Pax6 influences expression patterns of genes involved in neurodegeneration

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ABSTRACT

Background: Pax6, a highly conserved multifunctional transcription factor, has been critical for neurogenesis and neuronal plasticity. It is presumed that if level of Pax6 approaches either low or null, critical genes responsible for maintaining functional status of neurons or glia would be modulated.
Purpose: Therefore, it has been intended to explore possibility of either direct or indirect influence of Pax6 in neurodegeneration.
Methods: The cell lines having origin of murine embryonic fibroblast (Pax6-non expressing, NIH3T3-cell line), murine neuroblastoma (Pax6-expressing brain-derived, Neuro-2a-cell line), and human glioblastoma-astrocytoma (U87MG) were cultured and maintained in a CO_2 incubator at 37°C and 5% CO_2 in DMEM containing 10% fetal bovine serum. The knockdown of endogenous Pax6 in Neuro-2a cells was achieved through siRNA based gene knock-down approach. The efficiency and validation of knock-down was done by real time PCR. The knock-down of Pax6 was successfully achieved.
Results: The levels of expression of transcripts of some of the proposed putative markers of neurodegeneration like Pax6, S100β, GFAP, BDNF, NGN2, p73α, p73δ, LDH, SOD, and Catalase were analyzed in Pax6 knockdown condition for analysis of role of *Pax6* in neurodegeneration. Since the Pax6 has been proposed to bind to promoter sequences of catalase, and catalase suppresses TGFβ, relative lower levels of *catalase* in Neuro-2a and U-87MG as compared to NIH-3T3 indicates a possible progressive dominant negative impact of Pax6. However, presence of SOD and LDH indicates alternative protective mechanism.
Conclusion: Presence of BDNF and TGFβ indicates association between them in glioblastoma-astrocytoma. Therefore, Pax6 seems to be involved directly with p53 and TGFβ mediated pathways and indirectly with redox-sensitive pathway regulation. The neurodegenerative markers S100β, GFAP, BDNF, NGN2, p73α, p73δ, observed downregulated in Pax6 knockdown condition suggest Pax6-mediated regulation of these markers. Observations enlighten Pax6-mediated influences on cascades of genes involved in growth, differentiation and maturation of neurons and glia.

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Introduction

Progressive excitotoxicity, deregulation of mitochondrial (Mt) functions, and apoptosis have been observed as major causes for pathological conditions, aging, and neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple Sclerosis (MS) and amyolotrophic lateral sclerosis (ALS).[1] Neurodegenerative diseases display loss of nerve cells from brain and spinal cord. They have been manifested by either functional loss, ataxia, sensory dysfunction, or dementia. The Pax6, a highly conserved, multifunctional transcription factor, has been known to influence specification of neuronal subtypes in the retina and spinal cord, dorsoventral patterning, neurogenic specification, proliferation and migration in the developing cortex. The Pax6-KO (knockout) mice show embryonic lethality with loss of CNS, eyes and pancreatic alpha-cells. Studies suggest its dual role as both an activator and repressor of different target genes.[2–4] The mutations in Pax6 result in neuronal anomalies like defect in the CNS leading to cell death,[5] Polymicrogyria, absence of the pineal gland,[6] Psychiatric disorder, cognitive defects[7] and cerebral malfunctions.[8] Most of the phenotypes also match aging-associated pathological conditions. A large number of upstream effectors and downstream targets of Pax6 have either been proposed or confirmed.[9–19]

The BDNF, a neurotrophin, supports the development, maintenance and plasticity of brain throughout life.[20] The levels of BDNF have been found low in brain[21] of patients having neurodegenerative diseases. The p73, a transcription factor of p53 family has been implicated in many biological processes including neuronal development. The p73-KO mice revealed developmental defects in the central nervous system including congenital hydrocephalus and hippocampal dysgenesis. It also has defects in the both embryonal and adult neurogenesis suggesting that p73 isoforms may be survival factor for neural stem cell. p73α is essential for neuronal differentiation and maintenance of neural stem cells. p73δ plays a major role in neuronal survival.[22] The Ngn2 is a neuronal basic helix-loop-helix transcription factor which contributes to many distinct neuronal types during CNS development. It is known that ectopic expression of Ngn2 is sufficient to induce and promote neuronal differentiation of embryonic stem cells towards the appearance of mature and functional neurons.[23] In the absence of Ngn2, both cell cycle progression and neuronal output are significantly affected, leading to an overall reduction of the mature cerebellar volume.[24] In the case of Alzhiemer's and Parkinson's diseases, transcription factor Ngn2 expressions was significantly decreased.[25] The GFAP constitute intermediate filaments as a part of cytoskeleton in astrocytes. Reactive gliosis is a response of astrocytes to a variety of brain insults that are characterized by hypertrophy of the cell bodies and processes, altered gene expression, increased

