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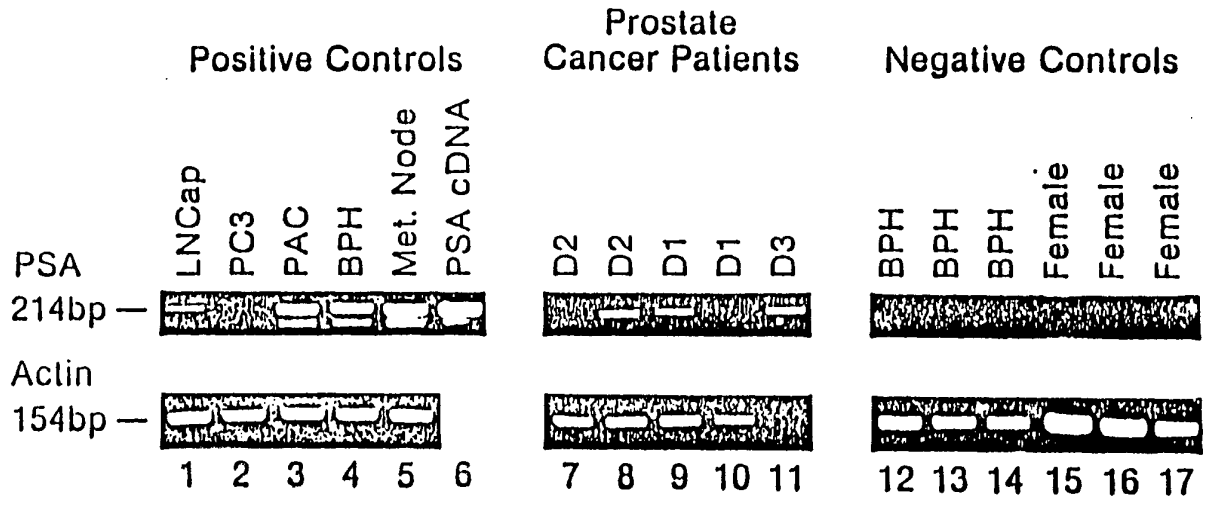
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(54) Title: METHODS OF DETECTING MICROMETASTASIS OF PROSTATE CANCER



(57) Abstract

A method of diagnosing prostate metastasis is provided by the present invention whereby RNA from a patient's blood is isolated and amplified using a pair of primers which are complementary to regions of the prostate specific antigen gene. The presence or absence of amplified RNA is detected and the presence of amplified RNA is indicative micrometastasis of prostate cancer.

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METHODS OF DETECTING MICROMETASTASIS OF PROSTATE CANCER

FIELD OF THE INVENTION

This invention is directed to methods of detecting prostate cancer.

BACKGROUND OF THE INVENTION

Prostate cancer metastasis will claim the lives of over 30,000 Americans this year. Boring et al., *Cancer Statistics* **1991**, 19. The mode of dissemination however, remains very poorly understood. An almost dogmatic view of metastasis holds that prostate cancer cells first spread through the prostatic capsule then into the lymphatics, and eventually hematogenously travel to bone. Byar et al., *Cancer* **1972**, 30, 5; Winter, C.C., *Surg. Gynecol. Obstet.* **1957**, 105, 136; Hilaris et al., *Am. J. Roentgenol.* **1974**, 121, 832; McLaughlin et al., *J. Urol.* **1976**, 115, 89; Jacobs, S.C., *Urology* **1983**, 21, 337; Batson, O.V., *Ann. Surg.* **1940**, 112, 138; Saitoh et al., *Cancer* **1984**, 54, 3078-3084; Whitmore, W.F., Jr., *Cancer* **1973**, 32, 1104. However, this model has been based on histopathologic studies which have significant limitations, and in actuality the sequence of metastatic events remain unknown. Solid tumor animal experiments suggest that only 0.01% of circulating cancer cells eventually create a single metastatic deposit. Fidler et al., *Science* **1982**, 217, 998-1001; Liotta et al., *Cancer Res.* **1974**, 34, 997; Schirrmacher, B., *Adv. Cancer Res.* **1985**, 43, 1-32. Ostensibly, a single bone metastasis from human prostatic adenocarcinoma (PAC) could be generated by

10,000 circulating cancer cells (2 cells/1 ml blood). In the past, detection of such a low concentration of cells has been difficult or impossible. Recently, however, Wu et al. used keratin-19 (K-19) mRNA PCR to detect breast cancer micrometastasis in patient lymph nodes and bone marrow. Wu et al., *Lab. Inv.* **1990**, 62, 109A. Miyomura et al., also reported the detection of minimal residual acute lymphoblastic leukemia by PCR in patients harboring the Philadelphia chromosome. Miyomura et al., *Blood* **1992**, 79, 1366-1370.

