



(11) **EP 2 436 763 A1**

(12) **EUROPEAN PATENT APPLICATION**  
published in accordance with Art. 153(4) EPC

(43) Date of publication:  
**04.04.2012 Bulletin 2012/14**

(51) Int Cl.:  
**C12N 15/09** (2006.01) **C12Q 1/34** (2006.01)  
**C12Q 1/68** (2006.01) **G01N 33/53** (2006.01)

(21) Application number: **10780625.9**

(86) International application number:  
**PCT/JP2010/059049**

(22) Date of filing: **27.05.2010**

(87) International publication number:  
**WO 2010/137671 (02.12.2010 Gazette 2010/48)**

(84) Designated Contracting States:  
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB  
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO  
PL PT RO SE SI SK SM TR**

- **MIURA, Masatomo**  
Akita-shi  
Akita 010-8543 (JP)
- **OGAWA, Junichi**  
Akita-shi  
Akita 010-8543 (JP)

(30) Priority: **27.05.2009 JP 2009128323**

(71) Applicant: **National University Corporation Akita  
University**  
Akita-shi  
Akita 010-8502 (JP)

(74) Representative: **HOFFMANN EITLE**  
Patent- und Rechtsanwälte  
Arabellastraße 4  
81925 München (DE)

(72) Inventors:  
• **MOTOYAMA, Satoru**  
Akita-shi  
Akita 010-8543 (JP)

(54) **METHOD FOR ASSESSING LYMPH NODE METASTASIS OF CANCER OR THE RISK THEREOF,  
AND RAPID ASSESSMENT KIT FOR SAID METHOD**

(57) The objective of the present invention is to provide a method and a means of rapidly and reliably detecting lymph node metastasis in cancer or the risk of lymph node metastasis. Specifically, the present invention provides a method and a rapid determination kit for detecting lymph node metastasis in cancer or its risk by identifying a certain genetic polymorphism of the human

CRP gene, and it is clinically significant in determining the treatment strategy, because effective prediction/determination can be made regarding lymph node metastasis, which is an important phenomenon in cancer progression.

**EP 2 436 763 A1**

**Description**

## TECHNICAL FIELD

5 **[0001]** The present invention relates to a method for determining lymph node metastasis in cancer and a rapid determination kit for the same.

## BACKGROUND ART

10 **[0002]** Cancer cells from a primary focus metastasize all over the body through blood vessels and lymphatic vessels. Because the primary focus is removed as much as possible in cancer surgery, the accurate detection of metastasis and the appropriate treatment depending on the degree of metastasis are necessary. Therefore, diagnosing lymph node metastasis by cancer cells is extremely important for selecting the appropriate treatment for cancer.

15 **[0003]** The diagnosis of lymph node metastasis by cancer cells is broadly divided into pretreatment diagnostic imaging and posttreatment (postoperative) pathological diagnosis. The diagnostic imaging methods that are used for detecting lymph node metastasis in cancer (inspection for the presence of lymph node metastasis) include computed tomography (CT); positron emission tomography (PET); PET-CT, which uses an apparatus integrating PET and CT; and endoscopic  
20 ultrasonoscopy (EUS); however, the diagnostic imaging methods have difficulty or only limited availability in detecting microscopic lymph node metastasis. On the other hand, pathological diagnosis method utilizes specimens prepared from a number of excised lymph node tissues under a microscope and is a highly accurate and reliable diagnostic method; however, the diagnosis can only be made using excised lymph nodes as posttreatment (postoperative) diagnosis and therefore cannot be used for selecting the optimum treatment in advance. The diagnosis of lymph node metastasis by cancer cells is problematic in that the pretreatment diagnosis depends on the diagnostic imaging, which is currently  
25 less accurate, whereas reliable diagnosis is made in the posttreatment (postoperative) pathological diagnosis.

30 **[0004]** Therefore, molecular diagnostic techniques using molecular markers are important in the diagnosis of lymph node metastasis by cancer cells, and several techniques have been developed. Many conventionally known molecular diagnostic techniques utilize a protein (target protein) that is not expressed or expressed at a lower level in normal cells and is highly expressed in cancer cells or a nucleic acid (target nucleic acid, as a general term for DNA, mRNA, cDNA, etc.) included in a gene encoding the target protein. Specifically, a target protein included in lymph node tissues resected/  
35 excised from a living body is detected using an immunoassay, or conversely, a target nucleic acid is amplified using loop-mediated isothermal amplification (LAMP) or polymerase chain reaction (PCR) to detect the amplification product using a known method to determine the presence of metastatic cancer cells.

40 **[0005]** Regarding molecular diagnostic techniques, for example, Patent Literature 1 (Japanese Laid-Open Patent Publication No. 2007-175021) proposed a method for determining the presence of lymph node metastasis by colon cancer cells using the mRNA or a fragment of a gene encoding at least one protein selected from the group consisting of PIGR, CLDN3, LGALS4, AGR2, TACSTD1, GPX2, RAI3, TSPAN1, CKB, ELF3, FXYD3, CDH1, REG4, GDF 15, CLDN4, OLFM 4, CD9, CDH17, SELENBP, LCN2, TMPRSS4, CFTR, TM4SF3, ID1, CYP2S1, TFF3, EHF, FAT, KLF5, SLC9A3R2, HOXB9, ATP1B1, PCK1, and FCGBP. Patent Literature 2 (Japanese Laid-Open Patent Publication No. 2007-037421) described the determination of lymph node metastasis in colon cancer by entering the value of expression  
45 of a gene set represented by the database access numbers (serial numbers) NM\_003404 (G1592), NM\_002128 (G2645), NM\_052868 (G3031), NM\_005034 (G3177), NM\_001540 (G3753), NM\_005722 (G3826), and NM\_015315(G4370) into a mathematical function. Patent Literature 3 (Japanese Laid-Open Patent Publication No. 2008-020438) describes that lymph node metastasis, e.g., from breast cancer, can be determined with higher reliability by determining the expression of a polypeptide related to cytokeratin in a sample prepared from lymph node tissue.

50 **[0006]** On the other hand, it was recently determined that inflammatory responses promote carcinogenesis by damaging DNA, stimulating angiogenesis and cell proliferation, and inhibiting apoptosis. In this regard, serum C-reactive protein (CRP) has been investigated as a risk factor and a prognostic factor in colon (Non Patent Literature 1: Erlinger T.P. et al., JAMA 2004;291;585-590), esophageal (Non Patent Literature 2: Shimada H. et al., J. Surg. Oncol. 2003;83; 248-252), hepatocellular (Non Patent Literature 3: Hashimoto K. et al., Cancer 2005;103;1856-1864), renal (Non Patent Literature 4: Miyata Y. et al., Urology 2001;58;161-164), and ovarian (Non Patent Literature 5: Hefler L.A. et al., Clin. Cancer Res. 2008;14;710-714) cancers.

55 **[0007]** A higher serum CRP level is considered to be associated with a higher risk of developing cancer. For example, Non Patent Literature 6 (Nozoe T. et al., Am. J. Surg. 1998;176(4):335-8) describes that liver metastasis and lymph node metastasis in colon cancer patients are associated with preoperative increases in serum CRP levels, Non Patent Literature 7 (Nozoe T. et al., Am. J. Surg. 2001; 182(2), 197-201) describes that lymph node metastasis in esophageal cancer patients is associated with preoperative increases in serum CRP levels, and Non Patent Literature 8 (Ines G. et al., World J. Gastroenterol. 2006;12(23),3746-3750) describes that a higher serum CRP level in esophageal cancer patients is associated with lymph node metastasis.

**[0008]** It has been reported that genetic polymorphisms are strongly related to serum CRP levels (Non Patent Literature 9: Carlson C.S. et al., Am. J. Hum. Gen. 2005; 77;64-77 and Non Patent Literature 10: Szalai A.J. et al., J. Mol. Med. 2005;83;440-447).

