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(54) **OLIGONUCLEOTIDES FOR GENOTYPING THYMIDYLATE SYNTHASE GENE**

OLIGONUKLEOTIDE ZUR GENOTYPISIERUNG DES THYMIDYLAT-SYNTHASE GENS

OLIGONUCLEOTIDES POUR LE GENOTYPAGE DU GENE DE THYMIDYLATE SYNTHASE

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- **HORIE N ET AL: "FUNCTIONAL ANALYSIS AND DNA POLYMORPHISM OF THE TANDEMLY REPEATED SEQUENCES IN THE 5'-TERMINAL REGULATORY REGION OF THE HUMAN GENE FOR THYMIDYLATE SYNTHASE" CELL STRUCTURE AND FUNCTION, JAPAN SOCIETY FOR CELL BIOLOGY (JSCB), KYOTO, JP, vol. 20, no. 3, June 1995 (1995-06), pages 191-197, XP001008554**

EP 1 546 376 B9

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Description

Technical Field

[0001] The present invention relates to the genotyping of the thymidylate synthase gene. The present invention also relates to the prediction of the responsiveness of a subject towards an antitumor agent based on the thymidylate synthase genotype.

Background Art

[0002] 5-Fluorouracil (5-FU) is a compound that has been utilized as an antitumor agent for a long time (Heidelberger C, Chaudhuri NK, Danenberg PV, Mooren D, et al. (1957) Fluorinated pyrimidines: a new class of tumor inhibitory compounds. Nature 179:663.666). The antitumor effects of 5-FU against various tumors have been reported.

[0003] The cytotoxic effect of 5-FU is based on the inhibition of DNA synthesis in cells. 5-FU inhibit even the DNA synthesis in non-tumor tissue not only that in tumor tissue. However, since usually a far more active DNA synthesis takes place in tumor tissues compared to non-tumor tissues, the manifested influence of the 5-FU-mediated inhibitory action is thought to be comparatively larger in tumor tissues. It is through this mechanism that 5-FU exerts an inhibitory action on tumor tissues.

[0004] On the other hand, the administration of 5-FU, which is a cytotoxic agent, often accompanies adverse effects that cannot be ignored. The cytotoxic effect of 5-FU disables not only tumor tissues, but also non-tumor tissues. 5-FU sensitivity in 5-FU administered patients is considered to be closely related to the magnitude of the adverse effects of the drug.

[0005] 5-FU is a DNA synthesis inhibitor that targets thymidylate synthase. Thymidylate synthase catalyzes the intracellular conversion of deoxyuridylate to deoxy thymidylate. Deoxythymidylate is the only de novo source of thymidylate, an essential precursor for DNA synthesis (Danenberg PV (1997) Thymidylate synthase. a target enzyme in cancer chemotherapy. Biochim Biophys Acta 473:73.92).

[0006] The promoter of thymidylate synthase gene has been demonstrated to be polymorphic (Nobuyuki H, Masahiko C, Ryushi N, Keiichi T (1993) Characterization of the regulatory sequences and nuclear factors that function in cooperation with the promoter of the human thymidylate synthase gene. Biochim Biophys Acta 1216:409.416). Furthermore, it has been shown that the polymorphism of the thymidylate synthase gene promoter i s related to the response of a subject towards 5-FU. Human thymidylate synthase gene has a polymorphism comprising two or three tandem repeats of a 28-bp sequence in its regulatory region. Marsh et al. 1999 (Genomics 58: 310-312) evaluated the influence of ethnicity on the thymidylate synthase promoter enhancer region (TSER) genotype, describing that both TSER genotype and allele frequency were nearly identical in Caucasian and Southwest Asian populations, whereas, in contrast, the TSER genotype was significantly different in Chinese and Caucasian subjects. Kawakami et al. 1999 (Anticancer Research 19: 3249-3252) investigated the association of the thymidylate synthase (TS) genotype with its protein expression in clinical specimens by determining the relationship between the TS polymorphism and the number of 5-fluoro-dUMP binding sites using fresh gastrointestinal cancer tissues, describing that appearance of polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. WO 01/36686 relates to the use of genetic polymorphism to provide individualized therapeutic regimens to treat patients suffering from diseases such as cancer, and discloses in particular methods for screening therapeutic regimens, which comprise determining a patient' s genotype at a 28 base pair region in the thymidylate synthase gene' s 5' -untranslated region. EP 1 207 210 relates to a method for analysis of a target nucleic acid consisting of repetitive and non-repetitive sequences and being present in a sample, wherein the number of repeat sequences is determined by means of melting temperature analysis.

[0007] The expression level of the thymidylate synthase gene which is homozygous for three tandem repeats, is 3.6 times that of the thymidylate synthase gene which is homozygous for two tandem repeats. As a result, subjects carrying the three tandem repeats have significantly fewer adverse effects (Pullarkat, ST, Stoehlmacher J, Ghaderi V, Xiong Y, et al. (2001) Thymidylate synthase gene polymorphism determines clinical outcome of patients with colorectal cancer treated with fluoropyrimidine chemotherapy. Pharmacogenomics J 1:65.70).

[0008] Thymidylate synthase is an important target of not only 5-FU, but also other antitumor agents. For example, capecitabine, which was developed as an oral prodrug of 5-FU, also targets thymidylate synthase. This suggested that the polymorphism in the regulatory region of the human thymidylate synthase gene is a useful marker for determining the responsiveness of a subject towards antitumor agents.

Disclosure of the Invention

[0009] An objective of the present invention is to provide a method for genotyping the thymidylate synthase gene. Especially, the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 1 suitable for genotyping the thymidylate synthase gene is provided. Another objective of the present invention is to provide a method for predicting the responsiveness of a subject towards an antitumor agent that targets thymidylate synthase based on the thymidylate synthase genotype.

[0010] It has been demonstrated that the polymorphism of tandem repeats in the promoter region of the thymidylate synthase gene is related to the responsiveness of a subject against antitumor agents that target thymidylate synthase. Therefore, the effectiveness or the degree of adverse effects of an antitumor agent can be predicted by analyzing this polymorphism. Polymorphism is generally determined by amplifying genomic DNA and analyzing amplicon size. The size of amplicons amplified by PCR is analyzed by gel electrophoresis. However, gel electrophoresis is a laborious and time-consuming analytical technique. Antitumor agents that target thymidylate synthase are important drugs in the chemotherapy of cancer. Therefore, a method that more conveniently yields information regarding the responsiveness of a subject against an antitumor agent that targets thymidylate synthase is desired.