A method of detecting the micrometastasis of prostate cancer would be greatly desirable.

SUMMARY OF THE INVENTION

In accordance with the present invention, methods of detecting prostate cancer micrometastasis in a patient are provided comprising the steps of obtaining a sample of RNA from a patient's blood and amplifying said RNA with polymerase chain reaction. The polymerase chain reaction is performed using a pair of primers which are complementary to separate regions of the prostate specific antigen gene. These primers may have the sequences GAGGTCCACACACTGAAGTT (SEQ ID NO: 1) and CCTCCTGAAGAATCGATTCCT (SEQ ID NO: 2). Thereafter, the presence or absence of amplified RNA is detected wherein the presence of amplified RNA indicates micrometastasis of prostate cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an agarose gel in which micrometastasis is indicated by the presence of a 214 base pair (bp) band.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with methods of the present invention, methods of detecting micrometastasis of prostate cancer in a patient is provided comprising the step of obtaining a sample of RNA from the patient's blood. Preferably the RNA is obtained from a blood sample such as a peripheral venous blood sample. A whole blood gradient may be performed to isolate nucleated cells and total RNA is extracted such as by the

RNazole B method (Tel-Test Inc., Friendswood, Texas) or by modification of methods known in the art such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Thereafter, a polymerase chain reaction may be performed on the total extracted RNA. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art. Innis et al., *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990. Polymerase chain reaction primers may be designed to be complementary to separate regions of the prostate specific antigen (PSA) gene. Henttu et al., *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. By separate regions is meant that a first primer is complementary to a 3' region of the PSA gene and a second primer is complementary to a 5' region of the PSA gene. Preferably, the primers are complementary to distinct, separate regions and are not complementary to each other.

PSA is an important marker produced exclusively by prostatic epithelial cells and almost always expressed by prostate cancer. Stamey et al., *J. Urol.* **1989**, 141, 1076-1083. Thus, PSA2 (5-GAGGTCCACACACTGAAGTT, SEQ ID NO: 1) and PSA3 (5-CCTCCTGAAGAATCGATTCCT, SEQ ID NO: 2) oligonucleotide primers were designed to have high specificity to the PSA gene. A Gene Bank version-70 (Mountain View, CA) search confirmed the specificity of these primers to PSA and not to the human glandular kallikrein (HMGK) gene which has high homology to the PSA gene. Henttu et al, *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. PSA2 and PSA3 bind sequences that span intron III of the PSA gene such that PCR amplification yields a 360 bp DNA and a 214 bp RNA product, thereby eliminating the possibility of false positives from DNA contamination. Oligonucleotide primers may be prepared by methods known in the art such as by standard phosphoramidite chemistry. (See Sambrook et al., *supra*). Following amplification, the presence or absence of mRNA amplification product may be detected. Preferably, the PCR product may be run on an agarose gel and

visualized using a stain such as ethidium bromide. (See Sambrook et al., *supra*).

The following examples are illustrative but are not meant to be limiting of the invention.

EXAMPLES

Example 1 Patient Specimens

Selection of cases was based on the following criteria. Prostate cancer patients were chosen for analysis if they had: (1) clinically and/or surgically staged DO-D2 disease (D0 = elevated tumor markers with no demonstrable metastasis, D1 = pelvic lymph node involvement, D2 = disseminated disease usually to bone) without having received prior hormonal therapy and who had an elevated serum PSA, or (2) stage D3 disease (D2 disease that is refractory hormonal therapy) with an elevated PSA Negative control patients consisting of female volunteers, and patients with benign prostatic hypertrophy (BPH) proven by biopsy or men who were on a BPH study protocol. Patients who had surgical manipulation of the prostate during the previous year were excluded from the study. Positive controls included a lymph node from a patient with known metastatic PAC tissue from pathologically proven BPH and cDNA PSA plasmid. Henttu et al, *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. The protocol was IRB approved and written consent was obtained. LNCAP and PC3 human cell lines were obtained from The American Type Culture Collection, (Rockville, MD).

Example 2 Blood Preparation for RNA Extraction

Approximately six ml of venous blood were obtained with a standard venipuncture technique using heparinized tubes. Whole blood was mixed with an equal volume of phosphate buffered saline (PBS) which was then layered over eight ml of Ficoll (Pharmacia Uppsala, Sweden) in a 15 ml polystyrene tube. The gradient was centrifuged at 200 g for 30 minutes at 5°C. The lymphocyte and granulocyte layer (approximately 5 ml) was carefully aspirated and re-diluted up to 50 ml with PBS in a 50 ml tube which was then centrifuged at 1800 g for 20 minutes a

5°C. Supernatant was discarded, and the pellet containing nucleated cells was used for RNA extraction using the RNazole B method, as described by the company (Tel-Test Inc., Friendswood, Texas).

