

METHOD OF DETERMINING EXPRESSION LEVEL OF T315I VARIANT OF
ABL1

TECHNICAL FIELD

[0001] This application claims the benefit of priority of Japanese Patent Application No. 2017-087578, the entire contents of which are incorporated herein by reference.

The disclosure relates to a method of determining the expression level of the T315I variant of ABL1 or a kit therefor.

BACKGROUND

[0002] The *BCR-ABL1* fusion gene is a chromosomal abnormality found in the patients of chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphocytic leukemia (Ph positive ALL). The chromosomal translocation of chromosome 9 and chromosome 22, which is designated as t(9;22), causes the fusion of the *ABL1* gene on chromosome 9 (band region q34) and the *BCR* gene on chromosome 22 (band region q11). The fusion gene encodes the chimeric *BCR-ABL1* protein. The *BCR-ABL1* protein has a tyrosine kinase activity and causes unlimited proliferation of hematopoietic stem cells and associated abnormalities by constitutively stimulating cell proliferation signals and suppressing apoptosis.

[0003] Since the *BCR-ABL1* chimeric protein is one of the causative proteins of CML and Ph positive ALL, tyrosine kinase inhibitors (TKIs) targeting the *BCR-ABL1* protein have been developed as the therapeutics. Imatinib, a representative TKI, exerts a remarkable therapeutic effect for CML and Ph positive ALL, but the effect of imatinib was found to decrease in the patients having some *BCR-ABL1* variants. Further TKIs which are effective for many imatinib-resistant variants of *BCR-ABL1*, nilotinib and dasatinib, were developed. However, they are not

effective for the variant BCR-ABL1 in which threonine at amino acid position 315 of the ABL region of the BCR-ABL1 protein is substituted with isoleucine (T315I variant) (Non-Patent Literatures 1 to 3). It used to be believed that TKI therapy for the patients having the T315I variant resulted in poor prognosis, and allogeneic stem cell transplantation was the first-line therapy. Detection of the T315I variant before hematological recurrence was demanded.

[0004] More recently ponatinib, which can inhibit tyrosine kinase activity of the T315I variant of BCR-ABL1, was developed. Ponatinib is effective for the patients of CML and Ph positive ALL who are resistant or intolerant to the prior TKI therapy, in particular who have the T315I variant (Non-Patent Literature 4 or 5).

[0005] Accordingly, once patients are found to have the T315I variant, any prior TKI therapy should be immediately changed to the ponatinib therapy. The conventional methods for assaying mutations in the *BCR-ABL1* gene include direct sequencing, pyrosequencing, DHPLC, high resolution melt analysis, and PCR using a specific primer. However, it is difficult to detect the T315I variant at an early stage because the sensitivity of the conventional methods is not very high (Non-Patent Literatures 6 to 8). If the detection of the T315I variant is delayed and the disease stage proceeds, the possibility that even ponatinib cannot ameliorate the disease is increased. Earlier detection of the T315I variant is an important issue in clinical therapy.

[0006] The therapeutic effect of the TKI therapy on the patients having the *BCR-ABL1* gene can be monitored by determining the expression level of the *BCR-ABL1* mRNA. Similarly, since the T315I variant is resistant to many TKIs and causes poor prognosis, monitoring the expression level of the *BCR-ABL1* mRNA having the T315I mutation should have a

clinical benefit and be useful for evaluating the effect of ponatinib. However, the existing methods for detecting the T315I variant are not suitable for this purpose because they lack a sufficient quantitativity.

REFERENCES

[0007]

NON-PATENT LITERATURE

[Non-Patent Literature 1] Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408-17.

[Non-Patent Literature 2] Modugno M. New resistance mechanisms for small molecule kinase inhibitors of Abl kinase. *Drug Discov Today Technol*. 2014;11:5-10.

[Non-Patent Literature 3] Zabriskie MS, Eide CA, Tantravahi SK, Vellore NA, Estrada J, Nicolini FE, et al. BCR-ABL1 Compound Mutations Combining Key Kinase Domain Positions Confer Clinical Resistance to Ponatinib in Ph Chromosome-Positive Leukemia. *Cancer Cell*. 2014;26:428-42.

[Non-Patent Literature 4] Goldman JM. Ponatinib for chronic myeloid leukemia. *N Engl J Med*. 2012; 367: 2148-2149.

[Non-Patent Literature 5] Cortes JE, Kantarjian H, Shah NP, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2012; 367: 2075-2088.

[Non-Patent Literature 6] Hofmann WK, Jones LC, Lemp NA, de Vos S, Gschaidmeier H, Hoelzer D, et al. Ph(+) acute lymphoblastic leukemia resistant to the tyrosine kinase inhibitor STI571 has a unique BCR-ABL gene mutation. *Blood*. 2002;99:1860-2.

[Non-Patent Literature 7] Yamamoto M, Kakihana K, Ohashi K, Yamaguchi T, Tadokoro K, Akiyama H, et al. Serial monitoring of T315I BCR-ABL mutation by Invader assay combined with RT-

PCR. Int J Hematol. 2009;89:482-8.

[Non-Patent Literature 8] Yin L, Dittman D, Chenn A. Rapid quantitative detection of the T315I mutation in patients with chronic myelogenous leukemia. Diagn Mol Pathol. 2012;21:34-39.

SUMMARY

[0008] An object of the disclosure is to provide a method of determining the expression level of the T315I variant of ABL1 in a subject or a kit therefor.

[0009] The inventors have found that the expression level of the T315I variant of ABL1 can be determined by reverse-transcribing an RNA sample obtained from the subject with two reverse primers that hybridizes to an ABL1 mRNA in regions upstream and downstream from the position of the T315I mutation in the presence of a modified nucleic acid that specifically hybridizes to a wild-type ABL1 mRNA, and comparing the amounts of the reverse transcripts generated with each primer.

[0010] Accordingly, an aspect of the disclosure provides a method of determining the expression level of the T315I variant of ABL1 in a subject comprising:

(1) reverse-transcribing an RNA sample obtained from the subject in the presence of a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type ABL1 mRNA by using (a) a reverse primer that hybridizes to an ABL1 mRNA in a region downstream from the position of the T315I mutation and (b) a reverse primer that hybridizes to an ABL1 mRNA in a region upstream from the position of the T315I mutation in one container; and

(2) calculating the expression level of the T315I variant of ABL1 on the basis of the ratio of the reverse transcript generated with the primer (a) to the reverse transcript generated with the primer (b).

[0011] Another aspect of the disclosure provides a kit for

determining the expression level of the T315I variant of *ABL1* in a subject comprising:

(a) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation;

(b) a reverse primer that hybridizes to an *ABL1* mRNA in a region upstream from the position of the T315I mutation; and

(c) a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA.

[0012] According to the disclosure, the expression level of the T315I variant of *ABL1* in a subject can be determined. This may give information useful for treating the patients having the T315I mutation.

BRIEF DESCRIPTION OF DRAWINGS

[0013]

Fig. 1-1 shows the nucleic acid sequence of SEQ ID NO: 1. The cytosine base at the nucleotide position 947 is boldfaced and underlined.

Fig. 1-2 shows the nucleic acid sequence of SEQ ID NO: 1 (continued).

Fig. 2-1 shows the nucleic acid sequence of SEQ ID NO: 3. The thymine base at the nucleotide position 947 is boldfaced and underlined.

Fig. 2-2 shows the nucleic acid sequence of SEQ ID NO: 3 (continued).

Fig. 3 is a schematic drawing of the reverse transcription of the wild-type *ABL1* mRNA using two reverse primers.

Fig. 4 is a schematic drawing of the reverse transcription of the *ABL1* mRNA having T315I mutation using two reverse primers.

Fig. 5 is a schematic drawing of the reverse transcriptions.

Fig. 6 is a schematic drawing of the quantitative real-time PCR of the *ABL1* mRNAs.

Fig. 7 is a schematic drawing of the quantitative real-time PCR of the *ABL1* mRNA having T315I mutation.

Fig. 8 shows the amplification curves of the cDNA generated with T315I reverse primer.

Fig. 9 shows the amplification curves of the cDNA generated with ABL reverse primer.

DETAILED DESCRIPTION

[0014] Unless otherwise defined, the terms used herein are read as generally understood by a skilled person in the technical fields such as organic chemistry, medical sciences, pharmaceutical sciences, molecular biology, and microbiology. Several terms used herein are defined as described below. The definitions herein take precedence over the general understanding.