[0011] Extensive research was carried out by the present inventors on a method for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene. As a result, they discovered that the number of tandem repeats in genomic DNA can be identified by using an oligonucleotide having a specific nucleotide sequence as a probe, and detecting mismatches therein. Furthermore, the present inventors confirmed that the genotype of the subject could be determined based on the number of tandem repeats elucidated as above. Furthermore, the present inventors discovered that it is possible to design a strategy for treating a cancer in a patient by relating thymidylate synthase genotype, which is determined by the present invention, with the responsiveness of the subject against antitumor agents targeting thymidylate synthase.

[0012] Namely, the present invention provides the isolated oligonucleotide consisting of the nucleotide sequence of SEQ ID NO:1 that:

(a) is complementary to a region consisting of:

(i) the central repeat unit of three repeat units composing a tandem repeat in the promoter region of the thymidylate synthase gene, and
(ii) the repeat unit located downstream of the central repeat unit, and

(b) hybridizes to the region of (a) under highly stringent hybridization conditions.

[0013] As mentioned earlier, the tandem repeat in the promoter region of the thymidylate synthase gene is polymorphic. Namely, the presence of two kinds of tandem repeats, a tandem repeat consisting of two repeat units, and a tandem repeat consisting of three repeat units, has been elucidated. "Tandem repeat" as mentioned herein refers to a region in which two or more similar nucleotide sequences repeat successively. Similar repeating nucleotide sequences are called repeat units. Generally, the number of repeats is 2 or more. In the present invention, the number of repeats to be identified is 2 and 3. Hereinafter, a polymorphic form in which three repeat units compose a tandem repeat will be referred to as 3R. Furthermore, a polymorphic form in which two repeat units compose a tandem repeat will be referred to as 2R. These polymorphic form nucleotide sequences can be found in a DNA database (3R: GenBank accession number AF279906, 2R: GenBank accession number AF279907). The oligonucleotide of this invention has the nucleotide sequence of SEQ ID NO:1 that is complementary to the nucleotide sequence constituting a region comprising two units of these polymorphic forms, which are the central repeat unit of 3R, and the repeat unit located downstream of the central repeat unit. More specifically, 132-193 of the nucleotide sequence disclosed in GenBank Accession No. AF279906 is the region indicated in the above-mentioned (a). The complementary nucleotide sequences also described herein specifically include the following two nucleotide sequences:

(1) a nucleotide sequence determined to be complementary to a certain nucleotide sequence according to the Watson-Crick rule, or
(2) a nucleotide sequence having a homology of 80% or more with the nucleotide sequence of (1).

[0014] Preferably, (2) includes a nucleotide sequence having a homology of 90% or more, more preferably, 95% or more, and even more preferably, 97% or more with the nucleotide sequence of (1). Algorithms for determining nucleotide sequence homology are well known. For example, programs for calculating nucleotide sequence homology using BLAST are in practical use. These programs can be used via the Internet.

[0015] The present inventors completed this invention by discovering that the two polymorphic forms can be distinguished when the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO:1 having such a nucleotide sequence is hybridized to genomic DNA under the same conditions. That is, the oligonucleotide hybridizes to a 3R tandem repeat, but does not hybridize to 2R under the same conditions.

[0016] Furthermore, the oligonucleotide consisting of SEQ ID NO:1 provided by this invention hybridizes to the region under highly stringent hybridization conditions. In the present invention, "highly stringent hybridization conditions" can be achieved by simultaneously fulfilling the following conditions of (1) and (2). Incidentally, "does not substantially hybridize" means that no hybridization is detected under the same conditions as (1) described below:

(1) the oligonucleotide consisting of SEQ ID NO: 1 hybridizes to the region of (a), and
(2) the oligonucleotide consisting of SEQ ID NO: 1 does not substantially hybridize to the tandem repeat consisting of two repeat units, which is another polymorphic form of the gene.

[0017] In the present invention, the oligonucleotide consisting of SEQ ID NO: 1 hybridizes to the 3' end repeat unit of the two repeat units composing a tandem repeat in the promoter region of the thymidylate synthase gene, under hybridization conditions that are less stringent than (b). The oligonucleotide consisting of SEQ ID NO:1 is useful for the melting curve analysis of the present invention.

[0018] The oligonucleotide consisting of SEQ ID NO:1 fulfilling the above-mentioned conditions is sometimes referred to as the mutation probe in this invention. The repeat units constituting the promoter of the thymidylate synthase gene are not completely identical. The nucleotide sequence of each of the three repeat units composing a tandem repeat in the promoter region of the thymidylate synthase gene is shown below.
5'-ccgcgccacttggcctgcctccgtcccg
ccgcgccacttcgcctgcctccgtcccg
ccgcgccacttcgcctgcctccgtcccccgcccg-3'
Therefore, an oligonucleotide that hybridizes to a specific repeat unit may not hybridize to other repeat units. The oligonucleotide consisting of SEQ ID NO:1 provided by this invention was designed by utilizing such a phenomena. A preferable oligonucleotide of this invention is an oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 1. A method for synthesizing an oligonucleotide having a nucleotide sequence of interest is known to those skilled in the art.

[0019] The oligonucleotide consisting of SEQ ID NO:1 provided by this invention can be used to identify the number of tandem repeats in the promoter region of the thymidylate synthase gene. That is, the present invention relates to a method for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene comprising the steps of:

(a) amplifying a genomic DNA that comprises tandem repeats in at least the promoter region of the thymidylate synthase gene,
(b) hybridizing the oligonucleotide consisting of SEQ
ID NO:1 to the amplified genomic DNA of step (a) under stringent conditions,
(c) detecting a hybridization between the oligonucleotide and the genomic DNA, and
(d) identifying the number of tandem repeats as "two" when a hybridization is not detected, identifying the number of tandem repeats as "three" when a hybridization is detected.

[0020] Preferably, the method of the present invention further comprises:

(e)hybridization of the oligonucleotide consisting of SEQ ID NO:1 to the amplified genomic DNA of step (a) under hybridization conditions that are less stringent than (b),
(f) detecting a hybridization between the oligonucleotide and the genomic DNA, and
(g) identifying the number of tandem repeats as "two" when hybridization is not detected in (c) but is detected in (f).

