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13. ABSTRACT *(Maximum 200 Words)*

The purpose of our research was to characterize the function of the novel gene Clar1 as it relates to the progression of human prostate cancer. The scope of this work included generating antibodies to Clar1, their use in characterizing the level of this protein in prostate cancer, examining the relationship of Clar1 protein expression to tumor progression, and determining the biological function of Clar1. We have successfully developed, tested, and utilized antibodies to the Clar1 protein to characterize Clar1 expression in prostate cancer tissues. We have characterized Clar1 expression in variety of human tissues using western analysis and demonstrated predominantly cytoplasmic staining of the protein by immunohistochemistry (IHC). We have also performed IHC analysis on tissue sections obtained from 23 different prostate tumors and we are currently completing IHC on an additional 27 samples. In the 23 samples analyzed we found that 65% of the prostate cancers exhibited some level of Clar1 expression. We have created Clar1 plasmid constructs for use as "bait" proteins to identify possible Clar1 binding partners. We employed a modified yeast two-hybrid, dual bait procedure that minimized non-specific protein-protein interactions. Using this method we have identified beta-tubulin 2 as a binding partner for the Clar1 SH3 domains, suggesting that Clar1 may participate as an adaptor protein at the cell surface, and participate in membrane modifications and/ or signal transduction.

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James V. Swede 3/29/01

PI - Signature Date

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INTRODUCTION

Recently, there has been a proliferation of novel genes asscoiated with prostate cancer initiation and progession and efforts are underway to validate these markers and determine their role in this disease. CLAR1 is a novel gene that was cloned in our laboratory using a differential display method that compared transcript expression patterns from early and late stage human prostate cancers (1). CLAR1 expresses two transcripts of 2.6 and 2.0 kb that are increased in prostate tumors of later pathologic stage and higher Gleason grade. These transcripts are also expressed to high levels in the PC-3, DU145, LNCaP and TSUPr-1 prostate cancer cell lines, with the predominant transcript being 2.0 kb. However, CLAR1 transcripts are also present in a variety of other human adult and fetal tissues and therefore it's expression is not prostate-specific. CLAR1 maps to chromosome 19q13.3 and is highly conserved among mammals. The gene encodes a 276 amino acid proline-rich protein of 30-32 kDa. The shorter 2.0 kb transcript encodes a truncated 256 amino acid version of the protein from a second in-frame methionine start site. The Clar1 protein contains several PXXP sites that are consensus sequences for binding SH3 domains. This suggests that Clar1 may be involved in signal transduction as an adaptor or an effector protein. Clar1 also contains a PPSSP consensus phosphorylation site suggesting its function could be regulated by phophorylation.

From this information we hypothesized that the Clar1 protein contributes to the progression of prostate cancer though enhanced expression and the participation in one or more signal transduction pathways. The purpose of this study funded by the DOD was to confirm and characterize the expression of Clar1 at the protein level in human prostate cancer and to begin to investigate it's potential function. To accomplish this we proposed to develop and test antibodies to the Clar1 protein and utilize them in western and immunohistochemical analysis of prostate tumors. This would allow us to determine whether the protein expression pattern mirrors the transcript expression pattern in these tumors. To determine the potential role of Clar1 in prostate tumor progression we proposed to use antisense oligonucleotdes to block expression in prostate cancer cell lines and observe the effects on tumor growth in *scid* mice. In order to elucidate the biological function of Clar1 we proposed using yeast two-hybrid analysis to identify specific binding partners for Clar1 that would provide clues to the signal transduction pathways it may participate in.

The following final report summarized the progress and accomplishments of the work supported by DOD grant DAMD17-98-1-8599 over the 30 month period funding period.

