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Connexin evolution ameliorates the risk of various cancers

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Abstract. – OBJECTIVE: Connexins can affect many cancers, but the relationship of many connexins is confused and the functions in cancers are unknown.

MATERIALS AND METHODS: With conservative domains of connexins, the phylogenetic tree was constructed and all connexins could be divided into five groups (I, II, III, IV and V). The clock analysis showed that group V appeared earlier than group IV, which was earlier than group III, which was earlier than group I and II in the evolution. Group I involves in colorectal, lung, breast, pancreatic, gastric, colon, bladder and ovarian cancers. Group II affects bladder, breast, lung, gastric, colorectal, prostate, esophageal, renal, head and neck cancers. Group III affects bladder and breast cancer. The function of group IV and V has not been reported.

RESULTS: When HT1376 bladder cancer cells were transfected with Cx31.9 (Group IV), the growth rate was inhibited by 17%. Inversely, when HT1376 cells were transfected with Cx31.9 RNAi, the growth rate was increased by 21%. For Cx23 (Group V), it could not affect the growth rate.

CONCLUSIONS: The results suggested that ancient connexins did not involve in cancers. Recent connexins have developed the functions for inhibiting the progression of cancers in the evolution.

Key Words:
Connexins, Evolution, Phylogenetic relationship, Cancers.

Introduction

Cancer is the leading cause of death in economically developed and developing countries[1], while the causes of cancers remain unknown in most cases. Early diagnosis is critical for the prevention and treatment of various cancers[2-5]. To meet the end, it is necessary to explore the molecular mechanism for the risk of cancers. connexins have been hotly reported to be the repressors of various cancers[6-9]. However, the relationship of these connexins is deadly confused and the functions of most connexins in the development of various cancers are widely unknown.

Biological processes of animals involve in much information exchange. Gap junction, connexins, is the most important channel for the information exchange between adjacent cells, including ions flow, messengers import, and small molecules import and export, which are the basic activities in the cell life[10]. To maintain cell homeostasis, the signals for the contact inhibition and apoptosis are often transferred from one cell to adjacent one via connexins. However, the uncontrollable growth and will enhance the risk of various cancers. Thus, the dysfunction of connexins can affect the growth control of the cells, which will result in tumorigenesis. The decreasing levels of connexins are related with several carcinogens[11] while high levels of connexins can inhibit the growth rate of various cancers[12,13].

To understand the functions of connexins at whole, exploring the evolution of the molecules is often considered. Most connexins are widely existed from low- to high-grade animals[14]. Although many invertebrate systems also can exchange much information between adjacent cells via gap junctions but no connexin has been found in invertebrate organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*[15]. Invertebrates display direct cell-cell communication via a family of proteins termed innexins, which play a connexin-like role[15,16]. Actually, the connexin has been reported in Tunicates[17].

Connexins are difficult to be found in invertebrates mainly due to the more divergent sequences of connexins and beyond to be recognized. Detailed evolutionary history of these proteins may help us to know more clues and functions of these proteins while dating the protein evolution is almost a prerequisite concept for understanding the distinguished functions of these proteins. Although there are more divergent sequences for these connexins, it is possible to ex-

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plore the phylogenetic relationship of these connexins proteins using the conserved truncated sequences.

The other problems with connexin studies are the present nomenclature depending on their molecular weights, which can cause deadly confusion when the molecules are compared in different species. Therefore, we hope to find the relationship of these connexins based upon the phylogenetic tree. Based upon the relation, the effects of the connexin members on the development of cancers may be explored.

Materials and Methods

Phylogeny Estimation Using Connexins Protein Sequences

A blast protein database search is performed using 21 annotated connexins in human at Genbank after referring to previous report18. More than 260 connexins protein sequences were collected from vertebrates (*Homo Sapiens, Pan troglodytes, Mus musculus, Rattus norvegicus, Gallus gallus, Anolis carolinensis, Xenopus tropicalis, Danio rerio, Tetraodon nigroviridis*) and invertebrates (*Ciona intestinalis, Halocynthia pyriformis* and *Oikopleura dioica*). The amino acid sequences were aligned using Clustal W, and edited by hand. The phylogeny tree of 262 taxa was reconstructed under JTT+G model based on Bayes factors. All similar sequences are specified to one group. All the more divergent sequences are specified to last one group. The sequences with high divergence on both ends were removed before the alignment. The phylogenetic relationships among all the groups are checked by bootstrap value. The bootstrapped confidence interval is based on 1000 replications.

