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BRIEF COMMUNICATION

# Functional splicing analysis in an infantile case of atypical hemolytic uremic syndrome caused by digenic mutations in *C3* and *MCP* genes

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## Abstract

Pathogenic variants in specific complement-related genes lead to atypical hemolytic uremic syndrome (aHUS). Some reports have indicated that patients with digenic variants in these genes might present severer phenotypes. Upon detecting novel intronic variants, transcriptional analysis is necessary to prove pathogenicity; however, when intronic variants are located in intron 1 and, as a result, no transcript is produced, no appropriate method had been established to reveal the pathogenicity. Recently, the minigene assay was used to assess the pathogenicity of intronic variants. Here, we report an infantile case of aHUS caused by digenic mutations in two different complement-related genes, *C3* and *MCP*. Targeted sequencing detected a known variant in *C3* and a novel variant in the intron 1 splicing donor site of *MCP*. To assess the pathogenicity of this intronic variant, we conducted functional splicing assay using a minigene construct and quantitative PCR analysis of the *MCP* transcript, revealing the pathogenicity of the intronic variant. In conclusion, the minigene assay revealed the pathogenicity of the intron 1 splicing donor site variant for the first time. This case showed a severe phenotype of infantile-onset aHUS associated with digenic variants in two complement-related genes.

## Introduction

Atypical hemolytic uremic syndrome (aHUS) is a rare disease characterized by microangiopathic hemolysis, thrombocytopenia, and renal failure. In recent years, it has been shown that over 50% of cases are associated with an abnormality affecting complement, such as pathogenic mutations in the genes encoding complement regulators (CFH, CFI, CD46, and thrombomodulin) or activators (CFB and C3), or autoantibodies against CFH. In addition, some reports indicated that patients with aHUS with digenic variants in these genes tend to show severer phenotypes [1, 2].

Transcriptional analysis is necessary to determine the effects of variants located outside of the splicing consensus sequence. However, this is often difficult because of the influence of nonsense-mediated mRNA decay or low expression of target mRNA in analyzed samples. When a variant is located in the intron 1 splicing donor site, the transcript will not be produced by the allele and mRNA analysis cannot reveal the pathogenicity. Fortunately, functional splicing analysis using a minigene construct was recently developed and successfully applied to investigate novel intronic variants in various inherited diseases.