[0015] When a numerical value is accompanied with the term "about", the value is intended to represent any value within the range of $\pm 10\%$ of that value. For example, "about 20" means "a value from 18 to 22." A range defined with a value of the lower limit and a value of the upper limit covers all values from the lower limit to the upper limit, including the values of the both limits. When a range is accompanied with the term "about", the both limits are read as accompanied with the term. For example, "about 20 to 30" is read as "18 to 33."

[0016] The *ABL1* gene is located on chromosome 9 (band q34). The chromosomal translocation of chromosome 9 and chromosome 22, which is designated as t(9;22), causes the fusion of exon 1, exons 1 to 13, or exons 1 to 14 of the *BCR* gene and exons 2 to 4 of the *ABL1* gene, resulting in the *BCR-ABL1* gene. The *BCR-ABL1* gene is found in the patients of chronic myelogenous leukemia and Philadelphia chromosome-positive acute

lymphocytic leukemia.

[0017] The nucleic acid sequence of the *ABL1* gene is typically represented by SEQ ID NO: 1, and a number of variants are known. The *ABL1* gene herein may have a polynucleotide sequence that hybridizes to the polynucleotide having the nucleic acid sequence complementary to the sequence of SEQ ID NO: 1 under a stringent condition. Regarding "hybridizes under a stringent condition", the hybridization may be carried out using a standard method, for example that described in "Molecular Cloning, T. Maniatis et al., CSH Laboratory (1983)". An example of the stringent condition is such that a hybrid is formed in a solution containing 6 × SSC and 50% formamide at 45°C and is washed with 2 × SSC at 50°C, wherein 10 × SSC is the solution containing 1.5 M NaCl and 0.15 M trisodium citrate (see "Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6"). Conditions that achieve the equivalent stringency also may be employed.

[0018] The *ABL1* gene may have a nucleic acid sequence having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity with the nucleic acid sequence of SEQ ID NO: 1. The term "sequence identity" means the degree of the similarity between two oligonucleotide sequences. The identity is determined by comparing two sequences that are optimally aligned with each other over the region subjected to the comparison. The term "optimally aligned" means the two sequences are aligned so that the number of the matched nucleotides is maximized. The numerical value (%) of the sequence identity is calculated by identifying the matched bases in the both sequences, determining the number of the matched bases, dividing the number by the total number of the bases in the region subjected to the comparison, and multiplying the derived numerical value by 100. For making the optimal alignment and calculating the sequence identity,

various algorithms that are commonly available to those skilled in the art, e.g., BLAST algorithm and FASTA algorithm, may be used. The sequence identity may be determined using a software for sequence analysis such as BLAST and FASTA.

[0019] In the disclosure, the term "wild-type *ABL1* allele" means an *ABL1* allele in which the nucleotide at the position of the T315I mutation is cytosine. An mRNA of a wild-type *ABL1* allele is referred to as "wild-type *ABL1* mRNA." A wild-type *ABL1* mRNA typically has the nucleic acid sequence of SEQ ID NO: 1 and encodes the protein having the amino acid sequence of SEQ ID NO: 2. The term "position of the T315I mutation" means the position in an *ABL1* allele that corresponds to the nucleotide position 947 of SEQ ID NO: 1 when the sequence of the *ABL1* allele is optimally aligned with the nucleic acid sequence of SEQ ID NO: 1.

[0020] If the cytosine nucleotide of a wild-type *ABL1* allele is substituted with a thymine nucleotide, the threonine residue of the encoded protein at the position corresponding to the amino acid position 315 of the *ABL1* region of the BCR-*ABL1* protein is substituted with an isoleucine residue. The substitution in the *ABL1* gene or the *ABL1* protein is herein referred to as "T315I mutation." The term "*ABL1* allele having T315I mutation" means an *ABL1* allele that has the T315I mutation, and the mRNA thereof is referred to as "*ABL1* mRNA having T315I mutation." An *ABL1* mRNA having T315I mutation typically has the nucleic acid sequence of SEQ ID NO: 3 and encodes the protein having the amino acid sequence of SEQ ID NO: 4.

[0021] The term "expression level of the T315I variant of *ABL1*" as used herein means the expression level of any *ABL1* gene or allele having the T315I mutation, including the *ABL1* gene that is not translocated and a translocated *ABL1* gene such as the BCR-*ABL1* gene.

[0022] The subject is typically a human being. The subject may be a subject suffering from or suspected to be suffering from chronic myelogenous leukemia (CML) or Philadelphia chromosome-positive acute lymphocytic leukemia (Ph+ALL). The subject may have the *BCR-ABL1* gene.

[0023] The term "RNA sample" as used herein means RNAs extracted from a specimen of the subject containing hematopoietic stem cells, leukocytes, or leukemia cells, for example, RNAs extracted from blood, bone marrow aspirate, or lymph. The RNA sample may be obtained from isolated hematopoietic stem cells, blood cells, leukocytes, or leukemia cells. In an embodiment, the RNA sample is obtained from leukocytes in peripheral blood or nucleated cells in bone marrow aspirate. In an embodiment, the RNA sample is obtained from leukocytes in peripheral blood.

[0024] RNAs may be extracted from a specimen by any known method, for example, by acidifying a solution containing RNAs and extracting them from an aqueous layer in PCI (phenol-chloroform-isoamyl alcohol extraction), by using a commercially available RNA extraction kit, or by any other known method. The RNAs may be total RNAs or purified mRNAs.

[0025] Step (1) of the method uses a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA comprising the position of the T315I mutation. The modified nucleic acid is herein referred to as "T315I clamp." The modified nucleic acid comprises at least one artificial nucleotide, hybridizes to an mRNA more strongly than the DNA having the same nucleic acid sequence, and is not degraded by the exonuclease activity of a reverse transcriptase. The reverse transcriptase stops the elongation reaction at the site where the modified nucleic acid is bound to the template mRNA.

[0026] The T315I clamp is designed so that it binds to a

wild-type *ABL1* mRNA, in which the nucleotide at the position of the T315I mutation is cytosine, but does not bind to an *ABL1* mRNA having T315I mutation, in which the nucleotide is thymine. In other words, the T315I clamp is designed so that it can suppress reverse transcription of a wild-type *ABL1* mRNA, but cannot suppress reverse transcription of an *ABL1* mRNA having T315I mutation. Specifically, the T315I clamp may be designed to have a nucleic acid sequence complementary to about 10 to 22, about 12 to 20, about 14 to 18, or about 15 to 17, e.g., about 16, continuous nucleotides of a wild-type *ABL1* mRNA comprising the nucleotide at the position of the T315I mutation. In an embodiment, the T315I clamp comprises the nucleic acid sequence of 5'-ATGAACTCAGTGATGA-3' (SEQ ID NO: 5). In an embodiment, the T315I clamp consists of the nucleic acid sequence of SEQ ID NO: 5.

[0027] The T315I clamp comprises at least one artificial nucleotide. The term "artificial nucleotide" means a nucleotide that is different from natural nucleotides in its structure due to a modification in the nucleoside (the base or sugar moiety). The artificial nucleotide may enhance the nuclease resistance and the binding affinity with the target sequence of the T315I clamp. For example, artificial nucleotides used herein include those described in Deleavey, G. F., & Damha, M. J. (2012). Designing chemically modified oligonucleotides for targeted gene silencing. *Chemistry & biology*, 19(8), 937-954, the entire contents of which are incorporated herein by reference. Examples of the artificial nucleotides include abasic nucleosides; arabinonucleosides, 2'-deoxyuridine, alpha-deoxyribonucleosides, beta-L-deoxyribonucleosides, and nucleosides having any other sugar modification; peptide nucleic acids (PNA), peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), 2'-O,4'-C-ethylene-bridged nucleic acids (ENA), constrained

ethyl (cEt), and morpholino nucleic acids. Examples of the artificial nucleotides having sugar modifications include those having substituted pentoses such as 2'-o-methyl ribose, 2'-o-methoxyethyl ribose, 2'-deoxy-2'-fluoro ribose, or 3'-o-methyl ribose; 1',2'-deoxyribose; arabinose; substituted arabinoses; hexoses, and alpha-anomers. Examples of the modified bases include pyrimidines such as 5-hydroxycytosine, 5-methylcytosine, 5-fluorouracil, or 4-thiouracil; purines such as 6-methyladenine and 6-thioguanosine; and other heterocyclic bases. The T315I clamp may comprise artificial nucleotides of the same type or two or more different types.

[0028] In an embodiment, the T315I clamp comprises at least one PNA portion as the artificial nucleotides. PNAs have peptide bonds between N-(2-aminoethyl)glycines and thus have DNA-like structures in which phosphodiester bonds of DNA are replaced with peptide bonds (Nielsen et al. 1991 Science 254, 1457-1500). PNAs are resistant to various nucleases and hybridize to DNAs or RNAs with the molecular recognition similar to that of DNAs and RNAs. The affinity between a PNA molecule and a DNA molecule is stronger than between a DNA molecule and a DNA molecule or between a DNA molecule and an RNA molecule. In an embodiment, all the nucleotides in the T315I clamp are those of PNAs.

