

Original Article

P53 Mutation at Codon 249 is Uncommon in Neonatal Kotb Disease Biliary Atresia

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Abstract:

Background: Hepatocellular carcinoma (HCC) is known to result from aflatoxin B1 that induces p53 mutation at codon 249. Aflatoxins are also known to cause The Kotb disease Biliary atresia (BA) variant which is characterized by congenital aflatoxicosis B1 in neonates with null glutathione S transferase M1.

Aim of the Work: We aimed to search for the aflatoxin B1 induced HCC 249 codon p53 mutation among neonates with Kotb disease BA variant and their mothers.

Patients and Methods: This study included 13 neonates and infants with confirmed BA who presented to Hepatology Clinic, New Children Hospital, Cairo University, Egypt during January-May 2019. All subjects and their mothers underwent detection of aflatoxins from peripheral blood. BA cases underwent detection of mutation from liver biopsy tissue as well.

Results: The studied cohort with confirmed BA comprised 9 (69.2%) girls and 4 (30.8%) boys, with mean ages \pm standard deviation (SD) at onset, presentation, diagnosis and portoenterostomy of 8.1 ± 5.7 days, 44.8 ± 11 days, 57 ± 14.53 and 64.5 ± 21.34 days respectively. All 13 and their mothers were found to have elevated blood levels of aflatoxin B1 with a mean of 8.56 ± 4.2 ng/ml and 14.75 ± 16.78 ng/ml respectively. The mean \pm SD duration of follow up was 259.1 ± 141 days. None of the mothers had abnormal levels of bilirubin or liver aminotransferases. All samples tested negative for p53 mutation at codon 249 except for one infant who tested negative for the mutation in his whole blood and had heterozygous mutation in DNA from his liver tissue. None of the studied cohort or their mothers had HCC. Cholestasis resolved in 2 children, 7 had progressive course and 4 died. There was no correlation between outcome and neonate/maternal aflatoxin B1 level ($p=0.299$; $p=0.443$), age at portoenterostomy ($p=0.93$), hepatic fibrosis degree ($p=0.56$), or other lab or liver biopsy findings.

Conclusion: p53 mutation at codon 249 is uncommon in infants with Kotb disease BA variant, despite the aflatoxicosis they suffer from. The cause remains to be studied. Screening for p53 mutation at codon 249 cannot be used as a diagnostic test for Kotb disease.

Level of Evidence of Study: IV (1).

Keywords: Aflatoxin B1; biliary atresia; hepatocellular carcinoma; Kotb disease; p53 mutation at codon 249

Abbreviations: AFT-B1: aflatoxin B1; ALT: alanine aminotransaminase; AST: aspartate aminotransaminase; BA: biliary atresia; CYP: cytochrome P450; GGT: gamma glutamyl transferase; GST: glutathione S transferase; HCC: hepatocellular carcinoma; PCR: polymerase chain reaction; SD: standard deviation.

Introduction

Biliary atresia (BA) results from ingoing inflammation and adhesions of major bile ducts, that rapidly progresses to obstruction of major bile ducts and liver cirrhosis during the early months of life. Pathogenesis can start as early as intrauterine gestational period (2). Outcome of BA depends on early prompt intervention, as beyond 90 days of life surgical removal of obstruction does not change the lethal irreversible hepatic damage (3). Etiology remained obscure for over 100 years, with many suggested hypotheses, but none could explain all aspects



of BA. Diagnosis of BA relied mainly on triangular cord (TC) sign detection by imaging, invasive procedures as percutaneous liver biopsy and intraoperative cholangiography (4–6). Newborn screening by watchful observation of stool color was found relatively reliable for early detection and prompt diagnosis. We have reported The Kotb Disease BA variant which is characterized by congenital aflatoxicosis B1 in neonates and infants with null glutathione S transferase M1 (GST M1) (7, 8). The Kotb Disease BA variant is characterized by the fact that only infants with null GST M1 suffer from congenital aflatoxin-induced cholangitis, neutrophil elastase induced liver damage and disruption of p53. All mothers of studied infants were heterozygous for GST M1; hence they do not exhibit clinical manifestations of aflatoxicosis. Aflatoxin B1 is the number one carcinogen in the world, it is hepatotoxic, and its hepatotoxicity is increased 20 folds by *Escherichia coli* toxins (9). Aflatoxin B1 is normally detoxified within hours by first tier cytochrome P450 (CYP)1A2, and second tier GST M1 detoxification enzymes (10). The CYP1A2 is ontogenically not functional during the early first 1-3months of life (11), hence all aflatoxin detoxification during early life would be handled by GST. In the presence of aflatoxins, the neonates with null GST M1 and dysfunctional GST Pi would present by BA phenotype, i.e. The Kotb disease.

