Neural Tube Formation Determinants of PAX3 By Non-Canonical WNT, ROR2 And Its Effect on Neural Crest Specifiers Like SOX10, RET Genes.

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Abstract:

Introduction: Anencephaly occurs due to the complete absence of cranial vault and subsequent disruption of the cerebral cortex with a severely damaged brain. In anencephaly, the forebrain and brain stem are exposed. Forebrain either does not develop or is destroyed, leading to the absence of cerebrum and cerebellum.

Material and method: NTD were taken in the study group. During the autopsy clinical findings, external examination, internal examination and photography were done along with the histopathology of the specimens. Total RNA from NTD patient and control was extracted using acid guanidinium thiocyanate-phenol-chloroform extraction

Result: Demonstrated here the differential expression of specific genes like WNT11, ROR-2, PAX3/PAX7, SOX10, RET associated with development of the neural tube affected and normal adjacent tissues.

Keywords: Neural tube defects, Anencephaly, PAX, SOX 10, WNT11

Abbreviations: NTD: Neural tube defects, PAX: Paired box, SOX: SRY-box transcription factor

Introduction:

The development of neural tube takes place during 28th day of gestation. During gastrulation mesoderm formation and anterior-posterior (A-P) axis elongation occurs (Andre et al., 2015). Cells of anterior-posterior axis lead to formation of axial mesoderm which results in formation of notochord (Lawson et al., 1991). Wnt signaling pathway is known to regulate gastrulation during development. Both Wnt5a and Wnt11 regulate planar cell polarity signaling which is known to play important role in neural tube closure and anterior posterior axis closure (Kibar et al., 2001; Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2006; Ybot-Gonzalez et al., 2007; Gao et al., 2011). Study done on zebrafish has shown that Ror2 receptor is known to mediate Wnt11 ligand signaling which is known to affect convergence and extension movements in vertebrate gastrulation (Bai et al., 2014). The ligand binding site of ROR-2 interact with Wnt 11 leading to induction of a signal which stimulate neural plate border specifier like PAX3 resulting in development of neural tube. After the development of neural tube neural crest cell migration occurs.

PAX3/PAX7 which is a neural plate border specifier leads to formation of neural crest marker like SOX10. The ectopic expression of Sox10 occurs in neural tube in migrating neural crest cells

(McKeown et al., 2005). This Sox10 on cooperation with Pax3 is known to activate *c-ret* gene (Lang and Epstein, 2003a). The protein domain of PAX3 interacts with HMG domain of SOX10 (Lang and Epstein, 2003a). Study show that c-RET enhancer has binding sites for activation of PAX3 and SOX10 (Lang et al., 2000). By deletion assay, it was found that Pax3 interacts with the HMG domain of Sox10 in activation of c-Ret (Lang and Epstein, 2003b). The neural plate border (PAX3) on interaction with neural crest specifiers (SOX10) activates the neural crest effector (cRET). cRET is expressed in peripheral nervous system and enteric nervous system during embryonic development. The activation of cRET prevents complication like paraplegia, bladder and bowel involvement etc.
The hypothesis of the research was to trace the gene regulatory network pathway and check the expression of genes that control embryonic neural tube development and neural crest specifiers which may be a factor for complication like paraplegia, bladder and bowel involvement etc.

Material and Method

Sample Collection

Tissue sample of patient (20) and control group (20) were obtained from Department of Pediatric surgery, S S Hospital, Banaras Hindu University, Varanasi stored in trizol at -20 to -80°C.

RNA extraction

Total RNA from NTD patient and control was extracted using acid guanidinium thiocyanate-phenol-chloroform extraction. The RNA extracted was dissolved in nuclease free water and quantitated spectrophotometrically (NanoDrop ND-1000).

Primers

Primers for amplification of Bcl-2, Bax, P53, P21, PAX3 and PAX7 were determined using Primer 3 (v. 0.4.0) computer program (PE Applied Biosystems, USA) and their sequence homology were checked using BLAST (http:// www.ncbi.nlm.nih.gov/blast). Primers sequence shown in table 1 below.