**[0009]** Therefore, the present inventors examined whether CRP genetic polymorphisms act as cancer progression factors in esophageal cancer patients. Although we consequently revealed the potential association of CRP-717T>C genetic polymorphisms with lymph node metastasis (Non Patent Literature 11: Motoyama et al., The Japanese Journal of Gastroenterological Surgery, vol. 41, No. 7, pp. 1169, July 2008), a technique for detecting lymph node metastasis by cancer cells using the CRP-717T>C genetic polymorphism suffered from lower determination accuracy and was not put into practical use because metastasis was not statistically significant.

CITATION LIST

PATENT LITERATURE

**[0010]**

- [Patent Literature 1] Japanese Laid-Open Patent Publication No. 2007-175021
- [Patent Literature 2] Japanese Laid-Open Patent Publication No. 2007-037421
- [Patent Literature 3] Japanese Laid-Open Patent Publication No. 2008-020438

NON PATENT LITERATURE

**[0011]**

- [Non Patent Literature 1] Erlinger T.P. et al., JAMA 2004;291;585-590
- [Non Patent Literature 2] Shimada H. et al., J. Surg. Oncol. 2003;83;248-252
- [Non Patent Literature 3] Hashimoto K. et al., Cancer 2005;103;1856-1864
- [Non Patent Literature 4] Miyata Y. et al., Urology 2001;58;161-164
- [Non Patent Literature 5] Hefler L.A. et al., Clin. Cancer Res. 2008;14;710-714
- [Non Patent Literature 6] Nozoe T. et al., Am. J. Surg. 1998;176(4):335-8
- [Non Patent Literature 7] Nozoe T. et al., Am. J. Surg. 2001; 182(2), 197-201
- [Non Patent Literature 8] Ines G. et al., World J. Gastroenterol. 2006;12(23), 3746-3750
- [Non Patent Literature 9] Carlson C.S. et al., Am. J. Hum. Gen. 2005;77;64-77
- [Non Patent Literature 10] Szalai A.J. et al., J. Mol. Med. 2005;83;440-447
- [Non Patent Literature 11] Motoyama et al., The Japanese Journal of Gastroenterological Surgery vol. 41, No. 7, pp. 1169, July 2008

SUMMARY OF INVENTION

TECHNICAL PROBLEM

**[0012]** Regarding methods to detect lymph node metastasis by cancer cells using a conventionally known molecular diagnostic technique, as described in the methods of Patent Literatures 1 to 3, an attempt to reliably detect lymph node metastasis requires a comprehensive investigation of multiple molecular markers as factors for determination and the necessity of using lymph nodes, which greatly torment patients and require considerable time and effort for sample preparation. Serum CRP levels often fluctuate due to the influences of age, smoking, and inflammation. Moreover, the association of the CRP-717T>C genetic polymorphism with lymph node metastasis by cancer cells, as described by Non Patent Literature 11, was later denied. Therefore, a new molecular marker for rapidly and reliably detecting lymph node metastasis by cancer cells and a molecular diagnostic technique using the molecular marker are desired.

SOLUTION TO PROBLEM

**[0013]** As described above, a higher serum CRP level is associated with a certain CRP genetic polymorphism. However, the present inventors have found that the usage of the SNP rs1205 (also referred to as CRP1846C>T or rs1205 in this description) as a molecular marker dramatically improves the determination accuracy of lymph node metastasis in cancer and is extremely useful, which is not assumed from the conventional prediction that a CRP genetic polymorphism related to a high serum CRP level may act as a cancer progression factor, thereby completing the present invention. The SNP ras1205 is a one-base mutation in a nontranscribed region of the CRP gene that has been reported to be correlated

with a decrease in serum CRP levels.

**[0014]** The present invention provides the following determination method and determination kit:

[1] A method of determining lymph node metastasis in cancer or the risk thereof by identifying a genetic polymorphism in the human C-reactive protein (CRP) gene

[2] The method according to item 1 above, wherein lymph node metastasis in cancer or the risk thereof is determined by identifying a genetic polymorphism SNP rs1205

[3] The method according to item 2 above, wherein the risk is considered to be high when the genotype of SNP rs1205 is T/T

[4] The method according to any one of items 1 to 3 above, wherein the genotype is identified by restriction fragment length polymorphism (RFLP) or by analyzing its binding to a corresponding complementary strand sequence

[5] The method according to item 4 above, wherein the genotype is identified by PCR-RFLP

[6] The method according to item 5 above, wherein a forward primer, 5'-CTT ATA GAC CTG GGC AGT-3'(SEQ ID No.1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3'(SEQ ID No. 2), are used for PCR and Bst4CI is used as a restriction enzyme

[7] The method according to any one of items 1 to 6 above, wherein the cancer is a solid cancer

[8] A rapid determination kit for PCR-RFLP for detecting lymph node metastasis in cancer or the risk thereof, in which the kit comprises primers for amplifying a region containing SNP rs1205 of base sequence of human C-reactive protein gene and a restriction enzyme for detecting the genotype of SNP rs1205 by RFLP

[9] The rapid determination kit according to item 8 above, comprising a forward primer, 5'-CTT ATA GAC CTG GGC AGT-3' (SEQ ID No.1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3' (SEQ ID No.2), as a primer pair

[10] The rapid determination kit according to item 9 above, comprising the restriction enzyme Bst4CI

[11] A nucleic acid for analyzing bases of the SNP identification number rs1205 of human C-reactive protein gene, the nucleic acid specifically hybridizing to a DNA fragment derived from a region containing the bases of the SNP identification number rs1205 of the human C-reactive protein gene, the region being amplifiable by a PCR method using primers of SEQ ID Nos. 1 and 2.

[12] The method according to any one of items 1 to 7 above, wherein the sample used in identifying the genotype of the human CRP gene is selected from the group consisting of whole blood, leukocytes, the primary focus of cancer, lymphatic vessels, and lymph node tissue.

#### ADVANTAGEOUS EFFECTS OF INVENTION

**[0015]** Although it is known that the production of CRP is associated with various cytokines (interleukins, tumor necrosis factors, interferons, transforming growth factors), the method of the present invention is independent of the levels of various cytokines and can by itself effectively predict/detect lymph node metastasis by cancer cells. The method of detecting lymph node metastasis in cancer using SNP rs1205 is simple compared to conventional methods and yet remains extremely accurate. Therefore, the usage of SNP rs1205 enables the detection of lymph node metastasis with statistical significance. Because effective prediction/detection can be made on the basis of lymph node metastasis, which is an important phenomenon in cancer progression, the method enables the selection of the most reliable and least invasive therapy from options such as surgery involving lymph node dissection, endoscopic resection without lymph node dissection, chemoradiotherapy, chemotherapy, and radiation therapy, making the present invention clinically significant in determining the treatment strategy.

**[0016]** As the current diagnosis of lymph node metastasis of cancer cell has problems in that the pretreatment diagnosis depends on diagnostic imaging, which is currently less accurate, and that reliable diagnosis is as achieved only as a pathological diagnosis, which is a posttreatment (postoperative) diagnosis, the method of the present invention can solve both problems simultaneously.

**[0017]** Moreover, because this method does not require lymph tissue (lymph nodes or lymphatic vessel) to detect the presence of SNP rs1205 (peripheral blood and other substances/tissues can be used instead), patients suffer less, and work load of laboratory personnel is reduced because of the reduced requirements of sample preparation.

#### DESCRIPTION OF EMBODIMENTS

##### A. Method of Detecting Lymph Node Metastasis and the Risk Thereof

**[0018]** The CRP gene used in this invention is a gene corresponding to CRP (C-reactive protein). CRP is a type of acute-phase protein produced predominantly by hepatocytes in response to inflammation, and its serum levels are conventionally used as a marker for various acute and chronic inflammatory diseases. The name is derived from a serum protein (present in the  $\beta$ -globulin fraction) causing a precipitation reaction with the C-polysaccharide of *Diplococcus*

## EP 2 436 763 A1

*pneumoniae*, and its expression drastically increases in blood from 0.2 µg/mL by a factor of several hundred to one thousand due to infection, inflammation, and tissue damage. CRP is a homopentamer having a molecular weight of one hundred thirty thousand daltons, and its amino acid sequence is homologous to a portion of serum amyloid P-protein, complement C1.