[0029] The amount of the T315I clamp can be any amount sufficient to suppress reverse transcription of a wild-type *ABL1* mRNA. For example, the concentration of the T315I clamp in the final reaction solution of the reverse transcription may be in the range about 0.1 μ M to 10 μ M or about 0.5 μ M to 5 μ M, for example, about 2 μ M.

[0030] The step (1) of the method uses two reverse primers: (a) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation and (b) a reverse primer that hybridizes to an *ABL1* mRNA in a

region upstream from the position of the T315I mutation for the reverse transcription. The reverse primer (a) is used for reverse-transcribing a part of an *ABL1* mRNA comprising the region to which the T315I clamp is expected to bind. The reverse primer (a) is herein referred to as "T315I reverse primer." The reverse primer (b) is used for reverse-transcribing a part of an *ABL1* mRNA upstream from the region to which the T315I clamp is expected to bind. The reverse primer (b) is herein referred to as "ABL reverse primer." Methods for designing primers suitable for reverse transcription are well known to those skilled in the art.

[0031] In the step (1) the reverse transcriptions with the T315I reverse primer and the ABL reverse primer are carried out in one step, i.e., simultaneously or sequentially in one container. In an embodiment, the reverse transcriptions with the T315I reverse primer and the ABL reverse primer are carried out simultaneously in one container. Schematic drawings of the reverse transcriptions using the primers and the T315I clamp are shown in Figs. 3 and 4.

[0032] Reagents such as reverse transcriptases, dNTPs, and buffers, may be those commonly used in the art. Reaction conditions such as the amount of each reagent, the reaction durations, and the reaction temperatures can be appropriately determined in a usual manner, for example according to the description of the package insert of the reverse transcriptase or protocols generally used in the art. For example, the reverse transcriptase may be any known reverse transcriptase used in molecular biological experiments, including Tth DNA polymerase, rTth DNA polymerase, AMV reverse transcriptase, MMLV reverse transcriptase, HIV reverse transcriptase, and derivatives thereof.

[0033] The step (2) calculates the expression level of the T315I variant of *ABL1* on the basis of the ratio of the reverse

transcript generated with the primer (a) to the reverse transcript generated with the primer (b). For example, the measurement of the reverse transcript generated with the primer (a) may be divided by the measurement of the reverse transcript generated with the primer (b) to give the measurement of the expression level of the T315I variant of ABL1. The quantification of the reverse transcripts can be carried out by any known method, for example, by quantitative PCR; electrophoresis of the nucleic acids after amplification; hybridization with a nucleic acid probe labeled with a detectable label; staining of double-stranded DNAs with an intercalating fluorescent dye; or using a fluorescent probe.

[0034] In an embodiment, the step (2) further comprise (2-1) quantifying the reverse transcript generated with the T315I reverse primer (a) by quantitative PCR, and (2-2) quantifying the reverse transcript generated with the ABL reverse primer (b) by quantitative PCR. The quantitative PCR may be a quantitative real-time PCR. Various fluorescent PCR techniques may be used for the quantitative real-time PCR. Examples of the fluorescent PCR techniques include, but not limited to, intercalator assays, which use a fluorescent nucleic acid binding dye such as SYBR GREEN I and an instrument such as LightCycler (registered trademark) of Roche or ABI Prizm 7700 Sequence Detection System (registered trademark) of Perkin Elmer Applied Biosystems; TaqMan probe assays, which monitor the amplification in real-time by virtue of 5' exonuclease activity of DNA polymerase; and cycling probe assays, which use RNase activity of RNaseH and a specialized chimeric RNA probe.

[0035] In an embodiment, a TaqMan probe assay is employed for the quantitative real-time PCR. In a TaqMan probe assay generally an oligonucleotide labeled with a fluorescent substance at the 5' end and a quencher at the 3' end (TaqMan

probe) is added to the PCR reaction system. The TaqMan probe specifically hybridizes to the template DNA in the annealing step. At this step the fluorescence emission is suppressed due to the quencher on the probe even if excitation light is irradiated. At the elongation step the 5' exonuclease activity of Taq polymerase degrades the TaqMan probe hybridized to the template and releases the fluorescent substance from the quencher, allowing the fluorescence emission. The TaqMan probe can be designed by any method known in the art as a probe capable of binding somewhere in the PCR product. Any combination of fluorescent substances and quenchers may be used.

[0036] Amplification curves of the fluorescence may be created in the quantitative real-time PCR. A sample solution containing a cDNA at an unknown concentration and standard solutions containing the cDNA at known concentrations are simultaneously used in the quantitative real-time PCR. The amplification curves are generated by plotting the cycle numbers on the x-axis and the log-transformed fluorescence intensities from the reporter substance on the y-axis. The median value of the fluorescence intensities is determined in the range where the amplification curves are linear. A line parallel to the x-axis is drawn at the level near the median value. The cycle numbers at which the parallel line and the amplification curves cross are determined. Furthermore, a standard curve of the cDNA concentration is created by plotting the log-transformed cDNA concentrations in the standard solutions on the x-axis and the above-determined cycle numbers of the standard solutions on the y-axis. The cDNA concentration in the sample solution can be determined by using the above-determined cycle number of the sample and the standard curve of the cDNA concentration.

[0037] The primer pairs used in the steps (2-1) and (2-2)

are designed so that they can amplify the reverse transcripts obtained in the step (1). Methods for designing primers suitable for PCR are well known to those skilled in the art. The primer pairs may be designed so that each PCR product has a length suitable for quantification, for example, about 10 to 1000, about 20 to 500, about 50 to 300, about 100 to 200, e.g., about 150 nucleotides in length.

[0038] The primer pair used in the step (2-1) is designed so that it can amplify a region of the reverse transcript comprising the position of the T315I mutation. In other words, the forward primer has a nucleic acid sequence of a region comprising the position of the T315I mutation of the *ABL1* gene or a region upstream therefrom, and the reverse primer has a nucleic acid sequence complementary to a region comprising the position of the T315I mutation of the *ABL1* gene or a region downstream therefrom. The forward primer used in the step (2-1) may have a nucleic acid sequence of a region comprising the position of the T315I mutation and have a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation. In an embodiment, the reverse primer used in the step (2-1) and the T315I reverse primer used in the step (1) have the same structure. In an embodiment, the reverse primer used in the step (2-2) and the ABL reverse primer used in the step (1) have the same structure.

[0039] In the step (2-1), the reverse transcript generated with the T315I primer may be quantified by RNase H-dependent quantitative PCR specific to the T315I mutation. RNase H-dependent quantitative PCR is a sequence-specific PCR technique using an RNase H (Boucard AA, et. al. J Biol Chem, 289(1):387-402; Dobosy JR, et al., BMC Biotechnol, 11(80):1-18). RNases H recognize an RNA/DNA heteroduplex portion and cleave a phosphodiester bond between an RNA portion and a DNA portion at the 5'-end of the RNA portion. The primer used in

RNase H-dependent quantitative PCR comprises at least one ribonucleotide, which forms an RNA/DNA heteroduplex portion with the template DNA. Furthermore, the primer has a blocking region at the 3' end of the RNA portion. DNA polymerases cannot bind to the blocking region. The PCR proceeds only after the RNases H recognize an RNA/DNA heteroduplex portion and cleave the blocking region of the primer. RNases H require that the ribonucleotide in the primer is complementary to, i.e., not mismatched with, the paired deoxyribonucleotide in the template DNA for cleaving the phosphodiester bond between the RNA portion and the DNA portion in the primer.

[0040] Where the RNase H-dependent quantitative PCR is carried out in the step (2-1), it uses a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation, having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation, and having a blocking region at the 3' end. The forward primer is herein referred to as "T315I forward primer." When a wild-type *ABL* cDNA is the PCR template, the uracil ribonucleotide in the T315I forward primer causes a mismatch with the paired nucleotide in the template, the blocking region is not cleaved, and thus the PCR does not proceed. In contrast, when an *ABL1* cDNA having T315I mutation is the PCR template, the uracil ribonucleotide causes no mismatch, the blocking region is cleaved, and thus the PCR proceeds. Consequently, the RNase H-dependent quantitative PCR using the T315I forward primer can specifically amplify the *ABL1* cDNA having T315I mutation.

