neurogenetics

9q34.3 microduplications lead to Neurodevelopmental Disorders through EHMT1 overexpression

--Manuscript Draft--

Manuscript Number:	
Full Title:	9q34.3 microduplications lead to Neurodevelopmental Disorders through EHMT1 overexpression
Article Type:	Original Article
Keywords:	9q34 duplication syndrome, Kleefstra syndrome, EHMT1, 9q34.3 microduplications, neurodevelopmental disorders, autism
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Funding Information:	
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	the available clinical and molecular cytogenetic data, we were able to discover that similar neurodevelopmental disorders (NDDs) were shared by patient carriers of even very differently sized duplications. Moreover, some facial features of the 9q34 DS were more represented than those of KS. However, an accurate in silico analysis of the genes mapped in all the duplications allowed us to support EHMT1 as being sufficient to cause a NDD phenotype. Wider patient cohorts are needed to ascertain whether the rearrangements have full causative role or simply confer the susceptibility to NDDs and possibly to identify the cognitive and behavioral profile associated with the increased dosage of EHMT1.

9q34.3 microduplications lead to Neurodevelopmental Disorders through *EHMT1* overexpression

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Abstract

Both copy number losses and gains occur within subtelomeric 9q34 region without common breakpoints. The microdeletions cause Kleefstra syndrome (KS), whose responsible gene is *EHMT1*. A 9q34 duplication syndrome (9q34 DS) had been reported in literature, but it has never been characterized by a detailed molecular analysis of the gene content and endpoints.

To the best of our knowledge, we report on the first patient carrying the smallest 9q34.3 duplication containing *EHMT1* as the only relevant gene.

We compared him to 21 reported patients described here as carrying 9q34.3 duplications encompassing the entire gene and extending within ~3 Mb. By surveying the available clinical and molecular cytogenetic data, we were able to discover that similar neurodevelopmental disorders (NDDs) were shared by patient carriers of even very differently sized duplications. Moreover, some facial features of the 9q34 DS were more represented than those of KS. However, an accurate *in silico* analysis of the genes mapped in all the duplications allowed us to support *EHMT1* as being sufficient to cause a NDD phenotype.

Wider patient cohorts are needed to ascertain whether the rearrangements have full causative role or simply confer the susceptibility to NDDs and possibly to identify the cognitive and behavioral profile associated with the increased dosage of *EHMT1*.

Key words

9q34 duplication syndrome, Kleefstra syndrome, *EHMT1*, 9q34.3 microduplications, neurodevelopmental disorders, autism

Introduction

Chromosome 9q34 subtelomeric region shows genomic instability, as both copy number losses and gains – terminal or interstitial– have been reported to occur without common breakpoints [1].

Deletions of distal 9q34 encompassing *EHMT1* (euchromatic histone methyltransferase 1) gene (MIM*607001), as well as loss of function point mutations, are responsible for Kleefstra syndrome (KS, MIM #610253), characterized by facial characteristics, (childhood) hypotonia, developmental delay (DD), intellectual disability (ID), and other variable clinical features [2-4]. Based on multiple reports, 23–100% of subjects with KS have autism spectrum disorder (ASD) [5-7]. Moreover, a pathogenic variant has been recently identified in a patient with autism and normal intelligence [8].

EHMT1 is involved in chromatin remodeling during neurodevelopment and homeostatic plasticity through synaptic scaling [9].

A 9q34 duplication syndrome (9q34 DS) has been reported [10], involving both interstitial [11-14] and terminal [1, 10, 15-29] duplications, with different sizes. The associated manifestations include initial poor feeding and thriving, hypotonia, DD, mostly affecting speech and language, ID, craniofacial dysmorphisms and other musculoskeletal anomalies. Behavioral problems have been rarely described, and include hyperactivity, attention deficit hyperactivity disorder, and ASDs [27].

Starting from the identification of a small microduplication encompassing the dosage-sensitive *EHMT1* gene in a boy diagnosed in the autism spectrum, our aim was to understand whether the gene over-dosage may cause a recognizable phenotype, and whether this overlaps and/or mirrors that of KS.

Materials and methods

Patients

Informed consent was obtained from all individual participants included in the study or their legal guardians. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article. The study was approved by the Ethical Clinical Research Committee of IRCCS Istituto Auxologico Italiano.

Patient 1 (P1) belongs to a cohort of 325 Italian ASD patients that underwent chromosomal microarray analysis (CMA). After his clinical and molecular cytogenetic characterization we went on to retrieve detailed clinical and molecular data of comparable patients reported in public databases, i.e. SFARI (https://gene.sfari.org/) and DECIPHER (http://www.sanger.ac.uk/PostGenomics/decipher/). For this purpose, we used a uniform questionnaire. By

means of this investigation further clinical information has been made available for the two DECIPHER patients referred to as P2 and P4.