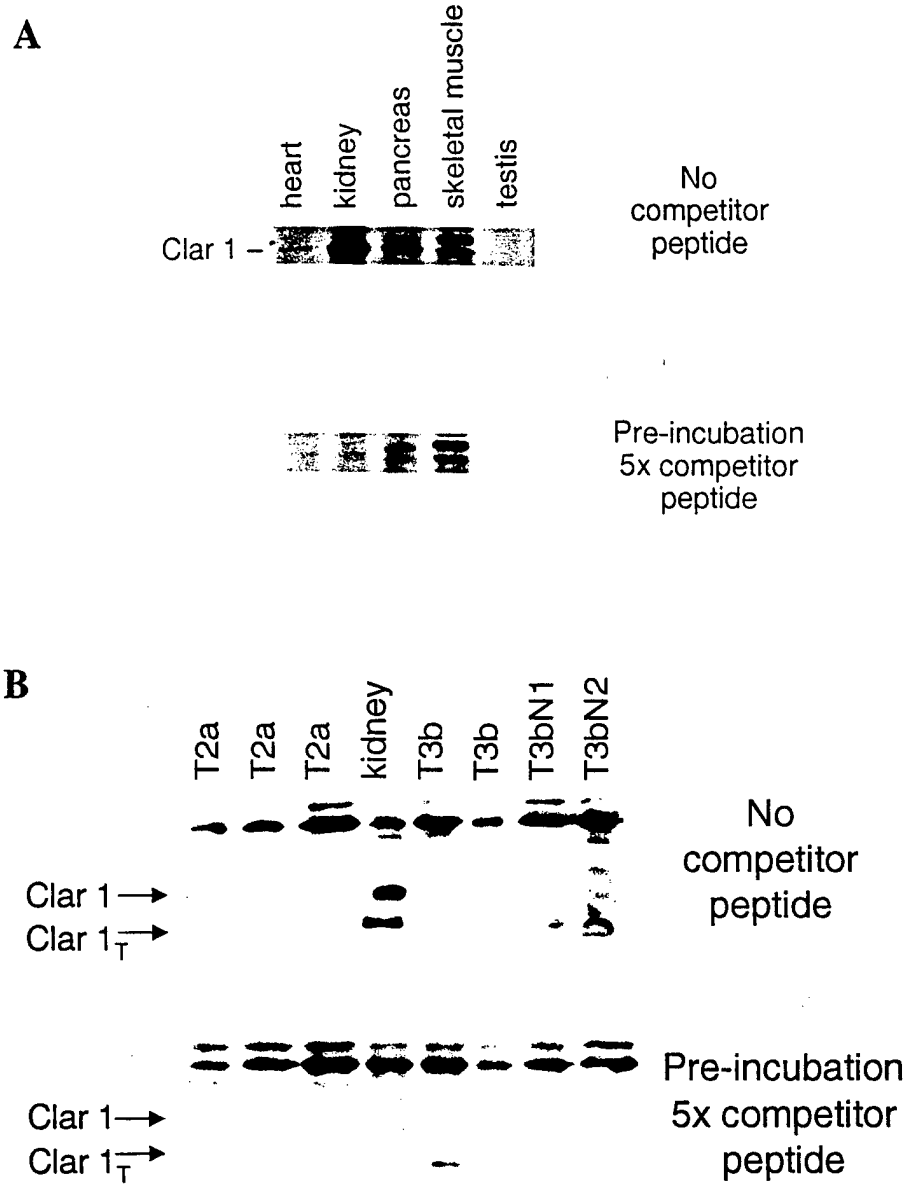

Figure 1: Western blot analysis of Clar1 expression in human tissues (A) and human prostate cancers (B). The western was performed at a 1:5000 dilution of Clar1 antibody in the absence (upper panels), or presence (lower panels) of a 5x competitor Clar1 peptide to antibody concentration.

REPORT BODY

Progress on Research Task 1: To determine the expression pattern of the Clar1 protein with regard to disease progression and cellular location in prostate cancer cells.

Characterization of Clar1 Transcript Expression in Prostate Carcinoma

Funding from this project allowed us to complete our analysis of Clar1 transcript expression in human prostate cancer by northern and quantitative reverse-transcriptase polymerase chain reaction. The results showed an increasing level of both the 2.6 and 2.0kb transcripts as a function of increasing pathologic stage and Gleason grade. This provided our initial link between Clar1 and prostate tumor progression and is included in one of our publications (1).

Production of Clar1 Antibodies

In order to conduct this task we had to first successfully develop and test antibodies to the Clar1 protein. The antibodies were raised in New Zealand white rabbits against a 15 amino acid peptide from a region in the N-terminal portion of Clar1. The purified antibody has a titer of 0.64 mg/ml and was tested at dilutions of 1:2500, 1:5000, and 1:10,000 by western analysis. Optimal results were obtained at a dilution of 1:5000. For each western analysis performed a control panel was run in which antibody was pre-incubated with a five-fold excess of competitor peptide. Initial studies using western analysis on whole tissue homogenates identified a 30-32 kDa protein that is most abundant in kidney and pancreas, with little expression in the heart, and no expression in the testis (Figure 1A). We also found significant expression of Clar1 protein in human liver, colon and spleen. In one kidney sample we detected a protein band of approximately 26-27 kDa that may correspond to the truncated form of Clar1 encoded by the 2.0 kb transcript. In all cases the bands corresponding to Clar1 protein did not appear when antibody was pre-incubated with competitor peptide. We examined homogenates of the prostate cancer cell lines PC-3, DU-145, LNCaP and TSU-Pr1 for Clar1 protein and found no detectable expression. These cell lines contain predominantly the 2.0 kb transcript, therefore the results suggest that this splice variant, for reasons we do not currently understand, is not always translated. When we analyzed 16 prostate cancer tissue homogenates by western to our surprise there was also little or no detectable Clar1 protein present (Figure 1B). One possible reason for this may be that only about 20% of the prostate is luminal epithelium. Thus, if only a portion of these epithelial cells were expressing Clar1 protein, and the protein was not being produced by the stroma, then we may not detect it in the whole tissue homogenates used on the westerns. In order to test this theory we utilized IHC to