5 **[0019]** The entire sequence of the CRP gene has been identified (Woo P, Korenberg JR, Whitehead AS, J. Biol. Chem., 260:13384-13388, 1985) and can be retrieved, for example, as Accession No. NG\_013007 in the NCBI web site ([http://www.ncbi.nlm.nih.gov/nuccore/NG\\_013007.1?report=gbwithparts&log\\$=seqview&from=5000&to=7300](http://www.ncbi.nlm.nih.gov/nuccore/NG_013007.1?report=gbwithparts&log$=seqview&from=5000&to=7300)) (see Table A for the entire base sequence).

10

15

20

25

30

35

40

45

50

55

[Table A]

1 taaggcaaga gatctaggac ttctagcccc tgaactttca gccgaataca tcttttccaa  
 5 61 aggagtgaat tcaggccctt gtatcactgg cagcaggacg tgaccatgga gaagctgttg  
 121 tgtttcttgg tcttgaccag cctctctcat gcttttggcc agacaggtaa gggccacccc  
 181 aggctatggg agagatttga tctgaggtat gggggtgggg tctaagactg catgaacagt  
 241 ctcaaaaaaa aaaaaaaaaa actgtatgaa cagaacagtg gagcatcctt catggtgtgt  
 10 301 gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtggtgtgta actggagaag gggtcagtct  
 361 gtttctcaat cttaaattct atacgtaagt gaggggatag atctgtgtga tctgagaaac  
 421 ctctcacatt tgcttgtttt tctggctcac agacatgtcg aggaaggctt ttgtgtttcc  
 481 caaagagtcg gatacttcct atgtatccct caaagcaccg ttaacgaagc ctctcaaagc  
 15 541 cttcactgtg tgccctcact tctacacgga actgtcctcg acccgtgggt acagtatttt  
 601 ctcgtatgcc accaagagac aagacaatga gattctcata ttttggctta aggatatagg  
 661 atacagtttt acagtgggtg ggtctgaaat attattcgag gttcctgaag tcacagtagc  
 721 tccagtacac atttgtacaa gctgggagtc cgccctcaggg atcgtggagt tctgggtaga  
 20 781 tgggaagccc agggtgagga agagtctgaa gaagggatac actgtggggg cagaagcaag  
 841 catcatcttg gggcaggagc aggattcctt cgggtgggaac tttgaaggaa gccagtcctt  
 901 ggtgggagac attggaaatg tgaacatgtg ggactttgtg ctgtcaccag atgagattaa  
 961 caccatctat cttggcgggc ccttcagtcc taatgtcctg aactggcggg cactgaagta  
 25 1021 tgaagtgcaa ggcgaaagtgt tcaccaaacc ccagctgtgg ccctgaggcc cagctgtggg  
 1081 tcctgaaggt acctcccgtt tttttacacc gcatgggccc cacgtctctg tctctggtac  
 1141 ctcccgcctt tttacactgc atggttccca cgtctctgtc tctgggcctt tgttccccta  
 1201 tatgcattgc aggcctgctc caccctctc agcgcctgag aatggaggta aagtgtctgg  
 30 1261 totgggagct cgtaactat gctgggaaac ggtccaaaag aatcagaatt tgaggtgttt  
 1321 tgttttcatt tttatttcaa gttggacaga tcttggagat aatttcttac ctacataga  
 1381 tgagaaaact aacaccaga aaggagaaat gatgttataa aaaactcata aggcaagagc  
 35 1441 tgagaaggaa gcgctgatct totatttaat tccccacca tgacccccag aaagcaggag  
 1501 ggcattgccc acattcacag ggctcttcag tctcagaatc aggacactgg ccaggtgtct  
 1561 ggtttgggtc cagagtgtc atcatcatgt catagaactg ctgggccag gtctcctgaa  
 1621 atgggaagcc cagcaatacc acgcagtccc tccactttct caaagcacac tggaaaggcc  
 40 1681 attagaattg ccccagcaga gcagatctgc tttttttcca gagcaaatg aagcactagg  
 1741 tataaatatg ttgttactgc caagaactta aatgactggg ttttgtttgc ttgcagtgtc  
 1801 ttcttaattt tatggctctt ctgggaaact cctcccctt tccacacgaa ccttgtgggg  
 1861 ctgtgaattc tttcttcac cccgcattcc caatataccc aggccacaag agtggacgtg  
 45 1921 aaccacaggg tgcctgtca gaggagcca tctccatct ccccagctcc ctatctggag  
 1981 gatagttgga tagttacgtg ttcttagcag gaccaactac agtcttocca aggattgagt  
 2041 tatggacttt gggagtgaga catcttcttg ctgctggatt tccaagctga gaggacgtga  
 2101 acctgggacc accagtagcc atcttgtttg ccacatggag agagactrtg aggacagaag  
 50 2161 ccaactgga agtggaggag ccaaggatt gacaaacaac agagccttga ccacgtggag  
 2221 tctctgaatc agccttgtct ggaaccagat ctacacctgg actgccaggg tctataagcc  
 2281 aataaagccc ctgtttactt g

55

**[0020]** In genetics, a genetic polymorphism is normally defined as an alteration (mutation) of certain bases present in one gene at a frequency of 1% or more in the population. A number of genetic polymorphisms have been identified in

the CRP gene, and the genetic polymorphism SNP rs1205 can preferably be utilized in the present invention. SNP rs1205 is a polymorphism in the 2148<sup>th</sup> nucleotide (base denoted by r) of the CRP gene sequence described in Table A. In this sequence, r indicates that the base is G (guanine) or A (adenine).

**[0021]** Table B describes the sequence around SNP rs1205 in a sequence complementary to the CRP gene sequence described in Table A.

[Table B]

```

10      1  CTTTAGTTTT TGCTCCTCAA ATTGGAATAA TGATAGAATG AGAGTACTAA AACCCCCACA
      61  ACTGGCCCTA CATGAATGGC CAGCTATCTC AAAAGAGGGA CTGTGCTTGT CAGAGGGAAT
     121  CCCTTCAGGG GACTCTTGGG CAGGTTAAAG TGCCATGGAT ATGTTGTGTA ATGGGAAGTG
     15  181  TAAACTTACA GGGACTTGAT TTCAAAGGTC ATTAGAGAAG TTAGCCACAA CTTCTAAAAG
      241  AACTATCAGA AAACAGCTTG GACTCACTCA AGTAAACAGG GGCTTTATTG GCTTATAGAC
      301  CTGGGCAGTC CAGGTGTAGA TCTGGTTCCA GACAAGGCTG ATTCAGAGAC TCCACGTGGT
      361  CAAGGCTCTG TTGTTTGTCA ATCCCTTGGC TCCTCCACTT CCAGTTTGGC TTCTGTCCCTC
     20  421  AYAGTCTCTC TCCATGTGGC AAACAAGATG GCTACTGGTG GTCCCAGGTT CACGTCCCTCT
      481  CAGCTTGGAA ATCCAGCAGC AAGAAGATGT CTCACTCCCA AAGTCCATAA CTCAATCCTT
      541  GGAAGACTG TAGTTGGTCC TGCTAGGAAC ACGTAACTAT CCAACTATCC TCCAGATAGG
     25  601  GAGCTGGGGA GATGGGAGAT GGGCTCCTCT GACAGGACAC CCTGTGGTTC ACGTCCACTC
      661  TTGTGGCCTG GGTATATTGG GAATGCGGGG ATGAAGAAAG AATTCACAGC CCCACAAGGT
      721  TCGTGTGGAA AAGGGGAGGA GTTCCCAGA AGAGCCATAA AATTAAGAAA GCACTGCAAG
      781  CAAACAAAAA CCAGTCATTT AAGTCTTTGG CAGTAACAAC ATATTTATAC CTAGTGCTTC
     30  841  ATTTTGCTCT GGAATAAAG CAGATCTGCT CTGCTGGGGC AATTCATAATG GCCTTTCCAG
      901  TGTGCTTTGA GAAAGTGGAG G

```

**[0022]** SNP rs1205 denotes a polymorphism in the 422nd base (base denoted by Y) of the base sequence described in Table B. In this sequence, Y indicates that the base is C (cytosine) or T (thymine).