Molecular Dating Analyses

Dating analyses were predominantly performed in BEAST v1.7.2 (Drummond et al. 2012). We used BEAUTi to generate BEAST XML input files, specifying an uncorrelated exponent-distribution 'relaxed' molecular clock model. When analyzing amino acid data, we used a JTT model with gamma-distributed rate heterogeneity among sites, and a Yule tree prior for all analyses. The calibration date was set at the Mammalian-Bird divergence, 300 Ma, Mammalian-Amphibian, 360 Ma, Mammalian-fish, 500 Ma and appeared period of multi cellular organisms since 1000 Ma[19].

The Evolution of Protein Structure

To compare the evolution of protein structure between different connexins, we use the program $(PS)^2$, which is an automated homology modeling server. The method uses an effective consensus strategy by combining PSI-BLAST, IMPALA, and T-Coffee in both template selection and target-template alignment. The final three dimensional structure is built using the modeling package MODELLER[20]. Other software, the PSIPRED Protein Structure Prediction Server aggregates several of structure prediction methods into one location and can be found at http://bioinf.cs.ucl.ac.uk/psipred/.

Searching for the Involvement of Connexin Isotypes in Cancer

From the taxonomy on the phylogenetic tree, the effects of connexins on various cancers were searched from PubMed database. All connexins were chosen from each group. All the results were listed in Table II.

Reconstruction of the Plasmids with Cx31.9 and Cx23

The biological functions of Cx31.9 standing for Group IV and Cx23 for Group V are still unknown in the development of various cancers. The biological functions of Cx31.9 and Cx23 in the development of bladder cancer were examined here. Cx31.9 gene (Accession number: AY093445.1) was amplified using the primers (Forward primer, 5'-GTGAGCTAG-CATGGGGGAGTGGGCGTTCCTG-3'; Reverse primer, 5'-CTGAGAATTCTTAGATGGCCA-GATCTCGGCGG-3'), generating about a 900-bp product. The PCR product was cloned into the *Nhe*I-*EcoR*I sites of pcDNA3.1 vector (TOPO TA Expression Kit, Applied Biosystems China Limited, Beijing, China), which was named as pcDNA3.1-Cx31.9. In the same way, Cx23 gene (Accession number: XM_003311518.2, also called gap junction protein, epsilon 1 gene) was amplified using the primers (Forward primer, 5'-GT-GAGCTAGCATGATGTCTCTAAATTACAT-CAAAAAC-3'; Reverse primer, 5'-CTGA-GAATTCTTATTGTCTGAATGGAAAG-TATAATC-3'), generating a 627-bp product. The PCR product was cloned into the *Nhe*I-*EcoR*I sites of pcDNA3.1 vector, which was named as pcDNA3.1-Cx23. Plasmid pcDNA3.1-Cx31.9 and-Cx23 was amplified in *E. coli*, isolated using QIAprep Miniprep Kit (QIAGEN China Co., Ltd., Shanghai, China) and verified by DNA se-

quencing. Transfection was performed when the cells (bladder cancer cell lines HT1376, purchased from the Cell Center of Chinese Academy of Sciences, Shanghai, China) confluence reached 60%. The plasmid and Lipofectamine 2000 were dissolved in 50 μL culture medium, separately. After incubation for 5 min, the plasmid and Lipofectamine 2000 were mixed for 20 min, and added into HT1376 cells. After 24 h transfection, cells on the plate were dispersed and cultured at the ratio of 1:10; after 48 h, G418, at an initial concentration of 600 μg/ml, was added to screen positive clones. When a positive clone appeared, the concentration of G418 was adjusted to 100 μg/ml and sustained for 3 days.

Cx31.9 and 23 Gene Silencing

Cx31.9 and 23-specific siRNA (Cx31.9 RNAi, ACTACCGCTTCTGGCTCTTC; Cx23 RNAi, GATGC481ATGGTTCCAGAACAC) was chemically synthesized and transfected into cell lines HT1376.

Quantitative RT–PCR

Total RNA was extracted from the HT1376 cells with TaKaRa MiniBEST RNA Extraction Kit (Takara Biotechnology (Dalian) Co., Ltd, Dalian, China). CDNA was synthesized from the total RNA with the RT-PCR kit (Shanghai ZJ Bio-Tech Co., Ltd., Shanghai, China). The mRNAs levels were evaluated by quantitative RT-PCR using the following primers: Cx31.9, 5'-GTCGCCAGA CCTGCTACGAC-3' and 5'-GAAGACGGTCTTCTCGGTGG -3'; Cx23, 5'-AATCTCTTCTGTTACAATCAG-3' and 5'-CACAGGTAAAGAGATTTTAC-3'; GAPDH, 5'-CCCTTCATTGACCTCAACTAC-3' and 5'-CCACCTTCTTGATGTCATCAT-3'. GAPDH was used as an internal control.

