inc, Friendswood, TX). The first and last tissue section of the series was stained with hematoxylin and eosin (H&E) for histological evaluation.

All samples were treated with DNase. Since the viral sequences of interest lack introns, cDNA synthesis reactions were performed with (+) and without (−) addition of the reverse transcriptase enzyme according to standard procedures using both oligo(dT) and random hexamers as described before.[22] The results were only interpreted when the (−) sample lacked amplification products. PCR was performed on the equivalent of 125 ng of total RNA for the core protein gene (*gag*) (primers 5′-AGAAGGAAAAGGTCCAGAATTA-3′ AND 5′-AGACTTGTATCTGGCCTCAACT-3′; T_{anneal} = 62°C, 35 cycles), the transmembrane region of the envelope gene (*env*-TM) (primers 5′-GCTGTAGCAGGAGTTGCATTG-3′ and 5′-TAATCGATGTACTTCCAATGGTC-3′; T_{anneal} = 58°C, 35 cycles), and the protease (*prt*) (primers 5′-TACAAGCAGTCTCTCTGCTTC-3′ and 5′-GCATGGTGATTTCCGCACCC-3′; T_{anneal} = 62°C, 35 cycles), resulting in PCR products corresponding to nucleotides 1600 to 2037, 7900 to 8362, and 3124 to 3765, with reference to the HERV-K10(+) sequence,[23] respectively. For RNA quality control, amplification of transcripts of the human hypoxanthine phosphoribosyltransferase gene was performed on the same cDNA batch (primers 244 and 243[24] (T_{anneal} = 62°C, 28 cycles), generating a fragment of 387 bp). All experiments were performed at least twice.

Sequencing and Subcloning

For direct sequencing of the RT-PCR products, the commercially available AmpliCycle kit (Roche Molecular Systems, Inc., Branchburg) was applied, using ^{33}P-labeled primers. Fragment separation was performed on a 6% acrylamid gel (mono:bis = 29:1). All experiments were performed twice.

Amplification products were subcloned by TA cloning, using a prokaryotic TA cloning kit (Invitrogen, San Diego, CA).

Results

We developed a specific RT-PCR to detect expression of the HERV-K genes *gag*, *env*, and *prt*. The primers for amplification of the *gag* and *env* sequences were designed such that the amplification products show overlap with at least one of the probes used for mRNA *in situ* hybridization as described by Herbst et al.[20] Using this approach, we studied samples of normal testicular parenchyma; pathological parenchyma with and without CIS; and invasive TGCTs of different histologies, including seminoma, embryonal carcinoma, yolk sac tumor, teratoma, and nonseminomatous TGCT with mixed histologies. Representative examples of the different histologies included in this study are demonstrated in Figure 1. Representative examples of RT-PCR analysis for the *gag* and *env* transcripts are given in Figure 2, and the results are summarized in Table 1. To exclude DNA contamination, all RNA samples were pretreated with DNase before cDNA synthesis (see Materials and Methods), which is crucial because the HERV-K genes lack introns. Absence of DNA was checked by RT-PCR analysis of the samples with and without reverse transcriptase treatment. Because no amplification products were observed in the samples without reverse transcriptase (not shown), the PCR products in the samples treated with reverse transcriptase indeed represent RNA. All samples were positive for the *prt* and *gag* transcripts. Although our method does not allow a quantitative interpretation of the levels of expression, there seems to be a tendency toward higher expression of the *gag* gene in invasive TGCTs and in testicular parenchyma containing CIS compared with normal testicular parenchyma. Because of the possible decline in RNA quality of samples obtained from autopsies, we also tested a parenchyma sample adjacent to a Leydig cell tumor, which was immediately snap frozen upon surgical removal, like the other pathological specimens. This sample showed the same level of expression as normal parenchyma, which also demonstrates that the level of expression is not related to the presence of a

