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Short Communication

Synonymous Mutations in the X-Linked Disease Genes UBA1 and HADH2 Affect Binding of the Splicing Regulatory Proteins SRSF2, SRSF6 and hnRNP F/H

Claus Lenski[1], Steffen Erkelenz[2], Juliane Ramser[1], Marek Widera[2], Jan Otto Peter[2], Heiner Schaal[2]* and Alfons Meindl[1#]

[1]*Department of Obstetrics and Gynecology, Technical University Munich, Germany*
[2]*Institute for Virology, Medical Faculty, Heinrich Heine University Düsseldorf, Germany*

***Corresponding author**
Heiner Schaal, Institute for Virology, Medical Faculty, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany, Tel: 0049-211-8112393; Email: schaal@uni-duesseldorf.de

Alfons Meindl, Department of Obstetrics and Gynecology, Technical University Munich, 81675 Munich, Germany, Tel: 0049-89-41406750; Email: alfons.meindl@lrz.tum.de

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Abstract

Previously, we associated two mutations with the diseases X-linked congenital spinal muscular atrophy (SMAX2) and a syndromic form of X-linked mental retardation (MRXS10). These mutations are synonymous, meaning that they do not influence the amino acid sequence of the respective protein. They are localized in exon 15 of the gene ubiquitin activating enzyme 1 (*UBA1*) and exon 5 of the hydroxyacyl-coenzyme A dehydrogenase-II gene (*HADH2*). As quantitative PCR (qPCR) revealed, these mutations caused a strong decrease of the mRNA expression of the both genes. Herewith, we show that each mutation affects the functionality of exonic splicing enhancers (ESE) critical for proper splice site usage. Firstly, the wild type exonic*UBA1* and *HADH2* sequences appeared to promote pre-mRNA splicing within an ESE dependent reporter, whereas both mutant sequences exhibited an impaired recognition of the respective 5´ splice site (5´ss). Secondly, RNA pull-down assays revealed reduced binding of the splicing regulatory SR-proteins SRSF2 and SRSF6 to the mutant RNA sequences of *UBA1* and *HADH2*. Remarkably, we found that the mutant *HADH2* pre-mRNA interacts with hnRNP F/H known to repress splicing from exonic positions. Therefore, we propose that functional ESEs in *UBA1* and *HADH2* are disabled by synonymous mutations and, in case of *HADH2*, an hnRNP F/H dependent exonic splicing silencer (ESS) is created. Our findings emphasize the substantial influence of synonymous mutations on gene expression and stress their impact on comprehensive genetic diagnostics and counseling.

ABBREVIATIONS

MRXS10: X-Linked Mental Retardation; SMAX2: X-Linked Spinal Muscle Atrophy; SRE: Splicing Regulatory Element; ESE: Exonic Splice Enhancer; ESS: Exonic Splice Silencer; 5´Ss: 5´-Splice Site; qPCR: Quantitative PCR.

INTRODUCTION

After a systematical screening for mutations associated with two severe X-linked neurological disorders, SMAX2 (MIM #301830) and MRXS10 (MIM #300220), by sequence analysis, we identified two distinct synonymous mutations in the genes *UBA1* (NM_003334) and *HADH2* (NM_004493) in the DNA taken from patients of six SMAX2 families and the MRXS10 family. In the SMAX2 patients, the mutation c.1731C>T in exon 15 of *UBA1* [1] was detected, and c.574C>A in exon 5 of *HADH2* in the MRXS10 family [2] (exons are numbered like in (1) and (2)). Both mutations are associated with considerably reduced mRNA expression levels of the affected genes within patient cells compared to healthy male controls as we showed by qPCR. In both cases the expression of the full-length transcripts was reduced [1,2]. Additionally, the expression of transcript variants lacking the exon 5 containing mutation showed increased levels in case of *HADH2* [2].Considering also the *in-silico* analyses using the ESE finder tool, which predicted different ESE motifs here [1], it seemed to be obvious that important splicing regulatory

elements (SRE) are targeted by these synonymous mutations. Now we present the identification of exonic SREs in both genes, *UBA1* and *HADH2*, whose malfunctioning explains the observed reduced expression of the full-length transcripts. SREs modulate the recognition of splice sites in addition to their intrinsic strength [3,4]. The character of this modulation depends on the relative position of a SRE to the respective splice site [5]. Here, we could find two effects of the synonymous mutations in *UBA1* and *HADH2*, the loss of function of ESEs in both genes as well as the creation of an ESS in *HADH2*.