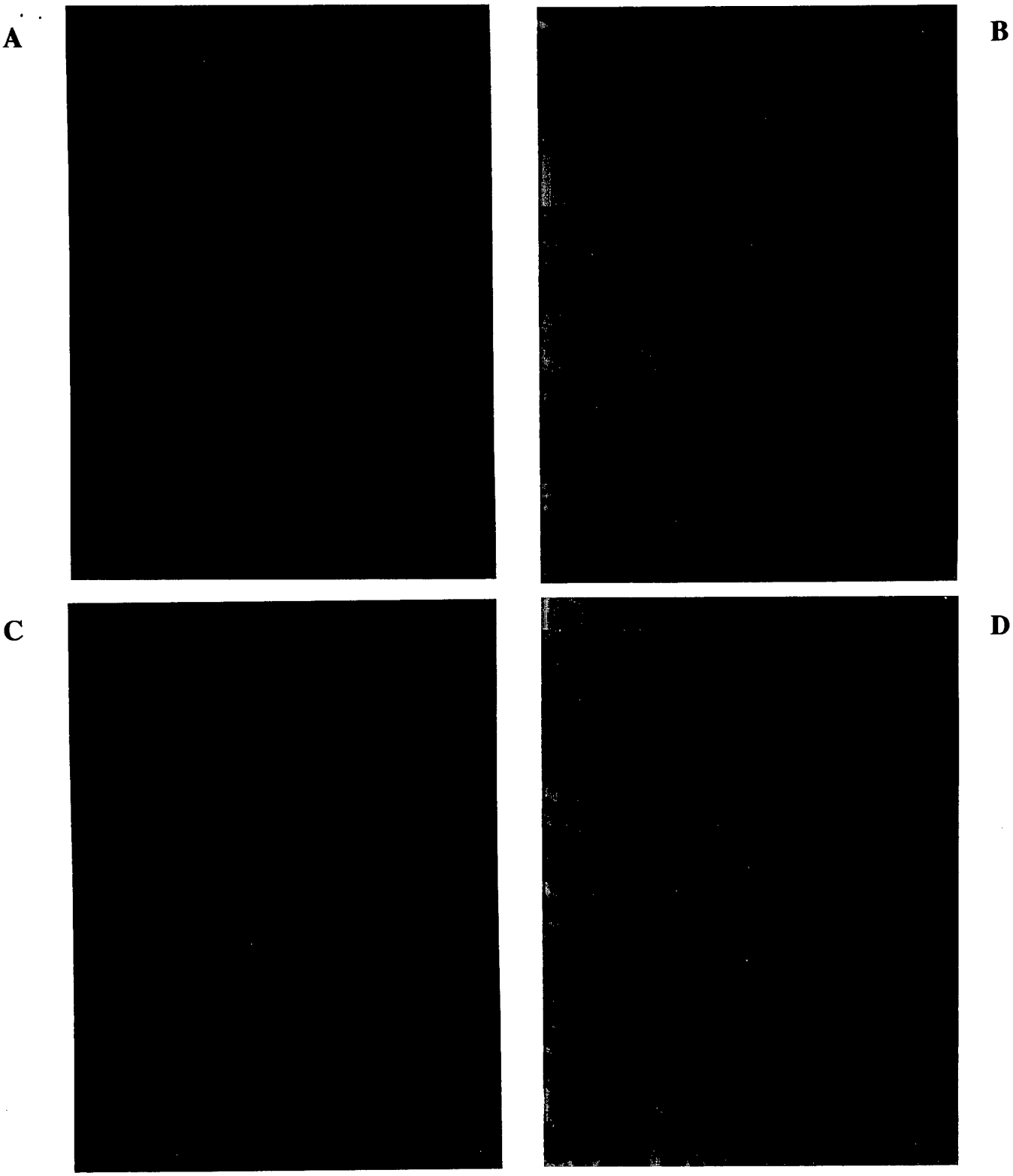

Figure 2: Immunohistochemical staining of prostate cancer tissues using Clar1 antibody. 2A and 2B, prostate cancer tissue from two different patients stained with Clar1 antibody (100x magnification); 2C and 2D, adjacent sections stained in an identical fashion except using rabbit IgG in place of Clar1 antibody (100X magnification). These served as negative controls and were performed on all samples analyzed.

examine Clar1 expression in human prostate tissue sections obtained from paraffin-embeded material.

Immunohistochemical Analysis

We began our immunohistochemical studies by examining the staining pattern and intensity of Clar1 in adult human kidney and liver tissues, since these gave robust signals on westerns. Therefore, we would predict that the Clar1 staining intensity would be high in these tissues. We found that both kidney epithelium and liver tissue showed intense staining, confirming our prediction that this tissue has abundant levels of Clar1 protein. Interestingly, the kidney glomeruli do not stain at all. We then proceeded to perform IHC analysis on tissue sections obtained from 49 different prostate tumors and detected staining in the malignant epithelium of the majority of these tumors. Each sample was scored using an established procedure that combined a percent positive glands score (0-5), and staining intensity score (0-3), to assign an immunoreactive score (0-8). The results of this analysis are shown in Table 1. Immunohistochemical staining in the 5uM prostate tissue sections revealed that the Clar1 protein is localized in the cytoplasm with no detectable nuclear staining, and no staining in the stroma (Figure 2). While too few prostate samples have been analyzed to date to draw any statistically significant correlations, several observations can be made. First, 65% of the prostate cancers in this group exhibit some level of Clar1 expression. The second is that of the 12 samples with an IRS of 6.5 or greater 7 were of stage T3a or greater and 7 were Gleason grade 7 or higher. These results support our observations at the transcript level that the Clar1 protein is more frequently expressed to greater levels in later stage and higher grade prostate tumors. This provides further support for our hypothesis that Clar1 is involved in some aspect of human prostate cancer progression. A manuscript describing this work is in preparation (2).

Progress on Research Task 2: To investigate the potential role of Clar1 in prostate tumor progression and to determine its biological function.

Yeast Two-Hybrid Analysis

We completed the construction of "bait" vectors containing Clar1 for use in the dual bait yeast two-hybrid system for the purpose of identifying binding factors to Clar1. This was accomplished using specific DNA primers to simultaneously amplify the SH3 binding region fragment of the Clar1 cDNA and incorporate EcoR1 and BamH1 restriction sites on either side of

the Clar1 fragment to allow directional cloning into the pEG202 vector. The primer also includes an in-frame stop codon. The constructs containing the LexA-Clar1/SH3 domain fusion protein were used to transform the yeast strain EGY48 that contains the *LEU2* and *lacz* reporter genes under the control of a LexA operator. We utilized a modified dual bait yeast two-hybrid method designed to minimize artifactual protein-protein interactions. We used a HeLa cell cDNA library to identify Clar1 binding partners since it had been used successfully in the past by our collaborators. We screened 48 clones for interaction with the LexA-Clar1/SH3 bait. Of these 34 were true positives containing an identifiable insert on PCR. The remaining 14 contained no insert sequence and thus were false positives. Sequencing of the positive cDNA clones revealed that all encoded regions of the beta-tubulin 2 gene. Based on these results we propose that Clar1 may be acting at the cell surface to influence membrane architecture and/or facilitate signal transduction pathways. There is a precedent for the interaction of SH3 target regions with elements of the cytoskeleton as exemplified by the protein dynamin, a 100k Da GTPase. Dynamin, like Clar1 has proline-rich sequences and target sequences for SH3 binding domain proteins. Studies have shown that dynamin can bind both microtubules and SH3 binding domains (PNAS 90: 11468-11472, 1993). Clar1 may act in a similar manner although we have no evidence for and enzymatic activity for this protein at this time. Future studies will utilize other cDNA libraries as a target for the Lex A-Clar1/SH3 bait protein. A manuscript describing these studies is in preparation.