Array CGH analysis

Array Comparative Genomic Hybridization (a-CGH) analysis was performed on genomic blood DNA of P1 and his parents, using the SurePrint G3 Human CGH Microarray Kit 244K in accordance with the manufacturer's instructions (Agilent Technologies, Palo Alto, CA).

Detected Copy Number Variants (CNVs) were classified according to guidelines reported in Koolen et al. [30], Miller et al. [31] and Kearney [32]. The following public databases were consulted: University of California Santa Cruz (UCSC) (http://genome.ucsc.edu/, GRCh37/hg19), Online Mendelian Inheritance in Man (OMIM) (www.ncbi.nlm.nih.gov/OMIM), ClinVar (www.ncbi.nlm.nih.gov/clinvar/), SFARI, and Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/, released in March 2016). A CNV was classified as rare if unreported or reported at a very low frequency (≤0.05%) according to the DGV.

Fluorescent In Situ Hybridization (FISH) analysis

FISH analysis was performed on P1's metaphases and interphase nuclei from peripheral blood lymphocytes as previously described [33, 34]. The BAC probes RP11-644H13 (Invitrogen Ltd., Carlsbard, CA), which maps at 9q34.3 and covers *EHMT1* from IVS1 to IVS25 (NM_024757), was nick-translation labelled with Cy3-dUTP (Amersham, Chalfont St. Giles, UK). At least 20 metaphases and 100 nuclei were screened.

Gene expression analysis

Total RNA of P1, his father and 10 healthy controls was collected and isolated using the Tempus Blood RNA tubes and Spin RNA Isolation kit (Thermo Fisher Scientific, Waltham, MA), and reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The expression of *EHMT1*, *CACNA1B*, *SYF2* and *IL1RAPL2* was first verified on a control blood RNA as well as on commercial RNAs of human adult and fetal brain (Clontech Laboratories, Inc., Mountain View, CA) by using specific pairs of primers.

RT-qPCR based on the TaqMan methodology was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The amounts of *EHMT1* and *SYF2* mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA box binding protein (*TBP*) as the endogenous-normalizing genes. All assays were provided by Thermo Fisher Scientific (TaqMan Gene Expression Assays: ID# Hs00964325_m1 *EHMT1* (ex 14-15, isoforms NM_024757 and NM_001145527); Hs01548694_m1 SYF2 (ex 6-7, isoform NM_015484.4); Hs99999905_m1 *GAPDH*; Hs00427620_m1 *TBP*). Real Time data were analysed using the RQ Manager 1.2 software (Thermo Fisher Scientific).

We established the proper range of gene expression in 10 healthy controls calculating the mean value ±2 standard deviations (SD). If the expression level in the patient was out of the control range, a dysregulation of the index gene could be inferred.

Gene annotation

Genes included in the 9q34.3 microduplications were studied taking into account their: i) function and possible involvement in neurodevelopmental disorders (NDDs), according to Gene Cards (https://www.genecards.org), OMIM and SFARI; ii) brain expression; iii) prediction to be Intolerant to Loss of function variants according to a pLI score [35].

As for ii), RNAseq expression data have been queried from two public repositories, namely the Genotype-Tissue Expression (GTEx: https://gtexportal.org//home/datasets) and BrainSpan (http://www.brainspan.org/static/download.html). Specifically, on the GTEx database we averaged the expression of the human post-mortem adult brain tissues, whereas on the BrainSpan repository we only considered the prenatal expression, after grouping and averaging the data into four temporal categories (fetal, infancy, childhood, and adolescence/adulthood). We classified a gene as not brain expressed either when the average expression was <0.1, or when postnatally expressed only, whereas genes with unavailable prenatal data were considered as brain expressed.

As for iii), we inferred as possible over-dosage sensitive genes those showing a pLI≥0.90.

Results

Clinical reports

P1

P1, who was referred for genetic assessments at 26 months because of psychomotor delay, was a 13-year-old boy at his last follow-up. In Fig. 1 (a-d) his mild dysmorphic facial features, i.e. wide forehead, cowlick, flat nasal bridge, anteverted nostrils, and folded helix are shown. With age, eyebrows became pronounced (Fig. 1d). He was the second born to healthy unrelated parents after 38 weeks of gestation and delivered with C-sec because of the umbilical cord around his neck. His birth weight was 2,620 g (9-25th centile), birth length 47 cm (2nd centile), and occipitofrontal circumference (OCF) 34 cm (25-50th centile). Apgar score was 8 and 9 at 1 and 5 min. At the time of his birth, his mother was 42 and his father 56 years old.

