Deletion of the ANKRD15 gene at 9p24.3 causes parent-of-origin-dependent inheritance of familial cerebral palsy

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A four-generation family was studied in which nine children had congenital cerebral palsy (CP), characterized by quadriplegia and mental retardation. All the affected children were born to healthy, related fathers, whereas the children of their healthy female relatives were unaffected. Linkage analysis attributed the condition to chromosome 9p24.3, where a 225 kb deletion was identified. The deletion spans a single gene, ANKRD15 (ankyrin repeat domain 15), which is ubiquitously expressed. In the affected children, the ANKRD15 is not expressed in lymphoblastoid cells, whereas in their healthy fathers, who harbor the same deletion, the expression of ANKRD15 did not deviate from controls. This expression pattern can be interpreted as a maternal imprinted gene that is expressed only from the paternal allele. The expression of ANKRD15 in lymphoblastoid cells from the control group was monoallelic but not imprinted. The monoallelic expression was restricted to the ANKRD15 gene, whereas biallelic expression was found in the DOCK8 gene, which resides at the telomeric side of the deletion. No correlation was found between the expression of the ANKRD15 gene and the pattern of DNA methylation in the CpG islands 5' of the gene. However, differences in methylation pattern were found in the CpG islands flanking the DMRT1 gene, which is located at the 3' side of the ANKRD15 gene. In the affected individuals, as in the control group, the CpG islands were hypomethylated, whereas in the healthy fathers, the CpG islands were hyper-methylated in cis with the deletion. This unique family demonstrates a phenomenon of a deletion that creates imprinting-like inheritance. The implication of this family to sporadic CP is discussed.

INTRODUCTION

Cerebral palsy (CP) is a group of non-progressive chronic disorders impairing control of movement and posture that results from an insult to the developing central nervous system (1,2). The characteristic signs of CP are spasticity, movement disorders, muscle weakness, ataxia and rigidity. The clinical classification of CP is based on the nature of the motor deficit as well as other symptoms accompanying it, such as mental retardation, optic atrophy, hearing loss and speech impediments. CP has been linked to unusual stresses that infants may undergo at birth and the susceptibility of an immature central nervous system to injury from a variety of agents such as cerebral anoxia, traumatic injury to the brain, fetal infection, hyperbilirubinemia, hypoglycemia, hypothyroidism as well as an inborn error in amino acid metabolism (3,2).

CP is the most common cause of severe physical disability in childhood, occurring in 0.15–0.25% of live births (1,4). The vast majority of CP patients are sporadic and only 2% are considered to be inherited. Recently, a new analysis of a Swedish CP children database (Goeteborg data) indicated that genetic causes might be the etiology in 40% of the children (5). Although familial aggregation of CP is very unusual, it may be of utmost importance in understanding the pathogenesis of idiopathic CP, which has no obvious explanation.

Here, we present a family in which nine children were born with typical CP to related fathers and the mechanism underlying its inheritance.
RESULTS

Description of BS family

An Israeli family (BS) of Jewish Moroccan origin was referred to genetic counseling because several children were affected with congenital neurodegenerative disease resembling CP (Fig. 1). The affected children were born to unrelated healthy parents following normal pregnancies and were delivered with no reported complications. Congenital hypotonia appeared at first, and over the first year, it evolved to spastic quadriplegia with accompanying transient nystagmus. All affected individuals had various degrees of mental retardation and most of them were institutionalized in special institutions for severely retarded children. Neuroimaging studies showed brain atrophy and ventriculomegaly. Extensive biochemical laboratory studies did not reveal any possible explanation for the severe phenotype. High-resolution chromosome analysis indicated normal karyotype; subtelomeric FISH analysis was normal.

