Molecular Analysis, Pathogenic Mechanisms, and Readthrough Therapy on a Large Cohort of Kabuki Syndrome Patients

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ABSTRACT: Kabuki syndrome (KS) is a multiple congenital anomalies syndrome characterized by characteristic facial features and varying degrees of mental retardation, caused by mutations in KMT2D/MLL2 and KDM6A/UTX genes. In this study, we performed a mutational screening on 303 Kabuki patients by direct sequencing, MLPA, and quantitative PCR identifying 133 mutations, three of them are novel. We found that a number of KMT2D truncating mutations result in mRNA degradation through the nonsense-mediated mRNA decay, contributing to protein haploinsufficiency. Furthermore, we demonstrated that the reduction of KMT2D protein level in patients’ lymphoblastoid and skin fibroblast cell lines carrying KMT2D-truncating mutations affects the expression levels of known KMT2D target genes. Finally, we hypothesized that the KS patients may benefit from a readthrough therapy to restore physiological levels of KMT2D and KDM6A proteins. To assess this, we performed a proof-of-principle study on 14 KMT2D and two KDM6A nonsense mutations using speciﬁc compounds that mediate translational readthrough and thereby stimulate the re-expression of full-length functional proteins. Our experimental data showed that both KMT2D and KDM6A nonsense mutations displayed high levels of readthrough in response to gentamicin treatment, paving the way to further studies aimed at eventually treating some Kabuki patients with readthrough inducers.
Introduction

Histone methylation is an epigenetic mechanism by which spatial and temporal expression of distinct genes and pathways are regulated at precise developmental stages. Different histone lysine methylation states (mono-, di-, or tri-methylation) are associated with gene transcriptional activation or repression depending on the location of the lysine residue.

In general, histone H3 lysine 4 (H3K4) di- and tri-methylation are linked to active transcription, whereas H3K27 di- and tri-methylation are associated with gene silencing [Santos-Rosa et al., 2002]. Aberrations in the histone modifiers have been associated with genetic diseases, such as Kleeblatts syndrome, Sotos syndrome, Weaver syndrome, and Schinzel–Giedion syndrome [Berdasco and Esteller, 2013]. The discovery of histone methyltransferase (HMT) KMT2D (MIM #602113; RefSeq NM_003482.3, also known as nonsense mutations and showed that 11 genes [Ansari and Mandal, 2010; Eissenberg and Shilatifard, 2012; Miyake et al., 2013a; Miyake et al., 2013b] that encode a Histone H3 lysine-27 demethylase. KDM6A plays a crucial role in general chromatin remodeling [Hong et al., 2007; Lan et al., 2007] and interacts with KMT2D, in a conserved SET-1-like complex that trimethylates H3K4 [Issavea et al., 2007]. The inactivation of the zebrafish kdm6a orthologue by morpholino is associated with severe and diverse structural defects and developmental abnormalities [Lindgren et al., 2013]; this inactivation resulted in the misregulation of HOX genes leading to a posterior developmental defect, whereas Kdm6a-deficient mice showed severe defects in heart development and embryonic lethality [Lee et al., 2012].

The majority of KMT2D and KDM6A nucleotide changes are truncating mutations (nonsense, frameshift, or splice site) that produce premature termination codons (PTCs), which are potentially deleterious. In the last few years, there have been several attempts to develop mutation-specific pharmacological approaches to restore sufficient levels of functional proteins. One approach that has been gaining prominence is that of using pharmacological agents to promote nonsense suppression or readthrough of PTCs thus enabling re-expression of full-length functional proteins [Nakamura et al., 2005; Bellais et al., 2010]. The potential of aminoglycosides and non-aminoglycosides as therapeutic tools has been demonstrated in several genetic disorders such as hemophilia, β-thalassemia, and spinal muscular atrophy, but most extensively in Duchenne muscular dystrophy and cystic fibrosis [Lee and Dougherty, 2012]. Interestingly, this treatment was successfully applied on an Ataxia-telangiectasia patient with heterozygous nonsense mutation, thereby demonstrating therapeutic ability despite the presence of a nonsense mutation in just one allele [Nakamura et al., 2011].