In the present invention, lymph node metastasis in cancer or the risk thereof is determined by identifying the genotype of this base (C/C (wild-type), C/T (heterozygous), and T/T (homozygous)).

rs1205 is the identification number of the SNP registered in the SNP database of the National Center for Biotechnology Information (dbSNP, NCBI) in the US, and information about rs1205 is available from the NCBI website

(<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

**[0023]** SNP rs1205 can be identified with various known methods capable of detecting genetic polymorphisms, such as polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-single strand confirmation polymorphism (PCR-SSCP) (e.g., Orita, M. et al., Proc. Natl. Acad. Sci., U.S.A., 86, 2766-2770 (1989)), PCR-specific sequence oligonucleotide (PCR-SSO), an allele-specific oligonucleotide (ASO) hybridization method (e.g., Saiki, Nature, 324, 163-166 (1986)) combining the PCR-SSO method and a dot hybridization method, Taq-Man-PCR (Livak, KJ, Genet Anal., 14, 143 (1999), Morris, T. et al., J. Clin. Microbiol., 34, 2933 (1996)), an Invader method (Lyamichev et al., Nat. Biotechnol., 17, 292 (1999)), a MALDI-TOF/MS (matrix) method (Haff LA, Smimov IP, Genome Res., 7, 378 (1997)) using primer extension, a rolling circle amplification (RCA) method (Lizardi PM et al., Nat Genet 19, 225 (1998)), a method using DNA chip or microarray (e.g., Wang DG et al., Science 280,1077 (1998)), primer extension, a Southern blot hybridization method, and a dot hybridization method (e.g., Southern, E., J. Mol. Biol. 98, 503-517 (1975)), and this identification is not particularly limited. The corresponding sequence portion may be analyzed by direct sequencing. These methods can be used in arbitrary combinations. When the method is implemented, primers and probes can be designed from the base sequences described in Tables A and B as needed.

**[0024]** When the identification method is implemented, if the number of target DNA is small, it is preferable to use PCR-based method, e.g., PCR-RFLP, for the identification in terms of detection sensitivity and accuracy. Any of the identification methods may be applied after the test DNA is amplified in advance by PCR or a PCR-based gene amplification method. Conversely, if the identification is performed for a number of target DNA, it is particularly preferable to use methods incorporating DNA chips or microarrays, the Invader method, TaqMan-PCR, and the MALDI-TOF/MS

(matrix) method using primer extension or RCA method.

Among the identification methods described above, the preferred method in the case of a small number of target DNA and the preferred method in the case of identification for a large number of target DNA will be described by using representative methods as examples.

5 **[0025]** The preferred method in the case of a small number of target DNA is preparing a DNA sample from a patient with a method well known to those skilled in the art, followed by cleaving the prepared DNA sample with a restriction enzyme, separating DNA fragments depending on their size, and then comparing the sizes of the detected DNA fragments with that of a control. Typically, a DNA sample is first prepared from a patient and DNA containing the CRP gene is then amplified. The amplified DNA is cleaved by restriction enzyme. DNA fragments are then separated depending on its  
10 size and the size of detected DNA fragment is compared with a control.

**[0026]** Such methods include RFLP and PCR-RFLP. In other words, if mutation exists in the recognition site of a restriction enzyme or if a base insertion or deletion exists in a DNA fragment generated by a restriction enzyme, the size of the fragment generated after restriction enzyme treatment is changed compared to that of the control. The portions including this mutation can be amplified by PCR and treated with respective restriction enzymes to detect the mutation as a difference in the mobility of the bands after electrophoresis. Alternatively, after a DNA sample (genomic (chromosomal) DNA is usable) is prepared from a patient, treated with restriction enzymes, and electrophoresed, the presence of a polymorphism (mutation) can be detected by Southern blotting using a DNA probe that may hybridize with a target nucleic acid. The restriction enzymes used can be selected depending on the respective mutations as needed. In this method, in addition to genomic DNA, RNA prepared from patients can be converted into cDNA using reverse transcriptase and directly cleaved with a restriction enzyme to perform Southern blotting. DNA containing the CRP gene can be amplified by PCR using this cDNA as a template and cleaved with a restriction enzyme to examine differences in mobility.  
20

**[0027]** Primers used in the present invention include all of the primers that can amplify DNA containing the CRP gene. The preferred base length of the primers is 10 or more bases, most preferably 15 or more bases. Each of the primers may be a single oligonucleotide or a mixture of a number of oligonucleotides. Examples of the primers used in PCR are  
25 a forward primer, 5'-CTT ATA GAC CTG GGC AGT-3'(SEQ ID No. 1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3' (SEQ ID No. 2). The restriction enzyme may be Bst4Cl. Materials other than the primers and conditions in PCR, application of the restriction enzyme, electrophoresis, detection, and other conditions may be the same as those of commonly used methods.

**[0028]** The DNA probes that can be used in Southern blotting are not particularly limited, provided that the DNA probe can hybridize with the target nucleic acid. An example of a DNA probe hybridizable to the target nucleic acid is that for SNP rs1205 of the human CRP gene, which specifically hybridizes with a DNA fragment containing rs1205 and which is derived from a region amplifiable by PCR using SEQ ID Nos. 1 and 2.  
30

**[0029]** The CRP gene can be acquired from blood cells, peripheral blood leukocytes, skin cells, mucosal cells, liver, kidney, adrenal gland, brain, and uterine tissues, hair, and other tissues of patients by using known extraction and purification methods. A partial- or full-length sequence of the CRP gene may be utilized in the present invention as long as it contains the target base. In other words, a DNA fragment of any length is usable as long as it contains SNP rs1205.  
35

**[0030]** The preferred method in the case of identification for a large number of target DNA is preparing DNA containing the CRP gene derived from a patient and a substrate fixed to nucleotide probes (synonymous with the DNA probes) hybridizing to the DNA, followed by bringing the DNA into contact with the substrate and subsequently detecting DNA  
40 (target nucleic acid) hybridizing to the nucleotide probes fixed to the substrate to detect PCR genetic polymorphisms.

**[0031]** Such a method can be well illustrated by a DNA chip method (microarray method). A DNA sample from a patient containing the CRP gene can be prepared with a method well known to those skilled in the art as described above. In the preferred method of DNA sample preparation, DNA is prepared from genomic (chromosomal) DNA extracted from blood, peripheral blood leukocyte, cells such as skin cell, and mucosal cell, tissues such as liver, kidney, adrenal gland, brain, and uterus, hair of patients as described above. To prepare a DNA sample of this method from genomic (chromosomal) DNA, for example, a primer hybridizing to DNA containing the CRP gene can be used for preparing DNA containing the CRP gene with PCR using the genomic (chromosomal) DNA as a template. The prepared DNA sample can be labeled as needed for detection with a method well known to those skilled in the art.  
45

**[0032]** In the DNA chip method, a plurality of DNA probes is aligned and fixed on a substrate of glass or another material to perform hybridization of a labeled DNA sample, and a method of detecting a label (such as fluorescence) signal on the probe is utilized to distinguish and detect complete matches and one-base mismatches through hybridization to detect genetic polymorphisms such as SNPs.  
50

**[0033]** The preferred methods in the case of identification for a large number of target DNA will hereinafter be summarized.

55 The TaqMan PCR method utilizes PCR of fluorescently labeled allele-specific oligos and Taq DNA polymerase.

**[0034]** The Invader method is the combination of i) the hybridization to template DNAs of two reporter probes specific to respective alleles of genetic polymorphisms such as SNPs and one invader probe, and ii) the cleavage of DNA by an enzyme exhibiting special endonuclease activity that recognizes and cleaves a specific structure of DNA.

**[0035]** The SniPer method can be employed as a method utilizing primer extension. A basic principle of the SniPer method is a technique called rolling circle amplification (RCA), and circular single-stranded DNA is used as a template for DNA polymerase to continuously synthesize complementary-strand DNA. With this method, genetic polymorphisms such as SNPs can be determined by measuring the presence of a color reaction generated when DNA amplification occurs.

