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# IGFBP7's susceptibility to proteolysis is altered by A-to-I RNA editing of its transcript

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ABSTRACT

**The selective deamination of adenosines (A) to inosines (I) in messenger RNAs (mRNAs) can alter the encoded protein's amino acid sequence, with often critical consequences on protein stability, localization, and/or function. Insulin-like growth factor-binding protein 7 (IGFBP7) supports cell-adhesion and stimulates fibroblast proliferation with IGF and insulin. It exists in both proteolytically processed and unprocessed forms with altered cell-extracellular matrix interactions. Here we show that editing of IGFBP7 transcripts impacts the protein's susceptibility to proteolytic cleavage, thus providing a means for a cell to modulate its functionality through A-to-I RNA editing.**

*Structured summary of protein interactions:*
**MT-SP1** cleaves **IGFBP7** by protease assay (View interaction).



## 1. Introduction

Posttranscriptional modifications of protein-coding RNA molecules increase the functionality of the proteome. The selective deamination of adenosines (A) to inosines (I) in double-stranded RNA molecules by Adenosine deaminases acting on RNA (ADARs) changes the sequence with consequences for downstream events, as the gene expression machinery recognizes inosine as guanosine [1]. In protein-coding transcripts, the edited codon may convey a different amino acid compared to its genomically encoded original, which can affect protein stability, localization, and function. Such recoding editing was first discovered in brain-specific mRNAs [2–4]. However, editing is required for the proper development and functionality of many tissues [5,6].

Insulin-like growth factor-binding protein 7 (IGFBP7) is a secreted protein with a N-terminal cysteine-rich domain, a heparin binding site, a Kazal-type trypsin inhibitor domain and a C-terminal Ig-like type C repeat [7,8]. While early studies elucidated its mitogenic effects in synergy with IGF and insulin [9], more recent reports illustrate its role as a putative tumor suppressor with functions in apoptosis and senescence [10–12]. IGFBP7 also appears to be involved in promoting [13] or blocking [14] angiogenesis. In addition there are reports of increased serum levels of IGFBP7 in insulin resistant diabetics [15] and that IGFBP7 presents chemokines to extravasating lymphocytes [16].

Bioinformatics screens predicted the mRNA of IGFBP7 to be a target of ADARs, and indeed it is edited, changing codon 78 from AGG (arginine) to IGG (glycine), and codon 95 from AAG (lysine) to AIG (arginine), henceforth termed R78G and K95R, respectively [17,18]. We hypothesized that the vast functional range of IGFBP7 may in part be facilitated by editing at these two sites, which would allow for the production of four protein isoforms from one allele.

Proteolytic processing of IGFBP7 modulates its biological activity: intact IGFBP7 stimulates growth of DLD-1 colon carcinoma cells in synergy with insulin/IGF-I, but cleaved IGFBP7 completely abolishes this growth-stimulatory activity, possibly due to the loss of insulin/IGF-I binding [19]. Heparin binding to IGFBP7 is decreased by proteolysis, while syndecan-1 binding is increased [19]. Thus, the intact and cleaved forms of IGFBP7 have different biological activities. Lysine 95 lies directly within the protease cleavage site, which overlaps with the heparin binding site (Fig. 1), and we hypothesized that editing would have functional consequences for the ensuing protein isoforms, specifically with regard to proteolytic processing. Here we show that IGFBP7 is edited at the two sites independently, in a tissue-specific manner. Moreover, the resulting protein isoforms are distinctly susceptible

*Abbreviations:* ADAR, adenosine deaminase acting on RNA; IGFBP7, insulin-like growth factor binding protein 7; MT-SP1, membrane-type serine protease 1