In this report, we have expanded the spectrum of mutations of KMT2D and KDM6A genes by analyzing our cohort of 303 Kabuki patients by direct sequencing, MLPA and quantitative PCR (qPCR). Based on KMT2D biological role, we designed functional studies that highlighted the haploinsufficiency of KMT2D as one of the mechanisms underlying the pathogenesis of the disease. Moreover, we evaluated the readthrough efficiency of 14 KMT2D and two KDM6A nonsense mutations and showed that 11 KMT2D and one KDM6A nonsense mutation responded to gentamicin treatment suggesting that this strategy can be effective to restore functional endogenous protein level and biological activity of KMT2D and KDM6A.

Material and Methods

Patients

In this study, 303 KS patients were included following the inclusion criteria reported in Micale et al. (2011). Patients were enrolled after obtaining appropriate informed consent by the physicians in charge and approval by the respective local ethics committees.

Cell Cultures, Nonsense-Mediated mRNA Decay Assay, and E2 Treatment

Lymphoblastoid cell lines (LCLs) were established from fresh peripheral blood leukocytes, infected by Epstein Barr Virus and cultured in RPMI 1640 supplemented with 10% of fetal bovine serum (FBS; Life Technologies, USA), L-glutamine, and 1% antibiotics mixture (penicillin and streptomycin 10,000 UI/ml; Life Technologies). Primary skin fibroblasts were grown in minimum essential medium supplemented with 1% L-glutamine, 10% FBS, and...
antibiotics. Nonsense-mediated mRNA decay (NMD) was assayed by treating fibroblast and lymphoblast cell lines with puromycin at a concentration of 200 µg/ml. After 8 hr of incubation, total RNA was obtained using the RNaasy mini Kit (Qiagen, Germany) according to manufacturer instructions and Quantitect Reverse Transcription kit (Qiagen, Düsseldorf, Germany) was used for cDNA synthesis. Fibroblast cell lines were treated with 200 nM of 17B-estradiol (E2), incubated for 8 hr and then harvested for RNA and protein extraction.

MLPA, Long PCR, and Genomic Real-Time qPCR

Genomic DNAs were extracted from fresh and/or frozen peripheral blood leukocytes of the probands and their parents following standard procedures. MLPA analysis was performed as reported in Priolo et al. (2012) using probe mixture (SALSA MLPA KIT P389-A1 KMT2D; MRC-Holland, Amsterdam, The Netherlands) that contains 27 probes targeting exons across the KMT2D gene.

Long PCR was carried out with Expand Long Template PCR system (Roche, Mannheim, Germany) with combinations of primer pairs spanning the KMT2D exons not covered by MLPA. qPCR reactions were carried out with primers designed to amplify all the 29 KDM6A exons as described in Priolo et al. (2012).

Real-Time qPCR Assays

Total RNA was extracted from peripheral blood leukocytes using TRIZOL reagent (Life Technology) and reverse transcribed using the Quantitect Transcription kit (Qiagen), according to the manufacturer’s instructions. Oligos for qPCR were designed using the Primer express program [Rozen and Skaletsky, 2000] with default parameters. EEFL1A1 and GAPDH were used as references genes. qPCR reactions and calculations were made as reported in Ferrero et al. (2010). Significance was determined by a two-tailed unpaired t-test for means.

Dual Luciferase Reporter Vector System

A dual gene reporter pCRFL (gently provided by Prof. J-P Rousset) was used to quantify the effect of gentamicin on stop mutation readthrough in culture cells. Sequence to be analyzed spanning 27 nucleotides centered on the different stop mutations were inserted in-frame between the Renilla and Firefly Luciferase coding sequences through site-directed mutagenesis by using the QuickChange II kit (Stratagene, La Jolla, California, USA). All target DNA sequences were assessed by sequencing. Plasmids were purified using QIAgen Midiprep Kit according to the manufacturer’s specifications. HEK293 cells were culture in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 1% antibiotics mixture, and incubated at 37ºC in humidity saturated 5% CO2. Cells were transfected using FuGene HD Transfection Reagent (Promega) with each pCRFL construct. Culture medium was replaced 24 hr later with fresh medium at a concentration of gentamicin (Hospira, Lake Forest, Illinois, USA) varying from 0 to 1,200 µg/ml. After 48 hr, the cells were lysed in passive lysis buffer and assayed for both Firefly and Renilla luciferase activity using the Dual-GLO® Luciferase Assay System (Promega, Madison, WI, USA).

