Bioinformatic analysis reveals potential properties of human *Claudin-6* regulation and functions

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Abstract. Claudin-6 (CLDN6) is an integral component of the tight junction proteins in polarized epithelial and endothelial cells and plays a crucial role in maintaining cell integrity. Deregulation of CLDN6 expression and distribution in tumor tissues have been widely documented and correlated with cancer progression and metastasis. However, a complete mechanistic understanding of CLDN6 regulation and function remains to be studied. Herein, we show new potential properties of CLDN6 regulation and functions from bioinformatics analysis. Using numerous algorithms to characterize the CLDN6 gene promoter elements and the CLDN6 protein structure, physio-chemical and localization properties, and its evolutionary relationships. CLDN6 is regulated by a diverse set of transcription factors (SP1, SPR, AML-1a, CdxA, CRE-BP and CREB) and associated with the levels of methylation of CpG islands in promoters. The structural properties of CLDN6 indicate that it promotes cancer cell behavior via the ASK1-p38/JNK MAPK secretory signaling pathway. In conclusion, this information from bioinformatics analysis will help future attempts to better understand CLDN6 regulation and functions.

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Abbreviations: CLDN, claudin; GC, cytosine and guanine; CpG, cytosine-phosphoric acid-guanine; ASK1, Apoptosis signal regulating kinase 1; JNK, c-Jun NH2-terminal kinase; ERα, estrogen receptor α; MMP-2, matrix metalloproteinase-2; TFBS, transcriptional factor binding sites; TBP, TATA binding protein; SP1, specificity protein 1; CBF, C-repeat binding factor; AML-1a, acute myeloid leukemia 1a; CREB, cAMP-response element bingding protein; CRE-BP, CREB-binding protein; ER, endoplasmic reticulum; EST, expressed sequence tag

Key words: CLDN6, CpG island, transcriptional factor binding sites, amino acid sequence, molecular structure, bioinformatics analysis

Introduction

Claudins (CLDNs) are critical transmembrane proteins in tight junction function primarily as a barrier against paracellular transport between epithelial cells and the CLDN family consisting of 27 members that are mostly 20-34 kDa and have four transmembrane helices with amino- and carboxyl-terminal tail extending into the cytoplasm and play a crucial role in cellular adhesion, polarity, permeability and glandular differentiation (1,2). It is reported that altered expression and mislocalization of CLDNs such as CLDN6 appear to be tissue specific in embryo epithelial development and several cancers (3-5).

CLDN6 gene is located on 16p13.3 and its expression is mainly found in mouse embryonic stem cells, epithelial lineage cells during early development and primitive germ cell tumors such as spermatocytic seminoma, embryonal carcinoma, mature teratoma and classic seminoma (6). Its expression is very weak or absent in mouse and tumor tissue (7-9). CLDN6 inhibits cancer cell growth and induces apoptosis (10-12). It is reported that CLDN6 expression is associated with ERα expression and MMP-2 and ASK1. Although some functions of CLDN6 are known, a complete understanding of CLDN6 regulation and function remains to be studied. Bioinformatic analysis to predict regulatory mechanism of the gene and protein expression greatly solves these problems.

Bioinformatics is an interdisciplinary field, which combines computer science, statistics, mathematics, and engineering to develop methods and software tools for processing and understanding biological data (13-15). In the field of genetics and genomics, it aids in sequencing and annotating genomes and their observed mutations. Sequence analysis for DNA elements helps to explain the biological meaning and functin of the gene. In addition, protein structure prediction is another important application of bioinformatics. The amino acid sequence of a protein can be easily determined from the sequence on the gene that encodes it. This primary structure uniquely determines a structure in its native environment. Knowledge of the structural information that is usually classified as one of secondary, tertiary and quaternary structure, is vital in understanding the function of the protein (16). Moreover, network analysis seeks to understand the relationships within biological networks such as metabolic or protein-protein, small molecular interaction networks. Therefore, bioinformatics tools can aid in the comparison of genetic and genomic data and more generally in the understanding of evolutionary aspects of molecular biology as well as, at a more integrative level, anlayzing and cataloguing of the biological pathways and networks that are an important part of systems biology (16).

In this study, we used bioinformatics tools to examine the *CLDN6* sequence to characterize the gene TATA-box, GC-box and CAAT-box, promoter, CpG islands, potential transcriptional factors binding sites (TFBS), encoded protein structure and its structure, subcellular localization, secondary and tertiary structures, and even evolutionary relationship. These characteristics will help define the basis for *CLDN6* regulation and differential expression in cancer. These various bioinformatics tools are among the common tools of molecular biology helping investigators finding leads to investigate genes/proteins.

Materials and methods

Bioinformatics databases and online software. The following were used: NCBI (http://www.ncbi.nlm.nih.gov); Neural network promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html); Promoter 2.0 prediction server (http://www.cbs.dtu.dk/services/Promoter/); TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html); EMBOSS and CpG island searcher (http://www.ebi.ac.uk/Tools/emboss/); expasy (http://www.expasy.org); Protparam (http://www.expasy.org/tools/protparam.html); compute pI/mw (http://www.expasy.org/tools/pi_tool.html); ProtScale (http://www.expasy.org/tools/protscale.html); Clustalx (http://www.clustal.org/download/current/); treeview (http://www.taxonomy.zoology.gla.ac.uk/rod/rod.html); GOR4 (http://npsa-pbil.ibcp.fr/cgi-bin/ npsa_automat.pl? page=npsa_gor4.html); TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/), SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/); TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/); Pfam24.0 (http://pfam.sanger.ac.uk/search); SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html); Swiss-model (http://www.expasy.ch/swissmod/SWISS-MODEL.html); KEGG (http://www.genome.jp/kegg/).

Prediction methods. The following prediction methods were used for CLDN6 regulatory elements, structure and function: promoter (Neural Network Promoter Prediction), CpG island (EMBOSS and CpG Island Searcher), TFBS (TFSEARCH), the relatively molecular, amino acid sequences, protein relatively molecular quality, mass of amino acids, theoretical isoelectric point, PI, half-life, unstable factor, the total average hydrophilic (ProtParam); hydrohobicity or hydrophilicity (Prot Scale); the secondary structure (ExPASy-SOPMA and GOR4); signal lead peptide (TargetP1.1 Server) and signal peptide cutting locus (SignalP4.1Server); nuclear localization signal prediction (NLStradamus); the subcellular localization (WOLF PSORT and PSORT II); transmembrane area and across the membrane (TMpred program and TMHMM2.0); structure (SWISS-MODEL); protein structure and function (InterPro); transmembrane helices (TMpred program); evolutionary tree and homology analysis (Clustalx program and BLAST pairwise alignments); the signal pathway analysis (KEGG).