We are not aware of the frequency, incidence or prevalence of Kotb disease variant as we are the only center that reported that aflatoxins cause BA among neonates with GSTM1 deficiency (12, 13). The Kotb Disease BA variant results from congenital aflatoxin-induced cholangiopathy in neonates with null for GST M1, yet they do not regularly symptomatize with the hepatocellular carcinoma (HCC) phenotype (7), which is notoriously known to be induced by aflatoxins. Aflatoxin B1 induced HCC is also known to cause 249 codon p53 mutation in situ, and in blood (14, 15). We aimed to study aflatoxin B1 induced 249 mutation of p53 among BA neonates with cholestasis and their mothers.

Subjects and Methods

This prospective descriptive study included 13 neonates and infants with confirmed BA who presented to Hepatology Clinic, New Children Hospital, Cairo University, Egypt during January-May 2019 and followed up to identify their outcome. The study was approved by Pediatric Surgery Department Councils, Cairo University, Egypt and Tanta University. (Tanta University IRB approval number: 35882/9/22). The study conformed with the Helsinki Declaration of studies (16).

Participants

All subjects and their mothers underwent detection of aflatoxins from peripheral blood and detection of mutation of codon 249 of p53 gene from peripheral blood and liver biopsy tissue from confirmed BA cases.

Methods

BA diagnosis was confirmed by assessment of liver transaminases (alanine and aspartate) (ALT, AST), gamma glutamyl transpeptidase (GGT), bleeding pro-file, imaging as abdominal sonography, liver biopsy and operative cholangiography. Other lab or imaging done were dictated by clinical judgement. Outcome grading was cleared jaundice, improvement within four folds of upper level of normal of bilirubin, progressive disease, and death of liver cell failure due to BA (17). The cohort was followed regularly clinically, by laboratory and sonographic imaging. Detection of hepatocellular carcinoma relied upon ultrasonography; alfa fetoprotein was only tested in cases where a suspicious mass was detected by imaging during the follow up of 12 months.

Detection of Aflatoxin B1

Aflatoxin B1 (AFT-B1) was detected by using AFT-B1 Elisa kit (NOVA) (Cat no FT0011), (USA) according to the manufacture instruction. The kit is an AFT-B1 quantitative detection of serum sample, it uses a direct competitive ELISA method in microplate coated with AFT-B1 coupled antigen. The samples compete for AFT-B1 antibody enzyme marker color with TMB substrate; AFT-B1 standard or samples, free AFT-B1 and pore strips pre-coated AFT-B1 conjugated antigen. The stop solution is added after the color turns from blue to yellow. The sample absorbance value is read by microplate reader detection wavelength at 450nm. AFT-B1 content is calculated inversely proportional to the sample through the standard curve AFT-B1 content. We followed the recommended steps of keeping all reagents before the start of the experiment at room temperature. The sample dilution was (10x), concentrated washing liquid (20x) was diluted by distilled water into the working fluid. A 50 µl 0.0 ng/ml standard solution was added in the hole B0, 50 µl of the standard holes was added in the standard solution, 50 µl



sample solution was added in the hole in each sample and in all holes by adding 50 µl of anti-AFT-B1 antibody conjugate. The incubation was for 30 min at 37 °C temperature. We got rid of holes in the liquid, washed with a lotion microplate 5 times, then added 50 µl of solution A and 50 µl of solution B (color solutions) in each well, and with mild shake of the plate to mix thoroughly and incubate at 37 °C for 10 min. For each well we added 50 µl stop solution, mixed and read absorbance at 450nm. The AFT-B1 concentration was read on the standard curve graph where the value of x axis, y axis is percentage of absorbance.