expression of GFAP in some neurodegenerative diseases. GFAP null mice have been demonstrated to be sensitive to spinal cord injury to cerebral ischemia and to neurotoxicity indicating a protective role of GFAP.[26,27] The S100β is a low molecular weight Ca^{2+} binding protein composed of two isomeric subunits found predominantly in astrocytes and Schwann cells. It plays important role in normal CNS development and recovery after injury. At nanomolar concentration, S100β stimulates neurite outgrowth in cerebral cortex neuron and enhance survival of neurons in various systems during development but at micromolar concentration, S100β may have deleterious effects i.e., it stimulates the expression of pro-inflammatory cytokines and induces apoptosis. In Alzheimer's, S100β protein levels are significantly increased when β-amyloid interact with S100β and stimulate synthesis of both S100β mRNA and S100β protein in astrocytes cultures. Significant immune response to S100β suggests that it may reflect neurodegenerative brain damage occurring in Parkinson's disease.[28,29] PCNA plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. It is a 36kDa polypeptide whose expression and synthesis is linked with cell proliferation. In neurodegenerative diseases, presence of cell cycle markers has raised the possibility that aberrant activation of the cell cycle machinery in postmitotic neuron could be lethal and contributes to neurodegeneration.[30] The PCNA has a triple function in life and death of the cells. When not engaged in DNA replication, PCNA under p53 control commits cells to cell cycle arrest and repair of DNA damage, or when repair is not possible, absence or low levels of functional PCNA may drive cells into apoptosis.

It is presumed that Pax6 regulates the neurodegeneration either through regulation of its own transcription or through the regulation of a large no. of targets involved in the maintenance of the neuronal functions. Since Pax6 is the master regulator and regulates the transcription of the genes involved in the neurogenesis and plasticity, we intended here to explore that does Pax6 involve directly or indirectly in neurodegeneration.

Methods

Maintenance of cell-lines

The cell lines having origin of murine embryonic fibroblast (Pax6-non expressing, NIH3T3-cell line), murine neuroblastoma (Pax6-expressing brain-derived, Neuro-2a-cell line), and human glioblastoma-astrocytoma (U87MG) were cultured and maintained in a CO_2 incubator at 37°C and 5% CO_2 in DMEM containing 10% fetal bovine serum. The knockdown of endogenous *Pax6* in Neuro-2a cells was achieved through siRNA based gene knockdown approach. The study was ethically approved by IBSC.

Knockdown of Pax6 by siRNA and analysis of neurodegeneration-associated markers

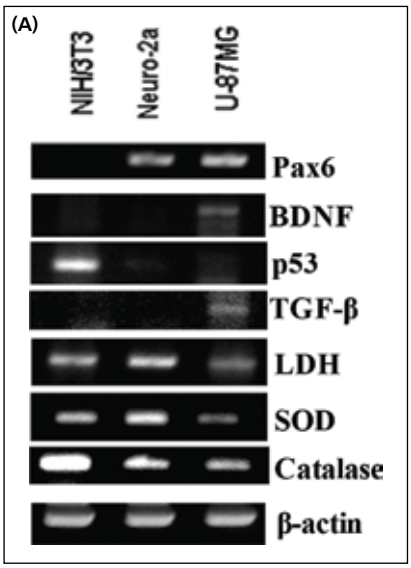
The siRNA based gene-silencing approach was used to knock down the endogenous transcripts of Pax6 (Pax6_2, Pax6_4, Pax6_5, and Pax6_7). The siRNAs targeting transcripts of *Pax6* were procured from Flexi tube Gene Solution (Qiagen Inc., GmbH, Germany), and suspended to yield 10μM stock solution. The Neuro-2a was transfected with *Pax6* specific siRNAs and control siRNAs using Human/mouse RNAi starter kit (Qiagen Inc., GmbH, Germany) and Lipofectamine RNAi MAX (Invitrogen, Life Technologies, USA) as per the manufacturer's instructions. The 5 nM of each of siRNAs/well of the 12-well plate and/or 250 ng per well/6-well plate were observed effective. After 72-hours of Post-transfection or After transfection total RNA was isolated from different sets of siRNA transfected-cells using Pure Link RNA mini kit (Ambion, Life Technologies, USA). One microgram of the total RNA was reverse transcribed into first strand cDNA using first strand cDNA synthesis kit (High capacity cDNA synthesis kit, Applied Biosystems, Life technologies, USA). The levels of putative markers *Pax6, PCNA, S100beta, GFAP, Ngn2, p73alpha, p73gama, BDNF, p53, TGF-β, LDH, SOD, and Catalase*, under *Pax6*-knockdown background, were assessed using Maxima SYBR Green qPCR Master Mix (Fermentas, USA) on ABI 7500 Real Time thermal cycler manufacturer details. Following gene specific primer sets were used:

