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Structural and Quantitative Expression Analyses of HERV Gene Family in Human Tissues

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Human endogenous retroviruses (HERVs) have been implicated in the pathogenesis of several human diseases as multi-copy members in the human genome. Their gene expression profiling could provide us with important insights into the pathogenic relationship between HERVs and cancer. In this study, we have evaluated the genomic structure and quantitatively determined the expression patterns in the env gene of a variety of HERV family members located on six specific loci by the RetroTector 10 program, as well as real-time RT-PCR amplification. The env gene transcripts evidenced significant differences in the human tumor/normal adjacent tissues (colon, liver, uterus, lung and testis). As compared to the adjacent normal tissues, high levels of expression were noted in testis tumor tissues for HERV-K, in liver and lung tumor tissues for HERV-R, in liver, lung, and testis tumor tissues for HERV-H, and in colon and liver tumor tissues for HERV-P. These data warrant further studies with larger groups of patients to develop biomarkers for specific human cancers.

INTRODUCTION

Human endogenous retroviruses (HERVs) entered genomic DNAs via the infection of germ-line cells by exogenous retroviruses during primate evolution. After infection, they were transmitted from parents to offspring in a Mendelian manner and formed mobile genetic elements in the human genome (Blikstad et al., 2008). Full-length genomic structures of HERVs harbor coding regions for gag, pro, pol, and env proteins. LTR elements exist at the termini of both sides, and primer binding sites (PBS) are located between the sides of the 5′LTR and gag gene. HERVs are commonly categorized by their unique PBS, which is similar to that of one of the human tRNA complement sequences (Tristem, 2000). However, the majority of HERVs in the human genome exist in truncated form, characterized by multiple stop codons, insertions, and deletions (Kim et al., 2004; Ruprecht et al., 2008a). They contribute to genome instability arising via mutation events such as rearrangement and retrotransposition. Thus, HERVs have been previously proposed to perform a function in human pathology (Ruprecht et al., 2008b). Most studies concerning the pathological potential of HERV have attempted to assess the expression of HERV RNA or protein. Recently, env genes of HERV-K, E and ERV3 have been shown to be abundantly expressed in ovarian cancer tissues as compared to normal tissues (Wang-Johanning et al., 2007). In particular, the HERV-K family members harbor full-length intact ORFs, and their protein and particles have been detected in breast cancer (Frank et al., 2008; Golan et al., 2008; Wang-Johanning et al., 2008), as well as testicular cancer (Goedert et al., 1999; Ruprecht et al., 2008b). Evidence collected in previous studies points to the possibility that HERV elements may perform a critical biological function in the oncogenic process leading to the development of specific human tumors.

In this study, we analyzed the genomic structures of a variety of HERVs, and conducted a quantitative analysis of the expression of the HERV env gene located on six specific loci, via real-time RT-PCR amplification using human tumor/normal adjacent tissues.

MATERIALS AND METHODS

Human RNA samples

Human tumor/normal adjacent (lung, uterus, colon, liver, and testis) total RNAs were obtained from Ambion. Total RNAs from normal human tissues (adrenal gland, bone marrow, cerebellum, brain, fetal brain, fetal liver, heart, kidney, liver, lung, placenta, prostate, salivary gland, skeletal muscle, spinal cord, testis, thymus, thymus, thyroid, trachea, and uterus) were purchased from Clontech.

Reverse-transcription reaction

In order to eliminate possible DNA contamination of the purchased RNA samples, Turbo DNA-free™ (Ambion, USA) was utilized in accordance with the manufacturer's instructions. A no-RT control was also amplified in order to double-check for the absence of DNA contamination. The RNA samples were quantitated using a ND-1000 UV-Vis spectrophotometer (NanoDrop, USA). Moloney-Murine Leukemia Virus reverse transcriptase with an annealing temperature of 42°C was utilized for the reverse transcription reaction with RNase inhibitor (Promega, USA).