Results

Properties of the TATA-box, GC-box, CAAT-box motifs in the 5' regulatory region of CLDN6. To determine whether there are TATA-box, GC-box, CAAT-box motifs in the 5' regulatory region of human CLDN6 to which the transcription fators, TBP, SP1, and CBF, respectively, can bind we BLAST searched *CLDN6* mRNA (NM_021195.4) and human genomic sequences between -2000 bp to 200 bp from the transcriptin start site, for the motifs TATAWAW (where W represents A or T), GGGCGG and CCAAT. We identified three GC-box fragments, but no TATA- or CAAT-boxes, suggesting that expression and transcriptional activity of CLDN6 is regulated by SP1.

Promoter, CpG island and TFBS prediction in the 5' regulatory region of CLDN6. The *CLDN6* promoter sequence and TFs that bind to this sequence determine the temporal and spatial expression pattern of the gene. Therefore, defining of TFBS is important in the study of gene regulation. We used online programs, such as neural network promoter prediction, EMBOSS, CpG island searcher and TFSEARCH to predict promoters, CpG islands and TFBS in 5' regulatory region sequences of human *CLDN6*. We identified five promoters shown in Table I, and three CpG islands with Obs/Exp ratio >0.60, percent C + G >50% and length >200. These CpG islands have different length and location as shown in Fig. 1A. CpG Island Searcher program also identified three different CpG islands (Fig. 1B). TFSEARCH program predicted 432 potential TFBS with a score higher than 85 points, 156 potential TFBS with a score higher than 90 points, 66 potential TFBS with a score higher than 95 points, 24 potential TFBS, including SPR, AML-1a, CdxA, CRE-BP and CREB with a score over 99 points (Fig. 2). Together, these findings suggest that *CLDN6* expression is associated with the levels of methylation of CpG islands in its promoters. Different transcription start sites makes *CLDN6* transcription in a different way, then produce a variety of different biological functions of a transcription product.

The amino acid sequence and physicochemical properties of CLDN6 protein. To predict the protein structure of CLDN6, we used ProtParam online software (http://au.expasy.org/tools/) to analyze amino acid composition, molecular formula, molecular weight and isoelectric point. CLDN6 consists of 220 amino acids with 20 different amino acids, including alanine (10.90%), glycine (9.50%), leucine (14.50%), serine (8.20%) and valine (10.90%) (Table IIA). CLDN6 has the following properties: protein formula $C_{1054}H_{1682}N_{268}O_{291}S_{16}$, molecular weight 23,2775 kDa, theoretical PI 8.32, estimated half-life *in vitro* 30 h, instability index 42.03 and the hydrophilic residue ratio is lower than that of the hydrophobic residues. The threshold value of CLDN6 hydrophilicity is

