



# A novel Romani microdeletion variant in the promoter sequence of *ASS1* causes citrullinemia type I

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## ABSTRACT

**Background:** Citrullinemia type I (CTLN1, MIM #215700) is an autosomal recessive urea cycle disorder caused by deficiency of argininosuccinate synthase (ASS). CTLN1 is characterized by life-threatening hyperammonemia and risk for resulting neurocognitive impairments. The diagnosis of CTLN1 is confirmed by the identification of biallelic pathogenic variants in the *ASS1* gene. However, there are a small percentage of CTLN1 patients with a characteristic biochemical phenotype without identifiable variants in *ASS1*. We describe the molecular characterization of two related Romani children with biochemically diagnosed CTLN1, whose clinical genetic testing failed to detect any pathogenic variant in *ASS1*.

**Methods:** Genomic DNA was extracted from peripheral blood lymphocytes collected from both patients. Sanger sequencing was performed after PCR amplifications of 5'- and 3'-untranslated regions of the *ASS1* gene. A luciferase reporter assay was performed using the human malignant melanoma A2058 cell line and the human liver cancer cell line HepG2.

**Results:** We interrogated the non-coding regions of *ASS1* by targeted PCR amplification and identified a homozygous 477-bp microdeletion in the promoter region of the *ASS1* gene in both patients. Heterozygosity of the variant was confirmed in their parents. Sanger sequencing confirmed the microdeletion contained the entire sequence of the non-coding exon 1 of *ASS1* that includes promoter elements of GC-box, E-box, AP2-binding site, and TATA-box. Luciferase reporter assay using an expression plasmid containing the wild-type or mutant *ASS1* sequences showed robust reporter expression from the wild-type sequence and significantly reduced expression driven by the mutant insert (3.6% in A2058 cells and 3.3% in HepG2 cells). These findings were consistent with the hypothesis that the microdeletion identified in the patients disrupted an essential promoter element and resulted in deficiency of *ASS1* mRNA expression.

**Conclusions:** This is the first report of CTLN1 patients caused by a Romani microdeletion variant affecting the non-coding upstream sequence of *ASS1*. Ablation of the promoter sequence can cause CTLN1 by the reduction of *ASS1* expression. Currently available clinical sequencing methods usually do not cover the promoter sequence including the non-coding exon of *ASS1*, highlighting the importance of evaluating this region in genetic testing for CTLN1.

## 1. Introduction

The *ASS1* gene encodes a cytosolic urea cycle enzyme, argininosuccinate synthase (ASS), which plays a crucial role in ammonia detoxification. This enzyme is expressed predominantly in the liver and kidney [1,2]. Urea cycle metabolism mainly occurs in the periportal hepatocytes [3], and involves several enzymes including ASS, carbamoyl-phosphate synthetase 1, ornithine transcarbamylase, argininosuccinate lyase, and arginase 1 [4]. Ammonia detoxification through

the urea cycle is the essential route of nitrogen disposal by converting ammonia to urea, which can be excreted in urine [4]. Deficiencies of the enzymes or proteins associated with the urea cycle cause urea cycle disorders which are characterized by hyperammonemia, which in severe forms leads to profound metabolic disturbances and neurocognitive impairments [5]. Currently, mainstays of the management of urea cycle disorders includes protein restricted diet, ammonia scavenger medications, and supplements of deficient metabolic intermediates such as citrulline or arginine [6]. For patients with poor ammonia

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control, liver transplantation has been successful in providing a cure of the hyperammonemic aspects of the disease [6].

