Expression and clinical importance of a newly discovered alternative splice variant of the gene for acrosin binding protein found in human brain tumors

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Abstract

Background: Acrosin binding protein (ACRBP) is a member of the cancer–testis antigen (CTA) family. Normally, ACRBP mRNA is expressed only in seminiferous tubules, while abnormally it is expressed in various types of cancers in tumor tissues, such as brain tumor.

Objectives: To determine the expression and clinical impact of a newly discovered splice variant of ACRBP in brain tumor.

Methods: Total RNA was extracted and reverse transcribed from 92 tumor specimens and 3 cell lines. Primers were designed to determine the expression of the new splice variant in all the samples. Quantitative real-time PCR (qPCR) was conducted for samples positive in reverse transcriptase-PCR. Association of the expression of ACRBP with the clinicopathological features of the various brain tumors was assessed statistically.

Results: The primers identified a newly discovered splice variant of ACRBP named ACRBP-V5a. The proportions of samples of the various brain tumor types positive for the ACRBP-V5a splicing variant were as follows: astrocytoma 10/33 (30%), glioblastoma 10/30 (33%), medulloblastoma 14/29 (48%), all tumors 34/92 (37%). Although we did not find a significant difference in the proportions of samples of various types of brain tumor tissues positive for the new splice variant (P > 0.05), levels of expression of the ACRBP-V5a splice variant were significantly different for tumor grade (P = 0.01) and tumor type (P = 0.02).

Conclusions: A newly discovered splice variant, ACRBP-V5a, is present in brain tumor. The new splicing variant may have discriminative value and potential importance in molecular-targeted therapy for brain tumors.

Keywords: ACRBP protein, human; alternative splicing; antigens, neoplasm; brain neoplasms; cancer–testis antigen, human

The gene encoding acrosin binding protein (ACRBP) is also known as CT23; SP32 and OY-TES-1 is located on chromosome 12p12-p13, and includes 10 exons [1]. ACRBP is a precursor and intermediate binding protein of serine protease, which is specifically located in the acrosome of germ cells and sperm [2, 3]. ACRBP was asserted as a Pem (placenta and embryos) expression gene and functions by binding to proacrosin to package and condense the acrosin zymogen in the
acrosomal matrix [4]. As an additional member of the cancer–testis antigen (CTA) family, it became known as a human homolog of the precursor of human myosin binding protein SP32 [3–5]. The expression profile of ACRBP shows that the protein has the characteristics of a CTA and causes a strong immune response in some cancer patients [1]. For example, the mRNA of ACRBP is highly expressed in gliomas and polyclonal antibody for the ACRBP antigen was found in the serum of 5/36 (14%) of patients with glioma, but absent in all the serum samples from 107 healthy donors [6]. ACRBP is rarely expressed in normal adult tissues (except, of course, in testis tissue) [1].

Alternative splicing offers the largest potential for molecular diversity and controlled regulation in the cell [7]. Alternative splicing events may occur in up to 50% of human genes, leading to the enormous variety of potential coding regions of the genome [8]. Splicing events can lead to faults and can cause various diseases, including cancers [9]. Some alterations are relevant to tumor progression, metastasis, therapy resistance, and oncogenic processes [10].

A 55 kDa porcine proacrosin precursor protein of ACRBP is modified posttranslationally to produce a 32 kDa mature form [2]. In mice, 2 different types of ACRBP are identified: wild-type ACRBP-W and variant ACRBP-V5. Investigators have found that ACRBP-W and ACRBP-V5 presented and played a role in the process of acrosome formation in mice. At first, ACRBP-V5 controls the formation and configuration of the acrosomal granule; subsequently, ACRBP-W maintains the inactivation of proacrosin [11].

To our knowledge, alternative splicing in human ACRBP has not yet been reported. Therefore, our study sought to determine whether there was alternative splicing of ACRBP, thus ACRBP-V5a, in human brain tumors.