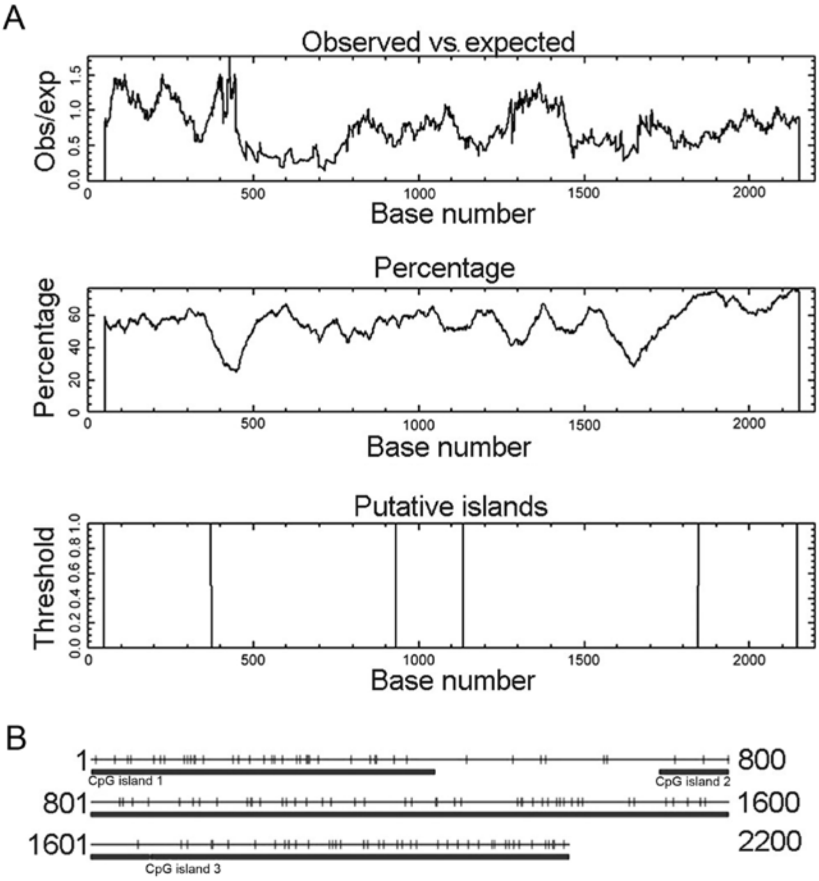


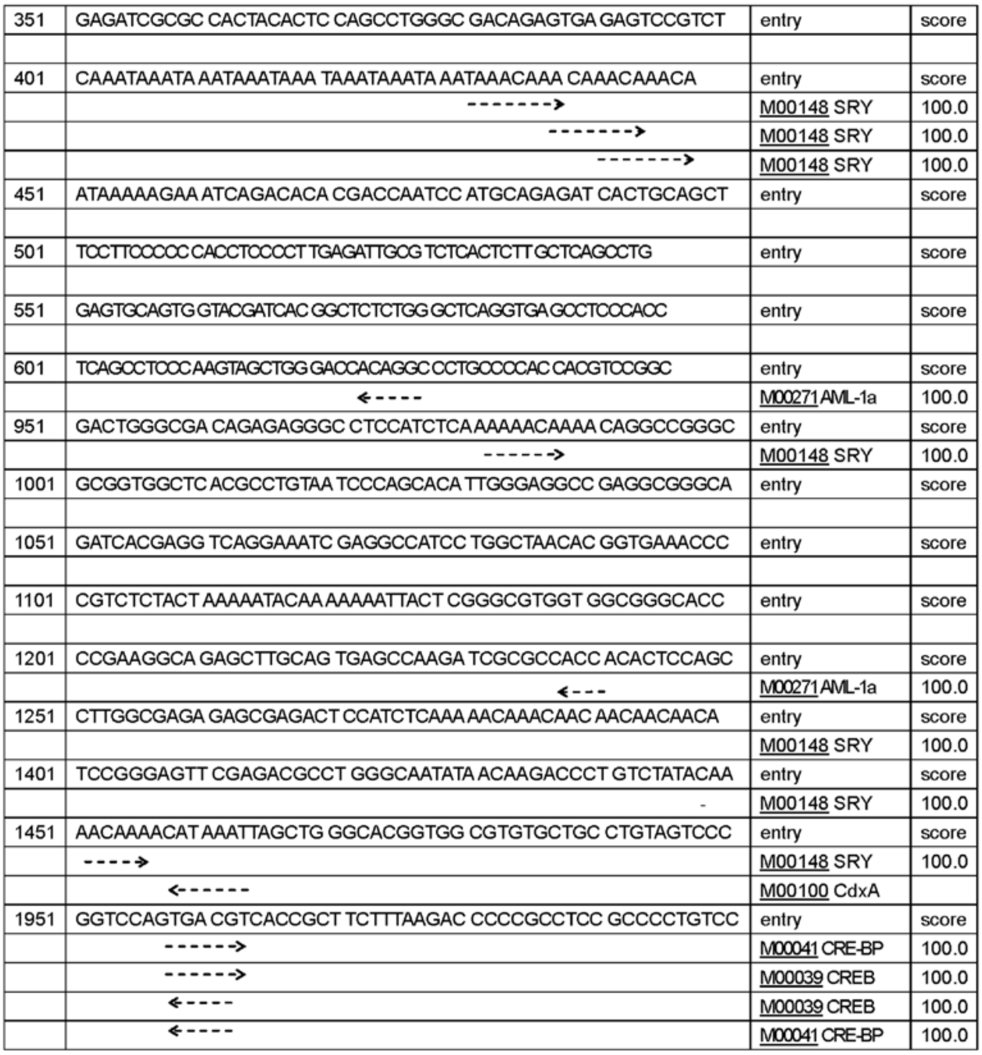
Figure 1. CpG island prediction for *CLDN6* using two prediction programs. (A) CpG island prediction using online EMBOSS. These CpG islands were 325 bp in length (located at 49-373 bp), 204 bp (932-1135 bp) and 299 bp (1847-2145 bp). (B) CpG island prediction using CpG Island Searcher program. CpG Island Searcher program identified three different CpG islands of 432 bp (1-432 bp), 962 bp (713-1674 bp) and 526 bp (1675-2200 bp). Select lower limits: % GC=50, obsCpG/expCpG = 0.60, length = 200, distance = 100. CpG island 1 star = 1, end = 432, % GC=53.9, obsCpG/expCpG = 1.024, length = 432. CpG island 2 star = 713, end = 1674, % GC=52.8, obsCpG/expCpG = 0.735, length = 962. CpG island 3 star = 1675, end = 2200, % GC=65.4, obsCpG/expCpG = 0.711, length = 526.

Table I. CLDN6 promoter site prediction using online Neural Network Promoter Prediction program.

Software	Start sites	End sites	Score	Sequence
Neural network promoter prediction	237	287	0.98	ACTCGAAATACAAAAATTAGCCGGGCGTGGTGGCG CGCGCCTGCAATCCG
	975	1025	0.84	ATCTCAAAAAACAAAACAGGCCGGGCGCGGTGGCT CACGCCTGTAATCCC
	1414	1464	0.82	GACGCCTGGGCAATATAACAAGACCCTGTCTATACA AAACAAAACATAAA
	1450	1500	0.93	AAACAAAACATAAATTAGCTGGGCACGGTGGCGTG TGCTGCCTGTAGTCC
	1965	2015	0.99	ACCGCTTCTTTAAGACCCCCGCCTCCGCCCCTGTC CCGACACTCGGCCTA

highest at: 105-118 bp, 153-161 and 205-216 bp (Fig. 3A and Table IIB), and the threshold value of CLDN6 polarity is highest at: 106-116, 142-145 and 154-161 bp (Fig. 3B and Table IIB), the threshold value of turn-back coefficient is highest at 14-22, 26-34, 43-55, 60-71, 80-89, 92-97, 100-115, 133-151, 154-156, 179-186, 195-200 and 213-216 bp (Fig. 3C and Table IIB). The overlapping range of these three parameters is shown in Table IIB at 106-115 and 154-156 bp. Taken together, these data suggest that CLDN6 mainly contains hydrophilic amino acid and polar amino acids, functional overlapping structure ranges from 106 to 156 bp, and that CLDN6 protein maybe a hydrophobic and unstable protein.

Secondary structure prediction for CLDN6 protein. The arrangement of atoms in space of the main polypeptide chain (α helix, extended strand, β turn and random coil) determines



351	GAGATCGCGC CACTACACTC CAGCCTGGGC GACAGAGTGA GAGTCCGTCT	entry	score
401	CAAATAAATA AATAAATAAA TAAATAAATA AATAAACAAA CAAACAAACA	entry	score
	------->	M00148 SRY	100.0
	------->	M00148 SRY	100.0
	------->	M00148 SRY	100.0
451	ATAAAAAGAA ATCAGACACA CGACCAATCC ATGCAGAGAT CACTGCAGCT	entry	score
501	TCCTTCCCCC CACCTCCCCT TGAGATTGCG TCTCACTCTT GCTCAGCCTG	entry	score
551	GAGTGCAGTG GTACGATCAC GGCTCTCTGG GCTCAGGTGA GCCTCCCACC	entry	score
601	TCAGCCTCCC AAGTAGCTGG GACCACAGGC CCTGCCCCAC CACGTCCGGC	entry	score
	<----	M00271 AML-1a	100.0
951	GACTGGGCGA CAGAGAGGGC CTCCATCTCA AAAAACAAAA CAGGCCGGGC	entry	score
	----->	M00148 SRY	100.0
1001	GCGGTGGCTC ACGCCTGTAA TCCCAGCACA TTGGGAGGCC GAGGCGGGCA	entry	score
1051	GATCACGAGG TCAGGAAATC GAGGCCATCC TGGCTAACAC GGTGAAACCC	entry	score
1101	CGTCTCTACT AAAAATACAA AAAAATTACT CGGGCGTGGT GGCGGGCACC	entry	score
1201	CCGAAGGCA GAGCTTGCAG TGAGCCAAGA TCGCGCCACC ACACTCCAGC	entry	score
	<---	M00271 AML-1a	100.0
1251	CTTGGCGAGA GAGCGAGACT CCATCTCAAA AACAAACAAC AACAACAACA	entry	score
		M00148 SRY	100.0
1401	TCCGGGAGTT CGAGACGCCT GGGCAATATA ACAAGACCCT GTCTATACAA	entry	score
	-	M00148 SRY	100.0
1451	AACAAAACAT AAATTAGCTG GGCACGGTGG CGTGTGCTGC CTGTAGTCCC	entry	score
	---->	M00148 SRY	100.0
	<------	M00100 CdxA	
1951	GGTCCAGTGA CGTCACCGCT TCTTTAAGAC CCCCGCCTCC GCCCCTGTCC	entry	score
	------>	M00041 CRE-BP	100.0
	------>	M00039 CREB	100.0
	<----	M00039 CREB	100.0
	<----	M00041 CRE-BP	100.0