Citrullinemia type I (CTLN1, MIM #215700) is an autosomal recessive urea cycle disorder caused by a functional defect of ASS [7]. Classical CTLN1 is characterized by life-threatening hyperammonemia, poor feeding, neurological impairments, markedly elevated plasma citrulline, low plasma arginine, and orotic aciduria [7]. Onset of this condition varies from the neonatal period to adulthood depending on the residual ASS activity [8,9]. Since the introduction of expanded newborn screening (NBS) using tandem mass spectrometry, CTLN1 has become amenable to detection by NBS. Based on population screening data, its current incidence is estimated at approximately 1 in 250,000 newborns in the US [10]. The diagnosis of CTLN1 is confirmed by decreased ASS enzyme activities measured in cultured fibroblasts or identification of biallelic pathogenic variants in the *ASS1* gene [7]. Of interest, it has been observed there is a small subset of CTLN1 patients without pathogenic variants in *ASS1* identified even with the presence of the characteristic biochemical phenotype [7,8,11]. Here, we describe two Romani children whose biochemical profiles were consistent with the diagnosis of CTLN1, while their molecular confirmation tests using all clinically available genetic sequencing failed to detect a pathogenic *ASS1* variant. Further molecular analysis identified, a novel microdeletion involving the non-coding upstream sequence of *ASS1*.

## 2. Materials and methods

### 2.1. Variant detection

Sanger sequencing of the 5'- and 3'-untranslated regions (UTRs) of the *ASS1* (NM\_000050.4), was carried out using PCR products amplified from genomic DNAs obtained from peripheral blood lymphocytes of each subject. Primer sets for Sanger sequencing were designed to cover the entire UTRs containing intronic sequences of at least 100 bp (Supplemental Table 1).

### 2.2. Co-segregation analysis

The zygosity status of available family members for the microdeletion encompassing exon 1 of *ASS1* was confirmed based on PCR analysis (Fig. 1A). We designed two primer sets, 1 (F1/R) and 2 (F2/R), as shown in Fig. 1B (Supplemental Table 1). The second set was required because it was difficult to amplify the wild-type allele in the heterozygotes with the primer set 1 due to PCR bias. Using the primer set 1, two PCR products (1148 bp and 671 bp) were designed to amplify from wild-type and the mutant allele with the microdeletion, respectively. The wild-type allele was also confirmed by the detection of a 611 bp band using primer set 2, which did not amplify the mutant allele.

### 2.3. Luciferase reporter assay

Measurement of the *ASS1* promoter activity was done by luciferase reporter assay to examine the transcriptional effect of the microdeletion of the non-coding upstream sequence of *ASS1*. The upstream sequences covering the entire exon 1 of *ASS1* were prepared by PCR using genomic DNAs from peripheral lymphocytes (Fig. 2A). The PCR primers, which are modified from the ones described by Dr. Tsai et al. [12] were designed as follows:

forward 5'-AATGAGCTCCCTGTGAGAGGGCTGAGGGG-3'.

reverse 5'-AATCTCGAGCGGGCGGGCGTCTTCTAC-3'.

After purification of the PCR fragments, they were digested with restriction enzymes, *SacI* and *XhoI*, and cloned upstream of the luciferase reporter gene in the pGL4.12 [luc2CP] vector. We used the pGL4.74 [hRluc/TK] vector as an internal control. A2058 and HepG2 cell lines were obtained by ATCC and were maintained in Dulbecco's modified Eagle's GlutaMAX™ medium (Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL) and 1% penicillin-

streptomycin (Gibco-BRL) at 37 °C and 5% CO<sub>2</sub>. 1.8 µg of pGL4.12 (either empty, wild-type, or mutant) along with 0.2 µg of the pGL4.74 were transiently co-transfected using X-tremeGENE9 DNA transfection reagent (Roche) into cells cultured on 60-mm dishes. 24 h after transfection, these cells were lysed with 200 µl of 1× passive lysis buffer (Promega). Luciferase activity in each lysate was measured using Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Relative luciferase activities were normalized and calculated by protein concentration of each lysate and the expression levels of Renilla luciferase from the pGL4.74 control. All the above experiments were performed independently three times.

### 2.4. Research ethics

Written informed consent was obtained from parents of the subjects. This research was approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai.

