CLARI, a Novel Gene That Exhibits Enhanced Expression in Advanced Human Prostate Cancer

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ABSTRACT

The molecular events involved in prostate cancer progression are, at present, poorly understood. Using a differential display technique, we identified a cDNA fragment that is present in greater abundance in stage D prostate tumors compared to stage B tumors. Northern analysis was used to confirm that transcripts for this gene are expressed at higher levels in prostate tumors of later pathological stage and higher Gleason grade compared to tumors of earlier stage and lower grade. These transcripts were also expressed at high levels in all four human prostate cancer cell lines, the neonatal prostate cell line FNC 267b1, and in a variety of other normal human adult and fetal tissues. The cDNA fragment obtained by differential display was used as a probe to clone the full-length cDNA for this gene from a fragment obtained by differential display was used as a probe to clone the full-length cDNA for this gene from a human heart cDNA library. DNA sequence analysis confirmed that the cDNA was novel, and we have named this human heart cDNA library. DNA sequence analysis confirmed that transcripts for this gene are expressed at higher levels in prostate tumors of later pathological stage and higher Gleason grade compared to tumors of earlier stage and lower grade.

INTRODUCTION

Prostate cancer is the second leading cause of male cancer death in the United States (1). However, the etiology of this disease is unclear, and most prostate cancer patients have no known risk factors for prostate cancer development or progression. There appear to be at least two different prostate cancer patient populations, in that some patients never progress or do so very slowly, whereas others progress very rapidly. In one study, 84% of nonpalpable cases that were identified by early screening methods were clinically significant tumors, with at least 44% of these tumors having already progressed to advanced cancers characterized by capsular penetration, lymph node, and/or seminal vesicle involvement (2). By the time these tumors are palpable, many may have already progressed to the point at which they are beyond cure. Although high Gleason grade tumors are associated with systemic disease, most prostate tumors are of moderate grade, and the risk for development of advanced disease is unpredictable (3).

There are several examples of gene expression correlated with Gleason grade and aggressive growth (4–12). The best examples are the e-cadherin/α-catenin genes, which are significantly decreased in a large percentage of high Gleason grade human prostate tumors (8–11), and KAI1, a human metastasis suppressor gene (12). Thus, it is possible that other consistent gene expression differences exist between tumor types of the slow progressing and aggressive prostate cancer patient populations. The purpose of this study was to identify novel genes that may provide insights into the molecular mechanism(s) of prostate tumor progression. We have used a modified RT-PCR differential display method (13, 14) to compare early- and late-stage primary human prostate tumors for differences in gene expression patterns, and we have isolated the full-length cDNA to one of these differentially expressed genes. We show that the cDNA encodes a novel gene, CLARI, that is expressed at higher levels in human prostate tumors of later pathological stage and higher Gleason grade. In addition, we have characterized the expression pattern of CLARI in human prostate cancer cell lines and in normal fetal and adult organs. The deduced amino acid sequence of Clar1 suggests a possible role for this protein in signal transduction.

MATERIALS AND METHODS

Prostate Tumor Tissue and Total RNA Extraction. A total of 31 radical prostatectomy tumor specimens were analyzed. All tumor specimens were grossly dissected from surrounding normal tissue, and adjacent frozen sections of each tumor sample were stained with H&E and reviewed by a pathologist to verify the presence and extent of malignancy. Only samples with >70% tumor tissue were used for RNA extraction. Total RNA was extracted from the tumor tissues using guanidinium isothiocyanate, as described previously (15). Ten µg of each RNA were treated with 2.5 units of DNase (Promega, Madison, WI) at 37°C for 1 h.
Cell Lines and Culture Conditions. All cell culture media and supplements were purchased from Life Technologies, Inc. (Gaithersburg, MD). PC-3, DU145, LNCaP and TSUPr1 cells were cultured as described previously (16). FNC 267b1 cells were cultured in keratinocyte-SFM medium supplemented with 50 µg/ml bovine pituitary extract, 5 ng/ml human epidermal growth factor, 50 units/ml penicillin, and 50 µg/ml streptomycin. Total RNA was extracted from the exponentially growing cell lines using RNeasy (Qiagen, Chatsworth, CA), according to the manufacturer’s protocol. Eight µg of total RNA from each of the cell lines were treated with 2.5 units of DNase (Promega, Madison, WI) at 37°C for 1 h prior to Northern analysis.