Figure 2. There are 24 potential TFBSs prediction for CLDN6, including for SPR, AML-1a, CdxA, CRE-BP and CREB with a score of 99 using TFSEARCH program prediction.

basic protein secondary structure. Determination of this arrangement can help predict functions, and protein modifications. We used ExPASy-SOPMA and GOR4 secondary structure prediction module to calculate CLDN6 secondary structure, and to draw the structure model. Module output prediction results can be shown as a peak figure or the diagram can be simplified to show as the random of coiled and folded areas. The SOPMA method identified 104 (47.27%) α helixs, 48 (21.82%) extended strands, 14 (6.36%) β turns and 54 (24.55%) random coils and irregular coiled and folded structures located mainly at 1-70, 96-114, 120-150 and 176-220 bp between the peak figure and simplified diagram (Fig. 4A and Table III). The GOR4 method identified 56 (25.45%) α helixs, 67 (30.45%) extended strands, and 97 (44.09%) random coils and irregular coiled and folded structures located mainly at 26-42, 102-112, 131-139, 147-156, 173-194 and 196-220 bp (Fig. 4B and Table III). The comparison of two secondary structure prediction results is shown in Table III. CLDN6 secondary structure mainly consists of the irregular curl overlapping areas at 26-42, 102-112, 131-139, 147-150, 222-238, 176-194 and 196-220 bp, suggesting that these areas are mainly composed of α helix, extended strand and random coil structure. Taken together, the functional domain of CLDN6 protein is likely to be limited to these overlapped areas.

Analysis of signal peptide cleavage site, subcellular location, transmembrane domains in the CLDN6 protein. Signal peptides direct protein localization in cells and usually consists of 15-30 N-terminal amino acid residues. To analyze the CLDN6 signal peptide, we first used the Anthprot signal peptide cutting locus analysis module and the result showed CLDN6 has a short signal peptide composed of 21 amino acid residues, and its sequence is shown in Figs. 5A and 6. Consistent with previous results, the CLDN6 isoelectric point is approximately near 8.0, and the physiological state of CLDN6 molecules is closest to pH 7.3, with a positive charge of 2.184, as shown in Fig. 5B.

We also used SignalP4.1 to predict the signal peptide and its cleavage site. As shown in Fig. 5C and Table IV the cleavage site is between amino acids 21 and 22: VNG-LV. We also used NLStradamus, a simple Hidden Markov Model for nuclear localization signal prediction, to show that there were no nuclear localization signal sequences, suggesting that CLDN6

Table II. The basic properties of CLDN6 analyzed by using ProtParam online software.

A, Amino acid composition of CLDN6.

Amino acid	Abbreviations	Number	Composition (%)
Alanine	Ala(A)	24	10.90
Arginine	Arg(R)	6	2.70
Asparagine	Asn(N)	4	1.80
Aspartate	Asp(D)	4	1.80
Cystine	Cys(C)	10	4.50
Glutamine	Gln(Q)	9	4.10
Glutamate	Glu(E)	6	2.70
Glycine	Gly(G)	21	9.50
Histidine	His(H)	2	0.90
Isoleucine	Ile(I)	9	4.10
Leucine	Leu(L)	32	14.50
Lysine	Lys(K)	7	3.20
Methionine	Met(M)	6	2.70
Phenylalanine	Phe(F)	4	1.80
Proline	Pro(P)	9	4.10
Serine	Ser(S)	18	8.20
Threonine	Thr(T)	11	5.00
Tryptophan	Trp(W)	6	2.70
Tyrosine	Tyr(Y)	8	3.60
Valine	Val(V)	24	10.90

B, Molecular formula, molecular weight, isoelectric point and other basic properties of CLDN6.

Parameters	Prediction results
Formula	C1054H1682N268O291S16
Molecular weight	23277.5
Theoretical pI	8.32
Total number of atoms	3311
Number of amino acids	220
Total number of negatively charged residues	10
Total number of positively charged residues	13
Estimated half-life (mammalian reticulocytes, *in vitro*)	30 h
Instability index	42.03
Hydrophilic	105-118, 153-161, 205-216
Polarity	106-116, 142-145, 154-161
Turn-back coefficient	14-22, 26-34, 43-55, 60-71, 80-89, 92-97, 100-115, 133-151, 154-156, 179-186, 195-200, 213-216
The predicted results of the overlapping area	106-115, 154-156

Table III. Comparison of CLDN6 secondary structure prediction results between SOPMA and GOR4 methods.

Secondary structure prediction methods	Prediction results
SOPMA	1-70, 96-114, 120-150, 176-220
GOR4	26-42, 102-112, 131-139, 147-156, 173-194, 196-220
The predicted results of the overlapping area	26-42, 102-112, 131-139, 147-150, 222-238, 176-194, 196-220

Table IV. Results of CLDN6 protein signal peptide using SignalP-4.1 euk predictions.

# Measure	Position	Value	Cut off	Signal peptide
Max. C	22	0.545		No
Max. Y	22	0.570		No
Max. S	6	0.795		No
Mean S	1-21	0.635		No
D	1-21	0.596	0.500	Yes

Table V. Subcellular localization prediction of CLDN6 using TargetP1.1.

Name	Len	mTP	SP	Other	Loc	RC
Sequence	220	0.022	0.987	0.023	S	1
Cut off		0.000	0.000	0.000		

The location assignment is based on the predicted presence of any CLDN6 N-terminal presequences: mitochondrial targeting peptide (mTP) or secretory pathway signal peptide; other, other localization within cells. Number of query sequences: 1, cleavage site predictions are not included using Non-Plant networks.

Table VI. CLDN6 protein may locate in the endoplasmic reticulum (ER, 66.7%) and mitochondria (33.3%) using PSORT II Prediction.