## 3. Results

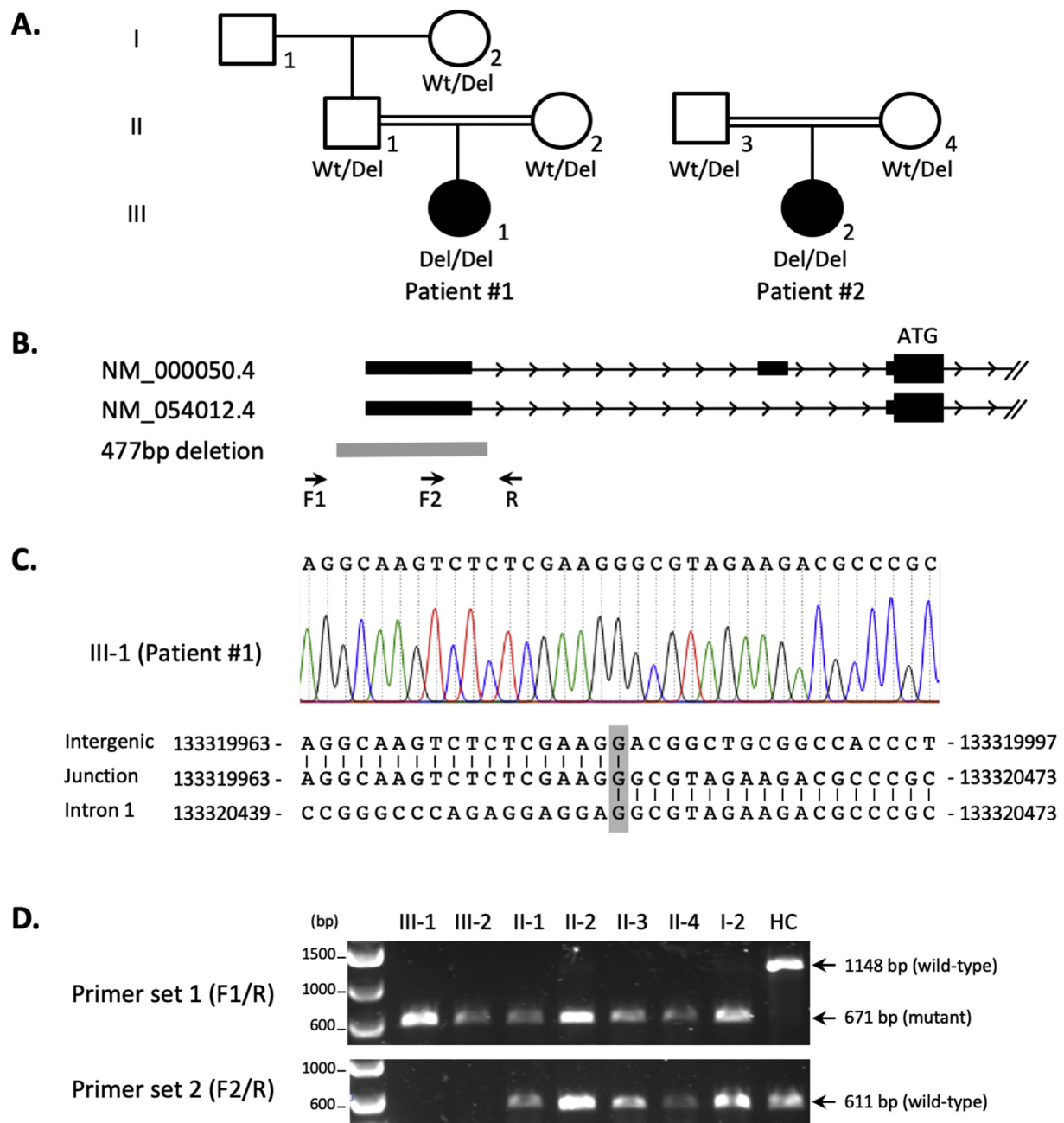
### 3.1. Case descriptions

Patient #1 is a currently 3-year-old female (III-1 in Fig. 1A) who was born to consanguineous healthy parents of Romani descent. She screened positive by NBS with an elevated citrulline and biochemically diagnosed with CTLN1. Since then, she has been managed by dietary protein restriction, sodium phenylbutyrate, and arginine supplementation. She had her first hyperammonemic crisis that required an ICU admission at 4 weeks of age. Since then, she has had only one non-ICU admission due to hyperammonemia and liver dysfunction. Her ammonia and citrulline have ranged between 40–70 µM and 1000–3500 µM, respectively. For genetic confirmation, next generation sequencing (NGS)-based targeted sequencing tests for *ASS1* and *SLC25A13* were done but did not detect any clinically significant variants. Whole exome sequencing was also performed using genomic DNA extracted from blood leukocytes of the Patient #1 by GeneDx Inc., with no likely pathogenic or pathogenic variants identified. Comparative genomic hybridization (aCGH) with single-nucleotide polymorphism (SNP) probes was done and detected multiple areas of absence of heterozygosity (AOH) totaling 83.0 Mb and ~ 2.8% of the genome.

Patient #2 is currently a 13-year-old Romani female, who is related to Patient #1. They shared common ancestors several generations prior. She was diagnosed with CTLN1 based on biochemical findings of an elevated plasma ammonia and citrulline at the age of 9 months. She has also been managed by dietary protein restriction, sodium phenylbutyrate, and arginine supplementation. Her ammonia and citrulline have generally ranged between 30–50 µM and 1000–3000 µM, respectively. Except for the initial hyperammonemic admission, she has not had any significant hyperammonemic episodes until she turned 3 years of age when she was admitted to our pediatric ICU. For the past 10 years, she has had three admissions for hyperammonemia, but no ICU admission was required. NGS-based targeted sequencing of *ASS1* was done, but also did not detect any clinically significant variants.

