The HCCR Oncoprotein as a Biomarker for Human Breast Cancer

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Abstract

**Purpose:** HCCR oncoprotein is reported to be related to tumorigenesis, including breast cancer, functioning as a negative regulator of p53. Mice transgenic for HCCR developed breast cancers. The objective of this study was to validate the HCCR oncoprotein as a candidate biomarker for breast cancer.

**Experimental Design:** HCCR expression in breast cancer cells was analyzed by quantitative PCR, ELISA, immunohistochemistry, Western blotting, fluorescence-activated cell sorting, and confocal microscopy. Epitope areas were determined using mass spectrometry through the analysis of time-dependent tryptic fragment patterns of HCCR. HCCR expression profiles in breast cancer patient sera were analyzed, and correlations with clinicopathologic data and carbohydrate antigen 15-3 (CA15-3) levels were determined.

**Results:** HCCR was up-regulated in breast cancer cells and tissues. The epitope regions of HCCR recognized by monoclonal antibody (BCS-1) were HFWTPK and QQTDFLDIYHAFR. According to fluorescence-activated cell sorting and confocal microscopic analysis, BCS-1 was bound to HCCR antigen on the cell surface. Serum HCCR concentrations were measured using ELISA from 299 subjects, including 129 patients with breast cancer, 24 patients with benign breast disease, and 158 normal volunteers, and comparisons were made to CA15-3. Serologic studies revealed an 86.8% sensitivity for HCCR in breast cancer, which was higher than 21.0% for CA15-3. Eighty-six of 98 (87.8%) patients with breast cancers that were negative for CA15-3 were positive for HCCR-1. A positive response rate of 83.3% was identified even at early stages for pathologic factors in breast cancer.

**Conclusions:** The HCCR assay has an advantage over CA15-3 in diagnosing breast cancer and detecting early stages of the disease.

Breast cancer is the most frequent malignancy among women in western countries with an incidence rate in the United States of 111 cases per 100,000 woman-years and a mortality rate of 24 deaths per 100,000 woman-years (1, 2). Whereas the incidence of breast cancer seems to be increasing, mortality rates are now declining in some western countries (3). This decrease in mortality is most likely due to both the increased use of screening for the disease and the widespread administration of systemic adjuvant therapies.

The two best established markers for breast cancer are carbohydrate antigen 15-3 (CA15-3; ref. 4) and carcinoembryonic antigen (5). Nevertheless, the American Society of Clinical Oncology has stated in the Clinical Practice Guidelines for the use of tumor markers that neither CA15-3 nor carcinoembryonic antigen is recommended for routine use in diagnosing breast cancer and that new powerful markers for breast cancer are needed (5, 6).

We recently identified two new oncogenes associated with human cervical cancer that are also overexpressed in other human tumors, including breast and liver cancers (7, 8). The HCCR gene is classified into two isoforms, HCCR-1 (Genbank accession no. AF195651) and HCCR-2 (Genbank accession no. AF315598) according to molecular characteristics. Comparison of both sequences reveals that HCCR-2 corresponds to amino acids 57 to 360 of HCCR-1.

Experimental Design: HCCR expression in breast cancer cells was analyzed by quantitative PCR, ELISA, immunohistochemistry, Western blotting, fluorescence-activated cell sorting, and confocal microscopy. Epitope areas were determined using mass spectrometry through the analysis of time-dependent tryptic fragment patterns of HCCR. HCCR expression profiles in breast cancer patient sera were analyzed, and correlations with clinicopathologic data and carbohydrate antigen 15-3 (CA15-3) levels were determined.

Results: HCCR was up-regulated in breast cancer cells and tissues. The epitope regions of HCCR recognized by monoclonal antibody (BCS-1) were HFWTPK and QQTDFLDIYHAFR. According to fluorescence-activated cell sorting and confocal microscopic analysis, BCS-1 was bound to HCCR antigen on the cell surface. Serum HCCR concentrations were measured using ELISA from 299 subjects, including 129 patients with breast cancer, 24 patients with benign breast disease, and 158 normal volunteers, and comparisons were made to CA15-3. Serologic studies revealed an 86.8% sensitivity for HCCR in breast cancer, which was higher than 21.0% for CA15-3. Eighty-six of 98 (87.8%) patients with breast cancers that were negative for CA15-3 were positive for HCCR-1. A positive response rate of 83.3% was identified even at early stages for pathologic factors in breast cancer.

Conclusions: The HCCR assay has an advantage over CA15-3 in diagnosing breast cancer and detecting early stages of the disease.
results indicate that HCCR is an oncoprotein that is related to breast cancer development and regulation of p53.

In the present study, we investigated the expression levels of HCCR in breast cancers and the sensitivity of HCCR as an useful biomarker for breast cancer.