He sat and stood alone at 11 and 21 months, respectively, and walked unassisted at 36 months. He was able to say his first words between 26 and 36 months. Then his expressive language improved further so that at 5 years and a half it was structured in sentences and rich in vocabulary, although not always consistent with the context. He was toilet

trained at 44 months; however, anal and bladder sphincter accidents could still occur until the age of 7 and 13 years, respectively, when he was tense or not able to ask for the toilet. At the age of 4,9 years his development was measured by Griffiths scales to be equivalent to that of a 3,8-year-old boy; total IQ was equal to 78 with strengths in the areas of hearing and language and weakness in the performance area. The diagnosis was of pervasive developmental disorder, unspecified and motor dyspraxia. As shown in Supplementary Table 1, at the age of 6 years and 6 months he met the criteria for autism diagnosis at the Autism Diagnostic Observation Schedule (ADOS), module 3 (for children/adolescents with fluent speech) [36].

At the age of 10 years he showed a high arch club feet equinovarus deformity leading to impairment of balance; leg surgery was carried out when he was 11 years and 3 months old (Fig. 1e, f), but he still had walking difficulties. X-ray showed a moderate wide-ranging right-convex lumbar scoliosis with vertebrae rotation, whereas neurological examination as well as brain and spinal cord MRI showed no abnormalities. At the time of clinical evaluation at 13 years, his weight was 38,7 Kg (>25th centile), height 161 cm (>75th centile), and OFC 53,7 cm (9-25th centile). Cognitive as well as emotional and social skills continued to be more impaired than speech and language.
Routine chromosomal analysis from amniotic liquid and postnatal blood showed a normal male karyotype; fragile X syndrome was ruled out.

P2

P2 was the second son of Caucasian non consanguineous parents. He was born at term (37 weeks) with spontaneous and uncomplicated delivery after a pregnancy complicated by threatened abortion; conception was spontaneous. His birth weight was 3,100 g (25th centile), birth length 48 cm (25th centile).

Family history disclosed epilepsy in a maternal uncle and ID in two maternal cousins.

He showed psychomotor delay, sitting at 9 months, walking independently at 15 months, and producing first words at 30 months. At the age of six years a severe ID was diagnosed. He had behavioral problems, with poor concentration, hyperactivity and distractibility.

EEG, brain MRI, ECG, echocardiogram, hearing and ophthalmologic evaluation were normal. Puberty occurred at the expected age. FMR1 analysis excluded Fragile X syndrome.

At 17 years, age of the last follow-up, clinical evaluation showed weight 75 kg (75-90th centile), length 181,5 cm (75-90th centile) and OFC 58 cm (>97th centile). He had narrow and horizontal palpebral fissures and large ears (+2SD).

P4

She was the second child born to Caucasian New Zealand non-consanguineous parents. She was born at 35 weeks after a pregnancy complicated by threatened miscarriage in the first trimester and the development of intrauterine growth restriction and oligohydramnios in later gestation. Her birth weight was 2,040 g (10-25th centile), crown heel length 42 cm (10th centile) and OFC 31,5 cm (50th centile).

She was admitted to the neonatal intensive care unit for supportive care. She required nasogastric feeding for two weeks until oral feeding was fully established. She was noted to be hypotonic in infancy and had plagiocephaly. A brain MRI scan showed no structural abnormalities. She had feeding difficulties when solids were introduced, which resolved around the age of 2 years. She had mild-moderate global developmental delay: she sat with support at 12 months and walked at 26 months; she did not have any recognizable words until 27 months. She had mild learning difficulties.

She developed signs of puberty when she was between 8 and 9 years old. There was mild but proportional deceleration of all her growth parameters during adolescence.

She was diagnosed with mild non-progressive myopia when she was 10 years old. When she was 16 years old she was noticed to have a mild pigmentary retinopathy. A diagnosis of Cohen syndrome was considered, but a proper diagnosis was provided by microarray analysis when the patient was 17 years old.

At last follow up she was 24 years old and was completing tertiary level study with additional assistance.

Clinical data of P1, P2 and P4 are summarized in Supplementary Table 2a. It is noteworthy that patients' developmental milestones, especially inherent speech and language, are overlapping.

P3, *P5→P7*, *P8*, *P9→P14*, *P15→P22*

Clinical data of these patients are reported in Supplementary Table 2a.

Genetic findings

P1

a-CGH analysis revealed a *de novo* heterozygous 527 kb microduplication at 9q34.3 (chr9:140498690-141025921) encompassing the *ARRDC1*, *EHMT1*, and *CACNA1B* genes (Fig. 1g), which was classified as pathogenic.

BAC-FISH analysis identified two signals specific for *EHMT1* at 9q34.3 on both metaphases (Fig. 1h) and nuclei (Fig. 1i), one of which was enlarged in size, indicating that *EHMT1* duplication was *in tandem*.

Quantitative gene expression assays on blood evidenced an increase of the *EHMT1* transcripts in P1 compared to controls (Fig. 1l), whereas *CACNA1B* expression on blood could not be tested as the gene was found to be specifically expressed in the central nervous system (data not shown).