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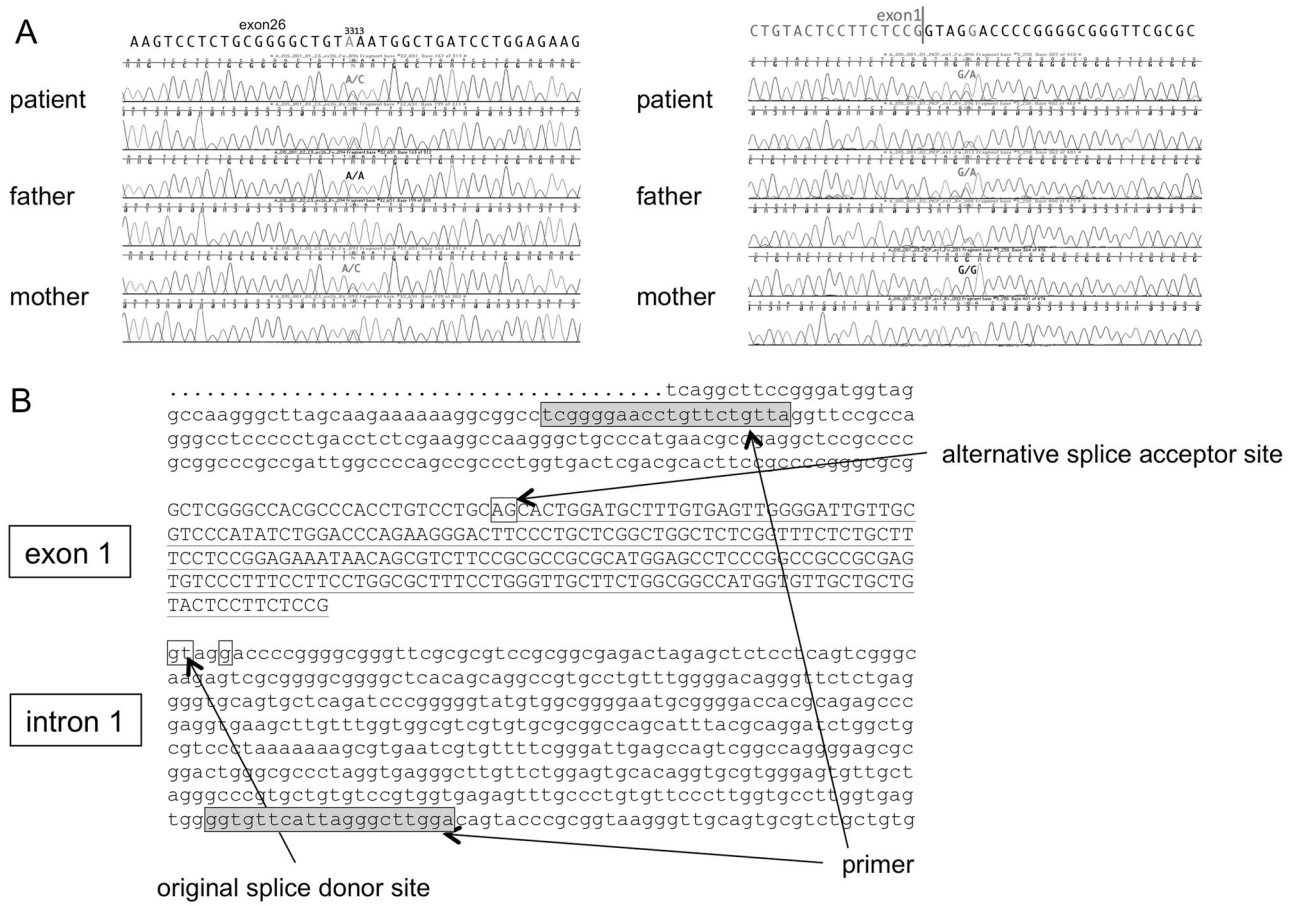


**Fig. 1** Mutations detected in the *C3* and *MCP* genes. **a** Left: A heterozygous single-base substitution of A to C in exon 26 (c.3313A>C) of the *C3* gene was detected in the maternal allele. Right: A heterozygous single-base substitution of G to A in intron 1 (c.97+5G>A) was detected in the paternal allele. **b** Normal sequence of exon 1 and intron 1 in the *MCP* gene. The original splice donor site was interrupted by the mutation (c.97+5G>A) and the sequence of "AG" in exon 1 was used preferentially as an alternative splice acceptor site in minigene assay

However, in the field of inherited kidney diseases, only a few reports have been published [3–6].

In this study, we conducted a functional splicing assay using a minigene construct and quantitative analysis of target mRNA for an infantile case of aHUS with suspected digenic gene variants. We proved the presence of digenic pathogenic variants in this case and showed their association with a severe phenotype of infantile-onset aHUS.

## Materials and methods

An 8-month-old Japanese boy was referred to our hospital for genetic analysis of aHUS and targeted sequencing using next-generation sequencing was performed. The detailed medical history and analytical method are described in the supplementary data.

In this study, hybrid minigene constructs were created by inserting a test sequence fragment consisting of exon 1 of the *MCP* gene into the multicloning site (Supplementary Fig. 1). This minigene was transfected into human derived cells and mRNA was analyzed as described elsewhere [7–9]. Detailed method was described in the supplementary data.

Quantitative mRNA analysis of the *MCP* gene was conducted by capillary electrophoresis using the Agilent 2100 Bioanalyzer (semiquantitative analysis) and SYBR® green real-time RT-PCR amplification using a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The analytical method is described in the supplementary data.

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine. Informed consent was obtained from the parents.