Materials and Methods

Cell lines. Mammalian cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF-7, MDA-MB-231, MDA-MB-361, SK-BR-3, and BT-474 are human breast carcinoma cell lines from mammary gland. MCF-7 and MDA-MB-231 cells express low HER-2, MDA-MB-361 cells express intermediate HER-2, and SK-BR-3 and BT-474 cells express high HER-2 (10, 11). MCF-7, MDA-MB-361, SK-BR-3, and BT-474 cells are estrogen receptor (ER)–positive and progesterone receptor (PR)–positive cells. MDA-MB-231 is a ER-negative and PR-negative cell line (12, 13). MCF-7 and MDA-MB-361 cells have wild-type p53, and MDA-MB-231 cells have null-type p53. SK-BR-3 and BT-474 cells have mutated p53 (14, 15).

Quantitative PCR. Total RNAs were isolated from breast cancer cell lines MDA-MB-468, MCF-7, and T47D and lung carcinoma cell lines NCI-H292 and 16-HBE, which is an immortalized bronchial epithelial cell line (kindly provided by Dr. Grunen Dieter, University of Vermont, Burlington, VT), as well as primary mammary epithelial cells. Human normal mammary epithelial cells were obtained from Chembrex (East Rutherford, NJ). These epithelial cells were obtained from normal female breast tissue, excluding the nipple and areola, from a 30-year-old donor. Cells were isolated by enzymatic digestion and >95% expressed cytokeratin-14 and cytokeratin-18 but not cytokeratin-19. All other cell lines were obtained from American Type Culture Collection. Total RNAs were isolated using RNeasy with on-column DNase I digestion (Qiagen, Valencia, CA). For real-time PCR analysis, total RNA (2 μg) was used to synthesize cDNA using an Omniscript reverse transcription kit in a 20 μL reaction (Qiagen). An aliquot containing 2 μL of the reverse transcription reaction was used as template for quantitative PCR reactions, which were carried out in a total volume of 25 μL, using an iQ SYBR Supermix (Bio-Rad, Hercules, CA) at 200 nmol/L primer concentration. Primers for quantitative PCR were designed by using a Beacon Designer (Biosoft International, Palo Alto, CA). The HCCR-1 forward primer (AF195651; 58-79) was 5′-CAGCTCA-CCCTGTCGACTTGTG-3′ and the reverse primer (AF195651; 175-152) was 5′-AATCTCTCCTACATGCTTTGAGGA-3′. The HCCR-2 forward primer (AF315598; 1081-1000) was 5′-GGAGGAGGAGAGAGGAGGAG-3′ and the reverse primer (AF315598; 1841-1616) was 5′-AGCCAGAGGGTGGTGTCTGTT-3′. This primer set amplifies both HCCR-1 and HCCR-2 mRNAs. The internal control glyceraldehyde-3-phosphate dehydrogenase was determined by a quantitative PCR assay using an iCycler instrument (Bio-Rad, Hercules, CA). For real-time PCR analysis, total RNA (2 μg) was used to synthesize cDNA using an Omniscript reverse transcription kit in a 20 μL reaction (Qiagen). An aliquot containing 2 μL of the reverse transcription reaction was used as template for quantitative PCR reactions, which were carried out in a total volume of 25 μL, using an iQ SYBR Supermix (Bio-Rad) at 200 nmol/L primer concentration. Primers for quantitative PCR were designed using a Beacon Designer (Biosoft International, Palo Alto, CA). The HCCR-1 forward primer (AF195651; 58-79) was 5′-CAGCTCA-CCCTGTCGACTTGTG-3′ and the reverse primer (AF195651; 175-152) was 5′-AATCTCTCCTACATGCTTTGAGGA-3′. The HCCR-2 forward primer (AF315598; 1081-1000) was 5′-GGAGGAGGAGAGAGGAGGAG-3′ and the reverse primer (AF315598; 1841-1616) was 5′-AGCCAGAGGGTGGTGTCTGTT-3′. This primer set amplifies both HCCR-1 and HCCR-2 mRNAs. The internal control glyceraldehyde-3-phosphate dehydrogenase forward primer (BC014085; 1087-1107) was 5′-CCGCTCACCATCCATGACGAGG-3′ and the reverse primer (BC014085; 1371-1351) was 5′-GAGATGCTCATGACGAGGAGG-3′. An initial denaturation step started with 3-minute incubation at 95˚C. PCR cycles (n = 40) consisted of 30-second melt at 95˚C followed by annealing for 20 seconds at 60˚C and an extension for 30 seconds at 72˚C. Amplification products were analyzed by melt curve data analysis and agarose gel analysis (3.0%). All reactions were done in duplicate. The threshold cycle (Ct), defined as the number of PCR cycles required for the specific fluorescence signal to exceed a detection threshold, was automatically set by the iCycler IQ real-time detection system software. The fold differences of the HCCR-1 and HCCR-2 mRNA were calculated by 2^-ΔΔCt method (16).