MATERIALS AND METHODS

The *UBA1* and *HADH2* wild type and mutant sequences (Metabion) as well as a neutral splicing regulatory sequence [6] and a known splicing enhancer as positive control [7] were inserted each into an enhancer-dependent HIV-1 based splicing reporter for 5'ss activation upstream of the 5'ss (*Eco*RI and *Sac*I sites, Figure 1A) [8,9]. HeLa cells were transiently transfected (*Trans*IT-LT1, MIRUS) with the respective splicing reporters together with pCL-dTOM and pXGH5 containing the human growth hormone factor *GH1* for qPCR analysis as transfection controls [5]. Twenty-four hours post transfection, HeLa cells were analyzed by direct fluorescence microscopy (Nikon ECLIPSE TS100-F, Nikon NIS-Elements imaging software suite) and flow cytometry using FACSCalibur (BD, Franklin Lakes).

For qPCR, total RNA was extracted and reverse transcribed into cDNA (DNAse I, Roche; Superscript III, Invitrogen). qPCR was performed on the LightCycler 1.5 (Roche) using LightCycler DNA Master SYBR Green I kit (Roche) and primers specific for *eGFP* and *GH1* as control gene (primers available on request). Each analysis was repeated three times independently.

RNA pull-down experiments were performed in three technical replicates according to a well-established protocol [5,10] using agarose-beads coupled to RNA oligonucleotides (Metabion; adipic acid dihydrazide-Agarose, Sigma) (Figure 2A), representing either wild type or mutant sequence fragments of *UBA1* and *HADH2*. These complexes were incubated in a HeLa-nuclear extract (Cilbiotech) for protein interaction. After separation on a polyacrylamide gel (12%), mass spectrometry was performed to identify the bound proteins (ZMMK, Cologne, Germany).

