

[0056]

In this context, the mutants of the plasmids mean vectors in which the DNA, for example, CD-encoding DNA, incorporated in the plasmids has been mutated, which are plasmids that can be used in the same manner as or more preferably than the unmutated original vectors. For example, a mutant of pBLES100-S-eCD means a mutant of plasmid DNA derived from pBLES100-S-eCD, which is a plasmid that can be used in the same manner as or more preferably than pBLES100-S-eCD in the present invention. Alternatively, a mutant of pAV001-HU-eCD means a mutant of plasmid DNA derived from pAV001-HU-eCD, which is a plasmid that can be used in the same manner as or more preferably than pAV001-HU-eCD in the present invention.

[0057]

Such mutants of the plasmids can be preferably exemplified by a plasmid pAV001-HU-eCD-M968 (SEQ ID NO: 27) which is a plasmid in which a single-nucleotide mutation has been introduced in a CD coding region of the plasmid pAV001-HU-eCD. In the plasmid pAV001-HU-eCD-M968, aspartic acid ~~encoded by a nucleotide sequence from the 2433rd to 2435th nucleotides~~ as the amino acid corresponding to the 315th position in the amino acid sequence shown in SEQ ID NO: 44 28 has been substituted by alanine. As a result, a CD activity is remarkably improved.

[0058]

The gene delivery carrier of the present invention is not particularly limited as long as it is a gene delivery carrier consisting of an anaerobic microorganism transformed with an expression vector having a fragment of a plasmid of a bacterium of the genus Bifidobacterium,

CLAIMS

1. A method of constructing a gene delivery carrier consisting of an anaerobic microorganism capable of growing in a tumor tissue in an anaerobic environment and capable of expressing (A) a protein having an antitumor activity or (B) a protein having an activity of converting an antitumor substance precursor to an antitumor substance, comprising the steps of:

(1) preparing a fusion plasmid having a fragment of a plasmid of a bacterium of the genus *Bifidobacterium* and a fragment of a plasmid of *Escherichia coli*;

(2) incorporating a DNA fragment comprising a promoter and a terminator of a gene encoding a histone-like DNA-binding protein derived from a bacterium of the genus *Bifidobacterium* to the fusion plasmid;

(3) incorporating, between the promoter and the terminator, (a) a DNA encoding a protein having an antitumor activity or (b) a DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance to prepare an expression vector; and

(4) transforming an anaerobic microorganism with the expression vector, wherein

(a) the DNA encoding a protein having an antitumor activity or (b) the DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance, which is incorporated in the step (3) of expression vector preparation further comprises on the 5'-terminal side thereof, a DNA fragment comprising a nucleotide sequence

~~encoding a peptide consisting of an amino acid sequence from the 1st to at least 4th amino acids at the N-terminus of the histone-like DNA-binding protein.~~

2. The method of constructing a gene delivery carrier according to claim 1, wherein the peptide consisting of an amino acid sequence from the 1st to at least 4th amino acids at the N-terminus of the histone-like DNA-binding protein is a peptide consisting of an amino acid sequence from the 1st amino acid to any one of the 4th to 18th amino acids in SEQ ID NO: 29.

3. The method of constructing a gene delivery carrier according to claim 1 or 2, further comprising a step (5) of mutating the DNA (a) encoding a protein having an antitumor activity or the DNA (b) encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance before the step (4).

4. The method of constructing a gene delivery carrier according to claim 3, wherein the step (5) of mutating (a) the DNA encoding a protein having an antitumor activity or (b) the DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance is a step (5') of mutating (a) the DNA encoding a protein having an antitumor activity or (b) the DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance, which has been incorporated in the expression vector prepared in the step (3).

~~5. The method of constructing a gene delivery carrier according to any one of claims 1 to 4,~~

wherein the gene delivery carrier is a gene delivery carrier consisting of an anaerobic microorganism capable of growing in a tumor tissue in an anaerobic environment and capable of expressing a protein having an activity of converting an antitumor substance precursor to an antitumor substance.

6. The method of constructing a gene delivery carrier according to any one of claims 1 to 5,

wherein the protein having an activity of converting an antitumor substance precursor to an antitumor substance is cytosine deaminase.

7. The method of constructing a gene delivery carrier according to claim 6, wherein the gene delivery carrier consisting of an anaerobic microorganism is a cytosine deaminase-expressing 5-fluorouracil-resistant bacterium having a resistance to 5-fluorouracil at a concentration at least effective for an antitumor activity.

