



## RESEARCH PAPER

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*In silico* MicroRNAs target identification within the *GJB2*  
*Gene*

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**Abstract**

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Regulation of gene activity is a multi-factorial procedure that requires coordination of complex regulatory network. MicroRNA is a novel gene regulator that requires exposition of open questions concerning selection of targets and mechanisms. MicroRNA target prediction using bioinformatics tool is often the first line of approaching the multifarious system of gene regulation. Such methodologies will helps in setting search priorities for experimental validation of gene controlling mechanisms. But finding a functional miRNA target is still a challenging task. In the present study we focused on gene regulation potentiality of miRNAs to investigate their target sites within *GJB2* (a candidate gene for hearing impairment that codes for connexin 26). Mapping was made for miRNAs to establish collaborative regulatory profiles. This computational setting will subsequently allow better *in vitro* confirmation of the regulatory networks in cellular systems and possibly later on in transgenic animal models for this severe human anomaly of hearing loss. It is thus concluded that this is the first pilot approach to investigate predicted miRNA involved in *GJB2* gene regulation.

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## Introduction

MicroRNAs (miRNAs) are a broad class of small non-coding RNAs of about 22 nucleotides in length that target and suppress multiple genes across diverse signalling pathways comprising biologically meaningful networks. Most human mRNAs have binding sites for miRNAs (Friedman *et al.*, 2009; Landgraf *et al.*, 2007). MiRNAs are expressed in specific tissues and in particular developmental stages (Ambros, 2004; Bartel, 2004; Wienholds and Plasterk, 2005). Besides, miRNAs are deregulated in numerous human diseases (Alvarez-Garcia and Miska, 2005). Recent study have shown the role of miRNAs is as a repressor of mRNA levels and translation (Fabian *et al.*, 2010). MiRNAs originate from genome-encoded primary transcripts contain several 60 to 80 nt long distinctive hairpin stem-loop structures which are excised by the Drosha–DGCR8/Pasha complex to form the precursor miRNA (pre-miRNA) (Han *et al.*, 2006) which are further trimmed by the ribonuclease Dicer (Gregory *et al.*, 2004; Han *et al.*, 2004) to imperfect ~22-nt duplexes (miRNA:miRNA\*). One strand of the duplex is incorporated into RNA-induced silencing complex (RISC) (Liu *et al.*, 2004; Meister *et al.*, 2004) and becomes a functional miRNA. A single miRNA can deregulate the expression levels of many target genes by a post-transcriptional mechanism (Lim *et al.*, 2005). MiRNAs have been shown to be crucial for the majority of physiological processes; development, cell proliferation and cell death (Boehm and Slack, 2005; Esau *et al.*, 2004; Sokol and Ambros, 2005; Sonkoly and Pivarcsi, 2009; Wang *et al.*, 2007; Xu *et al.*, 2004). MiRNA deregulate gene expression mostly by imperfect binding to complementary sites within transcript sequences and suppresses their translation, stimulate their deadenylation and degradation or induce their cleavage (Bartel, 2004; Perron and Provost, 2008). Therefore, microRNAs displaying deregulated expression in the context of specific diseases are of particular interest as therapeutic targets especially if they can be shown to coordinate such disease networks.

Hearing loss (HL) or hearing impairment (HI) is a

common neurosensory disorder in human populations. It is partial or complete loss of hearing which results in impaired speech, language and communication skills. Hearing impairment (HI) is the most frequent sensory disorder involving a multitude of factors, at least 50% of the cases are due to genetic etiology. The pattern of inheritance can be dominant, recessive, X-linked and mitochondrial. Multiple genes are involved in different types of deafness (syndromic and non-syndromic). Non-syndromic hereditary deafness which constitutes about 80% are due to recessive genes (or mutations). It is believed that more than one hundred genes could be involved in hearing impairment. The gene involved in this type of deafness is *GJB2*, which encodes the gap junction protein connexin 26 (Cx26). The *DFNB1* locus at chromosome 13q11-q12 includes the *GJB2* and *GJB6* genes, which respectively encode connexin 26 (Cx26) and connexin 30 (Cx30). These connexin proteins are co-expressed and co-localized in the cochlea, where they create heteromeric gap junctions (Ahmad *et al.*, 2003) and make important contributions to cochlear homeostasis (Zhao *et al.*, 2006). Altered *GJB2* gene leads to dysfunctional gap channels and potassium cycle, affect hearing causing deafness, skin and other disorders (Abe *et al.*, 2000; Morell *et al.*, 1998) which could disrupt the conversion of sound waves to nerve impulses. These deletions lead to an aberrantly small protein that cannot form functional gap junctions. Mutations in *GJB2* cause a major fraction of hearing loss worldwide, with loss-of-function mutations of *GJB2* estimated to be responsible for more than half of all recessively inherited NSHL (non syndromic hereditary hearing Loss) in developed countries (Hilgert *et al.*, 2009).