[0041] Those skilled in the art can design primers used in the RNase H-dependent PCR, for example, referring to the protocols provided by Integrated DNA Technologies, Inc. The T315I forward primer is designed so that it comprises a nucleic acid sequence of an *ABL1* allele upstream from the position of

the T315I mutation and works as a forward primer in PCR after the removal of the ribonucleotide and the blocking region. For example, the T315I forward primer comprises a nucleic acid sequence of about 8 to 60, 10 to 30, 15 to 25, or 19 to 23 nucleotides, e.g., about 20, 21, or 22 nucleotides, of a region comprising the position of the T315I mutation and upstream thereof of an *ABL1* mRNA. The T315I forward primer has a uracil ribonucleotide at the position corresponding to the position of the T315I mutation. The 3'-blocking region comprises a mismatched deoxyribonucleotide and a blocking moiety such as a C3 spacer. For example, the blocking region consists of four deoxyribonucleotides having the same bases as the nucleotides from nucleotide position 945 to 948 of the *ABL1* gene, one deoxyribonucleotide having a base different from that of the nucleotide at nucleotide position 949 of the *ABL1* gene, and a blocking moiety. For another example, the blocking region consists of one deoxyribonucleotide having the same base as the nucleotide of nucleotide position 945 of the *ABL1* gene, two blocking moieties, one deoxyribonucleotide having the same base as the nucleotide of nucleotide position 946 of the *ABL1* gene, and one deoxyribonucleotide having a base different from that of the nucleotide at nucleotide position 947 of the *ABL1* gene.

[0042] In an embodiment, the T315I forward primer is described as 5'-GAGCCCCCGTTCTATATCATCARUT/iSpC3//iSpC3/GC-3' (SEQ ID NO: 6), wherein rU indicates a uracil ribonucleotide and iSpC3 indicates a spacer C3.

[0043] The steps (2-1) and (2-2) may be carried out in the presence of the T315I clamp. Hence the steps (2-1) and (2-2) may be carried out by adding reagents required for the steps (2-1) and (2-2), respectively, to a whole or a part of the product of the step (1). For example, the steps (1) and (2-1) or the steps (1) and (2-2) may be carried out in one step,

i.e., simultaneously or sequentially in one container.

[0044] When the steps (1) and (2-1) are carried out in one step and the step (2-1) is RNase H-dependent PCR, sometimes a part of the T315I clamp and a part of the T315I forward primer may hybridize. In general, the ability of an oligonucleotide to distinguish one base mutation is maximized when the position of the mutation is located at the center of the oligonucleotide. However, to decrease the hybridization, the T315I clamp may be designed so that the position corresponding to the T315I mutation is shifted from the center of the T315I clamp toward the 3' end. In other words, the T315I clamp may be designed so that the complementary region between the T315I clamp and the T315I forward primer is shorter than 50% of the total length of the T315I clamp. For example, the T315I clamp consisting of the nucleic acid sequence of SEQ ID NO: 5 is optimized so that the hybridization with the forward primer used in RNase H-dependent PCR is decreased and the clamp can distinguish the mutation.

Meanwhile, such adjustment of modified nucleic acids may be applied for detecting mutations other than the T315I mutation. When one intends to inhibit reverse transcription of a wild-type mRNA with a modified nucleic acid and to inhibit the following PCR by RNase H-dependent PCR, it is recommended that the complementary region between the modified nucleic acid and the forward primer of RNase H-dependent PCR is shorter than 50% of the total length of the modified nucleic acid.

[0045] Reagents such as DNA polymerases, RNases H, dNTPs, and buffers, may be those commonly used in the art. Reaction conditions such as the amount of each reagent, the reaction durations, and the reaction temperatures can be appropriately determined in a usual manner, for example according to the description of the package insert of the enzyme or protocols generally used in the art. For example, the DNA polymerase may

be any known DNA polymerase used in molecular biological experiments, including rTth DNA polymerase, Taq polymerase, and derivatives thereof. For example, the RNase H may be any known RNase H used in molecular biological experiments, including RNase H2.

[0046] In an embodiment, the primers, clamp, and probes shown in the table below are used. All of them may be used in combination or at least one of them may be used.

[Table 1]

Gene		
ABL T315I	Forward primer	SEQ ID NO: 6 5'- GAGCCCCCGTTCTATATCATCArUT/iSpC3//iSpC3/GC- 3'* *rU: RNA uracil, iSpC3: spacer C3
	Reverse primer	SEQ ID NO: 7 5'-CCCCTACCAGGCAGTTTCG-3'
	Probe	SEQ ID NO: 8 5'-/HEX/CAGGAGGTGAACGCCGTGGTGC/3IABkFQ/-3' * IABkFQ: Iowa Black FQ * HEX: hexachlorofluorescein
	Clamp	SEQ ID NO: 5 5'-ATGAACTCAGTGATGA-3' *PNA in which all nucleotides are linked via peptide bonds
ABL	Forward primer	SEQ ID NO: 9 5'-CGTTGGAACCTCCAAGGAAAAC-3'
	Reverse primer	SEQ ID NO: 10 5'-ATTCCCCATTGTGATTATAGCCTAAG-3'
	Probe	SEQ ID NO: 11 5'- /HEX/CTGGACCCA/ZEN/GTGAAAATGACCCCAAC/3IABk FQ/-3' * IABkFQ: Iowa Black FQ * HEX: hexachlorofluorescein * ZEN: ZEN Quencher

[0047] An aspect of the disclosure provides a kit for conducting the method disclosed herein. The kit comprises at least;

- (a) a reverse primer that hybridizes to an *ABL1* mRNA in

a region downstream from the position of the T315I mutation;

(b) a reverse primer that hybridizes to an *ABL1* mRNA in a region upstream from the position of the T315I mutation; and

(c) a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA.

[0048] The kit may further comprise at least one of forward primers and additional reverse primers for amplifying the reverse transcripts of the *ABL1* mRNA generated with the reverse primers (a) and (b). The primers may be for RNase H-dependent PCR. The kit may further comprise a probe for quantitative PCR. The kit may further comprise at least one standard, e.g., a known amount of a wild-type *ABL1* mRNA or an *ABL1* mRNA having T315I mutation.

[0049] The kit may further comprise at least one of the reagents necessary for conducting the method disclosed herein, for example, the reagents for reverse transcription, PCR, quantitative PCR, or RNase H-dependent PCR, including reverse transcriptases, DNA polymerases, RNases H, dNTPs, and buffers. The kit may further comprise any component preferred for marketing or using the kit, for example, a package insert (e.g., a document or a storage medium) containing description for using the kit.

[0050] The components of the kit are provided individually or, if possible, in a mixture. Each component may be provided in a solution in water or a suitable buffer, or in the lyophilized form, contained in a suitable container. Examples of the suitable containers include bottles, vials, test tubes, tubes, plates, and multi-well plates. The container may be made of at least one material, such as glass, plastic, or metal. The container may have a label.

[0051] Without wishing to be bound by any theory, a mechanism of an embodiment of the method and kit disclosed

herein is illustrated below with some schematic drawings.

[0052]

1. Overview of the measurement mechanism

The expression level of an *ABL1* mRNA having T315I mutation, in which the base of nucleotide position 944 of a wild-type *ABL1* mRNA is substituted from cytosine to thymine, in an RNA sample extracted from leukocytes in peripheral blood or nucleated cells in bone marrow aspirate is measured. The measurement is carried out by two-step quantitative RT-PCR including a step of reverse transcription (RT) to synthesize a complementary DNA (cDNA) along with an RNA template and a step of real-time PCR to quantitate the cDNA with a fluorescence-labeled probe. An *ABL1* mRNA having T315I mutation is amplified so that a region comprising the position of the T315I mutation is amplified, and all *ABL1* mRNAs are amplified so that a region not comprising the position is amplified. The measurement of the former is divided by the measurement of the latter, and the resulting ratio is reported.

[0053]

1.1 Reverse transcription

A schematic drawing of the reverse transcription is shown in Fig. 5. An *ABL1* mRNA in which the base at nucleotide position 944 is cytosine is defined as the wild-type *ABL1* mRNA. An *ABL1* mRNA in which the base is thymine is defined as the *ABL1* mRNA having T315I mutation. Two primers, ABL reverse primer and T315I reverse primer, are used for reverse transcribing two regions in the *ABL1* mRNAs; the former is for quantitating all *ABL1* mRNAs and the latter is for quantitating the *ABL1* mRNA having T315I mutation.