Differential Display Analysis. Complementary DNAs from five early-stage B and four late-stage D primary prostate tumor samples were prepared using the SuperScript II Pre-amplification System (Life Technologies, Inc.) and amplified with 25–30 primer combinations using GeneAmp (Perkin-Elmer Corp., Foster City, CA) and 1 µM primer. The degenerate decamer primers used to detect CLAR1 in the differential display analysis were: LG 27, 5'-GAACCAACGG-3'; and LG 153, 5'-TACAACGAGG-3'. The PCR cycling conditions used were: 95°C for 5 min; then 45 cycles at 95°C for 1 min, 34°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The resulting PCR products were analyzed on 2.5% Meta-Phor-1× TBE agarose gels. RT-PCRs were performed three times to verify the reproducibility of suspected marker fragments. Stage-specific PCR marker fragments were isolated from the agarose gels using Qiagen II (Qiagen) and were then subjected to a second PCR amplification using the same primer set and cloned into the TA Cloning vector, pCR II (Invitrogen, Carlsbad, CA). OneShot INV-alphaF7 competent cells (Invitrogen) were transformed with the TA vector-PCR fragment ligated products and selected on Luria-Bertani broth, 50 µg/ml ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates. At least five white colonies from each transformation were grown in 2× YT-50 µg/ml kanamycin medium overnight, plasmid DNA was isolated from these clones using the Perfect Prep system (5'-TBE gel and transferred to Maximum Strength Nytran (Schleicher & Schuell). The blot was hybridized sequentially with random-primed, 32P-labeled probes to CLAR1 and β-actin and analyzed as described above.

Human multiple organ Northern blots (Clontech, Palo Alto, CA) that contain 2 µg of poly(A)+ RNA from fetal kidney, liver, lung, and brain and adult peripheral blood leukocyte, colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart were hybridized sequentially with random-primed, 32P-labeled probes to CLAR1 and β-actin and analyzed as described above. All three blots were hybridized simultaneously with the same batch of 32P-labeled probe (either for CLAR1 or β-actin), so that transcript levels for each could be compared directly between the blots by phosphorimaging analysis.

Quantitative CLAR1 RT-PCR. cDNA was prepared from 1 µg total RNA from 20 primary human prostate tumors using Superscript II (Life Technologies, Inc.). The cDNAs were amplified using CLAR1 cDNA-specific primers (CLAR1 forward, 5'-GGGCCCTTTTGATGTTGAGG-3'; and CLAR1 reverse, 5'-TTGGGAATGGGAGAGCAAG-3') with 0.25 µM primer, 1× PCR Buffer II, 1.5 mM MgCl₂, 2 mM dNTPs, and 0.6 units of AmpliTaq (GeneAmp kit; Perkin-Elmer Corp.) and the following PCR cycling conditions; 20 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min. The 515-bp PCR products were analyzed on a 2% agarose-1× TBE gel and transferred to Maximum Strength Nytran (Schleicher & Schuell). The Southern blots were hybridized with a random-primed, 32P-labeled probe that represented the cloned fragment of CLAR1, stringently washed, and autoradiographed. To normalize for equivalent amounts of cDNA added to the PCR assay, we performed a quantitative RT-PCR assay for cellular N-ras gene expression that we described previously (16) under the same reaction and cycling conditions as the CLAR1 RT-PCR, except that the number of cycles was extended to 25. The products were analyzed on 2% agarose-1× TBE gels and transferred onto Maximum Strength Nytran. An N-ras oligonucleotide probe was end-labeled with [α-32P]dATP (Redivue, 5000 Ci/mmole; Amersham) using a Multiprime DNA labeling system (Life Technologies, Inc.) and purified on a BioSpin6 column (Bio-Rad). Hybridization with the N-ras probe was performed at 42°C, followed by two washes at low stringency in 2× SSC-0.1% SDS.
at ambient temperature for 15 min and a third wash in 0.2× SSC-0.1% SDS at 42°C for 15 min. The blots were autoradiographed and scanned on a BAS 1000 phosphorimager (Fuji).

The quantitative RT-PCR assay was performed at least three times for each tumor RNA sample to verify the reproducibility of the CLAR1 expression level.

**Relative CLAR1 Signal Intensity Calculation.** To normalize for RNA loading, we divided the phosphorimaging data from each sample (pixels/mm²) by the corresponding β-actin or N-ras phosphorimaging data from each sample (pixels/mm²) to yield a ratio of CLAR1/β-actin or CLAR1/N-ras expression. The sample with the highest normalized CLAR1 ratio was assigned a relative signal intensity of 1.00 (100%). All other samples within the group were then divided by the CLAR1/β-actin or CLAR1/N-ras ratio of this highest expressing sample to produce a relative CLAR1 signal intensity for each sample analyzed.

