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Original Article

Evidence for the Novel Expression of Human Kallikrein-related Peptidase 3, Prostate-Specific Antigen, in the Brain

Jeremy G. Stone[1], Raj K. Rolston[1], Masumi Ueda[1], Hyoung-Gon Lee[1], Sandy L. Richardson[1], Rudy J. Castellani[2], George Perry[1, 3] and Mark A. Smith[1]

[1]Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA; [2]Department of Pathology, University of Maryland, Baltimore, Maryland, USA and [3]College of Sciences, University of Texas at San Antonio, San Antonio, Texas, USA

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Abstract: Human kallikrein-related peptidase 3 (hK3), also known as prostate-specific antigen (PSA), is a 33 kDa single chain glycoprotein belonging to the kallikrein family of serine proteases. With chymotrypsin-like enzymatic activity, hK3 is directly and indirectly involved in a number of diverse biological functions including male fertility, the regulation of cell proliferation, and the inhibition of angiogenesis. The gene encoding hK3, *hKLK3*, is located on chromosome 19 and its expression has been shown to be regulated by steroid hormones through androgen receptor-mediated transcription. hK3 was once thought to be exclusively expressed and secreted by prostatic epithelial cells, hence the initial name of prostate-specific antigen, but has since been isolated in several non-prostatic tissues and ongoing characterization of alternative splicing variants has found at least 13 distinct mRNA transcripts. The detection of hK3 in cerebrospinal fluid prompted the hypothesis that hK3 may be produced in the brain. To test this notion, in this study we used RT-PCR amplification of brain tissue total RNA and examined hK3 protein by immunohistochemical, and immunoblot analysis. RT-PCR revealed several hK3 mRNA transcripts in the brain. Confirming these findings, both immunohistochemical staining and western immunoblotting showed evidence for hK3 protein in neuronal cells. Taken together, our findings support the expression of hK3 in neuronal cells reinforcing the concept of hK3 as a ubiquitous protein with more multifarious biological activity than previously believed. Ongoing research seeks to elucidate the functional significance of hK3 in brain cells.
Key Words: hK3, immunocytochemistry, mRNA, neuron, PCR, prostate specific antigen, protease, protein

Introduction

Human kallikrein-related peptidase 3 (hK3), also known as prostate-specific antigen (PSA), is one of 15 serine proteases encoded within a 300-kb region of chromosome 19q13.3-4 [1], and its expression has shown to be primarily regulated by steroid hormones through androgen receptor-mediated transcription [2, 3]. hK3, a chymotrypsin-like enzyme [4], is directly or indirectly linked to several biological functions including male fertility [5], cell proliferation regulation [6], and inhibition of angiogenesis [7].

The European Bioinformatics Institute currently recognizes 13 alternatively spliced mRNA transcripts of the *hKLK3* gene and isolation of additional variants is ongoing [8-12]. Characterization of *hKLK3* splice variants and the alternative proteins they encode is incomplete [13], but their presence suggests a complex and diverse *in vivo* activity of hK3.

Originally only three members of the serine protease family known as kallikreins were recognized. Of these, *KLK1* is transcribed in the telomere to centromere orientation, while the other two (*KLK2*, *KLK3*) are transcribed in the opposite orientation [14]. Now, there are 15 known kallikrein proteins encoded by tandemly located genes on chromosome 19 [15]. Although this family of genes shares a great deal of homology at the DNA and mRNA level [16], and have been shown to be comparably regulated by steroid hormones

[17], they are differentially expressed throughout the body, including some in the brain. In this latter, high expression levels of *KLK5*, *KLK6* and *KLK8* were measured in adult brain, and *KLK8* isoforms were differentially expressed in adult and fetal brains [18]. Also, *KLK11* was found in hippocampus and cerebellum with two alternatively spliced forms, one brain specific and one prostate specific. At the mRNA level, 12 of 15 kallikreins (excluding *KLK3*, *KLK13* and *KLK15*) have been found to be expressed in the CNS, and several have been linked to Alzheimer disease, Parkinson's disease and multiple sclerosis [19].

