IDENTIFICATION OF NOVEL INVERTED TERMINAL REPEAT (ITR) DELETIONS OF HUMAN ADENOVIRUS (Ad) FROM INFECTED HOST: VIRULENT Ads CONTAINING MIXED POPULATIONS OF GENOMIC SEQUENCES?

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ABSTRACT

There are a total six different species of human Adenoviruses, designated A through F. All Ads implicated in US military ARD infections are confined within two Ad species, B and E. It was demonstrated and confirmed that the clinical human Ad isolates from natural infections examined in this study carrying random terminal ITR deletions. Whereas, laboratory adapted human Ads including Ad human vaccine strains, such as Wyeth Ad4 and Ad7 strains contain mostly homogeneous and intact ITR sequences. The finding of novel ITR deletions in clinical Ads of natural infections could have significant impacts on the future Ad research and vaccine development.

INTRODUCTION

Acute respiratory disease (ARD) in military personnel is the most significant cause of morbidity, hospitalizations, and work-time loss among military recruits (Gray et al. 1999). There are a total six different species of human Ads, designated A through F. The principal etiological agents for ARD among US military recruits have been Ad4 and Ad7 and occasionally Ad3, Ad21, Ad14 (Hilleman et al. 1954; Berge et al. 1955; Van derVeen et al. 1962; McNeill et al. 1999). All Ads implicated in US military ARD infections are confined within two Ad species, B and E. Ad4 is the sole serotype of specie E, and the rest of Ad3, Ad7, Ad14 and Ad21 belong to subgroup B. Human Ads contain linear, double-stranded DNA genomes with sizes of about 30s kilo-basepairs. All human Ad genomes carry various inverted terminal repeats (ITR) ranging from the 100s to low 200s bps (basepairs) in size (Houng, et al., 2006; Shinagawa, M et al 1980; Shinagawa, M et al 1982). The representative ITRs for human Ad serotypes from different Ad species, such as A (Ad12, Ad18, Ad31), B (Ad3, Ad7, Ad11), and C (Ad1, Ad2, Ad5) are readily available in GenBank databases. Based on most of the available human Ad ITR sequences, the ITRs of the same Ad specie are highly conserved. For instance, the ITRs of Ad2 and Ad5 (specie C) had been determined and shown to contain an identical 103-bp in length and sequence. Similar conserved ITR patterns were also observed for the 137-bp ITRs of specie B (Ad3, Ad7 and Ad11), and the 162-bp ITRs of specie A (Ad12, and Ad18). A novel Ad4 genotype isolated from a recent infected military recruit contains a distinct ITR sequence as compared to prototype Ad4 isolate (Houng, et al., 2006). In contrast to other Human Ad species, Ad4 is the sole serotype in the specie E containing two distinctively different ITR sequences. After the loss of US military adenovirus vaccination program in 1990s, recurrent epidemic outbreaks of human adenovirus-associated acute respiratory diseases (ARD) caused mainly by the new Ad4 genotype and occasionally by other known Ads serotypes of specie B, such as Ad3 and Ad7 among military populations (Gray et al. 2000; Barraza et al. 1999).

The unique ITR feature of Ad allows it to replicate by a strand displacement mechanism from the origins of replication that are located within the ITR at each end of the linear genome. The viral DNA is covalently linked to a terminal protein, pTP that is encoded by an early transcription region, E2B. Ad viral DNA-protein

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complex could act as a replication template using either homologous or heterologous nuclear extract of Ad2 infected cells (Stillman et al, 1982). The homologous Ad-protein complex and Ad infected extract clearly yielded the highest DNA replication activity. These data suggested that the subgroup-specific ITR sequences of Ads play an important role in the initiation of viral replication (Garon et al. 1973). The recently discovered novel Ad4 genotype containing a distinctively different ITR from the Ad4 prototype might represent an emergent pathogen of much higher replication efficiency that has displaced most of the Ad4 prototype in human ARD infections (Houng, et al, 2006; Blasiole et al., 2004; McNeill et al., 1999; McNeill et al., 2000). In parallel, since 1999 there have been only a few known Ad4 prototype isolates containing the classic ITR.