Inhibition of Clar1 Expression Using Antisense Oligonucleotides

We stated in our 12 month progress report that our efforts at inhibiting Clar1 transcript expression in LNCaP cells using antisense oligonucleotides targeted at the first and second ATG sites were unsuccessful. In no case was inhibition of the Clar1 transcript or protein level observed. This may have been due to poor binding of the oligonucleotides to their targets or their rapid degradation despite the use of serum-free media and oligonucleotides protected by phosphorothioates. There is little of the 2.6kb transcript in LNCaP cells, which may explain the failure to detect a reduction in its intensity. However, why we did not see a reduction in the 2.0kb transcript in these experiments is still unknown. As an alternative to the antisense experiments we have decided to utilize a regulated expression system to express high levels of Clar1 in PC-3 cells and determine the effects on cell morphology and doubling times. PC-3 cells, like the other prostate cancer cell lines, contain high levels of the 2.0kb transcript, but very little of the 2.6kb full-length form. The Tet-Off system utilizes a hybrid protein known as a tetracycline-controlled transactivator that binds the TRE element in the pTRE expression plasmid and thereby activates transcription of the desired gene inserted into the pTRE plasmid from a CMV promotor. This

complex fails to form in the presence of either tetracycline (Tet) or doxycycline (Dox) resulting in the shut-down of gene expression. We have successfully transformed the Tet-Off plasmid into PC-3 cells and tested their response to Dox using a luciferase reporter construct. We are now ready to transfect the pTRE-Clar1 plasmid into these cells. However, the results of these experiments will not be known prior to the end of the phase I grant period.

CONCLUSIONS

The work accomplished during this phase I proposal was innovative in that it explored the biology of a novel gene associated with prostate cancer progression. Our work made important contributions by generating antibodies to Clar1, demonstrating differential immunostaining in prostate cancer tissues, and greater immunostaining in higher grade and later stage tumors. These results suggest that Clar1 protein may be useful as a clinical marker for prostate tumor pathology. Correlations with disease-free survival may also reveal a role for Clar1 in predicting disease course. The identification of beta-tubulin 2 as a potential binding partner for Clar1 is exciting and provides insights into the potential mechanism this protein may play in tumor progression. This work opens an entirely new set of experiments that can be performed to further elucidate the role of Clar1 at the cell surface. Unfortunately, we will be unable to pursue these studies since our request for phase II funding to support this work has been denied.

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- Cvetkovic, D. and Tricoli, J.V. (2001) Clar1 expression in advanced prostate cancer and interaction with beta-tubulin (manuscript in preparation).

- Development of Clar1 antibodies.

- Patent filed on the Clar1 cDNA sequence

PERSONNEL RECEIVING PAY

• James V. Tricoli, Ph.D.	Principal Investigator	40%
• Dusica Cvetkovic, M.D.	Postdoctoral	100%
• Tahseen Al-Saleem, M.D.	Pathologist	5%

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3. Cvetkovic, D., Serebriiskii, I, Kotova, E. and Tricoli, J.V. (2001) Clar1: Expression in prostate cancer and potential role in signal transduction. American Association for Cancer Research Meeting, New Orleans, LA.

Table 1

Clar1 Immunohistochemical Staining Scores in 49 Prostate Cancers

Sample	Tumor stage	Gleason grade	Age	PP	SI	IRS
1)	T4a	7	75	5	1.5	6.5
2)	T3b	7	58	5	1.5	6.5
3)	T2c	5	63	5	1	6.0
4)	T3a	8	50	0	0	0.0
5)	T3b	7	55	4	0.5	4.5
6)	T2a	4	65	4	0.5	4.5
7)	T3c	8	53	5	2	7.0
8)	T3a	6	69	5	1	6.0
9)	T3a	7	63	0	0	0.0
10)	T2b	9	69	0	0	0.0
11)	T3a	7	65	0	0	0.0
12)	T3c	7	56	2	0.5	2.5
13)	T2c	6	44	0	0	0.0
14)	T2c	6	68	0	0	0.0
15)	T3a	6	55	0	0	0.0
16)	T3a	7	68	3	1	4.0
17)	T3a	6	49	4	1	5.0
18)	T2c	7	63	5	1.5	6.5
19)	T3a	7	59	0	0	0.0
20)	T3a	6	64	5	1	6.0
21)	T3bN1	9	62	4	1.5	5.5
22)	T3a	6	67	5	1	6.0
23)	T3a	9	52	5	1.5	6.5
24)	T2b	6	49	0	0	0.0
25)	T2c	6	62	2	0.5	2.5
26)	T3a	6	65	0	0	0.0
27)	T3a	6	61	5	3	8.0
28)	T2c	6	54	2.5	1.5	4.0
29)	T3aN2	8	55	4	1.5	5.5
30)	T3b	7	59	4	0.5	4.5
31)	T3c	7	56	5	1	6.0
32)	T3a	6	71	0	0	0.0
33)	T3b	6	61	0	0	0.0
34)	T2b	6	53	0	0	0.0
35)	T1a	6	54	3	0.5	3.5
36)	T3a	6	55	4.5	2	6.5
37)	T2b	6	68	5	1.5	6.5
38)	T2a	6	61	5	2	7.0
39)	T2b	6	64	0	0	0.0
40)	T3a	6	62	4.5	1.5	6.0
41)	T2b	7	67	4	1	5.0
42)	T2a	7	51	4	1.5	5.5
43)	T2b	7	47	5	3	8.0
44)	T2b	6	59	5	2	7.0
45)	T3a	7	60	5	1.5	6.5
46)	T2b	6	57	4	1	5.0
47)	T1a	6	57	4	2	6.0
48)	T2b	6	49	0	0	0.0
49)	T2b	7	56	0	0	0.0

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CLAR1, a Novel Gene That Exhibits Enhanced Expression in Advanced Human Prostate Cancer[1]

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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 267β1, 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 human heart cDNA library. DNA sequence analysis confirmed that the cDNA was novel, and we have named this gene *CLAR1*. The gene displays two transcripts of 2.6 and 2.0 kb in all tissues examined. *CLAR1* maps to chromosome 19q13.3 and appears highly conserved among mammals. The deduced amino acid sequence of *CLAR1* encodes a proline-rich protein that contains several SH3-binding domains and a serine phosphorylation site. The presence of these motifs suggests a possible role for *CLAR1* in one or more signal transduction pathways. The enhanced expression of this novel gene in more advanced forms of prostate cancer and its potential role in signal transduction both argue that this gene should be further investigated.