Once thought to be produced and secreted exclusively by prostatic epithelial cells [20], hK3 has since been shown to be neither organ, tissue, nor gender specific [21, 22]. In fact, hK3 is detected in cerebrospinal fluid [23] where it was hypothesized that the protein may have originated from brain tissue. In partial support of this notion, hK3 expression was discovered and characterized in neuroblastoma cell lines SK-N-BE-2 and SK-N-MC [24]. However, it is unclear if hK3 is produced *in vivo* in the brain, and if so, from what cell types. To address this issue, in this study we evaluated the presence of hK3 mRNA and protein in brain tissue by qualitative RT-PCR and immunohistochemical analyses.

Materials and Methods

Tissues

Using an approved IRB protocol, hippocampal or cortical tissue samples were obtained at autopsy (n = 8, ages 42-87, mean = 69) with post-mortem intervals (3.6-24 h, mean = 14) from the Alzheimer Disease Research Center at Case Western Reserve University and from the Cuyahoga County Coroner (See **Table 1** for details). As a positive control, surgical prostate tissue was also obtained (n = 3, ages 64-84, mean = 74).

For immunocytochemistry, brain samples were fixed in either routine formalin or methacarn (methanol; chloroform; acetic acid; 6:3:1 v/v/v) at 4°C overnight. Following fixation, tissue was dehydrated through ascending ethanol, embedded in paraffin, and 5 µm sections were cut.

RNA Extraction and RT-PCR

RNA extraction and RT-PCR was performed as previously described [25] with modifications. Briefly, total RNA was extracted from frozen cortex, hippocampus, and prostate tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the instructions of the manufacturer. RNA was treated with DNase (Turbo DNase, Ambion, Foster City, CA) to remove trace DNA. cDNA was amplified from 100 ng total RNA using the RetroScript RT-PCR kit (Ambion). Initial mRNA concentrations were kept constant through preliminary assessment with a NanoDrop ND-1000 spectrophotometer. Amplification of the cDNA was performed in a DNA thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler). An initial denaturation step (95°C for 5 min) was followed by 32 cycles (95°C for 30s, 57°C for 50s, 72°C for 60s) and a final extension period (72°C for 5 min). The hK3 mRNA was amplified in a 50 µl

Table 1 Information on brain and prostate tissue used in this study

Case No	Tissue Type	Diagnosis	Age (yr)	PMI (hr)	Sex
1	Prostate	No evidence of malignancy	84	NA	M
2	Hippocampus	Diffuse Lewy body disease	70	10	M
3	Cortex	Alzheimer disease	81	3.6	F
4	Cortex	Lupus, pulmonary thrombosis, congestive heart failure	56	9	F
5	Cortex	Non-metastatic prostatic adenocarcinoma	87	21	M
6	Hippocampus	Alzheimer disease, diffuse Lewy body disease	74	4.5	M
7	Cortex	Bronchopneumonia, hypertensive and vascular disease, carcinoma	79	24	M
8	Cortex	Accident	42	16	M
9	Hippocampus	Accident	64	24	M

reaction containing 2.5 U of Taq DNA polymerase (Roche, Indianapolis, IN), 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotide triphosphates, and 200 nmol/L of each primer. Positive control PCR was completed with primers targeted to the constitutively expressed β-actin gene (forward: 5'-gctcgtcgtcgacaacggctc-3', and reverse: 5'-caaacatgatctgggtcacctt-3') to assess the quality of isolated total RNA. hK3 primers, forward: 5'- gaggagttcttgaccccaaag -3', and reverse: 5'-tatttccaatgacgtgtgtgcg-3' yielded a 69 bp sequence as expected. Negative controls were run omitting reverse transcriptase during reverse transcription. PCR products were run on a 1.7% agarose gel stained with ethidium bromide.

