In light of the foregoing, the well-designed detection tools targeting the aforesaid V384D mutation shall be helpful and valuable medical industry and clinical use. Although several researches have presented some primers and probes for the aforesaid purpose, the goal of the present invention is to provide a primer set and/or a probe being more specific and sensitive than the conventional ones.

SUMMARY

In light of the foregoing, one of the objects of the present invention is to provide a kit for cancer detection by using a promising biomarker.

Another object of the present invention is to provide a kit for evaluating the efficacy of an EGFR-TKI treatment and/or the progression-free survival of a subject after the same so that the follow-up treating strategies can be set up as soon as possible after an EGFR-TKI treatment.

In order to achieve the above-mentioned objects, the present invention provides a kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject, comprising:

a first primer comprising SEQ ID NO 06; and a second primer comprising SEQ ID NO 07; and/or

a first primer comprising SEQ ID NO 08; and a second primer comprising SEQ ID NO 09.

Preferably, said kit further comprises a third primer comprising SEQ ID NO 05.

Preferably, said kit of claim 1, further comprising a DNA polymerase.

Preferably, said kit further comprises a double strands DNA binding dye.

Preferably, said double strands DNA binding dye is SYBR® Green I, SYBR® Green II, SYBR® Gold, Oxazole Yellow, Thiazole Orange, PicoGreen, EvaGreen®, a combination thereof.

The present invention also provides a kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject, comprising:

a first primer comprising SEQ ID NO 08; and

a second primer comprising SEQ ID NO 09.

The present invention also provides kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject, comprising:

at least one mutation probe comprising SEQ

ID NO 10; wherein said probe is conjugated with a fluorophore; and/or

at least one wild-type probe comprising SEQ ID NO 11; wherein said probe is conjugated with a fluorophore.

Preferably, said at least one mutation probe and/or said at least one wild-type probe is further conjugated with a quencher. Preferably, said fluorophore is conjugated at the 5' end of said probe and said quencher is conjugated at the 3' end of said probe. Preferably, said at least one mutation probe comprises SEQ ID NO.17. Preferably, said at least one wild-type probe comprises SEQ ID NO.16.

Preferably, said at least one mutation probe and/or at said at least one wild-type probe comprises at least one nucleotide thereof being modified with a bridge connecting the 2' oxygen and 4' carbon thereof. Preferably, said mutation probe comprises SEQ ID NO 19 having the following sequence:

5'-CACCAGATGGATCGTACA-3'.

Preferably, said mutation probe has the 5th, 8th, 11th, and 14th nucleotides thereof counted from 5' to 3' being modified with a bridge connecting the 2' oxygen and 4' carbon.

Preferably, said wild-type probe comprises SEQ ID NO 18 having the following sequence:

5'- CACCAGATGGTTCGTACA -3'.

Preferably, said mutation probe has the 5th, 8th, 11th, and 14th nucleotides thereof counted from 5' to 3' being modified with a bridge connecting the 2' oxygen and 4' carbon.

Preferably, said kit further comprises an anchor probe conjugated with a donor fluorophore. Preferably, said at least one mutation probe comprises SEQ ID NO.21 and is conjugated with a receptor fluorophore. Preferably, said at least one wild-type probe comprises SEQ ID NO.20 and is conjugated with a receptor fluorophore. Preferably, said anchor probe comprises SEQ ID NO. 22

Preferably, said kit further comprises a primer set for amplifying at least a part of said *MLH1* gene to obtain an amplicon; wherein said amplicon comprises a site corresponding to said V384 alteration.

Preferably, said primer set is:

a forward primer comprising SEQ ID NO 12, and a reverse primer comprising SEQ ID NO 13; and/or

a forward primer comprising SEQ ID NO 14, and a reverse primer comprising SEQ ID NO 15.

Preferably, said kit further comprises a DNA polymerase.

To sum up, the present invention provides a novel primer set or a probe for identifying a V384 alteration of a *MLH1* gene and a kit having the same. By using the primer set and/or the kit of the present invention, the identification of the V384 alteration can be performed more specifically and sensitively than the conventional tools.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure. 1 shows the results of the Real-time polymerase chain reaction of the Example 1 of the instant specification (n=2); wherein the results of primer set of **SEQ ID NO 07** and **SEQ ID NO 05** were marked as curve (1), the results of primer set of **SEQ ID NO 07** and **SEQ ID NO 06** were marked as curve (2), and the results of negative control were marked as curve (3). (A) Genomic DNA of a subject without V384D mutation. (B) Genomic DNA of a subject with V384D mutation (heterozygote). (C) Artificial plasmid constructed with a partial sequence of a wild-type *MLH1* gene (homozygote). (D) Artificial plasmid constructed with a partial sequence of a *MLH1* gene with the T1151A mutation (homozygote).