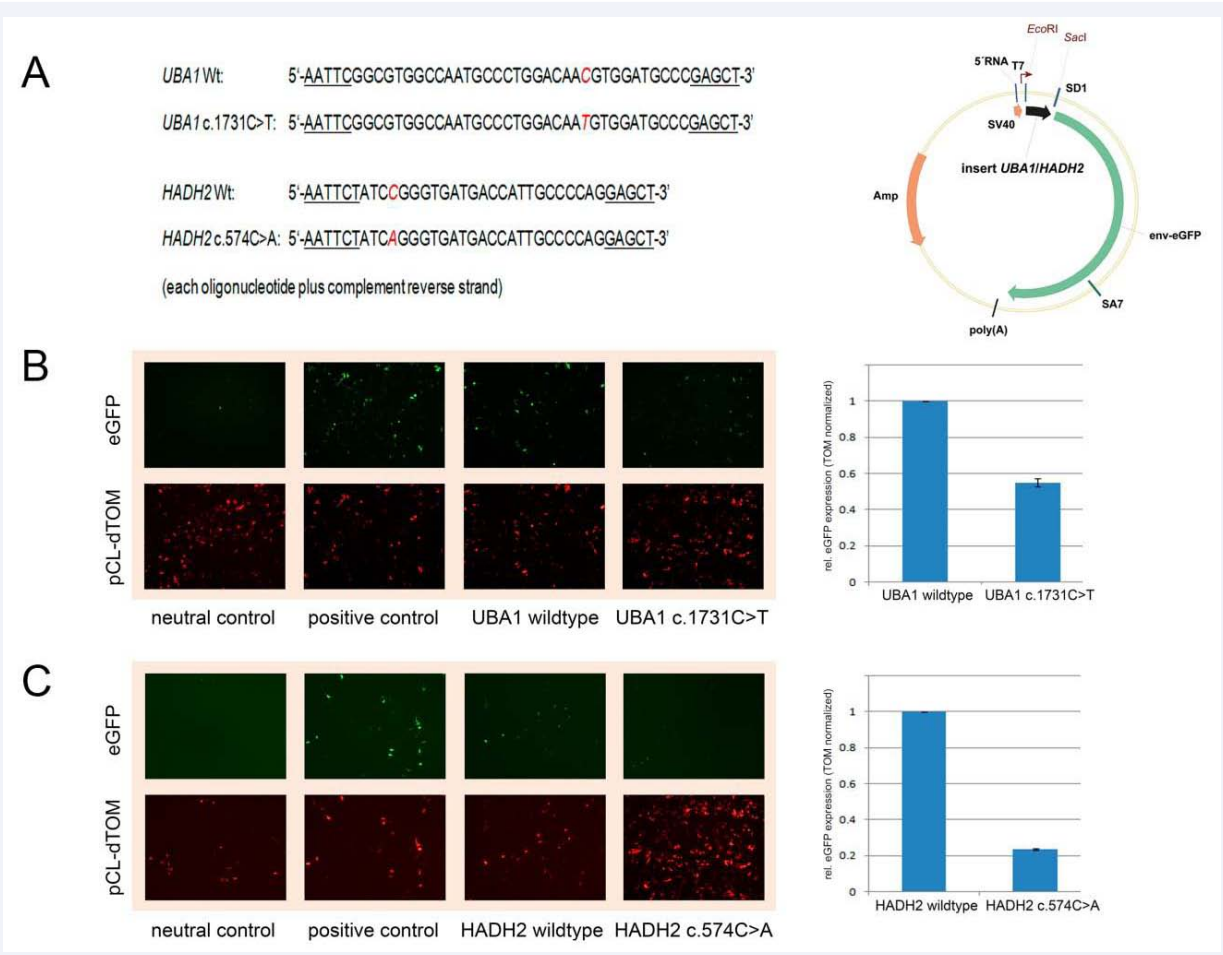

Figure 1 Analyses of the ESE/ESS activity of *UBA1* and *HADH2*
(A) The DNA oligonucleotides and the HIV-based splice enhancer dependent env-eGFP expression vector used in these studies.
(B-C) Analyses of the splice activity of the *UBA1* and *HADH2* constructs expressed in the env-eGFP-splice reporter by fluorescence microscopy and flow cytometry. As expression control pCL-dTom was co-transfected.
(B and C) Analyses of the ESE activity

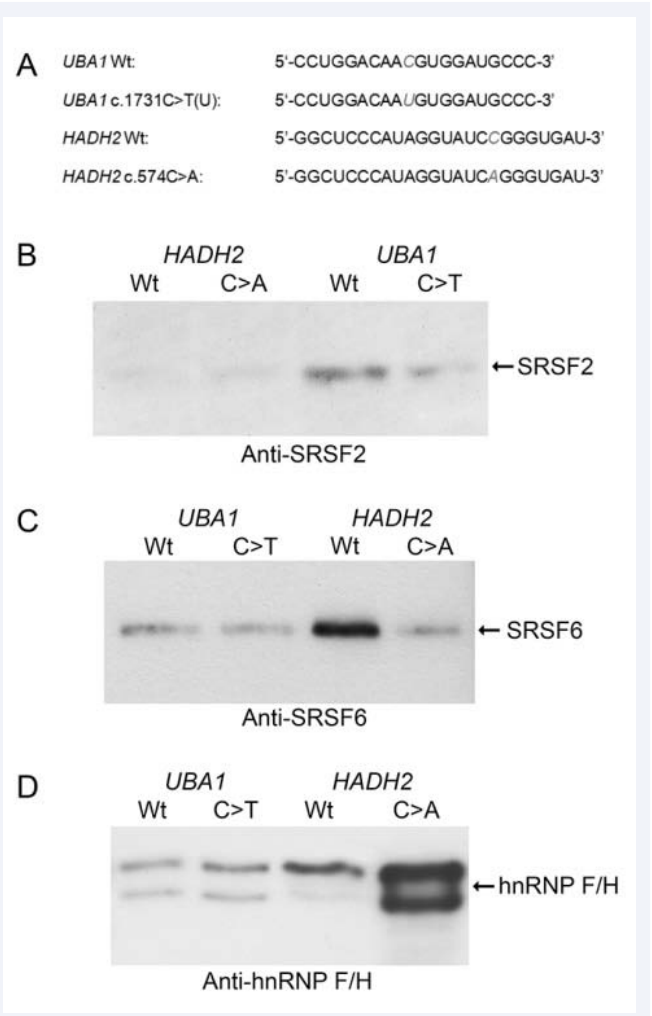

Figure 2 Analyses of the interactions between RNA fragments of *UBA1* and *HADH2* and SR proteins
(A) Analyses of binding proteins: The RNA oligonucleotides used in the RNA pull down experiments to interact with proteins from a HeLa nuclear extract. The synonymous mutations in the fragments of *UBA1* and *HADH2* are written in Italics.
(B-D) Western blots of proteins interacting with the RNA fragments of *UBA1* and *HADH2* after the RNA pulldown reaction.

For Western blotting analyses, anti-SRSF2 (ab28428, Abcam), anti-SRSF6 (WH0006431M1, Sigma-Aldrich), anti-hnRNP F/H (ab10689, Abcam), anti-SRSF1 (sc-10255, Santa Cruz), anti-SRSF7 (sc-10245, Santa Cruz) and anti-hnRNP-A1 (sc-56700, Santa Cruz) were used.