K=9/23

66.7%: Endoplasmic reticulum
33.3%: Mitochondrial
>> Prediction for QUERY is end (k=9)

does not enter the nucleus. TargetP1.1 predicts CLDN6 to be located in the secretory pathway (98.7%) (Table V), while WoLF PSORT predicts CLDN6 to be a multi-pass membrane protein with subcellular localization to tight junction or the cell membrane. However, PSORT II Prediction indicated that CLDN6 may locate to the endoplasmic reticulum (ER, 66.7%)

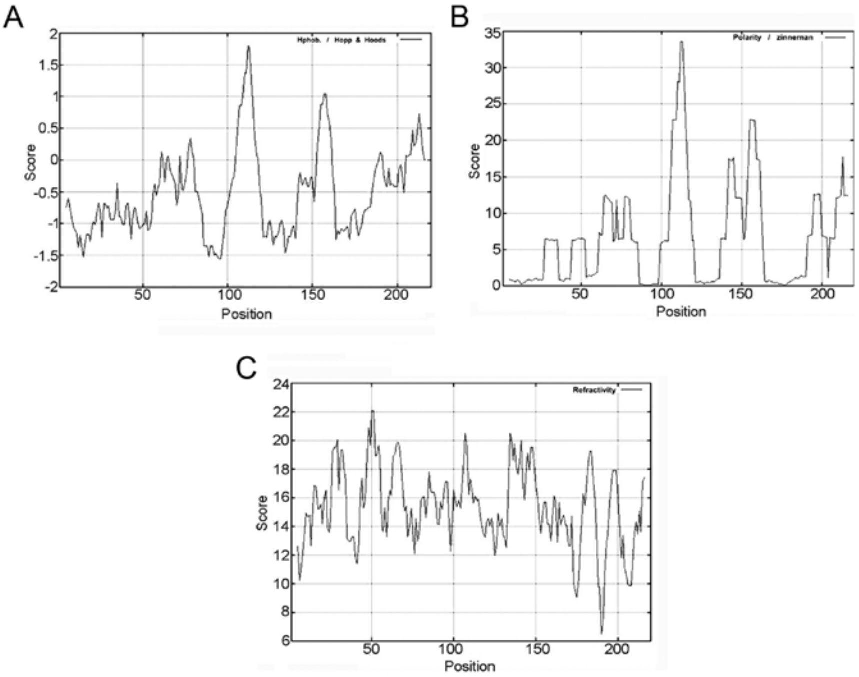


Figure 3. Nucleotide and deduced amino acid sequences of human *CLDN6* analyzed using Proparam. (A) It encodes a 220 amino acid polypeptide with a predicted molecular mass of 23,2775 kD. (B) Hydrophilicity plot analysis of CLDN6. The plot records the average hydrophilicity along the sequence over a window of 10 residues. Hydrophilic and hydrophobic residues are in the lower and upper part of the frame, respectively. The axis is numbered in amino acid residues. (C) Polarity analysis of CLDN6.

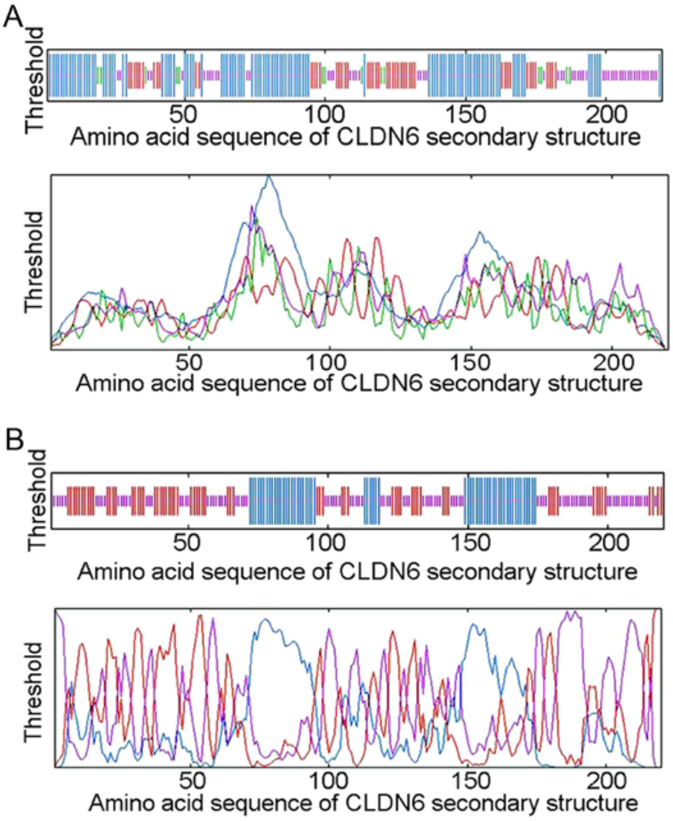


Figure 4. Secondary structure analysis of CLDN6 protein using SOPMA (A, up: schematic illustration; down: the peak figure) and GOR4 (B, up: schematic illustration; down: the peak figure) prediction software. Helix (blue), spiral structure; Sheet (red), folding; Turn (green), corner structure; Coil (purple), irregular curly structure.

and mitochondria (33.3%) (Table VI). The review by Koval on differential pathways of claudin oligomerization and integration into tight junctions described the three potential models for claudin oligomerization (cis interactions) occurring in the

