

Supplementary Appendix

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Supplement to: Wang J, Cortina G, Wu SV, et al. Mutant neurogenin-3 in congenital malabsorptive diarrhea. *N Engl J Med* 2006;355:270-80.

Supplementary Data

METHODS:

Isolation of Genomic DNA and Sequencing of *NEUROG3* Gene

DNA was isolated from blood samples using the QIAamp DNA Blood Mini kit and measured by optical density at 260 nm. The two exons of the *NEUROG3* were amplified, isolated and sequenced using standard methods.¹ The oligonucleotides used to amplify the exons were; exon 1: 5'-ACCCATTCTCTCTTCTTTTCTCC-3' (sense) and 5'-TACAAGCTGTGGTCCGCTATGC-3' (α -sense); exon 2: 5'-CAATCGAATG-CACGACCTCAAC-3' (sense) and 5'-AGATTATGGGGTGGTGGCAGAG-3' (α -sense). Polymerase chain reaction (PCR) were performed using 30 ng of genomic DNA by standard methods, for 35 cycles at 95°C for 30s, 62°C for 30s and 72°C for 60s. PCR-amplified exons were gel purified and sequenced bidirectionally with the previously described oligonucleotides.

Cloning, Site-Directed Mutagenesis, mRNA and Protein Synthesis

Expression vectors containing murine *NEUROG3* and human E47 were previously described and cloned in the pCR3.1 vector.² The human *NEUROG3* isoform was amplified (sense: 5'-GGATGACGCCTCAACCCTCGGGTG and α -sense: 5'-TCACAGAA-AATCTGAGAAAGCCAG), isolated and subcloned into the pCR2.1 vector. The insert was released by *Xba* I/*Hind* III digestion and subcloned into a similarly digested pCDNA3.1 (-) vector.

To simulate the identified mutations, site-directed mutagenesis was performed using the QuickChange™ (Stratagene) kit on both the human and murine transcript clones using the following oligonucleotides: R93L- human 5'- AACGACCGCGAGCTCAATC-GAATGCAC-3' (sense) and 5'- GTGCATTCGATTGAGCTCGCGGTCGTT-3' (α -sense); R93L- murine 5'- AATGATCGGGAGCTCAATCGCATGCAC-3' (sense) and 5'- GTGCATGCGATTGAGCTCCCGATCATT-3' (α -sense); R107S - human 5'- CTGGACGCCCTGAGCGGTGTCCTGC-3' (sense) and 5'- GCAGGACACCGCTCAGGGCGTCCAG-3' (α -sense); R107S - murine 5'- CTGGATGCGCTGAGCGGTGTCCTGC-3' (sense) and 5'- GCAGGACACCGCTCAGCGCATCCAG-3' (α -sense). To add a FLAG peptide sequence at the C terminus of NEUROG3, PCR was performed with each expression vector using T7 sense and FLAG specific α -sense oligonucleotide (5'- CGGGATCCTCACTTGTCATCGTCATCCTTGTTAGTCCAAGAAGTCTGAGAACACCAG-3'). The PCR product was digested with *Hind* III-*Bam*HI and subcloned into the corresponding site of pCR3.1.

In vitro transcription was performed on *AFL* III linearized murine NEUROG3 (wild-type, R107S and R93L) using the MEGAscript® T7 Kit (Ambion) and capped as recommended by the manufacture. *In vitro* translation was performed on human NEUROG3 (wild-type, R107S and R93L) and wild-type E47 linearized with *Hind* III using the Promega TNT coupled transcription-translation® kit. Parallel reactions with [³⁵S] methionine-labeled or unlabeled methionine were performed, and labeled samples were assessed by sodium dodecyl sulfate-polymerase gel electrophoresis (SDS-PAGE) on a 10% gel to confirm the relative size and abundance of the translated products.

Transient Transfections

A previously described luciferase reporter vector containing 155-bp of the immediate upstream region of the *NeuroD1* promoter, including three putative E boxes (labeled E1 to E3) and subcloned in pGL3-TATA was used for transient transfection assays.² HeLa cells which do not express NEUROG3, were grown in Dulbecco's modified Eagles media and transfected with Lipofectamine™ (Invitrogen) according to manufacture's protocol. Cotransfections were performed with the murine NEUROG3 wild-type and mutant pCR3.1 expression vectors. Negative control experiments were performed with a clone containing mutated (R93L) murine NEUROG3 subcloned in an anti-sense orientation. Protein stability following the transfection was assessed by Western blot using FLAG-tagged wild-type and mutant NEUROG3 vectors using commercially available anti-FLAG and anti- β -actin antibodies.

