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Research Paper

Integration Mapping of *piggyBac*-Mediated CD19 Chimeric Antigen Receptor T Cells Analyzed by Novel Tagmentation-Assisted PCR

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ABSTRACT

Insertional mutagenesis is an important risk with all genetically modified cell therapies, including chimeric antigen receptor (CAR)-T cell therapy used for hematological malignancies. Here we describe a new tagmentation-assisted PCR (tag-PCR) system that can determine the integration sites of transgenes without using restriction enzyme digestion (which can potentially bias the detection) and allows library preparation in fewer steps than with other methods. Using this system, we compared the integration sites of CD19-specific CAR genes in final T cell products generated by retrovirus-based and lentivirus-based gene transfer and by the *piggyBac* transposon system. The *piggyBac* system demonstrated lower preference than the retroviral system for integration near transcriptional start sites and CpG islands and higher preference than the lentiviral system for integration into genomic safe harbors. Integration into or near proto-oncogenes was similar in all three systems. Tag-PCR mapping is a useful technique for assessing the risk of insertional mutagenesis.

1. Introduction

The first successful application of gene therapy, retroviral gene therapy for patients with X-linked severe combined immunodeficiency, resulted in sustained reconstitution of the T cell pool and protection from infections [10]; however, the risk of development of T cell acute lymphoblastic leukemia raised concerns about the safety of gene therapy [11]. A number of approaches have been developed to reduce the potential risk associated with insertional mutagenesis, including systems based on the self-inactivating (SIN) retroviral vector [28] and the SIN lentiviral vector [3], and on non-virus vectors such as the *Sleeping Beauty* [7] and *piggyBac* [31] transposon systems. It is expected that these will provide safer gene transfer for clinical use.

During gene therapy for primary immunodeficiencies, if a vector becomes integrated into a part of the genome that contains a proto-oncogene such as *LMO2*, this can result in the development of leukemia [11]. Because of this, it is important to perform an integration site analysis to assess the potential insertional mutagenesis of a gene therapy. Various polymerase chain reaction (PCR)-based technologies have been developed to determine gene sequences in unknown DNA regions that flank a known sequence; these include inverse PCR [23], ligation-mediated PCR [21], and linear amplification-mediated PCR (LAM-PCR) [27]. However, because these methods use restriction enzymes to fragment the DNA, they can retrieve only a fraction of all the genomic integrations [12]. Nonrestrictive LAM-PCR (nrLAM-PCR), a high-throughput technique that does not involve restriction enzymes, was developed to overcome this drawback; it has been reported to be a comprehensive vector integration mapping method [25]. Target capture sequencing has also been reported to be an accurate, time-saving, and cost-effective method to determine the integration sites of viral vectors in human genes [29]. However, despite their improvements, these methods remain time-consuming. Therefore, we developed a new tagmentation-assisted PCR (tag-PCR) method to analyze the integration

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sites of genetically modified cells, which can analyze the integration sites of transgenes accurately and more comprehensively than restriction enzyme-based methods, and takes smaller number of steps to prepare next-generation sequencing (NGS) libraries because of the small number of sample preparation steps involved.

Therapy using genetically modified CD19-specific chimeric antigen receptor (CAR)-T cells (CD19 CAR-T cells) has been reported as a breakthrough treatment for CD19-positive B-cell lineage hematological malignancies. The initial clinical successes of CD19 CAR-T cell therapy have been achieved by SIN retroviral [2] and SIN lentiviral vector systems [9], but CD19 CAR-T cell engineering using non-viral vectors has also been developed in pre-clinical studies and phase I clinical trials [1,16,19].

In the present study, we applied the new tag-PCR method to the integration mapping of CD19 CAR-T cells to compare the cells produced by a *piggyBac*-mediated system with those from retroviral vector and lentivirus vector systems.

2. Materials and Methods

2.1. Vector Construction

The *piggyBac* transposase plasmid (pCMV-*piggyBac*) has been previously described [14,22,31]. The transposon plasmid for CD19-specific chimeric antigen receptor (pIRII-CAR.CD19) was produced by subcloning FMC63-28z receptor protein gene [17] into *Eco*RI and *Kpn*I-digested pIRII-*piggyBac* transposon vector backbone [22]. pIRII-CAR.CD19 encodes CD19 CAR (FMC63-28z) comprising a single-chain variable fragment from an anti-CD19 antibody derived from the FMC63 mouse hybridoma, a portion of the human CD28 molecule, and the intracellular component of the human T cell receptor ζ molecule. Both vectors are transcriptionally regulated by the cytomegalovirus (CMV) immediate early gene enhancer/promoter sequence.