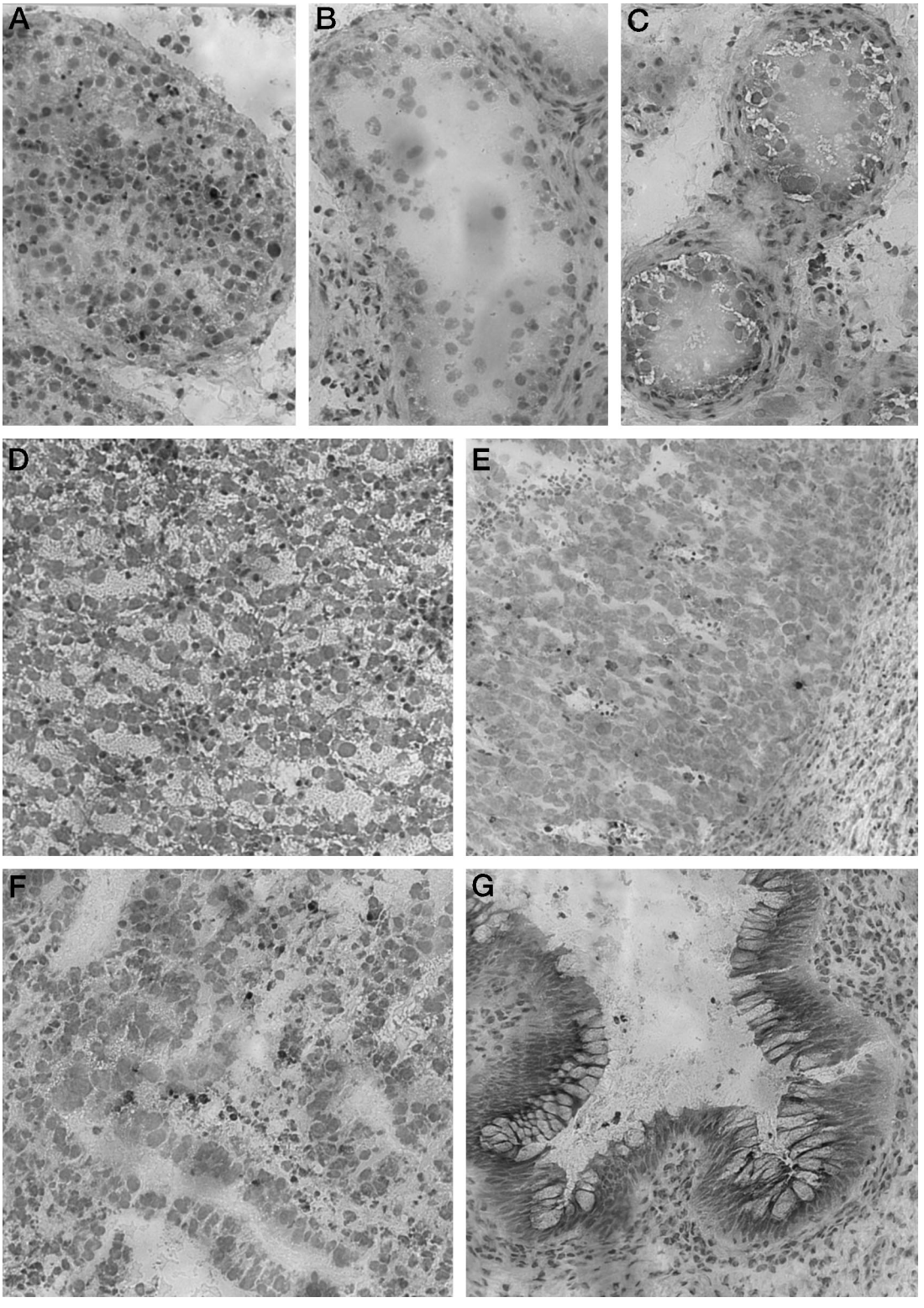

Figure 1. Representative examples of the various histologies included in this study, stained with H&E: normal testicular parenchyma (A), atrophic testicular parenchyma (B), testicular parenchyma with CIS (C), seminoma (D), embryonal carcinoma (E), yolk sac tumor (F), and mature teratoma (G). Magnification, ×160.

malignancy in that testis *per se*. Testicular parenchyma adjacent to a spermatocytic seminoma[25] showed a somewhat higher expression, which might be due to the presence of intratubular spermatocytic seminoma demonstrated in this sample. *env* transcripts were detected in all testicular parenchyma samples containing varying amounts of CIS, as well as all invasive TGCTs, except for the three pure teratomas. These transcripts were also found in the majority of normal testicular parenchyma samples.