Western Blot Analysis

The extracted proteins from the cultured cell lines were subjected to western blot analysis using Cx31.9, Cx23 and β-actin antibodies, which were kindly provided by National engineering research center for antibody medicine (Shanghai, China). Proteins were separated by SDS-PAGE and transferred to a PVDM (Millipore, Billerica, MA, USA). The PVDM were blocked with 5% skim milk and incubated with the corresponding antibodies overnight at 4°C. The membrane was treated with Horseradish peroxidase-conjugated detection antibodies (Dingguo, Beijing, China).

Results

Phylogenetic Estimation of Connexins

With the sequenced genome, it is time to investigate the evolution of the connexins. From the phylogenetic tree, the connexins can be divided into five subfamilies, including Group I, II, III, IV and V (Figure 1). As the data of genomes increased, it is clear for that most invertebrates' connexins belong to the fifth group with more divergent sequences (Figure 1). We also find many different results with those previous reported. For instance, the paper reports that only four connexin groups existed in fish[14] but all the groups can be found in mammalians (Figure 1 and Table I). Another example, after the divergence from the Reptilia, the genes Cx39.9 and Cx43.4 are lost among fish and mammals[14]. Actually, both genes are 61% and 72% homology to Cx46 and 45 gene in *Homo sapiens*.

Molecular Dating Analyses

Many connexins in human have their counterparts in other vertebrates including fish (Table I and Figure 1). Most vertebrate connexins are included in from group I to IV (Figures 1, 2 and Table I). In group V, human Cx23 and zebrafish Cx23 are the only vertebrate connexins. Most invertebrate connexins belong to group V, which is more divergent than other groups. The relax clock provided good date estimates: group I and II diverged at 540 Ma, group I, II and III at 585 Ma, group I, II, III and IV at 630 Ma; The split date of Group V with other groups was about 700 Ma (Figure 2). Group V appeared earlier than group IV, which was earlier than group III, which was earlier than group I and II during the long-history evolution.

The Evolution of Protein Structure

Connexins have the common structure of transmembrane protein, including double alpha-helical structures, outside loops, an inside loop, and cytoplasmic N- and C-end domains[21]. There are at least two possible ways for the evolution of connexins via the comparison of sequence and structure alignments for the protein domains between vertebrates and invertebrates or connexins and a kind of kinase in *Gallus gallus*. The first way, its distant ancestor might be alpha-helical transmembrane domains, so connexins may experience strand-to-helix transition during long-term evolution (Figure 3). The second way, recent connexins may evolve from the N-terminal of a kind of kinase (Figure 3).