Immunocytochemistry

Immunocytochemistry was performed as described previously [26] with modifications. Briefly, tissue sections were deparaffinized in xylene, hydrated through descending ethanol, and endogenous peroxidase activity was quenched by 30 minute incubation in 3% hydrogen peroxide in methanol. Non-specific binding sites were blocked with 30 minute incubation in 10% normal goat serum (NGS) in Tris buffered saline (50 mM Tris, 150 mM NaCL, pH = 7.6), then overnight at 4°C with primary antibodies. 1:100 dilutions of rabbit polyclonal antibody to hK3 and mouse monoclonal to hK3 (A0562, and M0750 respectively, Dako, Carpinteria, CA) were utilized. Following incubation with species specific secondary antibodies and peroxidase anti-peroxidase complexes (Jackson ImmunoResearch Laboratories, West Grove, PA), the antibodies were detected with 3, 3'-diaminobenzidine (DAB, Dako, Carpinteria, CA), as the chromogen. As a negative control,

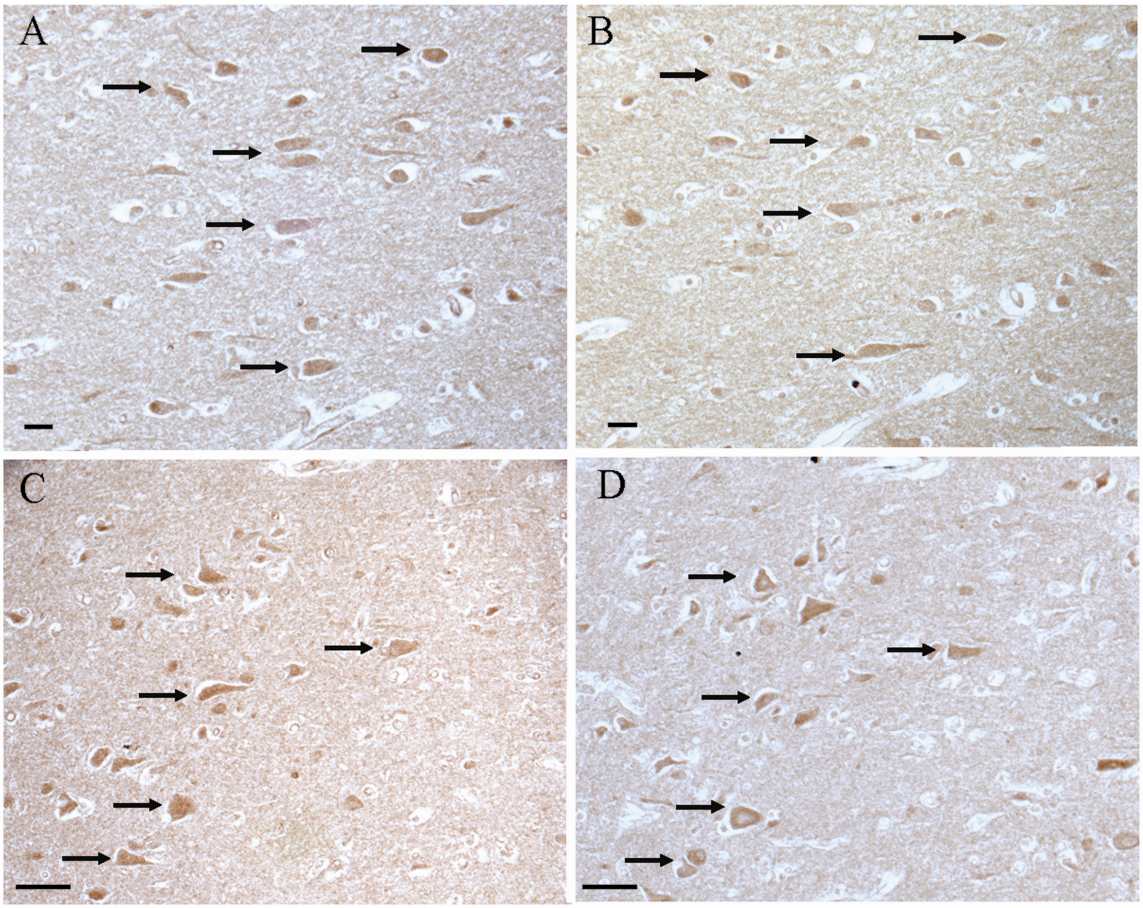

Figure 1 Adjacent tissue sections from different brain tissue cases (**A**, **B** and **C**, **D**) showing positive neuronal staining with rabbit polyclonal antibody to hK3 (**A**, **C**) and mouse monoclonal antibody to hK3 (**B**, **D**). Arrows highlight the similar staining pattern revealed by both antibodies and scale bars represent 20 μm (**A**, **B**) and 50 μm (**C**, **D**).