[0054] The ABL reverse primer and the T315I reverse primer hybridize to the complementary sequences in the sample (Fig. 5(1a) and (1b)). The first strand cDNA is synthesized from the reverse primers by reverse transcriptase activity of Tth DNA

polymerase contained in the reaction mixture (Fig. 5(2a) or (2b)). The reaction mixture contains a T315I clamp, which has a sequence complementary to the wild-type *ABL1* mRNA. During the synthesis of the first strand cDNA, the T315I clamp specifically binds to the wild-type *ABL1* mRNA and inhibits the reverse transcription (Fig. 5(2a)), decreasing the synthesis of the cDNA from the T315I reverse primer (Fig. 5(3a)). In contrast, the T315I clamp does not bind to the *ABL1* mRNA having T315I mutation and does not inhibit the reverse transcription (Fig. 5(2b) and (3b)). Since the T315I clamp does not affect the synthesis of the cDNA from the ABL reverse primer, the cDNA synthesis equivalently occurs for the wild-type *ABL1* mRNA and the *ABL1* mRNA having T315I mutation.

[0055]

1.2 Quantitative real-time PCR of *ABL1* mRNAs

A schematic drawing of quantitative real-time PCR of the *ABL1* mRNAs is shown in Fig. 6. The cDNAs of the *ABL1* mRNAs synthesized by the reverse transcription are amplified by real-time PCR (Fig. 6). During the amplification, a fluorescence-labeled probe (ABL probe) hybridized to one strand of the double-stranded cDNA is degraded by 5'-3' exonuclease activity of Tth DNA polymerase and a reporter substance is released. By measuring the fluorescence intensities of the released substance for each cycle of the PCR, the increase of the cDNAs derived from the *ABL1* mRNAs is determined in real-time (Fig. 6(6)).

[0056]

1.3 Quantitative real-time PCR of *ABL1* mRNA having T315I mutation

In the amplification of the cDNA derived from the *ABL1* mRNA having T315I mutation, the principle of RNase H-dependent PCR (rhPCR) is used for increasing the specificity of the reaction. RNase H2, the nuclease used in the rhPCR, recognizes

an RNA/DNA heteroduplex portion and cleaves the phosphodiester bond between the RNA portion and the DNA portion at the 5'-end of the RNA portion. An RNA/DNA heteroduplex portion is formed between the template DNA and the primer by replacing a deoxyribonucleotide in the primer with a ribonucleotide. The primer further has a blocking region, to which DNA polymerases cannot bind, at the 3' end of the RNA portion. Accordingly, the RCR proceeds only after the RNase H2 recognizes and cleaves the RNA/DNA heteroduplex. This strategy allows that the cDNAs derived from the *ABL1* mRNA having T315I mutation, in which the base at nucleotide position 944 of the *ABL1* mRNA is thymine, is solely amplified. A schematic drawing of the quantitative real-time PCR of the *ABL1* mRNA having T315I mutation is shown in Fig. 7.

[0057] The T315I forward primer has an RNA portion (uracil) in place of the DNA portion (thymine) at the nucleotide corresponding to nucleotide position 944 of the *ABL1* mRNA having T315I mutation (Fig. 7(1a)). Since the RNA portion does not form an RNA/DNA heteroduplex portion with the cDNA derived from the wild-type *ABL1* mRNA, the blocking region is not removed by RNase H2 and thus PCR does not proceed for the wild-type *ABL1* mRNA (Fig. 7(2a)).

[0058] In contrast, since the RNA portion forms an RNA/DNA heteroduplex portion with the cDNA derived from the *ABL1* mRNA having T315I mutation, the blocking region is removed by RNase H2 and thus the specific amplification proceeds for the *ABL1* mRNA having T315I mutation (Fig.7 (1b), (2b)). The specific amplification is repeated by PCR (Fig.7 (3) to (9)). During the amplification, a fluorescence-labeled probe (ABL T315I probe) hybridized to one strand of the double-stranded cDNA is degraded by 5'-3' exonuclease activity of Tth DNA polymerase and a reporter substance is released. By measuring the fluorescence intensity of the released substance for each cycle

of the PCR, the increase of cDNA derived from the *ABL1* mRNA having T315I mutation is determined in real-time (Fig. 7(6)).

[0059] Another aspect of the disclosure provides a method of detecting the T315I variant of *ABL1* in a subject, comprising:

- (1) reverse-transcribing an RNA sample obtained from the subject in the presence of a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA by using a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation;

- (2) amplifying the reverse transcript obtained in the step (1) in RNase H-dependent quantitative PCR by using a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation, having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation, and having a blocking region at the 3' end; and

- (3) determining that the subject is expressing the T315I variant of *ABL1* when the nucleic acid amplification is occurred in the step (2).

[0060] Another aspect of the disclosure provides a kit for conducting the method disclosed immediately above, comprising:

- (a) a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation, having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation, and having a blocking region at the 3' end;

- (b) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation; and

- (c) a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type

ABL1 mRNA.

[0061] The details of the method and kit are similar to those of the method and kit for determining the expression level of the T315I variant of *ABL1* in a subject described earlier.

[0062] For example, the disclosure provides the following embodiments.

[1] A method of determining the expression level of the T315I variant of *ABL1* in a subject, comprising:

(1) reverse-transcribing an RNA sample obtained from the subject in the presence of a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA by using (a) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation and (b) a reverse primer that hybridizes to an *ABL1* mRNA in a region upstream from the position of the T315I mutation in one container; and

(2) calculating the expression level of the T315I variant of *ABL1* on the basis of the ratio of the reverse transcript generated with the primer (a) to the reverse transcript generated with the primer (b).

[2] The method according to item 1, wherein the step (2) further comprising:

(2-1) quantifying the reverse transcript generated with the primer (a) by quantitative PCR; and

(2-2) quantifying the reverse transcript generated with the primer (b) by quantitative PCR.

[3] The method according to item 2, wherein the quantitative PCR in the step (2-1) uses

(X) a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation and having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation; or

(Y) a forward primer having a nucleic acid sequence of a region upstream from the position of the T315I mutation of an *ABL1* mRNA.

[0063]

[4] The method according to item 3, wherein the quantitative PCR in the step (2-1) uses a forward primer (X).

[5] The method according to item 4, wherein the forward primer has a blocking region at the 3' end.

[6] The method according to any one of items 2 to 5, wherein the quantitative PCR in the step (2-1) is RNase H-dependent quantitative PCR specific to the T315I mutation.

[7] The method according to any one of items 2 to 6, wherein the quantitative PCR in the step (2-1) uses a forward primer comprising the nucleic acid sequence of SEQ ID NO: 6.

[8] The method according to any one of items 2 to 7, wherein the quantitative PCR in the step (2-1) uses a forward primer consisting of the nucleic acid sequence of SEQ ID NO: 6.

[9] The method according to any one of items 3 to 8, wherein a part of the modified nucleic acid is complementary to a part of the forward primer (X), and the length of the complementary region is shorter than 50% of the total length of the modified nucleic acid.

[10] The method according to any one of items 1 to 9, wherein the position corresponding to the T315I mutation in the modified nucleic acid is shifted from the center of the modified nucleic acid toward the 3' end.

[11] The method according to any one of items 1 to 10, wherein the modified nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 5.

[12] The method according to any one of items 1 to 11, wherein the modified nucleic acid consists of the nucleic acid sequence of SEQ ID NO: 5.

[13] The method according to any one of items 1 to 12, wherein

the modified nucleic acid comprises a PNA portion.

[0064]

[14] The method according to any one of items 1 to 12, wherein the reverse primer (a) comprises the nucleic acid sequence of SEQ ID NO: 7.

[15] The method according to any one of items 1 to 14, wherein the reverse primer (b) comprises the nucleic acid sequence of SEQ ID NO: 10.

[16] The method according to any one of items 1 to 15, wherein the reverse primer (a) consists of the nucleic acid sequence of SEQ ID NO: 7.

[17] The method according to any one of items 1 to 16, wherein the reverse primer (b) consists of the nucleic acid sequence of SEQ ID NO: 10.

[18] The method according to any one of items 2 to 17, wherein the step (2-1) is conducted in the presence of the modified nucleic acid used in the step (1).

[19] The method according to any one of items 1 to 18, wherein the subject is a human being.

[20] The method according to any one of items 1 to 19, wherein the RNA sample is RNAs extracted from leukocytes in peripheral blood or nucleated cells in bone marrow aspirate.

[21] The method according to any one of items 1 to 19, wherein the RNA sample is RNAs extracted from leukocytes in peripheral blood.

[22] The method according to any one of items 1 to 21, wherein at least one of the primer, clamp, and probe shown in Table 1 is used.

[23] The method according to any one of items 1 to 22, wherein the primer, clamp, and probe shown in Table 1 are used.