In Silico Analysis

We analyzed the missense variants using the latest version of the server Polyphen-2 version 2.2.2 (http://genetics.bwh.harvard.edu/pph/) (Adzhubei et al., 2010), Align GVGD (http://gvgd.iarc.fr/ gvgd_input.php) [Tartgian et al., 2006], PROVEAN v1.1 (http://provean.jcvi.org/index.php) [Choi et al., 2012], and SIFT v1.03 (http://sift.jcvi.org/) [Kumar et al., 2009], UMD-predictor (http://www.umd.be/) [Frederic et al., 2009], using default parameters. Splice-site variants were evaluated for putative alteration of regulatory process at the transcriptional or splicing level with NetGene2 (http://www.cbs.dtu.dk/services/NetGene2) [Brunak et al., 1991] and NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html) [Reese et al., 1997].

Protein Extraction and Western Blot Analysis

Normal and patient lymphoblastoid and fibroblast cell lines were treated with concentration of gentamicin varying from 800 to 1,200 µg/ml. After 48 hr, cells were lysed in 10-mM HEPES, 1.5-mM MgCl2, 10-mM KCl, 0.5-mM DTT, 1.5-mM Phenylmethylsulfonyl fluoride (PMSF), and 2 N KCl. Proteins were separated on a SDS-polyacrylamide gel. Western blots were prepared as reported in Caratuzzolo et al. (2012) and probed with anti-KMT2D (Abnova, Taïpei, Taiwan). Bound primary antibodies were visualized using ECL western blotting or ECL plus Western blotting detection reagents (GE Healthcare, UK).
Results

Mutation Screening of KMT2D and KDM6A

Since 2011, we performed a comprehensive mutational screening on 303 Kabuki patients by direct sequencing, MLPA, and qPCR on KMT2D and KDM6A genes, respectively. Of these 303 patients, 79 were described in previous studies where 53 pathogenic KMT2D mutations were identified in 51 patients (Supp. Table S1) [Micale et al., 2011; Makrythanasis et al., 2013; Ratbi et al., 2013].

Here, we extended the KMT2D mutational analysis to a new cohort of 224 individuals clinically diagnosed as KS identifying 82 (82/224, 36%) patients carrying causative KMT2D mutations.

Overall, in our whole KS cohort, we identified 133/303 (44%) patients with KMT2D mutations and 140 different KMT2D mutations; of them, 87/140 (62%) were identified in this study with 66/140 (47%) never described before. The following types of KMT2D mutations were identified: 37 nonsense (37/140, 26%), 42 frameshift (42/140, 30%), 46 missense (46/140, 33%), eight splice site (8/140, 6%), and seven indel (7/140, 5%) (Supp. Tables S1 and S2). Almost all identified mutations occurred de novo; only 16 missense and four indel variants were inherited from an apparently asymptomatic parent.

To explore the occurrence of intragenic deletions and duplications, we screened KMT2D exons by MLPA analysis on 207 KS patients: 164 KMT2D point mutation negative, 37 with KMT2D missense, and six indels. We identified the KB43 patient carrying a singleton deletion covering from exon 49 to a part of exon 51 (Supp. Fig. S1A). Long PCR, followed by direct sequencing, allowed us to map the boundaries of the deletion as c.15785-238_16168del2425insTTGTATCTCAA mutation (Supp. Fig. S1B). KMT2D mutations were submitted to the Leiden Open Variation Database (LOVD v.3.0; http://www.lovd.nl/3.0/home) public database.