Numerous miRNA target prediction algorithms exploiting different approaches have been recently developed, and many methods of experimental validation have been premeditated. MicroRNA targets are considered difficult to predict in animal genomes because microRNAs generally display only partial complementarity to their target mRNA (Martin *et al.*, 2007), in contrast to plants where microRNAs can bind with almost perfect complementarity to their

targets<sup>22</sup>. All these shortcomings collectively suggest that the interactions of microRNAs with their mRNA counterparts are complex and poorly understood. Keeping the basic algorithm of miRWalk which is based on a computational approach that identifies the longest consecutive complementary between miRNA and gene sequences, we used miRWalk *in silico* based miRNA targets identification within *GJB2* gene.

### Materials and methods

In the present study, attention was drawn toward to practical aspects of computational miRNA target analysis and presents our approach to miRNA target prediction using one of the strong candidate genes of *GJB2* for hearing impairment. In this regards, miRWalk is one of the wide-range database that offers information on miRNA from human, mouse and rat on their predicted as well as validated binding sites on their target genes. miRWalk was used database for investigating predicted targets of microRNAs in the complete sequences (5' UTR, CDS and 3' UTR) of *GJB2* gene in the human genome. In the present study, specific miRNAs targeting *GJB2* gene was collected by 8 established miRNA prediction programs on 3' UTRs of all known genes of human, mouse and rat i.e. RNA22, miRanda, miRDB, TargetScan, RNA- hybrid, PITA, PICTAR, and Diana-microT.

### Results and discussion

Currently, thousands of microRNAs have been identified across variety of different species;

therefore, identification of microRNA targets has become an important step towards understanding these regulatory mechanisms. Computational based prediction presently remains the only source for rapid identification of a putative microRNA target. It helps in prioritizing predictions and efficiently allocating experimental resources towards validating the most promising targets. An increasing number of experimentally validated microRNAs targets are now available; utilizing this additional information in the search for further targets may help to improve the specificity of *in silico* based methods for target prediction. All computer-based microRNA target prediction programs are based on specific parameters where slight variation results for the same target input. Such weakness of single *in silico* studies however can be partially compensated by predicting targets using multiple programs. Scoring methods using dynamic programming (John *et al.*, 2004; Kiriakidou *et al.*, 2004; Lewis *et al.*, 2003) and a complementarity-based strategy (Lewis *et al.*, 2003; Rajewsky and Socci, 2004) are generally preferred to rank the prediction results. These approaches have been quite successful for a few top ranked results. Therefore microRNAs targets calculated from multiple prediction methods significantly improved target prediction accuracy. Keeping this in view, 8 key programs were used to optimize our search and to uncover microRNA targets in sequences of the *GJB2* gene cluster with high accuracy.

**Table 1.**

Gene Name	RefSeqID	MicroRNA	StemLoop ID	Seed Length	Star t	Sequence	End	Region	P-value
<i>GJB2</i>	NM_004004	hsa-miR-1228*	hsa-mir-1228	7	206	UGGGCGG	200	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1285	hsa-mir-1285-1	7	208	UCUGGGC	202	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1285	hsa-mir-1285-2	7	208	UCUGGGC	202	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1307	hsa-mir-1307	8	154	CUCGGCGU	147	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-132*	hsa-mir-132	7	25	CCGUGGC	19	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1469	hsa-mir-1469	7	154	CUCGGCG	148	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1539	hsa-mir-1539	8	126	UCCUGCGC	119	5 UTR	0.0033

