American Journal of Medical Genetics Part A

WILEY

AMERICAN JOURNAL OF medical genetics PART A

CASE REPORT OPEN ACCESS

Nanopore Sequencing Solves an Elusive Case of Sotos Syndrome

Pasquale Di Letto[1] | Alberto Budillon[1,2] | Sarah Iffat Rahman[1] | Francesca Del Vecchio Blanco[1] | Mariateresa Zanobio[1] | Margherita Scarpato[1] | Margherita Russo[1] | Maria Elena Onore[1] | Giulio Piluso[1] | TUDP Study Group | Vincenzo Nigro[1,3] | Gioacchino Scarano[4,5] | Annalaura Torella[1,3]

[1]Department of Precision Medicine, University of Campania Luigi Vanvitelli, Naples, Italy | [2]Department of Medicine, University of Trieste, Surgery and Health Science, Trieste, Italy | [3]Telethon Institute of Genetics and Medicine, Pozzuoli, Italy | [4]Inherited and Rare Cardiovascular Diseases Unit, Department of Translational Medical Sciences, University of Campania "Luigi Vanvitelli," Monaldi Hospital, Naples, Italy | [5]Medical Genetics Unit, AORN "San Pio,", Hospital "G. Rummo", Benevento, Italy

Correspondence: Vincenzo Nigro (vincenzo.nigro@unicampania.it)

Received: 20 November 2024 | **Revised:** 18 February 2025 | **Accepted:** 21 February 2025

Funding: This work was supported by Fondazione Telethon (TUDP Grant No. GSP1500124 to V.N.), European Union-Next Generation EU-NRRP M6C2-Investment 2.1 Enhancement and strengthening of biomedical research in the NH (PNRR-MR1-2022-12376412; CUP61H22000130001 to V.N.), the Ministry of University and Research (MUR; PRIN2022 code 2022L4F87B to V.N.) and "Genoma mEdiciNa pERsonalizzatA—GENERA" (T3-AN-04 to V.N.).

Keywords: alu-mediated rearrangement | Long-read sequencing | NSD1 | Sotos Syndrome | structural variant

ABSTRACT

Sotos syndrome is a rare genetic disorder characterized by distinctive facial features, including a broad and prominent forehead, dolichocephaly, and learning disabilities ranging from mild to severe intellectual impairment. Affected individuals often show overgrowth in height and head circumference over two standard deviations. The syndrome is caused by haploinsufficiency of the *NSD1* gene, with no evidence of genetic heterogeneity to date. Here we describe the unsolved case of a child of 4 years of age with a clinical diagnosis of Sotos syndrome. However, trio exome sequencing (ES) and exon chromosomal microarray (CMA) analysis excluded both small and large mutations in the *NSD1* gene. As part of the Telethon Undiagnosed Programme, we used additional tools to investigate the possibility of new genes or elusive mutations that may have been missed by previous molecular diagnostic approaches. Therefore, we performed Nanopore long-read sequencing. This revealed a 447 bp insertion in exon 13 of the *NSD1* gene. mRNA analysis confirmed in-frame skipping of exon 13 that encodes for two PHD domains. The genomic insertion shows 100% identity with an intronic region, although inverted, containing an *AluSx1* element 2 kb upstream of the skipped exon, which may drive the event by masking the splice acceptor site of exon 13. Interestingly, this is the first case of Sotos syndrome linked to a pathogenic mechanism involving an insertion enclosing a transposable element generating a protein devoid of two PHDs, which are required for reading histone post-translational modifications.

1 | Introduction

Sotos syndrome is a rare genetic disorder characterized primarily by excessive physical growth in the early years of life, along with distinctive facial features and varying degrees of learning disabilities. Individuals with the condition grow rapidly in infancy and childhood, resulting in above-average height and head circumference. Characteristic facial features include a long, narrow face, a high forehead, flushed cheeks, and a pointed chin. Other physical symptoms may consist of large hands and feet,

Pasquale Di Letto and Alberto Budillon contributed equally to this work.