the protocol was replicated but without the primary antibody.

Immunoblotting

Western immunoblotting was carried out as previously described [27]. In brief, brain tissue was homogenized in 10 volume of lysis buffer [50mM Tris-HCl (pH 7.6), 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 2 mg/mL antipain, and 1 mM sodium orthovanadate], and centrifuged at 11,745 g for 10 minutes at 4°C. Protein concentrations of each sample's supernatant were assessed by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Separation of proteins was completed by SDS-polyacrylamide gel electrophoresis (20 µg/lane), and subsequently, proteins were electroblotted onto polyvinylidene difluoride membrane as previously described [28]. Transferred blots were incubated in sequence with blocking agent (10% nonfat milk in Tris-buffered saline solution with Tween 20), mouse monoclonal antibody to hK3, and affinity purified goat anti-mouse immunoglobulin peroxidase conjugate preabsorbed to eliminate human cross-reactivity. Development of blots was completed by the enhanced chemiluminescence technique (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions.

Results

Immunocytochemistry

In all cases analyzed, marked hK3 immunostaining was detected in hippocampal pyramidal neurons (**Figure 1**). In support of the specificity of our findings, labeling was seen in a similar pattern with both rabbit polyclonal (**Figure 1A**, **C**) and mouse monoclonal antibodies to hK3 (**Figure 1B**, **D**). Omission of the primary antibody revealed only light background reaction (data not shown). Notably, staining was absent in microglia and other differentiated brain cells, only showing presence in neuronal cells. While slight differences in neuronal labeling were found among the different cases analyzed, no qualitative differences were observed as a function of age, post mortem interval, disease state, or gender. Hallmark pathological markers of Alzheimer disease including neurofibrillary tangles, amyloid plaques, neuropil threads, and dystrophic neurites were not labeled in immunostained AD cases. Moreover, Lewy body dementia cases showed no labeling of Lewy neurites or Lewy bodies.

Western blot analysis of brain homogenates confirmed the presence of hK3 in the brain (**Figure 2**). Further, only one robust band was revealed at 33 kD (the expected molecular weight of hK3), again indicating high antibody specificity to hK3.

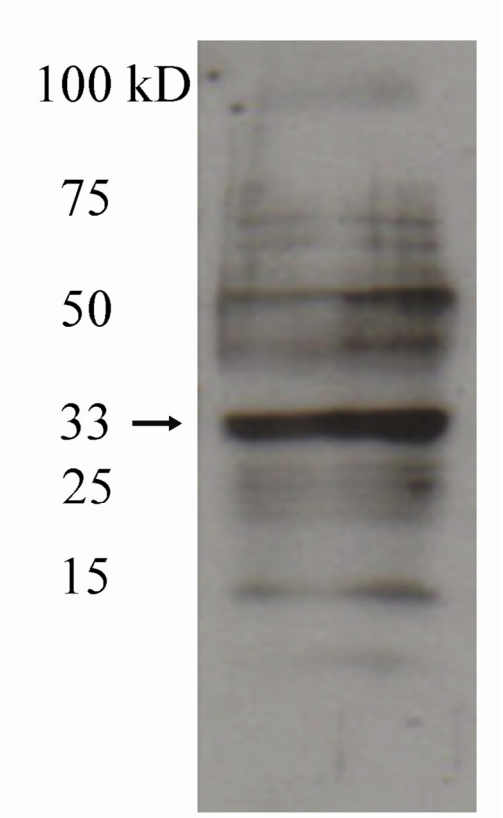

Figure 2 Presence of hK3 in one of the brain protein homogenates analyzed by western blot.