Nine of the 20 paternal offspring were affected, whereas all 12 maternal offspring were healthy. All the affected children were born to related clinically healthy fathers (II-2, II-10, III-9, III-10; Fig. 1), indicating that the inheritance is dependent on the parental gender ($\chi^2 = 7.51, P < 0.01$). A possible explanation for this mode of inheritance is a maternal imprinted gene expressed only from the paternal allele.

Linkage study pointed to deletion at 9p24.3

Linkage analysis was performed assuming that the mode of inheritance in the family is autosomal dominant with incomplete penetrance. On the basis of the affected individuals and the obligatory carriers, a candidate region in the distal end of the short arm of chromosome 9p24 was identified, with maximum LOD score values of 3.53 and 3.45 for D9S1779 and D9S917, respectively. All the affected individuals and carriers share the same haplotype, which was inherited from the mother I-1 who transmitted it to four of her healthy children (II-2, II-5, II-10 and II-12). The affected individuals and obligate carriers were hemizygous for the marker D9S1858, indicating a deletion at this locus. Recombination

Figure 1. Pedigree of BS family and the segregation analysis. For each polymorphism, the numbers 1–10 represent the different alleles and (−) indicates hemizygosity of D9S1858. The arrow points to the recombination between D9S288 and D9S1858. Black squares or circles are affected, dark gray ones are carriers, individuals I-1, II-12, III-22 and III-23 are carriers that were found through the linkage study.
between D9S1858 and D9S288 in individual III-3 narrowed the candidate region to 4 Mb.

On the basis of the linkage analysis and the deletion presence, prenatal diagnosis was offered for at-risk pregnancies. Eight prenatal diagnoses were performed; in four of them, the pregnancies continued and healthy children were born and in the remainder, the pregnancies were terminated.

**Characterization of the deletion**

Segregation analysis was performed using polymorphic dinucleotide repeats in proximity to D9S1858, retrieved from the sequence of the contig NT_008413. The polymorphic repeats 587410(ca) on the telomeric side and 821991(gt) on the centromeric side were outside the deletion and provided...
an estimation of the deletion size from 100 to 240 kb
(Fig. 2A). Serial Southern hybridizations detected differences
in the intensity of the relevant restriction fragments when
compared with control fragments (Fig. 2B) and allowed local-
ization of the breakpoints within 5–10 kb from each side.

Primers designed from the boundaries of the deletion gener-
ated in a long-range PCR a 7 kb fragment from family
members with the deletion (data not shown), thus enabled us
to identify the breakpoints at two Alu elements: AluSq
(located in position 817 061–817 211) and AluSx (located in
position 590 601–590 750), which share 90% of sequence
homology, resulting in a deletion of 225 kb. PCR primers
were generated to amplify the junction fragment and it was
found only in BS family members who have the deletion
(Fig. 2C): it was not found among DNA samples of 210
anonymous control individuals, including 130 Jewish indivi-
duals of a similar ethnic background.

The only gene within the deletion is ANKRD15 (ankyrin
repeat domain 15). The DOCK8 gene (dedicator of cytokin-
esis 8) is located telomeric to the ANKRD15 gene and the
DMRT1 gene (doublesex and mab-3 related transcription
factor 1) lies on its centromeric side. The 5’ upstream
sequences of the DMRT1 gene might be included in the
deletion (Fig. 3A).

Expression study of the ANKRD15 gene

Spanning 275 kb, the ANKRD15 gene is ubiquitously
expressed in various tissues (GeneCards). It includes at least
12 exons (Fig. 3A) and two transcripts that code for the
same protein (1194 amino acids) but use different promoters
and are designated variant A, exons 2–12 (NM_153 186.2),
and variant B, exons 1, 3–12 (NM_015 158.1) (6). Other
alternative transcripts may exist according to the analysis of
the identified cDNAs clones and the genomic DNA
(AceView, NCBI).