Example 3 Oligonucleotide primers and probes

PSA2 (5-GAGGTCCACACACTGAAGTT, SEQ ID NO: 1) and PSA3 (5-CCTCCTGAAGAATCGATTCCT, SEQ ID NO: 2) oligonucleotide primers were custom designed with high specificity to the PSA gene; a Gene Bank version-70 (Mountain View, CA) search confirmed the specificity of these primers to PSA and not to the human glandular kallikrein (HMGK) gene which is 75-85% homology to the PSA gene. Henttu et al, *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. All primers were synthesized and gel purified by the City of Hope DNA Synthesis Laboratory (Duarte, California). PSA2 and PSA3 bind sequences that span intron III such that PCR amplification yielded a 360 bp DNA and a 214 bp RNA product. Previously published actin PCR primer sequences were used to rule out degraded RNA, and amplification with actin oligonucleotide primers A1 and A2 yielded a 154 bp RNA and a 250 bp DNA product. Ben-Ezra et al., *J. Histochem Cytochem.* **1991**, 39, 351-354.

Example 4 Polymerase Chain Reaction

The reverse transcriptase reaction and PCR amplification were performed sequentially without interruption in a Perkin Elmer 9600 PCR machine (Emeryville, CA). 400 ng of total RNA in 20 μl DEPC (Diethyl-pyrocarbonate) treated water were placed in a 65°C water bath for five minutes then quickly chilled on ice immediately prior to the addition of PCR reagents. The 50 μl total PCR volume consisted of 2.5 units Taq polymerase (Perkin Elmer, Emeryville, CA), 2 units AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), 200 μM each of dCTP, dATP, dGTP, and dTTP (Perkin Elmer, Emeryville, CA), 18 pM each primer, 10 mM Tris-HCL, 50 mM KCl, 2 mM $MgCl_2$ (Perkin Elmer, Emeryville, CA). PCR conditions were as follows: cycle 1 was 42°C for 15 minutes, then 97°C for 15

seconds (one cycle); cycle 2 was 95°C for one minute, then 60°C for one minute and 72°C for 30 seconds (15 cycles); cycle 3 was 95°C for one minute, then 60°C for one minute, and 72 degrees for one minute (10 cycles); cycle 4 was 95°C for one minute, then 60 for one minute and 72°C for two minutes (8 cycles); cycle 5 was 72°C for 15 minutes (one cycle); and the final cycle was a 4°C hold until sample was taken out of the machine. The 50 μl PCR products were concentrated down to 10 μl with vacuum centrifugation and the entire sample was then run on a thin three percent Tris-borate-EDTA (TBE) agarose gel containing ethidium bromide. All specimens were analyzed at least twice to confirm a positive or negative outcome.

The potential risk of false positives from cross contamination was avoided by performing RT PCR in a single tube without interruption and using filtered pipet tips. Sensitivity was enhanced by using high amounts of Taq polymerase, progressively increasing extension times, and analyzing the entire 50 μl PCR product on thin ethidium bromide agarose gels. These measures ensured a high fidelity assay while maintaining technical simplicity.

Prostate human tissue specimens, tissue culture cell lines and a PSA cDNA plasmid, cloned and described by Henttu and Vihko; Henttu et al., *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910, were used as positive controls, and they demonstrated the 214 bp bands as shown in fig.1 top panel. A pelvic lymph node with metastatic PAC, a primary prostate cancer, and a BPH specimen all produced strong PSA PCR signals. The LNCAP and PC-3 human prostate cell lines produced weaker signals.

EXAMPLE 5 Sequencing

Specificity of these primers to the PSA gene was confirmed with DNA sequence analysis of the amplified 214 bp fragment (Figure 1 bottom panel) which in this segment had very little homology to the HMGK gene. The 214 bp product was purified with a Qiagen PCR Product Purification kit (Qiagen, Chatsworth, CA) as described by the manufacturer. One microgram

of the PCR product underwent a PCR sequencing reaction by using the Taq DyeDeoxy Terminator Cycle sequencing kit in a Perkin-Elmer 9600 PCR Machine, as described by Applied Biosystems (Applied Biosystems, Foster, CA). The sequenced product was purified using centri-sep columns (Princeton Separations, Adelphia, New Jersey) as described by the company. This product was then analyzed with a ABI Model 373A DNA sequencing system (Applied Biosystems, Foster, CA) integrated with a Macintosh IIci computer.