mRNA RT-PCR

RT-PCR showed 69 and 212 bp bands in prostate cDNA (case 1, **Table 1**), a 69 bp fragment in four brain cases analyzed (cases 3, 4, 5, and 8, **Table 1**), and as well alternative transcripts in five of the cases (**Figure 3**). The alternative fragments, detected in cases 2, 4, 6, 7 and 9, were located at about 40, 60, and 100 bp (**Figure 3**). Negative controls consistently produced no bands, and positive

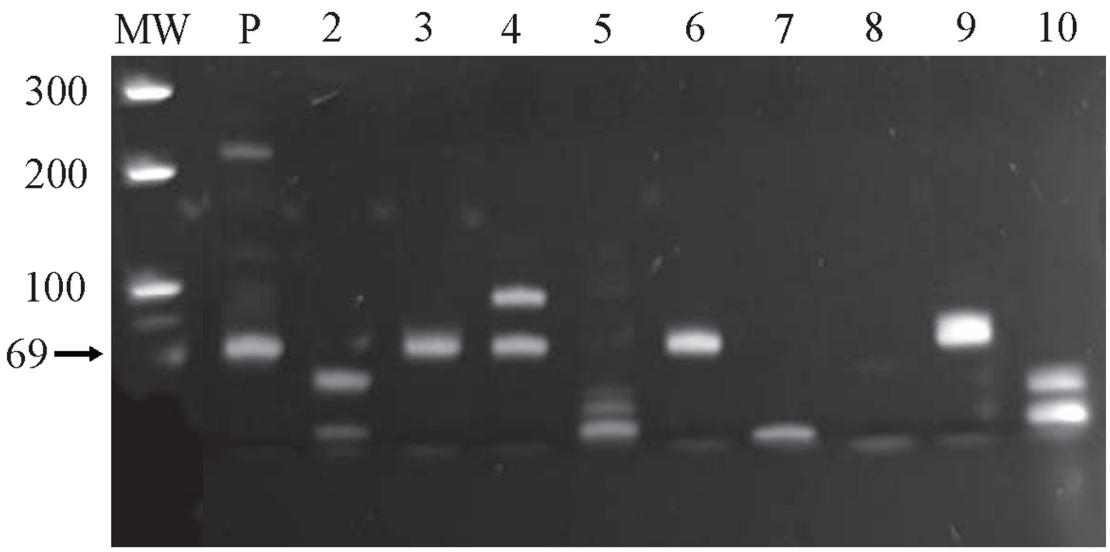

Figure 3 Ethidium bromide stained 1.7% agarose gel showing hK3 RT-PCR products from brain. Lane MW is a 1 kb plus DNA ladder; lane P is prostate sample for positive control (case 1, **Table 1**), subsequent lane numbers correspond to brain tissue cases in **Table 1**.

control PCR with β-actin primers revealed uniform bands of expected size in all cases (data not shown).

Discussion

The goal of this investigation was to determine whether hK3 mRNA and protein were expressed in brain tissue. Immunohistochemical staining of brain tissue sections showed positive detection of hK3 in neurons. We are confident the antibodies utilized did not demonstrate cross-reactivity with non-specific sites because similar staining was observed with both the monoclonal and polyclonal antibodies to hK3, and western immunoblotting of brain protein homogenate revealed one robust band at the 33 kD molecular weight of hK3.

While immunohistochemical staining detected hK3 protein in a similar pattern in all cases analyzed, RT-PCR analysis revealed more varied results with respect to number and types of mRNA transcript variants in particular brain cases. Originally, our PCR primers were designed to amplify only a 69 bp transcript from a region highly conserved between the various hK3 splice variants. Following RT-PCR amplification, closer scrutiny of the 13 alternative hK3 mRNA transcripts accepted by the European Bioinformatics Institute and analysis of the annealing ability of the primers [29] revealed that the utilized primers had the capacity to amplify sequences of varying length depending on which particular mRNA transcript was present in solution. **Table 2** reveals the binding position(s) of the primers on each hK3 transcript variant and the length of the amplified transcripts that would result. The analysis presented in **Table 2** accounts for all bands seen in **Figure 2** except the band around 100 bp in case 6. Since discovery and characterization of alternative hK3 mRNA transcripts are ongoing, this 100 bp band may present a novel transcript variant yet to be sequenced or, alternatively, may represent an incompletely spliced transcript.