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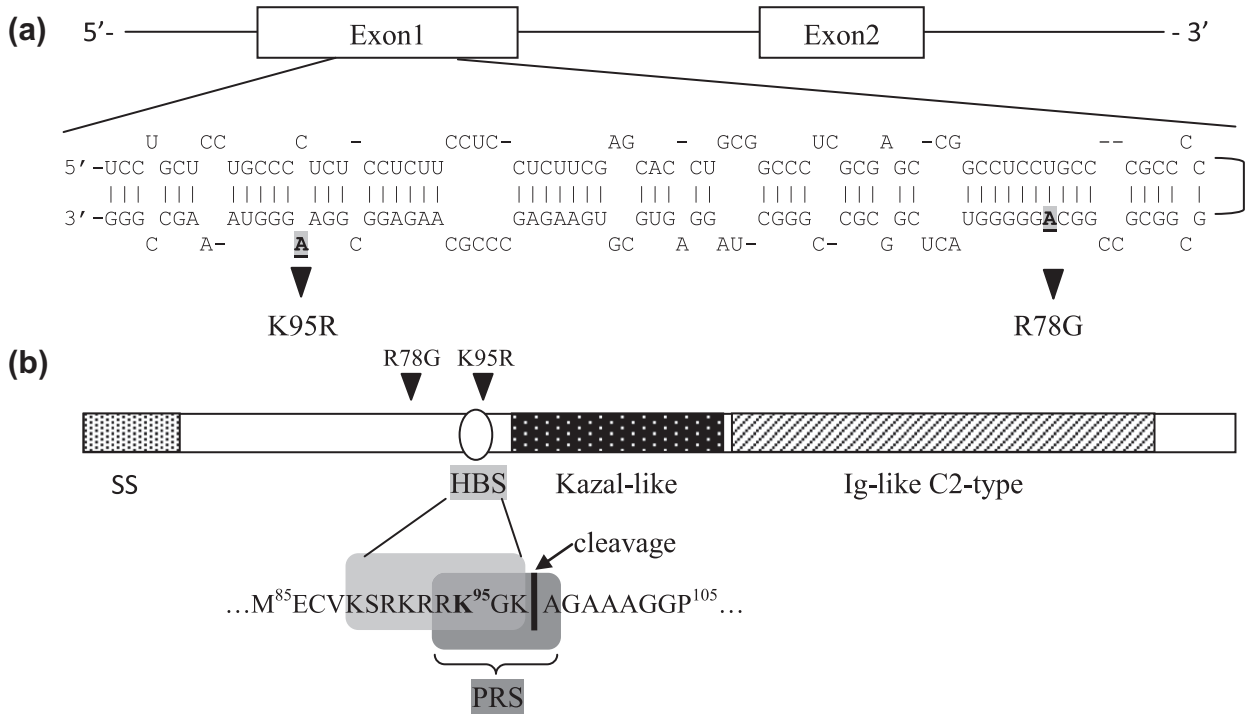


**Fig. 1.** (a) RNA fold encompassing IGFBP7 editing sites (underlined, bold) within exon 1 predicted by Mfold [17,31]. (b) Schematic representation of functional domains of the IGFBP7 protein [32]. The approximate positions of the two amino acid changes resulting from RNA editing are indicated. SS = secretion signal (aa1 1–26); HBS = heparin binding site (aa 89–97) [30]; cleavage = protease processing site at amino acid position 97 [20], PRS = protease recognition site [33].

to proteolytic cleavage by the specific Membrane-type serine protease 1 (MT-SP1) [20].

## 2. Materials and methods

### 2.1. IGFBP7 RNA editing analysis

Specimen-matched human RNA and gDNA was obtained from Biochain and RNA was reverse transcribed. An optimized PCR protocol contained 400nM of each primer (forward primer 5′-GTGCGGCTGCTGCCCTATGTGC-3′; reverse primer (cDNA) 5′-CGATGACCTCACAGCTCAAGTAC-3′; reverse primer (gDNA) 5′-CAGGTGCCCTTGCTGACCTG-3′), 2U Phire™ Polymerase (NEB), 0.4 mM dNTPs, 1/100 volume cDNA. Cycling conditions were 98 °C for 2 min, 35 cycles of 98 °C for 10 s, 68 °C for 5 s, and 72 °C for 8 s, followed by a final step of 72 °C for 1 min. Products were subjected to Sanger sequencing (Geneway Research) and editing levels determined as the ratios of the guanosine divided by the sum of both adenosine and guanosine signals at the specific location in the sequence tracks. For more accurate determination of editing levels, PCR amplicons were cloned into a pBluescript vector (Stratagene), individual recombinant clones isolated and purified plasmid DNA sequenced.