[0065]

[24] A kit for determining the expression level of the T315I variant of ABL1 in a subject, comprising:

(a) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation;

(b) a reverse primer that hybridizes to an *ABL1* mRNA in a region upstream from the position of the T315I mutation; and

(c) a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA.

[25] The kit according to item 24, further comprising a forward primer for amplifying the reverse transcripts that are generated by reverse transcriptions with the reverse primers (a) and (b).

[26] The kit according to item 25, wherein the forward primer is

(X) a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation and having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation; or

(Y) a forward primer having a nucleic acid sequence of a region upstream from the position of the T315I mutation of an *ABL1* mRNA.

[27] The kit according to item 26, wherein the forward primer is a forward primer (X).

[28] The kit according to item 27, wherein the forward primer has a blocking region at the 3' end.

[29] The kit according to any one of items 25 to 28, wherein the forward primer comprises the nucleic acid sequence of SEQ ID NO: 6.

[30] The kit according to any one of items 25 to 29, wherein the forward primer consists of the nucleic acid sequence of SEQ ID NO: 6.

[0066]

[31] The kit according to any one of items 25 to 30, wherein a part of the modified nucleic acid is complementary to a part

of the forward primer (X), and the length of the complementary region is shorter than 50% of the total length of the modified nucleic acid.

[32] The kit according to any one of items 24 to 31, wherein the position corresponding to the T315I mutation in the modified nucleic acid is shifted from the center of the modified nucleic acid toward the 3' end.

[33] The kit according to any one of items 24 to 32, wherein the modified nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 5.

[34] The kit according to any one of items 24 to 33, wherein the modified nucleic acid consists of the nucleic acid sequence of SEQ ID NO: 5.

[35] The kit according to any one of items 24 to 34, wherein the modified nucleic acid comprises a PNA portion.

[35] The kit according to any one of items 24 to 35, wherein the reverse primer (a) comprises the nucleic acid sequence of SEQ ID NO: 7.

[36] The kit according to any one of items 24 to 35, wherein the reverse primer (b) comprises the nucleic acid sequence of SEQ ID NO: 10.

[37] The kit according to any one of items 24 to 36, wherein the reverse primer (a) consists of the nucleic acid sequence of SEQ ID NO: 7.

[38] The kit according to any one of items 24 to 37, wherein the reverse primer (b) consists of the nucleic acid sequence of SEQ ID NO: 10.

[39] The kit according to any one of items 24 to 38, further comprising a probe for quantitative PCR.

[40] The kit according to any one of items 24 to 39, further comprising at least one of a wild-type *ABL1* mRNA and an *ABL1* mRNA having the T315I mutation.

[41] The kit according to any one of items 24 to 40, comprising

at least one of the primer, clamp, and probe shown in Table 1.

[42] The kit according to any one of items 24 to 41, comprising the primer, clamp, and probe shown in Table 1:

[0067] The entire contents of the documents cited herein are incorporated herein by reference.

The embodiments described above are non-limiting and may be modified without deviating from the scope of the invention as defined by the appended claims. The following example does not restrict or limit the invention and is for illustrative purposes only.

EXAMPLES

[0068]

Design of primers and probes

The following primer sets and probes were designed and synthesized.

The fluorescence-labeled probes had HEX (6-carboxyfluorescein) at the 5' end and Iowa Black FQ (Integrated DNA Technologies, Inc.) as a quencher at the 3' end.

The details of the primers and probes used in the test examples are shown in Table 2.

[Table 2]

Gene		
ABL T315 I	Forward primer	SEQ ID NO: 6 5'- GAGCCCCCGTTCTATATCATCArUT/iSpC3//iSpC3/GC- 3' * *rU: RNA uracil, iSpC3: spacer C3
	Reverse primer	SEQ ID NO: 7 5'-CCCCTACCAGGCAGTTTCG-3'
	Probe	SEQ ID NO: 8 5'-/HEX/CAGGAGGTGAACGCCGTGGTGC/3IABkFQ/-3' * IABkFQ: Iowa Black FQ * HEX: hexachlorofluorescein
ABL	Forward primer	SEQ ID NO: 9 5'-CGTTGGAAGTCCAAGGAAAAC-3'
	Reverse primer	SEQ ID NO: 10 5'-ATTCCCCATTGTGATTATAGCCTAAG-3'
	Probe	SEQ ID NO: 11 5'- /HEX/CTGGACCCA/ZEN/GTGAAAATGACCCCAAC/3IABkF Q/-3' * IABkFQ: Iowa Black FQ * HEX: hexachlorofluorescein * ZEN: ZEN Quencher

[0069]

Test Example 1

(1) Preparation of standard (ABL1 RNA having T315I mutation)

A synthesized RNA comprising a sequence of an *ABL1* mRNA having the T315I mutation was used as the standard. For generating a DNA fragment used as the template for the RNA synthesis, a plasmid vector comprising T7 promoter, which works as the origin of the RNA synthesis, and the regions of the wild-type *ABL1* mRNA that are amplified with the above-mentioned primers was prepared. One base mutation was introduced into the sequence derived from the *ABL1* mRNA in the plasmid vector at the position corresponding to the T315I mutation by site specific mutagenesis. *Escherichia coli* was transformed with the mutated plasmid vector. A large amount of the plasmid vector was prepared by culturing the bacteria. The plasmid

vector was digested at one site with a restriction enzyme to give a linear DNA fragment encoding the *ABL1* mRNA having the T315I mutation.

[0070] By using the DNA fragment as the template, an RNA comprising the sequence of the *ABL1* mRNA having the T315I mutation, i.e., the *ABL1* RNA having the T315I mutation, was synthesized with a T7 RNA polymerase. The synthesized RNA was diluted with a TE buffer containing 100 ng/ μ L of *E. coli* transfer RNA to give the RNA standard. The concentration of the RNA standard was adjusted at 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1.0×10^6 , or 1×10^7 copies/test. A TE buffer containing 100 ng/ μ L of *E. coli* transfer RNA was used as the negative control.

[0071]

(2) Reverse transcription

(2-1) Preparation of reaction solutions

The total volume of each reaction solution was 50 μ L. Reaction solutions containing 0.2 μ M each of T315I reverse primer and ABL reverse primer (Integrated DNA Technologies, Inc.), 0.1 mM of dNTPs (TOYOBO CO., LTD.), 2.4 mM of MnOAc (TOYOBO CO., LTD.), 2 μ M of PNA clamp (Panagene), 2.5 U of rTth DNA polymerase (TOYOBO CO., LTD.), and 1 μ g of an RNA to be tested were prepared. The standard RNA was tested once for each concentration. PNA clamp had the nucleotide sequence of ATGAACTCAGTGATGA (SEQ ID NO: 5) and all the nucleotides of the PNA clamp were linked via peptide bonds.

(2-2) Reaction condition:

The reverse transcriptions were conducted at 60°C for 60 minutes.

[0072]

(3) PCR

The cDNAs generated with T315I reverse primer and the cDNAs generated with ABL reverse primer (control) were

amplified by PCR in separate tubes.

(3-1) Quantification of cDNA generated with T315I reverse primer

(a) Preparation of reaction solutions

The total volume of each reaction solution was 30 μ L. Reaction solutions containing 0.3 μ M each of T315I forward primer and T315I reverse primer (Integrated DNA Technologies, Inc.), 0.15 μ M of fluorescence-labeled T315I probe (Integrated DNA Technologies, Inc.), 0.2 mM of dNTPs (TOYOBO CO., LTD.), 2.4 mM of MnOAc (TOYOBO CO., LTD.), 100 mU of RNase H2 (Integrated DNA Technologies, Inc.), 1.25 U of rTth DNA polymerase (TOYOBO CO., LTD.), and 25 μ L of one of the reverse transcripts obtained in the step (2) above were prepared. The reverse transcripts were used without purification. The reverse transcript of each concentration was quantified once.

(b) Reaction

Applied Biosystems 7500 Fast Realtime PCR system (Life Technologies Corporation) was used. After the reaction at 95°C for 10 seconds, the reaction cycle at 95°C for 10 seconds and then at 60°C for 60 seconds was repeated 40 times.

[0073]

(3-2) Quantification of cDNA generated with ABL reverse primer

(a) Preparation of reaction solutions

The total volume of each reaction solution was 30 μ L. Reaction solutions containing 0.3 μ M each of ABL forward primer and ABL reverse primer (Integrated DNA Technologies, Inc.), 0.2 μ M of fluorescence-labeled ABL probe (Integrated DNA Technologies, Inc.), 0.4 mM of dNTPs (TOYOBO CO., LTD.), 2.0 mM of MnOAc (TOYOBO CO., LTD.), 1.125 U of Tth DNA polymerase, and 5 μ L of one of the reverse transcripts were prepared. The reverse transcript of each concentration was quantified once.