**[0036]** The MALDI-TOF/MS method utilizes a mass spectrometer to detect changes in mass due to one-base substitution for genotyping SNPs. Methods utilizing PCR amplification and multiplex PCR are available.

**[0037]** The sequencing method can be used to amplify a region containing a genetic polymorphism using PCR and a dye terminator to sequence DNA to analyze the frequency of genetic polymorphisms such as SNPs.

**[0038]** The determination method of the present invention is applicable to various stages and is particularly useful in the decision of the treatment strategy. For example, for patients with esophageal cancer with submucosal invasion, detection of lymph node metastasis using conventional methods is difficult. On the other hand, the present invention can detect lymph node metastasis or the risk thereof with high accuracy and can therefore avoid the deterioration of QOL due to unnecessary lymph node dissection and prevent cancer from progressing without necessary lymph node dissection.

**[0039]** No particular limitation exists on the types of cancer for which this method is applicable, and the method is applicable to all the solid cancers. Specifically, the method is applicable to cancers with primary focus in the esophagus, lung, breast, head and neck, stomach, colon, biliary tract, pancreas, uterus, ovary, bladder, kidney, urothelium, and prostate gland.

#### B. Rapid Determination Kit for Lymph Node Metastasis or the Risk Thereof

**[0040]** The present rapid determination kit for lymph node metastasis and the risk thereof can be prepared with a method well known to those skilled in the art. Various reagents necessary for detecting the CRP genetic polymorphism by using the primers of the present invention can be packaged in advance into the kit. Specifically, the reagents provided as a kit include various oligonucleotides used as the primers or loop primers designed for the present invention, four types of dNTP (dATP, dCTP, dGTP, and dTTP) acting as substrates for nucleic acid synthesis, the template-dependent nucleic acid synthesis enzyme exhibiting strand displacement activity, buffer solution providing preferred conditions for the enzyme reaction, salts (e.g., magnesium salt or manganese salt) as cofactors, protectants stabilizing enzymes and templates, and restriction enzyme as well as reagents necessary for detecting reaction products as needed. DNA probes hybridizable to the target nucleic acid may be included in a kit as a component reagent.

**[0041]** Although terms used in this description have normally used meanings, "lymph node" in the "method of detecting lymph node metastasis in cancer or the risk thereof" may be used as a general term for both "lymph node" and "lymphatic vessel" or a term for "lymphoid tissue"; "detection of lymph node metastasis" includes detecting the presence/possibility of cancer cells existing in a lymph node; and "determination of the risk of lymph node metastasis" includes determining the presence/possibility of metastasis of cancer cells from a primary focus to a lymph node if a certain individual has a cancer.

#### EXAMPLES

**[0042]** Although the present invention will hereinafter be described in more detail with examples, it is not limited to these examples. The following study including experiments/examinations was performed with the approval from the Ethics Committee of Akita University School of Medicine. All the subjects provided informed consent.

#### EXAMPLE 1

**[0043]** This example was performed using 113 patients (all Japanese) with thoracic esophageal squamous cell cancer. Thirty-eight of the patients underwent esophagectomy over 1 year beginning in April 2007 after confirmation (e.g., by pathological diagnosis) of esophageal cancer. The remaining 75 patients were selected at random from among those undergoing esophagectomy between 2000 and 2007 and were observed for subsequent cancer progression. The disease was classified in accordance with the International Union against Cancer Tumor-Node-Metastasis (TNM) classification of malignant tumors, 6th edition.

**[0044]** After collecting peripheral blood from the patients, DNA was extracted using a QIAamp Blood Kit (Qiagen) and stored at -80°C until analysis. The investigation of the association with lymph node metastasis in cancer was performed for CRP1846C>T(rs1205), the example of the present invention, and for 18 other genetic polymorphisms, i.e., CRP polymorphisms CRP-717C>T(rs2794521), CRP1059G>C(rs1800947), and CRP1444C>T(rs1130864); tumor necrosis factor polymorphisms TNF- $\alpha$ -238G>A, TNF- $\alpha$ -308G>A, TNF- $\alpha$ -1031T>C; and TNF- $\beta$ 250G>A, INF- $\gamma$ 874A>T, TGF- $\beta$ 1 29T>C, IL-1 $\beta$ -31C>T, IL-1 $\beta$ -511C>T, IL-1 receptor antagonist, IL-2-330T>G, IL-4-590C>T, IL-6-634G>C, IL-6 receptor

48892A>C, IL-10-592A>C, and IL-12 $\beta$ -1188A>C.

**[0045]** PCR for amplifying target nucleic acids was performed by thermal denaturation of extracted DNA at 95°C for 15 min, 35 cycles of reaction at 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and heating at 72°C for 5 min. A forward primer, 5'-CTT ATA GAC CTG GGC AGT-3'(SEQ ID No.1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3'(SEQ ID No. 2), were used as primers for amplifying the CRP1846C>T (rs1205) genetic polymorphism. Bst4CI was added to the PCR amplification product acquired from the operation, and after incubation at 65°C for 8 h, RFLP was performed by electrophoresis.

CRP polymorphisms (a total of four genetic polymorphisms, i.e., the CRP1846C>T (rs1205) genetic polymorphism and three genetic polymorphisms mentioned above) were also investigated in 139 patients (all Japanese) treated for ailments other than cancer in Akita University Hospital as controls.

The frequencies of the appearance of the polymorphisms are consistent with those expected for Hardy-Weinberg equilibrium. The results were similar to those of the SNP500 database of the National Cancer Institute.

**[0046]** Of the 113 esophageal cancer patients studied, 62 patients (55%) had lymph node metastasis, whereas 51 patients (45%) did not have lymph node metastasis. Although patients with pathologically identified lymph node metastasis had significantly ( $P < 0.05$ ) deeper invasion by the cancer than the patients without lymph node metastasis, no significant correlation existed between the presence of lymph node metastasis and age, sex, preoperative nutritional state, tumor markers, tumor site and size, squamous cell and intramural metastasis (see Table 1 "Clinical characteristics of patients with or without lymph node metastasis").

[Table 1]

Clinical characteristics of patients with or without lymph node metastasis			
	Lymph node metastasis		<i>P</i>
	Negative ( <i>N</i> = 51)	Positive ( <i>N</i> = 62)	
Age (years)	65 $\pm$ 8	63 $\pm$ 8	0.106
Gender			
Male	47	52	
Female	4	10	0.2538
Hemoglobin (g/dL)	13.6 $\pm$ 1.5	13.7 $\pm$ 1.5	0.7718
Albumin (g/L)	43 $\pm$ 3	43 $\pm$ 3	0.4647
SCC (ng/mL)	1.1 $\pm$ 2.9	1.1 $\pm$ 1.5	0.8970
CEA (ng/mL)	3.5 $\pm$ 2.1	43 $\pm$ 2.8	0.1022
Preoperative serum CRP (mg/L)	4.0 $\pm$ 8.3	4.7 $\pm$ 8.1	0.6514
Tumor location			
Upper third	2	5	
Middle third	31	31	
Lower third	28	26	0.4305
Tumor size (mm)	49 $\pm$ 29	54 $\pm$ 25	0.3725
Depth of tumor invasion (pT)			
T1	27	14	
T2	4	11	
T3	18	31	
T4	2	6	0.0078*
Tumor differentiation			
Well-moderately	41	48	
Poorly	10	14	0.8184
Lymphatic invasion			
Positive	45	62	
Negative	6	0	0.0071*
Venous invasion			
Positive	34	55	

EP 2 436 763 A1

(continued)

Clinical characteristics of patients with or without lymph node metastasis			
	Lymph node metastasis		P
	Negative (N = 51)	Positive (N = 62)	
Negative	17	7	
Intramural metastasis			
Positive	4	8	
Negative	47	54	0.5422
Number of involved lymph nodes	0	3.9 ± 4.9	<0.001*

SCC squamous cell carcinoma antigen, CEA carcinoembryonic antigen, CRP C-reactive protein

**[0047]** As a result of the analysis of the association between various genetic polymorphisms and pathologically identified lymph node metastasis, only the CRP1846C>T (rs1205) genetic polymorphism which is utilized in the present invention was significantly associated with lymph node metastasis (Fisher's exact test, P = 0.0043). Regarding the CRP1846C>T (rs1205) genetic polymorphism, lymph node metastasis was found in 25 patients and not found in 35 patients with the C/C or C/T genotype, whereas lymph node metastasis was found in 37 patients and not found in 16 patients with the T/T genotype (see Table 2 "Relationship between CRP genetic polymorphism and lymph node metastasis").