Methods

Specimens and cells

After the use of human tissue for this study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (approval No. KY-E-011, January 13, 2018) and after patients included in this study signed written informed consent forms, we obtained 92 surgical tissue specimens (33 primary astrocytomas, 30 glioblastomas, and 29 medulloblastomas) from the patients. All samples were preserved in liquid N\textsubscript{2}. This study was conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its contemporary amendments. All patients underwent primary tumor resection and had no radiotherapy or chemotherapy before their surgery. Stable human cell lines of glioma (SHG-44) and liver cancers (hep G2 and SMMC7721) were cultured with Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal bovine serum of premium quality (Wisent, Canada).

cDNA primer design

The sequences of splicing variants of ACRBP (NCBI Gene ID: 84519) were predicted by using alternative splicing sequence databases from Homo sapiens, sourced from NCBI Genome (http://www.ncbi.nlm.nih.gov/genome/), the Ensembl Alternative Database (http://asia.ensembl.org/), and TCGA SpliceSeq Database (http://projects.insilico.us.com/TCGASpliceSeq/index.jsp). Primers for ACRBP-V5a (ENST00000536350.5) were designed using Primer Premier (version 5.0, Premier Biosoft) and aligned using BLAST (https://blast.ncbi.nlm.nih.gov/Blast/). Forward primer 5′-TGAAGTCTCACCCACCACGATG-3′ and reverse primer 5′-GCTAGGAAAATGGGCTTCTCA-3′ were designed to amplify a 600-bp segment of ACRBP-V5a, between exons 4 and 5a (Figure 1), and synthesized by Sangon Biotech Co. (Shanghai, China).

RNA and cDNA preparation

All samples were handled in a frozen state. Total RNA was extracted using a FastPure Cell/Tissue Total RNA Isolation Mini Kit (Vazyme Biotech Co., Nanjing, China). cDNA was prepared by reverse transcription of 1 μg total RNA (HiScript II 1st Strand cDNA Synthesis Kit; Vazyme Biotech Co.), following the instruction manual from the manufacturer. RNA integrity was confirmed spectrophotometrically (Tiangen Biotech Co., China). All experimental materials and reagents were RNA-free.

RT-PCR

Reverse-transcribed cDNA was amplified with the designed primers using a RT-PCR reverse transcription kit (Eppendorf) using the following cycling parameters: 1 min at 94 °C; 1 min at 59 °C; 2 min at 72 °C for 35 cycles; and a final extension at 72 °C for 8 min [12]. A positive-control experiment was performed using the same primers for the human liver cancer cell line, SMMC7721 (Figure 2). The cell line fragment was subcloned and sequenced by Sangon Biotech Co. (China) to confirm its identity (Figure 3). Another experiment was performed to identify the quality of cDNA with
a pair of primers for the gene for human glyceraldehyde 3-phosphate dehydrogenase, \textit{GAPDH}, 5′-CAAGGTCATC
-CATGACAACTTTG-3′ and 5′-GTCCACCACCCTGTTG
-CTGTAG-3′, using the following cycling parameters: 30 s at
94 °C; 30 s at 55 °C; 1 min at 72 °C for 30 cycles; and a final
extension at 72 °C for 8 min (Figure 2). All PCR products
were examined by electrophoresis (Bio-Rad Laboratories) on
2% agarose gels (7 cm × 10 cm) in 1× Tris/borate/EDTA
(TBE) buffer at 100 V for 30 min and observed using a Gel
Documentation and Analysis system (Alpha Innotech). DNA
Zheng markers (catalog Nos. MD101-01 and MD104-01) were from Vazyme Biotech Co.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was run with 34 positive samples and the SMMC-7721 cell line (positive control) using a ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech) following the instructions provided by the manufacturer. Thermocycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Experiments were run in triplicate, and the average value of the quantification cycle (Cq) was calculated for ACRBP-V5a, ACRBP, GAPDH (reference gene), and the No Template Control (NTC), respectively. qPCR efficiency was about 90%–100%. Linear dynamic range (LDR) was about 10^5–10^10 DNA copies/mL, and R^2 was >0.99. The limit of detection (LOD) was >10^3. Details of primer sequences and amplicons are presented in Figures 1 and 4. The resulting relative increases in reporter SYBR Green I emission were analyzed using LightCycler 480 software (Roche).

Statistical analyses

The data were analyzed using IBM SPSS Statistics for Windows (version 20.0) and the results are described as percentages or mean ± SE (standard error). A χ^2 test was used to determine the differences in the number of mRNA positive samples and clinicopathological categories of sex, age, or Karnofsky Performance Status, and one-way analysis of variance (ANOVA) was used to determine any difference for the various tumor types. Student t tests were used to determine differences in the levels of expression between the various clinicopathological categories and a Kruskal–Wallis test was used to determine differences between the various tumor types. A Mann–Whitney U test was used to determine whether there was a significant association between the ratio of ACRBP to ACRBP-V5a for each sample and tumor type. P < 0.05 was considered significant.