The members of TUDP Study Group are listed in Appendix A.

hypotonia, and scoliosis (Tatton-Brown and Rahman 2007). Additionally, children with Sotos syndrome often face developmental delays, speech impairments, and learning difficulties. Behavioral problems such as attention deficit hyperactivity disorder and autism spectrum disorder are also common (Baujat and Cormier-Daire 2007). Sotos syndrome is primarily associated with de novo variants in the *NSD1* gene, which encodes a nuclear receptor-binding SET domain protein that is central to chromatin modification and transcriptional regulation. Both single nucleotide variants (SNVs) and large copy number variations (CNVs) encompassing part or all of the *NSD1* gene have been implicated in the manifestation of the syndrome (Tatton-Brown et al. 2004).

Here we report a case of a 4-year-old child with a very striking clinical diagnosis of Sotos syndrome, which was negative on all genetic tests. In fact, Trio ES and Exon Array CGH analysis had already excluded both small and large mutations in the NSD1 gene, and this case was classified as undiagnosed. As part of the Telethon Undiagnosed Programme, we have included this family in a diagnostic pathway of additional genomic tools to investigate the possibility of new genes or elusive mutations that may have been missed by previous molecular diagnostic approaches.

2 | Material and Methods

2.1 | Exome Sequencing (ES)

DNA was extracted from peripheral blood using the FlexiGene DNA kit (Qiagen) and quantified with NanoPhotometer (IMPLEN). Libraries were prepared following the SureSelectQXT protocol (Agilent) and enriched using SureSelect Human All Exon v7 (Agilent). Sequencing was performed on NovaSeq6000 (Illumina) with paired-end 2×150 nt runs. An in-house pipeline analyzed the data, achieving ≥100× average coverage, with 90% of bases covered by ≥40 reads.

2.2 | Copy Number Variants (CNVs) Analysis

CNVs were assessed using a custom exome-based SurePrint G3 1X1M CMA array (Agilent). DNA labeling and hybridization followed manufacturer protocols. Cytogenomics software analyzed the data.

2.3 | Long-Read Sequencing (LRS)

High molecular weight DNA was extracted using the PureGene Blood Core kit (Qiagen), quantified with Qubit (Thermo Fisher), and quality-checked using TapeStation (Agilent). Libraries were prepared using the ONT Ligation Sequencing Kit (SQK-LSK114) and sequenced on the PromethION with chromosome 5 enrichment via Adaptive Sampling.

2.4 | RNA Transcript Analysis

RNA was extracted using the Tempus Spin RNA Isolation Kit (Applied Biosystems) and quantified with NanoPhotometer. cDNA synthesis was performed with SuperScript VILO (Thermo Fisher). The NSD1 canonical transcript (NM_022455.5) was amplified by PCR, and exons 12–14 were sequenced with BigDye v3.1 on the 3500 Genetic Analyzer (Applied Biosystems).

2.5 | Bioinformatics

Exome data were aligned to GRCh38/hg38 using BWA (Li and Durbin 2009) and processed with GATK v4.4.0.0 (McKenna et al. 2010) for SNVs and indels. Variants were annotated using VEP release/109 (McLaren et al. 2016). ONT data were processed with MinKNOW for FASTQ/BAM generation and analyzed with EPI2ME labs (v2.2.0) for SNVs, structural variants, and CNVs.

3 | Results

3.1 | Clinical Case

Here we present the case of a 4-year-old child born to unrelated, healthy parents (Figure 1A,B). The older sibling, a 9-year-old sister, has no health issues. The proband was delivered naturally at 39 weeks and 6 days of gestation, following a pregnancy marked by a Parvovirus B19 infection, which did not cause any complications. Prenatal ultrasound scans identified left pyelectasis measuring 8 mm, associated with hydronephrosis. Non-invasive prenatal testing (NIPT) indicated normal results. At birth, the proband's weight was 3920 g (79th percentile), length was 54 cm (92nd percentile), and head circumference was 39 cm (+3.5 SD). Apgar scores were 8 at 1 min and 9 at 5 min. Due to reduced urine output, the newborn was admitted to the Neonatal Intensive Care Unit (NICU) for 2 weeks.

Genetic counseling was initiated at birth because of observed macrocephaly and dysmorphic features, strongly suggestive of Sotos syndrome. A brain MRI performed at 11 days of age revealed multiple anomalies: mega cisterna magna, cysts of the septum pellucidum, bifrontal periventricular porencephalic cavities, skull base dysmorphia, and cerebellar hypoplasia. An EEG showed dysmature activity. The initial ultrasound-detected left pyelectasis was confirmed with a left anteroposterior diameter (APD) of 14 mm.