8. The method of constructing a gene delivery carrier according to claim 7, wherein the cytosine deaminase-expressing 5-fluorouracil-resistant bacterium having a resistance to 5-fluorouracil at a concentration at least effective for an antitumor activity is prepared using a 5-fluorouracil-resistant bacterium having a resistance to 5-fluorouracil at a concentration at least effective for an antitumor activity as a host.

~~9. The method of constructing a gene delivery carrier according to any one of claims 1 to 8,~~
wherein the DNA fragment comprising a nucleotide sequence encoding a peptide consisting of
an amino acid sequence from the 1st to at least 4th amino acids at the N-terminus of the
histone-like DNA-binding protein is a DNA fragment comprising a nucleotide sequence from
the 1482nd to at least 1493rd nucleotides in SEQ ID NO: 14 or 15.

10. The method of constructing a gene delivery carrier according to claim 9, wherein the DNA
fragment comprising a nucleotide sequence from the 1482nd to at least 1493rd nucleotides in
SEQ ID NO: 14 or 15 is a DNA fragment comprising a nucleotide sequence from the 1482nd
nucleotide to any one of the 1493rd to 1535th nucleotides in SEQ ID NO: 15.

11. The method of constructing a gene delivery carrier according to any one of claims 1 to 10,
wherein the anaerobic microorganism is a bacterium.

12. The method of constructing a gene delivery carrier according to claim 11, wherein the
bacterium is an enteric bacterium.

13. The method of constructing a gene delivery carrier according to claim 12, wherein the
enteric bacterium is a bacterium of the genus Bifidobacterium.

~~14. The method of constructing a gene delivery carrier according to claim 13, wherein the~~
bacterium of the genus *Bifidobacterium* is any bacterium of the genus *Bifidobacterium* selected
from *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium infantis*,
Bifidobacterium thermophilum, *Bifidobacterium pseudolongum*, *Bifidobacterium bifidum*,
Bifidobacterium breve, and *Bifidobacterium longum*.

15. The method of constructing a gene delivery carrier according to claim 13 or 14, wherein the
bacterium of the genus *Bifidobacterium* is *Bifidobacterium longum*.

16. The method of constructing a gene delivery carrier according to any one of claims 1 to 15,
wherein the expression vector used in the transformation in the step (4) is a single-nucleotide
mutation-introduced plasmid of a plasmid pAV001-HU-eCD.

17. The method of constructing a gene delivery carrier according to claim 16, wherein a CD-
encoding nucleotide sequence in the single-nucleotide mutation-introduced plasmid of a
plasmid pAV001-HU-eCD is a nucleotide sequence encoding an amino acid sequence in which
aspartic acid as an amino acid corresponding to the 315th position in an *Escherichia coli* CD
amino acid sequence described in SEQ ID NO: 28 has been substituted by another amino acid.

18. The method of constructing a gene delivery carrier according to claim 17, wherein the
another amino acid is alanine.

~~19. The method of constructing a gene delivery carrier according to claim 18, wherein the single-nucleotide mutation-introduced plasmid of a plasmid pAV001-HU-eCD is a plasmid pAV001-HU-eCD-M968.~~

20. A gene delivery carrier consisting of an anaerobic microorganism transformed with an expression vector having a fragment of a plasmid of a bacterium of the genus *Bifidobacterium*, a fragment of a plasmid of *Escherichia coli*, and a DNA fragment comprising a promoter and a terminator of a gene encoding a histone-like DNA-binding protein derived from a bacterium of the genus *Bifidobacterium*, having between the promoter and the terminator, (a) a DNA encoding a protein having an antitumor activity or (b) a DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance, and further comprising a DNA fragment comprising a nucleotide sequence encoding a peptide consisting of an amino acid sequence from the 1st to at least 4th amino acids at the N-terminus of the histone-like DNA-binding protein, on the 5'-terminal side of (a) the DNA encoding a protein having an antitumor activity or (b) the DNA encoding a protein having an activity of converting an antitumor substance precursor to an antitumor substance.

21. A gene delivery carrier consisting of an anaerobic microorganism being capable of growing in a tumor tissue in an anaerobic environment and having (a) a DNA encoding a protein having an antitumor activity or (b) a DNA encoding a protein having an activity of converting an

~~antitumor substance precursor to an antitumor substance, the gene delivery carrier being~~

constructed by a construction method according to any one of claims 1 to 19.

22. The gene delivery carrier according to claim 20 or 21, wherein the anaerobic bacterium is a bacterium of the genus *Bifidobacterium*.