To identify KDM6A mutations in KS patients, we first assessed KDM6A exon dosage by qPCR analysis on 139 samples with no KMT2D alteration without identifying any intragenic deletions and duplications. Then, we sequenced 29 coding exons of KDM6A along with its exon–intron boundaries in 98 patients (not enough DNA was available for the remaining 41 samples). We identified four KDM6A point mutations: one nonsense c.514C>T (p.Arg172X) [Banka et al., 2014], one frameshift c.1846_1849delACTCT (p.Thr616TyrfsX8), one missense c.2939A>T (p.Asp980Val), and one splicing mutation c.3284+3_3284+6delAAGT (p.Asn1070Lys1094del). Two of them occurred de novo; parental DNAs were unavailable for the others. KDM6A mutations were submitted to LOVD (http://www.lovd.nl/3.0/home) public database.

Characterization of KMT2D Splice-Site Mutations

We identified eight patients with KMT2D mutations in the proximity of the splice sites and we analyzed them by NetGene2 and Fruittly softwares to predict the molecular consequences of the observed nucleotide changes. The availability of the RNA from those KS patients, but one, allowed us to experimentally determine the effect at the transcriptional or splicing level (Supp. Fig. S2). All identified intronic KMT2D variants cause aberrant splicing of the corresponding transcripts that result in a frameshift with the generation of a premature stop codon as reported below:

- c.177-2A>C (p.Ser59ArgfsX86) affects the essential nucleotide −2 of the splice acceptor site of intron 2.

KMT2D Mutant Transcripts Suffer NMD

KMT2D nonsense mutations may result in the partial transcripts degradation through NMD pathway, contributing to protein haploinsufficiency. To investigate this, we measured by qPCR the levels of KMT2D mRNA in three KS skin fibroblasts and four KS LCLs following treatment with puromycin, a known indirect NMD inhibitor [Brichta et al., 2008]. After puromycin treatment, we observed a significant 2.18-, 2.35-, and 4.01-fold increase in the KMT2D transcript levels in KB186, KB153, and KB3 fibroblast cell lines, respectively (Supp. Fig. S3A and Supp. Table S3E), and 1.93- and 1.69-fold for KB48 and KB83 LCLs, compared with untreated cells, respectively (Supp. Fig. S3C and Supp. Table S3F). The reduced amount of KMT2D mRNA levels in mutated cell lines and its recovery following puromycin treatment indicate that endogenous KMT2D mutant transcripts are subject to NMD. The physiological NMD substrate SC-35 1.7 Kb was included as positive control (Supp. Fig. S3B–D) [Brichta et al., 2008]. We confirmed these data by direct sequencing of real-time PCR on mRNA derived from fibroblasts of patients KB186, KB153, KB3, showing that NMD inhibition restores the expression of the KMT2D-mutated alleles (Supp. Fig. S3E–G).

KMT2D Mutations Affect Its Activity in Patients Cell Lines

The large prevalence of KMT2D protein truncating mutations suggests loss of function, and therefore the haploinsufficiency of KMT2D as the likely mechanism for the KS phenotype. To assess this, we measured the protein level of KMT2D in six KS LCLs, and three KS fibroblast cell lines carrying truncating mutations, and we found a significant reduction of KMT2D protein level when compared with control cell lines (Fig. 1A–F and Supp. Table S3A).
KMT2D methyltransferase activity is impaired in KS lymphoblastoid and fibroblast cell lines with KMT2D-truncating mutations. (A–F): Immunoblotting analysis by using anti-KMT2D antibody on whole protein lysate from six (A and B) KS lymphoblastoid (KB41, KB44, KB45, and KB82, KB83, KB153, and KB186) and three (C) fibroblast cell lines (KB186, KB153, and KB3) with nonsense mutations and frameshift mutations, compared with control cell lines. Pool: pooled protein lysates from two control cell lines. D–F: The density of each band was determined by densitometer. The expression level of KMT2D was determined by calculating the protein level for each sample, normalized to the corresponding GAPDH level. G: qPCR was performed to measure the expression of known target genes of KMT2D (HOXC6, S100A2, S100A4, S100A5, S100A6) in six KS–LCLs, compared with control cell lines. Scale bars represent standard errors. *$P < 0.01$.  