During the first month, a voiding cystourethrography (VCUG) was conducted, revealing bilateral vesicoureteral reflux (VUR). Subsequently, the proband underwent endoscopic laser surgery to treat a posterior urethral valve. Post-surgical renal scintigraphy, evaluating renal parenchymal function and washout, showed normal results. The proband experienced a few episodes of urinary tract infections (UTIs), which were managed with Glazidin and prophylactic antibiotics.

Follow-up abdominal ultrasound examinations were normal. Initial echocardiographic findings of atrial and ventricular septal defects were not confirmed at the subsequent check-up 1 month later; however, mild mitral regurgitation was detected.

Our latest physical examination at the age of 4 years 9 months noted several features: macrocephaly, broad forehead, elongated

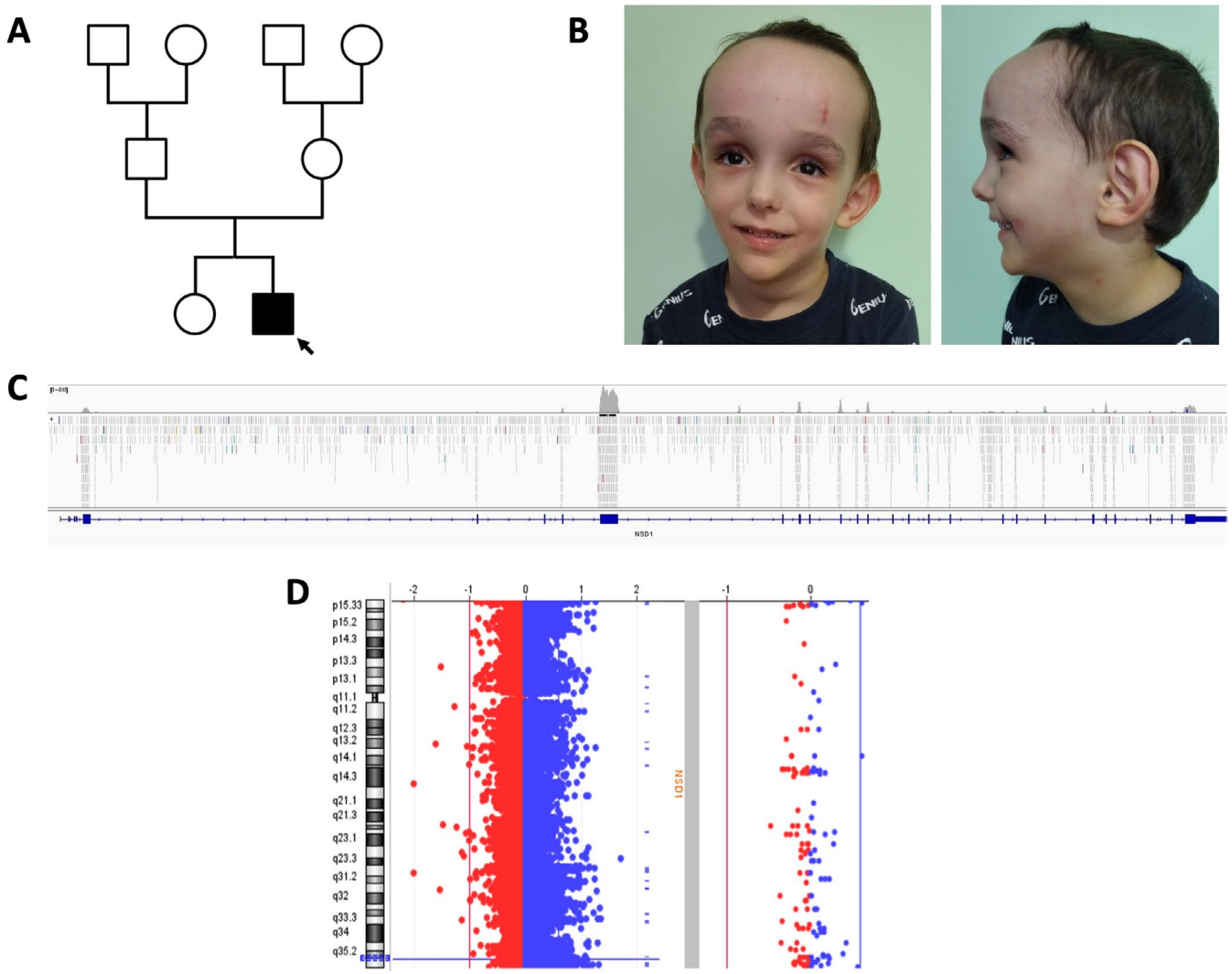

FIGURE 1 | (A) Family pedigree. (B) Proband's photos show the typical *facies* associated with Sotos syndrome, such as macrocephaly with a bitemporal narrow and prominent forehead. (C) IGV screenshot of ES reads on *NSD1* gene showing no variant calling. (D) Cytogenomics screenshot of aCGH analysis showing no CNVs detection.

face, large and protruding ears, pronounced plantar folds, and axial hypotonia. The proband's weight was 17,600 kg (25th percentile), height was 110 cm (50th percentile) and his head circumference was 58 cm (+5.6 SD). The echocardiogram revealed mild mitral valve insufficiency and pronounced trabeculation of the left ventricle. Auditory brainstem response (ABR) testing and an eye examination were both normal.

3.2 | Molecular Analysis