Example 6 Detection of Circulating Hematogenous Micrometastasis

Twelve prostate cancer patients and 17 control patients underwent RT PCR analysis on PSA and actin RNA extracted from blood, as described in Examples 1 through 4 (Table 1). All cases demonstrated satisfactory RNA quality by actin PCR (Figure 1, bottom row). Of the 12 human prostatic adenocarcinoma (PAC) patients with metastatic disease, four cases (33%) had positive PSA signals indicating the presence of prostatic epithelial cells in the peripheral venous blood. These four cases consisted of two stage D1 patients, one stage D2 patient, and one stage D3 patient (N=1) (Figure 1, top row). The 17 negative controls, which consisted of eight volunteer women and nine men with BPH, all had undetectable PSA mRNA by RT PCR. These data indicate that RT PCR of the PSA RNA gene can be used to specifically detect circulating hematogenous micrometastasis in patients with stage D1-D3 pathology. These findings are in agreement with studies by Hamby et al. who detected circulating PSA positive cells in patients with metastatic prostate cancer by flow cytology and immunohistology. Hamby et al., *Br. J. Urol.* **1992**, 69, 392-396.

Micrometastasis was not detected in eight of twelve prostate cancer patients consisting of two stage D3 patients, two stage D1 patients, and four stage DO patients. In order to enhance the detection of micrometastasis, analysis may focus on buffy coat cells. Results indicate that the prostate cancer cells may be more concentrated in the "buffy coat". The PSA

signal was stronger in the RNA extracted from cells obtained only from the "buffy coat" (Figure 1, lane 8) compared to those isolated from the entire Ficoll layer (Figure 1, lane 7) in the same prostate cancer patient. These findings are in agreement with those of Harty et al. who found that prostatic epithelial cells migrate into the "buffy coat". Harty et al., *J. Surg. Res.* **1979**, 26, 411-416.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Croce et al.

(ii) TITLE OF INVENTION: Methods of Detecting Micrometastasis Of Prostate Cancer

(iii) NUMBER OF SEQUENCES: 2
(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 19103
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: WORDPERFECT 5.1
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: n/a
(B) FILING DATE: Herewith
(C) CLASSIFICATION:
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(A) APPLICATION NUMBER:
(B) FILING DATE:
(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: Nucleic
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(iv) ANTI-SENSE: No
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
GAGGTCCACA CACTGAAGTT 20

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: Nucleic
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(iv) ANTI-SENSE: No
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CCTCCTGAAG AATCGATTCC T 21

What is claimed is:

1. A method of detecting prostate cancer micrometastasis in a patient comprising the steps of:

obtaining a sample of RNA from a patient's blood;

amplifying said RNA with polymerase chain reaction using a pair of primers which are complementary to separate regions of the prostate specific antigen gene; and

detecting the presence or absence of amplified RNA wherein the presence of amplified RNA indicates micrometastasis of prostate cancer.

2. The method of claim 1 wherein said primers have the sequences GAGGTCCACACACTGAAGTT (SEQ ID NO: 1) and CCTCCTGAAGAATCGATTCCT (SEQ ID NO: 2).

3. The method of claim 1 wherein said RNA is obtained from cells from the buffy coat of the Ficoll layer of the prepared blood sample.


PSA
214bp
Actin
154bp
Positive Controls
PSA cDNA
Met. Node
BPH
PAC
PC3
LNCap
Prostate
Cancer Patients
D3
D1
D1
D2
D2
Negative Controls
BPH
BPH
BPH
Female
Female
Female
1 2 3 4 5 6
7 8 9 10 11
12 13 14 15 16 17

FIGURE 1

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/US93/10331

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68
US CL :435/6, 91.1; 935/77, 78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, GENEBANK, EMBL, GENESEQ, CA, BIOSIS, MEDLINE, EMBASS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Cancer Research, Vol. 52, Number 21, 01 November 1992, MORENO ET AL., "Detection of Hematogenous Micrometastasis in Patients with Prostate Cancer", pages 6110-6112, see the entire document.	1-3
Y	British Journal of Urology, Vol. 69, issued 1992, HAMDY ET AL., "Circulating Prostate Specific Antigen-positive Cells Correlate with Metastatic Prostate Cancer", pages 392-396, see the Abstract and Figure 1.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"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 25 JANUARY 1994	Date of mailing of the international search report FEB 28 1994
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 17, No. 5, issued 1989, GOBLET ET AL., "One-step Amplification of Transripts in Total RNA Using the Polymerase Chain Reaction", page 2144, see Panel A.	1-3
Y	Journal of Surgical Research, Vol. 26, issued 1979, HARTY ET AL., "In Vitro Technique for Isolating Prostate Cells from Blood", pages 411-416, see Materials and Methods.	1-3
Y	Biochemical and Biophysical Research Communications, Vol. 159, No. 1, issued 28 February 1989, RIEGMAN ET AL., "Characterization of The Prostate-Specific Antigen Gene: A Novel Human Kallikrein-Like Gene", pages 95-102, see Figure 2.	1-3
Y	Current Opinion in Immunology, Vol.4, issued 1992, O'GARRA ET AL., "Polymerase Chain Reaction for Detection of Cytokine Gene Expression", pages 211-215, see Figures 1 and 2.	1-3