Two additional inherited heterozygous gains, of 400 kb~, paternal, at 1p36.11 (chr1:25320307-25719620), involving the entire *SYF2* (MIM *607090) and other four genes (*C1orf63*, *RHD*, *TMEM50A* and *RHCE*) not eligible candidates according to their function, and of 24 kb~, maternal, at Xq22.3 (chrX:104155507-104179536), involving part of the IVS2 of *IL1RAPL2 (*MIM *607090), were identified (Supplementary Fig. 1). These CNVs are, respectively, rare and unreported according to the DGV. Expression assays of the paternal CNV gene *SYF2* showed an increase of the corresponding transcripts in the patient's and father's blood compared to controls (Supplementary Fig. 1), whereas expression of the maternal CNV gene *IL1RAPL2 (*MIM *607090) could not be tested as the gene was not found expressed in blood but only in the central nervous system (data not shown). We therefore classified as likely benign the 1p36.11 CNV, and of uncertain significance the Xq22.3 CNV.

The identified CNVs were submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/, March 2019, date last accessed; accession numbers SCV000893133, SCV000893134 and SCV000893135).

P2, *P3*, *P4*, *P5→P7*, *P8*, *P9→P14*, *P15→P22*

Molecular cytogenetics data of these patients are provided in Supplementary Table 2b.

Genotype-phenotype correlation and SRO identification

In order to facilitate genotype/phenotype correlations, the NDDs exhibited by all 22 patients are shown in Fig. 2a on the left side of the bars indicating the microduplications. Clinical data from all patients of the literature and the above mentioned databases are reported in Supplementary Table 2a.

It is worth noting that patients carrying duplicated regions of very different sizes exhibit the same NDD phenotype, such as in the case of P1 compared to P10→P15. Moreover, the NDD phenotype of P4, presenting isolated learning disability, is even less severe than those exhibited by the patients carrying the smallest duplications, i.e. P1, showing DD, speech and language delay (SD), ID and ASD, and P2, who had isolated SD. Interestingly, the less severe NDD phenotype is exhibited by P4, whose opposite rearrangement causes KS in a nephew of her brother [23] (Supplementary Table 2a). Instead, only half of the series of patients P16→P23, who carry the largest duplications, exhibited DD/ID (Fig. 2a, Supplementary Table 2a). The distribution of NDD traits in the entire patient cohort is depicted in Fig. 2b.

As shown in Fig. 2a, breakpoints (bkps) involving the 9q subtelomeric region are highly variable, leading to differently sized microduplications; however, proximal and distal bkps underlying P6→P9, P10→P15, and P16→23 are recurrent. Furthermore, with the exception of P4, which is the result of an unbalanced reciprocal translocation [23], all the gains are interstitial (Supplementary Table 2a).

Within the largest 9q34.3 duplications of ~3.3 Mb, we annotated the 89 RefSeq included genes (Supplementary Tables 2b and 3). Among the 73 brain-expressed genes, eight are classified as OMIM-morbid genes for NDDs, namely *EHMT1*, *CACNA1B*, *GRIN1*, *KCNT1*, *INPP5E*, *MAN1B1*, *MRPS2*, and *PMPCA*. Out of them, only *GRIN1*, *CACNA1B* and *EHMT1* may be inferred as dosage-sensitive according to a pLI of 0.97, 0.98 and 1, respectively. However, *EHMT1* maps entirely within the SRO, whereas *CACNA1B* is only partially embedded.

9q34.3 microduplications cohort versus 9q34 DS and KS

As shown in Fig. 2c, in a subset of 4 patients, dysmorphisms described in 9q DS appeared more represented than those reported in KS, and some, namely ear dysmorphims and scoliosis, are shared by the two conditions. However, some of the features reported as distinctive of 9q DS, i.e. dolichocephaly, ptosis, microstomia and arachnodactyly, have not been found in the cohort. The same applies to brachy(micro)cephaly, synophrys, hypertelorism, upslanting palpepral fissures, flat face/midface hypoplasia, cupid bowed upper lip, everted lower lip, and macroglossia, that are characteristic of KS. It is noteworthy that no patient in the sample exhibited either heart or kidney malformations, that have been reported in both 9q34 DS and KS.

Discussion

We report on the patient (P1) who carries the smallest 9q34.3 duplication encompassing *EHMT1* reported to date and compare his clinical and molecular cytogenetic data to a sample of 21 further patients from a review of literature and databases (Fig. 2a). We selected the patients carrying a pure 9q34.3 microduplication ≤ 3.3 Mb in size encompassing the entire *EHMT1* according to the evidence that patients with an *EHMT1* pathogenic variant and those with a 9q34.3 deletion ≤ 3 Mb in size have highly comparable clinical findings [3, 40]. Complex 9q34.3 rearrangements [12; patients 47 in 1] were excluded from our survey.