Detection of p53 Mutation at Codon 249 (R249s)

DNA extraction

DNA was extracted from both EDTA anti-coagulated peripheral blood samples and portoenterostomy liver tissue by using QIAamp DNA blood Mini Kit (QIAGEN) (Cat no 51104) (Germany) according to the manufacture instruction.

Nested Polymerase chain reaction (PCR) amplification

Nested PCR amplification was performed on the 39 samples using 5 microns of extracted DNA was used for amplification of exon 7 of the TP53 gene with 0.2 mM primers (final concentration) flanking this exon. The first PCR reaction consisted of 0.5 µL of DNA template, 10 µL of Perfect Taq plus Master Mix Kit (5 prime GmbH, Hamburg, Germany), 1.25 mM outer forward primer (TP53-OS: 5'-CTTGCCACAGGTCTCCCCAA-3'), 1.25 mM outer reverse primer (TP53-OAS: 5'-AGGGGTCAGAGGCAAGCAGA-3'), and distilled water to final volume as 25 µL. The second PCR reaction was performed using nested primers, an inner forward primer (TP53-OS: 5'-AGGCGCACTGGCCTCATCTT-3') and inner reverse primer (TP53-OAS: 5'-TGTGCAGGGTGGCAAGTGGC-3'), first PCR product as DNA template. The PCR cycles for the first and second amplification were the same initially denatured at 94°C for 3 min, followed by 40 cycles at 94°C for 18 s (denaturing), at 56°C for 21 s (annealing), at 72 °C for 1.30 min (extension) and final 10 min extension at 72°C. The PCR-amplified products were examined by 2% agarose gel electrophoresis then stained with ethidium bromide and visualized under UV light. The size of the final PCR fragments were 237 bp and 177 bp for the first PCR product and the second PCR products respectively.

Restriction fragment length polymorphism (RFLP)

After that, the second PCR products were subjected to digestion with the restriction endonuclease, HaeIII, which recognizes the sequence CCGG that encompasses the 249 codon. The RFLP mixture contained 1 unit of HaeIII (New England BioLabs inc, Ipswich, MA, USA), 2 µl of 10X Buffer (New England BioLabs inc, Ipswich, MA, USA), 15 µl of second PCR product and dis-tilled water to a final volume of 20 µl. This mixture was then incubated at 37°C overnight and subsequently run on a 3% agarose gel. RFLP should cut wild-type DNA to producing three bands of 12, 61, and 92 bp by cutting at 3 sites, while RFLP would produce two bands in the mutant of 12 and 153 bp by cutting in 2 sites only.

Statistical Analysis

Statistical analysis of the data of the studied cohort was performed using statistical package for social science (SPSS) version 19 IBM Corp, USA. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp, USA. Data were tabulated and presented as simple mean and standard deviations. Correlation between studied parameters and outcome was also studied.

Results

Description of Studied Cohort

The studied cohort of cases with confirmed BA comprised 9 (69.2%) girls and 4 (30.8%) boys, 5 (38.4%) of them had consanguineous parents. All belonged to lesser advantaged classes and low-income families. None had family history of similar condition among sibs or family members. Among the studied infants with BA the mean ages ± standard deviation (SD) at onset, were 8.1±5.7 days, at presentation was 44.8±11 days, at diagnosis was 57±14.53 and at portoenterostomy was 64.5±21.34 days. The mean ± SD duration of follow up of the cohort was 259.1±141 days. At their clinical presentation all had clay stools, hepatomegaly, 11 had splenomegaly, 4 gave history of bleeding (ecchymosis in 2, bleeding umbilical stump, intracranial hemorrhage in 1), one had initial indirect hyperbilirubinemia necessitating phototherapy and one had umbilical hernia. Diagnosis relied upon typical lab findings, aflatoxin levels, abdominal sonar, and liver biopsy. The initial mean (± SD) total bilirubin level was 12.37±5.3 mg/dL, and



direct bilirubin 7.9 ± 2.89 mg/dL, 2.4 ± 1.4 folds of rise above upper limit of normal of ALT, 3.1 ± 2 folds of rise above upper limit of normal of AST, 1 ± 0.3 folds of rise above upper limit of normal of alkaline phosphatase and 4.9 ± 2.5 GGT folds of rise above upper limit of normal of at presentation. (Table 1) All had contracted gall bladder upon ultrasonography despite fasting and positive triangular cord sign. All had moderate to severe fibrosis of their percutaneous liver biopsy tissues, biliary plugs, von Kupffer cells hyperplasia, portal tract edema, few scattered giant cells, moderate to diffuse bile duct proliferation and hepatocyte balloon degeneration. Final visit labs are shown in Table 2.