Antibody production, Western blot analysis, ELISA, and immunohistochemistry. The COOH termini of human HCCR-1 cDNA encoding a polypeptide from 167 to 360 amino acid residues was cloned into the pMAL-p2X (New England Biolabs, Beverly, MA) vector. The recombinant HCCR-1 protein was purified using amylase resin. The fusion protein was cleaved by factor Xa protease, and the resulting HCCR-1 protein was used to generate polyclonal antibodies (7, 8). For monoclonal antibody (mAb) production, BALB/c mice were immunized twice by s.c. injection. The first immunization was done using 50 μg HCCR-1(167-360) in Freund’s complete adjuvant. Two weeks later, the second immunization was begun with HCCR-1(167-360). Mice showing the highest antibody titre received a boost of HCCR-1(167-360) before spleen removal. The splenocytes were fused to P3-X63-Ag8.65 myeloma cells using the procedure described elsewhere (17, 18). For Western blot analysis, the protein lysates were loaded on 8% SDS-polyacrylamide gels. Rabbit polyclonal anti-HCCR-1(167-360) Serum (1:1,000) or mouse anti-HCCR-1(167-360) BCS-1 mAb (1:2,000) was preincubated with 1 mg of the purified HCCR-1 COOH termini, and preblocked antibody was used for hybridization. The blots were incubated with anti-HCCR-1 or BCS-1 antibody and developed with the enhanced chemiluminescence detection system (Pierce, Rockford, IL). For ELISA, A549 and MCF-7 cells were grown for 4 days. The cultured supernatant was stored at 4˚C for ELISA analysis. A 96-well plate was coated with 100 μL of various concentrations of cultured supernatant in coating buffer [7.5 mmol/L Na2CO3, 17.5 mmol/L NaHCO3, 5.35 mmol/L MgCl2; 6H2O (pH 9.6)]. The plate was sealed and incubated at 4˚C overnight. The wells were washed thrice with 300 μL washing solution (PBS with 0.05% Tween 20) and incubated in blocking solution [washing solution containing 2% bovine serum albumin (BSA)] for 2 hours at 37˚C. After washing, polyclonal anti-HCCR-1 (1 μg/mL) antibody was allowed to interact with coated HCCR-1 for 4 hours at 37˚C. Then, the plate was incubated with 100 μL goat anti-rabbit IgG, horseradish peroxidase conjugate (1:10,000; Sigma) for 1 hour at 25˚C. Peroxide substrate (100 μL) was added and incubated for 10 minutes at room temperature. The absorbance was determined at 450 nm using a ELISA reader. For immunohistochemistry experiments, paraffin sections (5 μm thick) of normal human breast and cancer tissues were used. The sections were incubated with affinity-purified polyclonal anti-HCCR-1 antibody or BCS-1 mAb for 2 hours. Aminoethyl carbazole was used as the chromogen. After immunostaining, sections were counterstained with hematoxylin.

Epitope mapping by the analysis of time-dependent tryptic fragment pattern. Maltose-binding protein (MBP)-HCCR-1 (10 μmol/L) and BCS-1 mAb (5 μmol/L) were mixed in 3 μL PBS, and the same concentration of MBP-HCCR-1 alone was also prepared in PBS. By the addition of 8 μmol/L trypsin into preincubated samples for 30 minutes, digestion reaction was initiated, and at certain time points, each solution (0.5 μL) was withdrawn and mixed with matrix solution (0.5% trifluoroacetic acid in 70% acetonitrile containing matrix, c-cyano-4-hydroxycinnamic acid) to quench the cleavage reaction. Quenched samples were loaded directly onto matrix-assisted laser desorption/ionization plate or kept at –20˚C until later analysis. Ettan Pro matrix-assisted laser desorption/ionization time-of-flight (Amersham Biosciences, Piscataway, NJ) was used to collect spectra of digestion fragments. All spectra collected were acquired in positive-ion reflector mode. Typically, 500 shots with 400 to 500 power were added per spectrum. For analysis of spectra, theoretical tryptic fragments of MBP-HCCR-1 were calculated by ProteinProspector (http://www.prospector.ucsf.edu).

Flow cytometric analysis. Cells (4 × 10^6-5 × 10^6) were incubated with PBS containing 0.5% BSA alone or with BCS-1 mAb against HCCR-1 for 30 minutes at 4˚C. Cells were washed extensively with PBS buffer containing 0.5% BSA and incubated with FITC-conjugated secondary antibody for 30 minutes at 4˚C in the dark. After washing, cells were analyzed on a FACScalibur (Becton Dickinson & Co., Mountain View, CA). For each sample, data from 10,000 cells were collected. Cells for control were incubated with secondary antibody alone to show background fluorescence.