[0021] In the present invention, genomic DNA can be obtained from a biological sample from a subj ect whose number of tandem repeats in the promoter region of the thymidylate synthase gene is to be identified. For example, a method for obtaining genomic DNA from blood cells collected from a subject is well known. Any method that can amplify DNA in a nucleotide sequence specific manner can be utilized to amplify genomic DNA. Generally, the PCR method is used to amplify genomic DNA. When amplifying DNA, it is sufficient to amplify an arbitrary region containing tandem repeats in at least the promoter region of the thymidylate synthase gene. More specifically, genomic DNA of at least 90 bp that contains tandem repeats can be selected as the region to be amplified. For example, when detecting hybridization by melting curve analysis using LightCycler as described below, the length of the DNA to be amplified is usually 700 bp or less.

[0022] The method of this invention for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene includes the step of hybridizing the mutation probe to the amplified genomic DNA under stringent conditions. Among the polymorphic forms in the promoter region of the thymidylate synthase gene, the mutation probe hybridizes to 3R, but not to 2R. Therefore, using hybridization of the mutation probe as an index, the number of tandem repeats can be determined. To detect the hybridization of the mutation probe, an arbitrary method for detecting DNA hybridization can be used.

[0023] In the present invention, melting curve analysis is the preferred method for detecting differences in nucleotide sequences using DNA hybridization. Certain oligonucleotides hybridize to polynucleotides having complementary sequences. Although DNA hybridization is sequence-specific, it is difficult to completely exclude hybridizations towards very similar nucleotide sequences. Melting curve analysis is a method for detecting changes in hybridization based on changes in melting temperature (Tm). Double strand DNA (dsDNA) formed by hybridization of nucleotide sequences that are complementary to each other, gradually dissociate and become single strand DNA (ss DNA) when the temperature is raised. When the relationship between the change from ds DNA to ss DNA and the change in temperature is plotted on a graph, the change into ss DNA is not linear, and occurs abruptly at a certain temperature. The temperature at which this abrupt change to ss DNA occurs is Tm. Tm changes with various factors such as nucleotide sequence, and composition of the solution in which the DNA exists. However, under specific conditions, Tm clearly changes depending on the nucleotide sequence, when there is a difference in a nucleotide sequence. Therefore, differences in Tm of a certain oligonucleotide towards a target sequence can be detected easily, even if the difference in the target sequence is slight. Melting curve analysis is a method that facilitates sensitive detection of slight differences in nucleotide sequences based on differences in Tm detected as above.

[0024] To carry out the method of this invention based on melting curve analysis, the difference in Tms of the mutation probe towards 3R and 2R can be detected. In melting curve analysis, the hybridization of a mutation probe towards a target sequence must be observed. There are no limitations on the method for observing hybridization. In the present invention, a preferred method for observing hybridization includes the application of fluorescence resonance energy transfer (FRET). FRET is a method for detecting hybridization utilizing the fact that two oligonucleotides that hybridize to adjacent regions on a target sequence come in close proximity to each other due to hybridization. The ends of the two adjacent oligonucleotides are labeled with different fluorophores that function as a donor or an acceptor. When the two come into close proximity due two hybridization, a characteristic fluorescence emission can be detected due to the energy transfer between the fluorophores.

[0025] To apply the FRET to the methods of this invention, a second oligonucleotide that hybridizes to the region adjacent to the mutation probe consisting of SEQ ID NO:1 is necessary for detecting the hybridization of the mutation probe. The present inventors discovered that when using as the mutation probe the oligonucleotide consisting of SEQ ID NO:1 having the aforementioned properties (a) and (b) , an oligonucleotide that can hybridize to the 5' side thereof is useful as the second oligonucleotide. More specifically, also described herein is an isolated oligonucleotide that hybridizes to the region adjacent to the 5' side of the oligonucleotide consisting of SEQ ID NO:1 that:

(a) is complementary to a region consisting of:

(i) the central repeat unit of three repeat units composing a tandem repeat in the promoter region of the thymidylate synthase gene, and
(ii) the repeat unit located downstream of the central repeat unit, and

(b) hybridizes to the region of (a).

Such a second oligonulceotide is applied in the methods of the present invention. In the present invention, "the region adjacent to the 5' side of the oligonucleotide" refers to the 5' side region of the region to which the oligonucleotide hybridizes on the target nucleotide. "Adjacent to" includes the case where the ends of the oligonucleotide and the second oligonucleotide are - 0 to 10 bases apart, and preferably 0 to 5 bases. In the present invention, when using the second oligonucleotide for FRET, it is sometimes called an anchor probe. It is preferred that the Tm of the anchor probe is the same or more than the Tm of the mutation probe towards a 3R tandem repeat. The relationship between genomic DNA and each probe is indicated in Fig. 1.

[0026] Hybridization of the mutation probe can be observed by FRET while performing PCR. That is, hybridization of the mutation probe can be detected while amplifying genomic DNA. To detect hybridization of the mutation probe during PCR, it is preferable to design the Tm of the mutation probe and anchor probe in such a way that hybridization to the amplicon takes place during the annealing phase of PCR. To adjust the Tm to an appropriate range, mismatched bases can be included in the nucleotide sequences of the mutation probe and the anchor probe. Furthermore, it is preferred that the 3' end of each probe is modified to avoid extension of the probes by DNA polymerase. For example, an oligonucleotide labeled at its 5' end with a fluorophore can be modified at its 3' end by phosphorylation.

[0027] An instrument that uses FRET to detect hybridization of the mutation probe during PCR is commercially available. For example, LightCycler(TM) is equipped with the mechanism and software necessary for analyzing a PCR amplicon by FRET. The present invention can be carried out using such an instrument. A specific protocol for carrying out the method of this invention by LightCycler (TM) using the mutation probe and the anchor probe is described below.

[0028] Genomic DNA that comprises tandem repeats in at least the promoter region of the thymidylate synthase gene is amplified with specific primers from human genomic DNA. The amplicon is detected by fluorescence using the mutation probe and the anchor probe as a specific pair of Hybridization Probes. The Hybridization Probes consist of two different short oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the PCR cycle. One probe (mutation probe) is labeled at the 5'-end with LightCycler-Red 640, and to avoid extension, modified at the 3'-end by phosphorylation. The second probe (anchor probe) is labeled at the 3'-end with fluorescein. Only after hybridization to the template DNA do the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the LightCycler Instrument, and part of the excitation energy is transferred to LightCycler-Red 640, the acceptor fluorophore. The emitted fluorescence of LightCycler-Red 640 is then measured by the LightCycler Instrument.

