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Role of Myocilin in Glaucoma : Molecular Defects and Possible Functional Aberrations Leading to Pathogenesis

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Abstract : Glaucoma is a heterogeneous group of optic neuropathies, with a complex genetic basis. Among the established candidate genes known to be involved in the disease, *myocilin* has been reported to cause a small percentage of adult onset and a major percentage of juvenile onset cases of glaucoma. Mutations in different regions of the gene have been found to be associated with a wide spectrum of glaucoma phenotypes. The gene has also been implicated in primary congenital glaucoma as well as in digenic cases of the disease. The article intends to explore the functional aspects of the protein in normal trabecular meshwork (TM) and molecular basis of TM cell dysfunction as a result of mutation in the protein as revealed from the current studies. We also report occurrence in an Indian POAG family a mutation (Q368X), common among Caucasians, and the studies in progress on myocilin-related genes that could serve as candidates for glaucoma.

Introduction :

Glaucoma is a heterogenous group of optic neuropathies, with a complex genetic basis. It is a multifactorial optic disc neuropathy in which there is a characteristic acquired loss of retinal ganglion cells and atrophy of the optic nerve. It is the second largest blinding disorder after cataract (Resnikoff *et al.*, 2000). Untreated glaucoma is a leading cause of irreversible blindness.

Glaucoma could be classified according to etiology (primary vs. secondary), anatomy of the anterior chamber (open angle vs. closed angle) and time of onset (infantile vs. juvenile). In general glaucoma is broadly classified into three major groups : (i) Primary Open Angle Glaucoma, (ii) Primary acute closed angle glaucoma and (iii) Primary congenital glaucoma. In addition, a number of ocular conditions that are generally postulated to be

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the result of abnormal differentiation of neural crest cells are also reported to be associated with glaucoma.

The definition of glaucoma has evolved over time. It is believed nowadays that the primary and sufficient condition to diagnose a patient with glaucoma is not only the intra-ocular pressure and cupping of the optic disc but also the characteristic visual field damage and detection of retinal nerve fibre layer Loss. It is estimated that there are approximately one million nerve fibres in a normal eye. Normal individuals lose ganglion cells with age at an estimated rate as high as 5000 axons per year, which may translate to a considerable axon loss during a 70 years life span. In glaucomatous eye these cells are lost at an accelerated rate. However, the in-built redundancy in the visual system makes it difficult to ascertain retinal nerve fibre damage with traditional diagnosis methods, until a significant proportion of ganglion cells (>50%) has been lost (Quigley *et al.*, 1982). Recent advances in optical imaging technologies have resulted in techniques that provide measurements of optic disc and retinal nerve-fibre layer (RNFL) loss with micron scale sensitivity. Among these, SLP (Scanning Laser Polarimetry) is a better technology for RNFL analysis as a tool for diagnosis of glaucoma. SLP can detect early minimal loss of optic nerve axons among many high-risk patients who are apparently normal by standard procedures.

Primary Open Angle Glaucoma (POAG) is the most common of the glaucoma subtypes. The disease is known to be transmitted both as a monogenic as well as a complex disease. Among seven implicated chromosomal loci three underlying candidate genes have been identified – *Myocilin (MYOC), Optineurin (OPTN) and WDR36* (Stone *et al.*, 1997; Rezaie *et al.*, 2002; Monemi *et al.*, 2005).

In 1993, the first genetic locus for POAG (GLC1A) was identified (Sheffield *et al.*, 1993) and in 1997 the causal gene, M*yocilin (MYOC),* was discovered (Stone *et al.*, 1997). Polansky *et al*. (1997) identified the product of the same gene while studying the effects of steroids on the trabecular meshwork (TM) cells in culture. The cultured cells, when treated with steroids, secreted the same protein which Nguyen et al (Nguyen *et al.*, 1998) called TIGR (Trabecular Meshwork Inducible Glucocorticoid Response Protein). *Myocilin*, when mutated, cause severe open angle glaucoma mostly in its juvenile form and rarely the adult onset form. It is well documented that

MYOC mutation does not cause glaucoma through haplo-insufficiency but by a dominant negative effect as indicated by the presence of normal eye morphology in *MYOC* knock out mice (Lam *et al.,* 2000). Although the pathophysiology is unknown, it has been suggested that mutant MYOC obstructs the outflow of the aqueous humor through the TM resulting in an increased IOP, which is frequently associated with glaucoma.