### 3.2. Microdeletion in the *ASS1* gene

We identified a homozygous microdeletion of 477-bp (Chr.9; 133,319,979-133,320,456, hg19) with 1-bp (G) microhomology at the junction in both patients by Sanger sequencing (Fig. 1B and C). The deleted region encompasses the entire non-coding exon 1 and its flanking intronic sequence in the *ASS1* gene. This deleted region was predicted to contain multiple promoter elements including GC-box, E-box, activating protein-2 (AP2) binding site and TATA-box by computational analyses (Fig. 2A) [12–14]. Of note, it was previously confirmed that three GC-boxes and one E-box in the deleted sequence enhanced transcription of the *ASS1* gene through an interaction with



**Fig. 1.** CTLN1 caused by a microdeletion involving the *ASS1* promoter sequence. (A) family pedigrees and (B) illustrations of the 5'-UTR of the *ASS1* two isoforms. The 477-bp microdeletion encompasses the entire sequence of the non-coding exon 1 of both *ASS1* isoforms as indicated with a gray bar. The primer sets 1 (F1/R) was designed to amplify two independent PCR bands from both wild-type and mutant alleles, whereas the mutant allele can't be amplified by the primer set 2 (F2/R). (C) Electropherogram of a deletion junction fragment (upper) and junction sequences (lower) in Patient #1. Homologous nucleotide at the deletion junctions is highlighted in gray. (D) PCR analysis of the familial co-segregation of the microdeletion. HC: healthy control.

transcription factors, specificity protein (Sp) 4 or c-Myc in the A2058 cells [12,14,15]. Other than the microdeletion, no additional variations were detected in the 5'- and 3'-UTRs. Heterozygosity of the variant was confirmed in their parents and a grandmother of the Patient #1 (Fig. 1A and D).