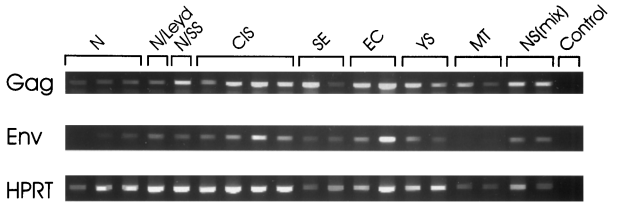

Figure 2. Representative examples of RT-PCR analysis of expression of the *gag* and *env* genes in normal testicular parenchyma (N), parenchyma adjacent to a Leydig cell tumor (N/Leyd) and a spermatocytic seminoma (N/SS), as well as parenchyma adjacent to a testicular germ cell tumor of adolescents and adults containing CIS. Also shown is RT-PCR analysis of *gag* and *env* expression in invasive tumor, with the histology of seminoma (SE), embryonal carcinoma (EC), yolk sac tumor (YS), mature teratoma (MT), and mixed nonseminomas (NS (mix)). In addition, a negative (H_2O) control is indicated. As RNA quality control, expression of the human hypoxanthine phosphoribosyltransferase (HPRT) is shown.

The identity of the amplification products was confirmed by direct sequencing. Specifically, the expressed *gag* sequences of all four normal testicular parenchyma samples and two seminomas were analyzed, and the *env* sequences of two of the normal parenchyma samples as well as both seminomas were determined. Within the region of the *gag* gene analyzed, a number of base differences were detected in comparison with the published HERV sequence,[23] which are indicated in Figure 3. Moreover, at several positions in this sequence, multiple bases were found, which suggests that several HERV transcripts were present. To test whether expression of specific HERV sequences could provide a basis to develop a strategy for early diagnosis of TGCTs, we sequenced several independent RT-PCR products. The *gag* products of one normal testicular parenchyma sample and one seminoma were cloned, and subsequently five and two individual subclones were sequenced, respectively. Representative findings are shown in Figure 4, demonstrating the presence of different sequences within the various subclones. This finding confirms the simultaneous presence of multiple HERV transcripts, both in normal parenchyma and seminoma. There was, however, no transcript found to be specific for the TGCT that might be suitable as marker for early diagnosis. In addition, the sequence analyses demonstrated that the extra G after position 1749 (indicated by an asterisk in Figure 4), which restores the open reading frame of the *gag* gene,[26] is not specific for TGCTs, but is also present in normal testicular parenchyma (see also Figure 3). In contrast to the *gag* sequences, no differences were found between the expressed *env* sequences determined by us in testicular parenchyma and seminoma and the published sequence.[23]

Table 1. Summary of the Results of RT-PCR Polymerase Chain Reaction-Based Detection of Expression of the *prt*, *gag*, and *env* genes in Normal and Pathological Testicular Parenchyma, as Well as Testicular Germ Cell Tumors of Adolescents and Adults of Various Histologies (Numbers of Positives Per Cases Tested Are Indicated)

Tissue*	*prt*	*gag*	*env*
N	4/4	7/7	5/7
N/Leyd		1/1	1/1
N/SS		1/1	1/1
CIS (low)		2/2	2/2
CIS (high)	3/3	4/4	4/4
SE	5/5	5/5	3/3
EC		3/3	3/3
YS		3/3	3/3
MT		3/3	0/3
NS (mix)		3/3	3/3

*Abbreviations are as indicated in the legend to Figure 2. CIS (low) indicates that fewer than 20% of the seminiferous tubules contain CIS cells, and CIS (high) accounts for more than 50%.