The gene (*MYOC/TIGR)*, located on chromosome 1 (1q24.3), spans about 17 kb region of genomic DNA, contains three exons and expressed as 2.3 kb transcript with the translated product predicted to contain 504 amino acids (Fingert *et al*., 1998). Exon 1 of myocilin codes for the amino terminal region of the protein which includes peptide signal sequence (amino acids 1-32) and a leucine zipper motif composed of 50 amino acid residues (amino acids 117-169) (Ortego *et al.*, 1997). The latter region, with periodic arginine and leucine repeats arranged along an alpha helix, gives rise to an amphipathic structure, which suggests that the protein might participate in molecular interactions (Coca-Prados *et al.*, 1999). Exon 3 of myocilin codes for an olfactomedin-like domain. Olfactomedins are a family of mucus proteins that are found predominantly in nasal mucus. However, it has been shown by *in silico* analysis that MYOC has a unique bipartite structure, α- helical structure in the N-terminal region and b-sheet in the C-terminal region. It has been further hypothesized that mammalian *MYOC* has evolved from fusion of genes for two different primordial proteins (Mukhopadhyay *et al*., 2002). *In vitro* experiments have provided evidence that myocilin forms homodimer through its leucine zipper domain (Fautsch *et al*., 2001).

Glaucoma represents a group of disorders, which includes both Mendelian as well as multifactorial traits. Parallel to the rush to characterize the implicated chromosomal loci to identify the candidate genes and understand the molecular basis of pathology for specific sub-types of glaucoma, efforts to understand the genetic basis of multifactorial traits of glaucoma has also begun. It has been appropriately suggested that single-gene mutations must reside in a permissive genetic background, modulated by modifier genes. Recent animal studies in mice provide supportive evidence that glaucoma may be digenic or polygenic disease with possible interaction of multiple genes (Chang *et al*., 1999). Hence interaction of MYOC with other proteins forms an important part in understanding glaucoma pathogenesis.

Molecular defects in *MYOC* in glaucoma patients.

Most of the mutations in *MYOC* causing POAG are listed in the Human Genome Mutation Database (HGMD; http://archive.uwcm.ac.uk/ uwcm /mg/hgmd0.html). It has been observed that among three exons of *MYOC*, mutations are mostly clustered in exon 3 (51 mutations), located rarely in exon 1 (6 mutations) and none has been detected so far in exon 2. Recently Aung *et al.* (2005) has screened *MYOC* in 106 Chinese Primary Angle Closure Glaucoma (PACG) patients and has not observed any defect in the gene (Aung, *et al*., 2005).

Among MYOC mutations identified in Indian POAG patients (Mukhopadhyay *et al*., 2002; Kanagavalli *et al*., 2003; Markandaya *et al*., 2004; Sripriya *et al*., 2004), Gln48His represents an allelic condition involving a spectrum of glaucoma phenotypes, and could be a potential risk factor towards disease predisposition among patients of Indian origin (Chakrabarti *et al*., 2005). In a study carried out in our lab, the mutation has been identified in 3 out of 56 unrelated POAG patients who were found to be heterozygous for the change (Mukhopadhyay *et al*., 2002). Recently Chakrabarty *et al.* (Chakrabarti *et al*., 2005) has reported this mutation in heterozygous condition in 4 out of 200 POAG patients. In addition, other studies from India have reported two other POAG cases harboring the same mutation (Sripriya *et al*., 2004). These observations clearly establish that Gln48His is a common mutation among Indian patients and has not yet been reported in other populations (Kaur *et al*., 2005).

The role of myocilin in pathogenesis of the disease continues to elude us. As already stated, myocilin does not cause glaucoma through haplo-insufficiency but through dominant negative effect (Lam *et al*., 2000). This is supported by the report (Lam *et al*., 2000) of absence of open angle glaucoma in an elderly woman homozygous for an early premature termination codon (Arg46stop) as well as lack of ocular phenotypes in both *MYOC* +/- and -/- mice (Kim *et al*., 2001). *Myocilin* mutations have been found to result in considerable phenotypic heterogeneity, e.g. Pro370Leu is reportedly associated with severe glaucoma phenotype, but Gly399Val located in the same protein domain has been reported to cause adult onset POAG. In combination with a *CYP1B1* mutation (Arg368His) the same *MYOC* mutation precipitates the disease condition much sooner. On the other hand, the most prevalent MYOC mutation (c.1102C.T; Gln368Stop) in Caucasians cause adult onset POAG

with variable penetrance (Craig *et al*., 2001). Incidentally we have detected this mutation in one familial POAG case from eastern India.

