·Basic Research·

Bioinformatics analysis and construction of eukaryotic expression plasmid of *Cx50* V64G mutation

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Received:2008-10-28 Accepted:2008-12-15

Abstract

• AIM: To construct and analyze eukaryotic expression plasmid inserted by *Cx50* with V64G mutation through bioinformatics software.

• METHODS: The full coding domain sequence of *Cx50* with V64G mutation was acquired from the blood of patients with cataract and was cloned into pcDNA3.1/Amp (+).The constructed plasmid was identified with PCR , enzyme digestion and sequencing. The analysis of *Cx50* with V64G mutation was performed with bioinformatics software.

• RESULTS: *Cx50* with V64G mutation was successfully amplified and its eukaryotic expression plasmid was constructed. Valine-64 is well conserved in the first extracellular loop of connexin 50 in different species and also in different human α -type gap junctional proteins.

• CONCLUSION: The successive reconstruction and verification of eukaryotic expression plasmid containing *Cx50* with V64G mutation established the foundation for further studying the mechanism of cataract.

• KEYWORDS: *Cx50*; bioinformatics analysis ; eukaryotic expression plasmid

Liu P, Lin Y, Yang YY, Zheng JQ, Hou Y, Jin D, Fu XB, Ma HM. Bioinformatics analysis and construction of eukaryotic expression plasmid of *Cx50* V64G mutation. *Int J Ophthalmol* 2009;2(1):16-18

INTRODUCTION

Congenital cataract, a clinically and genetically highly heterogeneous eye lens disorder, is one of the significant causes of visual impairment. In the world 20 million children under the age of 16 suffer from cataract and 1.4 million of them are blind [1]. Hereditary cataracts are most commonly inherited in an autosomal dominant manner and are phenotypically and genotypically heterogeneous, showing considerable inter- and intrafamilial variability [2]. At least 35 loci have been linked with various forms of congenital and developmental cataracts, and mutations in at least 15 genes have been identified for isolated congenital cataracts [3]. These genes encode crystallins, gap junction proteins , the major intrinsic protein (MIP) of lens fiber and cytoskeletal proteins [4].

Gap junctions are membrane specializations containing clusters of channels that allow intercellular passage of ions, metabolites, and secondary messengers up to 1 kDa [5]. The channels that make up a junction are formed from the contribution of one hemichannel or connexon from each apposed cell surface. Hemichannels are themselves oligomers formed of six subunit proteins of largely a-helical structure called connexins.

Connexin 50 encoded by *GJA8* located on chromosome 1q21.1 is comprised of two exons, the coding sequence being encompassed entirely by the second exon (NM-005267). It is an integral membrane protein containing four transmembrane domains, two extracellular loops, and an intracellular loop with both the amino and carboxyl termini located in the cytoplasm.

In the former study [6],we identified a five generation family having bilateral nuclear congenital cataract. Upon sequencing analysis of *GJA8* we identified a heterozygous mutation T>G at position 191 (c.191 T>G) resulting in the transition of valine to glycine at codon 64 (V64G). The change cosegregated completely with the disease phenotype, thus suggesting this as the causative mutation in the present family. This is a novel mutation which hasn't been reported previously with congenital cataract.

MATERIALS AND METHODS

Bioinformatics Analysis of Connexin50 V64G Mutation Using the program of the Clustal *et al* [7], we know the degree of the conservation of valine in the first extracellular loop of connexin 50 in different species (Figure 1) and also in different human α-type gap junctional proteins (Figure 1). Secondary structure predictions of the altered protein were analyzed using the Bioinformatics tool of the DeepView/ Swiss-PdbViewer3.7.

Cloning of Mutant Human *Cx50* DNA Mutant human

64

Cx 50 [Homo sapiens]	DFVCNTQQPGCENVCYDEAFPISHIRLWVLQIIFVSTPSLMYVGH
Cx 50 [Rattus norvegicus]	DFVCNTQQPGCENVCYDEAFPISHIRLWVLQIIFVSTPSLMYVGH
Cx 50 [Mus musculus]	DFVCNTQQPGCENVCYDEAFPISHIRLWVLQIIFVSTPSLMYVGH
Cx 43 [Gallus gallus]	DFVCNTQQPGCENVCYDEAFPISHIRLWVLQIIFVSTPSLVYFGH
Cx 43 [Homo sapiens]	AFRCNTQQPGCENVCYDKSFPISHVRFWVLQIIFVSVPTLLYLAH
Cx 38 [Homo sapiens]	DFICNTQQPGCTNVCYDQAFPISHVRYWVLQFLFVSTPTLIYLGH
Cx 46 [Homo sapiens]	DFTCNTQQPGCENVCYDRAFPISHIRFWALQIIFVSTPTLIYLGH
Cx 59 [Homo sapiens]	GFICNTEQPGCRNVCYDQAFPISLIRYWVLQVIFVSSPSLVYMGH

Figure 1 A multiple alignment of amino acid sequences of *Cx50* in different species and in different human α-type gap junction proteins

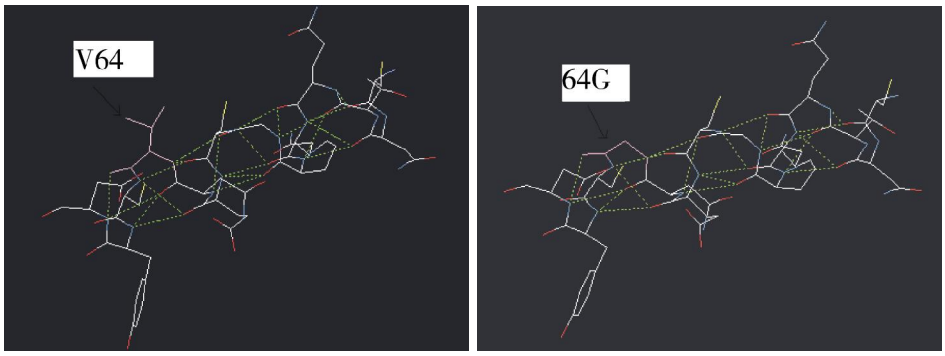

Figure 2 Secondary structure predictions of the amino from 54 to 67 in normal protein and altered protein. Green represents H-bond. Purple represents the site of 64