In the selected cohort, the 9q34.3 microduplications were inherited in 6 out of the 12 characterized trios (Supplementary Table 2a). However, in two among the apparently *de novo* gains, FISH analysis carried out on parents with 9q34.3 locus specific probes unveiled that the pure 9q34.3 microduplication was the result of the inheritance of a derivative chromosome as in P3 [23] or P4 [13] (Supplementary Table 2a). We would therefore recommend, in order to properly address genetic counseling, conducting FISH analysis in parents of patients carrying an apparently *de novo* 9q34.3 microduplication. Indeed, the presence of the two cousins of P3, both affected by ID, suggests a further balanced rearrangement segregating in the family.

Among the genes located within the 3.3 Mb 9q34.3 region that are brain-expressed and predicted to be dosage sensitive, only three turned out to be plausible candidates for the phenotype observed in our cohort (Supplementary Table 3). They include *GRIN1*, mapping outside the SRO, *CACNA1B* and *EHMT1*, respectively partially and entirely

located within the SRO. *GRIN1* (MIM *138249), encoding a subunit of the NMDA receptors, has a key role in the plasticity of synapses. Individuals carrying *GRIN1* loss of function mutations may exhibit a NDD with or without hyperkinetic movements and seizures (MIM #614254, #617820) (Supplementary Table 3). However, the presence of a comparable neurodevelopmental phenotype in P9→P14 and P1, carrying and not carrying, respectively, a duplication involving *GRIN1*, is evidence against its relevant contribution to the phenotype. As regards *CACNA1B* (MIM *601012), coding for a subunit of an N-type voltage-dependent calcium channel and involved in calcium-dependent neurotransmission, its partial duplication is present in P5→P8. Based on the orientation of the duplicated region, the rearrangement might have resulted in either the preservation of the reading frame or in a gene break, thus leading to a silent effect on gene transcription or haploinsufficiency primed by gene disruption, respectively. Unfortunately, these occurrences could not be evaluated in blood, as the gene has a brain-specific expression. However, Yatsenko et al. [4] reported on healthy family member carriers of a heterozygous *CACNA1B* deletion. In addition, the gene has so far been associated in humans with dystonia (MIM #614860) through dominant gain of function mutations (Supplementary Table 3) and the null-mutant mice showed no gross abnormalities and normal motor coordination [41]. The overall evidence so far does not permit attributing *CACNA1B* with a role in the phenotype associated with the 9q34.3 microduplications. Conversely, *EHMT1*, encoding a chromatin remodeling protein, can be appointed as the gene responsible for the comparable NDD phenotypes exhibited by patients carrying 9q subtelomeric microduplications. This view is supported by the demonstration, for the first time in P1, of an effective *EHMT1* overexpression in blood.
Evidence of a NDD phenotype effected by microduplication leading to overexpression of only *EHMT1* underlines the dosage sensitivity of this chromatin remodeling gene, consistent with the sharp developmental alterations observed in *Drosophila* models overexpressing *EHMT1* [42].

In P1 a strength of language skills compared to other cognitive skills, a feature witnessed in KS patients [6], was evident. As data retrieved on the patients at comparison were scarce, we cannot confirm the existence of such a cognitive profile in the overall cohort. In addition, it is likely that penetrance of DD (73%), ASD (36%), that had rarely been reported in association with 9q34.3 DS, and SD (14%) (Fig. 2b) is underestimated in the sample. For instance, we do not have information about development for P8 and ASD was recorded as absent in P5→P7, despite the possibility of exhibiting ASD [37] (Supplementary Table 2a). Similarly, we know that facial features, as well as skeletal defects, were among the indications for genetic testing in 3 patients of the series P15→P22 (Supplementary Table 2a). However, as the signs were not detailed, they could not be accounted for, so dysmorphic features shown in Fig. 2c are underestimated too.

Interestingly, the same developmental profile observed in P1 was found in the patient reported by Gijsbers et al. [12], whose cognitive level was lower. Indeed, this patient, who carries a ~2.9 Mb duplication at 9q34.3

encompassing *EHMT1*, was not included in our sample because of the complexity of the rearrangement, showing a triplication in the middle of two flanking duplicated regions. The hypothesis of a neurocognitive and behavioral profile may be supported by findings in models with haplo-insufficiency for the gene. For instance, mutant *Drosophilas* showed normal learning, but severe impairment in short- and long-term memory in a courtship conditioning paradigm [43]. Of relevance, P1 and the patient reported by Gijsbers et al. [12] developed feet deformities with walking difficulties, a feature witnessed (probably as congenital) in several KS patients [44]. It is noteworthy that a follow-up is crucial in order to observe all the possible skeletal anomalies, which might become evident with age, as demonstrated by examination of the feet at 13 years in P1 and at 16 years in that reported by Gijsbers et al. [12].