High expression of ANKRD15 was found by northern
hybridization of fetal and adult tissues probed with
ANKRD15 exon 3. A major band of 6.2 kb is characteristic
of the fetal tissues: brain, lung, liver and kidney (Fig. 3B).
In the adult, high expression was prominent in the heart, skel-
etal muscle and kidney, whereas a very faint band was
observed in the brain (data not shown).

The expression of the ANKRD15 gene in BS family
members was analyzed in lymphoblastoid cell lines (LCLs)
established from the affected individuals and their fathers by
RT–PCR. In the affected children (IV-3, IV-6, III-20), there
was no expression of variant A and very low expression of
variant B, whereas in the fathers (III-9, III-10, II-10), the
expression was similar to the normal controls (C1, C2), suggesting monoallelic expression of the paternal allele (Fig. 3C). In extra-embryonic tissues (chorionic villi) of fetuses with paternal deletion (V1573, V1574), the expression was the same as that of matched controls (V1 and V2) (Fig. 3C). These results indicate that the gene is not uniformly expressed.

The mode of expression of ANKRD15 in LCL established from control individuals was done utilizing SNPs in the coding region gene (1236 C/G-dbSNP: 912 175; 1255 G/T-dbSNP: 912 174) The expression in LCL was monoallelic: in individuals 16-3 and 123-4, both variants are expressed only from the paternal allele, whereas in individual 189-13, the maternal allele is expressed. Fibroblasts (Fib SA, Fib PA) showed monoallelic expression of variant B and biallelic expression of variant A. The expressed allele in Fib SA is maternal (G). The parental origin of the alleles in Fib PA is not informative. In amniotic cells (A32966, A32968), the expression is biallelic for both variants. m, mother; f, father.

### Methylation pattern in the deletion segment

In order to examine whether the mode of ANKRD15 expression is attributed to differential methylation, four CpG islands within the deleted segment and in its flanking regions were chosen for methylation analysis (Fig. 4). Regions b (688 221–688 685) and c (692 515–694 200) were within the deletion, whereas regions a (485 219–486 988) and d (822 641–824 211) were outside. In the regions a, b and c, no differences were found in methylation pattern of affected and healthy individuals: regions a and b were hypo-methylated, whereas region c was hyper-methylated [analyzed by PCR amplification after HpaII digestion of genomic DNA (Fig. 4B) or sequencing after bisulfite modification (Fig. 4C)].

Region d, which encompasses CpG islands flanking the DMRT1 gene, was found to be hypo-methylated in normal control individuals. In this region, a different methylation pattern was found within BS family members: in the affected individuals, region d was hypo-methylated, whereas in the healthy deletion carriers, this region was hyper-methylated (Fig. 5). Using heterozygosity to the SNP (T/A dbSNP: 3 739 583), we were able to demonstrate in the carrier brothers (III-9 and III-10) that the methylation occurred on their maternal chromosome, the chromosome bearing the deletion (Fig. 5A). To confirm this result, we used the polymorphic gt-repeat at nucleotide 821 991 (Fig. 2A) that is located in the proximity of the CpG islands. Following HpaII digestion, a 847 bp fragment containing both the repeat and the CpG islands was generated only in carriers of the maternally derived deletion (Fig. 5B). This PCR fragment served as a template in nested PCR for the repeat polymorphism. In all individuals (III-9, III-10, II-5, II-2, II-10, II-12, III-22 and III-23), the 138 bp allele (maternal) was co-amplified with the hyper-methylated CpG, indicating that the methylation occurred on the maternal allele in cis with the deletion (Table 2). The same allele is hypo-methylated through paternal transmission (IV-3, IV-6, III-3, III-7, III-19 and III-20). These results confirmed that hyper-methylation occurred only through maternal transmission of the deletion.

### DISCUSSION

In this study, we present a four-generation family with unique features: (a) the phenotype of the affected individuals is indistinguishable from CP, which is usually non-genetic; (b) the trait is inherited through carrier fathers, whereas the offspring of carrier mothers are healthy. At face value, the mode of inheritance implies an imprinted gene that is expressed from the paternal allele. Linkage analysis pointed at 9p24.3 as the gene location in which a 225 kb deletion spanning the ANKRD15 gene was identified. This deletion was not found among 210 individuals of a control group.