Discussion

Sequences of endogenous retroviruses, which are normally present within the genome, show structural and sequence similarities to exogenous retroviruses. Although a pathogenic potential has been shown for a number of exogenous retroviruses in both mice and humans, this has only been reported in mice for endogenous retroviruses.[27] The pathogenic importance of HERVs is questionable, because these sequences are defective due to multiple termination codons, preventing generation of functional proteins.[28,29] A possible exception is HERV-K, for which intact open reading frames and functional proteins have been identified.[18,26,30,31] Interestingly, proteins encoded by these sequences, in particular *gag*, have been found in TGCTs, and antibodies against *gag* and *env* proteins have been detected in serum of patients with these tumors.[18,19] Expression of HERV-K genes *gag* and *env* in TGCTs was recently confirmed using *in situ* hybridization.[20] This study added the finding that these transcripts are also present in CIS, the precursor of all TGCTs. If expression of these genes is specific for TGCTs, including CIS, they might be a proper target for early diagnosis of this cancer. Because mRNA *in situ* hybridization is not suitable for routine application in a clinical setting, we developed an RT-PCR to detect HERV-K-specific transcripts. Although a more heterogeneous pattern was identified for the *env* gene, we basically found expression of all tested HERV-K genes in normal testicular parenchyma. Therefore, we have to conclude that detection of HERV-K transcripts by means of PCR cannot be used for early diagnosis of TGCTs. Although the quality of RNA from autopsy samples can be argued, this is not of relevance in this particular context, because expression was found. The explanation for the observation that *in situ* hybridization, as performed by Herbst et al,[20] does not reveal expression of the *gag* and *env* genes in normal testicular parenchyma is most likely the lower sensitivity of the technique. This is in agreement with the lower level of expression we detected in normal testicular parenchyma compared with the level in parenchyma containing CIS and most invasive TGCTs. Although mRNA *in situ* hybridization demonstrated rather clear differences in *gag* and *env* expression levels between some histological variants of TGCTs and normal testicular parenchyma, our RT-PCR showed a less differentiated pattern. This can be attributed to the fact that total RNA is used for the latter approach. Whereas the *in situ* approach elegantly demonstrates the number of tran-

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gag  AGCAGGTCAG GTGCTCGTAA GATTACAACC TCAAAAGCAG GTTAAAGAAA ATAAGACCCA ACCGCAAGTA GCCTATCAAT
N1   ********** ****C***** S********* *****N**** ********** ********** *****C**** **Y*******
N2   ********** ****C***** S********* *****H**** ********** ********** *****C**** **Y*******
N3   ----****** ****C***** S********* *****H**** ********** ********** *****C**** **Y*******
N4   ********** ****CT**** C********* **GGGW**** ********** ********** *****C**** **Y*******

Se1  ---------- ---------- ---------- ---------- ---------- ---******* *****C**** **********
Se2  ********** ****C***R* C********* ********** ********** ********** *****C**** ********R*


gag  ACTG-CCGCT GGCTGAACTT CAGTATCGGC CACCCCCAGA AAGTCAGTAT GGATATCCAG GAATGCCCCC AGCACCACAG
N1   ****G**K*C ********** ********** ********** ********** ********** ********** **********
N2   ****G**K*C ********** ********** ********** ********** ********** ****------ ----------
N3   ****G**K*C ********** ********** ********** ********** ********** ********** **********
N4   ****G**K*C ********** ********** ********** ********** ********** ********** **********

Se1  ****G**T*C ********** ********** ********** ********** ********** ********** **********
Se2  ****G**T*C ******R*** ********** ********** ********** ********** ********** **********


gag  GGCAGGGCGC CATACCATCA GCCGC
N1   ********** ******---- -----
N2   ---------- ---------- -----
N3   ********** ******C*** ***TG
N4   ********** ******C*** *****