The two described variants c.574C>A (*HADH2*) and c.1731C>T (*UBA1*) were submitted to the LOVD-database (www.LOVD.nl).

RESULTS AND DISCUSSION

Using an enhancer-dependent, HIV-1 based splicing reporter, the *UBA1* and *HADH2* derived sequences (Figure 1A) function as strong ESEs critical for the required 5´ss recognition by the spliceosome [9, 11]. As observed by direct fluorescence microscopy, both wild type exon-derived sequences could promote activation of the splicing reporter 5'ss resulting in a high expression of eGFP comparable to the positive control (Figure 1 B-C). In contrast, the mutant sequences of *UBA1* and *HADH2* showed a decreased 5'ss activation resulting in lower levels of eGFP signals (Figure 1 B-C). For both sequences, flow cytometry confirmed the reduced enhancer-dependent eGFP expression: Compared to wild type fragments and the positive control, the expression levels of eGFP were below 60% and 25%, respectively (Fig. 1 B-C, bar charts). These results were in agreement with qPCR analysis on mRNA level, where the eGFP expression was reduced to 40%, if the expression was regulated by the mutated *UBA1* exon 15 sequence and to about 18% in case ofthe mutated *HADH2* exon 5 sequence (data not shown). Therefore, both exonic wild type sequences of *UBA1* and *HADH2* contain ESE activities and each of these ESEs were impaired by the synonymous mutations c.1731C>T in *UBA1* and c.574C>A in *HADH2*.

Next, performing RNA pull-down and mass spectrometric analyses, we could identify the known splicing factors SRSF1, SRSF2, SRSF6, SRSF7, hnRNP A1 and hnRNP F/H as candidate binding proteins of the *UBA1* and *HADH2* derived RNA substrates [12]. Western blotting analyses showed selective capabilities of the *UBA1* and *HADH2* fragments to bind either SR-proteins or hnRNPs. Using a specific anti-SRSF2 antibody, we demonstrated that binding of SRSF2 to the *UBA1* exon 15 fragment was decreased in case of the c.1731C>T carrying fragment (Figure 2B). As detection by the anti-SRSF6 antibody revealed, the wild type *HADH2* exon 5 fragment could be shown to interact with SRSF6, while this interaction was impaired by the c.574C>A mutation (Figure 2C). Moreover, the mutated *HADH2* exon 5 fragment showed an increased binding of hnRNP F/H (Fig. 2 D), known to repress splice site recognition when positioned upstream of a 5´ss [5,10]. Western blotting analyses using antibodies against SRSF1, SRSF7 and hnRNP A1 did not show any differences between wild type and mutant fragments (data not shown).

The combination of different analysis methods, an enhancer-dependent splicing reporter based analysis and RNA pull-down experiments, enabled a clear view of functional SREs in *UBA1* and *HADH2* and their interaction with the respective SR-protein. Two synonymous single base pair mutations within exons 15 and 5 of *UBA1* and *HADH2* could be shown to impair the ESE activities resulting in strongly reduced expression levels in an enhancer-dependent HIV-1 based splicing reporter. In accordance with these reduced expression levels, we could demonstrate by RNA pull-down analyses that the two well-known ESE-binding SR-proteins, SRSF2 and SRSF6, could not bind anymore to the mutated exonic fragments of *UBA1* and *HADH2*, efficiently. For the synonymous mutation in*HADH2*, we showed an even twofold effect on splice site recognition: The impairment of a so far unknown ESE and a de novo creation of an hnRNP F/H-dependent ESS which has numerously shown to interfere with functional spliceosome assembly, in this case across the *HADH2* patient target intron. Herewith, the clearly specifiable characteristics of an inactivated enhancer and a newly formed silencer could be illustrated exemplary.

CONCLUSION

Our studies illustrate the severe outcome of single synonymous mutations affecting normal pre-mRNA processing. So far unknown exonic splicing enhancers within exons 15 and 5 of *UBA1* and *HADH2*, respectively, could be found to be impaired

by synonymous mutations detected in patients suffering from the severe neurological diseases SMAX2 and MRXS10. Apart from an enhancer inactivation, we could also show clear evidence for the de novo creation of an active hnRNP F/H-dependent silencer. These findings are exemplary as the underlying mechanisms are in accordance with shown mechanisms affecting the splicing regulation and the gene expression in case of other inherited diseases. Therefore, they emphasize the importance of analyzing the splicing modified at the RNA level due to synonymous mutations, especially in the context of comprehensive diagnostics and potential therapeutics.

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