[0029] The oligonucleotide consisting of the nucleotide sequence of SEQ ID NO:1 provided by the present invention is also used to determine the genotype by performing a melting curve analysis after the amplification cycles are completed and the amplicon is formed.

[0030] The fluorescein-labeled oligonucleotides also described herein hybridize to a part of the target sequence that is not mutated and functions as an anchor probe.

[0031] The other oligonucleotide, labeled with Light Cycler-Red640, spans the repeat unit (mutation probe). The latter probe has a lower melting temperature (Tm) than the anchor probe, thus ensuring that the fluorescent signal generated during the melting curve analysis is determined only by the mutation probe. The Tm is not only dependent on the length and G+C content, but also on the degree of homology between the mutation probe and the template DNA. When a 2R type tandem repeat is present, the mismatch of the mutation probe with the target destabilizes the hybrid. With a 3R type tandem repeat, mismatches do not occur, and the hybrid has a higher Tm. The temperature is slowly increased and when the mutation probe melts off and the two fluorescent dyes are no longer in close proximity, the fluorescence will decrease. For mutated genotypes, this will occur at temperatures lower than that for the wildtype genotype. The 5R type tandem repeat of the the thymidylate synthase has been reported recently (Luo HR, Lu XM, Yao YG, Horie N, Takeishi K, Jorde LB, Zhang YP. (2002) Length polymorphism of thymidylate synthase regulatory region in hinese populations and evolution of the novel alleles. Biochem Genet 40(1-2): 41-51). The mutation probe consisting of the nucleotide sequence of SEQ ID NO:1 provided by the present invention will hybridize to the 5R type tandem repeat besides the 3R type one. However, it does not make significant difference whether the probe can distinguish between the 3R type and 5R type. The genotyping and prediction for responsiveness in the present invention can be carried out whenever the probe can distinguish the 2R type that possesses high responsiveness from other polymorphic types..

[0032] As described above, the genotype of the thymidylate synthase gene is elucidatedbased on the number of tandem repeats determined by the present invention. More specifically the present invention provides a method for genotyping the thymidylate synthase gene of a subject, the method comprising:

(a) identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene by the method of present invention, and
(b) determining that the thymidylate synthase genotype of the subject is "homozygous 2R/2R" when the number of tandem repeats is identified as only two, "homozygous 3R/3R" when the number of tandem repeats is identified as only three, or "heterozygous 2R/3R" when the number of tandem repeats is identified as both "two" and "three".

[0033] There is a report that used the LightCycler for genotyping of other genes (Nicolas Von Ahsen et al. Clinical Chemistry 46: 12, 1939-1945 (2000), DNA base bulge vs unmatched end formation in probe-based diagnostic insertion/ deletion genotyping: Genotyping the UGT1A1 (TA)n polymorphism by real-time fluorescence PCR). However, the Light-Cycler has not been used for genotyping the thymidylate synthase gene prior to the present invention.

[0034] Based on the thymidylate synthase genotype elucidated, the responsiveness of a subj ect towards an antitumor agent targeting thymidylate synthase can be predicted. More specifically, the present invention provides a method for predicting the responsiveness of a subject towards an antitumor agent targeting thymidylate synthase, the method comprising:

(a) determining the thymidylate synthase genotype of the subject by the method of the invention, and
(b) associating the thymidylate synthase genotype with the responsiveness of the subject towards an antitumor agent targeting thymidylate synthase.

[0035] In the present invention, "predicting the responsiveness of a subject towards an antitumor agent targeting thymidylate synthase" refers to the prediction of the degree of cytotoxic activity of an antitumor agent targeting thymidylate synthase towards a certain patient and/or a tumor tissue obtained from a patient. As mentioned earlier, thymidylate synthase genotype is a major factor determining the expression level of thymidylate synthase. Furthermore, the expression level of thymidylate synthase is related to the responsiveness of a subj ect towards an antitumor agent targeting thymidylate synthase. That is, the expression level of thymidylate synthase is inversely correlated to the responsiveness. Therefore, the genotype and the responsiveness can be correlated. Specifically, based on the present invention, a subject whose thymidylate synthase genotype has been determined to be homozygous 2R/2R is predicted to have high responsiveness. That is, in this subject, the cytotoxic activity of the antitumor agent targeting thymidylate synthase is predicted to be high. On the other hand, a subject whose genotype has been determined to be 2R/3R heterozygous, or 3R/3R homozygous is predicted to have a normal responsiveness. That is, in this subject, it is predicted that the antitumor agent targeting thymidylate synthase will have a normal cytotoxic activity. "Normal cytotoxic activity" refers to a condition in which the possibility of having severe adverse drug effects is not high when the drug is administered according to a usual administration protocol. Alternately, it refers to a condition in which the inhibitory action of a drug on tumor tissues cannot be expected unless the dose is based on a normal administration protocol.

[0036] In the present invention, the antitumor agent targeting thymidylate synthase includes an antitumor agent having an action to adjust the activity of thymidylate synthase directly or indirectly. One of the modes of action of a 5-FU-type antitumor agent is inhibiting the activity of thymidylate synthase by its metabolite, FdUMP. Thymidylate synthase is the direct target enzyme of 5-FU-type antitumor agents. On the other hand, responsiveness towards Methotrexate used for treating leukemia and such, is also thought to be related to the thymidylate synthase genotype (The Lancet Vol.359, 1033-1034, March 23, 2002). Methotrexate is an inhibitor of dihydrofolate reductase. On the other hand, the reaction catalyzed by thymidylate synthase requires reduction of dihydrofolate. That is, Methotrexate is an antitumor agent that indirectly inhibits thymidylate synthase. The method of this invention allows the prediction of the responsiveness towards such antitumor agents that have indirect inhibitory actions on thymidylate synthase. Examples of antitumor agents for which the responsiveness can be predicted by the method of this invention are 5-FU, Carmofur, Tegafur, UFT, S-1, Doxifluridine, Capecitabine, Fludarabine, Methotrexate, Leucovorin, and Levofolinate.

[0037] Based on responsiveness determined in this manner, a chemotherapy method for for cancer can be designed. More specifically, the present invention relates to a method for determining the dose and/or the type of an antitumor agent that targets thymidylate synthase for treating a cancer patient, the method comprising:

(a) determining the thymidylate synthase genotype of the patient by the method of the present invention, and
(b) for a "homozygous 2R/2R" patient, deciding to: (i) administer an antitumor agent dose that is lower than the normally used dose, or (ii) use an antitumor agent that has a different target.