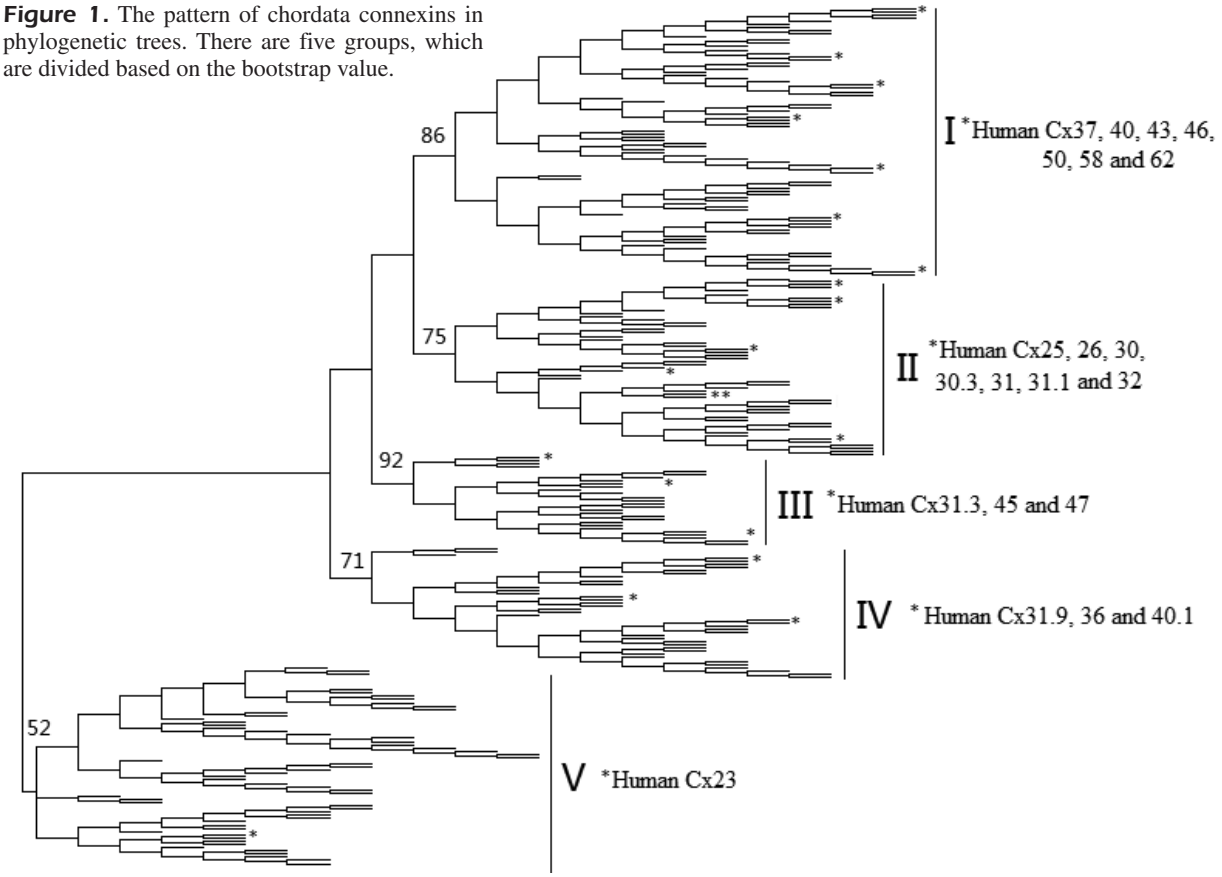

Figure 1. The pattern of chordata connexins in phylogenetic trees. There are five groups, which are divided based on the bootstrap value.

Involvement of Connexin Isotypes in Cancers

According to reported papers, the members from Group I involves in colorectal, lung, breast, pancreatic, gastric, colon, bladder and ovarian cancers. Group II affects bladder, breast, lung, gastric, colorectal, prostate, esophageal, renal, head and neck cancers. Group III affects bladder and breast cancer. The function of group IV and V in cancers has not been reported (Table II). The effects of Group IV and V on various cancer developments are needed to be determined in future.

The Levels of Cx31.9 and Cx23 in the Transfected and Non-Transfected HT1376 Cells

To explore the functions of Cx31.9 and Cx23 in bladder cancer, the both genes and the RNAi against each gene were transfected HT1376 cells respectively. The real-time RT-PCR analysis indicated that the transcriptional amounts of Cx31.9 and Cx23 were increasing when the cells were transfected with the both genes while the levels were decreasing when the cells were transfected with the RNAi (Figure 4A and B). Similarly, the western blot analysis showed the similar changing trend for the protein levels of Cx31.9 and Cx23 (Figure 4C and D). These results applied that the HT1376 strains were successfully harbored with the both genes and the RNAi against each gene.

The Effects of Cx31.9 and Cx23 on the HT1376 Strains

In the HT1376 strains containing pcDNA3-Cx31.9, the growth rate of cells was inhibited by 17% comparing with corresponding non-transfected cell lines after three-day culture (Figure 5A). In the HT1376 strains containing Cx31.9 RNAi, the growth rate of the cells was increased by 21% comparing with corresponding non-transfected cell lines after three-day culture (Figure 5A). The results suggest Cx31.9 can affect the growth of bladder cancer cells. Comparatively, Cx23 could not affect the growth of the cells (Figure 5B). For Cx23 (Group V), it cannot affect the growth of bladder cancer because Cx23 differs from all other connexins and does not form Gap-junction channels[22].

Table I. Connexins of Vertebrate species.