Confocal analysis. MCF-7 cells were cultured on the cover glasses in the six-well plate. After overnight culture, cells were fixed with fresh 4% paraformaldehyde solution for 15 minutes and washed thrice with PBS. Cells on the cover glasses were incubated for 2 hours at 37˚C with
primary antibody in PBS with 1% BSA. Concentration of each antibody was 200 µg/mL, and 100 µL of solution were used to cover the surfaces of the cover glasses. Diluted secondary antibody (anti-mouse IgG) conjugated with FITC was added on the cover glasses and incubated for 1 hour at 37°C in the dark. Washed cells were mounted on the slide glasses and fluorescence was monitored on a confocal microscope (LIF confocal microscope, Leica, Solms, Germany).

Subjects. From October 2002 to December 2003, a total of 287 subjects were enrolled at the Kangnam St. Mary’s Hospital at the Catholic University of Korea. There were 129 individuals (mean age 54.7 years, range 31-77) with histologically proven or clinically diagnosed breast cancer and 158 normal volunteers (mean age 48.2 years, range 20-81) who were undergoing a routine health care check were included. None of the patients had other malignancies or active pulmonary disease. Clinicopathologic factors were abstracted from the initial surgical pathology reports. Staging was determined according to the tumor-node-metastasis classification of malignant tumors set by the Union International Contre le Cancer (19). Sera from 129 female patients with newly diagnosed breast cancer were obtained before therapy. Twenty-four with benign breast disease (8 intraductal papillomas, 8 fibroadenomas, and 8 mastitis), 16 with benign lung disease, and 14 with benign colon disease treated in our hospital were also analyzed for HCCR-1. All patients and controls were subjected to the analysis with individual consent for the study. The use of blood samples was approved by the ethics committee of our institution.

Detection of HCCR-1 in serum by indirect ELISA. The HCCR-1 levels were measured using an indirect ELISA. Serial dilutions of purified HCCR-1, ranging from 20 to 320 ng/mL, were coated on 96-well Maxisorp microtiter plates in 100 µL of 10 mmol/L phosphate buffer and incubated. The standard curve was constructed with serial dilutions of HCCR-1, ranging from 20 to 320 ng/mL. Using 20, 40, 80, 160, and 320 ng/mL of HCCR-1, a calibration curve was prepared. The microtiter plate was then saturated with PBS containing 2 mg/mL BSA. Purified primary polyclonal antibody (100 ng/mL/well) was prepared in PBS containing 0.1% BSA and 0.05% Tween 20 and incubated. Serum samples diluted 1:100 were analyzed in duplicate. A 100-µL sample was dispensed into the wells and washed with PBS containing 0.05% Tween 20, and the primary antibody (100 ng/well) was added to each well and incubated. After washing, the secondary antibody (goat anti-rabbit antibody conjugated with horseradish peroxidase) was added to the wells. The microtiter plate was washed and a 100-µL aliquot of the color-developing reagent (TMB) was dispensed into the wells to measure the horseradish peroxidase activity. The reaction was stopped by addition of 100 µL of 2 N sulfuric acid and an absorbance at 450 nm was read using a SpectraMax 250 microplate reader ( Molecular Devices, Sunnyvale, CA). The data were processed with the Softmax software. A standard curve was prepared and a reference sample was included in each test run. The concentration of HCCR-1 in each serum sample was calculated by reference to the standard curve and expressed as nanograms of HCCR-1 per milliliter of serum. The intraassay and interassay coefficients of variation were 0.7% to 5.9% and 4.0% to 7.0% for seven concentrations, respectively. The reference ranges were obtained by receiver operating characteristic curve analyses: values of 10 µg/mL were used as a cutoff for breast cancer. CA15-3 was measured by a two-step sandwich immunoradiometric assay using a commercially available kit [CA15-3 RIA kit (TFB), Fujirebio Diagnostics, Inc., Malvern, PA].

Statistical analysis. Serum levels (log value, µg/mL) of HCCR-1 in both groups and in each stage of clinicopathologic factors were presented as mean ± SD and compared using Smith-Satterthwaite’s F test, ANOVA, and Scheffe’s multiple comparison, respectively. To determine positive responses, we used the value of 30 units/mL as the cutoff for CA15-3, and the cutoff value for HCCR-1 was determined by receiver operating characteristic curve analyses with randomly selected 64 breast cancer and 79 controls. To confirm the validity of cutoff value, further diagnostic analysis was done with the remaining 65 breast cancer and 79 controls. Positive response rates of CA15-3 and HCCR-1 were estimated and their 95% confidence intervals were compared by McNemar’s test. We did not have CA15-3 levels for 5 patients; only 124 patients were used in McNemar’s test. Sensitivity, specificity, and total accuracy of HCCR-1 were estimated and their 95% confidence intervals were presented. After dividing patients by the stage of clinicopathologic factors, including age, number of lymph node metastasis, distant metastasis, lymphatic invasion, vessel invasion, histologic grade, and tumor-node-metastasis stage, McNemar’s test was used to compare the positive rates of CA15-3 and HCCR-1 within each stage. Two-tailed significance level of 0.05 and SAS release version 8.1 were used for all statistical analyses.