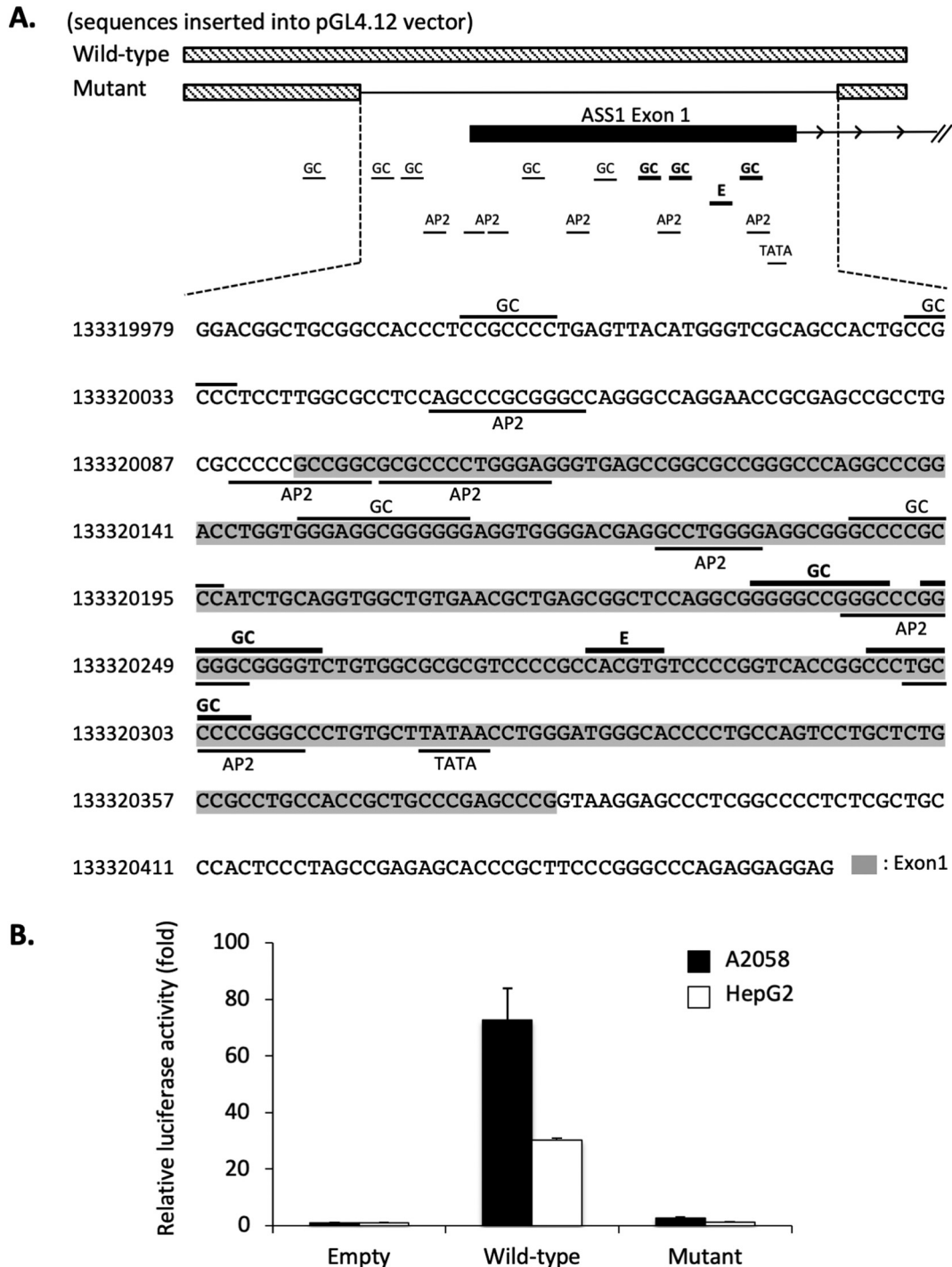
### 3.3. Luciferase reporter assay

Luciferase reporter assay using an expression plasmid containing the mutant sequence with the microdeletion detected a significantly decreased reporter expression compared with the wild-type control sequence (3.6% in A2058 cells and 3.3% in HepG2 cells) (Fig. 2B). The expression of luciferase from the mutant sequence was still elevated

compared to the negative control expression from the empty plasmid (Fig. 2B). These findings were consistent with the hypothesis that the microdeletion identified in the patients ablated an essential promoter element for *ASS1* mRNA expression.