Real-time RT-PCR amplification

The transcript products were detected via quantitative real-time RT-PCR using the primers HERV-K env: 5′-CACAACTAAA-

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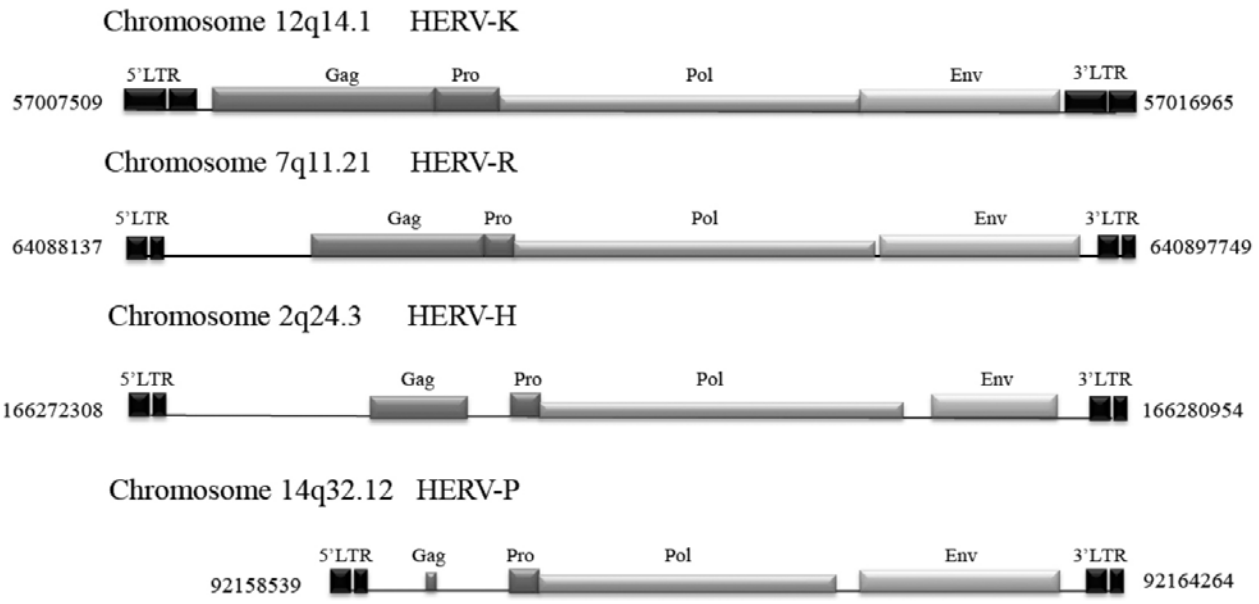

Fig. 1. Structural analysis of HERVs located in specific loci on human chromosomes. The schematic structures were analyzed using HERV sequences with the RetroTector10 program. Those HERVs harbor a 5′LTR-gag-pro-pol-env-3′LTR structure. Structural gene sizes differ depending on the HERV family.

GAAGCTGACG-3′ and 5′-CATAGGCCCAGTTGGTATAG-3′ (GenBank accession no. AC074261 from Chr12q14.1); HERV-R env:5′-CATGGGAAGCAAGGGAACT-3′ and 5′-CTTTCCC-CAGCGAGCAATAC-3′ (GenBank accession no. AC073210 from Chr.7q11.21); HERV-H env:5′-TTCACTCCATCCTTGG-CTAT-3′and 5′-CGTCGAGTATCTACGAGCAAT-3′ (accession no. AJ289711 from Chr.2q24.3);

HERV-P env: 5′-CAAGATTGGGTCCCCTCAC-3′ and 5′-CCTATGGGGTCTTTCCCTC-3′(accession no. DQ247958 from Chr.14q32.12). For normalization, the GAPDH gene was amplified using the primers GPH-QS (5′-GAAGATGGTGAT-GGGA-TTTC-3′) and GPH-QAS (5′-GAAGGTGAAGGTCGGA-GT-3′) from human GAPDH (GenBank accession no. NM_ 002046). The amplification efficiencies and correlation coefficients (R^2) of the env genes were generated using the slopes of the standard curves obtained by serial dilutions. Standard curves with a 10-fold dilution series were employed to calculate the amplification efficiency. The amplification efficiency was calculated by the following formula: efficiency (%) = $(10^{(-1/slope)}-1)*100$. Each primer pair exhibited a single, sharp peak, thereby indicating that the primers amplify only one specific PCR product. No primer dimers were observed.