DNA Binding Assay

Standard electrophoretic mobility shift assays (EMSA) were performed utilizing specific methods well known to our laboratory.^{3,4} EMSA was performed using a previously double-stranded oligonucleotide (5'-GATCGGACCGGGAAGACCATATG-GCGCATGCCGGG-3') representing the E-box 1 of the NeuroD1 promoter, that was labeled with Klenow fragments.² *In vitro* translated wild-type E47 and wild-type and mutant NEUROG3 proteins were used to assess relative binding affinities by standard techniques.⁵

Assessment of NEUROG3 function in *Xenopus* Embryos

The wild-type and mutant murine NEUROG3 transcribed and capped mRNA using standard commercially available reagents was injected in the animal region of the

prospective left blastomeres of the four-celled *Xenopus* embryos according to previously described methods.² In these experiments, the amount of RNA injected into the frogs was 600 pg/blastomere and rhodamine with 2.5-5ng dextran coated beads was co-injected as a lineage tracer or injected alone as a control for non specific effects due to the microinjection procedure. Embryos were scored by fluorescence microscopy, and those embryos that lack rhodamine were discarded. The rhodamine+ embryos (at least 10 embryos in each group) were hybridized with a digoxigenin-labeled *BETA2/NeuroD1* cDNA as previously described.² The uninjected side of the NEUROG3-injected embryos was used as a control.

RESULTS:

Supplementary clinical history of three children with generalized malabsorption

Case #1: The index case was a full term male product of a consanguineous relationship born to parents of Mexican-American descent. He was hospitalized on several occasions during the first month of life with vomiting, diarrhea and dehydration and a severe hyperchloremic metabolic acidosis. He was found to have severe intractable malabsorptive diarrhea that ceased only with fasting. He had normal 46 X, Y chromosomes, mitochondrial DNA and cystic fibrosis DNA screening. Sweat chloride test, serum amino acids and urine organic acids, and cholesterol panel were all normal. HIV1/2 antibody, T and B cell subsets and repetitive stool cultures were always normal. Stool trypsin activity was 4+, and mucosa lactase, maltase, sucrase, isomaltase, and glucoamylase activity were normal on two separate occasions. A barium upper GI and small bowel follow through study was normal.

An exhaustive trial of various formulas and individual nutrients was attempted. On an electrolyte and glucose-based oral rehydration solution the stool output was 100 gm day kg⁻¹ (normal is <20 gm day kg⁻¹). On a carbohydrate-free formula consisting of protein and fat, the diarrhea continued, and when either glucose or fructose was added, stool volumes increased even further. A trial of several amino acid-based formulas, either with (Neocate[®] and Vivonex[®]) or without (Mead Johnson 3232A[®]) carbohydrates, resulted in similar levels of diarrhea. The addition of pancreatic enzymes had no influence on the severity of his diarrhea. He had no evidence of diarrhea when consuming only water, however, his stool volume increased when either medium or long chain triglycerides were added. When amino acids (3%) were added to water, he had 80 gm day kg⁻¹ of stool and ~80% of the ingested amino acids were recovered in the feces.

After all attempts to provide enteral nutrition failed, he was discharged on parenteral nutrition and a small amount of oral feeds. His serum glucose level during this time was normal. Over the course of the subsequent 18 months, he developed progressive chronic liver disease and continued diarrhea with all challenges of enteral nutrients. At two years of age he underwent a native subtotal enterectomy and total hepatectomy and received an orthotopic en bloc liver-intestinal transplant. He had an unremarkable post-operative course and was doing well on full oral feeds without either diarrhea or glucose intolerance. Unfortunately he died unexpectedly for central venous line sepsis one month short of his third birthday.