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[3] 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, *CLAR1*, 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 *CLAR1* in four 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.

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[1] This work was supported in part by NIH Postdoctoral Training Grant CA09035 (to R. H. R.) and National Cancer Institute Public Service Grant CA57189 (to J. V. T.).

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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; TBE, Tris-borate EDTA; FISH, fluorescence *in situ* hybridization.

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 267β1 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 Preamplification 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′-GAACCAACCG-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% MetaPhor-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 Qiaex 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-alphaF′ competent cells (Invitrogen) were transformed with the TA vector-PCR fragment ligation 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′→3′, Boulder, CO), and the presence of the correct PCR fragment was verified by *Eco*RI (New England Biolabs, Beverly, MA) digestion and agarose gel electrophoresis. Clones containing the correct-size insert were sequenced by automated fluorescent sequencing. The marker fragment inserts were isolated from the sequenced plasmid clones by *Eco*RI digestion, 1% NuSieve-GTG agarose gel electrophoresis, and β-agarase I purification (New England Biolabs).

Northern Analysis of *CLAR1* Expression. Ten μg of DNase-treated total RNAs from stage B, C, and D prostate tumors were separated on denaturing formaldehyde-1% SeaKem LE agarose gels and transferred onto Maximum Strength Nytran (Schleicher & Schuell, Keene, NH). The 24 patient specimens were analyzed on three separate gels and were repeated several times. Random-primed *CLAR1* probe, β-actin, or desmin probes were labeled with [α-^{32}P]dCTP (Redivue, 3000 Ci/mmol; Amersham, Arlington Heights, IL) using a Multiprime DNA labeling system (Amersham). All probes were BioSpin-6 column-purified (Bio-Rad, Hercules, CA). The desmin cDNA was obtained from the American Type Culture Collection (Manassas, VA).

The three blots containing the human prostate tumor RNAs were hybridized sequentially with the ^{32}P-labeled probes to *CLAR1*, β-actin, and desmin in Rapid-Hyb buffer (Amersham). All three blots containing human prostate tumor specimens were incubated with the same probe preparation and hybridization solution to ensure that resultant phosphorimaging data could later be compared. Hybridization with *CLAR1*, β-actin, and desmin probes was performed at 65°C, followed by stringent washes in 2× SSC-0.1% SDS at ambient temperature for 15 min and then two washes in 0.2× SSC-0.1% SDS at 65°C for 15 min each. The blots were autoradiographed and scanned on a BAS 1000 phosphorimager (Fuji, Tokyo, Japan). Following each hybridization, the blots were washed in 55% formamide, 2× SSPE, and 1% SDS at 65°C for 1 h, followed by a wash in 1× SSC-0.1% SDS at 65°C for 15 min to remove bound probe.

Eight μg of DNase-treated total RNAs from the cell lines were separated on a denaturing formaldehyde-1% SeaKem LE agarose gel and transferred onto Maximum Strength Nytran (Schleicher & Schuell). The blot was hybridized sequentially with random-primed, ^{32}P-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, ^{32}P-labeled probes to *CLAR1* and β-actin and analyzed as described above. All three blots were hybridized simultaneously with the same batch of ^{32}P-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′-GGGCTCTTTGTGATGGATGAGG-3′; and *CLAR1* reverse, 5′-TTGGGAATGGGAGACGCAAG-3′) with 0.25 μM primer, 1× PCR Buffer II, 1.5 mM $MgCl_2$, 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, ^{32}P-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-^{32}P]ATP (Redivue, 5000 Ci/mmol; Amersham) using a 5′ DNA Terminus 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/mm^2 − background pixels/mm^2) by the corresponding β-actin or N-ras phosphorimaging data from each sample (pixels/mm^2 − background pixels/mm^2) 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 $P \leq 0.05$.

***CLAR1* cDNA Cloning.** The GeneTrapper oligonucleotide primer used to isolate *CLAR1* cDNA library clones was: 5′-dAAGGAGAAGAGGACAGAGG-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 multiprimed, ^{32}P-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).

***CLAR1* Gene Conservation.** Five μg of genomic DNA from human, cat, cow, dog, horse, mouse (BALB/c nude), pig, rat (Fisher), and yeast (*Schizosaccharomyces pombe*) were digested with *Eco*RI (Life Technologies, Inc.) and separated on a 0.8% agarose-1× TBE gel. The DNAs within the gel were denatured, neutralized, and transferred onto a MagnaCharge membrane (Micron Separations, Inc., Westborough, MA). The blot was hybridized for at 65°C with a random-primed, ^{32}P-labeled probe of *CLAR1*. The blot was washed twice in 2× SSC-0.1% SDS at an ambient temperature for 15 min and once in 0.2× SSC-0.1% SDS at 42°C for 15 min.