Table 1. Description of the studied cohort with Kotb Disease Biliary Atresia

	Mean	SD
Age at onset (in days)	8.1	5.7
Age at clinical presentation to medical attention (in days)	44.8	11
Age at diagnosis (in days)	57	14.53
Age at Kasai portoenterostomy (in days)	64.5	21.3
Aflatoxin Level in blood of neonate (ng/ml)	8.56	4.2
Aflatoxin Level in blood of mother (ng/ml)	14.75	16.8
Total Bilirubin (mg%)	12.37	5.3
Direct Bilirubin (mg%)	7.9	2.9
ALT (in folds of upper level of normal)	2.4	1.4
AST (in folds of upper level of normal)	3.1	2
GGT (in folds of upper level of normal)	4.9	2.5
	Number	%
Sex		
Female	9	69.2
Male	4	30.78
Outcome		
Resolved cholestasis	2	15.38
Improved	0	
Progression of liver disease	7	53.8
Death within 18 months of portoenterostomy	4	30.78
p53 mutation at codon 249 of exon 7		
Heterozygous mutation in whole blood of neonate	0	0
Homozygous mutation in whole blood of neonate	0	0
Heterozygous mutation in excised porta hepatis of neonate	1	7.6%
Homozygous mutation in excised porta hepatis of neonate	0	0
Heterozygous mutation in mother	0	0
Homozygous mutation in mother	0	0

ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyl transferase.

Aflatoxin B1 Detected Levels

All had elevated blood levels of aflatoxin B1 that ranged between 2.14-16.77 ng/ml with a mean of 8.56 ± 4.2 ng/ml. Their mother's aflatoxin B1 levels had a mean of 14.75 ± 16.78 ng/ml. None of mothers had abnormal levels of bilirubin or liver aminotransferases.

Table 2. Initial and final Lab values of the studied cohort with confirmed Kotb Disease Biliary Atresia

	Before Portoenterostomy	After Portoenterostomy	P value
Total Bilirubin (mg/dL)	12.37 ± 5.3	7.05 ± 6.49	0.044
Direct Bilirubin (mg/dL)	7.9 ± 2.89	5.2 ± 5.1	0.539
ALT (folds of upper level of normal)	2.4 ± 1.4	1.96 ± 1.4	0.26
AST (folds of upper level of normal)	3.1 ± 2	2.4 ± 1.26	0.33
Alkaline Phosphatase (folds of upper level of normal)	1 ± 0.3	1.2 ± 0.73	0.09
GGT (folds of upper level of normal)	4.9 ± 2.5	4.7 ± 4.13	0.004

ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyl transferase.

p53 mutation at codon 249 Findings

Twelve of the studied whole blood of infants, their respective liver tissue and their mothers tested negative for p53 mutation at codon 249. One infant tested negative for p53 mutation at codon 249 in his whole blood, and heterozygous in his liver tissue, but unfortunately, he died 3 months after portoenterostomy. None of the studied cohort or their mothers developed HCC.

Outcome of Studied Cohort with BA

All studied infants received fat soluble vitamins, 10 received sulfamethoxazole (20mg/kg/day) and trimethoprim (4mg/kg/day) and 2 received one-month prednisone. Eight received off-label ursodeoxycholic acid after initial improvement and restoration of bile flow, all had progression of their liver disease. All were exclusively breast fed except for one child. Complications were abundant, ranging from repeated attacks of cholangitis among all, portal hypertension in 8, hematemesis and melena in 3, spontaneous bacterial peritonitis in 3, repeated attacks of diarrhea among 3 and multiple hepatic abscesses in one. Initially 3 of studied cohort resolved their jaundice, yet one had a relapse of cholestasis 2 months later and only 2 maintained the resolution during the follow up period, none had an improved outcome, 7 had progressive course and 4 died. None of the studied infants or their mothers developed hepatocellular carcinoma. There was no correlation between outcome and aflatoxin B1 level in infants with BA ($r=0.312$, $p=0.299$) or their mothers aflatoxin B1 levels ($r=0.233$, $p=0.443$), (Table 3) age at Kasai portoenterostomy ($r= -0.036$, $p=0.93$), degree of hepatic fibrosis ($r= -0.018$, $p=0.56$), or other lab or liver biopsy findings.