Results

Identification and cloning of the novel splicing variant of human ACRBP retaining the intron

A new splicing variant of ACRBP, named ACRBP-V5a, was cloned from the cDNA of human brain tumor specimens and liver cancer cell lines. Gel electrophoresis of RT-PCR products amplified using the newly designed primers according to the special fragment and cDNA from the cell line as template were used to identify ACRBP-V5a. Sequence analysis shows that ACRBP-V5a originated from partial splice of intron 5 and ended the expression of the next 5 exons compared with the full-length cDNA. The predicted amino acid (AA) and ACRBP-V5a and the full-length gene were compared (Figure 1).

ACRP-V5a expression in the 3 types of brain tumors

To assess the presence of ACRBP-V5a mRNA in the tumor tissue samples, RT-PCR was conducted and the transcription products were examined (Figure 2). We found 34/92 (37%) of the tumor samples to be positive for ACRBP-V5a mRNA, with
Figure 3. Continued
Figure 3. Sequence of the TA cloning result for the variant of the gene for acrosomal binding protein, ACRBP-V5a.
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10/33 (30%) of astrocytoma, 10/30 (33%) of glioblastoma, and 14/29 (48%) of medulloblastoma samples positive for ACRBP-V5a mRNA. A one-way ANOVA found no significant differences in the proportions of positive samples between the 3 types of brain tumors (Table 1).

### Table 1. Analysis of ACRBP-V5a expression and clinicopathological characteristics

| Clinicopathological characteristic | No. of mRNA positive patient samples/total samples (%) | tP | Relative expression Mean ± SE | tP |
|------------------------------------|------------------------------------------------------|----|-------------------------------|----|
| Sex                                |                                                      |    |                               |    |
| Male                               | 21/63 (33.3)                                        | 0.29 | 46.7 ± 14.0               | 0.81|
| Female                             | 13/29 (44.8)                                        |    | 41.7 ± 11.3                |    |
| Age (years)                        |                                                      |    |                               |    |
| <34                                | 20/46 (43.5)                                        | 0.20 | 50.7 ± 9.5                  | 0.47|
| ≥34                                | 14/46 (30.4)                                        |    | 36.4 ± 19.1                |    |
| KPS§                               |                                                      |    |                               |    |
| <70                                | 15/34 (44.1)                                        | 0.28 | 57.6 ± 18.7                | 0.24|
| ≥70                                | 19/58 (32.8)                                        |    | 34.7 ± 8.5                 |    |
| Tumor grade                        |                                                      |    |                               |    |
| II                                 | 10/33 (30.3)                                        | 0.32 | 17.7 ± 3.8                  | 0.01*|
| IV                                 | 24/59 (40.7)                                        |    | 56.1 ± 12.8                |    |
| Tumor type                         |                                                      |    |                               |    |
| Astrocytoma                        | 10/33 (30.3)                                        | 0.30 | 17.7 ± 3.8                  | 0.02*|
| Glioblastoma                       | 10/30 (33.3)                                        |    | 50.4 ± 26.1                |    |
| Medulloblastoma                    | 14/29 (48.3)                                        |    | 60.2 ± 12.7                |    |

†χ² test and ‡Student t test, except for tumor type, which was calculated using a Kruskal–Wallis test. §Karnofsky performance status. ¶World Health Organization (2016). ¥One-way ANOVA. *P < 0.05.

Association between ACRBP-V5a and clinicopathological characteristics

The association between ACRBP-V5a mRNA transcription and the clinicopathological characteristics of patients, including categories of sex, age, Karnofsky performance status (KPS), and WHO tumor grade, was determined using RT-PCR and qPCR. The mean patient age was 33.5 years (range 2–78 years). All tumor tissues were classified according to World Health Organization (WHO) criteria [13], with 33 cases of WHO grade II and 59 cases of WHO grade IV identified. No significant differences were observed between expression of ACRBP-V5a and clinicopathological characteristics except for the level of expression in tumor grade and tumor type (Table 1). It is noteworthy that a one-way ANOVA only indicated a significant difference between level of expression between astrocytomas and medulloblastomas (P = 0.017).

Association between the ratio of ACRBP to ACRBP-V5a and the 3 types of brain tumors

ACRBP (exons 5–8) expression was examined in all the samples positive for ACRBP-V5a (34 cases) by qPCR, and was present in all the samples. ACRBP-V5a expression was determined as described previously. The ratio of ACRBP to ACRBP-V5a was calculated for each sample and the ratio was classified by different tumors and analyzed using a Mann–Whitney U test. We found significant differences in association between pairs for glioblastoma vs. medulloblastoma and astrocytoma vs. medulloblastoma, but not for astrocytoma vs. glioblastoma (Figure 5).