	Homo Sapiens	Pan troglodytes	Mus musculus	Rattus norvegicus	Gallus gallus	Anolis caro linensis	Xenopus tropicalis	Danio rerio	Tetraodon nigroviridis
I		α-4	37	37	37	α-4	41	34.5	N16
		37			39		N2	32.3	N15
								28.9	N14
								39.4	**N1**
	40	α-5	40	40	42	α-5	α-5	41.8	N4
								45.6	N7
	43	α-1	43	43		α-1	43		N25
			33	33					
	46	α-3		46	44	α-3	46		N17
					56		N3		N24
									N8
									N12
	49								
	50	α-8	50	50	45.6			44.1	N5
	59	α-9			α-9		α-9	55.5	N30
								52.9	
	62	α-10	57	57	α-10	α-10	α-3	52.6	N28
II	25	β-7				β-4	β-7		N13
	26	β-2	26	26	31				
	30	β-6	30	30	β-2	β-6	26	33.8	N23
						β-6-1			N18
	30.3	β-4-1	30.3	30.3				34.4	N21
	31			31		β-3	31	35.4	N22
	31.1	β-5	β-5	31.1	β-5	β-5	β-5	28.6	N20
								30.9	N2
	32	β-1	32	32	32	β-1	32	29	N9
									N11
III	31.3	γ-3	29	γ-3					
	45	γ-1	45	45	45	γ-1-1	γ-1	γ-1	N26
									N29
	47	γ-2		47	γ-2	γ-1	47	47.1	N19
					γ-1	γ-1-2	45	44.2	N31
IV	31.9	Δ-3	30.2	Δ-3		Δ-3	Δ-3		
		Δ-2	36	36	35.1	Δ-2	Δ-2	35	N27
							N1	34.7	N10
	36							36.7	
								α-8	
	40.1	Δ-4		39	Δ-4	Δ-4	40.1	Δ-4	N3
									N6
V	23							23	

All the connexins are placed in the same line when the location, of human connexins and the connexins from other vertebrates, is on the same branch on the phylogenetic tree. All the number stands for the connexin taxonomy of corresponding species.