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 decamer 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 ^{32}P-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. 1*A*. 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. 1*B*, 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. 2*A* demonstrate that by RT-PCR *CLAR1* expression is

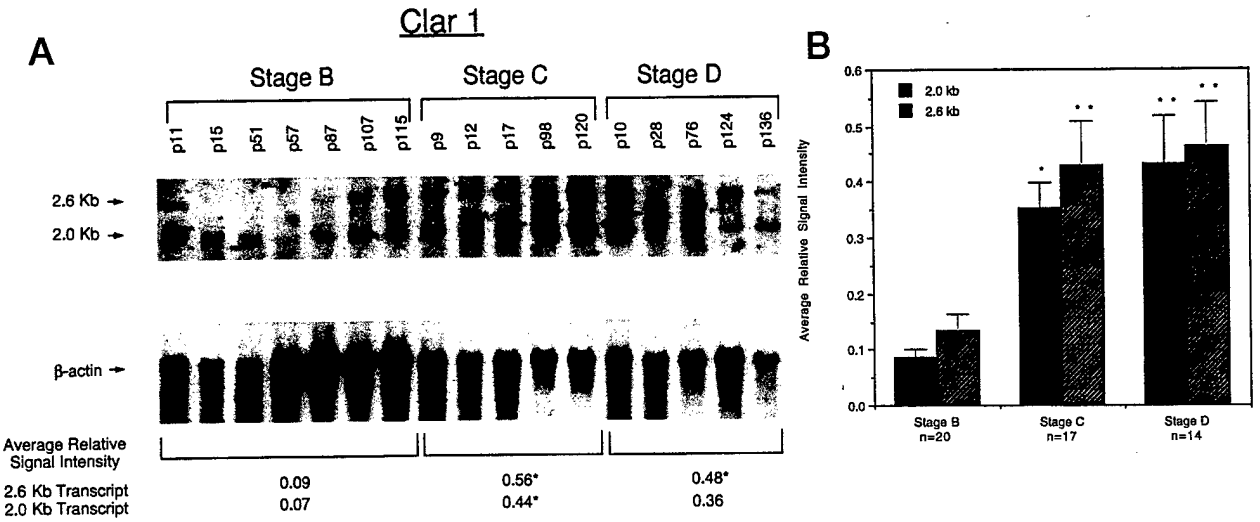


Fig. 1 Northern blot analysis of *CLAR1* expression in pathological stage B, C, and D primary human prostate tumors. *A*, a Northern blot containing total RNAs from stage B, C, and D prostate tumors was hybridized with ^{32}P-labeled probes to both *CLAR1* and β-actin. The two transcripts detected by the *CLAR1*-specific probe are indicated by the *arrows*. The average relative signal intensities of each detected transcript following β-actin normalization and phosphorimaging analysis are shown below each stage. *, statistically significant difference compared to stage B patients, $P \leq 0.05$. *B*, average relative signal intensities of *CLAR1* expression in 11 stage B, 8 stage C, and 5 stage D primary prostate tumors following β-actin normalization and phosphorimaging analysis of three independent northern analyses. *n*, total number of replicates observed. *, statistically significant increase in *CLAR1* transcripts in stage C and D tumors compared to stage B, $P \leq 0.01$. **, statistically significant increase in *CLAR1* transcripts in stage C and D tumors compared to stage B, $P \leq 0.001$.

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. 2*A* (data not shown). In Fig. 2*B*, 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 TSUPr1, DU145, LNCaP, and PC-3 cell lines as well as in FNC 267β1, an immortalized normal neonatal prostate cell line (19). We found that *CLAR1* was expressed to high levels in all of the prostate cancer cell lines examined (Fig. 3). *CLAR1* was expressed to similarly high levels in the neonatal prostate cells as well. Interestingly, all of these cell lines predominantly 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 ^{32}P-labeled probes to *CLAR1* and β-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 ^{32}P-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).

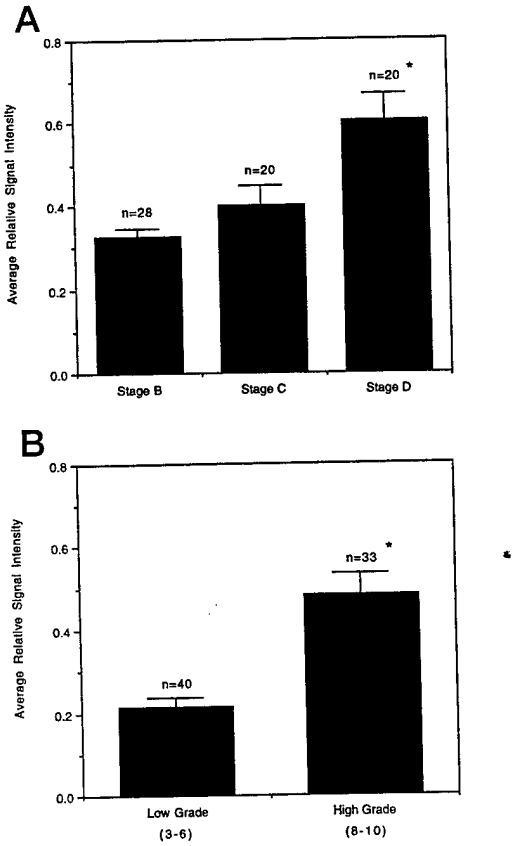


Fig. 2 Quantitative RT-PCR analysis of *CLAR1* in human primary prostate tumors of various pathological stages (*A*) and low and high Gleason grades (*B*). *A*, average relative *CLAR1* signal intensities following Southern analysis of CLAR1 and N-ras quantitative RT-PCR products in 17 primary human prostate tumors with regard to pathological stage on tumor samples. *B*, average relative *CLAR1* signal intensities following Southern analysis of CLAR1 and N-ras quantitative RT-PCR products in 20 primary human prostate tumors of low (3–6) and high (8–10) Gleason grades. *n*, total number of replicates observed. *, statistically significant increase in *CLAR1* transcripts in stage C and D tumors compared to stage B and in tumors of higher Gleason grade compared to those of lower grade, $P \leq 0.05$.