Cx50 alleles were PCR amplified from genomic DNA of affected individuals using primers (sense: 5'-CGGAA TTCGTGAGAAATGGGCGACTGGAG -3', and anti-sense: 5'-GCTCTAGATTTCCTTTCATCTTGCCCTACG-3') . The Eco RI and Xbal I restriction sites were introduced to the bi-side of *Cx50* by PCR. After being separated and purified by 10g/L agarose electrophoresis and QIA quick Gel Extraction Kit (Promega), the *Cx50*containing the restriction sites and pcDNA3.1 were incubated with Eco RI and Xbal I respectively, and were linked using T4 ligase to construct pcDNA3.1- *Cx50* V64G plasmid. The Escherichia coli (E. coli) strain JM109 was transformed by the plasmid and inoculated on an LB plate with ampicillin (Amp 0.1g/L), and incubated overnight at 37℃ . The inserted fragment was sequenced to exclude introduction of random mutations.

RESULTS

Bioinformatics Analysis of Connexin50 V64G Mutation Using the program of Clustal *et al*[7], we found that Valine-64 was well conserved in the first extracellular loop of connexin50 in different species (Figure 1) and also in human α-type gap junctional proteins (Figure 1). Using bioinformatic tool of DeepView/Swiss-PdbViewer3.7, we found there were changes of a-helix, H-bond and the energy (Figure 2) in the altered protein, which might be the pathogenesis. The transmembrane domains of the connexins are essential for the correct transport of the protein into the plasma membrane. It has been identified that pore lining residues lie in the first transmembrane domain and are essential for the formation of the pore and therefore channel permeability.

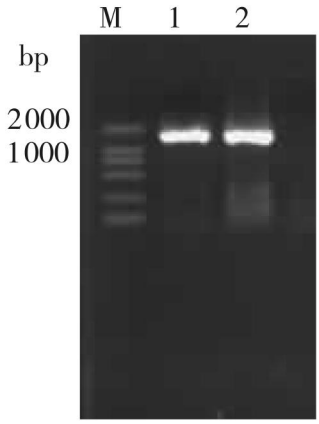

Figure 3 Agarose electrophoresis Lane 1 and lane 2 were patients. M was Marker DL2000

Cloning of Mutant Human *Cx50* DNA The full-length sequence of *Cx50* was amplified by PCR (Figure 3). The sequencing data indicated that the construction of expression plasmid with T>G at position 191was correct (Figure 4).

DISCUSSION

The connexin gene family encodes gap-junction channel proteins that mediate the intercellular transport of small biomolecules (<1kDa) including ions, metabolites, and second messengers in diverse vertebrate cell types, including cochlea cells, Schwann cells, epidermal cells, and lens fiber cells. At least 10 genes for connexins of varying molecular mass (26-50 kDa) have been identified in humans. Mutations in the genes for *Cx26 (GJB2), Cx31 (GJB3),Cx32 (GJB1),*

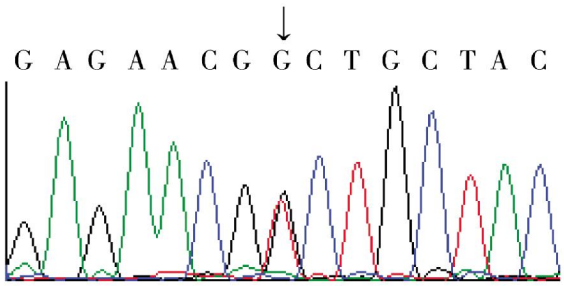

Figure 4 DNA sequence of a part of *Cx50* with V64G mutation A heterozygous change T>G at the second base of codon 64 (GTC-GGC) resulting in substitution of valine 64 by glycine (V64G)

Cx43 (GJA1), and Cx50 (GJA8) have been associated with certain types of deafness, skin disease, peripheral neuropathy, heart defects, and cataracts .

The former study described the identification of a novel V64G substitution in *Cx50* segregating solely in 7 affected members of a five generation family, having nuclear cataract with Y-sutural opacities. The observed V64G substitution lies within the first extracellular loop of *Cx50* and represents a non-conservative amino acid change as valine is a polar amino acid while glycine is a nonpolar amino acid. The transmembrane domains of the connexins are proposed to participate in the oligomerization into connexin hemichannels and are also essential for the correct transport of the protein into the plasma membrane. It has been identified that pore lining residues lie in the first transmembrane domain and are essential for the formation of the pore and therefore channel permeability [8]. The V64G mutation may influence the correct transport of proteins into the plasma membrane. Valine-64 is well conserved in the first extracellular loop of connexin50 in different species and also in different human α-type gap junctional proteins. So far, at least eight congenital cataract families have been linked with *Cx50* and significant inter-familial phenotypic variability has been observed. It seems that in cataract, one major gene is involved but variants in other genes, involved in lens development, growth, and maintenance. The variants might cause phenotypic variability.

The successive reconstruction and verifying of eukaryotic expression plasmid containing *Cx50* V64G gene established the platform to further pursue the mechanisms of *Cx50* gene mutation leading to cataract.

Acknowledgements: We gratefully acknowledge the numerous sample donors for making this work possible.

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