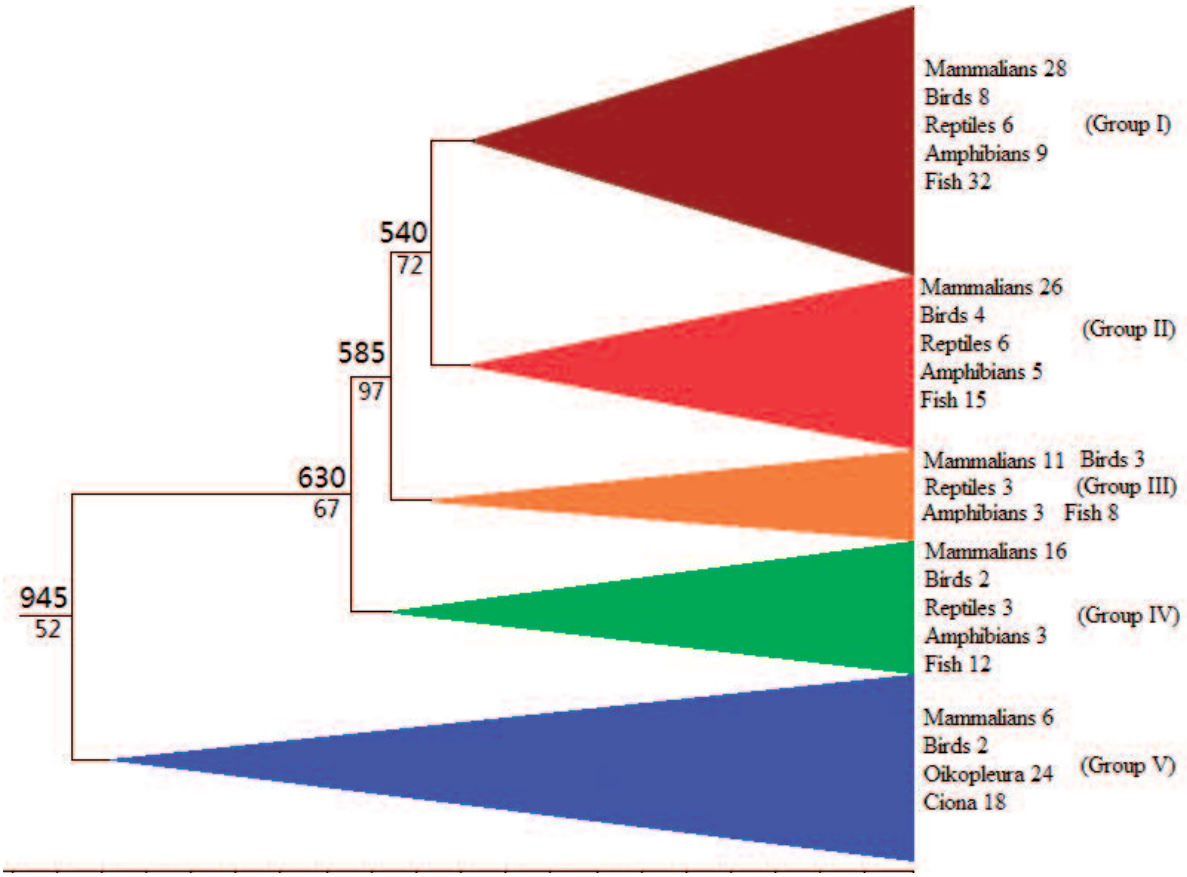

Figure 2. Quantitative RT-PCR and western blot analysis for the Cx31.9 and Cx23 levels in transfected and non-transfected HT1376 cells. **A**, Quantitative RT-PCR analysis showed highest levels of connexin mRNA in cells transfected with Cx31.9 while the lowest levels of connexin mRNA could be observed in the cells transfected with RNAi. **B**, Quantitative RT-PCR analysis showed highest levels of connexin mRNA in cells transfected with Cx23 while the lowest levels of connexin mRNA could be observed in the cells transfected with RNAi. **C**, Western blot analysis showed highest levels of connexin protein in cells transfected with Cx31.9 while the lowest levels of connexin protein could be observed in the cells transfected with RNAi. **D**, western blot analysis showed highest levels of connexin protein in cells transfected with Cx23 while the lowest levels of connexin protein could be observed in the cells transfected with RNAi.

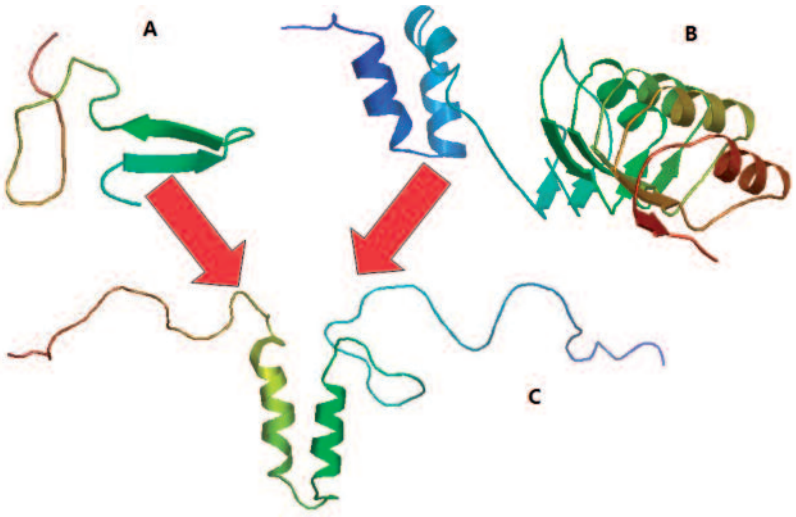

Figure 3. The effects of Cx31.9 and Cx23 on the growth rate of HT1376 strains. **A**, Cx31.9 can decrease the growth of HT1376 strains. **B**, Cx23 cannot affect the growth of HT1376strains. All values are the mean ± SD.

Table II. Connexin isotypes and involvement in various cancers.

Group	Connexin members (GenBank No.)	Involvement of cancers	References
I	Cx37 (AAF62342.1)	Gastric cancer	36
	Cx40 (AAA91833.1)		
	Cx43 (AAA52131.1)	Colorectal, lung, breast, pancreatic, gastric, colon, bladder and ovarian cancers	37-44
	Cx46 (AAI21138.1)	Breast cancer	6
	Cx49 (AAF01367.1)		
	Cx50 (EAW50926.1)		
	Cx59 (AAG09406.1)		
	Cx62 (AAK51676.1)		
	Cx25 (CAC93845.1)		
	Cx26 (AAL87696.1)	Bladder, breast, lung, gastric, colorectal, prostate, esophageal, head and neck cancers	6, 8, 45-50
II	Cx30 (EAX08258.1)	Gastric, head and neck cancers	51, 52
	Cx30.3(Q9NTQ9.1)		
	Cx31 (AAC95471.1)		
	Cx32 (AAV38136.1)	Lung, gastric, breast and renal cancers	46, 53-55
	Cx31.3 (AAP51161.1)		
III	Cx45 (AAH96214.1)	Bladder cancer	44
	Cx47(EAW69860.1)	Breast cancer	56
	Cx31.9 (AAM18801.1)		
IV	Cx36 (EAW92315)		
	Cx40.1 (Q96KN9.1)		
V	Cx23 (A6NN92.1)		

Discussion

The connexins can form the microchannels between adjacent cells. The microchannels play a critical role in cell-cell contact, which can allow the passage of ions, metabolites and many small molecules. They are widely existed in most vertebrates[14]. Invertebrates have been thought to utilize different "connexins", which has the similar secondary structure with that from the vertebrate, but the sequence is quite different[23]. We use the conservative domains of connexins to blast protein database and find connexins have their ana-