23. The gene delivery carrier according to claim 22, wherein the bacterium of the genus *Bifidobacterium* is any bacterium of the genus *Bifidobacterium* selected from *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium infantis*, *Bifidobacterium thermophilum*, *Bifidobacterium pseudolongum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium longum*.

24. The gene delivery carrier according to claim 22 or 23, wherein the bacterium of the genus *Bifidobacterium* is *Bifidobacterium longum*.

25. The gene delivery carrier according to any one of claims 20 to 24, wherein the gene delivery carrier is capable of growing in a tumor tissue in an anaerobic environment and is capable of expressing a protein having an activity of converting an antitumor substance precursor to an antitumor substance.

~~26. The gene delivery carrier according to claim 25, wherein the protein having an activity of converting an antitumor substance precursor to an antitumor substance is cytosine deaminase.~~

27. The gene delivery carrier according to claim 26, wherein the gene delivery carrier is a cytosine deaminase-expressing 5-fluorouracil-resistant bacterium having a resistance to 5-fluorouracil at a concentration at least effective for an antitumor activity.

28. The gene delivery carrier according to claim 26 or 27, wherein the gene delivery carrier is a single-nucleotide mutant plasmid of *Bifidobacterium longum* 105-A/pAV001-HU-eCD.

29. The gene delivery carrier according to claim 28, wherein the single-nucleotide mutant plasmid of *Bifidobacterium longum* 105-A/pAV001-HU-eCD is *Bifidobacterium longum* 105-A/pAV001-HU-eCD-M968.

30. A pharmaceutical composition comprising a gene delivery carrier according to any one of claims 20 to 29.

31. A pharmaceutical composition comprising a gene delivery carrier according to any one of claims 25 to 29 in combination with an antitumor substance precursor converted to an antitumor substance by the protein capable of being expressed by the gene delivery carrier.

~~32. The pharmaceutical composition according to claim 31, wherein the gene delivery carrier is a gene delivery carrier according to any one of claims 25 to 29, and the antitumor substance precursor is 5-fluorocytosine.~~

33. A therapeutic agent for solid tumor comprising a gene delivery carrier according to any one of claims 20 to 24 in an amount sufficient for expression of a therapeutically effective amount of a protein having an antitumor activity.

34. A therapeutic agent for solid tumor comprising a gene delivery carrier according to any one of claims 25 to 29 in an amount sufficient for expression of the protein in an amount capable of converting an antitumor substance precursor to a therapeutically effective amount of an antitumor substance, in combination with an antitumor substance precursor converted by the protein capable of being expressed by the gene delivery carrier, in an amount capable of being converted to a therapeutically effective amount of an antitumor substance.

35. The therapeutic agent for solid tumor according to claim 34, wherein the gene delivery carrier is a gene delivery carrier according to any one of claims 25 to 29, and the antitumor substance precursor is 5-fluorocytosine.

CLAIMS

1. A method of constructing a gene delivery carrier consisting of a bacterium of the genus Bifidobacterium transformed with an expression vector, comprising the steps of:

(1) preparing a fusion plasmid having a fragment of a plasmid of a bacterium of the genus Bifidobacterium and a fragment of a plasmid of Escherichia coli;

(2) incorporating a DNA fragment comprising a promoter and a terminator of the gene encoding the histone-like DNA-binding protein derived from a bacterium of the genus Bifidobacterium into the fusion plasmid;

(3) incorporating, between the promoter and the terminator, a DNA encoding cytosine deaminase to obtain the expression vector, wherein the DNA comprises:

(a) a DNA sequence encoding an amino acid sequence which: (i) deletes methionine at position 1; and (ii) replaces aspartic acid at position 315 with alanine from the amino acid sequence set forth in SEQ ID NO: 28; and

(b) a DNA sequence which encodes an amino acid sequence corresponding to positions 1 through at least 4 and up to a maximum of 18 amino acids of the histone-like DNA-binding protein as set forth in SEQ ID NO: 29 and which is linked to (a) on the 5'-terminal side of (a); and

(4) transforming the bacterium of the genus Bifidobacterium with the expression vector.

2. The method according to claim 1, wherein the bacterium of the genus Bifidobacterium is selected from Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium infantis, Bifidobacterium thermophilum, Bifidobacterium pseudolongum, Bifidobacterium bifidum, Bifidobacterium breve, and Bifidobacterium longum.

