

LENTIVIRAL VECTOR APPLICABLE TO GENE THERAPY OF THALASSEMIA AND SICKLE ANEMIA

Technical Field

5 The present invention belongs to the technical field of genetic engineering, and relates to a lentiviral vector, in particular to a lentiviral vector applicable to gene therapy of thalassemia and sickle anemia.

Background Art

10 To date, more than 7000 rare diseases have been found, but only 5% of which have medicines available. At present, almost all medicines for rare diseases require lifelong use. Gene therapy offers the potential of one-time cure for rare diseases. Thalassemia, a monogenic inherited hemoglobinopathy that is the most widespread in the world and affects the largest number of people, is the most common human
15 inherited disease among rare diseases.

 The good safety of gene therapy is a prerequisite for it to become a routine therapy and benefit more patients. Lentiviral vectors are inserted into the genome in an uncontrollable manner (approximately 75% integrated into transcriptionally active gene coding regions) with a higher risk of activating oncogenes and
20 inactivating tumor suppressor genes. Indeed, lentiviral vectors have activated the HMGA2 gene during gene therapy of thalassemia, resulting in the massive proliferation of a transduced hematopoietic stem cell clone. The abnormal proliferation of this clone is caused by an insulator element, namely, the core of chicken beta globin deoxyribonuclease I hypersensitivity site 4 (cHS4), used in a
25 lentiviral gene therapy vector (Cavazzana-Calvo, M. et al. Transfusion Independence and HMGA2 Activation after Gene Therapy of Human Beta-thalassaemia. Nature 467, 318-22 (2010).).

 The insulator element is a genetic element that functions to prevent gene silencing or to prevent the activation of genes in the region in which the insulator
30 element is located. The cHS4 insulator contains a cryptic RNA splicing signal, which results in clonal amplification, affects the safety of gene therapy, and causes a large reduction in lentivirus titer (Cavazzana-Calvo, M. et al. Transfusion Independence and HMGA2 Activation after Gene Therapy of Human Beta-thalassaemia. Nature 467, 318-22 (2010). Negre, O. et al. Preclinical Evaluation of

Efficacy and Safety of an Improved Lentiviral Vector for the Treatment of Beta-thalassemia and Sickle Cell Disease. *Curr Gene Ther* 15, 64-81 (2015).). The insulator element contained in the present invention is derived from a Foamy virus, has an insulator sequence of only 36 bp, does not contain a cryptic RNA splicing signal, and functions to maintain gene expression and prevent the activation of oncogenes in the region in which the insulator element is located.

The specific expression of beta globin gene (HBB) during development is regulated by a locus control region (LCR) located upstream of the HBB (Kim, A. & Dean, A. Chromatin Loop Formation in the Beta-globin Locus and its Role in Globin Gene Transcription. *Mol Cells* 34, 1-5 (2012).). This sequence is up to 16 kb in length, which far exceeds the carrying capacity of lentiviral vectors. To construct a beta globin gene expression cassette for use in gene therapy, it is necessary to select a core element that has an appropriate size and that functions to regulate beta globin gene expression from the LCR region. LCR consists of deoxyribonuclease I (DNase I) hypersensitive sites such as HS2, HS3 and HS4. These hypersensitive sites are still too large in length and must be further reduced for packaging into lentiviral vectors. Since these hypersensitive sites have different functions and must cooperate with each other to exert precise regulatory functions, it is necessary to find a combination that facilitates efficient and specific expression of the beta globin gene. For example, a minilocus control region (miniLCR) consisting of core elements of deoxyribonuclease I hypersensitive sites HS2, HS3 and HS4 results in a significant reduction in viral titer (Weber, L. et al. An Optimized Lentiviral Vector Efficiently Corrects the Human Sickle Cell Disease Phenotype. *Mol Ther Methods Clin Dev* 10, 268-280 (2018).). The screened minilocus of the present invention does not result in a significant reduction in viral titer.

At present, there are two reasons why the technique for treating thalassemia based on lentiviral vectors has not really achieved clinical use and commercialization: firstly, the titer of a therapeutic viral vector produced by the prior art is too low, which results in too large production scale and too expensive production cost, and therefore it is difficult to produce a sufficient amount of viral vectors for clinical use and commercialization; and secondly, after the treatment with a therapeutic viral vector produced by the prior art, the amount of the beta globin expressed is very limited, and therefore it is difficult to achieve a desired therapeutic effect. In the present invention, the vector structure is optimized, and it

has been found that the optimization of intron 2 of beta globin gene (HBB) not only increases gene expression to a certain extent, but also increases the viral packaging yield to a great extent, making the clinical use and industrialization of the lentiviral vector possible.