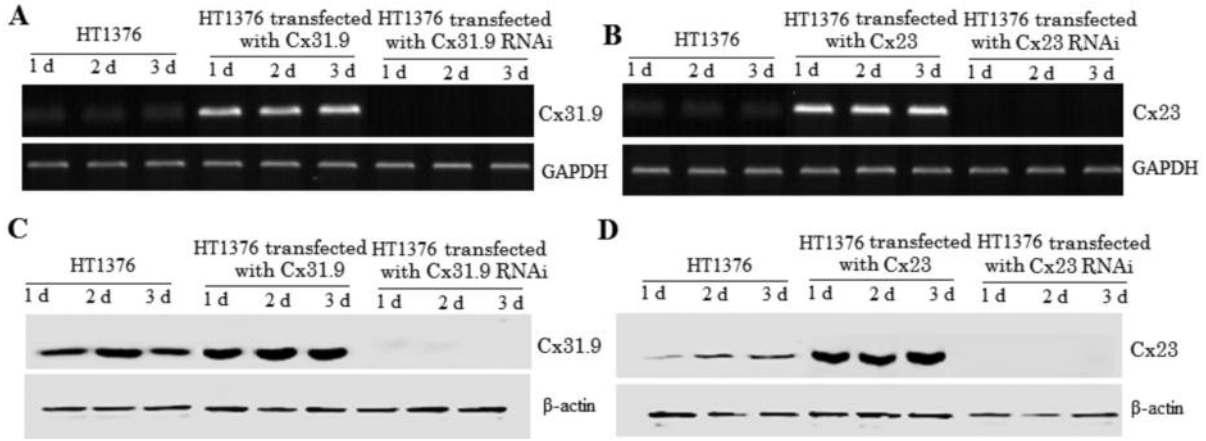

Figure 4. Phylogenetic tree of vertebrates and invertebrates connexins. The tree is constructed using the conserved sequences of 262 connexins from vertebrates (from fish to mammalian) and invertebrates (*Ciona intestinalis, Halocynthia pyriformis and Oikopleura dioica*). The program BEAST was used[24]. The tree is constructed by JTT and unrelated exponential relax clock model. The length of the triangle presents the difference of the sequences, and the width of the triangle stands for the amounts of sequences in the corresponding group. The number stands for the amounts of the species. The scale bar shows the splitting dates for important events. The number of upline at node is bootstrap value using 1000 bootstrap replications.

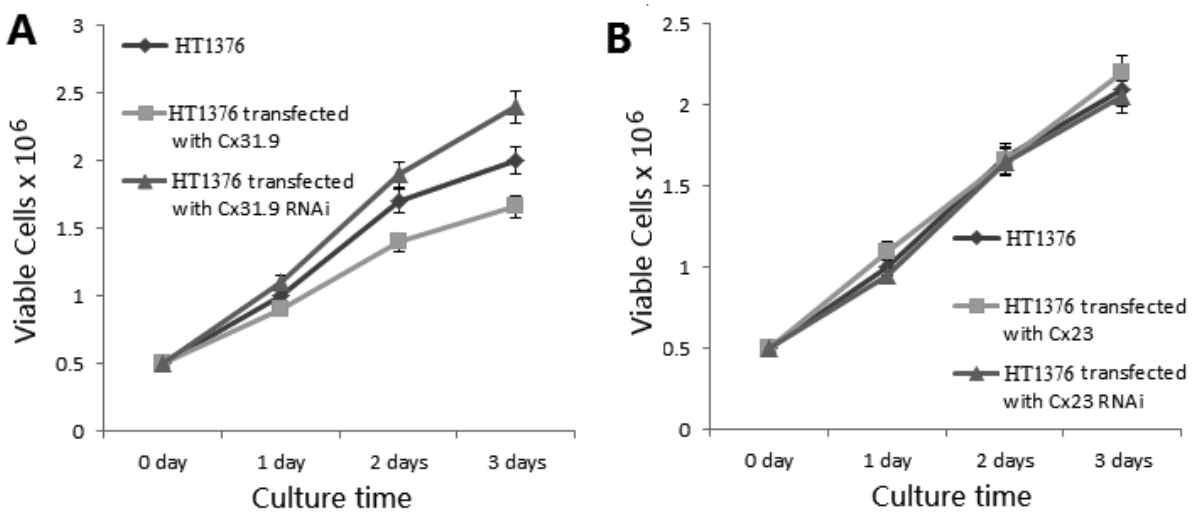

Figure 5. The evolution of connexin subunits. The blue color in the protein stands for N-terminal and red color for N-terminal. **A**, The distant ancestor of connexin is with beta-strands transmembrane domains, which is remolded according to the unnamed connexins in *Oikopleura dioica*. **B**, Another distant ancestor of connexin is a kind of kinase with alpha-helical transmembrane domains at N-terminal. C, the connexin is recent molecule with alpha-helical transmembrane domains.