In the past, the Ad ITR DNA sequences were mostly determined by the chemical degradation procedures of Maxam and Gilbert (Shinagawa et al., 1980; Stillman et al, 1982; Tokunaga et al., 1982). A simple DNA sequencing method was recently established to obtain ITR sequences by direct sequencing the PCR product of the ligated circular laboratory adapted Ad genomes (Houng, et al, 2006). The sequencing technique of ligated Ad genomes was used to confirm various laboratory derived Ad4 and Ad7 ITRs sequences showing a typical feature of inverted repeat sequence, complementary palindrome sequence (Houng, et al. 2006). In this study, we observed pre-mature terminations of the DNA sequencing reactions that occur at the ligated Ad DNA junctions of different clinical isolates from natural infections. In addition, we also will contrast the difference in terminal deletions, deletion frequencies among Ads isolates of clinical origins as well as Ads isolates adapted to tissue culturing.

MATERIALS & METHODS

Virus strains: Most of the wild type (clinical) Ad strains used in this study were isolated from US military recruits. These human Ad isolates were maintained and kept either as original throat swabs or low passage viral cultures by the Naval Health Research Center (NHRC) and Walter Reed Army Institute of Research (WRAIR). Direct ITR sequencing: All sequences except those of terminal ITR regions were determined by direct DNA sequencing of Ad PCR products via the Big Dye-terminator DNA sequencing method (Applied Biosystems). In order to obtain the complete ITR sequences of a linear DNA viral genome, the linear viral DNA genome has to be circularized through DNA ligation to allow generation of PCR product containing the ligated terminal junction of the genome. The Ad viral DNA has a terminal protein (TP) that is covalently linked to the 5'-terminus via a phosphodiester bond (Carusi et al, 1977; Roninson et al, 1980). The phenol-extracted viral DNA derived from Ad4 viral culture was separated (de-blocked) from the TP by incubating phenol extracted viral DNA with 0.3 N NaOH at 37 oC overnight (Arrand et al., 1979). After overnight NaOH deblocking, viral DNA was neutralized by adding an equal volume of 0.3 N HCl. The de-blocked Ad4 DNA was then used to form the circular Ad genome molecule through T-4 DNA ligation. The resultant DNA ligation mixture was used as a template for PCR amplification to obtain the ligated junction sequence.

RESULTS

Demonstrating complementary palindrome sequences of ITRs from different laboratory adapted Ad genomes. The ITR sequences of different Ad strains were determined by direct DNA sequencing of the ligated Ad viral preparations as described in Materials & Methods. Fig. 1 shows the complementary palindrome sequence patterns that reflect the joint left and right ITR junctions of the circular Ad4 genomes of Jax78 and Wyeth vaccine strains. For both viruses, the 1st nucleotide of the Ad4 ITR was noticed to be deoxy-C (Stillman et al, 1982). These two viruses also share an identical initial 22 nucleotides sequence starting from the 1st nucleotide of their linear ITR sequences. However, the divergent sequences evolved at the 23rd nucleotide of the ITR. The complete complementary ITR sequences (right and left ends) of Ad4 Jax78 display a perfect 208 bps inverted repeat sequence (GenBank accession AY878642 and AY878643). A shorter complementary 116 bps ITRs of the Wyeth Ad4 vaccine was also confirmed to be consistent with the known Ad4 ITR sequences (GenBank accession AY487947). Except for the identical initial 22 bps ITR sequences, there is poor homology (less than 50%) for the rest of ITR between Ad4 Jax78 and Ad4 Wyeth vaccine.