Figure 2 showed the result of the HRM assay in Example 2 (n=2). (A) Standard stage; (B) Melt curve stage. Curve 1: Artificial plasmid constructed with a partial sequence of a wild-type *MLH1* gene (homozygote). Curve 2: Artificial plasmid constructed with a partial sequence of a *MLH1* gene with the T1151A mutation (homozygote).

Figure 3 showed the result of the TaqMan assay in Example 3 (n=2). (A): Artificial plasmid constructed with a partial sequence of a wild-type *MLH1* gene (homozygote). (B) Artificial plasmid constructed with a partial sequence of a *MLH1* gene with the T1151A mutation (homozygote). Curve (1): wild-type probe (negative probe, ML-TP-Wt, **SEQ ID NO 16**); curve (2): mutation probe (positive probe, ML-TP-Mt, **SEQ ID NO 17**); curve (3): control (no probes used).

Figure 4 showed the result of the LNA assay in Example 4 (n=3). (A): Artificial plasmid constructed with a partial sequence of a wild-type *MLH1* gene (homozygote). (B) Artificial plasmid constructed with a partial sequence of a *MLH1* gene with the T1151A mutation (homozygote). Curve (1): wild-type probe (negative probe, ML-TP-Wt, **SEQ ID NO 18**); curve (2): mutation probe (positive probe, ML-TP-Mt, **SEQ ID NO 19**); curve (3): control (no probes used).

Figure 5 showed the result of the FRET assay in Example 5 (n=2). (A)(B): probes of

period, have no progress or are reduced in size.

The term of "analyzing" or "analyze" used herein is referred to as evaluating or examining a property of a subject of interest by at least of technical means. Said technical means include but not limited to polymerase chain reaction, Southern blot, and Western blot.

The description of "V384 alteration of a *MLH1* gene encoded protein" recited herein is referred to as an alternation existing in the sequence of said *MLH1* gene that results in an alteration at V384 position of a *MLH1* gene encoded protein. In an alternative embodiment of the present invention, said V384 alteration is a V384D mutation of a protein encoded by said *MLH1* gene. In an alternative embodiment, said V384D mutation can be identified as a T1151A mutation of a cDNA of said *MLH1* gene, or a T1349A mutation of a RNA of said *MLH1* gene. The term of "wild-type" is a comparative term for describing a gene and/or a protein without the aforesaid mutation/alternation.

The term of "V384" used herein is referred to as the 384th amino acid of the amino acid sequences of a *MLH1* protein (that is, a protein encoded by said *MLH1* gene); wherein "V" is the one-letter abbreviation of Valine (Val). The term of "V384 alteration" used herein is referred to as the 384th amino acid of Valine is altered to another amino acid other than Valine. The term of "V384D mutation" used herein is referred to as the 384th amino acid of Valine is altered to Aspartic acid; wherein "D" is the one-letter abbreviation of Aspartic acid (Asp). See also **SEQ ID NO 01** for the amino acid sequence having the aforesaid mutation and **SEQ ID NO 02** for the DNA sequence having the aforesaid mutation.

The term of "U1349" used herein is referred to as the 1349th nucleotide of the nucleotide sequence of a mRNA encoding a *MLH1* protein; "U" is an abbreviation of uracil. The term of "U1349 mutation" used herein is referred to as the 1349th nucleotide of thymine is altered/mutated to another nucleotide other than uracil. The term of "U1349A mutation" used herein is referred to as the 1349th nucleotide of uracil is altered to adenine; wherein "A" is an abbreviation of adenine. See also **SEQ ID NO 03** for the mRNA sequence having the aforesaid mutation.

The term of "T1151" used herein is referred to as the 1151th nucleotide of the nucleotide sequence of a cDNA; wherein said cDNA is from a mRNA of said *MLH1* gene; "T" is an abbreviation of thymine. The term of "T1151 mutation" used herein is referred to as the 1151th nucleotide of thymine is altered/mutated to another nucleotide other than

thymine. The term of "T1151A mutation" used herein is referred to as the 1151th nucleotide of thymine is altered to adenine; wherein "A" is an abbreviation of adenine. See also **SEQ ID NO 04** for the cDNA sequence having the aforesaid mutation.