## Results

Targeted resequencing and Sanger sequencing detected a reported heterozygous variant in exon 26 (c.3313A>C, p. Lys1105Gln) in the *C3* gene and a novel heterozygous

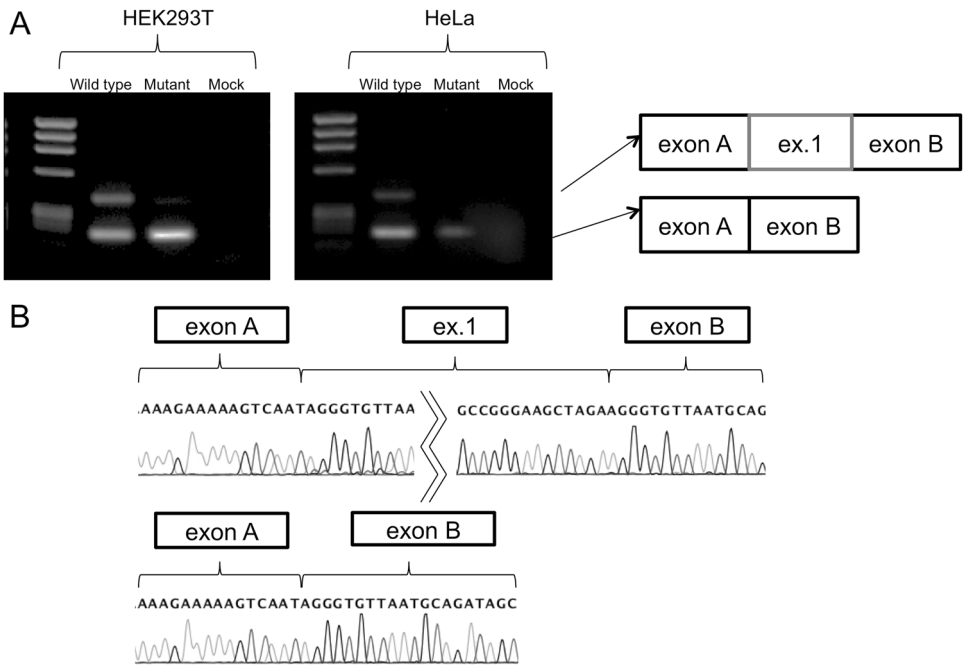


**Fig. 2** In vitro splicing assay using hybrid minigene construct. **a** RT-PCR-amplified products of hybrid minigene transcripts. As shown in the gel file, a comparatively large band was present in the wild-type construct in both HEK293T and HeLa cells. In contrast, in the minigene derived from the patient sequence, only a relatively small band was detected in both cell lines, which corresponds to exon B directly following exon A. **b** The RT-PCR product containing exon 1 between exon A and exon B was obtained from the minigene encoding the wild-type genomic DNA (top). In contrast, the product containing only exon A and exon B could be obtained from the hybrid minigene encoding the intronic mutation (bottom)

intronic variant at the fifth base of intron 1 (c.97+5G>A) in the *MCP* gene. These variants were derived from the maternal and paternal alleles, respectively (Fig. 1a). RNA was extracted from peripheral blood leukocytes for RT-PCR analysis. However, only the normal *MCP* transcript was detected because of the absence of transcript production from the variant allele. This was because its exon 1 was not recognized due to disruption of the intron 1 splicing donor site.

As a result of minigene assay, the RT-PCR product containing the exon 1 sequence between cassette exons A and B was obtained from the minigene encoding the wild-type sequence. In contrast, the product from the hybrid minigene containing the intronic variant of the patient did not contain exon 1 (Fig. 1b, 2a, b).

Quantitative mRNA analysis showed that comparatively weak bands were present in the mRNA extracted from the patient compared with those for the healthy control at all locations of the *MCP* gene by capillary electrophoresis. As a result of correction using *GAPDH* gene expression, the *MCP* gene expression of the patient was about 46–50% (Fig. 3a). In addition, SYBR® green RT-PCR analysis showed that the *MCP* gene expression of the patient was about half that of the control (Fig. 3b).