To address whether truncated KMT2D mutations affect its transcriptional activity, we measured the mRNA level of direct known transcriptional target genes of KMT2D complex, including HOXC6 [Ansari et al., 2011] and some members of the S100A family genes, such as $S100A2$, $S100A4$, $S100A5$, and $S100A6$. As shown in Figure 1G, KMT2D targets were downregulated in KS fibroblast cells (Supp. Table S3B). Overall, these data show that the transcriptional activity of KMT2D is impaired in KS patients.

**HOXC6 Expression Is Affected in Kabuki Patients**

Many of the KMT2D mutations mapped before the LXXLL domain required for KMT2D binding to estrogen receptors (ERs), ERα, and ERβ. Previous studies demonstrated that KMT2D, in coordination with ERα and ERβ, transcriptionally regulates HOXC6 in an Estradiol (E2)-dependent manner. We thus hypothesized that the loss of the KMT2D–ER interaction affects ER gene expression. qPCR showed that HOXC6 transcriptional level decreased in KS fibroblast cells, a reduction that persists even after E2 treatment (Fig. 2A and B and Supp. Table S3C). As KMT2D is associated with HOXC6 via binding to the estrogen receptor element (ERE), located in the HOXC6 promoter region, we assessed the ability of KMT2D to bind to the HOXC6 EREs in three KS patients by a luciferase reporter assay. We transfected the pGL3–ERE1–ERE2 luciferase-based reporter vector containing the HOXC6 EREs sequences (schematized in Fig. 2C) in KS and healthy fibroblast cell lines treated with E2.
**KMT2D and KDM6A Nonsense Mutations Are Responsive to Gentamicin-Induced Readthrough Treatment**

A large number of KMT2D and KDM6A mutations are nonsense mutations that produce aberrant PTCs that are potentially deleterious. We evaluated the potential ability of gentamicin in suppressing KMT2D and KDM6A nonsense mutations by using an in vitro readthrough reporter system [Floquet et al., 2011]. Fourteen KMT2D and two KDM6A nonsense mutations were selected and tested. The mutations were selected, when possible, following the hypothetical rule of a uracil immediately upstream and a cytosine just downstream the stop codon as associated with an optimal gentamicin-induced readthrough, respectively [Floquet et al., 2012]. For each mutation, sequences spanning 27 nucleotides centered on the different stop mutations were inserted into the dual reporter vector pCRFL in-frame between Renilla and Firefly luciferase coding sequences [Floquet et al., 2011]. Readthrough levels were quantified in HEK293 cells transiently transfected by the dual reporter vector in the presence of increasing amounts of gentamicin. Cytotoxicity experiments demonstrated that no signs of cytotoxicity were detectable after 48 hr of treatment with up to 1,200 μg/ml of gentamicin (data not shown). The difference between basal and gentamicin-induced readthrough level was statistically significant ($P < 0.01$) for 11 KMT2D and one KDM6A nonsense mutations (Fig. 3A and B and Supp. Table S4), although with a wide variability in strength. In agreement with the previous report [Floquet et al., 2012], we found that 11/12 (92%) of the mutations responsive to gentamicin have a uracil in –1 residue immediately upstream the stop codon and/or a cytosine in +4 position confirming that the presence of these residues promotes higher basal and gentamicin-induced readthrough than other nucleotides. To test the in vivo effectiveness of gentamicin, we evaluated the expression levels of two known targets of KMT2D, HOXC6, and S100A4 in LCLs from KS patients cultured in the presence of gentamicin. qPCR analysis revealed that KB41 LCL resulted in sixfold and threefold increase of HOXC6 and S100A4 mRNA levels, respectively (Fig. 3C). In agreement with the in vitro assay, a significant increase of S100A4 mRNA levels was observed in KB45 cells, whereas we did not detect any significant increase of HOXC6 expression (Fig. 3D). These findings are consistent with our in vitro observations about the effectiveness of readthrough that varies with respect to type of mutations and patient cell lines.