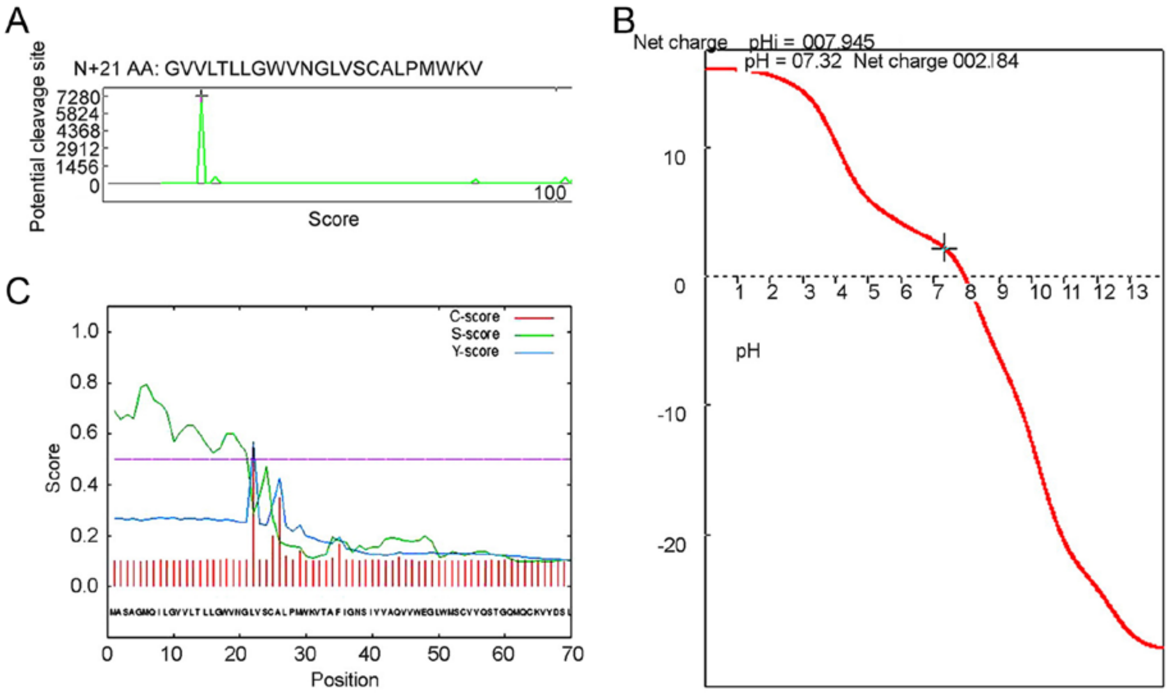
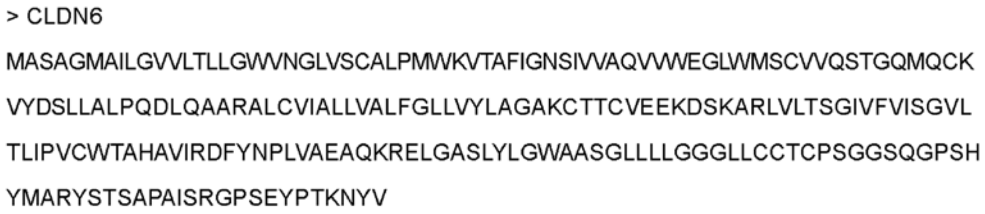


Figure 5. Signal peptide cleavage site and titration curve of CLDN6 using Anthprot and SignalP analysis. (A) Anthprot signal peptide cleavage site, subcellular localization prediction using TargetP1.1 and PSORT II Prediction. (B) Titration curve of CLDN6 using Anthprot analysis. (C) Curve of CLDN6 protein signal peptide using SignalP-4.1 euk predictions.


> CLDN6

MASAGMAILGVVLTLLGWVNGLVSCALPMWKVTAFIGNSIVVAQVVWEGLWMSCVVQSTGQMQCK

VYDSLLALPQDLQAARALCVIALLVALFGLLVYLAGAKCTTCVEEKDSKARLVLTSGIVFVISGVL

TLIPVCWTAHAVIRDFYNPLVAEAQKRELGASLYLGWAASGLLLLGGGLLCCTCPSGGSQGPSH

YMARYSTSAPAISRGPSEYPTKNYV

Figure 6. A short peptide composed of 21 amino acid residues.

endoplasmic reticulum (ER) (17). A requirement for CLDN quality control early in the secretory pathway and mutant CLDNs which are misfolded accumulate in the ER (18,19). CLDN oligomerization is more likely to happen in the TGN or another late secretory pathway and formation of stable claudin-claudin intermediates, however, given the structural diversity of different claudin family members, it is unlikely that all claudins will oligomerize via the same pathway. There is some evidence that CLDN16, CLDN3 and CLDN4 tagged with an ER retentin signal, His-Lys-Lys-Ser-Leu (HKKSL) are retained in the ER (20,21), while there is no reported for CLDN6 retaining in ER. We considered that CLDN6, as common protein functional maturation steps, oligomerizes in ER and is transported via the secretory pathway and integrates into tight junction.

Prediction of CLDN6 protein domain and function site. To predict CLDN6 structural domain and important functional sites, we used InterPro software. The results show that CLDN6 belongs to the PMP-22/EMP/CLDN superfamily (IPR004031) and CLDN (IPR006187) as shown in Fig. 7A. Its function is associated with structural molecular activity (GO: 0005198) and that the action contributes to cellular component in bicellular tight junction (GO: 0005923) and the structure integrity of a complex or assembly within or outside a cell (GO: 0016021).

CLDN6 has a four-element fingerprint that provides a signature for CLDNs. The fingerprint was derived from an initial alignment of two sequences. The motifs were drawn from conserved regions within the C-terminal half of the alignment, focusing on those sections that characterize CLDN6 and distinguish it from other family members: motif 1 lies in the intracellular loop; motif 2 resides within TM domain 3; and motifs 3 and 4 lie within the cytoplasmic C-terminal region. A single iteration on SPTR39_14f was required to reach convergence, no further sequences were identified beyond the starting set. CLDN6 possibly has four strong transmembrane helices according to the strongly preferred model (Fig. 7B). Taken together, CLDN6 is four-transmembrane tight junction protein.

Advanced structure of human CLDN6 protein. Analysis of the detailed structures of CLDN6 will further our understanding of its biological role. To obtain a high level protein structure