### 2.2. Recombinant protein expression and purification

Full-length IGFBP7 coding sequence in the pCMV6-XL4 expression vector was purchased from Origene. Three isoforms were created by site-directed mutagenesis, changing codon 78 from arginine (AGG) to glycine (GGG) and/or codon 95 from lysine (AAG) to arginine (AGG). All ORFs were C-terminally tagged with the HA sequence by PCR amplification and ligated into a pCI-neo vector. These vectors and an empty control were transfected into HEK293 cells using XtremeGene 9 transfection reagent (Roche). Stable transfectants were obtained after selection in MEM medium supplemented with 10% FCS containing 500 μg/ml G418 for 3 weeks and were maintained in 300 μg/ml G418 thereafter.

Culture medium of stably transfected HEK293 cell lines was collected, 1/20 volume 20×TBS and 1/250 volume EZview Red Anti-HA Affinity Gel (Sigma) was added and incubated at 4 °C with light agitation for 1 h. Beads were washed 3× with excess cold 1 × TBS. Bound IGFBP7 protein was eluted with 5 volumes of 1 × TBS, 0.05% SDS, 100 μg/ml HA peptide at 38.5 °C for 2 h with frequent vortexing. Eluates were diluted 1:5 with adjustment buffer (56.875 mM Tris, 28.75 mM NaCl, 0.01% Tween 20, pH 9) to convert the elution buffer into the MT-SP1 assay buffer. Diluted samples were ultrafiltrated using Amicon Ultra-0.5 Centrifugal filters (Millipore) for 15 min at 14,000*g* at 4 °C.

### 2.3. MT-SP1 proteolytic cleavage assay

100 ng/μl stock solutions of recombinant human matriptase (MT-SP1, R&D Systems) were prepared in sterile 50 mM Tris, 10% glycerol, pH 8.0. Relative concentrations of purified IGFBP7 isoforms were estimated from Western blots and input for the proteolysis reactions was normalized accordingly. Total reaction volume was equalized with 1× assay buffer (50 mM Tris, 50 mM NaCl, 0.01% Tween 20, pH 9). MT-SP1 was added to a final concentration of 325 nM (12.5 pg/μl). Proteolysis reactions were allowed to proceed for 15 min at 25 °C, samples were removed at 1, 2, 3, 5, 7.5, 10, 12.5, and 15 min, mixed with SDS sample buffer and boiled for 5 min at 90 °C to stop the reaction.

Proteins were separated on a 12% SDS–polyacrylamide gel and electrophoretically transferred to 0.2 μm nitrocellulose membranes. Membranes were incubated with primary antibody (rabbit anti-HA, Clontech, 1:300) over night at 4 °C and with secondary antibody (HRP-conjugated anti-rabbit, Promega, 1:25,000) for 2 h at room temperature. Bound antibody was detected with ECL Plus (Amersham) and exposure to film for 5–30 min.

The band intensities at 27 and 35 kDa were quantified using ImageJ software (available from http://rsbweb.nih.gov/ij). Percentage of cleaved IGFBP7 was plotted against time and linear regression was performed. Slope values denote percentage cleaved as a function of time (cleavage rate). Cleavage rates were normalized with respect to substrate input level of the unedited variant and averages were calculated over four independent experiments.