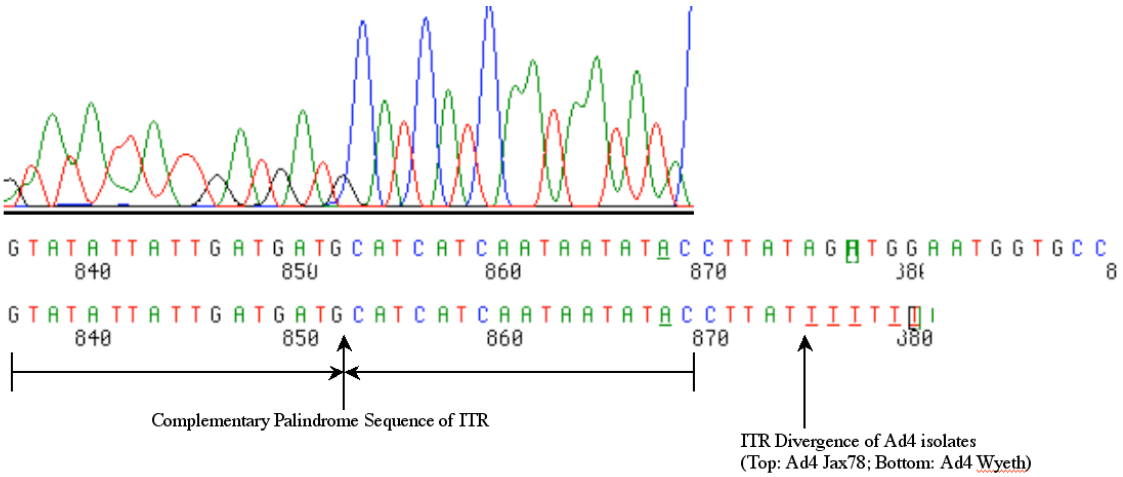

Fig.1. Typical complementary palindrome sequences of human Ads derived from the ligated junction regions of Ad4 Jax78 and Wyeth Ad4. The arrow indicates the ligated terminal junction of the Ad4 ITR. The complementary palindrome sequences for the Ad4 Jax78 (Top sequence) and Wyeth Ad4 vaccine (Bottom sequence) are shown beneath the electrogram derived from ABI 3100 automatic DNA sequencer. Starting from the arrow, the rightward DNA sequences (5'-CATCATCAATAATATA…..) and the leftward DNA sequences (5'-GTAGTAGTTATTATAT……) of these two viruses demonstrate identical complementary palindrome pattern for the initial 22 bp nucleotides. The ITRs divergence between the ITRs for these two viruses begins at the 23rd nucleotide.