**Statistical Analyses.** Statistical analyses on all relative CLAR1 signal intensity data were performed on a 486 IBM personal computer using the SPSS statistical software package for MS Windows 6.1. All data were first examined using the Levene test for homogeneity of variance. The β-actin-normalized relative CLAR1 signal intensity data required nonparametric analyses and were analyzed for statistical significance using Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney U-Wilcoxon rank sum post hoc comparisons. The N-ras-normalized relative CLAR1 RT-PCR signal intensity data were suitable for one-way ANOVA, followed by Fisher’s least significant difference post hoc comparisons for stage data. For all tests, the significance level was assigned at P ≤ 0.05.

**CLAR1 cDNA Cloning.** The GeneTrapper oligonucleotide primer used to isolate CLAR1 cDNA library clones was: 5'-dAAGGAGAGAGGACAGAGG-3'. The CLAR1 primer was biotinylated and hybridized to a prepared single-stranded adult human heart (female, 50 years old) cDNA library constructed in pCMV-SPORT (Life Technologies, Inc.). Following separation from the unhybridized library sequences using streptavidin-coated paramagnetic beads, the probe-magnetic bead complex was removed from the single-stranded CLAR1 cDNA target sequences, and the target sequences were repaired to double-stranded molecules using a nonbiotinylated oligonucleotide CLAR1 primer identical to that used to select the target. Following repair, this enriched plasmid sequence pool was used to transform ElectroMAX DH10B cells (Life Technologies, Inc.) by electroporation. Colony blots were prepared from these CLAR1 cDNA-enriched transformation plates on Nytran circles (Schleicher & Schuell) and hybridized with a multiprimer, 32P-labeled probe for CLAR1. Positive colonies were selected from the plate, grown in overnight cultures, and prepared with plasmid DNA, and the cDNA sequences were determined by automated fluorescent sequencing.

**CLAR1 Chromosomal Location.** Metaphase spreads from phytohemagglutinin-stimulated lymphocytes of a healthy female donor were prepared as described (17). The hybridization probe for chromosomal mapping was a 1.2-kb cDNA subclone of CLAR1 that was isolated from a human heart cDNA library. FISH and detection of immunofluorescence were carried out as described previously (18).

**RESULTS**

**Differential Display.** Using a recently described modified differential display technique (14), we compared the gene expression patterns between five organ-confined (stage B) and four metastatic (stage D) primary prostate tumors (not lymph nodes). Total RNA from pathological stage B and D prostate tumors were reverse-transcribed and amplified with multiple combinations of degenerate primer sets. One of the primer sets identified a 680-bp amplified cDNA fragment that exhibited late-stage specificity. The 680-bp cDNA fragment was cloned into a TA-cloning vector, and Southern blot analysis of the RT-PCR products from which the fragment was isolated confirmed that the correct differentially expressed fragment had been cloned (data not shown). The cloned fragment was designated CLAR1.

**Expression of CLAR1 in Human Prostate Cancer.** To confirm the stage specificity of CLAR1, we used the 32P-labeled purified insert from the 680-bp clone to hybridize three independent Northern blots containing total RNA from 11 stage B, 8 stage C, and 5 stage D human primary prostate tumors. A representative Northern blot is shown in Fig. 1A. The CLAR1 probe detected two transcripts of ~2.6 and 2.0 kb in size in all tumor RNAs examined.

We used β-actin gene expression to normalize for RNA loading and phosphorimaging analysis to determine the relative signal intensities of the Clar1 transcripts in prostate tumor RNAs. Tumor RNA samples in each stage category were analyzed several times each, and the signals from independent Northern blots were averaged. The average relative signal intensities of the Clar1 transcripts with respect to tumor stage from the three independent Northern blots are shown in Fig. 1B, where n represents the total number of replicates performed in each stage category. Our analysis demonstrates that the expression level of the 2.6-kb transcript was 3.3–3.5-fold greater in stage C and D tumors than in stage B tumors, whereas the 2.0-kb transcript was increased by 4.4–5.4-fold in stage C and D prostate tumors as compared to stage B tumors. In both cases, these increases were statistically significant.

We have also established a quantitative RT-PCR assay for CLAR1. The validity of this assay has been established previously (16). A subset of 17 of the 24 tumor samples examined by Northern analysis (Fig. 1) were reanalyzed using the RT-PCR method. Each sample was analyzed a total of four times, and the average was calculated. Thus, n represents the number of replicates examined for each tumor category. The results shown in Fig. 2A demonstrate that by RT-PCR CLAR1 expression is
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slightly greater in stage C than in stage B tumors and ~2-fold greater in stage D than in stage B prostate tumors. These results generally confirm the Northern blot results, although the magnitude of the increase in CLAR1 expression for stage C and D tumors, compared to that of stage B, is smaller by RT-PCR. Analysis of Clar1 expression by RT-PCR analysis on RNA from nine additional prostate tumors (two stage B, two stage C, and five stage D) resulted in a stage-specific expression pattern that was virtually identical to that seen in Fig. 2A (data not shown).