The ANKRD15 that contains ankyrin repeats in its C-terminus is evolutionary conserved; its orthologs are found among multi-cellular organisms. It was first obtained from human cDNA library and termed as KIAA0172 (7),
and later it was characterized as a tumor suppressor gene (KANK) in renal cell carcinoma (6). ANKRD15 is ubiquitously expressed in various tissues during fetal development and adult life (including in LCL). In BS family, the ANKRD15 gene is expressed in healthy individuals who carry the deletion on the maternal allele and a normal paternal allele, whereas in the affected individuals, who are carriers of the paternal deletion and a normal maternal allele, the ANKRD15 gene is repressed.

Recently, VAB-19, the nematode ortholog of ANKRD15, was characterized, and in the presence of a mutated gene, an interesting phenotype of arrest in the 2-fold epidermal elongation period was created (8). Shortly after muscle contraction begins, muscle detaches from the epidermis, the mutant embryos twitch normally but never roll; these were essentially paralyzed after the 2-fold stage. In this study (8), it was shown that the VAB-19 gene product is essential for a defined time during embryo development and is important in attaching muscles to the epidermis via intermediate filament junctions. Sarkar et al. (6) have also shown that in the kidney tumor cell line (G-402), there is a difference in the β-actin distribution, depending on whether the cells express the ANKRD15 (KANK) gene. This supports the view that the ANKRD15 protein is important for the cytoskeleton structure and may be involved in crucial adhesion complexes.

The ANKRD15 gene is expressed in the fetal brain; if the gene product is indeed essential in a specific threshold and in a precise time window during the development of the fetal brain, then its absence may explain the severe phenotype in the affected children.

The characterized deletion may be rare, but other factors can influence the level of ANKRD15 gene expression and lead to its association with sporadic CP. As there are several binding sites, GCGTG, for hypoxia-induced factor 1 at the upstream sequences of the ANKRD15 gene (position 451 268–451 648), the expression of the gene may be oxygen dependent. Hypoxia and low birth weight (fewer cells in the fetal brain during critical developmental processes) are associated with the etiology of CP (9,10) and may potentially affect the level of ANKRD15 expression. Thus, combinations of factors that may affect the level of ANKRD15 expression possibly explain the sporadic nature of CP.
The parent-of-origin-dependent inheritance in BS family raises the possibility that *ANKRD15* is imprinted and expressed from the paternal allele; this possibility was also raised by Sarkar et al. (6). Normal control LCLs and fibroblasts showed monoallelic expression rather than the parent-of-origin effect-dependent expression. Monoallelic and/or variable expression has been documented for other genes and may even be common (11–13). The expression study identified two haplotypes based on the SNPs 1236G/C and 1255T/G; the haplotype 1236G-1255T was preferentially expressed in heterozygous cases, suggesting that the monoallelic expression is dependent on crucial *cis* elements.

An exception to the monoallelic expression was in embryonic fetal tissues: in normal amniotic cells, the expression was biallelic and in the chorionic villi of fetuses with paternal deletion, there was expression of the maternal allele. The mode of the *ANKRD15* expression supports the view of a complex locus with more than one promoter and several different transcripts that may code for different proteins.