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Summary

In the prior art, the use of lentiviral vectors for gene therapy of thalassemia and sickle anemia has safety risks and is not very efficient; and in view of the following defects in the prior art, an objective of the present invention is to provide a lentiviral vector applicable to gene therapy of thalassemia and sickle anemia: too low vector titer to reach clinically useful levels; insufficient amount of gene expression, so as not to achieve a therapeutic effect; and lack of an insulator sequence in a vector, so as to easily activate the expression of adjacent genes, having a higher safety risk, etc.

15 The objective of the present invention is achieved by the following technical solutions:

one or more embodiments of the present invention relate to a lentiviral vector comprising a beta globin expression cassette, wherein the expression cassette comprises:

20 a, a minilocus control region, which is a micro regulatory element that is screened from a 16-kb locus control region of a beta globin and does not contain an HS1 region;

b. a gene sequence of the beta globin; wherein intron 2 of the beta globin gene is optimized, and the gene sequence of the beta globin gene is as shown in SEQ ID NO. 2;

25 c, a flanking promoter sequence at the upstream of the gene sequence of the beta globin;

d, a flanking sequence at the downstream of the gene sequence of the beta globin;

e, an insulator sequence from a Foamy virus.

30 As an embodiment of the present invention, the minilocus control region is formed by combining the sequences of deoxyribonuclease I hypersensitive sites HS2, HS3 and HS4 after screening; or is formed by combining the sequences of deoxyribonuclease I hypersensitive sites HS3 and HS2 after screening.

As an embodiment of the present invention, the minilocus control region is a minilocus regulatory element consisting of deoxyribonucleotide sequences truncated from the HS4, HS3 and HS2 regions, respectively, of the locus control region LCR of the beta globin.

5 As an embodiment of the present invention, the sequence of the minilocus regulatory element is as shown in SEQ ID NO. 1.

As an embodiment of the present invention, the promoter sequence is as shown in SEQ ID NO. 4.

10 As an embodiment of the present invention, the flanking sequence is as shown in SEQ ID NO. 5.

As an embodiment of the present invention, the insulator sequence is as shown in SEQ ID NO. 6.

15 The present invention relates to the use of a lentiviral vector comprising a beta globin expression cassette in the preparation of a drug for gene therapy of thalassemia and sickle anemia.

Brief Description of the Drawings

20 By reading the following detailed description made with reference to the drawings for non-limiting embodiments, the other features, objectives and advantages of the present invention will become more apparent:

FIG. 1 is a schematic diagram of the design of vectors; wherein, a is a schematic diagram of the design of LGO4 vector, and b is a schematic diagram of the design of LGO2 vector;

25 FIG. 2 is a schematic diagram of hemoglobin^{T87Q} relative expression after infection with various vectors;

FIG. 3 is a schematic diagram of comparison of the titers of various vectors;

FIG. 4 is a schematic diagram of the number of BFU-E (burst-forming unit-erythroid) formed after infection of stem cells with various vectors.

30 Detailed Description of Embodiments

The present invention will be described in detail below in conjunction with examples. The following examples help those skilled in the art to further understand the present invention, but do not limit the present invention in any way. It should be noted that a person of ordinary skill in the art could also make several adjustments

and improvements without departing from the spirit of the present invention. These would all fall within the scope of protection of the present invention.

Example

This example relates to a human beta globin expression cassette, thereby obtaining a lentiviral vector LGO4 comprising the expression cassette for gene therapy. The design of the LGO4 vector is as shown in FIG. 1(a), and the expression cassette comprises several elements: (1) a minilocus control region (miniLCR), which is a micro regulatory element that is screened from a 16-kb locus control region of the beta globin and does not contain an HS1 region, and in particular a minilocus regulatory element (sequence as shown in SEQ ID NO. 1) with a total length of 3 kb consisting of deoxyribonucleotide sequences with lengths of about 380 bp, 1900 bp and 720 bp truncated from the HS4, HS3 and HS2 regions, respectively, of the 16-kb locus control region (LCR) of the beta globin; (2) a gene sequence of the beta globin; wherein exon 2 contains a T87Q mutation, and the sequence of intron 2 was optimized; the gene sequence of the beta globin is as shown in SEQ ID NO. 2; and the design of LGO2 vector is as shown in FIG. 1(b), and the selected gene sequence of the beta globin is as shown in SEQ ID NO. 3; (3) a 265-bp flanking promoter sequence (sequence as shown in SEQ ID NO. 4) at the upstream of the gene sequence of the beta globin; (4) a 300-bp flanking sequence (sequence as shown in SEQ ID NO. 5) at the downstream of the gene sequence of the beta globin; and (5) placement of the expression cassette in the lentiviral vector; and addition of an insulator sequence (sequence as shown in SEQ ID NO. 6: aaggagacatctagtgtatataagtgtgaactacac) of only 36 bp from a Foamy virus to the LTR U3 region of the lentiviral vector. This insulator sequence has the function of maintaining long-term gene expression and preventing the activation of genes near the integration site without reducing the titer of the lentiviral vector. The sequence of the lentiviral vector comprising a minilocus control region of 3 kb is as shown in SEQ ID NO. 7. The vector plasmid (13 µg) of the present invention, a plasmid (such as 3.75 µg of pMD2G (sequence as shown in SEQ ID NO. 8)) expressing a membrane protein, a plasmid (typically 13 µg of pMDlg/pRRE (sequence as shown in SEQ ID NO. 9)) expressing a GagPol long-chain protein, and a plasmid (typically 3 µg of pRSV-REV (sequence as shown in SEQ ID NO. 10)) expressing a REV protein were co-transfected into 4×10^6 virus-producing cells (such as 293T) to produce a lentivirus LGO4 expressing the beta globin. The design of LGO2 vector