Case #2: The patient was a 2,720 gm male born to Mexican-American parents after a full-term uneventful pregnancy. Initially he was fed a combination of breast milk and a

cow's milk protein based formula, and he developed diarrhea by day 4 of life. At 6 days of age, he was admitted for diarrhea and dehydration with stools positive for occult blood. His white blood cell differential showed 17% eosinophils. A venous pH was 7.13 and electrolytes showed a marked hyperchloremic metabolic acidosis. All stool cultures and specimens for *C. difficile* toxin were negative. He was thought to have allergic enterocolitis, and after intravenous rehydration and correction of his acid-base abnormalities, he was discharged taking an elemental amino-acid based formula.

At five weeks of age, he was readmitted weighing 250 gm below his birth weight and appearing severely dehydrated and malnourished. Again, his serum electrolytes showed a profound hyperchloremic metabolic acidosis with no anion gap. Serum glucose, magnesium, calcium, aminotransferases, alkaline phosphatase, bilirubin, amylase, and lipase were normal. All stools examined for bacteria, *C. difficile* toxin, viruses, and parasites were negative. Stools were found to contain reducing substances but no obvious fat. The total protein was normal and the albumin mildly depressed at 3.0 gm/dl. Cholesterol was 89 mg/dl and triglycerides 33 mg/dl. Serum zinc levels were normal. A sweat test was negative as was cystic fibrosis DNA screening. Quantitative T and B cell studies were normal and there was no evidence of HIV. After rehydration, simultaneous lactate and pyruvate determinations were normal.

The diarrhea stopped promptly when the infant was fasted. Resumption of the amino acid based elemental formula resulted in the immediate resumption of diarrhea with positive reducing substances and a stool pH between 3.5 and 5.5. Measurement of brush border disaccharidases including lactase, maltase, sucrase, isomaltase, and glucoamylase were all within normal limits.

Empiric trials of cholestyramine, loperamide, pancreatic enzymes, and broad spectrum antibiotics for bacterial overgrowth were not helpful. A trial of a carbohydrate free formula was tolerated slightly better. However, the addition of fructose to the formula again resulted in worsening diarrhea. Because of persistent diarrhea, a central venous catheter was placed and parenteral nutrition begun. Over the course of 5 months in the hospital, the infant gained approximately 12 gm/day.

During the next year, the patient was admitted three times for septicemia associated with candida species, staphylococcus aureus, and gram-negative enteric bacteria, necessitating the removal and replacement of central venous lines. He also was treated for recurrent episodes of C.difficile infection with worsening diarrhea. During one admission, esophagogastroduodenoscopy and colonoscopy were performed and were unrevealing. During the procedure, a secretin stimulation test of pancreatic exocrine function was done and showed normal levels of baseline and stimulated lipase and trypsin, but low levels of amylase pre and post secretin infusion. A serum immunoreactive trypsinogen level was normal and long bone films showed no changes consistent with Shwachman's syndrome. Further investigations with normal results included anti-tissue transglutaminase antibodies, plasma total and free carnitine levels and acylcarnitine profile, plasma amino acids, urine organic acids, lipoprotein profile, apo-B levels, and fasting serum bile acids. He continued to grow, but both height and weight were below the 3rd percentile, and his weight for height was at the 5th percentile. Mild delay in both speech and motor development were thought to be secondary to prolonged hospitalization during his first year of life.

Over the course of the next two years, the patient consumed increasing amounts of normal food including cow milk and was weaned from parenteral nutrition. Diarrhea continued in spite of trials of probiotics including lactobacillus and saccharomyces boulardii to prevent recurrent colonization with *C. difficile*. However, he was able to maintain normal acid base balance and hydration with supplemental oral acetate solution. Because of persistently mildly elevated aminotransferases even one year after discontinuation of parenteral nutrition, tests for hepatitis A, B, and C were done and were negative. An abdominal ultrasound showed the presence of gallstones but no evidence of biliary obstruction. A liver biopsy revealed steatohepatitis with marked macro and microvesicular steatosis and a mixed portal infiltrate with neutrophils and lymphocytes. There was no evidence of cholestasis or fibrosis.

Now at the age of seven years, he consumes a regular diet and has five loose bowel movements per day. Monitoring has revealed low levels of vitamin E and vitamin D with normal levels of vitamin A and normal prothrombin and partial thromboplastin times. All other vitamin levels are normal as are serum electrolytes, total protein, albumin, calcium, phosphate, and aminotransferases. Serum magnesium is slightly depressed. Currently, the patient receives supplemental oral fat soluble vitamins, magnesium, and acetate. While receiving parenteral nutrition, his blood glucose levels were occasionally elevated, but since receiving all of his nutrition enterally, his blood glucose levels were normal. He just presented recently with evidence of hyperglycemia which is currently being evaluated.