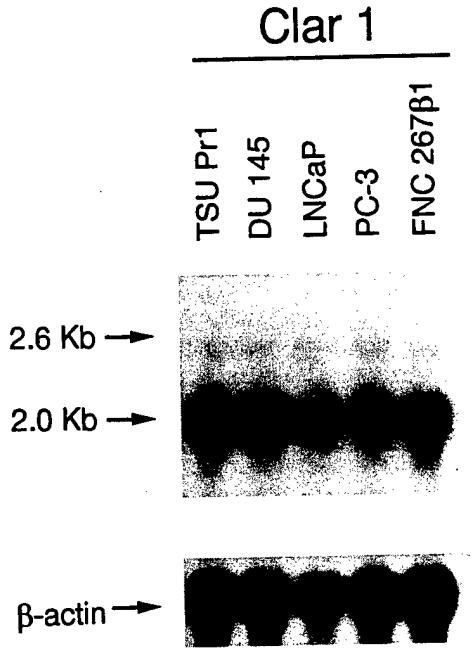


Fig. 3 Expression of *CLAR1* in human prostate cancer cell lines and a normal neonate prostate cell line. A Northern blot containing total RNA from exponentially growing cultures of TSU-Pr1, DU145, LNCaP, PC-3, and FNC 267β1 was hybridized with ^{32}P-labeled probes to both *CLAR1* and β-actin. The two transcripts detected by the *CLAR1*-specific probe are indicated by the *arrows*.

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. 5*A*). 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. 5*B*. 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 sequence (21) that is highly homologous to the 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. 5*A*). The two putative *CLAR1* splice variants predict an NH_2-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 kallikrein (*hGK-1* or *KLK2*), also map to this region of chromosome 19q (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 ^{32}P-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

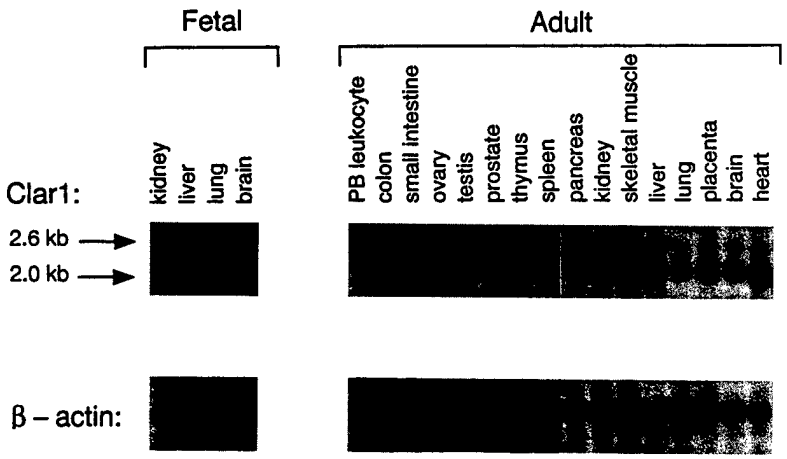
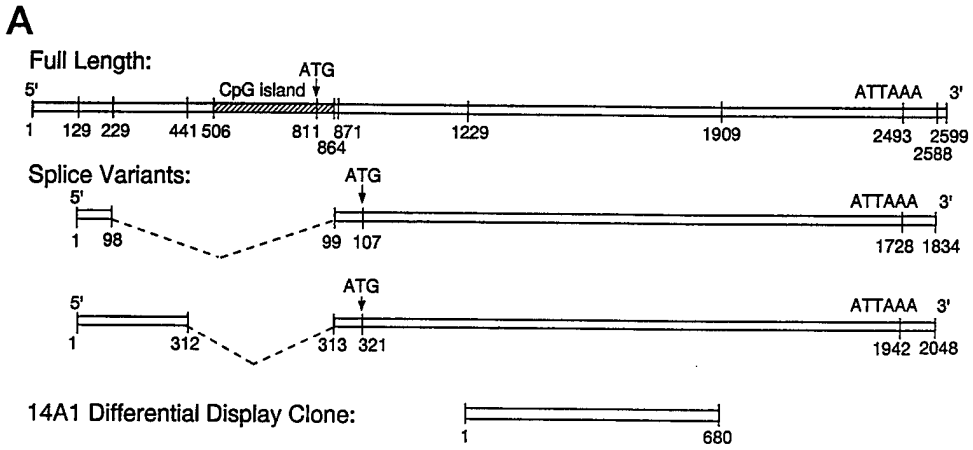


Fig. 4 Expression of *CLAR1* in human adult and fetal organs. Human multiple organ Northern blots (Clontech) that contain 2 μg of poly(A)$^+$ RNA from fetal brain, kidney, liver and lung and adult peripheral blood (*PB*) leukocyte, colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart. All three blots were hybridized and washed simultaneously with ^{32}P-labeled probes first for *CLAR1* and later for β-actin, so that transcript levels could be compared directly between the blots.



B

MSFEGGDGAG PAMLATGTAR MASGRPEELW EAVVGAAERF RARTGTELVL
LTAAPPPPPR PGPCAYAAHG RGALAEAARR CLHDIALAHR AATAARLPAP
PPAPQPPSPT PSPPRPTLAR EDNEEDEDEP TETETSGEQL GISDNGGLFV
MDEDATLQDL PPFCESDPES TDDGSLSEET PAGPPTCSVP PASALPTQQY
AKSLPVSVPV WGFKEKRTEA RSSDGENGPP *SSP*DLDRIAA SMRALVLREA
EDTQVFGDLP RPRLNTSDFQ KLKRKY

Fig. 5 Clar1 cDNA and Clar1 protein sequences. *A*, full-length Clar1 cDNA sequence and two Clar1 splice variant cDNAs were isolated from a human adult heart cDNA library using the GeneTrapper enrichment system (Life Technologies, Inc.). ▨, CpG island located within the full-length Clar1 cDNA. The position of the original Clar1 differential display clone (here designated 14A1) relative to the full-length and splice variant forms of *CLAR1* is shown. *B*, predicted Clar1 amino acid sequence was deduced from the full-length 2.6-kb cDNA sequence to *CLAR1* using the MacVector software package (Oxford Molecular Group, London, United Kingdom). *CLAR1* encodes a 276-amino acid, proline-rich protein that is expected to be ~33.8 kDa in molecular mass. The Clar1 protein contains several potential SH3 binding domains (*PXXP*), which are underlined, as well as a potential serine phosphorylation site (*PPSSP*), which is identified in *italics*.