[0038] For patients predicted to have a high responsiveness towards an antitumor agent that targets thymidylate synthase, lowering the dose of the antitumor agent, or selecting an antitumor agent having a different target is recommended. As a result, the danger of exposing a patient to adverse drug effects can be decreased.

[0039] Additionally, the present invention provides a kit for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene, the kit comprising:

(a) the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 1, and
(b) an oligonucleotide that hybridizes to the region adjacent to the 5' side of the oligonucleotide of SEQ ID NO:1.

[0040] As mentioned earlier, the oligonucleotides constituting the kit of this invention can be labeled with a fluorophore for FRET. Furthermore, additional factors can be combined with the kit of this invention. Examples of additional factors are:

hybridization buffer,
control sample that yields the result of 2R and/or 3R, and
DNA polymerase and substances for PCR.

<u>Brief Description of the Drawings</u>

[0041]

Fig. 1 shows the relationship between tandem repeats of 2R and 3R, and two probes that hybridize to the tandem repeats.

The nucleotide sequences in the figure indicate the anchor probe (top), the tandem repeats of genomic DNA (middle), and the mutation probe (bottom). The sequences of the repeat units are in italics. Each repeat unit is separated by a space. All sequences are shown as the sequence of the sense strand for easy verification of the sequences. In reality, either one of the genomic DNA and each probe is an antisense sequence.

Best Mode for Carrying out the Invention

1) Extraction of DNA

[0042] Genomic DNA was purified from 100 μl of human whole blood. For the purification, GFX™ Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech) was used.

2) Sequences of PCR Primer FW, PCR Primer REV, Hybridization Probe (Anchor), and Hybridization Probe (Mutation):

[0043]

PCR Forward Primer Sequence 5'-GTG GCT CCT GCG TTT CCC C-3'

PCR Reverse Primer Sequence 5'-TCC GAG CCG GCC ACA GGC AT-3'

Hybridization probe (Anchor) Sequence 5'-CGC GGA AGG GGT CCT GCC ACC GCG CCA CTT GGC CTG CCT CGG TCC CGC CG-FITC-3'

Hybridization probe (Mutation) Sequence 5'-LCRed640-CTT GGC CTG CCT CCG TCC CGC CGC GCC-phosphorylation-3'

[0044] Primers were synthesized by SAWADY Technology Co. , Ltd., and probes were synthesized by Nihon Gene Research Lab's, Inc.

3) Preparation of PCR mixture

[0045] LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) was used. The PCR mixture was prepared from the following compositions.

PCR Grade Distilled Water (attached to Kit)	5.4 μl	
10 μM Forward Primer	1 μl (final conc. 0.5 μM)	
10 μM Reverse Primer	1 μl (final conc. 0.5 μM)	
4 pmol/μl Hybridization probe (Anchor)	1 μl	
4 pmol/μl Hybridization probe (Mutation)	1 μl	
25 mM $MgCl_2$ (attached to Kit)	2.4 μl (final conc. 4 mM)	
DMSO	1.2 μl	
Hybridization master mix (attached to Kit)	2 μl	
Human Blood Genomic DNA solution	5 μl	Total volume 20 μl

4) PCR using LightCycler

[0046] Experimental Protocol for PCR using the LightCycler

[0047] Experimental Protocol

Program Segment Number	Denature Temperature Target (°C)	Hold Time (sec)	Type Slope (°C/sec)	None 2* Target Temp (°C)	Step Size (°C)	Cycles Step Delay (Cycles)	1 Acquisition Mode
1	95	300	20	0	0	0	None
Program Segment Number	PCR Temperature Target (°C)	Hold. Time (sec)	Type Slope (°C/sec)	Quantification Temp (°C)	2° Target Step Size (°C)	Cycles Step Delay (Cycles)	33 Acquisition Mode
1	95	15	20	0	0	0	None
2	58	5	20	0	0	0	Single
3	72	12	20	0	0	0	None

(continued)

Program Segment Number	Melting Temperature Target (°C)	Hold Time (sec)	Type Slope (°C/se c)	Melting Curve 2° Target Temp (°C)	Step Size (°C)	Cycles Step Delay (Cycles)	1 Acquisition Mode
1	95	3	20	0	0	0	None
2	77	30	0.5	0	0	0	None
3	70	30	0.2	0	0	0	None
4	56	30	0.2	0	0	0	None
5	95	0	0.1	0	0	0	Continuous

Program Segment Number	Cooling Temperature Target (°C)	Hold Time (sec)	Type Slope (°C/sec)	None 2° Target Temp (°C)	Step Size (°C)	Cycles Step Delay (Cycles)	1 Acquisition Mode
1	40	30	20	0	0	0	None

5) Melting Curve Analysis using LightCycler

[0048] Analysis was performed by using the melting curves program of LightCycler. Fluorescence was set to F2/F1. The "Calculation method" of "Step 1: Melting Peaks" was set to "Linear with Background Correction". To adjust the base line, the cursor at the low temperature side (Green) was set to around 62°C and cursor at the high temperature side was set to around 83°C. To calculate themeltingpeak area, "Step2: PeakAreas" was selected, and the number of peaks were chosen for each sample to obtain the Tm Value, peak area, and standard deviation.

6) Determination

[0049] A sequence whose peak Tm value was only 68-70°C was determined to be 2R/2R homozygous, and a sequence whose peak Tm value was only 76-79°C was determined to be 3R/3R homozygous. A sequence which had both Tm values was determined to be 2R/3R heterozygous.

<u>Industrial Applicability</u>

[0050] An oligonucleotide for genotyping the thymidylate synthase gene is provided. The number of tandem repeats in the promoter region of the thymidylate synthase gene can be identified based on the hybridization of the oligonucleotide consisting of SEQ ID NO:1 to the genomic DNA. The identification based on hybridization is simple and fast compared to gel electrophoresis. Using the oligonucleotide consisting of SEQ ID NO:1 provided by this invention, the number of tandem repeats can be identified easily using mismatches as indexes.