### 4. Discussion

With the introduction of expanded NBS, neonates with CTLN1 can be identified through an elevated citrulline [7]. In the majority of cases, affected individuals become symptomatic with overwhelming hyperammonemia due to catabolic stressors. Accurate diagnosis in a timely manner is critical for appropriate management of patients. Recently, clinical genetic testing has become more broadly accessible and the



**Fig. 2.** Overview of the deleted sequence and luciferase reporter assay. (A) Potential or functional promoter elements presented in the microdeletion modified from the data published by Husson et al. [14]. The microdeletion contains putative seven GC-boxes, six AP2-binding sites, one E-box and one TATA-box. Among them, three GC-boxes and one E-box highlighted in bold have been confirmed to be functionally promoter elements. (B) The effects of the microdeletion on transcriptional activity analyzed by luciferase reporter assay using the plasmid containing wild-type and mutant sequences. The *ASS1* promoter activity was normalized with protein concentration in each lysate and Renilla expression levels of the pGL4.74 vector. The experiment was performed independently three times, and the means ± SD (*n* = 3) are displayed.

identification of biallelic variants in the *ASS1* gene, along with a biochemical profile characterized by elevated plasma ammonia and citrulline concentrations, has become the standard of care for CTLN1 diagnosis. The mutation detection rate for CTLN1 patients is generally high; estimated to be over 90% by clinical genetic testing of *ASS1* [7,8,16,17]. While clinical severity is not always predictable, there are some existing genotype-phenotype correlations [18]. Therefore, genetic

testing of patients with CTLN1 is valuable for long-term management. Additionally, it is beneficial for families through establishing recurrence risk of disease and for facilitating prenatal diagnosis of future pregnancies if desired. Even with recent advances of genetic testing techniques, there have been a small subset of patients who were biochemically diagnosed CTLN1 without detectable *ASS1* pathogenic variants [7]. In these cases, it is predicted that they may have variants

affecting either regulatory regions or splicing of *ASS1* as currently available clinical sequencing tests mainly cover the coding regions and their exon/intron boundaries. Through genomic DNA sequencing throughout the non-coding sequences of the gene, we were able to successfully identify a novel pathogenic microdeletion affecting the non-coding promoter area, including the entirety of the non-coding exon 1 of the *ASS1* gene. This finding supports the hypothesis and it is speculated that variants in the non-coding regions of *ASS1* can account for some CTLN1 patients without detectable *ASS1* variants. During the genetic work up, Patient #1 had both NGS-based *ASS1* sequencing and whole exome sequencing. Upon reviewing the covered sequences in both tests, we retrospectively identified the enrichment processes excluded the 5'-UTR from sequencing. Previously, three variants affecting the region around the 5'-UTR have been reported. Those include, c.-4C > T [18,19], c.-5-10C > G [9] and a large deletion spanning from exon 1 to exon 2 [20]. The severity of disease of those patients is unclear due to the limited clinical information available. However, their clinical biochemical characteristics were consistent with CTLN1, highlighting the importance of the regulatory sequence of *ASS1*.

The enrolled patients were both of Romani descent and remotely related. They also have a family history of a common relative who has biochemically diagnosed CTLN1. According to the result of aCGH with SNP probes for the Patient #1, one of the AOH regions was 9q33.2q34.12 that contains the entire *ASS1* gene. Along with high consanguinity rates in this population, the obtained findings and medical history of those patients indicated that this new variant could possibly be a novel *ASS1* founder variant in the Roma population, while a larger scale screening of this variant in this population is needed to confirm that. Nevertheless, since conventional clinical sequencing tests currently available are unlikely to detect this Roma *ASS1* variant, investigation of this microdeletion is essential, particularly for Roma individuals with biochemically confirmed CTLN1.