To assess a possible mutation in the *NSD1* genomic region, we used a high-resolution, custom-designed CMA covering each individual exon, which did not reveal any major abnormalities (Figure 1D). In addition, ES analysis did not identify any potentially pathogenic SNVs (Figure 1C). Both first-level analyses did not reveal any variants in the *NSD1* gene or other genes associated with conditions resembling the clinical manifestations of Sotos syndrome. This case was therefore considered undiagnosed and analyzed as part of the activities of the TUDP.

The unambiguous clinical presentation drove us to further search for non-common causative variations related to the *NSD1* gene. Confident with this, long-read sequencing was performed using the ONT platform. With the Adaptive Sampling technology, the sequencing targeted specifically chromosome 5 and achieved a 100× depth. This approach allowed for high-resolution analysis of the region of interest, still maintaining comprehensive coverage of the whole genome, which was fully sequenced with shorter reads. This ensured no potential information loss across the genome. The Epi2me human-variation workflow for SVs detected a 447 bp insertion within exon 13 (chr5:177257134_177257135) in a heterozygous state (Figure 2A). We analyzed the inserted stretch, aligning it against the reference genome. Using UCSC Genome Browser BLAT, we found a 100% similarity with a deep intronic region (chr5:177254858–177,255,304) that includes an *Alu* sequence, precisely *AluSx1*, located 2 kb upstream of exon 13 of *NSD1*. The specific breakpoints for the insertion were validated with Sanger sequencing (Figure 2B,C). Interestingly, the inserted stretch was inverted compared to the intronic one.