The proband (Fig. 1, III-1) was diagnosed at an age of 55 years at an advanced stage of glaucoma suggesting onset of the disease at a much younger age. The patient had an IOP of 21 mm Hg in both the eyes before trabeculectomy and visual field analysis showed superior arcuate defect. Her left eye was detected with myopic chorioretinopathy and squint and both of her eyes were operated for cataract. She had a positive family history of glaucoma. Her mother, maternal uncle and grandfather were known to have the disease. Hence the family members of the proband were examined for the presence of the mutation and clinical status of the carriers assessed for genotype-phenotype correlation.

Analysis of DNA samples of the family members of the proband using TaaI RFLP assay (Baird, *et al*., 2001) led to identification of individuals (Fig. 1; III-7, III-9, IV-4 & IV-6) heterozygous for the mutation who were subsequently examined by SLP in order to detect thinning of retinal nerve Fibre layer (RNFL) as an early sign of glaucoma. The SLP of the left eye of III-7 (53 yrs) showed no thinning of RNFL. She was detected to have Coloboma in the right eye for which SLP of the eye was inconclusive. Coloboma is a congenital abnormality caused by defective closure of the embryonic fissure of the optic cup. However, visual field analysis test did not reveal any glaucomatous visual field damage. She had an IOP of 18 and 19 mm of Hg. Individual III-9 (52 yrs) had cataract in both the eyes, normal range of IOP (12 & 13 mm of Hg), and no thinning of RNFL. Individual IV-4 was also clinically examined and found to have IOP within normal range (13 & 12 mm of Hg) and no thinning of the RNFL. Excepting III-7 no other member of the family who were examined had coloboma.

This is the first case in our knowledge regarding occurrence of Q368X mutation in Asian population. Among the carriers, III-7 and III-9 showed no sign of disease onset, which provide evidence of a possible incomplete penetrance in the family. In a collaborative study with LVPEI, analysis of markers flanking GLC1A locus is in progress to determine whether the mutation is a *de novo* event in the family or the carriers share the same founder chromosome as the Caucasians.

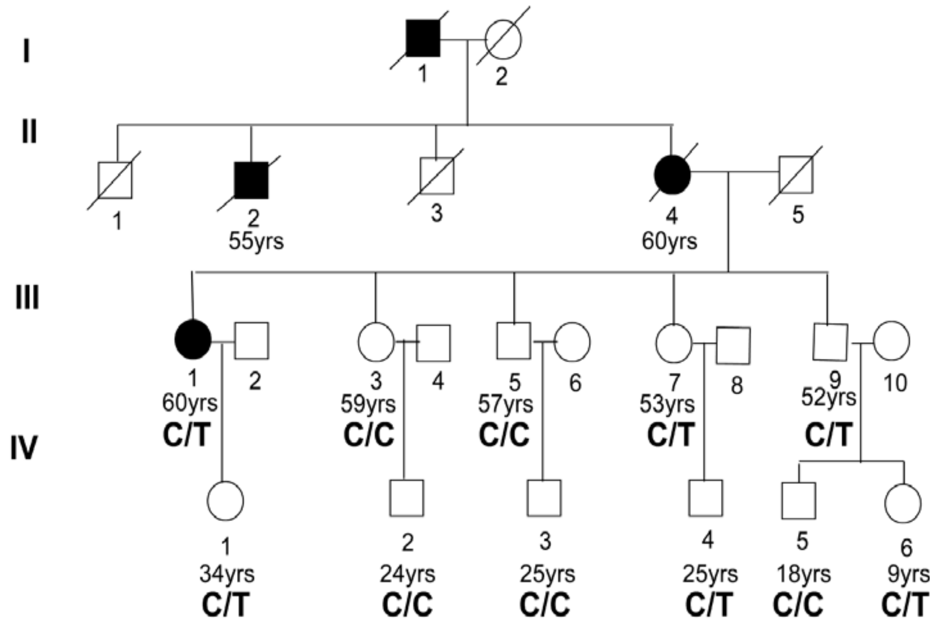

Fig. 1 : Pedigree of an eastern Indian family with Q368X mutation in *MYOC*. The proband (III-1) affected with POAG is identified by an arrow. Squares and circles represent males and females, respectively. Struck out symbols represent deceased individuals in the pedigree including those affected with POAG. In the pedigree, present age of all the participating individuals, and genotypes determined by allele specific restriction analysis have been shown.