In Fig. 2B, we present CLAR1 expression data on a set of 20 tumors, 7 of which were samples that were not previously analyzed for CLAR1 expression, as a function of Gleason grade. High Gleason grade prostate tumors are associated with poor clinical prognoses (4). Again, n represents the number of replicates examined in each category, not the number of tumors. These results demonstrate that CLAR1 expression was increased by nearly 2.5-fold in the group of tumors with Gleason grades between 8 and 10 compared to the group with grades between 3 and 6.

Expression of CLAR1 in Human Prostate Cancer Cell Lines. To investigate whether CLAR1 may also be expressed at high levels in human prostate cancer cell lines, we examined the expression pattern of CLAR1 in the TSUP1, DU145, LNCaP, and PC-3 cell lines as well as in FNC 267B1, an immortalized normal neonatal prostate cell line (19). We found that CLAR1 was expressed to similarly high levels in the neonatal prostate cells as well. Interestingly, all of these cell lines predominately expressed the 2.0-kb CLAR1 transcript and expressed very little of the 2.6-kb form.

CLAR1 Expression in Fetal and Adult Human Organs. To determine the organ distribution of CLAR1 expression and the relative ratio of the CLAR1 transcripts, we examined CLAR1 expression in normal human fetal and adult organs. Multiple organ Northern blots containing poly(A)⁺ RNA from several fetal and adult organs, including normal prostate, were hybridized together with 32P-labeled probes to CLAR1 and beta-actin (Fig. 4). This approach allowed us to directly compare CLAR1 expression in fetal organs to that in adult organs. CLAR1 expression was detected in all organs examined; however, the transcript levels were highly variable according to organ type. Phosphorimaging analysis (data not shown) demonstrated that fetal brain, adult skeletal muscle, and heart had the highest signals relative to the other normal organs. The remaining fetal organs and adult pancreas, kidney, liver, lung, and brain had moderate CLAR1 expression; however, normal prostate had a moderately low level of CLAR1 expression. In all of the normal organs, both Clar1 transcripts were detected, but the 2.0-kb transcript was predominant.

Skeletal muscle had relatively high CLAR1 expression (Fig. 4). Because skeletal and smooth muscle fibers are common within the fibromuscular stroma of the prostate (20), we addressed the possibility that the high CLAR1 levels detected for stage C and D tumors were a reflection of high muscle content instead of elevated CLAR1 expression within the cancer cells themselves. We, therefore, rehybridized the three Northern blots containing the prostate tumor RNAs with a 32P-labeled probe to desmin, which is expressed specifically in muscle. We found that desmin RNA levels did not correlate with tumor stage (P = 0.347), indicating that muscle content was not a confounding variable in the analysis of patient specimens (data not shown).
Cloning and Characterization of CLAR1 cDNA. On the basis of the results of the organ expression analysis, we screened an adult human heart SuperScript cDNA library for the full-length cDNA to CLAR1 using the original Clar1 cDNA fragment identified by differential display. The screen of the adult heart cDNA library identified 142 CLAR1-positive clones, from which we sequenced the 46 largest clones and identified 2.6 kb of overlapping CLAR1 cDNA sequence (Fig. 5A). The CLAR1 cDNA has a single open reading frame (nucleotides 811–1638) that predicts a protein of 276 amino acids with an approximate molecular mass of 33.8 kDa. The deduced amino acid sequence derived from the full-length CLAR1 cDNA sequence is presented in Fig. 5B. A BLAST search of the GenBank/EMBL and SwissProt databases revealed that CLAR1 shares no significant DNA, expressed sequence tag, or protein sequence homologies with any other known sequence, except for a CpG island found in CLAR1 (nucleotides 506–864). In addition to the full-length CLAR1 sequence, we isolated two smaller cDNAs that represent potential splice variants of CLAR1 (Fig. 5A). The two putative CLAR1 splice variants predict an NH2-terminal truncated Clar1 protein that results from the loss of the ATG start codon at nucleotide 811. These splice events create transcripts that encode a Clar1 protein that lacks the first 20 amino acids but is in-frame with the full-length protein. The two smaller cDNAs also lack the bulk of the CpG island sequence.