We hypothesize the existence of a 9q34.3 microduplication syndrome encompassing *EHMT1* characterized by a variable clinical spectrum ranging from non-syndromic to syndromic NDDs with certain facial and skeletal features mainly resembling either 9q34.3 DS or KS, or both, but very likely without a gestalt. As regards neurodevelopment, the core features may be speech delay and learning disabilities.

In conclusion, by the clinical and molecular description of a patient carrying a small duplication involving entirely the *EHMT1* gene compared to a reference cohort with duplications of increasing size, we have provided evidence that *EHMT1* is crucial for pathogenicity of the 9q34.3 microduplications. We propose to add further signs supporting the existence of a 9q subtelomeric microduplication syndrome. For this purpose, a deep cognitive-behavioral and clinical phenotyping is advisable for both the patients and the transmitting parents, taking into account that the rearrangement has been inherited in a high proportion of patients. We believe this approach will be useful to better characterize the hypothesized cognitive profile associated with *EHMT1* dosage variation, where language is slow to develop, but then becomes stronger than other cognitive skills, as well as to unveil NDDs also in apparently healthy carriers, as has been demonstrated for 16p11.2 recurrent deletion [45]. Moreover, differently from what occurs in Williams-Beuren syndrome and Williams-Beuren region duplication syndrome [46], both *EHMT1* dosage imbalances seem to express the same NDDs. This phenomenon is known for NDDs related recurrent CNVs, where gains usually show a reduced penetrance compared to their reciprocal losses [47].

Acknowledgments

This study makes use of data generated by the DECIPHER community. A full list of centres who contributed to the generation of the data is available from http://decipher.sanger.ac.uk and via email from decipher@sanger.ac.uk. Funding for the project was provided by the Wellcome Trust.

Funding: This study was funded by Ministry of Health 'Ricerca Corrente' (grant numbers 08C607_2006, 08C602_2006 and 08C204_2012) to IRCCS Istituto Auxologico Italiano.

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

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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Legend to Figures

Fig. 1 Photographs of patient 1 and molecular characterization of his 9q34.3 microduplication. P1 at the age of 26 months (a), 6 years and 6 months (b, left profile; c, frontal view), and 13 years (d). (e, f) Feet after surgery. (g) Array-CGH analysis identifies a *de novo* 527 kb duplication at 9q34.3 (chr9:140498690-141025921, hg19). (h, i) FISH analysis performed by using probe RP11-644H13 on proband's metaphases (h) and interphase nuclei (i) shows increased/double fluorescent signals on chromosome dup(9q34.3) (arrowed) as compared to those observed on normal chromosome 9. (l) Scatter plots, obtained using TaqMan probes, show increased *EHMT1* expression in P1 (circular dot) compared to 10 normal individuals (circular dots). The horizontal dotted bars in the control expression indicate the range between mean ±2 standard deviation values. CTRL, controls

Fig. 2 Chromosome 9q34.3 microduplications encompassing *EHMT1* and penetrance of developmental and clinical features exhibited by the carrier patients. (a) UCSC Genome Browser Genome viewer (hg19) comparing the extension of the novel duplication (P1, speckled bar) with those reported in literature and databases (black bars). The patient (P) or patient series P→P number is indicated on the right side of each duplication bar. On the left side of each microduplication's bar the associated neurodevelopmental disorders are shown: ASD, autism spectrum disorder; DD, developmental delay; H, hyperactivity; ID, intellectual disability; LD, learning disability; SD, speech delay. The light blue shaded rectangle represents the SRO, Smallest Region of Overlap across the duplications. RefSeq UCSC genes associated to a neurological phenotype are shown in green. (b) Penetrance of each developmental disorder exhibited by the 22 patients carrying a pure 9q34.3 duplication. (c) Penetrance of dysmorphisms exhibited by the 4 patients (P1→P4) for whom detailed information was available; asterisks, features typically exhibited by patients with 9q34 duplication syndrome (9q DS); small circles, features typically exhibited by patients with Kleefstra syndrome (KS): LAF, long/asymmetric face; PNB, prominent nasal bridge; NHPB, narrow/horizontal palpebral fissures; DE, deep-set eyes;

AN, anteverted nostrils; MRG, micrognatia/retrognatia; ED, ear dysmorphisms; HP, high palate; STF, slandered/tapered fingers; S, scoliosis ; TE, non-congenital talipes equinovarus

Supplementary Fig. 1 Array-CGH analysis identifies two rare inherited CNVs: a paternal 399 kb duplication at 1p36.11 (chr1:25320307-25719620, hg19) (upper panel), and a maternal 24 kb gain at Xq22.3 (chrX:104155507-104179536, hg19) (lower panel)

Supplementary Fig. 2 Scatter plots, obtained using TaqMan probes, show increased SYF2 expression in P1 (Pt) and his father (F) (circular dot) compared to 10 normal individuals (Ctrls, circular dots). The horizontal dotted bars in the control expression indicate the range between mean ±2 standard deviation values

To Professors G. W. J. Auburger, M. B. Greaeber, L. J. Ptacek
Editors- in-Chief of *Neurogenetics*

Milan, 11th April 2019

Dear Editors,

Please find uploaded the manuscript entitled **"9q34.3 microduplications lead to Neurodevelopmental Disorders through EHMT1 overexpression"** by Maria Teresa Bonati et al, as Original Article, to be considered for publication in *Neurogenetics*.