<i>GJB2</i>	NM_004004	hsa-miR-1539	hsa-mir-1539	7	125	CCUGCGC	119	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-185*	hsa-mir-185	7	87	AGGGGCU	81	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1908	hsa-mir-1908	8	175	CGGCGGGG	168	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-1908	hsa-mir-1908	7	174	GGCGGGG	168	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-1908	hsa-mir-1908	7	163	GGCGGGG	157	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-191*	hsa-mir-191	7	138	CUGCGCU	132	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-191*	hsa-mir-191	7	124	CUGCGCU	118	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-21*	hsa-mir-21	7	41	CAACACC	35	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-326	hsa-mir-326	8	209	CUCUGGGC	202	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-330-5p	hsa-mir-330	8	209	CUCUGGGC	202	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-423-5p	hsa-mir-423	7	88	GAGGGGC	82	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-542-5p	hsa-mir-542	7	75	UCGGGGA	69	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-566	hsa-mir-566	7	98	GGGCGCC	92	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-566	hsa-mir-566	7	21	GGCGCCU	15	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-593	hsa-mir-593	8	142	GUCUCUGC	135	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-615-5p	hsa-mir-615	7	160	GGGGGUC	154	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-663	hsa-mir-663	7	114	AGGCGGG	108	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-663	hsa-mir-663	8	174	GGCGGGG	167	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-663	hsa-mir-663	7	163	GGCGGGG	157	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-671-5p	hsa-mir-671	7	183	AGGAAGC	177	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-744	hsa-mir-744	8	51	UGCGGGG	44	5 UTR	0.0033
<i>GJB2</i>	NM_004004	hsa-miR-744	hsa-mir-744	7	173	GCGGGGC	167	5 UTR	0.013
<i>GJB2</i>	NM_004004	hsa-miR-744	hsa-mir-744	7	50	GCGGGGC	44	5 UTR	0.013

**Table 2.** Illustrated specific miRNA sequences within CDS of *GJB2* gene along with seed sequences, its location and size respectively.

Gene Name	RefSeqID	MicroRNA	StemLoop ID	Seed Length	Start	Sequence	End	Region	P-value
<i>GJB2</i>	NM_004004	hsa-miR-103-as	hsa-mir-103-1-as	7	445	CAUAGCC	439	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-103-as	hsa-mir-103-2-as	7	445	CAUAGCC	439	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-105*	hsa-mir-105-1	7	439	CGGAUGU	433	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-105*	hsa-mir-105-2	7	439	CGGAUGU	433	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-106a*	hsa-mir-106a	7	807	CUGCAAU	801	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-10a*	hsa-mir-10a	7	820	CAAAUUC	814	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1184	hsa-mir-1184	8	236	CUGCAGCG	229	CDS	0.0103

<i>GJB2</i>	NM_004004	hsa-miR-1184	hsa-mir-1184	8	236	CUGCAGCG	229	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1184	hsa-mir-1184	8	236	CUGCAGCG	229	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1205	hsa-mir-1205	7	237	UCUGCAG	231	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1205	hsa-mir-1205	9	386	CUGCAGGGU	378	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-1207-3p	hsa-mir-1207	7	459	UCAGCUG	453	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-122	hsa-mir-122	8	267	UGGAGUGU	260	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-122	hsa-mir-122	7	266	GGAGUGU	260	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1226	hsa-mir-1226	9	717	UCACCAGCC	709	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-1226	hsa-mir-1226	8	716	CACCAGCC	709	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1228	hsa-mir-1228	7	255	UCACACC	249	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1228	hsa-mir-1228	7	346	CACACCU	340	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1234	hsa-mir-1234	8	364	UCGGCCUG	357	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1234	hsa-mir-1234	7	363	CGGCCUG	357	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1236	hsa-mir-1236	7	527	CCUCUUC	521	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1237	hsa-mir-1237	7	591	CCUUCUG	585	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1238	hsa-mir-1238	7	530	CUUCCUC	524	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1244	hsa-mir-1244	7	421	AAGUAGU	415	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1245	hsa-mir-1245	7	417	AGUGAUC	411	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1248	hsa-mir-1248	7	592	ACCUUCU	586	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1254	hsa-mir-1254	7	393	AGCCUGG	387	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1265	hsa-mir-1265	7	827	CAGGAUG	821	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-128	hsa-mir-128-1	7	749	CACAGUG	743	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-128	hsa-mir-128-2	7	749	CACAGUG	743	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1301	hsa-mir-1301	7	397	UUGCAGC	391	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1301	hsa-mir-1301	7	336	UUGCAGC	330	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-1	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-1	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	8	742	UUGGGACA	735	CDS	0.0103