(b) Reaction

Applied Biosystems 7500 Fast Realtime PCR system (Life

Technologies Corporation) was used. After the reaction at 95°C for 10 seconds, the reaction cycle at 95°C for 10 seconds and then at 60°C for 60 seconds was repeated 40 times.

[0074]

(4) Results

As described, the RNA standard and the negative control were reverse-transcribed with (a) T315I reverse primer and (b) ABL reverse primer, and the obtained cDNAs were amplified. The fluorescence amplification curves and the cycle numbers were reported.

Fig. 8 shows the amplification curves for the cDNAs generated with (a) T315I reverse primer. Fig. 9 shows the amplification curves for the cDNAs generated with (b) ABL reverse primer. Table 3 shows the cycle numbers of the amplification for the cDNAs generated with T315I reverse primer and the cDNAs generated with ABL reverse primer. The test was able to quantify the cDNAs generated with T315I reverse primer and the cDNAs generated with ABL reverse primer in the wide range from 1×10^2 to 1×10^7 copies/test.

[0075]

[Table 3]

Concentration of RNA standard	Cycle numbers of the amplification of the cDNA generated with T315I reverse primer	Cycle numbers of the amplification of the cDNA generated with ABL reverse primer
1×10^7 copies/test	15.88	15.04
1×10^6 copies/test	19.99	18.63
1×10^5 copies/test	23.49	22.14
1×10^4 copies/test	27.29	25.54
1×10^3 copies/test	30.94	29.34
1×10^2 copies/test	34.45	33.29
Negative control	Not detected	Not detected

[0076]

Test Example 2: Evaluation of detection limit

The lower detection limit of the system for determining the expression level of the T315I variant of ABL1 was evaluated. Test samples were prepared by adding various concentrations of the ABL1 RNA having the T315I mutation (standard used in Test Example 1) to RNA extracted from cells of human leukemia cell line HL60. The cDNAs generated with T315I reverse primer and the cDNAs generated with ABL reverse primer were quantified, the measurements of the former are divided by the measurements of the latter, and the resulting ratios were reported.

[0077]

(1) Preparation of standard (ABL1 RNA having T315I mutation)

The standard was prepared according to Test Example 1.

[0078]

(2) Test samples

Test samples were prepared by adding various concentrations of the ABL1 RNA having the T315I mutation (standard) to RNA extracted from cells of BCR-ABL1-negative human leukemia cell line HL60. Specifically, total RNA was extracted from HL60 cells, 25, 50, or 100 copies/test of the ABL1 RNA having the T315I mutation (standard) were added, and the final concentration of the RNA was adjusted to 100 ng/ μ L with a TE buffer. The total RNA extracted from HL60 cells and adjusted at 100 ng/ μ L with a TE buffer was used as the control.

[0079]

(3) Reverse transcription

The reverse transcription was conducted according to Test Example 1. For the standard RNA, one reverse transcript was generated for each concentration. For the test samples, twelve reverse transcripts were generated for each concentration.

(4) PCR

The PCR was conducted according to Test Example 1. The reverse transcripts of the standard and the test samples were quantified once.

[0080]

(5) Results

Table 4 shows the measurements of the cDNAs generated with T315I reverse primer. Table 5 shows the measurements of the cDNAs generated with ABL reverse primer. Table 6 shows the ratios obtained by dividing the measurements of the cDNAs generated with T315I reverse primer with the measurements of the cDNAs generated with ABL reverse primer (ABL1 T315I variant/ABL1 ratios). The measurements of the cDNAs generated with T315I reverse primer for the samples containing 100, 50, and 25 copies/test of the ABL1 RNA having the T315I mutation were 109.4, 47.4, and 27.3, respectively, on average, being compatible with the theoretical values. Regarding the negative control, RNA extracted from HL60, which does not contain the ABL1 RNA having the T315I mutation, no fluorescence amplification was observed in the quantification of the cDNAs generated with T315I reverse primer.

[0081]

Since HL60 cells were expressing a certain amount of the ABL1 mRNA, the measurements of the cDNAs generated with ABL reverse primer were equivalent for every sample and the average was 2.08×10^5 . Even such samples containing more than 10^5 copies of the wild-type ABL mRNA were measured, the cDNAs generated with T315I reverse primer were not detected. This is compatible with that the samples were derived from HL60 cells, which do not contain the ABL1 RNA having the T315I mutation. These results indicate that the system used for the quantification has very high specificity to the ABL1 RNA having the T315I mutation.

[0082]

The ABL1 T315I variant/ABL1 ratios for the samples containing 100, 50, and 25 copies/test of the ABL1 RNA having the T315I mutation were 0.052%, 0.023%, and 0.013%,

respectively, on average. These results indicate that the system can detect the mutation with high sensitivity even if the percentage of the variant is as low as 0.01%.

[0083]

[Table 4]

Measurement of cDNA generated with T315I reverse primer				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	110.4	29.0	41.1	Not detected
2	97.7	53.6	26.3	Not detected
3	99.7	43.6	37.4	Not detected
4	100.0	44.7	25.3	Not detected
5	112.5	57.3	25.5	Not detected
6	108.1	60.1	33.9	Not detected
7	113.1	45.2	19.8	Not detected
8	107.9	41.2	24.1	Not detected
9	117.6	50.8	21.6	Not detected
10	101.9	40.7	12.7	Not detected
11	115.5	41.4	40.6	Not detected
12	128.2	61.5	19.4	Not detected
Average	109.4	47.4	27.3	-

[0084]

[Table 5]

Measurement of cDNA generated with ABL reverse primer				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	2.08×10^5	2.06×10^5	2.13×10^5	2.06×10^5
2	2.02×10^5	2.07×10^5	1.99×10^5	2.03×10^5
3	2.06×10^5	2.11×10^5	2.08×10^5	2.07×10^5
4	2.05×10^5	2.07×10^5	2.06×10^5	2.08×10^5
5	2.14×10^5	2.08×10^5	2.04×10^5	1.99×10^5
6	2.06×10^5	2.06×10^5	2.10×10^5	2.06×10^5
7	2.08×10^5	2.09×10^5	2.06×10^5	2.09×10^5
8	2.15×10^5	2.06×10^5	2.08×10^5	1.97×10^5
9	2.08×10^5	2.02×10^5	2.04×10^5	2.07×10^5
10	2.08×10^5	2.10×10^5	2.08×10^5	2.07×10^5
11	2.13×10^5	2.07×10^5	2.05×10^5	2.09×10^5
12	2.35×10^5	2.03×10^5	2.24×10^5	2.13×10^5
Average	2.11×10^5	2.07×10^5	2.08×10^5	2.06×10^5

[0085]

[Table 6]

ABL1 T315I variant/ABL1 ratio				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	0.053%	0.014%	0.019%	-
2	0.048%	0.026%	0.013%	-
3	0.048%	0.021%	0.018%	-
4	0.049%	0.022%	0.012%	-
5	0.052%	0.027%	0.012%	-
6	0.052%	0.029%	0.016%	-
7	0.054%	0.022%	0.010%	-
8	0.050%	0.020%	0.012%	-
9	0.057%	0.025%	0.011%	-
10	0.049%	0.019%	0.006%	-
11	0.054%	0.020%	0.020%	-
12	0.055%	0.030%	0.009%	-
Average	0.052%	0.023%	0.013%	-

[0086]

Test Example 3: Effect of PNA clamp to inhibit reaction

The purpose of the test is to confirm the effect of the PNA clamp in the reverse transcription.

(1) Preparation of standard (ABL1 RNA having T315I mutation)

The standard was prepared according to Test Example 1.

(2) Test samples

Test samples were prepared according to Test Example 2.

(3) Reverse transcription

The reverse transcription was conducted according to Test Example 2, but no PNA clamp was added.

(4) PCR

The PCR was conducted according to Test Example 2.

[0087]

(5) Results

Table 7 shows the measurements of the cDNAs generated with T315I reverse primer. Table 8 shows the measurements of the cDNAs generated with ABL reverse primer. Table 9 shows the ratios obtained by dividing the measurements of the cDNAs

generated with T315I reverse primer with the measurements of the cDNAs generated with ABL reverse primer (ABL1 T315I variant/ABL1 ratios). The measurements of the cDNAs generated with T315I reverse primer for the samples containing 100, 50 and 25 copies/test of the ABL1 RNA having the T315I mutation were 333.7, 235.3, and 202.3 copies/test, respectively, on average. The average of the measurements of the cDNAs generated with T315I reverse primer for the RNA extracted from HL60 cells (control) was 186.9 copies/test.