Primer Sets, Probes, and Kits Using the Same

First Aspect

In the first aspect of the present invention, the V384 alteration of a *MLH1* gene encoded protein is detected by using a primer set. Said "V384 alteration of a *MLH1* gene encoded protein" is defined as set forth in the preceded paragraphs. Preferably, the detection is conducted by real-time PCR with said primer set.

In the first aspect of the present invention, a kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject is provided. Said kit comprises a primer set, which is designed for analyzing a sequence of a *MLH1* DNA, a *MLH1* mRNA, and/or a cDNA sequence derived from said *MLH1* mRNA of a subject to identify a V384 alteration of a *MLH1* gene encoded protein.

In a preferable embodiment, said primer set comprises a first primer and a second primer. Said first primer is designed to comprise a sequence that is capable of hybridizing with a *MLH1* gene having said V384 alteration at V384 site. Said second primer is designed to comprise a sequence that is capable of hybridizing with a conserved region of said *MLH1* gene. The PCR product of said first primer and said second primer is detected representing the existence of the V384 alteration.

The term of "capable of hybridizing with a *MLH1* gene having said V384 alteration at V384 site" means said sequence is capable of base-pairing with at least one nucleotide of the codon encoding said V384 alteration in said *MLH1* gene. It is known that a codon contains three nucleotides. In a preferable embodiment, said sequence is capable of forming base-pairs with at least two nucleotides of the codon encoding said V384 alteration in said *MLH1* gene.

The term of "a conserved region of said *MLH1* gene" used herein is referred to a sequence of said *MLH1* gene that is conserved or unchanged between a wild-type *MLH1* gene and a *MLH1* gene having the aforesaid V384 alteration. The term of "wild-type" is a relative description to describe a subject without the aforesaid V384 alteration.

In an alternative embodiment, said kit further comprises a third primer. Said third primer is designed to comprise a sequence that is capable of hybridizing with a wild-type

of a protein encoded by said *MLH1* gene. For example, said segment may have the T1349A mutation of said mRNA or the T1151A mutation of said cDNA. Through examining the result of hybridization, the aforesaid V384 alteration can be identified. In this positive scenario, the detection of the signal of hybridization between said probe and the target represents the existence of the V384 alteration. In a preferable embodiment, said mutation probe comprises **SEQ ID NO 10**.

In a *negative* scenario of identifying V384 alteration, said probe (*wild-type* probe) is designed to hybridize with a segment of a *wild-type* genomic DNA, a mRNA and/or a cDNA of said *MLH1* gene. In this negative scenario, the inexistence of the signal of hybridization between said probe and the target represents the inexistence of wild-type *MLH1* gene, giving possibility of the existence of the V384 alteration. In a preferable embodiment, said wild-type probe comprises **SEQ ID NO 11**.

Nevertheless, those having skill in the art can realize that there is a possibility that the subject be tested may have a heterozygous genotype of *MLH1* gene. That is, an allele of the *MLH1* gene the subject has is wild-type while the other allele thereof is of a V384 alteration. In that circumstance, it is preferable to use a mutation probe or use both of the mutation probe and wild-type probe for detection.

Preferably, said probe (mutation probe and wild-type probe) is modified to fulfill the purpose of targeting the V384 alternation of said *MLH1* gene. In an embodiment of the present invention, said probe is conjugated with a fluorophore and/or isotope. In a preferable embodiment, said probe is conjugated with a fluorophore and a quencher. In an alternative embodiment, said probe is further modified with a bridge connecting the 2' oxygen and 4' carbon thereof.

Preferably, said fluorophore is conjugated at the 5' end of said probe and said quencher is conjugated at the 3' end of said probe. Said fluorophore includes, but not limited to 6-carboxyfluorescein, tetrachlorofluorescein, FAM, VIC, LC640, or a combination thereof Said quencher includes, but not limited to tetramethylrhodamine, BHQ1, or a combination thereof.

In a preferable embodiment, said kit is designed for a PCR-based **TaqMan assay**. Said kit comprises a mutation probe comprising **SEQ ID NO. 17**, and/or a wild-type probe comprising **SEQ ID NO. 16**. Both of said probe are conjugated with a fluorophore and a quencher respectively at both ends of said probe.