## 3. Results

### 3.1. Tissue-specific editing patterns of IGFBP7

Editing of IGFBP7 mRNA changes the amino acid encoded by codon 78 from arginine to glycine (R78G), and codon 95 from lysine to arginine (K95R) [17,18] (Fig. 1). Editing of other mRNAs has been shown to occur in a tissue-specific manner [21], allowing cells to modulate proteome functionality according to specific needs or in response to external cues. We thus first sought to determine the editing levels of the two sites in IGFBP7's mRNA in eight different human organs. After amplification and sequencing of cDNA, editing levels were estimated from sequence tracks as the ratio of the guanosine signal over the sum of guanosine and adenosine signals at that location. Editing at K95R is consistently higher than at R78G, with a wide range from 6% to 57% for R78G, and 30% to 85% for K95R, which may be indicative of tissue- and site-specific regulation of editing (Fig. 2a). Since each cDNA was derived from a different donor, with the exception of the two brain samples, this variability could also be due to individual differences rather than tissue-specificity. However, Li and co-workers determined IGFBP7 editing levels by deep sequencing and obtained a similar result for kidney (7% at R78G, 30% at K95R) and various brain regions (7–33% at R78G, 46–60% at K95R, respectively) [22]. We thus believe that the differences in editing seen across the tested human tissues reflect true tissue-specific editing and not individual differences.

### 3.2. Tissue-specific, site-independent editing in IGFBP7

Editing can produce four transcript versions of IGFBP7. To determine the distribution of the four versions, IGFBP7 cDNAs from brain, spleen and heart were cloned into a pBluescript vector and approximately 100 clones per sample sequenced. Editing leads to a distinct distribution of transcript variants in the three tissues (Fig. 2b). Chi-square analysis using a two-way classification reveals no significant difference between the editing levels of the two brain samples. Conversely, when comparing the data from brain with heart or spleen, respectively, editing levels are significantly different (brain: $P < 0.01$; spleen: $P < 0.025$). Comparison between heart and spleen also shows significantly different editing levels ($P$-value < 0.001). The consistency of these data with those obtained by sequence track analysis underscores the validity of the conclusion that editing in IGFBP7 occurs in a tissue-specific manner.

Editing at one adenosine is often accompanied by editing at additional adenosines lying on the same side of the RNA duplex, which is referred to as coupling [23]. Editing at the R78G could therefore be dependent on editing at the K95R site. To test this, a Pearson Chi-square analysis was performed. Coupling between the two sites is statistically significant only in the first brain ($P < 0.01$) and the heart samples ($P << 0.001$), but in the second brain and the spleen samples the percentage of transcripts edited at both positions simultaneously is statistically insignificant. This