[Table 2]

Relationship between CRP genotypes and lymph node metastasis			
CRP genotypes	Lymph node metastasis		P
	Negative (N = 51)	Positive (N = 62)	
CRP -717T/C (rs2794521) genotypes			
T/T	35	50	
T/C	15	12	
C/C	1	0	0.2302
T/T	35	50	
T/C + C/C	16	12	0.1891
CRP 1059G/C (rs1800947) genotypes			
G/G	48	58	
G/C	3	4	>0.9999
CRP 1444C/T (rs130864) genotypes			
C/C	49	56	
C/T	1	6	
T/T	1	0	0.1350
C/C	49	56	
C/T + T/T	2	6	0.2906
CRP 1846C/T (rs1205) genotypes			
C/C	6	7	
C/T	29	18	
T/T	16	37	0.0068*
C/C + C/T	35	25	
T/T	16	37	0.0043*

\* Significant difference

**[0048]** On the other hand, with regard to the CRP1459G>C (rs1800947) genetic polymorphism, lymph node metastasis was found in 58 patients and not found in 48 patients with the G/G genotype, whereas lymph node metastasis was found

EP 2 436 763 A1

in 4 patients and not found in 3 patients with the G/C genotype. Regarding the CRP-717T>C (rs2794521) genetic polymorphism discussed in Non Patent Literature 11 (Motoyama et al., The Japanese Journal of Gastroenterological Surgery vol. 41, No. 7, pp. 1169, July 2008), lymph node metastasis was found in 12 patients and not found in 16 patients with the T/C and C/C genotypes, and regarding the CRP144C>T (rs1130864) genetic polymorphism, lymph node metastasis was found in 56 patients and not found in 49 patients with the C/C genotype (see Table 2 "Relationship between CRP genetic polymorphism and lymph node metastasis").

**[0049]** As a result of multivariate logistic analysis using the CRP1846C>T (rs1205) genetic polymorphism and various clinical factors related to lymph node metastasis as covariates, it was revealed that the T/T genotype in a patient at rs1205 is significantly associated with lymph node metastasis (odds ratio  $\geq 3$ ). Conversely, as a result of similar analysis of preoperative serum CRP and SCC levels, tumor size, and age, the odds ratio was approximately 1 in each case. A comparison of the depth of tumor invasion (T2 to 4 versus T1) revealed lymph node metastasis involvement at an odds ratio of 2.571 (see Table 3 "Multivariate logistic regression analysis of lymph node metastasis").

[Table 3]

Multivariate logistic regression analysis of lymph node metastasis

	$\beta$	Wald $\chi^2$	<i>P</i>	Odds ratio	95% CI
CRP 1846C/T genotypes (T/T versus C/C + C/T)	1.112	5.615	0.0178	3.040	1.212-7.625
Preoperative serum CRP	-0.107	0.078	0.7799	0.898	0.423-1.907
Serum SCC	0.070	0.252	0.6160	1.072	0.816-1.409
Tumor location (upper versus middle-lower)	0.608	0.297	0.5855	1.836	0.207-16.312
Tumor size	-0.005	0.209	0.6472	0.995	0.975-1.016
Depth of tumor invasion (T2-4 versus T1)	0.944	2.700	0.1004	2.571	0.833-7.929
Tumor differentiation (well-moderately versus poorly)	-0.496	0.710	0.3995	0.609	0.192-1.930
Venous invasion (positive versus negative)	1.131	2.680	0.1016	3.099	0.800-12.003
Intramural metastasis (positive versus negative)	0.219	0.085	0.7705	1.245	0.285-5.439
Age	-0.049	2.246	0.1340	0.952	0.893-1.015
Gender (male versus female)	-0.821	1.278	0.2582	0.440	0.106-1.826

Likelihood-ratio  $\chi^2$  test,  $\chi^2 = 23.241$ (df = 11), *P* = 0.0163

With regard to the rs1205 genetic polymorphism, preoperative serum CRP levels were 0-5 mg/L for 43 of the patients with the C/C and C/T genotypes and 43 of the patients with the T/T genotype whereas the levels were greater than 5 mg/L for 16 of the patients with the C/C and C/T genotypes and 8 of the patients with the T/T genotype. (see Table 4 "Relationship between CRP1846C>T (rs1205) genotypes and preoperative serum CRP level, depth of tumor, and number involved lymph nodes").

[Table 4]

Relationship between CRP 1846C/T (rs1205) genotypes and preoperative serum CRP level, depth of tumor, and number of involved nodes

	CRP 1846C/T(rs1205)genotypes			<i>P</i>	CRP 1846C/T(rs1205) genotypes		<i>P</i>
	C/C (N=13)	C/T (N=47)	T/T (N=53)		C/C + C/T (N=60)	T/T (N=53)	
Preoperative serum CRP (mg/L) <sup>a</sup>	5.7 ± 5.8	4.6 ± 10.6	3.8 ± 6.0	0.737	4.8 ± 9.7	3.8 ± 6.0	0.0537
0-5 mg/L	6	37	43	0.0107*	43	43	0.1706
>5 mg/L	7	9	8		16	8	
Depth of tumor invasion (pT)							
T1	2	24	15		26	15	

EP 2 436 763 A1

(continued)

Relationship between CRP 1846C/T (rs1205) genotypes and preoperative serum CRP level, depth of tumor, and number of involved nodes

	CRP 1846C/T(rs1205)genotypes			P	CRP 1846C/T(rs1205) genotypes		P
	C/C (N=13)	C/T (N= 47)	T/T (N=53)		C/C + C/T (N = 60)	T/T (N= 53)	
T2-4	11	23	38	0.0153*	34	38	0.1184
Number of involved lymph nodes	1.4 ± 1.9	1.8 ± 3.7	2.7 ± 4.9	0.4605	1.7 ± 3.7	2.7 ± 4.9	0.2284
0-2	11	39	35		50	35	
>3	2	8	18	0.1038	10	18	0.0487*

Preoperative serum CRP level was not measured in one patient in C/T genotype group or in two patients in T/T genotype group

**[0050]** The diagnosability was extremely high after limiting the analysis to subjects with submucosal esophageal cancer (33 patients), in which diagnosis of lymph node metastasis is particularly difficult and approaches to therapy is significantly influenced by the presence of pretreatment lymph node metastasis. Although the lymph node metastasis diagnosis using the most advanced diagnostic imaging apparatus (CT and ultrasonography) achieved a sensitivity, specificity, positive predictive value, and negative predictive value of 50%, 79%, 54%, and 68%, respectively, the diagnosis using the CRP1846C>T (rs1205) genetic polymorphism achieved a sensitivity, specificity, positive predictive value, and negative predictive value of 64%, 79%, 69%, and 75%, respectively, which were more favorable (see Table 5 "Prediction of lymph node involvement in submucosal esophageal cancer using CRP1846C>T (rs1205) genetic polymorphism or the usual methods (CT and ultrasonography)").

[Table 5]

Prediction of lymph node involvement in submucosal esophageal cancer using CRP 1846C>T(rs1205) polymorphism or the usual methods (CT and ultrasonography)

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CRP 1846C>T (rs1205) polymorphism	64	79	69	75
Usual methods	50	79	54	68

EXAMPLE 2

**[0051]** Similarly, as a result of investigating the relationship between the CRP1846C>T (rs1205) genetic polymorphism and pathological lymph node metastasis in 152 lung cancer patients (all Japanese) who underwent surgery, a significant relationship between the two was revealed as observed for esophageal cancer (Fisher's exact test, P = 0.0312).