3. The method according to claim 2, wherein the bacterium of the genus Bifidobacterium is Bifidobacterium longum.

4. The method according to claim 1, wherein the expression vector is a plasmid pAV001-HU-eCD-M968.

5. A gene delivery carrier consisting of a bacterium of the genus Bifidobacterium transformed with an expression vector comprising:

- (i) a fragment of a plasmid of a bacterium of the genus Bifidobacterium;
- (ii) a fragment of a plasmid of Escherichia coli;
- (iii) a DNA fragment comprising a promoter and a terminator of the gene encoding the histone-like DNA-binding protein derived from a bacterium of the genus Bifidobacterium; and
- (iv) a DNA encoding cytosine deaminase positioned between the promoter and the terminator, wherein the DNA comprises:

- (a) a DNA sequence encoding an amino acid sequence which: (1) deletes methionine at position 1; and (2) replaces aspartic acid at position 315

with alanine from the amino acid sequence set forth in SEQ ID NO: 28;

and

(b) a DNA sequence which encodes an amino acid sequence corresponding to positions 1 through at least 4 and up to a maximum of 18 amino acids of the histone-like DNA-binding protein as set forth in SEQ ID NO: 29 and which is linked to (a) on the 5'-terminal side of (a).

6. The gene delivery carrier according to claim 5, wherein the bacterium of the genus Bifidobacterium is any bacterium of the genus Bifidobacterium selected from Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium infantis, Bifidobacterium thermophilum, Bifidobacterium pseudolongum, Bifidobacterium bifidum, Bifidobacterium breve, and Bifidobacterium longum.

7. The gene delivery carrier according to claim 6, wherein the bacterium of the genus Bifidobacterium is Bifidobacterium longum.

8. The gene delivery carrier according to claim 5, wherein the gene delivery carrier is capable of growing in a tumor tissue in an anaerobic environment.

9. The gene delivery carrier according to claim 8, wherein the gene delivery carrier is a cytosine deaminase-expressing 5-fluorouracil-resistant bacterium having a resistance to 5-fluorouracil at a concentration at least effective for an antitumor activity.

10. The gene delivery carrier according to claim 9, wherein the gene delivery carrier is a single-nucleotide mutant plasmid of Bifidobacterium longum 105-A/pAV001-HU-eCD, and wherein the single-nucleotide mutant plasmid of Bifidobacterium longum 105-A/pAV001-HU-eCD is Bifidobacterium longum 105-A/pAV001-HU-eCD-M968.

11. A pharmaceutical composition comprising a gene delivery carrier according to claim 5.

12. A pharmaceutical composition comprising a gene delivery carrier according to claim 8 in combination with 5-fluorocytosine converted to 5-fluorouracil by cytosine deaminase capable of being expressed by the gene delivery carrier.

13. A therapeutic agent for solid tumor comprising a gene delivery carrier according to claim 8 in an amount sufficient for expression of a cytosine deaminase in an amount capable of converting 5-fluorocytosine to a therapeutically effective amount of 5-fluorouracil, in

combination with 5-fluorocytosine converted by the cytosine deaminase capable of being expressed by the gene delivery carrier, in an amount capable of being converted to a therapeutically effective amount of 5-fluorouracil.

14. A cytosine deaminase-highly-expressing unit comprising a DNA fragment comprising:

(i) a promoter and a terminator of the gene encoding the histone-like DNA-binding protein derived from a bacterium of the genus Bifidobacterium; and

(ii) a DNA encoding cytosine deaminase positioned between the promoter and the terminator, wherein the DNA comprises:

(a) a DNA sequence encoding an amino acid sequence which: (1) deletes methionine at position 1; and (2) replaces aspartic acid at position 315 with alanine from the amino acid sequence set forth in SEQ ID NO: 28, and

(b) a DNA sequence which encodes an amino acid sequence corresponding to positions 1 through at least 4 and up to a maximum of 18 amino acids of the histone-like DNA-binding protein as set forth in SEQ ID NO: 29 and which is linked to (a) on the 5'-terminal side of (a).

15. A cytosine deaminase-highly-expressing unit comprising a DNA fragment comprising:

(i) a promoter and a terminator of the gene encoding the histone-like DNA-binding protein derived from a bacterium of the genus Bifidobacterium; and

(ii) a DNA encoding cytosine deaminase positioned between the promoter and the terminator, wherein the DNA comprises:

(a) a DNA sequence encoding an amino acid sequence which: (1) deletes methionine at position 1; and (2) replaces aspartic acid at position 315 with alanine from the amino acid sequence set forth in SEQ ID NO: 28, and

(b) a DNA sequence which encodes the amino acid sequence corresponding to positions 1 through 9 of the histone-like DNA-binding protein as set forth in SEQ ID NO: 29 and which is linked to (a) on the 5'-terminal side of (a).