Discussion

To summarize, this experiment describes a novel splice variant ACRBP-V5a that has not been previously studied in brain tumors, to our knowledge. The primers newly designed in the present study can specifically amplify ACRBP-V5a. All 3 types of malignant brain tumor samples and liver cancer cells studied can express ACRBP-V5a. We did not find a significant difference in the expression of ACRBP-V5a and in the clinicopathological characteristics of the patients or the proportion of samples positive for ACRBP-V5a expression between the 3 types of malignant brain tumor samples. However, in samples positive for ACRBP-V5a expression, the relative levels of
expression were significantly different for tumor types and grades. The ratio ACRBP/ACRBP-V5a was different between different tumor types, implying an association with different types of brain tumors.

A new database of sequencing analysis (GTEx) maintains 42,611 genes, of which 20,352 may potentially code protein and 22,259 may not, with 323,258 transcripts in total [14]. In general, every human gene translates 3 splicing variants; some of these will be regarded as nonsense-mediated mRNAs and be decomposed [15–17].

Alternative splicing confers genetic diversity in several ways, including competing 5′ splice sites, competing 3′ splice sites, exon skipping, mutually exclusive exons, and intron retention [18]. For human ACRBP, there are 9 types of splicing variants predicted, 4 that are protein coding, 4 protein noncoding, and 1 that is nonsense. It is important to identify the specific function of other potential ACRBP variants in the next stage of research.

Some variants of ACRBP have been reported for mammals. Polakoski and Parrish [19, 20] detected a 29 kDa protein in porcine sperm (similar to our 32 kDa protein), which combined with proacrosin (55 kDa) and a 49 kDa intermediate. In 1989, these investigators demonstrated a 55-kDa precursor protein of ACRBP in porcine spermatogenic cells [2]. After posttranslational modification, a 32 kDa mature form was produced by removing half of the N-terminal of the precursor during spermatogenesis. Baba et al. [2, 3] asserted that both ACRBP and proacrosin exist in the acrosomes of germ cells. However, they only identified the size of the ACRBP protein and its function in procreation.

We predicted the amino acid sequence of ACRBP-V5a and compared it with the porcine form (Figure 6). In general, the sequences are highly similar. Therefore, we propose that the 32 kDa protein originates from ACRBP-V5a. In mice, unlike in other mammals, 2 different splicing isoforms are found for ACRBP: a wild-type, ACRBP-W, and a variant, ACRBP-V5. ACRBP-V5 plays a role in the formation and configuration of the acrosome during early spermatogenesis [11]. We compared the structure of the human ACRBP variant with ACRBP-V5 of mice. We identified a distinctive characterization of the special splice variant of human ACRBP, which retained a part of intron 5 (exon 5a) and prevented the next translation; so, we named it ACRBP-V5a. Recently, we also identified and described ACRBP-V5a in liver and brain tumor cell lines. ACRBP is expressed in early embryonic development. In 2016, a medulloblastoma was removed from a glioma and divided into embryonic tumors as identified by the latest classification criteria [21]. Interestingly, even though we found no significant differences between the proportion of samples positive for ACRBP-V5a mRNA between the 3 types of brain tumors, the proportion of the positive medulloblastoma samples was higher than for glioblastomas or astrocytomas. This finding may provide evidence to support the latest classification for medulloblastoma [13]. It is also suggested that if the quantity of samples was increased, a significant difference may be found. Although no significant differences were found between the proportion of positive samples for age and tumor grade, an association should be suspected considering the high proportion and particularity of medulloblastoma [22].

The proportion of glioblastoma samples positive for ACRBP-V5a mRNA of 33% was higher than the 24% proportion predicted from the TCGA database (http://projects.insilico.us.com/TCGA Splice Seq/index.jsp). We may find a proportion closer to that predicted by increasing the number of samples. At the expression level, this implies that ACRBP-V5a is associated with ACRBP. The level of expression of ACRBP was higher than that for ACRBP-V5a. The ratio of ACRBP expression to ACRBP-V5a expression was significantly different between the 3 types of brain tumors. It was noteworthy that this phenomenon is not accidental or isolated. We found no such mechanism in the present study, but propose that the ratio ACRBP/ACRBP-V5a is necessary to clarify potential links between various tumor types. The ratio may discriminate between different types of brain tumors.

Our study of alternative splicing of ACRBP may provide a basis for the future research to investigate the function of ACRBP-V5a and identify other potential splice variants of ACRBP and their biological importance of the expression of the alternative splicing.