As the carriers did not show any sign of glaucomatous optic neuropathy in spite of having truncation mutation, the proband was screened for variations in other genes (*viz CYP1B1* and *OPTC*) to examine the possibility of digenic inheritance of glaucoma in the family. No nucleotide change was detected in the coding region of *CYP1B1* and *OPTC* of the proband.

MYOC mutations in the context of potential digenic forms of glaucoma

Although *MYOC* has been implicated in open angle glaucoma, recently (Chakrabarti *et al.,* 2005; Kaur *et al*., 2005) have examined the role of *MYOC* in primary congenital glaucoma, which is primarily caused by *CYP1B1* with a predominantly autosomal recessive mode of inheritance. They have identified the Gln48His mutation in MYOC in four PCG patients who did not harbor any *CYP1B1* mutation. Among them the mutation was found in homozygous condition in one PCG patient. Additionally, the mutation was also found in a patient who was heterozygous for Arg368His in CYP1B1

suggesting a digenic mode of inheritance of PCG. In another report the same group has observed the Gln48His mutation in MYOC to occur in a POAG patient who was also found to be heterozygous for a CYP1B1 mutation (Pro437leu) (Chakrabarti *et al*., 2005). Report of possible interaction of MYOC and CYP1B1 through a digenic mechanism has been reported earlier in JOAG (Vincent *et al*., 2002). We identified a JOAG patient with a homozygous mutation in *MYOC* together with two heterozygous mutations in *CYP1B1* suggesting causation of JOAG by a digenic or triallelic mode of inheritance (unpublished data).

MYOC-related genes as potential candidates for glaucoma

Despite the limited transmission of POAG in families following monogenic traits due to seven chromosomal loci, it is largely sporadic and transmitted as a complex disease with no knowledge of any biochemical pathway to choose candidate genes from. The identification and inclusion of the new genes in the repertoire of the candidates for eye diseases provides the opportunity to test these genes for the causation of the disorder. In this context, Torrado *et al.* (2002) identified an olfactomedin-related protein, Optimedin, that interacts with MYOC to form heterodimer *in vitro*. Based on this observation, it has been speculated that optimedin might have a role in eye pathogenesis (Torrado *et al*., 2002).

In a study carried out in our lab to identify other olfactomedin domain containing proteins that would have the potential of interacting with myocilin and thereby serving as putative candidate genes for eye diseases, three genes, *viz Noelin 1*, *Noelin 2* and *Noelin 3*, were identified. The structure of all the *Noelin* genes were determined and the splice variants characterized. For *Noelins* to serve as putative candidates for eye diseases they need to be expressed in the eye. Therefore, the expression profile of the *Noelin* splice variants were determined by electronic northern blot experiments. While *Noelin 1* and *Noelin 2* were found to be expressed primarily in the brain and eye, *Noelin 3* expression was evident in brain and other tissues but not in the eye (Mukhopadhyay *et al*., 2004). However, Torrado *et al.* (2002) have reported expression of *Noelin 3* in TM cells by RT-PCR. Lower level of expression of the gene might be responsible for negative finding in the EST database that is dependent on the number of submissions made to the site.

Our initial study could identify five variants for *Noelin 1* and six variants for *Noelin 3*. However, a single transcript was identified for Noelin 2, named

as Noelin 2_v1, with the speculation that additional splice variants will be identified with further submissions to the database and/or through experimental approaches. At present the updated version of AceView (http://www.ncbi.nih.gov/IEB/Research/Acembly/) contains four splice variants for *Noelin 2* including the variant reported by our lab (Mukhopadhyay *et al*., 2004). We evaluated three out of four cDNA entries as true splice variants including one we reported earlier (Mukhopadhyay *et al*., 2004).