Pax6PDF:5'GCATGCAGAACAGTCACAGCGGAG3',**Pax6PDR:**5'CTGTTGCTTTTCGCTAGCCAGGTT3';**BDNFMF:**5'CCGAGGTTCGGCTCACACCG3',**BDNFMR:**5'GCCCCTGCAGCCTTCCTTGG3';**P53F:**5'AGAGACCGCCGTACAGAAGA3',**P53R:** 5'GCATGGGCATCCTTTAACTC3';**Tg13F**:5'TACAACAGCACCCG3',**Tg13R:**5'CTGTCCACCTTGGG3';**LDHABMF:**5'CGGCTCAACCTGGT3',**LDHABMR:**5'TAGGCACTGTCCACCAC3';**SODF:**5'TGGGGACAATACACAAGGCTGT3',**SODR:**5'TTTCCACCTTTGCCCAAGTCA3';**CatF:**5'CCTCCTCGTTCAGGATGTGGTT3',**CatR:**5'CGAGGGTCACGAACTGTGTCAG3';**BactnF:**5'TGACGGGGTCACCCACACTGTGCCCATCTA3',**BactnR:**5'CTAGAAGCATTTGCGGTGGACGATGGAGGG3';**GFAPF:**5'ACATCGAGATCGCCACCTAC3',**GFAPR**:5'TCACATCACCACGTCCTTGT3';**PCNAF:**5'GCACGTATATGCCGAGACCT3',**PCNAR**:5'CAGTGGAGTGGCTTTTGTGA3';**p73αF:**5'CAAAGTGTCCACACCACCAC3',**p73αR:**5'CATACGGCACAACCACACTC3';**p73δF**:5'CAAAGTGTCCACACCACCAC3',**p73δR**:5'CATACGGCACAACCACACTC3';**NGN2F**:5'GTCCCCATACAGCTGCACTT3',**NGN2R**:5'CAAAGGGCCAACTTCTGTTC3';**S100βF:**5'GAGGAGCACAGCCACACTTA3',**S100βR**:5'CATTCCCCTCCTCTGTCCT3';

Results

The observations are important and interesting because they reveal several valuable aspects of understanding neuro-degeneration under Pax6 background. The *Pax6*, as expected was detected in Pax6 expressing cell-lines, the Neuro-2a and U-87MG cells, but not in NIH3T3 cells (Non-Pax6 expressing cell-line) (Fig. 1A). The levels of expression of putative markers of neurodegeneration S100β, GFAP, BDNF, NGN2, p73α, p73δ, and LDH, SOD, Catalase were detectable in these cell lines (Fig 1B). The siRNA mediated knockdown of Pax6 validated through semi quantitative RT-PCR (Fig. 2A) and real-time-PCR (Fig. 2 B-C) show effective knock-down of *Pax6* and modulation in *Pax6* and *Pax6 (5a)* transcript (Fig. 2A-C). The expression pattern and modulation (Table 1) of neuronal-glial degenerative markers in *Pax6*-knockdown background suggest association of the *Pax6* and these neurodegenerative markers.