Real-time RT-PCR amplification was conducted on a Rotor Gene 3000 (Corbett Research, Australia). In each run, 1 μl of cDNA was utilized as a template for amplification per reaction. Real-time RT-PCR was conducted using reactions containing a mixed cDNA template representing a combination of the different types of examined tissues. Additionally, an amplification reaction containing no template control was generated in order to establish non-specific background amplification. The sample was added to 19 μl of reaction mixture containing 7 μl of H_2O, 10 μl of QuantiTect® SYBR® Green PCR Master Mix (Qiagen, Valencia, CA) and 1 μl forward and reverse primers. Real-time RT-PCR amplification for HERVs env and housekeeping genes were conducted for 50 cycles of 94°C for 10 s, 58°C for 15 s, and 72°C for 15 s. Melting curve analysis was conducted for 30 s at 55-99°C. All samples were amplified in triplicate.

RetroTector 10 application

The RetroTector 10 program was employed for the determination of the genomic structure of HERV located on a specific locus of the human genome. It contains three basic modules: first, the detection of candidate long terminal repeats (LTRs); Second, the detection of chains of conserved retroviral motifs fulfilling the distance constraints and the attempted reconstruction of the original retroviral protein sequences; and third, combination of the alignment, codon statistics, and properties of the protein ends (http://www.kvir.uu.se/RetroTector/RetroTector-Project).

RESULTS AND DISCUSSION

In order to determine the HERV gene structure located on specific loci (HERV-K, R, H, P), we used the RetroTector10 program, with their sequences (Fig. 1). The HERV-K on chromosome 12q14.1 contained intact gag, pro, and env genes, but the pol gene containing the RT (reverse transcriptase) domain and the IN (integrase) domain was interrupted. In the case of HERV-R on chromosome 7q11.21 and HERV-H on chromosome 2q24.3, the interrupted ORF between the MA (matrix) and CA (capsid) of gag gene was observed, but the remnant genes harbor intact ORFs. The HERV-P on chromosome 14q32.12 evidenced truncated gag, pro, and pol genes, but not the env gene. Consequently, these HERVs were proposed to comprise an intact env ORF, which may potentially encode for a functional env protein. On the basis of these results, we focused on env gene expression in a variety of human samples, including tumor/normal adjacent tissues. The env gene of HERV-K was predominantly expressed in the kidney and testis among the 20 normal human tissue types, whereas the env gene of HERV-R was abundantly expressed in the placenta. This dominant expression in the placenta tissues was also noted in the HERV-W env gene located on human chromosome 7q21.2 (Kim et al., 2008a; 2008b). The env gene of HERV-H evidenced high expression levels in the fetal brain,