Fig. 1. Expression of HCCR-1 mRNA or protein in cell lines. A, quantitation of relative HCCR-1 and HCCR-2 mRNA levels in various cell lines using quantitative PCR. The relative levels of HCCR-1 and HCCR-2 mRNA in the various cells. B, level of HCCR-1 protein expressions in relation with different biological characteristics, including ER, PR, p53 genotype, and HER-2 status. Expression of HCCR-1 was measured by immunoblotting in a panel of breast cancer cell lines, including BT-474 (ER+/PR+/mutant p53/high HER-2), SK-BR-3 (ER+/PR+/mutant p53/high HER-2), MDA-MB-361 (ER+/PR+/wild-type p53/intermediate HER-2), MCF-7 (ER+/PR+/wild-type p53/low HER-2), and MDA-MB-231 (ER−/PR−/p53−/low HER-2) cells.
Fig. 2. Determination of HCCR-1 protein expressions. A, detection of HCCR-1 protein by antibodies. Polyclonal antiserum or BCS-1 mAb was specifically bound to immunogen HCCR-1-167-360. Equivalent volumes of bacterial protein lysates with pMAL-p2X vector alone (lane 1) or pMAL vector containing recombinant HCCR-1-167-360 (lane 2) were loaded on 8% SDS-polyacrylamide gels. B, ELISA. A549 lung cancer and MCF-7 breast cancer cells were grown for 4 days. The cultured supernatant was harvested and the amount of HCCR-1 released from cells was measured by ELISA. C, immunohistochemical staining analysis. The immunoreactivity was observed only in the breast cancer tissues with predominant nuclear staining. Magnification, ×400. Normal breast tissue and benign breast disease (breast fibroadenoma) showed no staining. Magnification, ×400. There were no expressions of HCCR-1 in normal tissues from uterine endometrium, ovary, and colon and in cancer tissues from squamous cell carcinoma of lung and stomach cancer. Magnification, ×400. D, Western blot analysis. The blot was incubated with purified polyclonal antibody and developed with the enhanced chemiluminescence detection system.
Results

Quantification of HCCR mRNA and expression of HCCR protein levels in various cell lines. To determine whether the HCCR mRNA isoforms HCCR-1 and HCCR-2 are overexpressed in breast cancer cell lines, human primary mammary epithelial cells and human breast and lung cells were isolated and the mRNA levels of HCCR-1 and HCCR-2 were measured using a quantitative PCR assay. The levels of both HCCR-1 and HCCR-2 were elevated to varying degrees in breast cancer cells compared with primary mammary epithelial cells (Fig. 1A). Up-regulation was greatest in T-47D cells (11.7-fold), intermediate in MCF-7 cells (4.4-fold), and moderate in MDA-MB-468 cells (2.2-fold). In the lung carcinoma cell line NCI-H292 and the immortalized bronchial epithelial cell line 16-HBE, the level of HCCR-1 was only moderately up-regulated (1.7- and 1.8-fold, respectively). The level of HCCR-2 mRNA closely paralleled the level of HCCR-1, with the exception that no up-regulation was seen in the MDA-MB-468 cell line. The levels of HCCR-1 and HCCR-2 mRNA were elevated in breast carcinoma cells when compared with noncancer cells. The level of elevation was variable depending on the cell line. These results support our previous findings that HCCR is overexpressed in breast cancer and play a causative role in breast cancer (7).

To elucidate whether HCCR protein expression in breast cancer is related to different biological characteristics, including ER, PR, p53 genotype, and HER-2 status, expression of HCCR was measured by immunoblotting in a panel of breast cancer cell lines. HCCR was detected in BT-474 (ER+/PR+/mutant p53/high HER-2), SK-BR-3 (ER+/PR+/mutant p53/high HER-2), MDA-MB-361 (ER+/PR+/wild-type p53/intermediate HER-2), and MCF-7 (ER+/PR+/wild-type p53/low HER-2) cells, whereas HCCR was not detected in MDA-MB-231 (ER−/PR−/p53−/low HER-2) cells (Fig. 1B). The expression level of HCCR was in an increasing order of BT-474, SK-BR-3, MCF-7, and MDA-MB-361.