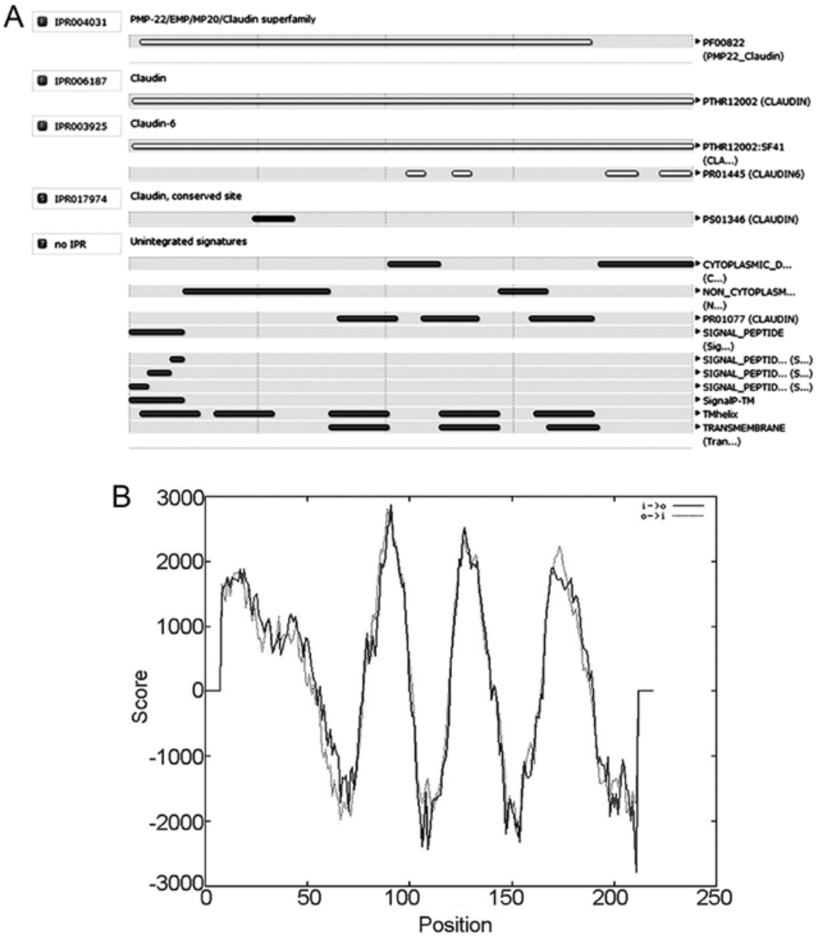


Figure 7. Transmembrane structural domain and function of CLDN6. (A) CLDN6 belongs to the PMP-22/EMP/CLDN superfaminly (IPR004031) and CLDN (IPR006187) according to the InterPro software. (B) CLDN6 possibly has four strong transmembrane helices in strongly preferred model.

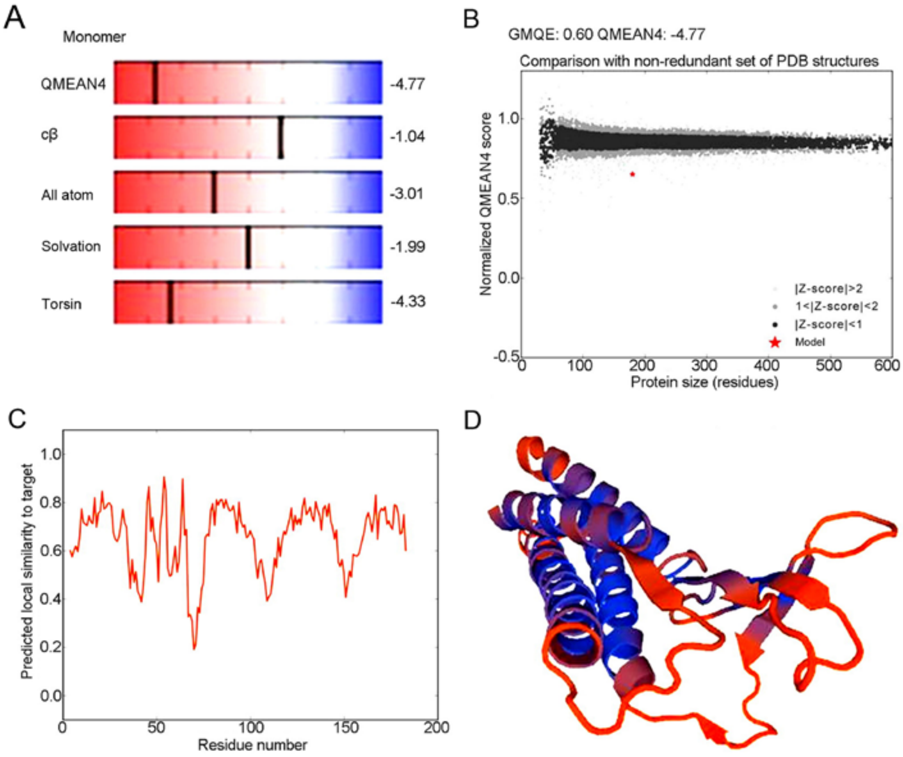


Figure 8. Predicted three-dimensional structure of CLDN6 with Swissmodel server. (A) CLDN6 protein and its structure database template 3x29.1.A. (B) CLDN6 protein has 42.62% amino acid sequence. (C) GMQE is 0.60 and QMEAN4 is -4.77. (D) The predicted 3D structure of CLDN6.

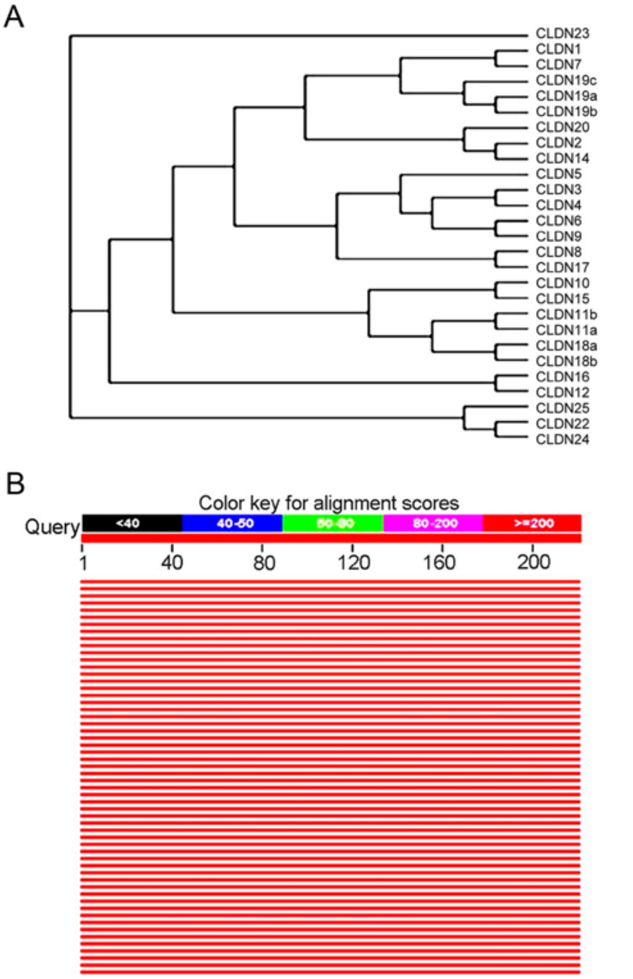


Figure 9. Evolutionary tree of the CLDN family. *Drosophila* Tap protein amino acid sequence with other species Phylogenetic tree between the two systems. (A) The family system evolutionary tree of CLDNs protein amino acid sequence was drawn (B) through the relevant data in the library collection download sequence similarity information encoding protein >50% of the species to build the system tree.