	004	1302	1302-2						
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-2	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-3	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-3	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-4	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-4	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-5	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-5	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-6	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-6	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-7	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-7	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-8	8	742	UUGGGACA	735	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-1302	hsa-mir-1302-8	7	741	UGGGACA	735	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-130b*	hsa-mir-130b	7	554	ACUCUUU	548	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1321	hsa-mir-1321	7	611	CAGGGAG	605	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1324	hsa-mir-1324	7	814	CCAGACA	808	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-138	hsa-mir-138-2	7	272	GCUGGUG	266	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-138	hsa-mir-138-1	7	272	GCUGGUG	266	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-140-3p	hsa-mir-140	9	615	ACCACAGG G	607	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-141	hsa-mir-141	8	796	AACACUGU	789	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-141*	hsa-mir-141	7	218	CAUCUUC	212	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181a	hsa-mir-181a-2	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181a	hsa-mir-181a-1	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181b	hsa-mir-181b-1	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181b	hsa-mir-181b-2	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181c	hsa-mir-181c	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-181d	hsa-mir-181d	7	835	ACAUUCA	829	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-1915	hsa-mir-1915	7	248	CCCCAGG	242	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-193a-3p	hsa-mir-193a	7	893	AACUGGC	887	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-193b	hsa-mir-193b	7	893	AACUGGC	887	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-197	hsa-mir-197	7	718	UUCACCA	712	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-200a	hsa-mir-200a	8	796	AACACUGU	789	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-214	hsa-mir-214	7	830	CAGCAGG	824	CDS	0.0407

<i>GJB2</i>	NM_004004	hsa-miR-220a	hsa-mir-220a	7	347	CCACACC	341	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-220b	hsa-mir-220b	7	617	CCACCAC	611	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-220c	hsa-mir-220c	7	613	CACAGGG	607	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-296-5p	hsa-mir-296	7	451	AGGGCCC	445	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-299-3p	hsa-mir-299	8	436	AUGUGGGA	429	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-30c-1*	hsa-mir-30c-1	7	433	UGGGAGA	427	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-30c-2*	hsa-mir-30c-2	7	433	UGGGAGA	427	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-330-3p	hsa-mir-330	7	759	CAAAGCA	753	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-338-5p	hsa-mir-338	9	870	AACAAUUAUC	862	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-338-5p	hsa-mir-338	8	869	ACAAUAUC	862	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-361-3p	hsa-mir-361	8	249	CCCCAGG	242	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-370	hsa-mir-370	7	361	GCCUGCU	355	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-375	hsa-mir-375	7	260	UUUGUUC	254	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-376c	hsa-mir-376c	7	682	ACAUAGA	676	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-383	hsa-mir-383	7	462	AGAUCAG	456	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-412	hsa-mir-412	8	720	ACUUCACC	713	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-412	hsa-mir-412	7	719	CUUCACC	713	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-433	hsa-mir-433	7	802	AUCAUGA	796	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-452*	hsa-mir-452	7	356	CUCAUCU	350	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-455-3p	hsa-mir-455	8	755	GCAGUCCA	748	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-455-3p	hsa-mir-455	7	754	CAGUCCA	748	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-489	hsa-mir-489	7	838	GUGACAU	832	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-505*	hsa-mir-505	7	609	GGGAGCC	603	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-517b	hsa-mir-517b	7	498	CGUGCAU	492	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-520g	hsa-mir-520g	7	370	ACAAAGU	364	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-520h	hsa-mir-520h	7	370	ACAAAGU	364	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-524-3p	hsa-mir-524	7	665	GAAGGCG	659	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-525-3p	hsa-mir-525	7	665	GAAGGCG	659	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-548p	hsa-mir-548p	7	857	UAGCAAA	851	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-575	hsa-mir-575	7	290	GAGCCAG	284	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-581	hsa-mir-581	7	628	CUUGUGU	622	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-588	hsa-mir-588	7	492	UGGCCAC	486	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-589*	hsa-mir-589	8	873	CAGAACAA	866	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-609	hsa-mir-609	8	382	AGGGUGUU	375	CDS	0.0103