Fig. 3. Epitope mapping analysis of HCCR-1. A, matrix-assisted laser desorption/ionization time-of-flight mass spectra of both samples, MBP-HCCR-1 alone and MBP-HCCR-1 with BCS-1 mAb, over various time points. Tryptic cleavage fragments of each sample, MBP-HCCR-1 alone and complex of MBP-HCCR-1 and BCS-1 mAb, were collected at 10, 20, 30, 60, and 120 minutes. As shown in box, relative intensity of 1,653 (m/z) was smaller at the early stages (10 minutes) than 1,584 (m/z) peak in both samples. However, the intensity of 1,653 peak of MBP-HCCR-1 alone increased rapidly, and at 120 minutes, its relative intensity became almost twice higher than 1,584 peak. Meanwhile, 1,653 peak in the complex of MBP-HCCR-1 and BCS-1 mAb increased slowly and remained similar to the intensity of 1,584 peak even after 120 minutes, which could mean 1,653 peak corresponding sequence 175 to 187 of MBP-HCCR-1 is protected by BCS-1 mAb. Similar pattern was shown for 815 peak corresponding sequence 169 to 174. B, epitope areas for BCS-1 mAb. Full sequence of HCCR-1 and epitope areas for BCS-1 mAb marked as bold underlined characters for 815 (169-174) and italic underlined characters for 1,653 (175-187).
Generation of antibodies to HCCR-1 and determination of HCCR-1 protein expression. Recombinant HCCR-1 protein was used as an immunogen for polyclonal and mAb production. Polyclonal antiserum and BCS-1 mAb directed against the 167 to 360 amino acids of HCCR-1 were generated. Western blot analyses using either polyclonal antiserum or BCS-1 mAb showed strong bands corresponding to recombinant HCCR-1 (Fig. 2A). These bands disappeared and in cell supernatants of MCF-7 cells compared with A549 cells that exhibited only minor expression of this protein as a negative control. HCCR-1 release was significantly enhanced in cell extracts and in cell supernatants of MCF-7 cells compared with A549 cells (Fig. 2B). Breast cancer and normal breast tissues from four patients and one benign breast disease sample (breast fibroadenoma) were subjected to immunohistochemical analysis. Results showed that HCCR-1 was overexpressed in all four breast cancer tissues. Immunoreactivity was observed mainly in breast cancer cells with a predominant nuclear localization (Fig. 2C). In normal breast tissues and in breast fibroadenoma tissue, there was no expression of HCCR-1. We also did immunohistochemistry of normal tissues from the uterine endometrium, the ovary, and the colon and other types of cancer tissues from squamous cell carcinoma of lung and stomach cancers (Fig. 2C). There was no expression of HCCR-1 in normal tissues from the uterine endometrium, the ovary, and the colon and in cancer tissues from squamous cell carcinoma of lung and stomach cancers (Fig. 2C). Western blot analysis showed that the HCCR-1 protein was overexpressed in breast cancer tissues and in breast cancer cell line compared with normal breast tissues (Fig. 2D).

Localization of an epitope in HCCR-1. The binding area on HCCR-1 for BCS-1 mAb was determined using mass spectrometry through analysis of the time-dependent tryptic fragment pattern of HCCR-1 (20–23). Tryptic digestion usually produces partial cleavage fragments at the beginning stage of digestion because structurally buried regions are shielded from trypsin. Similarly, protein complexes protect each other from tryptic cleavage in the interfacial areas. Well-protected regions are not cleaved or are cleaved slowly relative to exposed regions. Based on this concept, the pattern of cleaved fragments over time can be used to determine inaccessible or buried areas by structure or other interacting molecules. Tryptic digestion of MBP-HCCR-1 alone (control) and complex with BCS-1 mAb generated similar fragment patterns, except for the slow appearance rate of some fragments. Peaks at 1,584 (amino acid residues 260-273), 1,292 (amino acid residues 278-288), 1,256 (amino acid residues 293-304), 1,198 (amino acid residues 311-321), and 1,153 (amino acid residues 201-211) appeared at similar rates in both samples, but the relative intensities of distinctive peaks at 815 (amino acid residues 169-174) and 1,653 (amino acid residues 175-187) increased slowly over time in the spectrum of the complex between MBP-HCCR-1 and BCS-1 mAb. Mass spectra at the each time point are shown (Fig. 3A). Because slowly cleaved fragments compared with a control are supposed to be part of areas interacting with counter proteins, two fragments at 815 (amino acid residues 169-174) and 1,653 (amino acid residues 175-187) considered to be slowly cleaved fragments were expected to be epitopes of HCCR-1 interacting with BCS-1 mAb. The two fragments corresponded to sequences of HFWTLPK and QQTDFLDIYHAFR. Epitope areas with bold or italic underlined characters are indicated in the full sequence of HCCR-1 (Fig. 3B).