We report the smallest 9q34.3 microduplication containing the chromatin-remodelling *EHMT1* gene, whose overexpression is responsible for the NDD phenotypes of the carrier patient.

We survey 21 other patients with larger 9q34.3 microduplications, all including *EHMT1*, who have been reported in literature and/or the DECIPHER or SFARI database, to compare their gene content to the clinical phenotype of the patients.

Interestingly, we found that the spectrum of their graded NDD phenotypes is not related to the microduplication size, being primarily dependent instead on the increased dosage of *EHMT1*.

While the clinical consequences of loss of function *EHMT1* mutations are largely known, the effect of increased *EHMT1* dosage has been less documented.

Based on the above notes, I believe that our manuscript might be of interest to the readers of *Neurogenetics.*

The identified CNVs were submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/, March 2019, date last accessed; accession numbers SCV000893133, SCV000893134 and SCV000893135).

The manuscript includes 2 figures, and 5 additional files (4 tables and 2 figures).

I confirm that the manuscript is an original work, not previously published or under consideration by any other peer-reviewed journal.

All the authors have read and approved the manuscript and they accept responsibility for the manuscript's content. All the authors confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support that could have influenced its results. We furthermore declare that there are no impediments to publication of text, tables and figures.

I send you my very best regards and look forward to your comments.

Yours sincerely
Maria Teresa Bonati, MD

Figure 1

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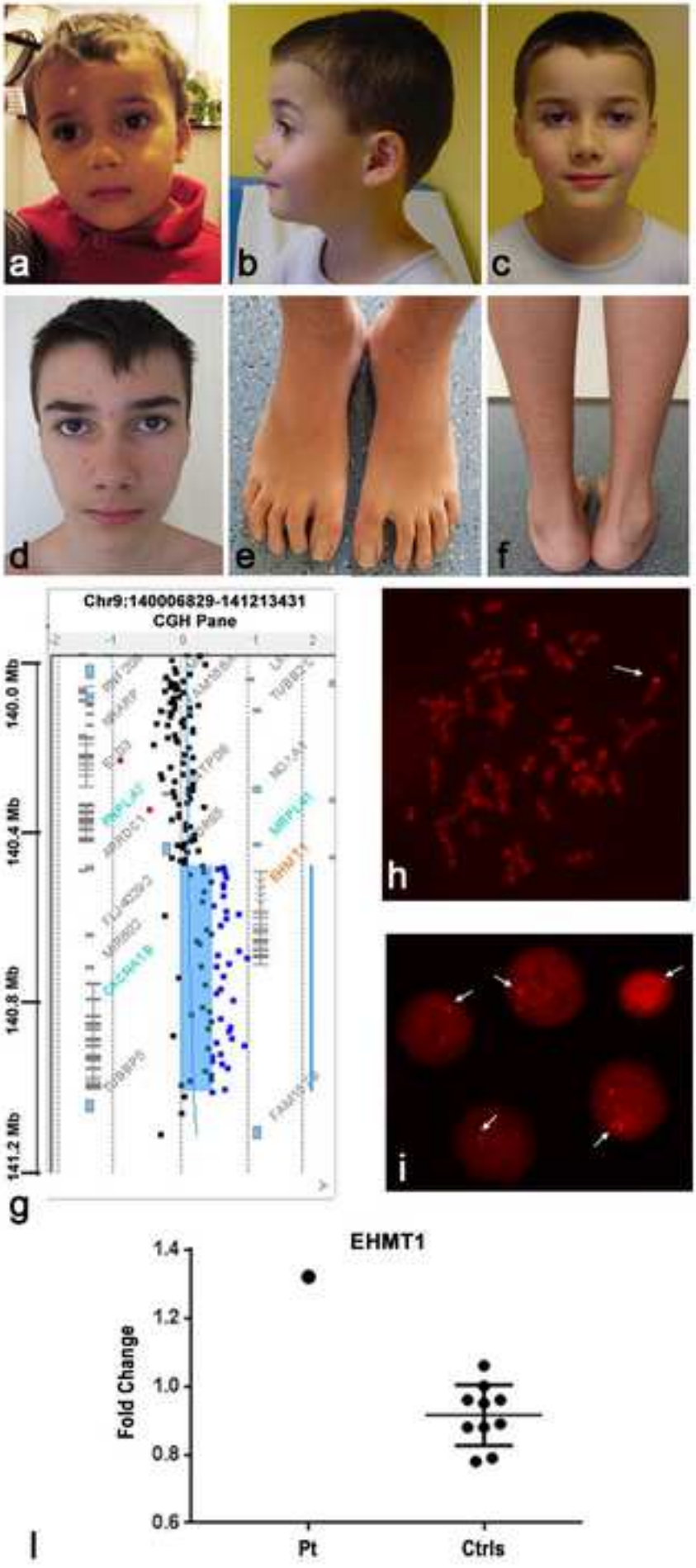