**[0052]** As indicated by the results above, according to the present invention, lymph node metastasis or the risk thereof can be detected with high accuracy. In particular, lymph node metastasis or the risk thereof can notably be determined with higher accuracy using the CRP1846C>T (rs1205) genetic polymorphism than with polymorphisms of other cytokines involved in CRP production.

EXAMPLE 3

**[0053]** The relationship between the CRP1846C>T (rs1205) genetic polymorphism and lymph node metastasis was analyzed in 64 patients having a wall invasion depth of pT1-2 among the 113 subjects of Example 1. Regarding the CRP1846C>T (rs1205) genetic polymorphism, lymph node metastasis was found in 6 patients and not found in 35 patients with the C/C or C/T, whereas lymph node metastasis was found in 18 patients and not found in 5 patients with the T/T genotype. The CRP1846C>T (rs1205) genetic polymorphism was significantly related to lymph node metastasis (Fisher's exact test, P = 0.0001). As lymph node metastasis can certainly be detected in early cancer, in which lymph

node metastasis may be overlooked by conventional diagnostic imaging methods, it was confirmed that the method of the present invention can meet clinical needs.

EXAMPLE 4

5

**[0054]** The relationship between the CRP1846C>T (rs 1205) genetic polymorphisms and lymphatic vessel invasion was analyzed in 144 patients implicated for lymphatic vessel invasion among the 152 subjects of Example 2. Regarding the CRP1846C>T (rs1205) genetic polymorphism, lymphatic vessel invasion was found in 36 patients and not found in 42 patients with the C/C or C/T genotype, whereas lymphatic vessel invasion was found in 45 patients and not found in 21 patients with the T/T genotype. The CRP1846C>T (rs1205) genetic polymorphism was significantly related to lymphatic vessel invasion (Fisher's exact test, P = 0.008). "Lymphatic vessel invasion" indicates the state in which the presence of cancer cells is observed in lymphatic vessel of the primary focus and suggests the possibility of future "lymph node metastasis" even if no "lymph node metastasis" actually exists. Therefore, identifying the genotype of the CRP1846C>T (rs1205) genetic polymorphism is useful in the determination (prediction in earlier stage) of the risk of "lymph node metastasis."

10

15

**[0055]**

**SEQUENCE LISTING**

20

<110> Akita University

<120> Determining method and quick determining kit of lymph node metastasis or the risk thereof

<130> A001-0001P

25

<160> 2

<170> PatentIn version 3.1

30

<210> 1

<211> 18

<212> DNA

<213> Artificial sequence

35

<220>

<223> Forward primer

<400> 1

cttatagacc tgggcagt

40

<210> 2

<211> 19

<212> DNA

<213> Artificial sequence

45

<220>

<223> Forward primer

<400> 2

ggagtgagac atcttcttg

50

**Claims**

55

1. A method of determining lymph node metastasis in cancer or the risk thereof, comprising identifying a genetic polymorphism in the human C-reactive protein (CRP) gene.
2. The method according to claim 1, wherein lymph node metastasis in cancer or the risk thereof is determined by identifying a genetic polymorphism, SNP rs1205.

## EP 2 436 763 A1

3. The method according to claim 2, wherein the risk is determined to be high when genotype of SNP rs1205 is T/T.
4. The method according to any one of claims 1 to 3, wherein genotype is identified by RFLP or its binding with a corresponding complementary strand sequence.
5. The method according to claim 4, wherein genotype is identified by PCR-RFLP.
6. The method according to claim 5, wherein a forward primer, 5'-CTT ATA GAC CTG GGC AGT-3'(SEQ ID No.1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3'(SEQ ID No. 2), are used as primers in PCR and Bst4CI is used as a restriction enzyme.
7. The method according to any one of claims 1 to 6, wherein the cancer is a solid cancer.
8. A rapid determination kit for PCR-RFLP for determining lymph node metastasis in cancer or the risk thereof, comprising primers for amplifying a region containing SNP rs1205 and a restriction enzyme for determining genotype of SNP rs 1205, of human CRP gene, by RFLP.
9. The rapid determination kit according to claim 8, comprising a forward primer, 5'-CTT ATA GAC CTG GGC AGT-3'(SEQ ID No. 1), and a reverse primer, 5'-GGA GTG AGA CAT CTT CTT G-3'(SEQ ID No. 2), as a primer pair.
10. The rapid determination kit according to claim 9, comprising the restriction enzyme Bst4CI.
11. A nucleic acid for analyzing bases of the SNP identification number rs1205 of a human CRP gene, the nucleic acid specifically hybridizing to a DNA fragment derived from a region containing the bases of the SNP rs1205 of the human CRP gene, the region being amplifiable by a PCR method using primers of SEQ ID Nos. 1 and 2.
12. The method according to any one of claims 1 to 7, wherein the sample used in identifying a genotype of the human CRP gene is selected from the group consisting of whole blood, leukocytes, the primary focus of cancer, lymphatic vessel, and lymph node tissue.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2010/059049

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> <i>C12N15/09(2006.01) i, C12Q1/34(2006.01) i, C12Q1/68(2006.01) i, G01N33/53(2006.01) n</i>  According to International Patent Classification (IPC) or to both national classification and IPC												
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) <i>C12N15/09, C12Q1/34, C12Q1/68, G01N33/53</i>  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched <table border="0"> <tr> <td>Jitsuyo Shinan Koho</td> <td>1922-1996</td> <td>Jitsuyo Shinan Toroku Koho</td> <td>1996-2010</td> </tr> <tr> <td>Kokai Jitsuyo Shinan Koho</td> <td>1971-2010</td> <td>Toroku Jitsuyo Shinan Koho</td> <td>1994-2010</td> </tr> </table> Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) <i>CA/BIOSIS/MEDLINE/WPIDS (STN), JSTPlus/JMEDPlus/JST7580 (JDreamII)</i>			Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2010	Kokai Jitsuyo Shinan Koho	1971-2010	Toroku Jitsuyo Shinan Koho	1994-2010		
Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2010									
Kokai Jitsuyo Shinan Koho	1971-2010	Toroku Jitsuyo Shinan Koho	1994-2010									
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
Y/A	Satoru MOTOYAMA et al., "Shokudogan Kanja no CRP Idenshi Tagata wa Shokudogan Shinten Inshi to naru", Japanese Journal of Gastroenterological Surgery, vol.41, no.7, 01 July 2008 (01.07.2008), page 1169 (O-1-134)	1, 4, 5, 7, 11, 12/2, 3, 6, 8-10										
Y/A	GOCKEL, I., et al., Significance of preoperative C-reactive protein as a parameter of the perioperative course and long-term prognosis in squamous cell carcinoma and adenocarcinoma of the oesophagus., World J. Gastroenterol., 2006, vol.12, no.23, p.3746-3750	1, 4, 5, 7, 11, 12/2, 3, 6, 8-10										
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.												
* Special categories of cited documents: <table border="0"> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"&amp;" document member of the same patent family</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention											
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone											
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art											
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family											
"P" document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search 09 August, 2010 (09.08.10)		Date of mailing of the international search report 17 August, 2010 (17.08.10)										
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer										
Facsimile No.		Telephone No.										