Localization of HCCR-1 in MCF-7 and HuH-7 cells. To determine whether BCS-1 mAb recognizes antigens expressed on the cell surface, different types of cancer cells, MCF-7, and human hepatoma HuH-7 were stained with BCS-1 mAb and FITC-conjugated secondary antibody. Fluorescence-activated cell sorting analysis showed that most BCS-1 binds to the cell surfaces of both cancer cell types with similar geometric mean ratios of 4.5 (145.96/32.76) and 5.4 (129.23/23.8) for MCF-7 and HuH-7, respectively (Fig. 4A). In addition, confocal microscopic analysis was done to visualize cell surface binding by BCS-1 mAb. Confocal microscopic images indicated that BCS-1 mAb bound to the cell surface of MCF-7 cells (Fig. 4B). Because cells were not permeable, we could only determine if
BCS-1 mAb recognizes the cell surface. HCCR-1 may not be localized only on the cell surface and it cannot be excluded that HCCR-1 is also expressed in other cell organelles. However, BCS-1 mAb clearly binds to the cell surface of cancer cells.

**Serum HCCR-1 levels in subjects.** The usefulness of serum HCCR-1 as a biomarker for breast cancer was evaluated. Serum levels of HCCR-1 were significantly higher in breast cancer (mean ± SD, 16.8 ± 6.8 μg/mL) than levels in normal controls (7.2 ± 2.6 μg/mL; P < 0.0001). Although receiver operating characteristic curve analyses resulted in a value of 9.5 μg/mL as the most efficient cutoff considering sensitivity, specificity, and total accuracy for 143 randomly selected subjects, we chose a value of 10 μg/mL as a more convenient cutoff from a clinical viewpoint. Using the cutoff value of 10 μg/mL, the positive rate of HCCR-1 in breast cancer was 82.3%, the negative rate in normal control was 93.7%, and the total accuracy was 88.8%. To confirm the validity of this cutoff level, further prospective analysis was done with the 144 remaining subjects. With the cutoff of 10 μg/mL, the positive rate of HCCR-1 in breast cancer was 90.8%, the negative rate in normal control was 87.3%, and the total accuracy was 93.7%, and the total accuracy was 88.8%, similar to previous results. In addition, for the 287 total subjects, the positive rate of HCCR-1 in breast cancer was 86.8%, the negative rate in normal control was 90.5%, and the total accuracy was 88.9% (Table 1; Fig. 5A and B). From these results for the three different subject groups, we used the value of 10 μg/mL as the cutoff for further analysis. To confirm the cancer sensitivity of HCCR-1, we analyzed 24 patients with benign breast diseases (8 intraductal papilloma, 8 fibroadenoma, and 8 mastitis). The mean value of the serum level was 7.86 ± 1.43 μg/mL and all patients showed results <9.08 μg/mL, except one (12.89 μg/mL) with mastitis. We also examined the specificity of HCCR-1 by analyzing the patients with benign inflammatory conditions and other malignancies. Two of 50 (4%) patients with chronic hepatitis were detected by HCCR-1. The false-positive rate for colitis patients were 7.4% (2 of 27 patients). One of 25 (4%) patients with uterine cervical cancer and 2 of 25 (8%) patients with lung cancer were detected by HCCR-1. Taken together, the average false-positive rate of HCCR-1 in benign breast cancers, inflammatory conditions, and other malignancies were 5.3% (8 of 151 patients).

One hundred nine of 124 (87.9%) patients were correctly detected by HCCR-1, whereas only 26 (21.0%) were detected by CA15-3 (cutoff, 30 units/mL). These results were significantly different (P < 0.0001). Eighty-six of 98 (87.9%) patients who were diagnosed as negative by CA15-3 were correctly identified by HCCR-1 (Table 1). The HCCR-1 levels according to each clinicopathologic factor and diagnostic results compared with CA15-3 are summarized in Table 2. The mean values of HCCR-1 levels were not significantly different among the different stages for all clinicopathologic factors. The positive response rates for HCCR-1 were higher than 83%, even in the early stages of the pathologic factors, whereas the rates for CA15-3 were, at most, 15%. All the positive response rates for HCCR-1 were significantly higher than the rates for CA15-3 at each stage of the pathologic factors, except grade 1 of histologic grade. Ninety percent of 22 patients with stage I cancer showed seropositivity (Table 2). All 22 patients underwent surgical resection and the serum concentrations of HCCR-1 in 13 of the 22 patients were examined after tumor resection. The HCCR-1 concentrations in the patient sera were decreased to the normal levels after tumor resection.