Table- 1: Primer Sequences used in study

GENE	PRIMER	SEQUENCE
B-actin	Forward	5’ ACCAGATCCTAAACAT GCGAG-3’
	Reverse	5’ ACCT T CTACACAATGAGCTGCG-3’
Wnt11R	Forward	5’ ACTCTGCTCAAGGACC CTCA -3
	Reverse	5’- GCTTCCAAGTGAAGGCAAAG -3
ROR-2	Forward	5’- GGGCAACCTTTCCAACTACA -3
	Reverse	5’- CGTTGCTCACATTGCTCACT -3
PAX3	Forward	5’ CCTG AGCGAGCGAGGTAAG-3
	Reverse	5’ -AGAGACACCGCGCATGAA -3’
PAX7	Forward	5’-GGACTCCTGCCTGTGGAAAC-3’
	Reverse	5’ –TCAGTTAGGGTTGGGCTGGGA- 3
SOX 10	Forward	5’ AGCCCAGGTGAAGACAGAGA -3
	Reverse	5’ ATAGGGTCCTGAGGGCTGAT-3
RET	Forward	5 CCTACGTGAAGAGGAGCCAG’

	Reverse	5’ GCAGGACACCAAAAGACCAT -3’

To confirm total gene specificity of the nucleotide sequences chosen as primers BLASTN was performed and it was found that the entire gene (Bcl-2, Bax, P53, P21, PAX3 and PAX7) showed no significant sequence similarity to each other, or to other known human gene sequences, as assessed by a Gene Bank database search.

Quantitative real-time RT-PCR:

The reaction mixture containing 1000µg of RNA (12.5µl), 10X/ml of random primer (2.5µl), 50U/ µl of reverse transcriptase (1.25µl Applied biosystem), 100 mM of each dNTPs (1µl), and 5 µl of nuclease free water to make a final volume of 25 ml. The reaction mixture was incubated for 2h 20 minutes at 37 ºC to synthesize cDNA. For real time RT-PCR, 0.5 µl of cDNA products were amplified in the reaction mixture (5 µl) containing SYBR Green master mix, 10pM of each primer (0.25 µl) in a Light Cycler instrument (Applied Biosystem) as instructed by the manufacturer and the fold change of mRNA was analyzed (Lee et al., 2012). Quantified transcripts of β-actin were used as endogenous RNA controls. All experiments were performed in triplicates for each data point.

The obtained results were recalculated to 1 µg of total RNA; subsequently, a profile for the entire genes was determined for each group examined. Numbers of Bcl-2, Bax, P53, P21, PAX3 and PAX7 mRNA copies were calculated based on calibration curve for β-actin standards. Data were expressed as mean+SD. The nonparametric Kruskal–Wallis One Way ANOVA test were used to evaluate variations of Bcl-2, Bax, P53, P21, PAX3 and PAX7 mRNA levels in normal and NTD patient were done to check the level of significance. Data are presented as means with 95% confidence intervals

Result

We demonstrated here the differential expression of specific genes like WNT11, ROR-2, PAX3/PAX7, SOX10, RET associated with development of the neural tube affected and normal adjacent tissues.

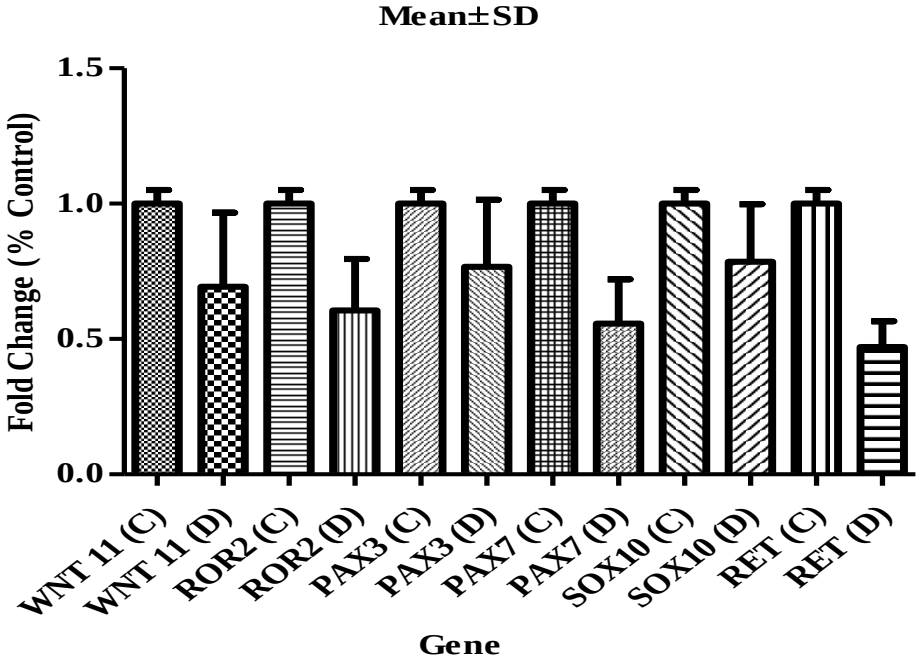

Fig. 1: Expression profile of genes (Wnt11, ROR2, PAX3, PAX7, SOX10 and RET) in normal and neural tube affected patient by RTPCR assay.