[0051] Therefore, the genotype of the thymidylate synthase gene can be determined based on the number of tandem repeats. The genotype relates to the responsiveness of a subject towards an antitumor agent targeting thymidylate synthase. Therefore, based on the present invention, it is possible to predict the responsiveness towards an antitumor agent targeting thymidylate synthase. Furthermore, based on the responsiveness predicted according to the present invention, a chemotherapy method for cancer can be designed. More specifically, for patients predicted to have a high responsiveness towards an antitumor agent targeting thymidylate synthase, lowering the dose of the antitumor agent, or selecting an antitumor agent having a different target is recommended. As a result, the danger of exposing a patient to adverse drug effects can be reduced.

SEQUENCE LISTING

[0052]

<110> F Hoffmann-La Roche AG

<120> OLIGONUCLEOTIDE FOR GENOTYPING THYMIDYLATE SYNTHASE GENE

<130> RCJ-A0213P

<160> 4

<170> PatentIn version 3.0

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Claims

1. An oligonucleotide consisting of SEQ ID NO: 1.

2. An in vitro method for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene, the method comprising:

   (a) amplifying a genomic DNA that comprises tandem repeats in at least the promoter region of the thymidylate synthase gene;
   (b) hybridizing the oligonucleotide of claim 1 to the amplified genomic DNA of step (a) under stringent conditions;
   (c) detecting a hybridization between the oligonucleotide and the genomic DNA; and
   (d) identifying the number of tandem repeats as "two" when hybridization is not detected, "and" identifying the number of tandem repeats as "three" when hybridization is detected.

3. The method of claim 2, further comprising:

   (e) hybridizing the oligonucleotide of claim 1 to the amplified genomic DNA of step (a) under hybridization conditions that are less stringent than those in step (b);
   (f) detecting a hybridization between the oligonucleotide and the genomic DNA; and
   (g) identifying the number of tandem repeats as "two" when hybridization is not detected in step (c), but is detected in step (f),
   (h) identifying the number of tandem repeats as "three" when hybridization is detected in step (c), but is not detected in step (f),
   (i) identifying the number of tandem repeats as "two" and "three" when hybridization is detected in step (c), and hybridization is detected in step (f).

4. The method of claim 2 or 3, wherein the hybridization is detected by melting curve analysis.

5. The method of claim 4, comprising the step of detecting fluorescence resonance energy transfer using

   (i) the oligonucleotide of claim 1, wherein the 5' end of the oligonucleotide is labeled with a fluorescent dye; and
   (ii) a second oligonucleotide that hybridizes to the region adjacent to the 5' side of the oligonucleotide of (i), wherein the 3' end of the second oligonucleotide is labeled with a different fluorescent dye that transfers fluorescence resonance energy to the fluorescent dye at the 5' end of the oligonucleotide of (i).

6. The method of claim 5, wherein the fluorescent dye that labels the oligonucleotide of (i) is RED640 or RED705, and the fluorescent dye that labels the oligonucleotide of (ii) is FITC.

7. An in vitro method for genotyping the thymidylate synthase gene of a subject, the method comprising:

   (a) identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene by the method of claim 3; and
   (b) determining that the thymidylate synthase genotype of the subject is "homozygous 2R/2R" when the number of tandem repeats is identified as only "two", "homozygous 3R/3R" when the number of tandem repeats is identified as only "three", or "heterozygous 2R/3R" when the number of tandem repeats is identified as both "two" and "three".

8. An vitro method for predicting the responsiveness of a subject towards an antitumor agent targeting thymidylate synthase, the method comprising:

   (a) determining the thymidylate synthase genotype of the subject by the method of claim 7; and

(b) associating the thymidylate synthase genotype with the responsiveness of the subject towards an antitumor agent targeting thymidylate synthase.

9. An in vitro method for determining the dose and/or the type of an antitumor agent targeting thymidylate synthase for treating a cancer patient, the method comprising:

(a) determining the thymidylate synthase genotype of the patient by the method of claim 7; and
(b) for a "homozygous 2R/2R" patient, deciding to:

(i) administer an antitumor agent dose that is lower than the normally used dose; or
(ii) use an antitumor agent that has a different target.

10. A kit for identifying the number of tandem repeats in the promoter region of the thymidylate synthase gene, the kit comprising:

(i) the oligonucleotide of claim 1; and
(ii) a second oligonucleotide that hybridizes to the region adjacent to the 5' side of the oligonucleotide of (i).

11. The kit of claim 10, wherein the 5' end of the oligonucleotide of (i) is labeled with the fluorescent dye RED640 or RED705, and the 3' end of the oligonucleotide of (ii) is labeled with the fluorescent dye FITC.

Patentansprüche

1. Oligonucleotid bestehend aus SEQ ID NO: 1.

2. In vitro-Verfahren zur Identifizierung der Anzahl an Tandemwiederholungen in der Promotorregion des Thymidylatsynthase-Gens, wobei das Verfahren umfasst:

(a) Amplifizieren einer genomischen DNA, welche Tandemwiederholungen mindestens in der Promotorregion des Thymidylatsynthase-Gens umfasst;
(b) Hybridisieren des Oligonucleotids nach Anspruch 1 an die amplifizierte genomische DNA von Schritt (a) unter stringenten Bedingungen;
(c) Nachweisen einer Hybridisierung zwischen dem Oligonucleotid und der genomischen DNA; und
(d) Identifizieren der Anzahl an Tandemwiederholungen als "zwei", wenn keine Hybridisierung nachgewiesen wird, "und" Identifizieren der Anzahl an Tandemwiederholungen als "drei", wenn Hybridisierung nachgewiesen wird.

3. Verfahren nach Anspruch 2, weiterhin umfassend:

(e) Hybridisieren des Oligonucleotids nach Anspruch 1 an die amplifizierte genomische DNA von Schritt (a) unter Hybridisierungsbedingungen, welche weniger stringent sind als die von Schritt (b);
(f) Nachweisen einer Hybridisierung zwischen dem Oligonucleotid und der genomischen DNA; und
(g) Identifizieren der Anzahl an Tandemwiederholungen als "zwei", wenn keine Hybridisierung in Schritt (c) nachgewiesen wird, aber in Schritt (f) nachgewiesen wird;
(h) Identifizieren der Anzahl an Tandemwiederholungen als "drei", wenn Hybridisierung in Schritt (c) nachgewiesen wird, aber in Schritt (f) nicht detektiert wird;
(i) Identifizieren der Anzahl an Tandemwiederholungen als "zwei" und "drei", wenn Hybridisierung in Schritt (c) nachgewiesen wird, und Hybridisierung in Schritt (f) nachgewiesen wird.