Expression of Brain-derived neurotrophic factor (BDNF) was observed lower in *Pax6*-knockdown background. It seems critical because the BDNF has been important for the survival, maintenance and regeneration of specific neuronal population in the adult brain. Its replacement strategies are considered as potential therapeutics for neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's diseases.[31–33] Lower levels of *Pcna* after *Pax6*- knock-down clearly indicate important association with the Pax6. Being *Pcna* being, a nuclear matrix protein, essential for multiple cell cycle pathways, has been associated as triple function in life and death of the cells. When not engaged in DNA replication, *Pcna* commits cells to cell cycle arrest and repair of DNA damage, or when repair is



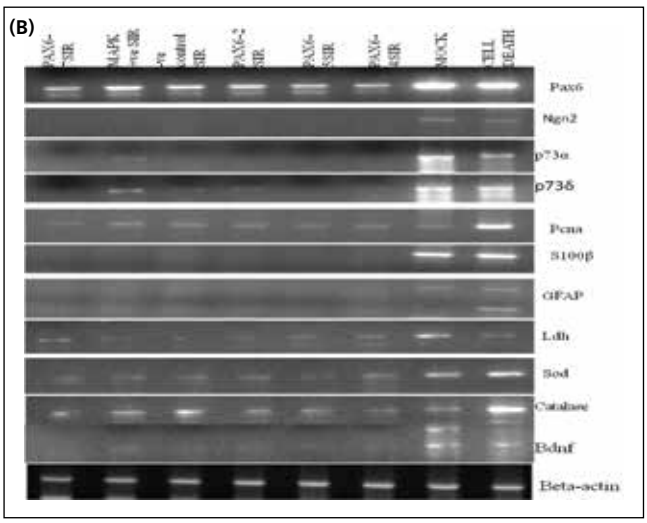
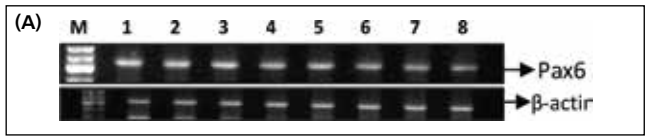
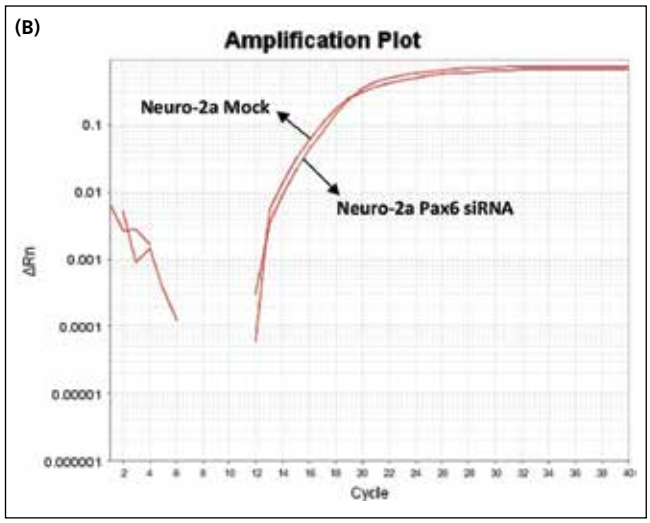


Fig. 1: Analysis of expression of putative neurodegenerative markers through RT-PCR. The β-actin was used as a loading control. **(A)** Analysis of expression of putative markers in cell lines. The β-actin was used as a loading control. **(B)** Analysis of expression of putative markers in Pax6 knock down condition in Neuro 2a.