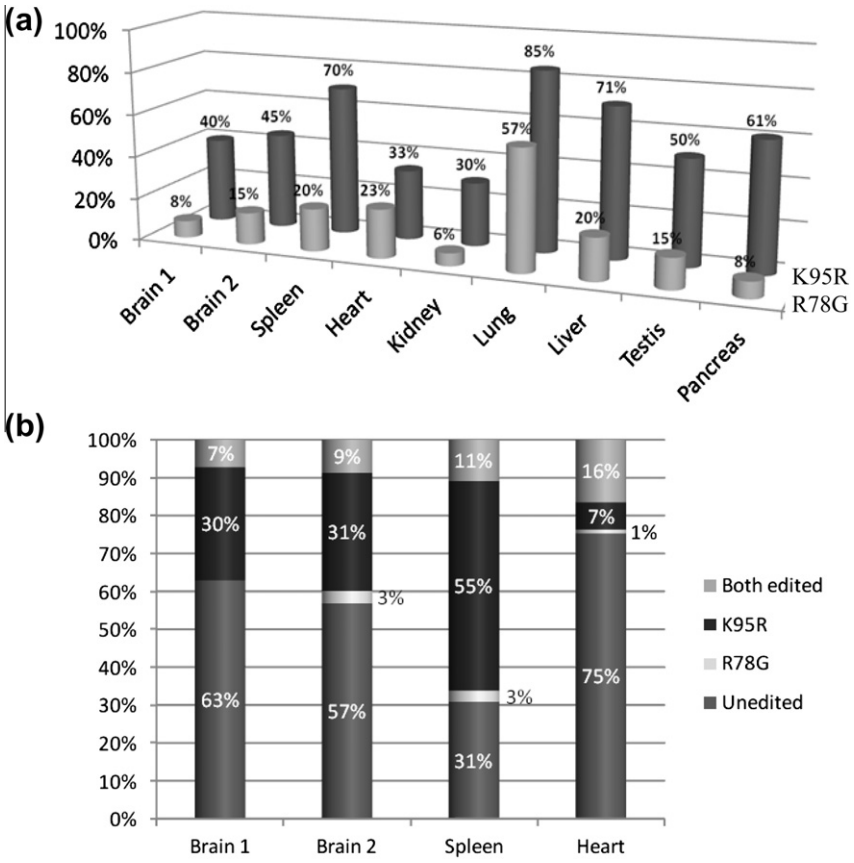


**Fig. 2.** (a) Editing levels at R78G (light bars) and K95R (dark bars) were estimated from sequence tracks obtained from eight different human tissues. Editing percentages determined from peak heights are indicated above the bars. (b) PCR products obtained from the indicated tissues were cloned, single clones isolated and sequenced, allowing detailed analysis of transcript versions present in different tissues. Exact percentages of editing are indicated.

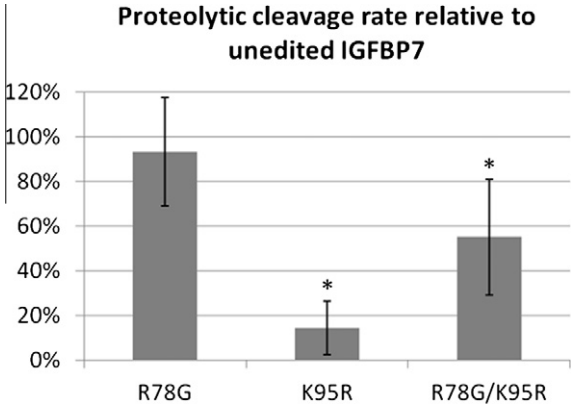


**Fig. 3.** Proteolytic cleavage rates relative to the unedited variant, normalized to the substrate input value of the unedited variant as determined by signal intensities. Displayed are the means and standard deviations of four independent experiments. A paired T-test shows statistically significant differences in cleavage rates of the K95R ($P = 0.001$) and the double mutant ($P = 0.041$) compared to the unedited variant. K95R and the double mutant are also significantly different from each other ($P = 0.046$).

is not surprising, as coupling is disrupted by bulges in the RNA helix, of which there are predicted to be five between R78G and K95R [17]. Therefore, mechanical coupling as described by Enstero and co-workers does not occur here and thus the two sites are edited independently of each other [23].

### 3.3. Proteolytic cleavage of IGFBP7 isoforms

Next, we wished to assess whether the changes in amino acid sequence caused by editing have a functional consequence with regard to proteolytic processing. We thus generated mutated versions of the IGFBP7 open reading frame (R78G, K95R, doubly mutated and wild type) and stably transformed HEK293 cells. Each IGFBP7 variant was C-terminal tagged with the hemagglutinin (HA) peptide sequence to facilitate purification of the protein isoforms. The editing competent double-stranded RNA structure of IGFBP7 is formed entirely by sequences within exon 1 and transcription of the open reading frame of IGFBP7 produces RNA structures amenable to editing. Editing levels in transcripts of the recombinant isoforms were determined to be 0% at the R78G and below 13% at the K95R positions (data not shown).

In vitro proteolysis of IGFBP7-HA isoforms by MT-SP1 results in 27 kDa C-terminal and 8 kDa N-terminal fragments, of which the full-length and the 27 kDa product are detectable on a Western blot using anti-HA antibody. The bands of cleaved and uncleaved protein were quantified by ImageJ software and cleavage rates determined over four independent experiments. Compared to the unedited variant the cleavage efficiency of the R78G isoform appears to be similar, but those of the K95R and double-edited variants, respectively, are statistically different (Fig. 3). The amino acid change elicited by editing at the K95R position lies directly within the protease recognition site, and in all experiments the K95R isoform is cleaved less efficiently than the other isoforms. The cleavage rate of the K95R variant is reduced by more than fourfold compared to that of the unedited IGFBP7 and the R78G variant. The double-edited version is cleaved at an intermediate rate. Cleavage is known to alter the protein's function and editing is thus likely to have an impact on IGFBP7 function.