## Discussion

In this case, targeted sequencing detected digenic variants in the *C3* and *MCP* genes. The pathogenicity of the former variant had already been proved by a quantitative hemolytic assay [10]. We conducted the same assay for the current case and showed the same result, confirming the pathogenicity. In contrast, the pathogenicity of the latter variant is unknown because it is a novel variant and located outside of the splicing consensus sequence. However, both variants were thought to work digenically since the case showed very early onset of aHUS, although the parents having just one of the variants were asymptomatic. In recent years, aHUS with combined complement gene mutations was reported and some such patients showed more severe phenotypes than those with a monogenetic variant [1, 2] .

To prove the pathogenicity of the novel intronic variant of the *MCP* gene, we conducted an in vitro splicing assay using a hybrid minigene construct, which revealed that exon 1 of the *MCP* gene was not translated in the minigene derived from the patient sequence. This finding indicated that the *MCP* mRNA from the variant allele was not produced, which is because only the normal *MCP* transcript was detected by standard RT-PCR analysis of the patient's mRNA. To confirm this result, we conducted quantitative

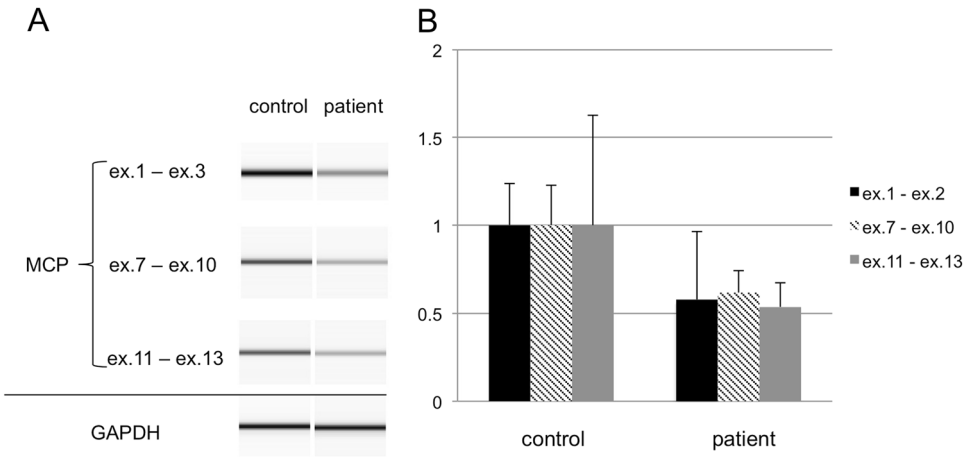


**Fig. 3** Quantitative mRNA analysis. **a** The semiquantitative analysis by electrophoresis using the Agilent 2100 Bioanalyzer. As shown in the gel file, comparatively weak bands were present in the mRNA extracted from the patient compared with mRNA extracted from the healthy control at all locations in the *MCP* gene. As a result of correction using GAPDH gene expression, the *MCP* gene expression of the patient was about 46–50%. **b** The SYBR® green RT-PCR analysis. The *MCP* gene expression of the patient was about half that of the control at all locations of the *MCP* gene

mRNA analysis of the *MCP* gene and successfully proved its pathogenicity.

Here, we report for the first time the use of an in vitro splicing assay using a minigene for the characterization of a mutation in aHUS, although this assay has previously been conducted for several inherited diseases [6–9, 11–14]. To the best of our knowledge, this is the first report showing the pathogenicity of an intron 1 splicing donor site variant. In this work, we confirmed the splicing abnormality using both the patient's sample and a minigene.

In conclusion, the combination of a minigene assay and quantitative analysis is a noninvasive and useful method for functional splicing assay of inherited diseases with an intron 1 splicing donor site variant, even if standard transcriptional analysis does not detect abnormal splicing. This study also indicated that the mutations in both *C3* and *MCP* worked digenically and resulted in the very early onset of aHUS in this case.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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