The CD19-encoding CAR retroviral and lentiviral vectors were produced by subcloning FMC63-28z into SFG retroviral [30] and CSII-

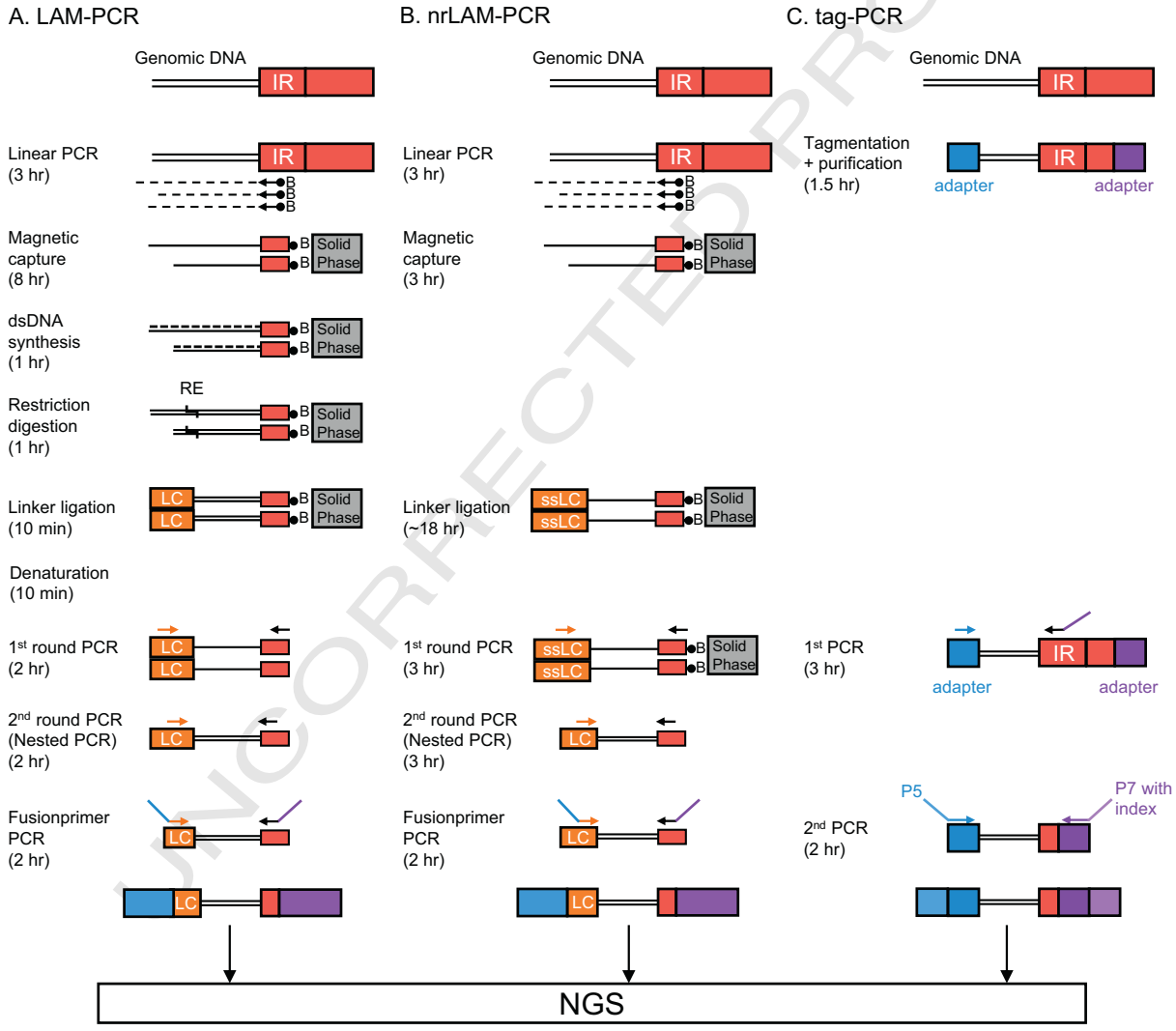

Fig. 1. Schematic outline of the tag-PCR method of integrated site mapping compared with standard LAM-PCR and nrLAM-PCR methods. Linear amplification-mediated polymerase chain reaction (LAM-PCR) (A), and non-restriction LAM-PCR (nrLAM-PCR) (B) start with the amplification of vector–genome junctions using biotinylated primers, hybridizing close to the end of the known vector DNA sequence. Biotinylated single strand (ss)DNA is captured on magnetic particles. LAM-PCR then uses restriction enzyme digestion and linker ligation after double strand (ds)DNA synthesis. The product is amplified by nested PCR with linker- and vector-specific primers. In nrLAM-PCR, the ssDNA linker sequence is directly ligated to the unknown end of the pre-amplified ssDNA following amplification by nested PCR with linker- and vector-specific primers. Additional PCR is performed to add sequencing specific adapters to these (nr) LAM-PCR products. In tag-PCR (C), the tagmentation enzyme randomly fragments genomic DNA and tags the fragments with adapters at both ends. DNA fragments containing the vector–genome junction are amplified with a primer that is complementary to the adapter sequence and the other primer that is complementary to the vector sequence and tagged with the additional adapter sequence. A second PCR is performed to add the complete adapter sequences necessary for sequencing. Finally, the libraries are analyzed by a next-generation sequencer. Estimated durations are indicated alongside each step. IR, inverted repeat; LC, linker cassette; ssLC, single strand linker cassette.

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