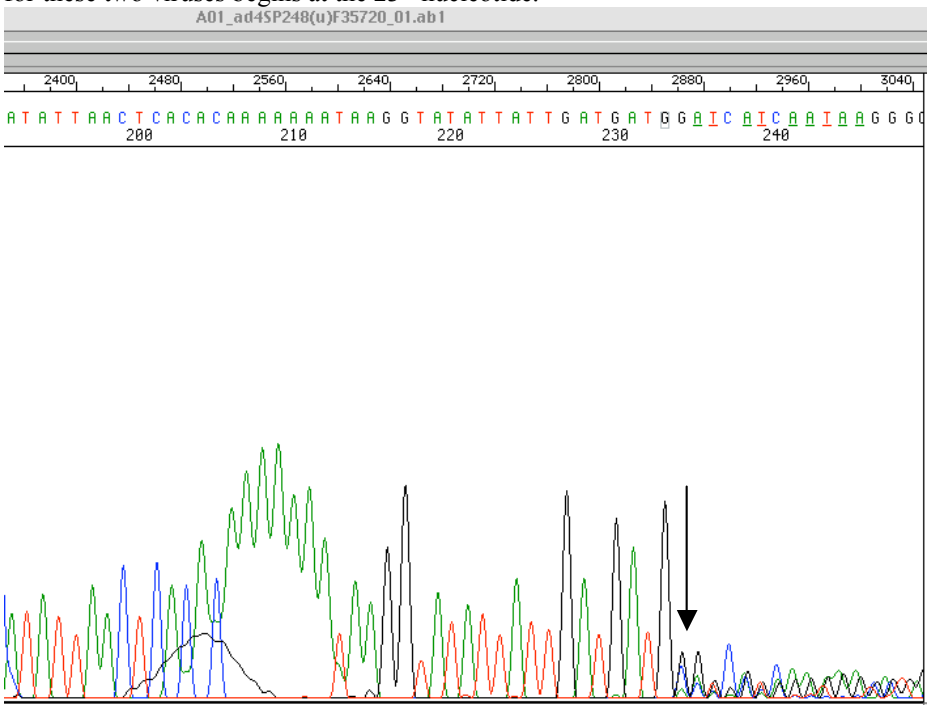

Fig. 2. Impairred complementary palindrome sequence due to premature termination or scrambling of the ligated DNA junction sequence of Ad4 SP248 derived from infected US military recruit in Cape May, NJ. Similar sequencing patterns could be obtained by using either reverse sequencing primer, Ad4R609 of the left Ad4 terminal region or forward sequencing primer, Ad4F35720 of the right Ad4 terminal region showing only half of a typical feature of inverted repeat sequence, complementary palindrome sequence. The arrow indicates the ligated terminal junction of the Ad4 ITR sequences (left-ITR nucleotides) that were scrambled beyond as readable DNA sequence by using Ad4F35720 sequencing primer. Similar pre-mature termination of the ligated DNA junction sequence (right-ITR nucleotides) of Ad4SP248 using Ad4R609 sequencing primer was also proven (data not shown).

Premature termination of DNA sequencing reaction of PCR product derived from ligated Ad genomes of clinical samples. In contrast to the perfect feature of inverted repeat sequences (complementary palindromes) found from the laboratory-adapted Ads, the DNA sequencing reactions derived from clinical Ad4 SP248 genome of natural infections were prematurely terminated or scrambled shown in Fig. 2. It was found that the starting point of DNA sequencing termination for Ad4 SP248 locates at the ligated Ad DNA junction of ITR. In addition to Ad4 SP248, it was further confirmed that other Ad serotyes, such as Ad3 and Ad7, isolated from natural infections contain similar premature termination patterns starting at the ligated Ad genome junctions. Identification of specific ITR deletions from the cloned ligated DNA terminal junctions of clinical Ad4. In order to verify the precise DNA sequence of the proposed ITR deletions of clinical Ad genomes, PCR amplification products of the ligated Ad DNA junctions were randomly cloned into a commercially available PCR cloning vector (TOPO PCR cloning vector, Invitrogen Inc.). The sizes of deletions derived from either the left or right Ad genomic terminus varied widely, ranging from 100 bp (SP248U3) to 500 bp (SP248L1). SP248U1 has a 330 bp deletion starting from the left ITR terminus of Ad4 (data not shown). The ITR deletions identified from this study were physically limited, or restricted to the boundary of the PCR primer pair selected. The maximum terminal deletion size of Ads is unknown.

Conversion of clinical Ad4 containing heterogeneous terminal deletions into homogeneous Ad genomes via repeated passage of virus in A549 cell culture. It was observed that clinical Ad isolates examined in this study including Ad4 and Ad3 contain heterogeneous terminal deletions in contrast to tissue-culture-adapted laboratory Ad strains that carry homogeneous intact ITR terminal sequences. One of clinical Ad4 strain containing heterogeneous ITR deletions, Ad4 SP248, could be transformed into viral stock containing homologous non-deletion ITRs over the course of two consecutive A549 culturing passages.

CONCLUSION

It was found that all clinical human Ads contain heterogeneous ITR deletions. In contrast, all laboratory-adapted human Ads, including vaccine strains such as the Wyeth Ad4 and Ad7 strains, were found to contain homogeneous, non-deletion ITR sequences. The existence of quasi Ad genomic sequences, i.e., ITR deletions were derived from natural infections in human hosts, could be used as a potential biomarker to differentiate the wild type Ads from the laboratory adapted or attenuated Ads, such as Wyeth Ad4 and Ad7 vaccines.

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