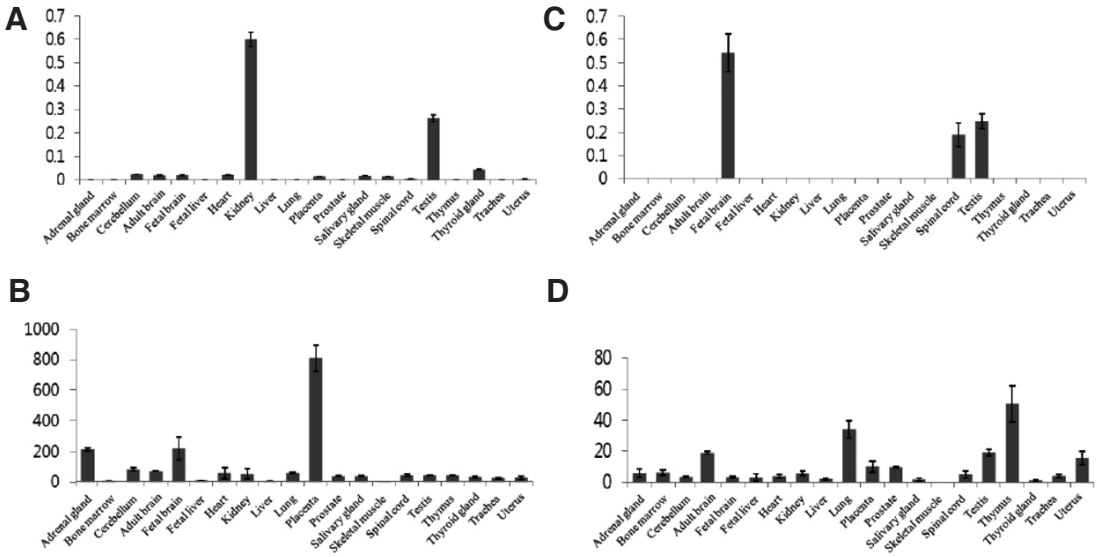

Fig 2. Quantitative real-time RT-PCR analysis of HERV env gene in 20 normal human tissues. The relative expression levels of the HERV env gene were normalized to the expression levels of the GAPDH gene. The error bar indicates the standard deviation. The experiments were repeated three times to ascertain reproducibility of results. The expression level of the HERV-K env gene located on chromosome 12q14.1 (A), HERV-R env gene located on chromosome 7q11.21 (B), HERV-H env gene located on chromosome 2q24.3 (C), HERV-P env gene located on chromosome 14q32.12 (D).

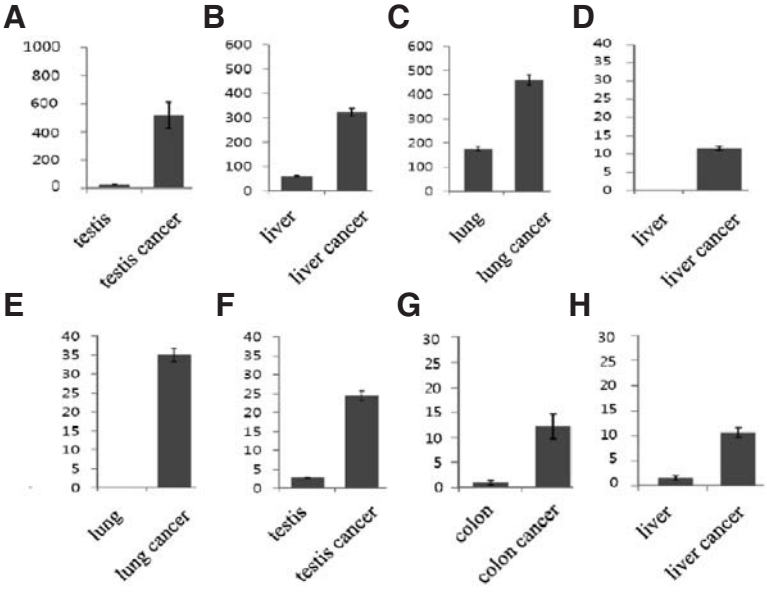

Fig 3. Quantitative real-time RT-PCR analysis between the cancer tissues and adjacent normal tissues in env gene of HERVs. Relative expression levels of the HERV env gene were normalized to the expression levels of the GAPDH gene. The error bar indicates the standard deviation. Experiments were repeated three times to ensure reproducibility of results. Expression level of HERV-K env gene located on chromosome 12q14.1 (A), HERV-R env gene located on chromosome 7q11.21 (B, C), HERV-H env gene located on chromosome 2q24.3 (D, E, and F), and HERV-P env gene located on chromosome 14q32.12 (G, H).

spinal cord, and testis, whereas the env gene of HERV-P was expressed predominantly in the adult brain, lung, and thymus (Fig. 2). These results were consistent with previously reported expression data (Blaise et al., 2005; de Parseval et al., 2003). Therefore, we applied these systems directly to human tumor/normal adjacent tissues in order to conduct a comparative analysis of quantitative expression (Fig. 3). The results of real-time RT-PCR amplification indicated that the tumor tissues evidenced a pattern of high expression as compared to the adjacent normal tissues in various HERV env genes. The HERV-K env gene evidenced abundant expression in testis tumor tissues as compared to the adjacent normal tissues (Fig. 3A), whereas the HERV-R env gene was expressed abundantly in the liver and lung tumor tissues (Figs. 3B and 3C). The HERV-H env gene exhibited abundant expression in the liver, lung, and testis tumor tissues (Figs. 3D, 3E, and 3F), whereas the HERV-P env gene was expressed abundantly in colon and liver tumor tissues as compared to the adjacent normal tissues (Figs. 3G and 3H).