Chromosomal Location and Conservation of the CLAR1 Gene. Using FISH of a CLAR1-specific probe to human lymphocyte metaphase spreads, we have determined the chromosomal location of the CLAR1 gene. A GeneTrapper-positive CLAR1 clone was used as a probe to hybridize to human metaphase spreads. Hybridization of the probe to human metaphase spreads revealed specific labeling on chromosome 19 in 20 of 21 metaphase spreads scored. Signals localized to 19q13.3–q13.4, with most being located at band 19q13.3 (data not shown). Interestingly, two other prostate-associated genes, prostate-specific antigen (PSA/APS) and human glandular kalikrein (hGK-1 or KLK2), also map to this region of chromosome 19 (22–25). To determine the extent to which CLAR1 is conserved among species, we have hybridized a Southern “zoo” blot containing human, cat, cow, dog, horse, mouse (BALB/c nude), pig, rat (Fisher), and yeast (Schizosaccharomyces pombe) DNA with a 32P-labeled CLAR1 cDNA fragment. The results revealed that CLAR1 is well conserved among mammals, hybridizing most strongly with human and cow DNA but also demonstrating visible bands in cat, dog, horse, mouse, pig, and...
rat DNA (Fig. 6). However, no hybridization signal was detected within yeast DNA, even upon a long (2-week) exposure (data not shown). In addition, a search of the *Saccharomyces cerevisiae* and *Caenorhabditis elegans* genome databases revealed no homologues to CLAR1.

**DISCUSSION**

We have used differential display and cDNA library screening to identify and clone the full-length cDNA for a novel gene that we have named CLAR1. Our analysis of 31 human prostate tumors demonstrated that CLAR1 expression was elevated in tumors of later pathological stage and higher Gleason grade. Upon Northern analysis, CLAR1 displayed two transcripts of 2.6 and 2.0 kb, the ratio of which were similar in most tumor specimens examined. Quantitative RT-PCR analysis with respect to tumor stage generally confirmed our Northern results and was very reproducible. However, both the fold increase in CLAR1 expression in later-stage tumors and the difference in
expression between tumors of stage B and C were less dramatic than that observed on Northern blot analysis. This discrepancy may be due to the intrinsic variability associated with the RT-PCR methodology that makes it less quantitative than Northern analysis and to the fact that a different mix of tumor samples was used for the RT-PCR analysis. However, overall, our data demonstrate that the level of Clar1 transcript expression is greater in later-stage and higher-grade prostate tumors. On the basis of these results, we predicted that Clar1 expression on prostate cancer etiology and progression remains to be determined, the enhanced expression of this novel gene in more advanced forms of prostate cancer and its potential role in disease advancement. The elucidation of a function for Clar1 will help to determine the role that this gene might play in prostate cancer progression.

Further studies will be required to establish that Clar1 is mechanistically involved in prostate cancer progression, to identify a function for the gene product, and to determine the significance of the alternatively spliced forms. We plan to use antisense constructs of Clar1 to reduce or eliminate its expression in the prostate cancer cell lines and observe the effect on their growth properties and tumorigenicity. In addition, we are currently using a yeast two-hybrid system to screen fetal brain cDNA libraries for proteins that are able to interact with Clar1. Indeed, proteins that are involved in cytoskeletal control (e-cadherin/α-catenin) have demonstrated expression level changes that correlate with prostate tumor progression (8–11) and may play a role in disease advancement. The elucidation of a function for Clar1 will help to determine the significance of the alternatively spliced forms. We plan to use antisense constructs of Clar1 to reduce or eliminate its expression in the prostate cancer cell lines and observe the effect on their growth properties and tumorigenicity. In addition, we are currently using a yeast two-hybrid system to screen fetal brain cDNA libraries for proteins that are able to interact with Clar1. Although the effect of elevated Clar1 expression on prostate cancer etiology and progression remains to be determined, the enhanced expression of this novel gene in more advanced forms of prostate cancer and its potential role in signal transduction both argue for its further investigation.

ACKNOWLEDGMENTS

We thank D. Winter, A. Cywinski, and M. P. Rondinelli for technical assistance; L. J. Goldstein for the gift of differential display primers; B. R. Nestok and T. Al-Saleem for characterization of patient specimen pathology; J. L. Mohler for supplying a stage D human prostate tumor; W. B. Isaacs and J. S. Rhim for the gift of the FNC 276B1 cell line; D. W. Bell and J. R. Testa for assistance in determining the chromosomal location of Clar1; and A. K. Godwin for the gifts of genomic DNAs.

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