	004								
<i>GJB2</i>	NM_004004	hsa-miR-609	hsa-mir-609	7	381	GGGUGUU	375	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-617	hsa-mir-617	9	880	GACUCCCCA	872	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-620	hsa-mir-620	7	703	AUGGAGA	697	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-622	hsa-mir-622	7	784	ACAGUCU	778	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-629	hsa-mir-629	7	586	UGGGUUU	580	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-647	hsa-mir-647	8	389	UGGCUGCA	382	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-664*	hsa-mir-664	7	892	ACUGGCU	886	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-767-3p	hsa-mir-767	8	359	CUGCUCAU	352	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-877*	hsa-mir-877	9	528	UCCUCUUCU	520	CDS	0.0026
<i>GJB2</i>	NM_004004	hsa-miR-877*	hsa-mir-877	8	527	CCUCUUCU	520	CDS	0.0103
<i>GJB2</i>	NM_004004	hsa-miR-892a	hsa-mir-892a	7	794	CACUGUG	788	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-892b	hsa-mir-892b	7	892	ACUGGCU	886	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-942	hsa-mir-942	7	780	UCUUCUC	774	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-942	hsa-mir-942	7	522	UCUUCUC	516	CDS	0.0407
<i>GJB2</i>	NM_004004	hsa-miR-96*	hsa-mir-96	7	803	AAUCAUG	797	CDS	0.0407

**Table 3.** Shows specific miRNA sequences within 3' UTR of *GJB2* gene along with seed sequences, its location and size respectively.

Gene Name	RefSeqID	MicroRNA	StemLoop ID	Seed Length	Start	Sequence	End	Region	P-value
<i>GJB2</i>	NM_004004	hsa-miR-124	hsa-mir-124-1	8	1397	UAAGGCAC	1390	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-124	hsa-mir-124-2	8	1397	UAAGGCAC	1390	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-124	hsa-mir-124-3	8	1397	UAAGGCAC	1390	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1244	hsa-mir-1244	10	1766	AGUAGUUGGU	1757	3 UTR	0.0014
<i>GJB2</i>	NM_004004	hsa-miR-126*	hsa-mir-126	8	2236	AUUAUUAC	2229	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1283	hsa-mir-1283-1	8	1434	CUACAAAG	1427	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1283	hsa-mir-1283-2	8	1434	CUACAAAG	1427	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1285	hsa-mir-1285-1	8	908	CUGGGCAA	901	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1285	hsa-mir-1285-2	8	908	CUGGGCAA	901	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-1304	hsa-mir-1304	9	1093	UUUGAGGCU	1085	3 UTR	0.0055
<i>GJB2</i>	NM_004004	hsa-miR-1304	hsa-mir-1304	11	1649	UUGAGGCUACA	1639	3 UTR	0.0003
<i>GJB2</i>	NM_004004	hsa-miR-1304	hsa-mir-1304	8	1092	UUGAGGCU	1085	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-140-3p	hsa-mir-140	8	1818	UACCACAG	1811	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-146a*	hsa-mir-146a	9	1499	CCUCUGAAA	1491	3 UTR	0.0055
<i>GJB2</i>	NM_004004	hsa-miR-146a*	hsa-mir-146a	8	1498	CUCUGAAA	1491	3 UTR	0.0219