Table 3. Aflatoxin Levels and Outcome of the studied cohort with Kotb Disease Biliary Atresia

Outcome	Number	Child Aflatoxin Level (Mean± SD)	P value
Resolved cholestasis	2	6.3±3.35	0.299 (r=0.312)
Improved	0		
Progression of liver disease	7	8.085±4.2	
Death within 18 months of portoenterostomy	4	11.72 ± 3.57	

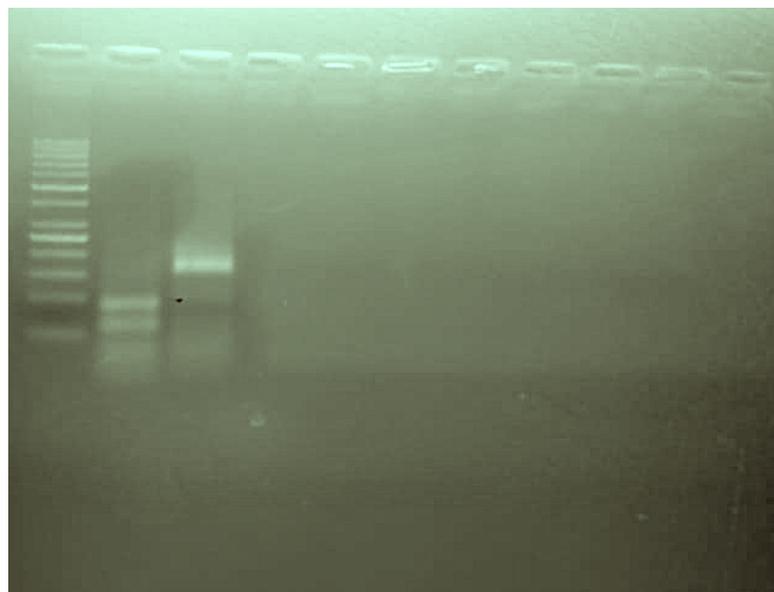


Figure 1. The only detected heterozygous p53 mutation of the excised porta-hepatis liver tissue among studied cohort of Kotb disease biliary atresia variant.

Heterozygous p53 mutation at codon 249 of exon 7 of extracted DNA from liver portoenterostomy core of a boy who had no mutation of DNA extracted from whole blood. This was the only mutation that we detected among our studied 36 samples from portoenterostomy removed hepatic liver tissue of infants, whole blood of infants and their mothers. First lane on the left: ladder 50bp. Second lane: wild type -no mutation- (bands at 12, 61 and 92). Third lane: mutant type (bands at 12 and 153). None of the studied cases or their mothers had any other heterozygous or homozygous mutation of p53 mutation at codon 249 of exon 7.



Discussion

Aflatoxin B1 and B2 in synergy with other factors induce disease phenotypes that has been known to be diverse among age groups. The reported phenotypes include biliary atresia the Kotb disease (12, 13), hepatitis, liver cell failure, hepatocellular carcinoma, failure to thrive, cholestasis, growth retardation, stroke and is known to damage kidneys, heart, brain and a very wide spectrum of other chronic diseases (18). Aflatoxins are known to cause acute tissue necrosis, organ failure and death, or interference with DNA repair and genotoxicity, or dysregulation of immune system and disruption of immune response (19–21).