The expression of all the genes was normalized on the expression level of the β-actin gene as an internal control. Fig. 1, showed the histogram of 6 genes associated with apoptosis in the neural tube affected and normal adjacent tissues. Our results demonstrated that RET, ROR-2, SOX10, WNT11, PAX3 and PAX7 were down regulated. Results revealed statistically significant ($P < 0.05$) differences for neural tube affected when compared to normal adjacent tissues. On comparing the gene expression between normal and neural tube affected cases it was found that gene WNT11, 1 vs. 0.69-fold; ROR-2, 1 vs. 0.606-fold; transcription factor PAX3, 1 vs 0.76-fold and PAX7, 1 vs 0.556-fold; RET, was 1 vs. 0.47-fold; SOX10, 1 vs. 0.786-fold change were observed.

Discussion:

In this study we show that the expression of non-canonical Wnt 11R and Ror2 are downregulated in neural plate. Also, there was a down regulation of neural plate border specifier like PAX3/PAX7. Due to down regulation of PAX3/PAX7 interaction of PAX3 in the HD region of SOX10 may not occur and as a result activation of neural crest effector cRET was not observed and complication like paraplegia, bladder and bowel dysfunction is seen **(Fig. 2).**

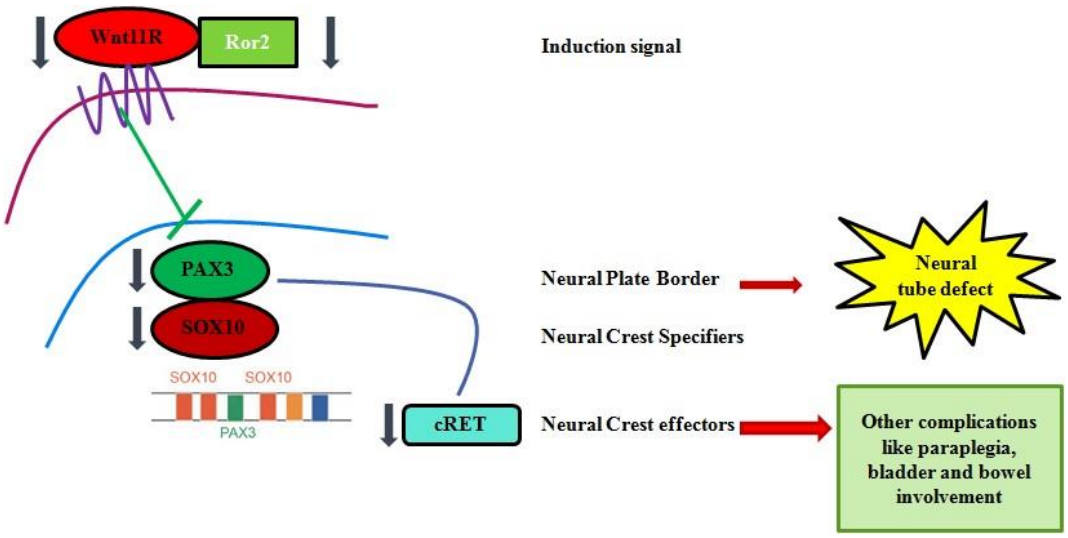

Fig. 2: Figure representing downregulation of different genes and their effect on neural plate border, neural crest specifier and neural crest effector.

In light of previous studies Wnt11R and Ror2 act as induction signal for neural plate (Matthews et al., 2008). The noncanonical Wnt signaling is essential regulator of ROR2. Previous study on mouse and *Xenopus* embryo reveal that Ror2 is expressed in early neuroectoderm at early neural plate border and later in migrating neural crest cells (Hikasa et al., 2002; Matsuda et al., 2001; Ossipova et al., 2002).
Previous studies show that non canonical pathway along with canonical pathway are involved in neural crest specification. Wnt signaling has been known to control the expression of PAX3 at early inductive phase (Bang et al., 1999). Pax3 is a transcription factor which is expressed in dorsal neural fold/ tube (Epstein et al., 1991; Goulding et al., 1991) and through Wnt signaling is involved in neural crest development (Degenhardt et al., 2010; Monsoro-Burq et al., 2005).
Study by McKeown, 2005, Lang and Epstein, 2003a has shown the interaction of PAX3 to activate cRET gene. Lang and Epstein, 2003b found that Pax3 interacts with the HMG domain of Sox10 for activation of c-Ret. During the study we observed that there was a decrease in expression of PAX3 gene due to which cRET is not activated. cRET play an important role in enteric ganglion formation and this may be a reason for complication like bladder and bowel involvement and paraplegia.