In a preferable embodiment, said kit is designed for a PCR-based LNA assay (locked nucleic acid). Said kit comprises a mutation probe comprising **SEQ ID NO. 19**, and/or a wild-type probe comprising **SEQ ID NO. 18**. Both of said probe are conjugated with a fluorophore and a quencher respectively at both ends of said probe.

Preferably, said probe of **SEQ ID NO. 19** or **SEQ ID NO. 18** has at least one nucleotide being modified with a bridge connecting the 2' oxygen and 4' carbon thereof to form a locked nucleic acid. For instance, the 5^{th}, 8^{th}, 11^{th}, and 14^{th} nucleotides of said probe (counted from 5' to 3') are modified with a bridge connecting the 2' oxygen and 4' carbon thereof.

In an alternative embodiment, said kit is designed for a PCR-based FRET assay (Fluorescence resonance energy transfer). In a practically embodiment, said kit comprises a mutation probe and/or a wild-type probe, and an anchor probe. Said mutation probe or wild-type probe is conjugated with a first fluorophore as a receptor and said anchor is conjugated with a second fluorophore as a donor. Preferably, said mutation probe comprises **SEQ ID NO.21**, said wild-type probe comprises **SEQ ID NO.20**, and said anchor probe comprises **SEQ ID NO.22**. In a preferable embodiment, said kit comprises said mutation probe, said wild-type probe, and said anchor probe.

In a preferable embodiment, said first fluorophore is FAM, HEX, LC Red 640, Cy5, or a combination thereof. In a preferable embodiment, said second fluorophore is fluorescein or a combination thereof.

In an alternative embodiment, said kit further comprises a DNA polymerase. Preferably, said DNA polymerase has exonuclease activity.

In a preferably embodiment, said kit further comprises a primer set for amplifying a part of said *MLH1* gene to obtain an amplicon; wherein said amplicon comprises a site corresponding to said V384. Said primer set may be:

- a first primer of **SEQ ID NO 12** and a second primer of **SEQ ID NO 13**; and/or
- a first primer of **SEQ ID NO 14** and a second primer of **SEQ ID NO 15**.

The following Table 1 lists the afore-mentioned primer sets and probes of the present invention together with the technology method therefor.

Example 1: Verification of the Primer Set Designed in the Present Invention (SYBR Green assay).

In this example, the primers of SB-MT-sF3 (**SEQ ID NO 06**), SB-sR3 (**SEQ ID NO 07**), and SB-WT-sF3 (**SEQ ID NO 05**) were used for a Real-time polymerase chain reaction in order to verify their capability in identifying the V384D mutation.

For the experiments, four templates were prepared including a genomic DNA extracted from a subject without V384D mutation, a genomic DNA extracted from a subject with V384D mutation (heterozygote), an artificial plasmid constructed with a partial sequence of a wild-type *MLH1* gene (homozygote), and an artificial plasmid constructed with a partial sequence of a *MLH1* gene with the T1151A mutation (homozygote). The templates and the primer set used in this example were listed in the following Table 2.

Table 2

Template	Primer Set		
	Curve (1), Fig. 1	Curve (2), Fig. 1	Curve (3), Fig. 1
Genomic DNA Subject w/o mutation	SEQ ID NO 05 SEQ ID NO 07	SEQ ID NO 06 SEQ ID NO 07	None (Ngative control)
Genomic DNA Subject w/ mutation	SEQ ID NO 05 SEQ ID NO 07	SEQ ID NO 06 SEQ ID NO 07	None (Negartive control)
Plasmid wild-type *MLH 1*	SEQ ID NO 05 SEQ ID NO 07	SEQ ID NO 06 SEQ ID NO 07	None (Negartive control)
Plasmid mutated *MLH 1*	SEQ ID NO 05 SEQ ID NO 07	SEQ ID NO 06 SEQ ID NO 07	None (Negartive control)

The experiments were conducted according to the product manual of the EZtime™ Real Time PCR premix (Yeastern Biotech Co., Ltd.). The PCR mixture contained: 1.0 μl of template (2.5μg/μl for genomic DNA template; 1μg/μl for plasmid template), 12.5μl of 2X EZtime Real-time PCR Premix, 1.0μl of forward primer (5μM), 1.0μl of reverse primer (5μM), and 9.5μl of ddH_2O was prepared. The reaction condition was: 95℃ denaturation for 10 min; 95℃ denaturation 20sec, 66℃ anneal/extension 1 min, 40 cycles; then melt curve stage: 95℃ 15 sec, 60℃ 1 min, 95℃ 30 sec, 60℃ 15 sec. The Real-time PCR instrument used was 7500 Fast (Applied Biosystem) equipped with Open 7500 Software v2.0.6.