Overall, these studies suggest that readthrough strategies can be effective to restore functional KMT2D and KDM6A activity, although testing additional mutations, many other compounds and
Figure 3. Identification of KMT2D and KDM6A nonsense mutations responsive to gentamicin treatment. A and B: Comparison between basal and gentamicin-induced readthrough level. Readthrough efficiency for 14 nonsense mutations in the KMT2D gene and for two elsewhere published \[Miyake et al., 2013b\] nonsense mutations in the KDM6A gene was assessed in HEK293 cells exposed or not to 600–800–1,200 μg/ml of gentamicin for 48 hr. A pCRFL reporter vector harboring the 319d Duchenne muscular dystrophy mutation was used as positive control (CNT). Each value corresponds to the mean of four to six independent experiments. Scale bars represent standard errors. C and D: Gentamicin restored the expression of HOXC6 and S100A4 in patient cultured cells with different efficiency. qPCR analysis was performed to evaluated HOXC6 and S100A4 expression level in two patients LCLs, KB41 (C) and KB45 (D), and two control cell lines treated or not with 800 μg/ml of gentamicin for 48 hr. The results are the mean from at least two experiments performed in duplicate. *P < 0.01.
different cell lines are mandatory to assess their potential therapeutic effectiveness.

Discussion

The orchestrated organization of epigenetic factors that control chromatin dynamism, including chromatin-remodeling proteins, is essential for the proper function of tissue homeostasis, cell identity, and development [Berdasco and Esteller, 2013]. KS is the most common of a growing group of multiple malformation syndromes associated to developmental delay that are caused by mutations in genes that encode proteins involved in histone methylation. In this study, we identified 133 KMT2D and four KDM6A mutations in 303 patients clinically diagnosed with KS. The mutation detection rates for KMT2D and KDM6A in KS suggest that other causative genes may be involved, although it can be taken into account that a number of these patients might have been misdiagnosed.

We demonstrated that KMT2D protein level is reduced and its activity impaired in KS cell lines. KMT2D through H3K4 methylation activity is a key epigenetic transcriptional regulator of the expression of multiple genes with related functions, including embryogenesis, development, and stem cell differentiation. Additionally, KMT2D-associated histone methyl transferase activity appears to be functional only in the context of its multiprotein complex, including ASH2L, RBBP5, WDR5, and other MLL family members [Issaeva et al., 2007; Dhar et al., 2012] and each KMT2D-interacting protein plays a distinct role in regulating MLL-mediated histone methylation and gene activation. In agreement with its biological function, the lack of KMT2D protein activity or of its ability to form a multiprotein complex might alter its role in histone methylation pathway with strong influences on global change in gene expression in many of the body’s organs and tissues, resulting in some of the abnormalities of postnatal development of KS.

In our screening, we found 46 missense variants distributed across the entire length of the KMT2D gene including residues in PHD and SET domain. The in silico analysis predicts that many of them might be pathogenic, although the exact role they play in the disease has not been addressed. Recently, it has been shown that KS C1430R and C1471Y missense mutations [Hannibal et al., 2011] in PHD_{4–6} domains reduce PHD_{4–6} binding ability and abrogate the nucleosomal methylation activity of KMT2D, although they do not affect the interaction of KMT2D with ASH2L, RBBP5, and WDR5 [Dhar et al., 2012]. These results offer a valuable and feasible assay to test the pathogenicity of KMT2D missense mutations and implicate that the missense mutations described here that lies in PHD domains may contribute to KS by reducing KMT2D enzymatic activity and consequently its mediated transcriptional activation.

The majority of genetic alterations detected in Kabuki patients result in truncated proteins upstream the LXXLL domain, which is involved in KMT2D–complex binding to ERα. Rationally, the loss of the KMT2D–ERα interacting region might result in a dramatic alteration of the ERα-mediated pathways. Notably, some of the clinical features associated to deregulated ERα-signaling pathways are included in the large spectrum of KS phenotypes, comprising immunological defects and cardiac anomalies [Deroo and Korach, 2006]. Moreover, more than 20% of Kabuki patients show early breast development consistent with the biological role of ERα in mammary gland formation. In this regard, it was recently reported that the homeobox (HOX)-containing gene HOXC6, a critical player in mammary gland development and milk production, is transcriptionally activated via coordination of KMT2D and ER in an estrogen environment in breast cancer and placental choriocarcinoma cells. Specifically, the HMTs, KMT2D, and MLL3, in coordination with ERs, ERα, and ERβ, play critical roles in histone H3 lysine-4 trimethylation and in the recruitment of general transcription factors and RNA polymerase II in the ERs regions of the HOXC6 promoter during E2-dependent transactivation, leading to HOXC6 transcriptional activation. Interestingly, the knockdown of KMT2D by using specific antisense oligonucleotides suppressed E2-induced expression of HOXC6 [Ansari et al., 2011]. In agreement with all these findings, we found that HOXC6 expression is impaired in KS patients’ cell lines.