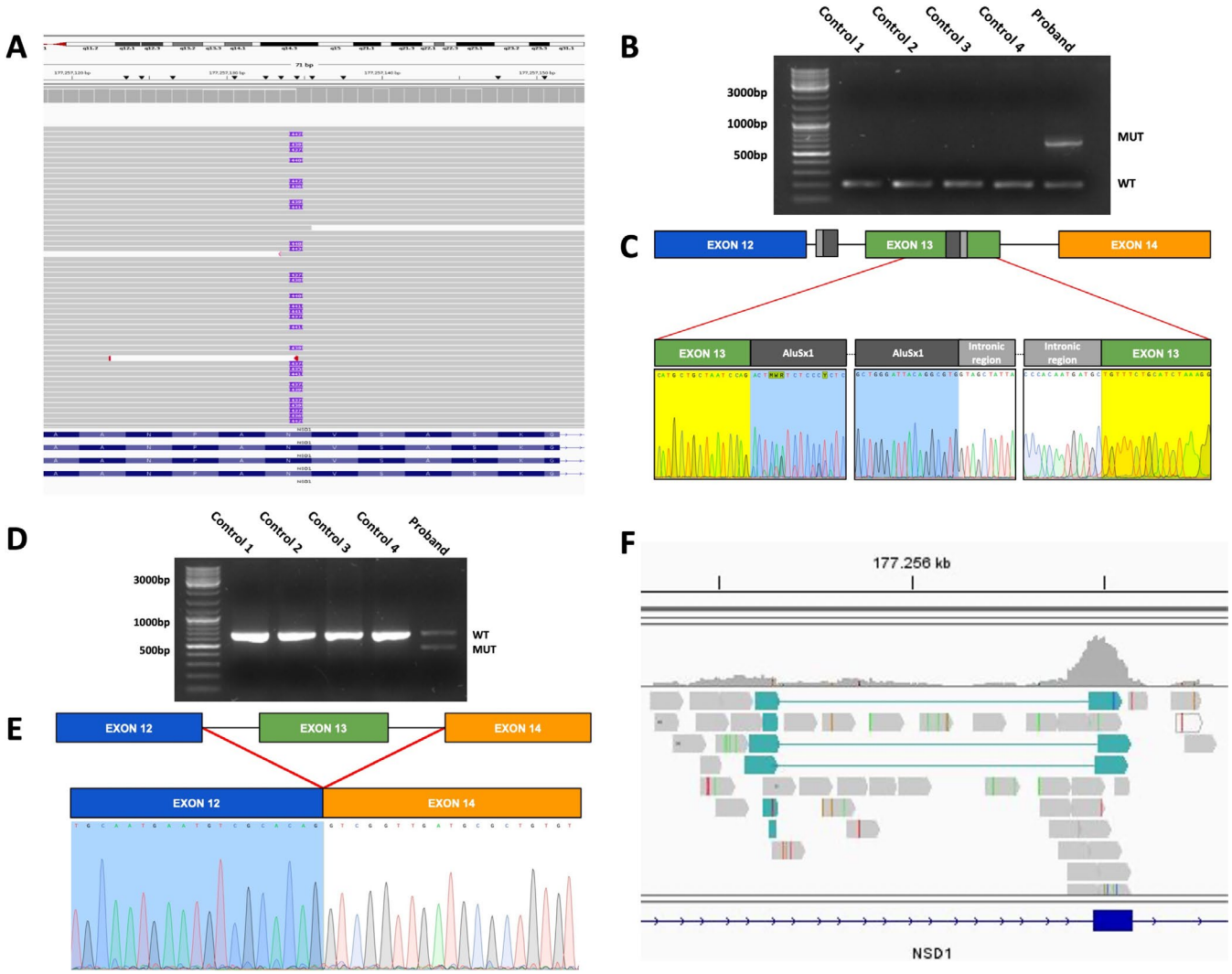

FIGURE 2 | (A) IGV screenshot on *NSD1* exon 13 displaying an insertion of ~440 bp at heterozygous state. (B) PCR on genomic DNA for the *NSD1* exon 13 region on proband and control samples. The electrophoresis displays an additional amplicon for the proband that is higher in molecular weight. (C) Schematic representation of the *Alu*-inserted element within the *NSD1* exon 13 with the breakpoint sequences of the *Alu*-inserted element and the intronic portion within the *NSD1* exon 13. (D) PCR on proband and control samples' cDNA (*NSD1* exons 12, 13 and 14). The electrophoresis displays an additional lower amplicon for the proband. (E) Schematic representation of the *NSD1* exon 13 skip at RNA level showing the direct junction between exon 12 and 14. (F) IGV screenshot of ES reads showing a putative inversion in *NSD1* exon 13.

Given the above, we decided to investigate how this insertion might affect RNA splicing. We analyzed the entire canonical *NSD1* transcript (NM_022455.5) using 14 overlapping primer pairs to ensure reliable readability of the PCR products for the cDNA sequence. An amplicon of about 500 bp, not present in the controls, was detected in the region encompassing exons 12, 13, and 14. The sequence of the aberrant PCR product showed the absence of exon 13 in the heterozygous state, suggesting a defective splicing mechanism consistent with disease onset (Figure 2D,E).

4 | Discussion

In this study, we encountered a diagnostically challenging case of Sotos syndrome for which the usual diagnostic workflow was insufficient. Our initial approach followed the established guidelines, incorporating SNVs and CNVs analyses. However, the patient's clinical features strongly suggested Sotos syndrome, prompting us to extend our investigation through Whole Genome Sequencing (WGS).

We employed long-read Oxford Nanopore Technology (ONT), which revealed a 447 bp insertion within exon 13 in a heterozygous state. A SINE (Short Interspersed Nuclear Element) belonging to the *AluSx* family was identified in this insertion. Interestingly, a retrospective revision of ES data revealed the presence of some poor-quality reads in exon 13, suggesting a potential rearrangement, such as inversion (Figure 2F). However, the data was difficult to interpret due to the intrinsic complexity of the genomic region, which is characterized by the presence of numerous similar *Alu* elements.