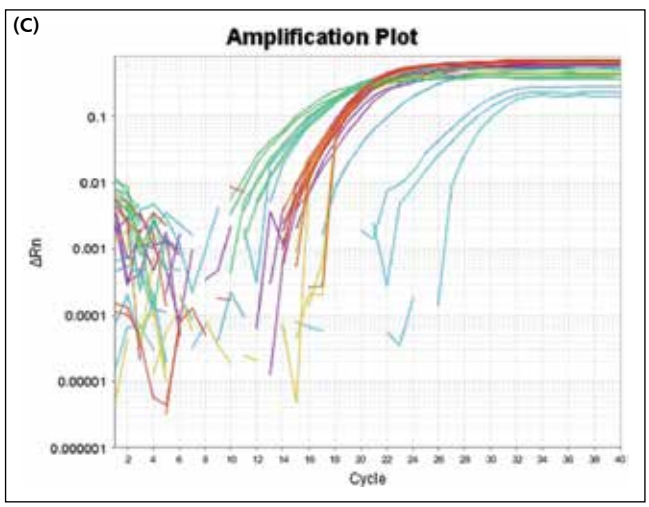


Fig. 2: (A) RT-PCR analysis to access the knockdown of Pax6. The β- actin was used as a loading control. M = Marker, 1 = Mock, 2 = Cell death siRNA, 3 = Negative control siRNA, 4 = +ve Control siRNA, 5 = Pax6_2siR, 6 = Pax6_4siR, 7 = Pax6_5siR, 8 = Pax6_7siR; **(B)** qPCR or Real time PCR RT-PCR analysis to access the knockdown of Pax6. The β- actin was used as a loading control. **(C)** Real time PCR data for Mock and different target Pax6-siRNA Transfected Neuro-2a cells.

not possible, absence or low levels of functional *Pcna* may drive cells into apoptosis.[34]

Lower levels of *Ngn2* indicate deregulation of neuronal growth and differentiation. The *Ngn 2* is necessary for the proper differentiation of excitatory glutamatergic projection neurons in the cerebral cortex. Proneural transcription factors are thus critical regulators for both the initiation of neuronal differentiation and the specification of neurons into distinct regional subtypes.[35] The S100β is a β homodimeric protein, expressed in astrocytes and oligodendrocytes[36] were also observed lower. Since the *S100β* is a potent marker of neuro-degeneration and its down-regulation in the *Pax6*-null background seems equally important in association with Pax6 in neuro-degeneration. Pax6 inhibits the growth and proliferation of astrocytes and help in the maturation of astrocytes.[37] The *S100β* also plays neurotrophic role in both development and repair by inhibition of cell growth either by cooperating with p53 directly or is able to inhibit its synthesis, resulting in decrease in the rate of cell proliferation.[38] Thus Pax6 seems to regulate the expression of *S100β,* and *S100β*- mediated pathways. Findings are in line of some reports the loss of TGFβ results in increased microgliosis and neuro-degeneration. However, its up-regulation influences silencing of neuro-inflammation.[39] The *Pax6* regulates cell proliferation, whereas p53 is critical for cell cycle regulation and cell death. Since, the interaction of Pax6 with p53 indicates Smad3 dependent auto-regulation,[40] this observation indicates critical Pax6-p53 associated regulation.