As Kabuki patients’ cell lines with KDM6A mutations were not available, it was not possible to study the activity of the endogenous KDM6A. However, it has been recently evaluated that the haploinsufficiency of Kdm6a in a zebrafish model. Kdm6a knockout fish exhibited abnormal craniofacial structures that included the absence of the branchial arches and otoliths, as well as the absence, clefting, or inversion of the ceratohyal and abnormal patterning or clefting of Meckel’s cartilages [Lindgren et al., 2013]. In addition, Kdm6a-deficient mice showed severe defects in heart development and embryonic lethality [Lee et al., 2012]. Very recently, it was demonstrated that Kdm6a knockout affects expression of master regulatory genes involved in adipogenesis and osteogenesis [Hemminger et al., 2014]. These observations further support the hypothesis that perturbation of a regulatory pathway shared by KDM6A is responsible for the clinical aspects of the KS.

The large size of the KMT2D cDNA hampers so far the development of any gene-therapy-based strategy. Mutation-based treatments are something of recent enough in genetic medicine, in which the nature of the mutation dictates the therapeutic strategy. As proof of principle, here we demonstrated the ability of gentamicin to induce the readthrough of naturally occurring stop mutations in the KMT2D and KDM6A genes using both in vitro and in vivo assays. Readthrough efficiency has been shown to also depend on the nature of the sequences surrounding the stop codon. Specifically, the consensus sequence U-STOP-C results in an optimal gentamicin-induced readthrough [Floquet et al., 2012]. However, the readthrough response to treatment is highly variable and little is known about the rules governing it and the response to different compounds. A wide variability in responsiveness to gentamicin was observed in our group of KMT2D and KDM6A mutations. Interestingly, the c.12844C>T mutation that exhibited the highest gentamicin-induced readthrough efficiency, follows the theoretical rule that a timine residue is located immediately upstream and a cystein downstream from the stop codon.

After confirming in vitro nonsense suppression in KS, our study goes one step further to investigate the in vivo effectiveness of gentamicin on two cultured patient LCLs carrying nonsense mutations. Consistent with our in vitro data, we showed that gentamicin treatment is able to partially restore a functional endogenous KMT2D protein confirming the use of the in vitro assay as a good model for evaluating drug-induced readthrough and selecting patient carrying nonsense mutations that could benefit from treatment. Nevertheless, studies on a larger number of KS cell lines are mandatory.

Our analysis indicated that some of KMT2D-truncating transcripts suffer NMD process contributing to instability of mutant mRNA and haploinsufficiency of KMT2D protein, thus it is possible that NMD minimizes the effect of readthrough treatment for some KMT2D transcripts. To overcome this undesirable contingent issue, the readthrough strategy might be combined with inhibition of NMD by specific inhibitors and/or siRNA directed against NMD key factors as UPF1 or UPF2.

Several other aminoglycosides and nonamino-glycosides agents are used for their nonsense suppression activity in various types of
cell culture with a different efficiency. Our preliminary results encourage further testing with other compounds including additional aminoglycoside such as G418 and amikacin, and nonaminoglycosides agents with improved biocompatibility, such as PTC124 and RTC13, RTC14, and NB30 [Bordeira-Carrico et al., 2012].

Conclusions

This study expanded the picture of KMT2D and KDM6A mutations that cause KS, adds some insight in the functional mechanisms that cause the disease, and finally provides the first preliminary proof-of-concept that naturally occurring nonsense mutations in KMT2D and KDM6A can be effectively suppressed providing a rational strategy for identifying patients likely to respond and therefore more likely to benefit from treatment with readthrough inducers.

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Disclosure statement

The authors declare no competing interests.

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