4. Verfahren nach Anspruch 2 oder 3, wobei die Hybridisierung durch Schmelzkurvenanalyse nachgewiesen wird.

5. Verfahren nach Anspruch 4, umfassend den Schritt des Nachweisens von Fluoreszenzresonanzenergietransfer unter Verwendung von

(i) des Oligonucleotids nach Anspruch 1, wobei das 5'-Ende des Oligonucleotids mit einem Fluoreszenzfarbstoff markiert ist; und
(ii) eines zweiten Oligonucleotids, welches an die Region angrenzend an die 5'-Seite des Oligonucleotids von

(i) hybridisiert, wobei das 3'-Ende des zweiten Oligonucleotids mit einem anderen Fluoreszenzfarbstoff markiert ist, welcher Fluoreszenzresonanzenergie auf den Fluoreszenzfarbstoff am 5'-Ende des Oligonucleotids von (i) überträgt.

6. Verfahren nach Anspruch 5, wobei der Fluoreszenzfarbstoff, welcher das Oligonucleotid von (i) markiert, RED640 oder RED705 ist, und wobei der Fluoreszenzfarbstoff, welcher das Oligonucleotid von (ii) markiert, FITC ist.

7. In vitro-Verfahren zur Genotypisierung des Thymidylatsynthase-Gens in einer Testperson, wobei das Verfahren umfasst:

(a) Identifizieren der Anzahl an Tandemwiederholungen in der Promotorregion des Thymidylatsynthase-Gens durch das Verfahren nach Anspruch 3; und
(b) Bestimmen, dass der Thymidylatsynthase-Genotyp der Testperson "homozygot 2R/2R" ist, wenn die Anzahl der Tandemwiederholungen als nur zwei identifiziert wird, "homozygot 3R/3R", wenn die Anzahl der Tandemwiederholungen als nur drei identifiziert wird, oder "heterozygot 2R/3R", wenn die Anzahl der Tandemwiederholungen sowohl als "zwei" als auch als "drei" identifiziert wird.

8. In vitro-Verfahren für die Vorhersage der Ansprechempfindlichkeit einer Testperson gegenüber einem Antitumor-Agens, welches gegen die Thymidylatsynthase gerichtet ist, wobei das Verfahren umfasst:

(a) Bestimmen des Thymidylatsynthase-Genotyps der Testperson durch das Verfahren durch Anspruch 7; und
(b) Assoziieren des Thymidylatsynthase-Genotyps mit der Ansprechempfindlichkeit der Testperson gegenüber einem Antitumor-Agens, welches gegen die Thymidylatsynthase gerichtet ist.

9. In vitro-Verfahren zur Bestimmung der Dosis und/oder des Typs eines Antitumor-Agens, welches gegen die Thymidylatsynthase gerichtet ist, für die Behandlung eines Krebspatienten, wobei das Verfahren umfasst:

(a) Bestimmen des Thymidylatsynthase-Genotyps des Patienten durch das Verfahren nach Anspruch 7; und
(b) Entscheiden im Falle eines "homozygoten 2R/2R"-Patienten, dass:

(i) ein Antitumor-Agens in einer Dosis verabreicht wird, welche niedriger ist als die üblicherweise verwendete Dosis; oder
(ii) ein Antitumor-Agens verwendet wird, welches gegen ein anderes Ziel gerichtet ist.

10. Kit zur Identifizierung der Anzahl an Tandemwiederholungen in der Promotorregion des Thymidylatsynthase-Gens, wobei der Kit umfasst:

(i) das Oligonucleotid nach Anspruch 1; und
(ii) ein zweites Oligonucleotid, welches an die Region angrenzend an die 5'-Seite des Oligonucletids von (i) hybridisiert.

11. Kit nach Anspruch 10, wobei das 5'-Ende des Oligonucleotids von (i) mit dem Fluoreszenzfarbstoff RED640 oder RED705 markiert ist, und das 3'-Ende des Oligonucleotids von (ii) mit dem Fluoreszenzfarbstoff FITC markiert ist.

Revendications

1. Oligonucléotide constitué de SEQ ID NO : 1.

2. Procédé in vitro d'identification du nombre de répétitions en tandem dans la région du promoteur du gène de thymidylate synthase, le procédé comprenant :

(a) l'amplification d'un ADN génomique qui comprend des répétitions en tandem dans au moins la région du promoteur du gène de thymidylate synthase ;
(b) l'hybridation de l'oligonucléotide selon la revendication 1 à l'ADN génomique amplifié de l'étape (a) dans des conditions stringentes ;
(c) la détection d'une hybridation entre l'oligonucléotide et l'ADN génomique ; et
(d) l'identification du nombre de répétitions en tandem comme de "deux" lorsque l'hybridation n'est pas détectée,

"et" l'identification du nombre de répétitions en tandem comme de "trois" lorsque l'hybridation est détectée.

3. Procédé selon la revendication 2, comprenant en outre :

(e) l'hybridation de l'oligonucléotide selon la revendication 1 à l'ADN génomique amplifié de l'étape (a) dans des conditions d'hybridation qui sont moins stringentes que ces de l'étape (b) ;
(f) la détection d'une hybridation entre l'oligonucléotide et l'ADN génomique ; et
(g) l'identification du nombre de répétitions en tandem comme de "deux" lorsque l'hybridation n'est pas détectée dans l'étape (c), mais est détectée dans l'étape (f),
(h) l'identification du nombre de répétitions en tandem comme de "trois" lorsque l'hybridation est détectée dans l'étape (c), mais n'est pas détectée dans l'étape (f),
(i) l'identification du nombre de répétitions en tandem comme de "deux" et "trois" lorsque l'hybridation est détectée dans l'étape (c), et l'hybridation est détectée dans l'étape (f).

4. Procédé selon la revendication 2 ou 3, dans lequel l'hybridation est détectée par l'analyse de la courbe de fusion.

5. Procédé selon la revendication 4, comprenant l'étape consistant à détecter un transfert d'énergie de résonance par fluorescence en utilisant

(i) l'oligonucléotide selon la revendication 1, où l'extrémité 5' de l'oligonucléotide est marquée avec un colorant fluorescent ; et
(ii) un second oligonucléotide qui s'hybride à la région adjacente au côté 5' de l'oligonucléotide de (i), où l'extrémité 3' du second oligonucléotide est marquée avec un colorant fluorescent différent qui transfère l'énergie de résonance par fluorescence au colorant fluorescent à l'extrémité 5' de l'oligonucléotide de (i).