Figure 5. The ratios of ACRBP to ACRBP-V5a in the 3 types of brain tumors. Data represent mean ± SE. A Mann–Whitney U test was used to determine the differences between the 3 different types. N.S.: not significant; **P < 0.01, ***P < 0.001.
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Author contributions: The author contributed substantially to the conception and design of the study, collection of data, and its analysis and interpretation. The author contributed substantially to drafting and critical revision of the manuscript, approved the final version submitted for publication, and takes responsibility for statements made in the published article.

Acknowledgments: The author thanks Professor Xiaoxun Xie, Department of Histology and Embryology, and Guangxi Colleges and Universities Key Laboratory of Preclinical Medicine Research, Guangxi Medical University, Nanning, Guangxi 530021, China, for contributions to drafting and critical revision of the manuscript, and Professor Shaowen Xiao, Department of Neurosurgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, China, for contributions to the conception and design of the study, funding acquisition and supervision of the project, collection of data, and its analysis and interpretation. The author thanks Ms. Fang Chen for technical assistance. The authors did not receive any specific grant for this research from any funding agency in the public, commercial, or not-for-profit sectors. The present address for Dr. Baolong Zheng is Clinic...

Figure 6. Comparison of porcine (full-length) and human (V5a) amino acid sequences for acrosomal binding protein (ACRBP; Clustal Omega alignment program, https://www.ebi.ac.uk/Tools/msa/clustalo/). An * (asterisk) indicates positions that have a single, fully conserved residue. A. (colon) indicates conservation between groups (porcine and human) of strongly similar properties. A. (period) indicates conservation between groups of weakly similar properties.

| Porcine | MRQLAAGSLLLKVLLLPLAAPAQDANASTPSGSLPETEYRFALLTPWKAETTC | 60 |
| Human | MRKPAAGFLPSLLKLLLPLAAPAQDSTQASTPSGSLPETEYRFALLTPWKAETTC | 60 |
| Porcine | RLATHGCRNPITLQYENHGLVPDGAVCSDLPYASWFFEFQFTOYRCNHYYAKR | 120 |
| Human | RLATHGCRNPITLQYENHGLVPDGAVCSNLPYASWFFEFQFTOYRCNHYYAKR | 120 |
| Porcine | VRCSEQPSILSPNLKEVDTTSVIPITMTSPVSSHTATGRQVFQWPWPERLNNVEELL | 180 |
| Human | VLCSSEQPSILSPNLKEIEASEAVSTTSIPSHPFTVTERQPTFQWPWPERLNNVEELL | 180 |
| Porcine | QSLSLGQEQEJQEHKQ---HKQEOQEQEHKQDEQEGEQEQQEQQEQEQQEEGGKQEQQGQTE | 236 |
| Human | QSLSLGQEQEJQEHKQDEQEQVEHRQEPQTEHQQEQGKQEQQEQQEQEQQEEGGKQEQQGQTE | 240 |
| Porcine | SLEAMSLQADSEPKQSEVFSSNPSPFTRPVRESTPMMENIQELIRSAQEMDGV | 296 |
| Human | GREAVSQQTSEPKHSELSNPSPAPFRVRESTPMENIQELIRSAQEMDGV | 300 |
| Porcine | VYEEEINWRAQPSGSLQLPHVDDLVLCSIVENTCVITPTAKWQYLEDVLFGKSV | 356 |
| Human | IYDENSYWRNQPGYRKF | 319 |
| Porcine | CDLSRGLHHACSLCDFSCLKEQCHSETNLQRQCNHKSPTFISPLASQMSGQT | 416 |
| Human | | 319 |
| Porcine | GTLSKGRFYGLGLGRMDWCLATKGCEDNVASWLQTELFLSFDQGDFTPKICDTE | 476 |
| Human | | 319 |
| Porcine | YVQYPNYCAFSSQCMRNDKVRSMRCRDQNETYVTQLQAKSEDVLWRSQFSTT | 536 |
| Human | | 319 |

Porcine QAG 539
Human --- 319
for Neurosurgery, University Hospital Düsseldorf, Düsseldorf 40225, Germany.

Conflicts of interest statement: The author has completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure form for Potential Conflicts of Interest. Baolong Zheng has no potential or actual conflict of interest to disclose in relation to the published article.

Data sharing statement: The data sets generated and analyzed during the present study are included in the published article. The data generated or analyzed during the present study are available in the National Center for Biotechnology Information ClinVar repository, with accession number: SCV001441593, and will be shared by the authors upon reasonable request after deidentification of data from any individual patient.

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