Case #3: The patient was born at 36 weeks gestation in the Emergency department at Wake Forest University Baptist Medical Center. Mother was a 22 year old Mexican-

American female who had no prenatal care. Birth weight was 2334 grams and APGAR's at 1 and 5 minutes were 9. The mother left the hospital against medical advice and never returned to claim the infant. The infant was initially fed Enfamil 24 cal/oz which he took well. He was discharged to the care of foster parents on day 6 of life with a weight of 2070 grams and report that he passed a stool with each feed.

He was readmitted to hospital on day 12 of life with clinical features of dehydration and a weight of 1970 grams. Lab work revealed a hyperchloremic ($Cl = 130$) metabolic acidosis with a CO_2 of 9 and a normal anion gap. Stools negative for rotavirus and positive (2+) for reducing substances. He received IV fluids and feeds were changed to LactoFree formula and subsequently to Pregestimil because of persistent reducing substance positive stools. Additional workup included stool and blood cultures and serum amino acids, urinary organic acids and acyl carnitine levels - all of which subsequently came back negative. He was discharged after 6 days with a weight of 2150 grams but with persistent loose stools.

He was readmitted on day 31 of life because of persistent diarrhea and poor weight gain. Admission weight was 2200 grams. He had a persistent metabolic acidosis with a CO_2 of 15 despite Polycitra t.i.d. Repeat stools for culture and rotavirus were negative. Additional work up included tests for HIV and sweat tests were all negative. Diarrhea persisted even on Pedialyte. A central line was placed and TPN was started. While NPO on the TPN there was a marked decrease in stool output. Neocate feeds were introduced and diarrhea returned. He developed central line sepsis on two occasions requiring treatment with antibiotics and subsequent removal of the central line. He was

discharged after 2 weeks in hospital with ongoing diarrhea on Pregestimil but was maintaining his weight.

At 4 months of age he was readmitted because of worsening diarrhea and poor weight gain. Admission weight was 3620 grams. Work up again showed only a metabolic acidosis. Additional work up included upper GI endoscopy with biopsies of the duodenum, stomach and esophagus and sigmoidoscopy with biopsies. Histology from all sites was normal. Disaccharidases (lactase, sucrase, isomaltase, palatinase and glucoamylase) were all within the normal range. He was treated with cholestyramine with minimal improvement. A central line was again placed for parenteral nutrition and on this he demonstrated good weight gain. With introduction of formula feeds his diarrhea increased. He was placed on RCF feeds and his stool output decreased somewhat. Subsequent addition of fructose to the feeds appeared to be tolerated and his diarrhea did not seem to increase any further. He was discharged on RCF with additional fructose feeds and parenteral nutrition. Parenteral feeds were discontinued at 6 months of age due to repeated line infection. At this stage he weighed 5.04 kg. He continued to ingest RCF with fructose and started a variety of solid feeds. Per his parents he passed 2-5 watery stools per day but showed continued slow weight gain. At 1 year of age he weighed 6.4 kg.

At 16 months of age he presented with tetany and was found to have a calcium of 6.2 mg/dl. X-ray of his wrists demonstrated features of rickets. 25-hydroxy vitamin D level was 5 (nl: 17-54). Further work up revealed marked fat malabsorption (3 day stool weight = 1438 grams, fecal fat 40 gm/day), repeat upper GI endoscopy with secretin stimulated pancreatic tests and biopsies revealed normal histology, normal pancreatic

enzymes (amylase, lipase, trypsin, chymotrypsin, carboxypeptidase A & B) and normal disaccharidases. Fat soluble vitamins A, D and E were all low. He was treated with dihydrotachysterol, calcium carbonate and ADEK and given a trial of pancreatic enzyme supplements with no change in his stool pattern.

He continued to make slow progress with regards to his growth pattern. At 4 years of age he weighed 13 kg and was 95 cm in height. At last visit, age 7.5 years he weighed 18 kg and his height was 109.4 cm. He still has loose stools. More recently, he has developed evidence of hyperglycemia while on a full enteral diet.

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