In order to better understand the control mechanism governing the monoallelic expression, we analyzed the methylation pattern of the CpG islands in the promoter region of the *ANKRD15* gene. Surprisingly, there were no differences between the affected individuals, their fathers and the control group. However, differences in the methylation pattern were found in the promoter region of the *DMRT1* gene. Hyper-methylation occurred only on the deleted chromosome when it was transmitted through the mother, but upon paternal transmission, this region was hypo-methylated, as in individuals without deletion. It is reasonable to assume that hypo-methylation of the promoter region of the *DMRT1* gene is essential for normal spermatogenesis (14); however, the connection between the hypo-methylated *DMRT1* promoter and the repression of *ANKRD15* expression has not yet...
Table 2. The parental origin of the hyper-methylated allele in the DMRT1 gene

<table>
<thead>
<tr>
<th>Individual</th>
<th>Polymorphic alleles (gt-repeat 821 991)</th>
<th>Paternal</th>
<th>Maternal</th>
<th>After HpaII</th>
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<tbody>
<tr>
<td>IV-3</td>
<td>138</td>
<td>140</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>III-9</td>
<td>159</td>
<td>138</td>
<td>138</td>
<td>–</td>
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<tr>
<td>III-10</td>
<td>159</td>
<td>138</td>
<td>138</td>
<td>–</td>
</tr>
<tr>
<td>IV-6</td>
<td>138</td>
<td>146</td>
<td>–</td>
<td></td>
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<td>II-5</td>
<td>165</td>
<td>138</td>
<td>138</td>
<td>–</td>
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<td>II-2</td>
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<td>138</td>
<td>138</td>
<td>–</td>
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<td>III-2</td>
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<td>III-3</td>
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<td>–</td>
</tr>
<tr>
<td>II-10</td>
<td>140</td>
<td>138</td>
<td>138</td>
<td>–</td>
</tr>
<tr>
<td>III-19</td>
<td>146</td>
<td>138</td>
<td>138</td>
<td>–</td>
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<tr>
<td>III-20</td>
<td>138</td>
<td>118</td>
<td>–</td>
<td></td>
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<tr>
<td>I-1</td>
<td>146/138</td>
<td>138</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>I-12</td>
<td>165</td>
<td>138</td>
<td>138</td>
<td>–</td>
</tr>
<tr>
<td>III-22</td>
<td>138</td>
<td>138</td>
<td>138</td>
<td>–</td>
</tr>
<tr>
<td>III-23</td>
<td>138</td>
<td>138</td>
<td>138</td>
<td>–</td>
</tr>
</tbody>
</table>

Fibroblast cultures and LCLs from control individuals and amniocyte cultures were obtained from the Human Genetics Department at Hadassah Hospital. Control DNA samples were taken from the genetic screening samples, which were stored anonymously (except for ethnic origin). DNA was extracted using the salting-out procedure.

**Linkage study**

Polymorphic DNA marker sequences were retrieved from the Genome Data Base. PCR was done using 0.5 μM primers (MWG Biotech AG), with 0.5 units of Taq polymerase (Bioline), in the presence of 0.2 mM DNTPs (Bioline) and 1.5 mM MgCl2. The annealing temperature range was 55–58°C. PCR products were separated on denatured polyacrylamide gel (8%) and detected with silver staining (26). The LOD score values were calculated with LIPED (J. Ott version 1988).

**Characterization of the deletion**

The deletion length was estimated by segregation analysis of dinucleotide repeats, which were retrieved from the published sequences of chromosome 9 (AL136979, AL136365, AL390279) and analyzed as in the linkage study (primers 1–6, Table 3). For Southern hybridization, 10 μg genomic DNA was digested with restriction enzymes (New England Biolabs) according to the manufacturer’s recommendations, run on 0.8% agarose gel and blotted to Sure Blot CHEMl membrane (Intergen). Probes, generated by PCR using primers 7–8 (Table 3), were labeled with 11dUTP dig (Roche Diagnostics). Hybridization was performed at 42°C for 18 h in the presence of 50% formamide. The last wash was done with 0.1× SSC—1% SDS for 15 min at 65°C. Detection was carried out using the Sure Blot CHEMI detection kit (Intergen). X-OMAT films (Kodak) were exposed for 30–120 min at room temperature.