The results were showed in Figure 1 (A) to (D); wherein the results of primer set of **SEQ ID NO 05** and **SEQ ID NO 07** were marked as curve (1), the results of primer set of **SEQ ID NO 06** and **SEQ ID NO 07** were marked as curve (2), and the results of negative control were marked as curve (3).

CLAIMS

1. A kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject, comprising:

a first primer comprising SEQ ID NO 06; and

a second primer comprising SEQ ID NO 07.

2. The kit of claim 1, further comprising a third primer comprising a sequence of SEQ ID NO 05.

3. The kit of claim 1, further comprising a DNA polymerase.

4. The kit of claim 1, further comprising a double strands DNA binding dye.

5. The kit of claim 4, wherein said double strands DNA binding dye is SYBR® Green I, SYBR® Green II, SYBR® Gold, Oxazole Yellow, Thiazole Orange, or PicoGreen.

6. A kit for identifying a V384 alteration of a *MLH1* gene encoded protein of a subject, comprising:

a first primer comprising SEQ ID NO 08; and

a second primer comprising SEQ ID NO 09.

7. The kit of claim 6, further comprising a DNA polymerase.

8. The kit of claim 6, further comprising a double strands DNA binding dye.

9. The kit of claim 6, wherein said double strands DNA binding dye is EvaGreen®, or a combination thereof.

10. A use of a kit for evaluating the efficacy of an EGFR-TKI treatment; wherein said kit comprises:

at least one mutation probe comprising SEQ ID NO 10; wherein said probe is conjugated with a fluorophore.

11. The use of claim 10, wherein said kit further comprises at least one wild-type probe comprising SEQ ID NO 11; wherein said probe is conjugated with a fluorophore.

12. The use of claim 10 or claim 11, wherein said at least one mutation probe and/or said at least one wild-type probe is further conjugated with a quencher.

13. The use of claim 12, wherein said fluorophore is conjugated at the 5' end of said probe and said quencher is conjugated at the 3' end of said probe.

14. The use of claim 12, wherein said at least one mutation probe comprises SEQ ID NO.17.

15. The use of claim 12, wherein said at least one wild-type probe comprises SEQ ID NO.16.

16. The use of claim 11, wherein said at least one mutation probe and/or at said at least one wild-type probe comprises at least one nucleotide thereof being modified with a bridge connecting the 2' oxygen and 4' carbon thereof.

17. The use of claim 16, wherein said mutation probe comprises SEQ ID NO 19 having the following sequence:

5'-CACCAGATGGATCGTACA-3'.

18. The use of claim 17, wherein said mutation probe has the 5^{th}, 8^{th}, 11^{th}, and 14^{th} nucleotides thereof counted from 5' to 3' being modified with a bridge connecting the 2' oxygen and 4' carbon.

19. The use of claim 16, wherein said wild-type probe comprises SEQ ID NO 18 having the following sequence:

5'- CACCAGATGGTTCGTACA -3'.

20. The use of claim 19, wherein said wild-type probe has the 5^{th}, 8^{th}, 11^{th}, and 14^{th} nucleotides thereof counted from 5' to 3' being modified with a bridge connecting the 2' oxygen and 4' carbon.

21. The use of claim 10, further comprising an anchor probe conjugated with a donor fluorophore.

22. The use of claim 21, wherein said at least one mutation probe comprises SEQ ID NO.21 and is conjugated with a receptor fluorophore.

23. The use of claim 21, wherein said kit further comprises at least one wild-type probe comprising SEQ ID NO.20 and being conjugated with a receptor fluorophore.

24. The use of claim 21, wherein said anchor probe comprises SEQ ID NO. 22

25. The use of claim 10, further comprising a primer set for amplifying at least a part of said *MLH1* gene to obtain an amplicon; wherein said amplicon comprises a site corresponding to said V384 alteration.

26. The use of claim 25, wherein said primer set is:

a forward primer comprising SEQ ID NO 12, and a reverse primer comprising SEQ ID NO 13; and/or

a forward primer comprising SEQ ID NO 14, and a reverse primer comprising SEQ ID NO 15.

27. The use of claim 24, further comprising a DNA polymerase.