6. Procédé selon la revendication 5, dans lequel le colorant fluorescent qui marque l'oligonucléotide de (i) est le RED640 ou le RED705, et le colorant fluorescent qui marque l'oligonucléotide de (ii) est le FITC.

7. Procédé in vitro de génotypage du gène de thymidylate synthase d'un sujet, le procédé comprenant :

(a) l'identification du nombre de répétitions en tandem dans la région du promoteur du gène de thymidylate kinase par le procédé selon la revendication 3 ; et
(b) la détermination que le génotype de la thymidylate synthase du sujet est "homozygote 2R/2R" lorsque le nombre de répétitions en tandem est identifié comme seulement de "deux", "homozygote 3R/3R" lorsque le nombre de répétitions en tandem est identifié comme seulement de "trois" ou "hétérozygote 2R/3R" lorsque le nombre de répétitions en tandem est identifié comme à la fois de "deux" et de "trois".

8. Procédé in vitro de prédiction de la réactivité d'un sujet envers un agent antitumoral ciblant la thymidylate synthase, le procédé comprenant :

(a) la détermination du génotype de la thymidylate synthase du sujet par le procédé selon la revendication 7 ; et
(b) l'association du génotype de la thymidylate synthase avec la réactivité du sujet envers un agent antitumoral ciblant la thymidylate synthase.

9. Procédé in vitro de détermination de la dose et/ou du type d'un agent antitumoral ciblant la thymidylate synthase pour traiter un patient cancéreux, le procédé comprenant :

(a) la détermination du génotype de la thymidylate synthase du sujet par le procédé selon la revendication 7 ; et
(b) pour un patient "homozygote 2R/2R", de décider :

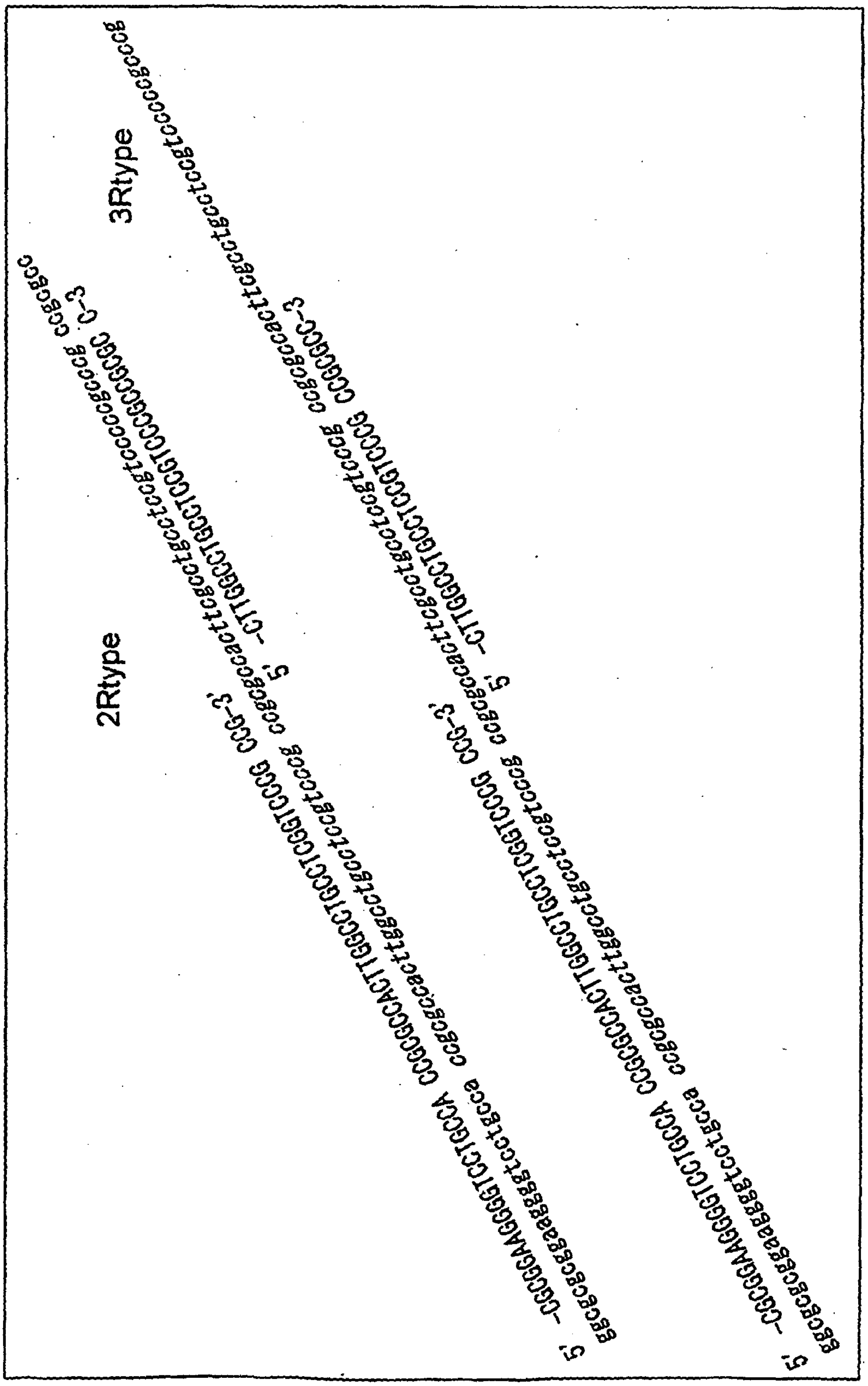
(i) d'administrer une dose d'agent antitumoral qui est inférieure à la dose normalement utilisée ; ou
(ii) d'utiliser un agent antitumoral qui a une cible différente.

10. Kit pour identifier le nombre de répétitions en tandem dans la région du promoteur du gène de thymidylate synthase, le kit comprenant :

(i) l'oligonucléotide selon la revendication 1 ; et
(ii) un second oligonucléotide qui s'hybride à la région adjacente au côté 5' de l'oligonucléotide de (i).

11. Kit selon la revendication 10, où l'extrémité 5' de l'oligonucléotide de (i) est marquée avec le colorant fluorescent RED640 ou RED705, et l'extrémité 3' de l'oligonucléotide de (ii) est marquée avec le colorant fluorescent FITC.

Fig. 1



REFERENCES CITED IN THE DESCRIPTION

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