## 4. Discussion

IGFBP7 is involved in diverse biological functions, from apoptosis [10,12], to inhibition or stimulation of growth and angiogenesis [9,13,14], to stimulation of prostacyclin production by endothelial cells [24] and increased serum levels correlate with insulin-resistance in diabetic men [15]. It associates with type IV collagen [25] and binds IGFs and insulin [9]. Only one type of post-translational protein modification of IGFB7 is known to date, the N-glycosylation of asparagine 171 [8], and thus regulation of its many activities through post-translational modification alone seems unlikely. The IGFBP7 pre-mRNA is alternatively spliced, but the functionality of the several splice variants remains to be evaluated [26]. Besides alternative splicing, transcript modification by A-to-I RNA editing might allow expanded functionality of this protein and/or alter transcript half-life or affect post-transcriptional processes such as translation efficiency.

Here we demonstrate that editing at the K95R site of IGFBP7 results in significant reduction of cleavage by the MT-SP1 protease in vitro. The reduction in cleavage efficiency of the K95R isoform might be even more pronounced in vivo, where binding to proteoglycans and/or IGFs and insulin may compete with recognition and cleavage of IGFBP7 by MT-SP1. While it remains to be determined whether inosine-containing IGFBP7 transcripts are processed like their unedited counterpart in vivo, our assay at least shows that transcripts with guanosine in place of adenosine at the respective locations are stable and translated normally. We further show that editing of IGFBP7 mRNA occurs in a tissue-specific manner and that the two ADAR target sites are edited independently of each other. Such tissue-specificity is indicative of regulated editing, especially as editing levels in spleen and lung significantly surpass those in brain, which generally displays the highest levels of editing [1]. Editing may thus allow a cell to regulate IGFBP7 function according to specific needs of the respective tissue, developmental time-point or in response to external cues.

The intermediate cleavage efficiency displayed by the double-edited isoform is unexpected. Only limited protein structure information is available from the related protein IGFBP5 [27,28], whose N-terminal domain is 57% similar (20% identical) to that of IGFBP7. Alignment of the homologous regions of IGFBP7 to this structure shows that both R78G and K95R are in loop-regions, separated by a β-strand. Since R78G and K95R are presumably in distinct regions of IGFBP7, it is unclear how the amino acid change at R78G in double-edited isoforms could increase MT-SP1 cleavage rate compared to the K95R single-edited variant. It is conceivable that the nucleotide change at codon 78 could alter translation rate or efficiency, which in turn might modify protein folding [29]. A change in protein folding could impact the local IGFBP7 domain structure and change binding affinity of MT-SP1 to the loop opposite to the R78G site.

Editing is likely to impact other functions of IGFBP7, as well. Heparin and heparan sulfates on cell surfaces bind to growth factors, cytokines, enzymes, and inhibitors, thereby modulating their biological activities. The motif $K_{89}$SRKRRKGK$_{97}$ was identified as heparin binding site on IGFBP7 [30], so amino acid changes elicited by editing might modulate cell surface binding. Also, IGFBP7 was shown to bind to IGFs and together with IGF and insulin to stimulate the growth of fibroblasts. IGFBP5 binds IGF-I with a domain homologous to one close to the K95R site in IGFBP7 [28]. Both binding of IGFBP7 to cell surfaces and IGFs and insulin could be affected by the amino acid changes elicited by editing.

A-to-I RNA editing in individual cells generates a pool of different IGFBP7 mRNAs, predicted to lead to the production of protein isoforms that differ not only in their amino acid sequences but also in their function as shown here. Only a limited number of site-specific editing events that effect a functional change in the resulting protein variant(s) have been identified so far. Our study shows that IGFBP7 may exist as functionally different isoforms in various organs and/or disease states, and as such studies involving IGFBP7 should take possible effects of editing into account.

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