Aflatoxins are not a normal constituent in blood, they form covalent adducts with albumin (22). Its maximum concentration peaks within half an hour of ingestion after which it starts declining during its elimination as the half-life is almost 15 hours in dairy cows. It is not clear however what are the determinants of when it causes disease? And which type of disease will be induced? And how long will it take to eliminate it after ingestion in humans (23)? Yet, we know that we have individualized detoxification genomics (24), and we know that at times we get exposed to more than a mycotoxin (25) in our meals, hence the response to aflatoxin load is not typical as a lot of factors and interaction governs the individual response to aflatoxin (26). All our studied cohort and their mothers had elevated aflatoxin B1 levels, hence, they all suffered from Kotb disease BA variant. The mothers did not have symptoms and signs of liver disease, yet their children had biliary atresia variant. Our earlier work has identified null glutathione S transferase as the determinant factor in development of BA, identified other factors responsible for severity of the variant, e.g. bacterial lipopolysaccharide, up-regulated CD34 + and disrupted p53 (7). It is very interesting however, that none of the studied cohort with confirmed BA lacked aflatoxin B1 in their blood. It is not clear how prevalent Kotb disease is, but thus far, aflatoxins were always detected whenever assessed among Egyptian infants with BA. Hence, it seems, Kotb disease is a recognized cause of BA among Egyptian neonates with BA. It remains to be studied if other variants of BA exists among Egyptian neonates with biliary atresia.

It is not clear if this variant is present in other geographic areas in the world, as international literature and studies of aflatoxins and GST M1 among those with BA are lacking.

Our results provide an explanation as to why we rarely encounter HCC among children with BA the Kotb disease, or among their mothers despite the aflatoxicosis. Yet, it is not clear why aflatoxins did not cause p53 mutation at codon 249 among our cohort and their mothers. It might be that the Kotb disease variant determinants protect against p53 mutation at codon 249, or that the typical p53 mutation needs more time to develop. It is interesting however, that all infants with biliary atresia tested for p53 were found to have p53 disruption (9). It remains to be seen if this child within situ mutation would develop HCC later in life.

Other crucial determinants of phenotype seem to be the neutrophil elastase attack on aflatoxin damaged hepatocytes and cholangiocytes, as children with Down syndrome who are known to have neutrophil function defects do not develop biliary atresia at all (27). It seems that more factors interact to produce the BA Kotb disease variant and the very poor outcome of native livers despite early prompt portoenterostomy.

Yet epigenetic modifiers of phenotype of BA await to be explored (28). Again it is important to highlight that only 10-50% with HCC have p53 mutation at codon 249 (29), hence the absence of p53 mutation at codon 249 does not rule out the susceptibility to develop HCC in BA (30, 31). During the 12 months of follow up, none developed HCC. The deceased neonate with heterozygous p53 mutation at codon 249 in excised porta hepatitis liver tissue but not in his whole blood sample poses a challenge. It is not clear if the boy would have developed homozygous mutation had he survived. Definitely underlying epigenetic factors and other genetic factors control the development or protect against the development of this mutation but are yet to be discovered. Unfortunately, the parents did not permit autopsy of this child.

The work also highlights the poor outcome associated with the Off-label use of ursodeoxycholic acid. It is not clear if the constant ursodeoxycholic acid deleterious effect among Egyptian children is a racial susceptibility. Its use among children with liver disease should be strictly within institutionally approved research premise (16) with frequent regular assessment of lithocholic acid (27, 32–34).

The small sample size of our studied group is a limitation, and the very poor outcome of our studied cohort despite the timely portoenterostomy did not allow for drawing conclusions about clear cut determinants of outcome. Another limitation of our study is the lack of infants from higher income classes; hence we cannot evaluate the effect of the low-income on the food they consume and lesser advantaged housing (35) as a determinant of the poor grave outcome of our studied BA cohort might. While screening for p53 mutation at codon 249 might be regarded as a useful molecular marker for early identification of HCC (36), screening for p53 mutation at codon



249 cannot be used as a diagnostic test for Kotb disease, or a screening test in neonatal screening for Kotb disease BA variant.

Conclusion

Infants with Kotb disease the biliary atresia variant rarely have p53 mutation at codon 249 despite the aflatoxicosis they suffer from. The cause of lack of aflatoxicosis induced p53 mutation at codon 249 among our studied cohort remains to be studied.

Author Contributions: All authors searched medical literature, databases, conceptualized, conducted the case review and reviewed the final manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in connection with the reported study. Authors declare veracity of information.

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