[0088]

Since HL60 cells were expressing a certain amount of the ABL1 mRNA, the measurements of the cDNAs generated with ABL reverse primer were equivalent for every sample and the average was 2.29×10^5 . The ABL1 T315I variant/ABL1 ratios for the samples containing 100, 50, and 25 copies/test of the ABL1 RNA having the T315I mutation were 0.147%, 0.107%, and 0.090%, respectively, on average. The average of the ABL1 T315I variant/ABL1 ratios for the RNA extracted from HL60 cells (control) was 0.077%.

[0089]

Unlike Test Example 2, in the absent of the PNA clamp, the average of the measurements of the cDNAs generated with T315I reverse primer for the RNA sample not containing the ABL1 RNA having the T315I mutation was 186.9 copies/test. No sufficient difference was found between the negative control and the samples containing the ABL1 RNA having the T315I mutation. The results indicate that the presence of the PNA clamp in the reverse transcription is important for the sensitive detection of the ABL1 T315I variant.

[0090]

[Table 7]

Measurement of cDNA generated with T315I reverse primer				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	338.4	224.1	163.4	176.1
2	328.1	209.7	180.9	175.0
3	346.3	221.7	218.9	187.3
4	322.6	246.4	205.8	191.7
5	336.8	249.9	214.8	199.3
6	308.9	214.8	197.0	207.3
7	311.3	222.3	213.5	182.1
8	325.2	250.6	219.4	183.3
9	353.4	265.9	198.2	178.4
10	327.4	254.7	209.7	177.4
11	354.1	217.4	192.7	193.9
12	351.5	246.4	213.1	191.2
Average	333.7	235.3	202.3	186.9

[0091]

[Table 8]

Measurement of cDNA generated with ABL reverse primer				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	2.19×10^5	2.07×10^5	2.19×10^5	2.40×10^5
2	2.15×10^5	2.03×10^5	2.17×10^5	2.31×10^5
3	2.20×10^5	2.08×10^5	2.30×10^5	2.39×10^5
4	2.34×10^5	2.23×10^5	2.17×10^5	2.86×10^5
5	2.11×10^5	2.12×10^5	2.19×10^5	2.40×10^5
6	2.09×10^5	2.06×10^5	2.13×10^5	2.48×10^5
7	2.16×10^5	2.04×10^5	2.48×10^5	2.55×10^5
8	2.17×10^5	2.15×10^5	2.08×10^5	2.51×10^5
9	2.36×10^5	2.30×10^5	2.23×10^5	2.39×10^5
10	2.44×10^5	2.37×10^5	2.39×10^5	2.25×10^5
11	2.47×10^5	2.38×10^5	2.42×10^5	2.33×10^5
12	2.53×10^5	2.50×10^5	2.37×10^5	2.40×10^5
Average	2.27×10^5	2.20×10^5	2.26×10^5	2.44×10^5

[0092]

[Table 9]

ABL1 T315I variant/ABL1 ratio				
No.	ABL1 RNA having T315I mutation (copies/test)			
	100	50	25	0
1	0.155%	0.108%	0.075%	0.073%
2	0.152%	0.104%	0.083%	0.076%
3	0.157%	0.107%	0.095%	0.078%
4	0.138%	0.111%	0.095%	0.067%
5	0.160%	0.118%	0.098%	0.083%
6	0.148%	0.104%	0.092%	0.083%
7	0.144%	0.109%	0.086%	0.071%
8	0.150%	0.116%	0.106%	0.073%
9	0.150%	0.115%	0.089%	0.075%
10	0.134%	0.108%	0.088%	0.079%
11	0.143%	0.091%	0.080%	0.083%
12	0.139%	0.098%	0.090%	0.080%
Average	0.147%	0.107%	0.090%	0.077%

INDUSTRIAL APPLICABILITY

[0093] The disclosure can provide a method of detecting the T315I variant of ABL1 or determining the expression level thereof more sensitively than the methods of the prior art. Accordingly, the method or the kit disclosed herein can determine the expression level of the T315I variant of ABL1 with high sensitivity, being useful for, e.g., diagnosing onset or recurrence of leukemia, predicting prognosis of leukemia, and determining timing of bone marrow transplantation.

What is claimed is

1. A method of determining the expression level of the T315I variant of *ABL1* in a subject, comprising:

(1) reverse-transcribing an RNA sample obtained from the subject in the presence of a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type *ABL1* mRNA by using (a) a reverse primer that hybridizes to an *ABL1* mRNA in a region downstream from the position of the T315I mutation and (b) a reverse primer that hybridizes to an *ABL1* mRNA in a region upstream from the position of the T315I mutation in one container; and

(2) calculating the expression level of the T315I variant of *ABL1* on the basis of the ratio of the reverse transcript generated with the primer (a) to the reverse transcript generated with the primer (b).

2. The method according to claim 1, wherein the step (2) further comprising:

(2-1) quantifying the reverse transcript generated with the primer (a) by quantitative PCR; and

(2-2) quantifying the reverse transcript generated with the primer (b) by quantitative PCR.

3. The method according to claim 2, wherein the quantitative PCR in the step (2-1) uses

(X) a forward primer having a nucleic acid sequence of a region of an *ABL1* mRNA comprising the position of the T315I mutation and having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation; or

(Y) a forward primer having a nucleic acid sequence of a region upstream from the position of the T315I mutation of an *ABL1* mRNA.

4. The method according to claim 3, wherein the quantitative PCR in the step (2-1) uses a forward primer (X) that has a blocking region at the 3' end.

5. The method according to any one of claims 2 to 4, wherein the quantitative PCR in the step (2-1) is RNase H-dependent quantitative PCR specific to the T315I mutation.

6. The method according to any one of claims 2 to 5, wherein the quantitative PCR in the step (2-1) uses a forward primer comprising the nucleic acid sequence of SEQ ID NO: 6.

7. The method according to any one of claims 2 to 6, wherein the quantitative PCR in the step (2-1) uses a forward primer consisting of the nucleic acid sequence of SEQ ID NO: 6.

8. The method according to any one of claims 3 to 7, wherein a part of the modified nucleic acid is complementary to a part of the forward primer (X), and the length of the complementary region is shorter than 50% of the total length of the modified nucleic acid.

9. The method according to any one of claims 1 to 8, wherein the position corresponding to the T315I mutation in the modified nucleic acid is shifted from the center of the modified nucleic acid toward the 3' end.

10. The method according to any one of claims 1 to 9, wherein the modified nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 5.

11. The method according to any one of claims 1 to 10, wherein

the modified nucleic acid consists of the nucleic acid sequence of SEQ ID NO: 5.

12. The method according to any one of claims 1 to 11, wherein the modified nucleic acid comprises a PNA portion.

13. The method according to any one of claims 1 to 12, wherein the reverse primer (a) comprises the nucleic acid sequence of SEQ ID NO: 7 and the reverse primer (b) comprises the nucleic acid sequence of SEQ ID NO: 10.

14. A kit for determining the expression level of the T315I variant of ABL1 in a subject, comprising:

(a) a reverse primer that hybridizes to an ABL1 mRNA in a region downstream from the position of the T315I mutation;

(b) a reverse primer that hybridizes to an ABL1 mRNA in a region upstream from the position of the T315I mutation; and

(c) a modified nucleic acid complementary to a region comprising the position of the T315I mutation of a wild-type ABL1 mRNA.

15. The kit according to claim 14, further comprising a forward primer for amplifying the reverse transcripts that are generated by reverse transcriptions with the reverse primers (a) and (b).

16. The kit according to claim 15, wherein the forward primer is

(X) a forward primer having a nucleic acid sequence of a region of an ABL1 mRNA comprising the position of the T315I mutation and having a uracil ribonucleotide in place of the nucleotide of the position of the T315I mutation, or

(Y) a forward primer having a nucleic acid sequence of a

region upstream from the position of the T315I mutation of an *ABL1* mRNA.

17. The method according to claim 16, wherein the forward primer is a forward primer (X) that has a blocking region at the 3' end.

18. The kit according to claim 16 or 17, wherein a part of the modified nucleic acid is complementary to a part of the forward primer (X), and the length of the complementary region is shorter than 50% of the total length of the modified nucleic acid.

19. The kit according to any one of claims 14 to 18, wherein the position corresponding to the T315I mutation in the modified nucleic acid is shifted from the center of the modified nucleic acid toward the 3' end.

20. The kit according to any one of claims 14 to 19, further comprising a probe for quantitative PCR.

21. The kit according to any one of claims 14 to 20, further comprising at least one of a wild-type *ABL* mRNA and an *ABL* mRNA having the T315I mutation.