Long-range PCR was done with the Expand Long system (Roche Diagnostics) using designed primers no. 9 (Table 3). The PCR fragment of ~7 kb was mapped by restriction enzyme analysis and the junction fragment was sequenced with the BigDye terminator kit using the ABI 310 sequencer (Applied Biosystems). On the basis of the sequence of the junction fragment, primers were designed to amplify a shorter fragment and a pair of primers was used as positive control (nos 10 and 11, Table 3).

**Expression study**

Northern hybridization was done on RNA blots from fetal and adult tissues (MTN Blot, Clontech). Exon 3 of the ANKR15 gene was labeled with 11dUTP dig (Roche Diagnostics) through PCR (using the primers no. 12; Table 3) and probed for 1 h at 68°C. Last wash was performed with 0.1× SSC—0.1% SDS for 30 min at the same temperature.

RT–PCR first-strand synthesis was carried out on 1 μg of total RNA (extracted with RNaseasy kit, Qiagen). The reaction mixture included oligo dT (1 μg), random primers (2 μg), 100 μM dNTPs (Roche Diagnostics) and 100 units of SuperScript reverse transcriptase (Invitrogen); the mixture was incubated for 2 h at 42°C. PCR on the first strand was performed.
with the Expand Long Enzyme system (Roche Diagnostics) according to the manufacturer’s instructions and included specific primers (nos 13 for variant A, 14 for variant B and 15 for the 3' of the NF1 gene which serves as a control, Table 3). PCR products were run on 2% agarose gel and visualized under UV illumination.

The expression pattern in samples without the deletion was evaluated by comparing genomic (primers 16) and cDNA (primers 13 and 14) sequences in the ANKRD15 utilizing the following SNPs: dbSNP, 912 175 and dbSNP, 912 174. Primers nos 17 and 18 (Table 3) were used for dbSNP, 2 297 079 and dbSNP, 1 887 957 in the DOCK8 gene. The mode of BRCA1, BRCA2 and NF1 expressions was analyzed, respectively, utilizing the following SNPs: dbSNP, 16 942; dbSNP, 1 801 406 and dbSNP, 1 801 052.

X-inactivation test
Genomic DNA cut with HpaII served as a template for PCR using primers from both sides of the trinucleotide repeats in the androgen receptor (no. 26, Table 3). PCR products were analyzed on ABI 3100 with GeneScan software.

Table 3. PCR primers used in the study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Nucleotide location</th>
<th>Sequence (5’–3’)</th>
<th>Purpose</th>
<th>PCR product (bp)</th>
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<tbody>
<tr>
<td>1</td>
<td>587370</td>
<td>ctttccccacagtatagca</td>
<td>Segregation</td>
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<tr>
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<td>3</td>
<td>635983</td>
<td>gatagacgatagaacacgtgt</td>
<td>Segregation</td>
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<td>636187</td>
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<td>182</td>
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<td>708846</td>
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<td>Segregation</td>
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<td>6</td>
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<td>cggcgtgactgtagagcagc</td>
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<td>Cen3-probe</td>
<td>662</td>
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<td>9</td>
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was performed with primers no. 22. A segment of region d was amplified using primers no. 21 and then nested PCR designed for the modified strand (reviewed in 28). Region c was amplified with the CpGenome DNA methylation study under UV illumination.

PCR products were run on 2% agarose gel and visualized with the relevant primers (nos 19, 20, 23, 25; Table 3). PCR products were cloned with the TA cloning kit (Invitrogen) and sequenced with M13 primers by the ABI 310 sequencer.

Methylation restriction analysis was performed by digestion of 100 ng genomic DNA with 20 units of HpaII for 18 h at 37°C. The restriction enzyme was inactivated at 95°C for 10 min and then the different regions (a, b, c, d) were amplified with the relevant primers (nos 19, 20, 23, 25; Table 3). PCR products were run on 2% agarose gel and visualized under UV illumination.

Databases

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Conflict of Interest statement. None declared.

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