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

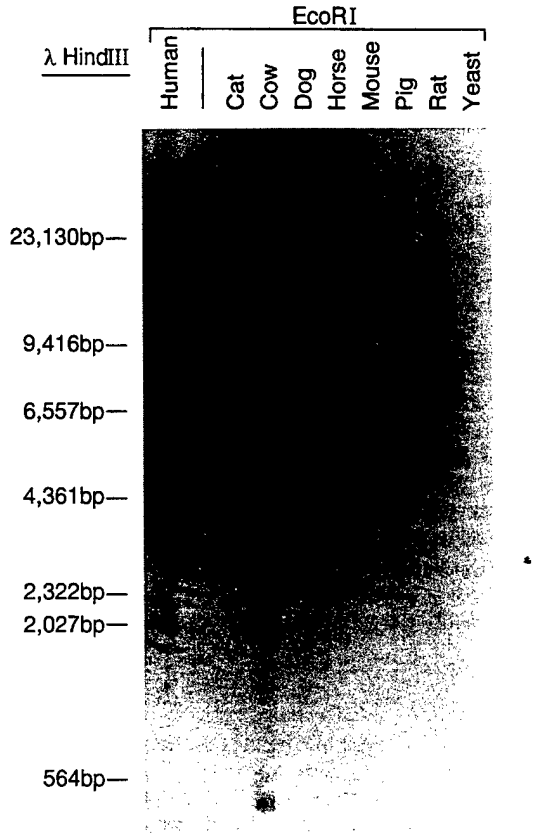


Fig. 6 Detection of *CLAR1* gene in other species. Genomic DNAs from human, cat, cow, dog, horse, mouse (BALB/c nude), pig, rat (Fisher), and yeast (*S. pombe*) were digested with *Eco*RI (Life Technologies, Inc.) and separated on a 0.8% agarose-1× TBE gel. The DNAs within the gel were denatured, neutralized, and transferred onto a MagnaCharge membrane (Micron Separations, Inc., Westborough, MA). The blot was hybridized with a random-primed, ^{32}P-labeled probe of *CLAR1*, washed, and autoradiographed for 18 h.

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 would be high in the metastasis-derived prostate cancer cell lines. As expected, all four human prostate cancer cell lines expressed robust levels of Clar1 transcript. The predominance of the 2.0-kb transcript in these cells could indicate a change in Clar1 transcript splicing efficiency and/or turnover rate that favors the shorter form. The expression of *CLAR1* in the neonate prostate cells is intriguing, with regard to the high proliferative nature of both fetal tissue and tumor cells. However, because these cells are transformed this may not reflect the actual expression pattern in the developing fetal prostate. Like e-cadherin/α-catenin and the metastasis suppressor gene *KAI1*, *CLAR1* is not expressed exclusively in the prostate and is detected in a variety of fetal and adult tissues including normal prostate (Fig. 4). The expression level of *CLAR1* in the prostate was moderately low compared to other normal adult tissues.

The Clar1 protein is proline rich (14%), a feature found in many proteins that are involved in protein-protein interactions and contains several PXXP sites which are consensus sequences for binding to SH3 domains (26–28). All high-affinity SH3 binding proteins contain this motif, and their binding specificity is conferred by the variable residues found within and flanking this consensus PXXP sequence. The presence of these sites in Clar1 suggests that the protein may function as a ligand for SH3 domain-containing proteins and could be involved in regulation or modification of these binding partners, many of which play significant roles within cytoskeletal localization and signal transduction pathways (29). In addition, the Clar1 protein contains a PPSSP site near its COOH-terminus that may be a potential site for serine phosphorylation by MAP kinases and cdc2 kinase (30). Therefore, *CLAR1* could potentially represent a new type or class of proteins that may be able to interact with SH3 domains and play a role in either cytoskeletal function or signal transduction. 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 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 and adult prostate 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.

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Clar1: Expression in Prostate Cancer and Potential Role in Signal Transduction. Dusica Cvetkovic, Ilya Serebriiskii, Elena Kotova and James V. Tricoli, *Fox Chase Cancer Center, Philadelphia, PA*

Clar1 is a novel gene that encodes a 34kDa protein containing multiple SH3-binding domains, suggesting that it may be involved in some aspect of signal transduction. Clar1 transcripts are expressed at higher levels in human prostate tumors of later pathological stage and higher Gleason grade. In order to determine the expression level of Clar1 protein in prostate tumors we have raised antibodies to Clar1. Western analysis demonstrates that Clar1 expression is most abundant in kidney and liver, with little or no detectable expression in prostate tumors or tumor cell lines. However, immunohistochemical analysis of prostate tumor tissue sections reveals nests of epithelial cells staining positive for Clar1. This staining is cytoplasmic, occurs predominantly in the epithelium, but does not occur in all malignant glands within a section. Quantitation of Clar1 staining reveals a positive correlation between the number of glands stained and prostate tumors of later stage and higher grade. This is consistent with our findings at the transcript level. Since Clar1 contains consensus sequences for SH3-binding, we are performing yeast two-hybrid analysis using a pEG202 "bait" vector containing 51 amino acids encompassing this region. We are using a HeLa cell cDNA library to identify clones that may interact with Clar1 in this system. The identity of any interacting clones and the possible role of Clar1 in signal transduction pathways will be discussed.

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