Figure 2

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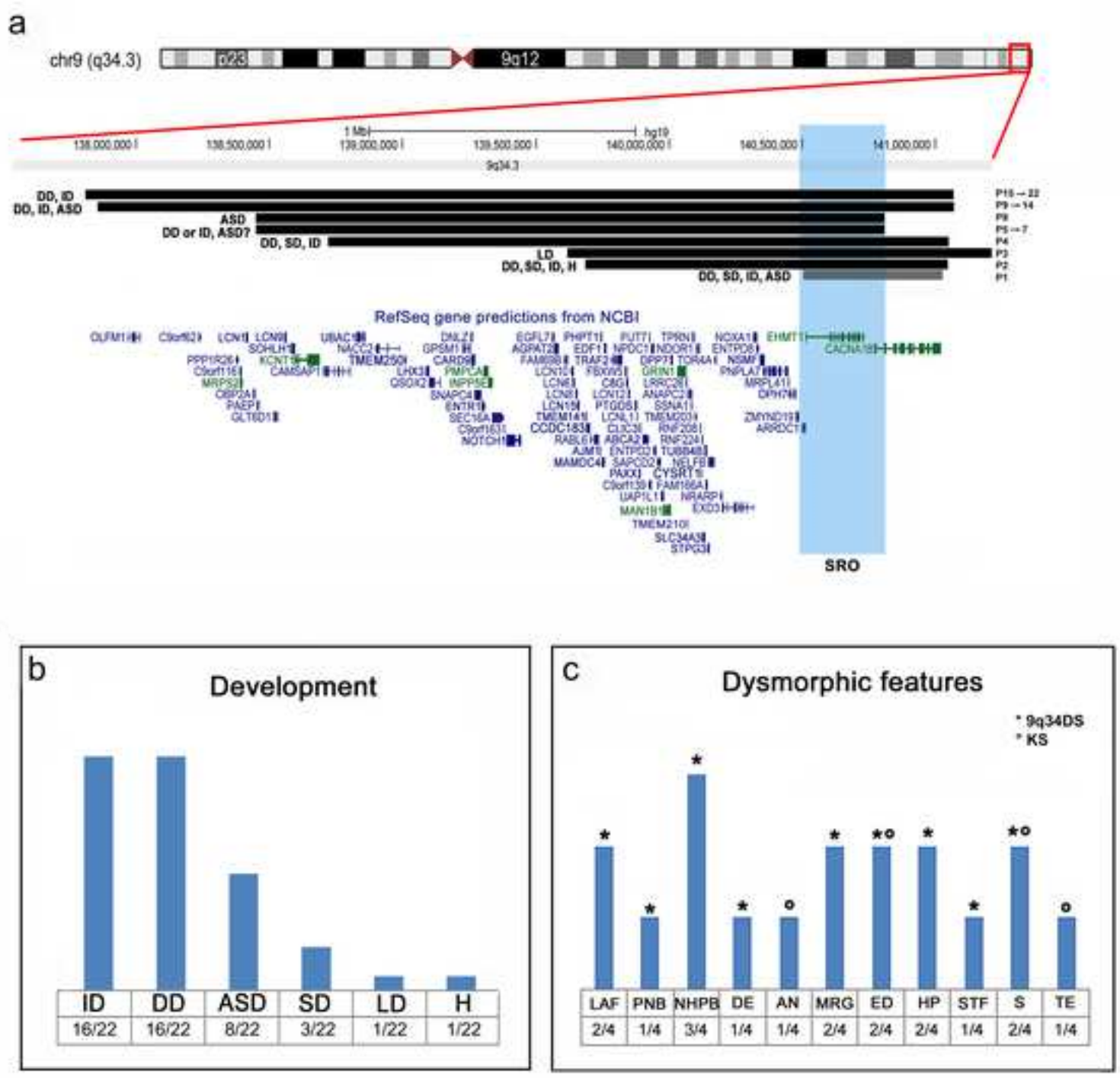

Table 1S

Table 2aS

Table 2bS

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