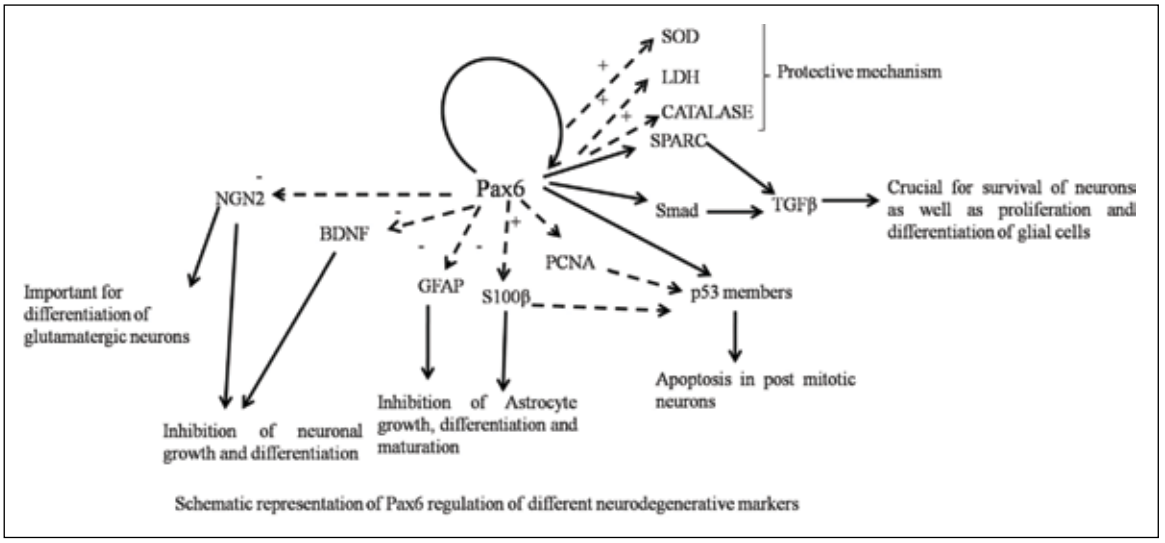


Fig. 3: Schematic diagram of Pax6 regulation of different neurodegenerative markers
PCNA = Proliferating Cell Nuclear Antigen
GFAP = Glial Fibrillary Acidic Protein
NGN2 = Neurogenin 2
BDNF = Brain Derived Neurotrophic Factor
TGFβ = Transforming Growth Factor β
SPARC = Secreted Protein Acidic and Rich in Cysteine
SOD = Superoxide Dismutase
LDH = Lactate Dehydrogenase
Pax6 = Paired Box 6

Table 1: Summary of gene expression in Mock and SiRNA mediated Pax6 knockdown background

Gene	Mock	Pax6_2sir	Pax6_ 4sir	Pax6_ 5sir	Pax6_ 7sir
Pax6	+++	-	-	-	-
LDH	++	+	+	+	+
SOD	++	+	+	+	+
CATALASE	+	+	+	+	+
PCNA	+	+	+	+	+
S100β	+++	-	-	-	-
GFAP	+	-	-	-	-
BDNF	++	-	-	-	-
Ngn2	+	-	-	-	-
p73α	++	+	+	+	+
p73δ	++	+	+	+	+
β-actin	+++	+++	+++	+++	+++

The other two members of the p53 family were also showed lower expression in the Pax6-null background. From the expression analysis of the p53 family genes suggested that Pax6 seems to be involved directly with p53- and TGFβ-mediated pathways and indirectly with redox-sensitive pathway regulation. The other markers like SOD, LDH and Catalase were also found down regulated in Pax6-knockdown background. The levels of expression of SOD, Catalase, and TGF-β were higher in Neuro-2a than in NIH/3T3, whereas Pax6 was exclusive to Neuro-2a cells. The increased levels of SOD and Catalase have been reported in cases of neurodegenerative disorders.[41] Down-regulation of *Catalase* in Pax6-expressing cell-lines shows association between them. Since the catalase suppresses *TGF-β*, lower level of *TGF-β* was observed in Neuro-2a and U-87MG. Similarly, there was a progressive lower expression of *catalase* in Neuro-2a and U-87MG as compared to NIH-3T3. Since the Pax6 has been proposed to bind to promoter sequences of *catalase*, progressive lower expression of *catalase* in Neuro-2a and U-87MG as compared to NIH-3T3 indicates a possible progressive dominant negative impact of Pax6. However, presence of SOD

and LDH indicates alternative protective mechanism. Almost similar expression patterns of BDNF and TGFβ indicates similar associated regulation in glioblastoma-astrocytoma.

Discussion

As neurodegenerative markers show altered expression with knockdown of different isoforms of *Pax6*, it clearly indicates that Pax6 critically regulates expression of neurodegenerative gene-expression (Fig. 3). Observations indicate that *Pax6* influences process of neuro-degeneration through all cascades of genes involved in growth, differentiation and maturation of neurons and glia. The functional analysis of Pax6 and its isoforms could be useful for exploring cascades and mechanisms of functions of Pax6-associated neuro-degenerative markers in differential diagnosis and managements of neurological problems. The neurodegenerative markers S100β, GFAP, BDNF, NGN2, p73α, p73δ, were observed down-regulated in Pax6 knockdown condition. The Pax6 seems influencing process of neuro-degeneration through cascades of genes involved in growth, differentiation and maturation of neurons and glia. It may be associated directly with p53 and TGFβ mediated pathways and indirectly with redox-sensitive pathway regulation. The functional analysis of Pax6-associated neuro-degenerative markers would be helpful in differential diagnosis and managements of neurological problems. It could be investigated that which isoform of *Pax6* is responsible for normal functioning/expression of particular neurodegenerative marker that will help in the diagnosis of neurological problems.

Authorship Contribution

Rajnikant Mishra: Planned experiments and mentored the progress of experiments, from initiation to completion of manuscript, **Sachin Shukla:** Initiated the work and did qPCR experiments following transfection, **Khushboo Srivastava:** Repeated transfection experiments and isolated RNA and prepared cDNA, **Shashank Kumar Maurya and Shuman Mishra:** Equally contributed for RT-PCR based experiments, compiled data and wrote their part of explanations

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