Alu elements are the most abundant transposable elements (TEs) in the human genome, comprising over a million copies. As SINEs, *Alu* elements replicate through RNA intermediates

in a "copy and paste" mechanism (Deininger and Batzer 1999). Their insertion plays a significant role in genetic variation and evolution (Feschotte and Pritham 2007). Furthermore, *Alu* rearrangements near gene loci can variably impact gene expression, from being silent to causing gene silencing, activation, or even creating new splice variants (Lee et al. 2024). These disruptions can affect normal gene function, contributing to genetic disorders and complex traits, thereby offering unique challenges and opportunities for genetic research and diagnostics.

To further elucidate the pathological mechanism of this in-frame insertion, we focus our attention on the RNA level, seeking the effect on mRNA production and maturation. The cDNA analysis revealed an aberrant PCR product lacking exon 13, indicative of a splicing defect.

Here, we propose a molecular mechanism for this aberrant splicing event. Indeed, the physiological upstream region, which includes the *AluSx1* element, with a 100% similarity with the insertion of interest, is approximately 2 kb from exon 13. This upstream *Alu*, in concert with the antisense-oriented *AluSx1* within exon 13, likely forms a double-stranded loop. This loop could mask the splice sites, prompting the spliceosome to skip exon 13 and join the donor splice site of exon 12 with the acceptor splice site of exon 14 (Figure S1). Indeed, it is well-known that divergently oriented *Alu* sequences, when placed in specific configurations, can act as splicing regulators, influencing transcript maturation both physiologically (Lev-Maor et al. 2008) and pathologically (Nakama et al. 2018; Payer et al. 2019).

In our case, the resultant transcript, although in-frame, lacks several critical domains essential for the NSD1 protein's catalytic function. Indeed, exon 13 contains essential PHD domains involved in recognizing and binding methylated histones, crucial for the gene's regulatory functions. The absence of the domain disrupts the protein's catalytic activity, which is consistent with the clinical presentation of Sotos syndrome (Tauchmann and Schwaller 2021). Notably, a pathogenic splicing variant at the donor site of exon 13 has been reported in the literature. This variant yields the same exon-skipping event, thus supporting the pathological role of this *Alu* insertion (Tei et al. 2006).

Overall, our findings suggest that the insertion of the *AluSx1* element caused the skipping of exon 13, a mechanism never before documented in Sotos syndrome, but already reported for other genetic disorders (Torella et al. 2023; Bouras et al. 2021; Awano et al. 2010).

5 | Conclusions

Our study extends the understanding of Sotos syndrome's genetic basis, illustrating that even rare and non-conventional molecular events can drive disease pathology. Given that Sotos syndrome is relatively common compared to many genetic disorders, there could be numerous undiagnosed cases with similar cryptic mechanisms, undetectable through standard diagnostic approaches. This highlights the necessity of combining multiple techniques and integrating advanced genomic technologies with a comprehensive clinical assessment to uncover these hidden aetiologies.

Author Contributions

A.T., G.S., and V.N. conceived and coordinated the project, reviewed and edited the manuscript. P.D.L. and A.B. performed and interpreted molecular and data analysis, drafted the manuscript, and figures. G.P. reviewed and edited the manuscript. M.Z., M.S., M.R., and M.E.O. performed NGS and CGH-array. S.I.R. generated the bioinformatic data. G.S. and F.D.V.B. characterized and collected clinical data.

