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Aberrant high expression of *NRG1* gene in Hirschsprung disease[☆]

Weibing Tang[a,c,1], Bo Li[a,c,1], Xiaoqun Xu[a,c], Zhigang Zhou[a,c], Wei Wu[a,b], Junwei Tang[a,c], Jingjing Qin[a,c], Qiming Geng[c], Weiwei Jiang[c], Jie Zhang[c], Jiahao Sha[a], Yankai Xia[a,b,*], Xinru Wang[a,b]

[a]*State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 210029, China*
[b]*Key Laboratory of Modern Toxicology (Nanjing Medical University), Ministry of Education, Nanjing 210029, China*
[c]*Department of Pediatric Surgery, Nanjing Children's Hospital Affiliated to Nanjing Medical University, Nanjing 210029, China*

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Abstract
Background/Purpose: Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of intramural ganglion cells along with variable lengths of the gastrointestinal tract. Recent studies have indicated the potential function of neuregulin-1 (*NRG1)* in HSCR, which encodes the heregulins and other mitogenic ligands for the *ErbB* family. The purpose of this study was to further clarify the role of *NRG1* in the pathogenesis of HSCR.
Methods: We examined the *NRG1* messenger RNA (messenger RNA) and protein expression levels in gut tissues of 63 patients with sporadic HSCR (both stenotic and dilated gut tissues) and 35 controls. Moreover, using the methylation-specific polymerase chain reaction, we examined the methylation pattern of exon 1 of the *NRG1* gene.
Results: The mRNA expression levels of *NRG1* were significantly higher in tissues of HSCR than those in controls, and the increased *NRG1* protein levels in HSCR were consistent with the mRNA levels. However, no methylation pattern change was observed in exon 1 of the gene among different groups.
Conclusions: Our study demonstrates that the aberrant expression of *NRG1* may play an important role in the pathology of HSCR. DNA methylation of the gene seems not to be involved in the mechanism of such aberrant expression, and other factors should be explored.
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Hirschsprung disease (HSCR), also called *aganglionosis*, is one of the most difficult diagnoses in pediatric surgery [1], characterized by the absence of ganglion cells in the lower digestive tract. It is the most frequent cause of a functional intestinal obstruction among newborns. The incidence of this disease is about 1:2000 to 1:5000. Hirschsprung disease usually occurs sporadically (up to70% of the cases), although

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* Corresponding author. State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing, China. Tel.: +86 25 86862939; fax: +86 25 86662863.
E-mail address: yankaixia@njmu.edu.cn (Y. Xia).
[1] These authors contributed equally.

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it can be familial, and is frequently associated with recognized syndromes, chromosome, or congenital anomalies [2,3]. Although many studies have been performed to elucidate the pathological process of this disease, the exact mechanism still remains unknown.

To date, more than 10 genes have been found to be involved in the pathogenesis of HSCR [2]. Among these genes, the *RET* proto-oncogene accounts for the highest proportion of both familial and sporadic cases [4]. It is now known that neural crest neuroblasts can colonize aganglionic and ganglionic gut in vivo, which brought a new prospect for the treatment of HSCR [5].

A previous genome-wide association study on Chinese patients with sporadic HSCR identified neuregulin-1 (*NRG1*) as a susceptibility locus for HSCR [6]. This study also found that there was a significant difference in *NRG1* expression level between the diseased and control individuals bearing a same-risk genotype [7]. In addition, not only common variants but also rare ones of the *NRG1* gene can contribute to HSCR [8].

Epigenetic alterations in DNA without concomitant changes in underlying genetic codes are now known to occur frequently in several kinds of human diseases [9-11]. Epigenetic silencing is achieved not only through DNA methylation but also by many other DNA, chromatin, and RNA modification mechanisms [12,13]. Changes in DNA methylation patterns impact several critical cellular processes including gene expression, X-inactivation, carcinogenesis, aging, and development [14-17]. Recently, it was shown that *NRG1* was unmethylated in normal breast tissues and methylated in breast tumor samples; hypermethylation of the gene is correlated with lower a *NRG1* gene expression level [18].

In the present study, we set out to investigate the role of *NRG1* in HSCR, initially by measuring expression of *NRG1* (both messenger RNA [mRNA] and protein) in human gut tissues (63 patients and 35 controls). We found that there were significant higher *NRG1* expression levels in patients with HSCR than those in controls. To find out whether aberrant expression of *NRG1* in HSCR is caused by abnormal DNA methylation and if there were any changes in the methylation pattern among different groups, we further examined the methylation pattern of the exon 1 of the *NRG1* gene. In this work, we report that there were no significant changes in the methylation pattern of *NRG1* among different groups tested. Future studies with a larger sample size are needed to confirm these results. The functional relevance of aberrant expression of *NRG1* to HSCR found in this work warrants further investigation.

1. Materials and methods

1.1. Patients and samples

This study was approved by the institutional review board of Nanjing Medical University (Nanjing, China), and all human subjects provided written informed consent. A total of 63 patients with HSCR were collected in Nanjing Children's Hospital affiliated to Nanjing Medical University from October 2009 to May 2011 (Nanjing Medical University Birth Cohort). All patients were confirmed with barium enema, anorectal manometry, and postoperative pathological examination. We took full-thickness tissues in the dilated segment (ganglionic bowel) and stenotic segment (aganglionic bowel) of the colon, and the tissues were immediately stored in liquid nitrogen. Tissue specimens in the normal colon of 35 patients without HSCR (intussusception, incarcerated hernia) were obtained as negative controls over the same period as that for the diseased samples.

1.2. Quantitative real-time polymerase chain reaction

Total RNA was obtained from tissues using TRIzol reagent as described by the manufacturer (Invitrogen Life Technologies Co, CA, USA). For NRG1 mRNA detection, RNA (500 ng) was reverse transcribed using a reverse transcription kit (Takala, Tokyo, Japan) under 37°C for 15 minutes and 85°C for 30 seconds. The expression level of the *NRG1* gene in each sample was measured by quantitative real-time polymerase chain reaction (PCR) in a volume of 10 μL with 384-well plates using ABI Prism 7900HT (Applied Biosystems, Foster City, CA). *β-Actin* was used as an endogenous control. Forward (F) and reverse (R) primer sequences were as follows: *NRG1*, (F) 5′-atgtgtcttcagagtctcccat-3′ and (R) 5′-tggacgtactgtagaagctgg-3′; *β-actin*, (F) 5′-ccaaccgcgagaagatga-3′ and (R) 5′-ccagaggcgtacagggatag-3′. The thermocycler program included a step of denaturation at 95°C (10 minutes) and 40 cycles of 95°C (15 seconds) and 60°C (1 minute).

1.3. Western blotting

Proteins were extracted from gut tissues using radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors (cOmplete, ULTRA, 132 Mini, EDTA-free, EASYpack; Roche, Basel, Switzerland). Protein concentrations were determined using the BCA (Bicinchoninic Acid) method. Equal amount of proteins (80-100 μg) were separated by 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Roche). Membranes were blocked using 5% skimmed milk and incubated with respective antibodies. Primary rabbit polyclonal rabbit anti-NRG1 antibody was purchased from Santa Cruz Biotechnology (SC-348; Santa Cruz, CA). The secondary antibody used was antirabbit from Santa Cruz Biotechnology. Blots were developed using electrochemiluminescence (ECL) (Millipore, Billerica, MA). Equal loading of protein amounts was confirmed using a *GAPDH* antibody. Integrated density values were calculated using AlphaImager 3400 (Alpha

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