Form PCT/ISA/210 (second sheet) (July 2009)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2010/059049

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y/A	CARLSON, C.S., et al., Polymorphisms within the C-reactive protein (CRP) promoter region are associated with plasma CRP levels., Am. J. Hum. Genet., 2005, vol.77, no.1, p.64-77	1, 4, 5, 7, 11, 12/2, 3, 6, 8-10
Y/A	SZALAI, A.J., et al., Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level., J. Mol. Med., 2005, vol.83, no.6, p.440-447	1, 4, 5, 7, 11, 12/2, 3, 6, 8-10
P,X	MOTOYAMA, S., et al., CRP genetic polymorphism is associated with lymph node metastasis in thoracic esophageal squamous cell cancer., Ann. Surg. Oncol., 2009.09, vol.16, no.9, p.2479-2485	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

## REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

## Patent documents cited in the description

- JP 2007175021 A [0005] [0010]
- JP 2007037421 A [0005] [0010]
- JP 2008020438 A [0005] [0010]

## Non-patent literature cited in the description

- Erlinger T.P. et al. *JAMA*, 2004, vol. 291, 585-590 [0006] [0011]
- Shimada H. et al. *J. Surg. Oncol.*, 2003, vol. 83, 248-252 [0006] [0011]
- Hashimoto K. et al. *Cancer*, 2005, vol. 103, 1856-1864 [0006] [0011]
- Miyata Y. et al. *Urology*, 2001, vol. 58, 161-164 [0006] [0011]
- Hefler L.A. et al. *Clin. Cancer Res.*, 2008, vol. 14, 710-714 [0006] [0011]
- Nozoe T. et al. *Am. J. Surg.*, 1998, vol. 176 (4), 335-8 [0007] [0011]
- Nozoe T. et al. *Am. J. Surg.*, 2001, vol. 182 (2), 197-201 [0007] [0011]
- Ines G. et al. *World J. Gastroenterol.*, 2006, vol. 12 (23), 3746-3750 [0007] [0011]
- Carlson C.S. et al. *Am. J. Hum. Gen.*, 2005, vol. 77, 64-77 [0008] [0011]
- Szalai A.J. et al. *J. Mol. Med.*, 2005, vol. 83, 440-447 [0008] [0011]
- Motoyama et al. *The Japanese Journal of Gastroenterological Surgery*, July 2008, vol. 41 (7), 1169 [0009] [0011]
- Woo P ; Korenberg JR ; Whitehead AS. *J. Biol. Chem.*, 1985, vol. 260, 13384-13388 [0019]
- Orita, M. et al. *Proc. Natl. Acad. Sci., U.S.A.*, 1989, vol. 86, 2766-2770 [0023]
- Saiki. *Nature*, 1986, vol. 324, 163-166 [0023]
- Livak, KJ. *Genet Anal.*, 1999, vol. 14, 143 [0023]
- Morris, T. et al. *J. Clin. Microbiol.*, 1996, vol. 34, 2933 [0023]
- Lyamichev et al. *Nat. Biotechnol.*, 1999, vol. 17, 292 [0023]
- Haff LA ; Smimov IP. *Genome Res.*, 1997, vol. 7, 378 [0023]
- Lizardi PM et al. *Nat Genet*, 1998, vol. 19, 225 [0023]
- Wang DG et al. *Science*, 1998, vol. 280, 1077 [0023]
- Southern, E. *J. Mol. Biol.*, 1975, vol. 98, 503-517 [0023]
- International Union against Cancer Tumor-Node-Metastasis (TNM) classification of malignant tumors [0043]
- Motoyama et al. *The Japanese Journal of Gastroenterological Surgery*, July 2008, vol. 41 (7), 1169 [0048]

专利名称(译)	评估癌症的淋巴结转移或其风险的方法，以及用于所述方法的快速评估试剂盒		
公开(公告)号	<a href="#">EP2436763A1</a>	公开(公告)日	2012-04-04
申请号	EP2010780625	申请日	2010-05-27
[标]申请(专利权)人(译)	国立大学法人秋田大学		
申请(专利权)人(译)	国立大学法人秋田大学		
当前申请(专利权)人(译)	国立大学法人秋田大学		
[标]发明人	MOTOYAMA SATORU MIURA MASATOMO OGAWA JUNICHI		
发明人	MOTOYAMA, SATORU MIURA, MASATOMO OGAWA, JUNICHI		
IPC分类号	C12N15/09 C12Q1/34 C12Q1/68 G01N33/53		
CPC分类号	C12Q1/6886 C12Q2600/118 C12Q2600/156 C12Q2600/112 C12Q2600/158		
优先权	2009128323 2009-05-27 JP		
其他公开文献	EP2436763A4 EP2436763B1		
外部链接	<a href="#">Espacenet</a>		

### 摘要(译)

本发明的目的是提供一种快速可靠地检测癌症中淋巴结转移或淋巴结转移风险的方法和手段。具体地，本发明提供了一种方法和快速测定试剂盒，用于通过鉴定人CRP基因的某种遗传多态性来检测癌症中的淋巴结转移或其风险，并且在确定治疗策略中具有临床意义，因为有效的预测/可以确定淋巴结转移，这是癌症进展中的重要现象。

### [Table A]

```

1 taaggacaga gatctaggac ttctagcccc tgaacttcca ggcgaataca tcttttccaa
61 aggggtgaat tcaggccett gtatcaactg cagcggagcg tgcacatgga gaagctgttg
121 tgtttcttgg tcttgaccag cctctctcat gcttttggcc agcacagtaa gggccacccc
181 aggctatagg agagatttga tctgagat atgggggggg tctaaactg catgacactg
241 ctcaaaaaa aaaaasaaa actgtctgaa ccaaacata gacatcctc catgtgtgtg
301 gctgtatgat cgtgtatgat gtgtgtatgt gtgtgtatata actggaaaag gggcagctct
361 gttctcaat cttaaatctc atacgtaagt gaggggatag actgtgtgta tctgcaaaa
421 cctcaaatc tgtgttctt tctggctcac agactgtcg aggaaggctt ttgtcttcc
481 caagatctg gtaactctc atgttctctc caagcaccg ttaacgagag ctctcaaaag
541 attcaactgt tgcctccaat tctaacgga actgtctcag acccgtgggt acagtatctt
601 ctcttatgac accaagagac aagacaatga gatctccta ttctgtata aggatatagg
661 atcacgtctt acagtgggtg ggtctgaact attatctcag gtctctgaa tccagctgag
721 tccagtcac atttgtcaaa gctggagtc cgtctcagag atgtgtgagt tctgtgtgaa
781 ggggaagccc aggtgagga aggtctgaa gaaggatac actgtggggg cagaagcaag
841 catcatcttg gggcaggaga aggttctctt cgggggaaa ttgagggaa gccagtccct
901 ggtggagacc atggaaaatp tgaacatgtg gaactctgtg ctgtcccaag atgagatcaa
961 aacatctat ctggggggc acttcaagtc aaigtctctg aactggggg cactgaaata
1021 tgaagtgcac gggcaggtt tcaaaaacc caagctgtgg cctgtaggcc cagctgtggg
1081 tctgaaggt acctccggt tttttacac gcatggccc cagctctctg tctctgttac
1141 ctcccgctt tttaactgc atgttccca cgtctctgtc tctggcctt tgttccccta
1201 tatgacttgc aggcctgtct caccctctc agcctctgag aatggagga aggtctctg
1261 tctggagct cgttaactat cgtggaaa ggtccaaaag aatcagaatt tgaagtgctt
1321 tgttttcaat tttattcaa gttggacaga tcttgagat aatttcttae ctcaataga
1381 tgaanaact aaaccocga aaggaatc gatgtatata aaaaactata aggcagagc
1441 tggagagaa cggctgact tctatttat tcccaccca tgaaccccaag aaagcaggg
1501 ggcattgcc acattcaag ggccttca gttcaaacatc agaacactgg ccaagtgctt
1561 ggtttgggtc cagaatgctc atcatcatg catagaactg ctggcccag gttctctgaa
1621 atggaaagcc cagcaatccc agcagctccc tccactctct caaagcaaac tgaanaagcc
1681 attagaatg ccccaagag cggatctg ttttttcca gacaaaatg aagcaataga
1741 taaaaatg ttgttactg caagaacta aatgactggt tttgttttgg ttgcaagtct
1801 tcttaattt tatgctctt ctgggaact cctcccctt tccacagaa ccttggtggg
1861 ctgtgaatto ttctctctc ccgcattcc caatacacc agcccaaac agtggccctg
1921 aaccacagg tttctgtca gggagccc tctctactc cccagctcc ctactgtgg
1981 gatagttga tagtctctg ttctcagag gcccaactac agtcttccca aggatlgat
2041 tatgcactt ggaatgaga catctctgt ctgtggat tccaagctga gaggactga
2101 acctggacc accagtagcc acttctctt ccaactgag agagacttg agacagag
2161 ccaacttga agtgaggag ccaagpatt gacacacac agactctga ccaagtgag
2221 tctctgaac agcctgtct ggaaccagat ctacaactgg actgcccagg tctataagcc
2281 aataagccc ctgttaact g

```