Acknowledgments

This work was supported by Fondazione Telethon (TUDP Grant No. GSP1500124 to V.N.), European Union-Next Generation EU-NRRP M6C2-Investment 2.1 Enhancement and strengthening of biomedical research in the NH (PNRR-MR1-2022-12376412; CUP61H22000130001 to V.N.), the Ministry of University and Research (MUR; PRIN2022 code 2022L4F87B to V.N.) and "Genoma mEdiciNa pERsonalizzatA—GENERA" (T3-AN-04 to V.N.). Vincenzo Nigro, Sandro Banfi, Annalaura Torella, Manuela Morleo, Nicola Brunetti Pierri, Giancarlo Parenti, Carmine Spampanato, Giulio Piluso, Michele Pinelli, Giuseppina Vitiello, Angelo Selicorni, Milena Mariani, Cecilia Daolio, Valeria Capra, Andrea Accogli, Marcello Scala, Francesca Nardecchia, Serena Galosi, Mario Mastrangelo, Donatella Milani, Corrado Romano, Pinella Failla, Donatella Greco, Chiara Pantaleoni, Claudia Ciaccio, Stefano D'Arrigo, Antonietta Coppola, Teresa Mattina, Marcella Zollino, Domizia Pasquetti, Federica L. Erario, Albina Tummolo, Claudia Santoro, Anna Grandone, Livia Garavelli, Carla Marini, Stefania Bigoni, Carmelo Piscopo, Antonio Trabacca, Marta De Rinaldis,

Ethics Statement

The study was approved by the local Ethics Committee "Comitato Etico Territoriale Campania 2" Prot. 0018726 of 04/07/2024. Patients provided written informed consent for the participation in the study and sharing images according to the Italian National Health System guidelines and the Declaration of Helsinki.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Additional data are available from the corresponding author on reasonable request.

References

Awano, H., R. G. Malueka, M. Yagi, Y. Okizuka, Y. Takeshima, and M. Matsuo. 2010. "Contemporary Retrotransposition of a Novel Non-Coding Gene Induces Exon-Skipping in Dystrophin mRNA." *Journal of Human Genetics* 55, no. 12: 785–790. https://doi.org/10.1038/jhg.2010.111.

Baujat, G., and V. Cormier-Daire. 2007. "Sotos Syndrome." *Orphanet Journal of Rare Diseases* 2: 36. https://doi.org/10.1186/1750-1172-2-36.

Bouras, A., M. Leone, V. Bonadona, M. Lebrun, A. Calender, and N. Boutry-Kryza. 2021. "Identification and Characterization of New *Alu* Element Insertion in the *BRCA1* Exon 14 Associated With Hereditary Breast and Ovarian Cancer." *Genes (Basel)* 12, no. 11: 1736. https://doi.org/10.3390/genes12111736.

Deininger, P. L., and M. A. Batzer. 1999. "Alu Repeats and Human Disease." *Molecular Genetics and Metabolism* 67, no. 3: 183–193. https://doi.org/10.1006/mgme.1999.2864.

Feschotte, C., and E. J. Pritham. 2007. "DNA Transposons and the Evolution of Eukaryotic Genomes." *Annual Review of Genetics* 41: 331–368.

Lee, K., J. Ku, D. Ku, and Y. Kim. 2024. "Inverted Alu Repeats: Friends or Foes in the Human Transcriptome." *Experimental & Molecular Medicine* 56: 1250–1262. https://doi.org/10.1038/s12276-024-01177-3.

Lev-Maor, G., O. Ram, E. Kim, et al. 2008. "Intronic Alus Influence Alternative Splicing." *PLoS Genetics* 4, no. 9: e1000204. https://doi.org/10.1371/journal.pgen.1000204.

Li, H., and R. Durbin. 2009. "Fast and Accurate Short Read Alignment With Burrows-Wheeler Transform." *Bioinformatics* 25, no. 14: 1754–1760. https://doi.org/10.1093/bioinformatics/btp324.

McKenna, A., M. Hanna, E. Banks, et al. 2010. "The Genome Analysis Toolkit: A MapReduce Framework for Analyzing Next-Generation DNA Sequencing Data." *Genome Research* 20, no. 9: 1297–1303. https://doi.org/10.1101/gr.107524.110.

McLaren, W., L. Gil, S. E. Hunt, et al. 2016. "The Ensembl Variant Effect Predictor." *Genome Biology* 17, no. 1: 122. https://doi.org/10.1186/s13059-016-0974-4.

Nakama, M., H. Otsuka, Y. Ago, et al. 2018. "Intronic Antisense Alu Elements Have a Negative Splicing Effect on the Inclusion of Adjacent Downstream Exons." *Gene* 664: 84–89. https://doi.org/10.1016/j.gene.2018.04.064.

Payer, L. M., J. P. Steranka, D. Ardeljan, et al. 2019. "Alu Insertion Variants Alter mRNA Splicing." *Nucleic Acids Research* 47, no. 1: 421–431. https://doi.org/10.1093/nar/gky1086.