**Discussion**

The useful markers for monitoring patients with breast cancer are CA15-3 and BR27.29 (24, 25). Assays for these markers detect the MUC-1 protein and both seem to provide similar clinical information (24, 25). A valuable marker in screening for breast cancer would have to detect early stages of breast cancer in asymptomatic populations. Summaries of multiple studies have shown that the sensitivities of CA15-3 in patients with stages I and II breast cancer were 9% and 19%, respectively (5). Although patients with early-stage breast cancer may have elevated circulating tumor marker levels, such as carcinoembryonic antigen, CA15-3, BR27.29, or the extracellular domain of c-erbB-2, no reports have shown the clinical utility of these markers in newly diagnosed breast cancer patients (26, 27). Early detection represents one of the most promising approaches to reducing the growing cancer burden. Early detection already is important in the management of cervical and breast cancers (28). Currently, the only

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<th>Table 1. Diagnostic results of HCCR-1 and CA15-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCCR-1 (cutoff = 10 μg/ml)</strong></td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Group Cancer</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diagnostic measures</th>
<th>Value (%)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>86.8</td>
<td>81.0-92.7</td>
</tr>
<tr>
<td>Specificity</td>
<td>90.5</td>
<td>85.9-95.1</td>
</tr>
<tr>
<td>Accuracy</td>
<td>88.9</td>
<td>85.2-92.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>CA15-3</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
</tr>
<tr>
<td>HCCR-1</td>
</tr>
<tr>
<td>Total 26 (21.0)</td>
</tr>
</tbody>
</table>

*Five patients were excluded due to missing value of CA15-3. P < 0.0001, McNemar test.
recommended predictive markers in oncology are ER and PR for selecting endocrine-sensitive breast cancers and HER-2 for identifying breast cancer patients with metastatic disease who may benefit from trastuzumab (6, 29). For malignancies other than breast cancers, validated predictive markers presently do not exist.

HCCR protein has been identified as an oncoprotein that is related to breast cancer development and a negative regulator of the p53 tumor suppressor gene (7, 9). In the present study, we investigated HCCR protein expression in breast cancer and the possibility of using HCCR as a useful biomarker for human breast cancer. We examined whether HCCR protein expression in breast cancer is related to different biological characteristics, including ER, PR, p53 genotype, and HER-2 status. The increasing expression level of HCCR was in the order of BT-474 (ER+/PR+/mutant p53/high HER-2), SK-BR-3 (ER+/PR+/mutant p53/high HER-2), MCF-7 (ER+/PR+/wild-type p53/low HER-2), and MDA-MB-361 (ER+/PR+/wild-type p53/intermediate HER-2). HCCR was not detected in MDA-MB-231 (ER−/PR−/p53−/low HER-2). HER-2 is expressed at a low level in MCF-7 and MDA-MB-231 cells, at an intermediate level in MDA-MB-361 cells, and at a high level in SK-BR-3 and BT-474 cells (10, 11). The breast carcinoma cell lines MCF-7, MDA-MB-361, and BT-474 with HER-2 expression (by flow cytometry) of 1:4:18 (11) were used to correlate the HCCR expression level with HER-2 positivity. The expression level of HCCR was high in BT-474 cells that highly express HER-2. However, results from Western blotting of MCF-7 and MDA-MB-361 indicate that HCCR expression and the HER-2 level were not correlated in these cell lines. The expression level of HCCR was high in cells with a mutated p53, such as BT-474 and SK-BR-3 cells (13–15), and MCF-7 and MDA-MB-361 cells that contain wild-type...
p53 showed low HCCR expression. MDA-MB-231 cells with the null-type p53 did not express HCCR. HCCR was expressed in all four ER-positive and PR-positive breast cancer cell lines (MCF-7, MDA-MB-361, SK-BR-3, and BT-474; refs. 12, 13). On the contrary, HCCR was not detected in the ER-negative and PR-negative cell line (MDA-MB-231). Our results show that HCCR is highly expressed in breast cancer cell lines with a high HER-2 level, with some exceptions, that have a mutated p53 and that express ER/PR. These data indicate that the level of HCCR in breast cancer cell lines is relatively well correlated with known breast cancer factors, including the HER-2 level, p53 mutation, and ER/PR expression (30–32).

We also examined the utility of serum HCCR-1 as a biomarker for breast cancer. Serum levels of HCCR-1 in breast cancer were ~2.3-fold higher than levels in normal controls. The HCCR serum level was relatively low in 24 patients with benign breast disease who were examined to determine the sensitivity. We also investigated the level of HCCR in 16 patients with benign lung disease and 14 patients with benign colon disease. The mean values of the serum level were 8.6 ± 1.17 and 7.25 ± 2.01 μg/mL, respectively. These data indicate that HCCR is sensitive for human breast cancer. We examined the false-positive rate of HCCR-1 against the patients with that HCCR is sensitive for human breast cancer. We also investigated the level of HCCR in 16 benign breast disease who were examined to determine the sensitivity. We also investigated the level of HCCR in 16 benign breast disease who were examined to determine the sensitivity. We also investigated the level of HCCR in 16 benign breast disease who were examined to determine the sensitivity.
The HCCR Oncoprotein as a Biomarker for Human Breast Cancer


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