Further studies are needed to examine the exact mechanism behind neural tube development and neural crest specification.

Conflict of interest

Authors have declared that and research was conducted in and the absence of any commercial or financial relationships without any conflict of interest.

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References

1. Andre P, Song H, Kim W, et al. Wnt5a and Wnt11 regulate mammalian anterior-posterior axis elongation. Development. 2015; 142(8):1516-27.
2. Lawson KA, Meneses JJ, Pedersen RA. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development. 1991; 113, 891-911.
3. Kibar Z, Vogan KJ, Groulx N, et al. Ltap, a mammalian homolog of Drosophila Strabismus/ Van Gogh, is altered in the mouse neural tube mutant Loop-tail. Nat Genet. 2001; 28:251-255.
4. Curtin JA, Quint E, Tsipouri V, et al. Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe NTDs in the mouse. Curr Biol. 2003; 13:1129-1133.
5. Curtin JA, Quint E, Tsipouri V, et al. Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe NTDs in the mouse. Curr Biol. 2003; 13:1129-1133.
6. Montcouquiol M, Rachel RA, Lanford PJ, et al. Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. Nature. 2003; 423:173-177.
7. Montcouquiol M, Rachel RA, Lanford PJ, et al. Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. Nature. 2003; 423:173-177.
8. Wang XD, Morgan SC, Loeken MR. Pax3 stimulates p53 ubiquitination and degradation independent of transcription. PLoS One. 2011; 6(12):e29379.
9. Ybot-Gonzalez P, Savery D, Gerrelli D, et al. Convergent extension, planar-cell-polarity signalling and initiation of mouse neural tube closure. Development. 2007; 134:789-799.
10. Gao B, Song H, Bishop K, et al. Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Dev Cell. 2011; 20:163-176.
11. Bai Y, Tan X,et al. Ror2 receptor mediates Wnt11 ligand signaling and affects convergence and extension movements in zebrafish. J Biol Chem. 2014; 289(30):20664-76.
12. McKeown SJ, Lee VM, Bronner-Fraser M, et al. Sox10 overexpression induces neural crest-like cells from all dorsoventral levels of the neural tube but inhibits differentiation. Developmental Dynamics. 2005; 233 (2):430–44.
13. Lang D, Chen F, Milewski R, et al. Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. J Clin Invest. 2000; 106:963-71.
14. Lang D, Epstein JA. Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. Human Molecular Genetics. 2003a; 12:937-945.
15. Matthews HK, Broders-Bondon F, Thiery JP, et al. Wnt11r is required for cranial neural crest migration. Dev. Dyn. 2008a; 237, 3404-3409.
16. Lang D, Lu MM, Huang L, et al. Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature. 2005; 433:884-7.

17. Lee JS, Mendez R, Heng HH, et al. Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. Am J Transl Res. 2012; 4:102-113.
18. Matthews HK, Broders-Bondon F, Thiery JP, et al. Wnt11r is required for cranial neural crest migration. Dev. Dyn. 2008a; 237, 3404-3409.
19. Hikasa H, Shibata M, Hiratani I, et al. The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development. 2002a; 129, 5227-5239.
20. Matsuda T, Nomi M, Ikeya M, et al. Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development. Mech Dev. 2001; 105:153-6.
21. Ossipova O, He X, Green J. Molecular cloning and developmental expression of Par-1/MARK homologues XPar-1A and XPar-1B from Xenopus laevis. Mech. Dev. 2002; 119, S143-S148.
22. Bang AG, Papalopulu N, Goulding MD, et al. Expression of Pax-3 in the lateral neural plate is dependent on a Wnt mediated signal from posterior nonaxial mesoderm. Dev. Biol. 1999; 212, 366-380
23. Epstein DJ, Vekemans M, Gros P. Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired HD of Pax-3. Cell. 1991; 67:767-74.
24. Goulding M, Sterrer S, Fleming J, et al. Analysis of the Pax-3 gene in the mouse mutant splotch. Genomics. 1993; 17:355-63.
25. Degenhardt KR, Milewski RC, Padmanabhan A, et al. Distinct enhancers at the Pax3 locus can function redundantly to regulate neural tube and neural crest expressions. Dev. Biol. 2010; 339, 519-527.
26. Monsoro-Burq AH, Wang E, Harland R. Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. Dev. Cell. 2005; 8,167-178.