logues in some invertebrates such as *Ciona intestinalis* and *Oikopleura dioica,* suggesting connexins may be widely existed in other invertebrates, but they are beyond to be recognized because of their high divergent sequences. Here, with relax clock model and Bayes factor, we use truncated connexins to date their evolution after cutting the more divergent sequences in connexins. Previous paper reports vertebrates connexins are divided into four groups[14]. We find vertebrates connexins can be divided into five groups (Figures 1, 2 and Table I). Most of invertebrate connexins and a fewer vertebrate connexins belong to the fifth group with highly divergent sequences. The result suggests the gap junctions for vertebrates and invertebrates may come from the same ancestor.

The functions of connexins are widely reported and found to be related with various cancers and other important chronic disease[11,12,24-29] but the mechanisms are seldom defined. Here, we find many interesting results. The protein sequences of proto-oncogene tyrosine-protein kinase LCK and Fyn in mouse are similar with that of connexin 48.5 in *Ciona intestinalis*, implying that connexin might evolve from the same ancestor or have the similar function with the kinase. A kind of kinase in *Gallus gallus*, ROS1 kinase and dual specificity mitogen-activated protein kinase 4 in mouse are also found to have their counterparts, connexin 43 in *Ciona intestinalis*. Actually, kinase plays an important role in the connexin assembly and the microchannel formation. For instance, connexin phosphorylation changes the signal pathway of adjacent cells, which causes the response to many cytokines, and disease mediators. Tyrosine protein kinases play an important role in such regulation of connexin[30]. The result indicates the similar sequences can develop different functions after long history evolution.

Furthermore, many novel functions can be revealed through the study of the evolution for connexins protein family. For examples, glucose transporter in mouse has been found to be existed in connexin group IV, suggesting connexin may be related with glucose transport in the cells. The relationship between Cx43 and the transporter GLUT1 has been investigated: the GLUT1 was mainly existed in the cell membrane close near with blood cells in non-pigmented epithelial cells. Comparatively, in non-pigmented cells, it GLUT1 was existed in the membrane near with aqueous humor; High level of Cx43 could be found in the ciliary body and mainly formed the gap junctions, from which the epithelial cells pigmented and non-pigmented could be connected; The localization of Cx43 and GLUT1 in the cells applied that Cx43 and GLUT1are closely related with glucose transport[31]. As matter of a fact, GLUT1 can be a biomarker of recurrence and metastasis of cancers[32]. Cx43 has also been reported to be a kind of factor for the intestinal cancer[33].

Human Cx31.9, forming functional hemichannels, is approximately 400 Da in molecular weight. Cx31.9 involves in the excitation of the atrioventricular and sinoatrial nodes via depolarizing the membrane and reducing the space constant[34]. Its connections to cancers have not ever been reported. Here we find that the connexin affects the growth of bladder cancer. For Cx23, its biological functions have been seldom reported. The most common inherited form of nonsyndromic deafness has been reported due to mutations in Cx23[35]. Its connexins with cancers is still unknown. Further work is needed to be done for addressing the problems.

Our results showed that the members from group I to IV may affect the progression of various cancers. For group V, much work needs to be done in the future. From above results, Group V appeared earlier than group IV, which was earlier than group III, which was earlier than group I and II during the long-history evolution. All the results suggested that ancient connexins might be lost or without involving in any cancer. Most recent connexins have developed the functions for inhibiting the progression of various cancers in the evolution of life.

Regarding for that various connexins has been reported in different species and a myriad of functions of the protein revealed while present nomenclature depending on molecular weight can cause dead confusion, we develop a phylogenetic tree based on the human connexins. We hope the new tree will promote the understanding of the involvement of connexin isotypes in cancer. More molecular mechanisms for the connexins inhibiting the development of various cancers will be defined with further study.

Conclusions

All connexin members can be divided into five groups: I, II, III, IV and V based on the phylogenetic tree. Group I involves in colorectal, lung, breast, pancreatic, gastric, colon, bladder and ovarian cancers. Group II affects bladder, breast, lung, gastric, colorectal, prostate, esophageal, renal, head and neck cancers. Group III affects bladder and breast cancer. Group IV also affects the development of bladder cancer. The effects of Group V on various cancers cannot be determined. Group V appeared earlier than group IV, which was earlier than group III, which was earlier than group I and II during the long-history evolution. The evolution of connexins may inhibit the progression of various cancers.

Acknowledgements

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Conflict of interest

The Authors declare that there are no conflicts of interest.

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