Tatton-Brown, K., T. R. P. Cole, and N. Rahman. 2004. *Sotos Syndrome*, edited by M. P. Adam, J. Feldman, G. M. Mirzaa, et al., 1993–2024. GeneReviews [Internet].

Tatton-Brown, K., and N. Rahman. 2007. "Sotos Syndrome." *European Journal of Human Genetics* 15, no. 3: 264–271. https://doi.org/10.1038/sj.ejhg.5201686.

Tauchmann, S., and J. Schwaller. 2021. "NSD1: A Lysine Methyltransferase Between Developmental Disorders and Cancer." *Lifestyles* 11, no. 9: 877. https://doi.org/10.3390/life11090877.

Tei, S., S. Tsuneishi, and M. Matsuo. 2006. "The First Japanese Familial Sotos Syndrome With a Novel Mutation of the NSD1 Gene." *Kobe Journal of Medical Sciences* 52, no. 1–2: 1–8.

Torella, A., A. Budillon, M. Zanobio, et al. 2023. "Alu-Mediated Insertions in the *DMD* Gene: A Difficult Puzzle to Interpret Clinically." *International Journal of Molecular Sciences* 24, no. 11: 9241. https://doi.org/10.3390/ijms24119241.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Appendix A

TUDP Study Group.

Vincenzo Nigro[1,3], Sandro Banfi[1,3], Annalaura Torella[1,3], Manuela Morleo[1,3], Nicola Brunetti Pierri[3,14], Giancarlo Parenti[3], Carmine Spampanato[1], Giulio Piluso[1], Michele Pinelli[6], Giuseppina Vitiello[6], Angelo Selicorni[7], Milena Mariani[7], Cecilia Daolio[8], Valeria Capra[9], Andrea Accogli[9], Marcello Scala[9], Francesca Nardecchia[10], Serena Galosi[10], Mario Mastrangelo[10], Donatella Milani[11], Corrado Romano[12], Pinella Failla[12], Donatella Greco[12], Chiara Pantaleoni[13], Claudia Ciaccio[13], Stefano D'Arrigo[13], Antonietta Coppola[14], Teresa Mattina[15], Marcella Zollino[16], Domizia Pasquetti[16], Federica L. Erario[16], Albina Tummolo[17], Claudia Santoro[18], Anna Grandone[18], Livia Garavelli[19], Carla Marini[20], Stefania Bigoni[21], Carmelo Piscopo[22], Antonio Trabacca[23], Marta De Rinaldis[23].

6. Department of molecular medicine and medical biotechnology, Federico II University, Naples, Italy.

7. Department of Pediatrics, ASST Lariana Sant'Anna Hospital, San Fermo Della Battaglia, Como, Italy.

8. MBBM Foundation, Monza, Italy.

9. Neuroscience Department, Giannina Gaslini Institute, Genoa, Italy.

10. Department of Human Neuroscience, Sapienza University of Rome, Italy.

11. Pediatric Highly Intensive Care Unit, Fondazione IRCCS Ca′ Granda, Ospedale Maggiore Policlinico, Milan, Italy.

12. Oasi Research Institute—IRCCS, Troina, Italy.

13. Developmental Neurology Unit, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy.

14. Department of Translational Medicine, Section of Pediatrics, Federico II University, Naples, Italy.

15. Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy.

16. Institute of Genomic Medicine, Catholic University, Gemelli Hospital Foundation, Rome, Italy.

17. Department of Metabolic Diseases, Clinical Genetics and Diabetology, Giovanni XXIII Children's Hospital, Bari, Italy.

18. Department of Woman, Child and of General and Specialized Surgery, Università degli Studi della Campania "Luigi Vanvitelli", Napoli, Italy.

19. Medical Genetics Unit, Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy.

20. Child Neurology and Psychiatric Unit, Pediatric Hospital G. Salesi, United Hospitals of Ancona, Ancona, Italy.

21. Medical Genetics Unit, Department of Mother and Child, University Hospital Sant'Anna of Ferrara, Ferrara, Italy.

22. Medical Genetics Unit, Cardarelli Hospital, Napoli, Italy.

23. Unit for Severe Disabilities in Developmental Age and Young Adults, Developmental Neurology and Neurorehabilitation, Scientific Institute IRCCS "*E. Medea*", Brindisi, Italy.