The HERV family has been proposed as a molecular genetic marker for various human cancers (Golan et al., 2008; Kim et al., 2009; Ruprecht et al., 2008b). Their value as genetic markers for tumors has been demonstrated repeatedly. The HERV-K env proteins were shown to be more abundantly expressed in ovarian epithelial tumors than in normal ovarian tissues, as evidenced by the detection of anti-HERV-K-specific antibody via immunohistochemical and ELISA assays (Wang-Johanning

et al., 2006; 2007). HERV-K gag and env transcripts derived from a provirus on chromosome 22q11.21 were selectively packaged into retroviral particles generated by the human germ cell tumor line Tera-1 (Ruprecht et al., 2008a). The HERV-K env proteins were expressed in invasive ductal breast carcino mas (Wang-Johanning et al., 2008). HERV-K-T47D-RT expression was also suggested as a prognostic marker for breast cancer (Golan et al., 2008). Recently, an experiment involving the downregulation of HERV-K expression by RNA interference showed that HERV-K was implicated in the malignant transformation of melanoma cells (Serafino et al., 2009). HERV transcripts have been also detected in normal, benign and malignant tissues and in cell lines of varying origins (Frank et al., 2008; Kim et al., 2006; Yi and Kim, 2004; 2007a; 2007b; Yi et al., 2004a; 2004b; 2006a; 2006b; 2007). Those HERV env genes produced proteins in the *in vitro* transcription-translation assay (de Parseval et al., 2003). Transcription patterns from specific HERV sequences varied among different malignant cell types. The HERV-R env gene showed transcript products in cancer cells--namely, RT4, BT-474, MCF7, OVCAR-3, LOX-IMVI, and AZ521--whereas the HERV-P env gene was expressed in cancer cells--namely, RT4, HCT-116, Jurkat, MCF7, OVCAR-3, PC3, and AZ521--in our previous study (Kim et al., 2006; Yi et al., 2007). Western blots using affinity-purified rabbit antiserum against a HERV-R env oligopeptide showed a 85-kDa band in the Harderian gland of ETR5, which expressed high levels of HERV-R mRNA (Tanaka et al., 2003). The GST-HERV-R env fusion proteins in E. coli BL21 were also detected via SDS-PAGE analysis (Kim et al., 2006). As is shown in Figs. 3D and 3E, no transcripts of the HERV-H env in the adjacent normal liver and lung tissues were detected, but the transcript products appeared in liver and lung tumor tissues. In our previous study, no bands were detected in the normal liver and lung tissues, but HERV-H env gene transcripts were detected in all examined cancer cells except for PFSK-1 (derived from primitive neuroectodermal tumor brain) (Yi et al., 2006b). HERV-H19 harboring a 1752-bp open env reading frame generated a 77-kDa protein in *in vitro* translation reactions, which could be detected in immunoblots using an anti-HA monoclonal antibody (Lindeskog et al., 1999). Likewise, the HERV-W env gene transcripts were detected in all cancer cells (Yi et al., 2004b). The HERV-W env gene was also expressed in astrocytoma tissues and a neuroblastoma cell line (Nellaker et al., 2006). The HERV-W and FRD env proteins (syncytin-1 and 2) exhibited fusogenic activities in several cell types (Blaise et al., 2003; 2005; Chang et al., 2005; Esnault et al., 2008). Although it currently remains to be determined whether this simply represents an epiphenomenon or indicates a sincerely relevant function in the process of tumorigenesis, it continues to be of considerable interest as a potential agent that may contribute to the pathogenesis of tumors or novel disease biomarkers. Additional experiments should be conducted using multiple patient samples in order to develop biomarkers for specific cancers.

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