is as shown in FIG. 1(a), the other steps are substantially the same as above (referring to CN 201910824134), so as to obtain a lentivirus LGO2 expressing the beta globin. The vector plasmid of the present invention was replaced with a vector plasmid (pLV/PGK-EGFP, cited document DOI: 10.1093/nar/gkt1163) expressing green fluorescent protein, and the other steps are substantially the same as above, so as to obtain a control viral vector. The lentiviruses were enriched or purified by ultracentrifugation or column chromatography.

Human hematopoietic stem cells were infected in vitro with the same amount of various viral vectors, the differentiation of the stem cells into erythroid cells was induced after the completion of the infection, RNA was extracted from each group of cells on day 14, and the expression level of integrated hemoglobin^{T87Q} mRNA was analyzed by RT-Q-PCR using quantitative fluorescence PCR, which reflected the hemoglobin^{T87Q} relative expression amount (FIG. 2), indicating that the expression amount after the infection with the vector LG04 was the highest.

293T cells were used to package the vector LG02 and vector LG04. After purification, the 293T cells were infected with the vectors respectively, and the viral infectious titers were detected (FIG. 3). The results show that the optimized intron can increase the viral packaging titer.

After infection of human hematopoietic stem cells with the same amount of various viral vectors, the stem cells were seeded into a solid medium containing characteristic cytokines, and after 14 days of culture, and the burst-forming unit-erythroid was counted under a microscope, wherein the colony number represents the differentiation capacity of the stem cells. As shown in FIG. 4, it could be seen that infection with the vector LG04 has substantially no effect on the differentiation capacity of the hematopoietic stem cells.

Compared with the prior art, the present invention has the following beneficial effects:

- 1) a safe and efficient gene therapy for thalassemia and sickle anemia is obtained;
- 2) the expression of the beta globin gene is increased;
- 3) the viral packaging efficiency is improved, and the viral titer is increased;
- 4) the safety of viral vectors is improved, and the safety risk in clinical use is reduced;

5) the insulator sequence from the foamy virus used in the present invention can prevent the activation of genes in the region in which the insulator element is located while maintaining gene expression.

Particular examples of the present invention have been described above. It
5 should be understood that the present invention is not limited to the particular embodiments described above, and that various variations or modifications may be made by those skilled in the art within the scope of the claims without affecting the substantive content of the present invention.

CLAIMS

1. A lentiviral vector comprising a beta globin expression cassette, characterized in that the expression cassette comprises:
 - 5 a, a minilocus control region, which is a micro regulatory element that is screened from a 16-kb locus control region of a beta globin and does not contain an HS1 region;
 - b, a gene sequence of the beta globin, wherein the gene sequence of the beta globin gene is as shown in SEQ ID NO. 2;
 - 10 c, a flanking promoter sequence at the upstream of the gene sequence of the beta globin;
 - d, a flanking sequence at the downstream of the gene sequence of the beta globin;
 - e, an insulator sequence from a Foamy virus.
- 15 2. The lentiviral vector comprising the beta globin expression cassette according to claim 1, characterized in that the minilocus control region is formed by combining the sequences of deoxyribonuclease I hypersensitive sites HS2, HS3 and HS4 after screening.
- 20 3. The lentiviral vector comprising the beta globin expression cassette according to claim 2, characterized in that the sequence of the minilocus regulatory element is as shown in SEQ ID NO. 1.
- 25 4. The lentiviral vector comprising the beta globin expression cassette according to claim 1, characterized in that the promoter sequence is as shown in SEQ ID NO. 4.
- 30 5. The lentiviral vector comprising the beta globin expression cassette according to claim 1, characterized in that the flanking sequence is as shown in SEQ ID NO. 5.

6. The lentiviral vector comprising the beta globin expression cassette according to claim 1, characterized in that the insulator sequence is as shown in SEQ ID NO. 6.

5 7. Use of the lentiviral vector comprising the beta globin expression cassette according to claim 1 in the preparation of a drug for gene therapy of thalassemia and sickle anemia.