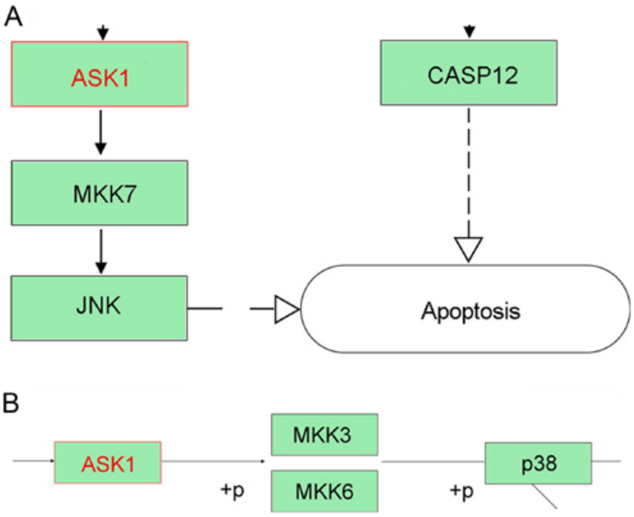


Figure 10. CLDN6-related signaling pathways, identified using KEGG pathway searches. (A) Cell apoptosis is induced by ASK1-MKK7-JNK signaling and casp12. (B) The effects of ASK1 on p38 expression is shown through downstream MKK3 and MKK6 molecules.

simulation map, the amino acid sequence was analyzed using Swissmodel server. CLDN6 protein and its structure database template 3x29.1.A, has 42.62% amino acid sequence, which is derived from the template CLDN19 that GMQE is 0.60 and QMEAN4 is -4.77, which 3D structure as shown in Fig. 8A-D.

The evolutionary tree of CLDN family amino acid sequence rendering system and homology analysis of human CLDN6 protein sequences. CLDN6 was identified by searching expressed sequence tag (EST) databases for sequences similar to *CLDN1* and *CLDN2* (22). It was subsequently cloned and expressed in cells, where it was shown to concentrate at tight junctions (22) and human and mouse isoforms have been identified. With Clustalx program construct phylogenetic tree, and with the Treeview software on the system of evolutionary tree edit and comparison, the family system evolutionary tree of CLDNs protein amino acid sequence was drawn. All members of CLDNs are clustered closely except CLDN23, and except CLDN5, CLDN19c, CLDN20 and CLDN25, 22 members of the others exist in pairs and CLDN6 in particular is paired with CLDN9, suggesting they are the closest (Fig. 9A). Comparison of the amino acid seuqences showed that CLDN6 shares 25-70% overall similarity with other CLDNs at the amino acid level with highest similarity to CLDN9. Through the relevant data in the library collection download sequence similarity information encoding protein >50% of the species to build the system tree, as shown in Fig. 9B, the CLDN6 protein with human and rodent coding product has high homology of other animals in the phylogenetic tree perimeter, they may play the same biological functions.

CLDN6 related signaling pathway analysis. To identify CLDN6-related signaling pathway, We focused on CLDN6-related molecular network information in KEGG pathway maps. CLDN6 is related to four signaling pathways including hsa04514; hsa04530; hsa04670; hsa05160 and has been identified as exosomal proteins of cancer cells such as ovarian cancer, and colorectal cancer, are closely related to CLDN6. CLDNs activated by miR-122 can activate RIP1, TRAF6, p38 and JNK in hsa05160, and apoptosis signal-regulating kinase 1 (ASK1) can activate downstream p38 and JNK-induced apoptosis (Fig. 10A and B). However, the other signaling pathways are not involved in apoptosis. ASK1, a member of the mitogen-activated protein kinase (MAPK) kinase family has been proved to positively correlate with the level of CLDN6 and associated with its pro-apoptosis effect in cervical carcinoma and breast cancer (12,23,24). Taken together, CLDN6 may induce cancer cell apoptosis via ASK1-p38/JNK MAPK pathway.

Discussion

CLDN6 on chromosome 16p13.3 encodes a 23 kDa four-transmembrane protein. CLDN6 has been shown to be expressed differentially in various tumor tissues and cells (25-27), and to a certain degree its alteration can inhibit or pomote tumor cell growth, apoptosis, invastion, migration and EMT *in vitro*, and methylation of the gene may be involved in tumorigenesis (7,26,28). Although we have clarified some functions of CLDN6, more questions remain to be answered, e.g. why it is differentially expressed in different cells, which signal pathway relates to it and how it is regulated, suggesting alternative ways are needed to answer the questions. Bioinformics may be a way complementary to experimental biology in understanding CLDN6 regulation and function, which necessarily must be validated by experiments *in vitro* or *in vivo*.

We studied the 5' regulatory region of *CLDN6* using bioinformatics tools and found that it contained three GC-boxes, three CpG islands and in the 5' regulatory region sequence 432 and 24 potential TFBS were predicted with a score of 85-99 and diverse TFs such as SP1 are predicted to bind to these TFBS. These results are supported by published literatures, e.g. Anelli and Sitia documented that the associations between CLDN6 expression and mRNA levels of SP1 in ovarian carcinoma effusions (18), as well as transcription factors such as CRE-BP and CREB (17), the expression of *CLDN6* is associated with methylating CpG islands; treatment with TSA and/or 5-aza or DMSO induced marked decreases in the levels of methylation of CpG islands in the promoters of *CLDN6*. The above are also supported by our experiments, which shows that DNA methyltransferase (DNMT1) inhibits the expression of CLDN6 to affect the function of tight junction in breast cancer MCF-7 cells in our unpublished work. In addition, it is reported that some TFBS, including those for AP-1, c-Jun, ATF-2, HNF-4α and COUP-TF have been shown to contribute to *CLDN6* regulation (29-34).

The amino acid sequence of CLDN6 was also analyzed by bioinformatics methods. The results showed that the isoelectric point was 8.32; leucine, valine, glycine, serine and alanine were the most abundant amino acids; CLDN6 was an unstable protein because of hydrophobic amino acids. Further, the investigation also found that CLDN6 was a transmembrane protein with leader peptides and a signal peptide at the N-terminus that was hydrophilic. CLDN conformation in the plane of the membrane shows the four transmembrane α-helical domain as a tightly packed complex, classes of claudin-claudin interactions within a tight junction strand and they can interact via head-to-head binding in the extracellular environment between adjacent cells and within the plane of the plasma membrane in the same cell (21). The main predicted secondary structures of CLDN6 are α helices, extended strands and random coils in our results. *CLDN6* encodes four-transmembrane domain protein components of tight junction strands (22). Our CLDN evolutionary tree shows CLDN6 and CLDN9 are most closely related, which is similar to a report by Lal-Nag *et al* (35) and it is reported that both CLDN6 and CLDN9 function as additional coreceptors for hepatitis C virus (25), suggesting they have common functions. CLDN6 related signaling pathway analysis results obtained from these tools are used to support CLDN6-induced apoptosis via regulating ASK1-p38/JNK signaling in breast cancer MCF-7 cells (36).

In summary, these results reveal that *CLDN6* gene may have diverse transcription start sites and its transcription is regulated by DNA methylation and transcription factors such as SP1. Additionally, CLDN6 may be oligomerized in ER and transported to cell membrane via secretory pathway and finally integrated into tight junctions.

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