Se1  ********** ******C*** *****
Se2  ********** ******C*** *****
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Figure 3. Summary of the sequence analyses of the expressed *gag* genes after direct sequencing of the amplification products derived from four independent normal testicular parenchyma samples (N1 to N4) and two seminomas (Se1 and Se2). Note the extra G base after position 1749. Abbreviations used: R = A/G, S = C/G, H = A/C/T, W = A/T, Y = C/T, K = G/T, and N = G/A/T/C.

scripts per cell in their histological context, RT-PCR results in information about the overall level of expression in a particular sample. Tissue samples are composed of a mixture of both malignant and normal host cells. Furthermore, there is usually a vast intrasample variation in expression of HERV-K genes, which might be due to cell cycle-dependent regulation, as suggested before.[20] These factors influence the overall expression level and result in relatively modest differences between the samples. The intensity of an immune response against HERV-K proteins, which is almost invariably found in patients with a TGCT, cannot directly be related to the expression level of their genes, because an immune response is determined by the proteins, not by the transcripts, and the immunogenicity of a protein is not determined by its level of presence only.

It has been estimated that, within the human genome, approximately 50 copies of HERV-K sequences are present.[32] They can differ in sequence and are potentially all expressed. Our sequencing results indeed demonstrate that multiple *gag* sequences are expressed simultaneously, both in TGCTs and normal testicular parenchyma. No conclusion can be drawn about *env*, because the region studied might lack differences between the various genes present. Our data therefore suggest that at least multiple *gag* genes are upregulated in TGCTs.

In agreement with the results described by Herbst et al,[20] we detected no *env*-specific transcripts in TGCTs that are somatically differentiated. However, we still detected expression of *gag* genes in these samples, which indicates that a selection of HERV-K genes is downregulated upon somatic differentiation. Inhibition of expression of genes of this kind in human cells upon somatic differentiation is not unique. For example, a similar observation has been made for LINE-1 sequences. These retrotransposons are only expressed in primary TGCTs and TGCT-derived cell lines with the histology of embryonal carcinoma and/or yolk sac.[33–36] No LINE-1 gene expression has been found in teratomas.[33,34] These data indicate that absence of expression of HERV-K genes in teratoma, in particular *env* (this study), might be a more general phenomenon related to differentiation. In our view, the hypothesis of Herbst et al[20] that teratoma is less closely related to embryonal carcinoma than seminoma, is not correct, which is supported both by clinical and experimental findings.[4,37–39]

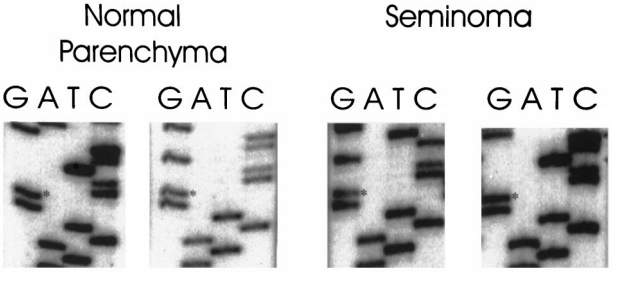

Figure 4. Representative examples of expressed *gag* sequences of one normal testicular parenchyma and one seminoma. Note the difference in sequence of the subclones. In addition, the extra G base after position 1749 of the original sequence is indicated with an asterisk.

An extra single base (G) after position 1749 of the *gag* gene found in a TGCT-derived cell line causes a frame shift necessary to generate a functional protein.[26] In accordance, this particular cell line shows expression of HERV-K genes and produces retrovirus particles. We found this particular G base not only in the *gag* transcripts of different independent TGCTs; it was also present in transcripts from normal testicular parenchyma. These data indicate that generation of a functional *gag* protein is not restricted to TGCTs.

In conclusion, our results show that detection of HERV-K transcripts by means of PCR cannot be applied for early diagnosis of TGCTs. In addition, we demonstrated that multiple HERV-K *gag* genes are expressed simultaneously in normal testicular parenchyma and TGCTs, and that not all HERV-K genes show downregulation upon somatic differentiation. Moreover, our results indicate that the formation of a functional *gag* protein is not restricted to TGCTs.

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