A qualitative estimation for the expression of *Noelin 2* splice variants was undertaken by use of human EST database using cDNA entries of different splice variants. The results suggest that Noelin 2_v1 and Noelin 2_v2 are expressed in the eye tissues among which Noelin 2_v2 has higher expression level. No human EST from eye origin was identified for Noelin 2_v3.

The opportunity to further explore the tissue specific expression of *Noelins* by *in silico* approaches exists in the newly created NEIBank EST database. But the tissue specific dataset at the NEIBank is not yet large enough to include genes expressed at low level. Hence work is in progress to examine the expression profile of the *Noelins* in different ocular tissues by wet lab experimental approaches.

Current concept on functional role of MYOC in glaucoma pathogenesis

Though myocilin has been established as a candidate gene for glaucoma (Stone *et al*., 1997), the function of the protein still remains largely unknown. But recent studies have partially unraveled the potential molecular basis of pathogenesis caused by MYOC. It is hypothesized that mutant forms of myocilin are not secreted from the cells and can diminish the secretion of the native protein when two forms are co-expressed (Jacobson *et al*., 2001). Zhou *et al.* (1999) investigated the Triton X-100 solubility characteristics of normal and mutant myocilin expressed by transiently transfected human embryonic kidney cells. Mutant protein was found to be Triton X-100 insoluble, while normal protein was completely soluble. Based on this assay it was hypothesized that normal myocilin can form dimers and, possibly multimers and that mutant protein might interfere with normal protein through formation of heteromultimers (Zhou *et al*., 1999).

Further studies have shown that myocilin containing pathogenic missence mutations are misfolded and accumulate in large aggregates in endoplasmic reticulum (ER) of human embryonic kidney cells and differentiated primary human TM cells which indicate that myocilin associated

glaucoma is an ER storage disease. In cells, under normal condition, ER monitors the folding of secretory proteins through association of ER chaperones with misfolded and even unfolded polypeptide chains (Ellgaard *et al*., 2003). Proteins unable to assume native structure fail to transit to the golgi compartments and are subjected to ER-associated degradation (ERAD) via retrotransport to the cytosol followed by ubiquitination and proteasomal degradation. Often proteins carrying mutations which affect the native folding are not efficiently degraded and form ER or cytoplasmic aggregates (Kopito *et al*., 2000). ER retention has been implicated in the pathogenesis of various diseases (Rutishauser *et al*., 2002). Similarly expression of misfolded, nonsecreted myocilin leads to trabecular meshwork dysfunction, TM cell death, and ultimately a dominant glaucoma phenotype (Liu *et al*., 2004).

In a recent study Aroca-Aguilar *et al.* have examined four pathogenic mutations (Glu323Lys, Gln368Stop, Pro370Leu, and Asp380Ala) in transiently transfected cell lines derived from monkey kidney COS 1, human embryonic kidney and cell lines from human ciliary muscle and iris pigmented epithelium. Their study revealed that wild type myocilin undergoes an endoproteolytic processing between amino acid residues Arg226 and Ile227. The cleavage occurs with the production of two fragments, one of 35 kDa containing C-terminal olfactomedin domain and another of 20 kDa containing the leucine-zipper like domain. Western immunoblot analysis suggests presence of the 35 kDa processed fragment in human aqueous humor and some other ocular tissues indicating that the endoproteolytic cleavage occurs *in vivo*. The study has clearly shown that ocular and nonocular cultured cell lines co-secrete full-length and the 35 kDa fragment which contains the entire olfactomedin domain. Pathogenic myocillin mutations inhibit this endoproteolytic processing which lowers the amount of processed myocilin in the extracellular matrix (ECM) of TM. It is hypothesized to result into altered interaction of myocilin with other ECM proteins which is otherwise required to maintain normal TM structure. Maintenance of normal TM structure is necessary to modulate the aqueous humor outflow and intraocular pressure (Aroca-Aguilar *et al*., 2005).