One of the challenges of urea cycle disorder research is the limited amount of resources available for *in vitro* analysis using cell models. The urea cycle occurs mainly in the liver and the ASS protein is predominantly expressed in the liver and some in the kidney [1,2]. Therefore, it is difficult to test the actual expression reduction of ASS protein from the deletion unless using liver tissues from patients. We attempted a quantitative reverse transcription-PCR assay to validate the mRNA reduction using cDNAs derived from lymphocytes of both patients, however, cDNA amplifications to compare the expression levels of *ASS1* mRNA was unsuccessful due to low ASS expression in the lymphocytes and existence of multiple pseudogenes (data not shown). Fortunately, transcriptional regulation of *ASS1* has been well-studied using melanoma cell lines, which are susceptible to arginine depletion [21]. One of the melanoma cell lines in particular, A2058, has an interesting characteristic that enhances *ASS1* mRNA transcription to gain tumor survival advantages from arginine depletion. This cell line has previously been used for analyzing the essential *ASS1* transcriptional elements [12]. It was reported that the 5' sequence of *ASS1* encompassing the microdeletion contains multiple essential elements that up-regulate mRNA expression [12]. By luciferase reporter assay, we confirmed the microdeletion led to a significant loss of the expression levels of the downstream gene. A similar finding was also obtained when we used a hepatoma cell line, HepG2, which is known to have relatively lower ASS protein expression compared to other hepatocellular cancer cell lines [22]. This overall suggests the microdeletion causes a reduction of *ASS1* mRNA levels.

Considering the homozygosity of this microdeletion, it is expected the ASS protein expression level and its resulting total enzymatic activity in the liver of patients are severely impaired. Most CTLN1 patients have a wide range of decreased ASS activities (2–62% compared to controls) measured by multiple methods [9]. The difference between clinical severity and the degree of biochemical abnormalities, including plasma citrulline levels, may be a result of the residual ASS activity [9,23]. Of interest, despite difficulties in adherence to dietary and

medical management, our patients have been relatively resistant to metabolic decompensation during intercurrent illnesses. Also, they did not have early neonatal-onset hyperammonemic crisis and have not required frequent hospital admissions with hyperammonemic episodes (once over 3 years for Patient #1 and four times over 16 years both excluding the initial hyperammonemic crisis). As shown by the luciferase reporter assay, the mutant allele may still have some minimal transcription activity in the tested cell lines. Particularly in the liver where the transcriptional activity for the *ASS1* mRNA is much more robust, it seems likely that there might be some residual ASS protein. Since there was no detectable variant in the coding sequence, even a small amount of ASS protein may be protective against severe hyperammonemic crisis, and this could be a partial explanation of the disease phenotype of these patients.

It has been proposed that *ASS1* mRNA expression is controlled by the regulation of transcription factors or DNA methylation of the promoter regions [22,24]. Previously, it was shown that the binding of transcription factors such as HIF1 $\alpha$  and Sp4 to the promoter elements down-regulate the *ASS1* transcriptional activity in A2058 cells [12]. The mechanism of *ASS1* mRNA expression regulation is complex and still incompletely understood. The results presented herein support the importance of the regulatory elements identified in the 5'UTR in driving liver expression of *ASS1* but the relatively milder clinical phenotypes of the affected patients suggest additional regulatory elements contribute to gene expression. Further studies to further characterize the regulatory elements involved in gene expression could potentially lead to novel therapies for CTLN1 patients with some *ASS1* variants.

In summary, we have identified a 5' microdeletion variant involving the non-coding exon of *ASS1* in Romani individuals affected with CTLN1. Our experimental data indicated that the microdeletion disrupts essential promoter elements and acts as a loss-of-function variant. This finding will enhance understanding of the genetic elements important for the regulation of ASS expression and their role in disease expression in CTLN1. Lastly, careful investigation of the 5'-UTR sequences including exon 1 of the *ASS1* gene is highly recommended for biochemically confirmed CTLN1 with unidentified *ASS1* variants.

## Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymgmr.2020.100619>.

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