Although RT-PCR analysis revealed a great deal of variability, it is important to note that hK3 transcripts were present in all cases analyzed which correlates with our immunohistochemical findings. Limited sample size makes demonstrating statistical significance difficult, but an interesting trend was seen by PCR. Specifically, all hippocampal tissue samples possessed transcripts which amplify the smaller products shown in **Figure 2** (transcripts 6, 9, and 10), while four of five cortical tissue samples only contained transcripts which amplify the 69 bp sequence (transcripts 1-5, 7, and 8). The incidence of region specific differences in gene products may be a result of differential transcriptional regulation and have a significant impact on

Table 2 Binding sites of primers used on each EBI hK3 splice variant and corresponding length of amplified transcripts

EBI Alternative Splice Database Pattern ID	Total mRNA Transcript Length (bp)	hK3 Forward Primer Binding Region(s)	hK3 Reverse Primer Binding Region	Amplified Sequence(s) Length (bp)
ENSG142515-SP1	990	528-548	575-596	69
ENSG142515-SP2	1130	950-970	997-1018	69
ENSG142515-SP3	778	383-403	430-451	69
ENSG142515-SP4	922	364-384	411-432	69
ENSG142515-SP5	1653	475-495	522-543	69
ENSG142515-SP6	1599	522-528, 672-685	712-733	62, 164, 212
ENSG142515-SP7	1035	508-528	555-576	69
ENSG142515-SP8	1115	936-956	983-1004	69
ENSG142515-SP9	817	442-448, 467-480	507-528	39, 62, 87
ENSG142515-SP10	586	498-504, 523-536	563-584	39, 62, 87
ENSG142515-SP11	704	542-548	None	None
ENSG142515-SP12	440	434-440	None	None
ENSG142515-SP13	417	411-417	None	None

biological function of resulting protein products. Ongoing experiments with greater sample size and primers designed against all of the currently sequenced transcripts to be transcript specific may reveal more about differences in brain site gene products of hK3.

The discovery of hK3 in brain tissue joins the growing body of evidence detecting hK3 in nonprostatic tissues and may explain the presence of hK3 in cerebrospinal fluid [23]. The majority of research on the biological functions of hK3 has focused on the role of hK3 in seminal plasma due to its relatively high concentrations in semen [30-32]. These investigations have found that hK3 quickly cleaves seminogelins I, II, and fibronectin [33] resulting in the liquefaction of seminal fluid following ejaculation. Although this appears to be the main function of hK3 *in vivo*, several other potential substrates hydrolyzable by hK3 have been revealed, including TGF-β, basement membrane, IGFBP-3, plasminogen, and parathyroid hormone-related peptide [34-37]. Several other members of the kallikrein gene family, which are significantly homologous to hK3, have been implicated in a number of biological activities in the CNS, including myelination and turnover of myelin proteins (hK6) [38], hippocampal plasticity [39], and neurite outgrowth during CNS development (hK8) [40]. The physiological relevance of hK3 in the CNS, presence of potential substrates, and possible role in pathological conditions are unclear at present. Nonetheless, taken together, our findings present many potential directions for future research including, the mode of expression regulation, possible differential distribution in discrete brain regions, enzymatic specificity, potential brain specific substrates, and relative expression levels in control vs disease states. Investigations currently in progress seek to elucidate the biological significance of hK3 in brain-derived cell lines as well as *in vivo*.

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Please address all correspondences to Mark A. Smith, Ph.D., Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, Ohio 44106, USA. Tel: 216-368-3670; Fax: 216-368-8964; Email: mark.smith@case.edu

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