	04								
<i>GJB2</i>	NM_0040	hsa-miR-187*	hsa-mir-187	8	1645	GGCUACAA	1638	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-194	hsa-mir-194-1	8	1409	UGUACACAG	1402	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-194	hsa-mir-194-2	8	1409	UGUACACAG	1402	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-26a-1*	hsa-mir-26a-1	9	2211	CUAUUCUUG	2203	3 UTR	0.0055
	04								
<i>GJB2</i>	NM_0040	hsa-miR-26a-1*	hsa-mir-26a-1	8	2071	CUAUUCUU	2064	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-26a-2*	hsa-mir-26a-2	10	2211	CUAUUCUUGA	2202	3 UTR	0.0014
	04								
<i>GJB2</i>	NM_0040	hsa-miR-26a-2*	hsa-mir-26a-2	8	2071	CUAUUCUU	2064	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-323-3p	hsa-mir-323	8	2285	ACAUUACA	2278	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-324-3p	hsa-mir-324	8	1908	CUGCCCCA	1901	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-421	hsa-mir-421	10	1972	AUCAACAGAC	1963	3 UTR	0.0014
	04								
<i>GJB2</i>	NM_0040	hsa-miR-421	hsa-mir-421	9	1971	UCAACAGAC	1963	3 UTR	0.0055
	04								
<i>GJB2</i>	NM_0040	hsa-miR-491-5p	hsa-mir-491	8	1319	GUGGGGAA	1312	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-495	hsa-mir-495	8	2007	AAACAAAC	2000	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-506	hsa-mir-506	8	1397	UAAGGCAC	1390	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-516b	hsa-mir-516b-2	8	1058	AUCUGGAG	1051	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-516b	hsa-mir-516b-1	8	1058	AUCUGGAG	1051	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-518a-5p	hsa-mir-518a-1	8	1584	CUGCAAAG	1577	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-518a-5p	hsa-mir-518a-2	8	1584	CUGCAAAG	1577	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-520d-5p	hsa-mir-520d	8	1434	CUACAAAG	1427	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-522	hsa-mir-522	8	1024	AAAUGGUU	1017	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-524-5p	hsa-mir-524	8	1434	CUACAAAG	1427	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-527	hsa-mir-527	8	1584	CUGCAAAG	1577	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-548l	hsa-mir-548l	8	1580	AAAGUAUU	1573	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-555	hsa-mir-555	8	2015	GGGUAAGC	2008	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-561	hsa-mir-561	8	1431	CAAAGUUU	1424	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-592	hsa-mir-592	10	1875	UGUGUCAUA	1866	3 UTR	0.0014
	04								
<i>GJB2</i>	NM_0040	hsa-miR-625	hsa-mir-625	8	1489	AGGGGGAA	1482	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-642	hsa-mir-642	8	942	UCCUCUC	935	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-665	hsa-mir-665	8	1294	CCAGGAGG	1287	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-767-3p	hsa-mir-767	8	2101	UCUGCUC	2094	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-892a	hsa-mir-892a	8	1877	ACUGUGUC	1870	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-9*	hsa-mir-9-1	8	2250	UAAAGCUA	2243	3 UTR	0.0219
	04								
<i>GJB2</i>	NM_0040	hsa-miR-9*	hsa-mir-9-2	8	2250	UAAAGCUA	2243	3 UTR	0.0219
	04								

<i>GJB2</i>	NM_004004	hsa-miR-9*	hsa-mir-9-3	8	2250	UAAAGCUA	2243	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-942	hsa-mir-942	9	1276	CUUCUCUGU	1268	3 UTR	0.0055
<i>GJB2</i>	NM_004004	hsa-miR-944	hsa-mir-944	8	2330	AAAUUAUU	2323	3 UTR	0.0219
<i>GJB2</i>	NM_004004	hsa-miR-944	hsa-mir-944	8	2277	AAAUUAUU	2270	3 UTR	0.0219

We used our well-established model of *GJB2* gene structure and expressional control to elucidate which microRNAs display a regulatory interaction with these 3 clustered target genes. Using an *in silico* based microRNA target prediction we found a number of potential target sites for microRNAs within the sequences of 5'-UTR (5'-untranslated region), CDS (coding DNA sequence) and 3' UTR (3'-untranslated region) of *GJB2* in the Human genome.

#### *Predicted microRNAs targets within the genomic sequence of GJB2 gene*

We further analysed functional regions of the *GJB2* gene cluster as possible sites for microRNA targeting. A unique target pattern was pointed within the genomic sequences representing the 5' UTR, promoter, CDS and 3' UTR of *GJB2* gene. Table 1, 2 and 3 presents specific sequences within 5' UTR, CDS and 3' UTR of *GJB2* gene along with seed sequences, its location and size respectively.

These pilot experimental data shows that the number of microRNA targets ranges differently in different regions of *GJB2*.

Currently, due to lack of high-throughput experimental methods for microRNA target identification, a collection of computational target prediction approaches have been developed and offer a first-line methodology. It should be noted that practical confirmation of the *in silico* data is still a laborious and time-consuming process. Nevertheless, computer based approaches have some limitations as biologically validated data that were suggested by *in silico* approaches are scarce, the power of all presently available target software is not ultimately proven, and the actual *in vivo* mechanism of microRNA mediated gene regulation remains unclear for most microRNAs making predictions less solid. Therefore, the complex

networks of microRNA regulation and their expected therapeutic potential remain to be revealed through consolidation of computational and experimental approaches.

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