Cell shape, volume, contractility, adhesion to neighboring cells and to ECM, and amount and composition of ECM have been long thought to control flow of aqueous humor through TM (Tian *et al*., 2000). Although there had been suspicion of a possible connection between mutant myocilin

Fig. 2: Schematic representation of the potential biological role of MYOC in the eye under normal condition and in glaucoma pathogenesis. The scheme has been postulated based on recent publications (Liu et al, Hum Mol Genet, 2004; Peters *et al.*, Exp Cell Res, 2004; Aroca-Aguilar et al, J Biol Chem, 2005)

protein and its effect on ECM, there was no experimental evidence to conclusively prove it. Recently, it has been demonstrated that myocilin produced from sf9 insect cells can interact with the HepII domain of fibronectin, an ECM protein. The particular domain of fibronectin is responsible for triggering the signaling pathways that regulate focal adhesion formation and recruitment of paxillin into the focal adhesions. When human skin fibroblasts are plated in presence of myocilin on substrates co-coated with myocilin and either fibronectin or its HepII domain, the cells are found to attach with the substratum but cell spreading does not occur. Thus myocilin probably does not interfere with the cell surface binding sites of fibronectin. This interaction of myocilin with fibronectin and its subsequent role in the regulation of paxillin incorporation in focal adhesion has been hypothesized to have important implications in controlling aqueous humor movement in human eye (Peters *et al*., 2005).

The studies on the role of myocilin in the maintenance of normal TM structure and consequently its role in causation of glaucoma have intensified in recent years. Till date there is still no compelling evidence regarding interaction of myocilin with ECM proteins. But myocilin has been found to be a secretory protein having olfactomedin domain at the C-terminal end with which it can interact with similar other proteins. Though myocilin is reported to interact with fibronectin, the precise region of myocilin which interacts with fibronectin is still not known. If myocilin is secreted as a processed 35kDa protein along with full length myocilin, whether it is the 35 kDa fragment which is responsible for the interaction with ECM proteins like fibronectin still remains a question. Moreover reported evidence of myocilin interacting with fibronectin is based on experiments done with sf9 insect myocilin where the predominant form is a 66 kDa protein which is grossly comparable to the size of full length myocilin (55-57 kDa) found in human. But experiments with protein secretion blocker like monensin suggests that under normal condition around 80% of intracellular soluble wild type myocilin corresponds to the 35 kDa processed fragment (Aroca-Aguilar *et al*., 2005). Moreover the precise role of interaction of myocilin with HepII domain of fibronectin in controlling aqueous humor outflow through trabecular meshwork is still unknown.

Although the first genetic locus for POAG to be identified was *GLC1A* with the causal gene being *MYOC*, the precise role of the protein in the normal eye and subsequently in disease pathogenesis continued to elude us. Mutations in different regions of the *MYOC* have been found to be

Table 1 : Clinical data of the proband and Q368X (c.1102 C>T) carriers

Family members	Age (Yrs)	Genotype	IOP (mm of Hg)	CD ratio (OD, OS)	Field analysis	Presence of Coloboma	History of cataract	Other eye problems	RNFL analysis
III-1 (Proband)	55	C,T	21 (BE, before trabeculec-tomy)	0.9, 0.8	Superior arcuate defect	No	Both eyes	myopic chorio retinopathy and Squint(LE)	Not done *
III-7	53	C,T	18, 19	0.8, 0.6	No visual field defect	Optic disc coloboma in RE	None	None	No thinning detected
III-9	52	C,T	12,13	0.4 (BE)	Not done	No	operated in RE and immature cataract in LE	posterior capsular opacity (RE)	No thinning
IV-5	25	C,T	13,12	0.4, 0.3	Not done	No	Examined	None	No thinning detected

* Glaucoma was detected in proband by visual field analysis test and not by RNFL

associated with a spectrum of phenotypic expression of the disease starting from complete absence of disease phenotype to severe glaucoma. *MYOC* mutations causing digenic cases of early onset glaucoma, point towards the multifactorial nature of the disease. Keeping in view this complex inheritance of glaucoma, identification of potential candidate genes may lead to better understanding of the disease. Identification of *MYOC* mutations in congenital cases of glaucoma raises the question regarding the role of the protein in eye formation, which is yet an unexplored territory. Lastly biological role of the protein in normal eye, its interaction with other eye proteins as well as the functional implications of such interactions may lead to better understanding of the role of the protein in adult onset glaucoma.

Table 2 : Different splice variants of *Noelin 2*

Proposed Name	cDNA (bp)	Protein (aa)	Accession number	
			protein	cDNA
Noelin 2_v1	2022	454	NP_477512	NM_058164
Noelin 2_v2	1147	480	NA	NA
Noelin 2_v3	1677	376	NA	NA

aa, amino acid; NA, Not Available

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