Elevated *SLC26A4* gene promoter methylation is associated with the risk of presbycusis in men

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Abstract. Presbycusis affects approximately one-third of people over the age of 65 and is a worldwide health problem. In the current study, whether the methylation level of solute carrier family 26 member 4 (*SLC26A4*) predicted an increased risk of presbycusis was investigated. Peripheral blood samples from 102 patients with presbycusis and 104 controls were collected, and the methylation of the CpG sites of *SLC26A4* was measured by applying pyrosequencing technology combined with sodium bisulfate DNA conversion chemistry. Within the *SLC26A4* promoter region, one CpG site (CpG3) exhibited a significantly (P<0.0001) greater methylation level in the patients with presbycusis (26.5±5.56%) compared with the controls (23.8±3.85%). Significantly different CpG3 methylation levels were observed between the patients with presbycusis and the controls among the male participants (P=0.0004). In addition, a significant decrease in the transcriptional level of *SLC26A4* in peripheral blood was observed in the patients with presbycusis compared with the controls. Furthermore, analyses of the receiver operating characteristic (ROC) curves indicated that CpG3 methylation at the *SLC26A4* promoter predicted the risk of presbycusis in the male participants (AUC=0.684, 95% CI=0.584-0.784, P=0.001). The results demonstrated the significance of the CpG site methylation level of *SLC26A4*, and thus provides a potential marker for the diagnosis of presbycusis.

Introduction

According to the World Health Organization, 360 million people are suffering disabling hearing loss, and approximately one-third of people over the age of 65 are affected (http://www.who.int/pbd/deafness/estimates/en/index.html). Noncongenital deafness has been reported to be closely associated with the environment (1). Noise exposure is known to induce excessive reactive oxygen species generation in the cochlea that damages macromolecules such as DNA. Presbycusis is also termed age-related hearing loss and is progressive bilateral sensorineural hearing loss that is primarily characterized by impairments in high frequency hearing (2-4). In China, presbycusis occurs in 30% of elders over the age of 60 (5). However, the pathogenesis of presbycusis remains unclear. Although environmental factors have been demonstrated to be associated, they cannot explain the differences in the onset and rate of development between different individuals from similar environments (2).

Epigenetics is the study of inherited changes in phenotype or gene expression that are caused by mechanisms other than changes in the underlying DNA sequence (6). Methylations that contribute to epigenetics can occur through DNA methylations within a gene particularly at CpG sites. The cytosines in CpG sites are often methylated to form 5-methylcytosine, which can influence the transcriptional activity of the promoter. Previous studies have demonstrated that the methylation levels of certain genes are associated with the risks of diseases, including type 2 diabetes, colorectal cancer, gastric carcinogenesis and breast cancer (7-9). Pendrin is encoded by the solute carrier family 26 member 4 (*SLC26A4*) gene and is an anion exchanger that exchanges Cl^- and $HCO3^-$ to ensure that the pH of the cochlear endolymph is higher than that of the perilymph (10-12). Abolished activation of *SLC26A4* results the acidification and enlargement of the membranous labyrinth and causes deafness (10,13,14). A nationwide survey in China suggested that mutations in *SLC26A4* are among the most common elements of congenital deafness (5). The *SLC26A4* mutation frequencies among Han Chinese, Hui, and Uyghur people are 14.3, 12.8 and 1.6%, respectively, which suggests that *SLC26A4* is a mutation hotspot gene across different regions and races (5). However, the methylation level of *SLC26A4* and the importance of its contribution to deafness remain to be thoroughly investigated.

In the present study, the contribution of the methylation level of the CpG site in the promoter region of *SL26A4* to presbycusis was investigated.

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Materials and methods

Sample collection. A total of 102 Han Chinese presbycusis patients (male:female, 56:46; mean age, 71) and 104 Han Chinese age-matched controls (male:female, 56:48; mean age, 70.1) were selected from Ningbo No. 7 Hospital (Ningbo, China). The patients were diagnosed by experienced physicians based on pure-tone threshold averages (PTAs). The PTA of the presbycusis cases was greater than 60 dB HL, and for the controls, this value was <26 dB HL. All of the included individuals were from Zhenhai, Ningbo and were free of genetic diseases, otitis media, cancer and metabolic disease including diabetes and hyperlipidemia. The peripheral blood samples were collected in 3.2% citrate sodium-treated tubes and then stored at -80˚C. The present study was approved by the clinical committees of Ningbo University (Ningbo, China) and Ningbo No. 7 Hospital, and written informed consent was obtained from all subjects.

Biochemical analyses. The website database of the University of California Santa Cruz (UCSC) human genome browser on Human February 2009 (GRCh37/hg19) Assembly (http://www.genome.ucsc.edu) was used to confirm the gene position and obtain the DNA sequence of *SLC26A4*. The *SLC26A4* gene is located on chromosome 7 and is 57,173 bp long. The 1-kb region upstream of the transcription start site (TSS) of *SLC26A4* was selected as promoter region for analysis. A nucleic acid extraction analyzer (Lab-Aid 820; Zsandx Co., Ltd., Xiamen, China) was used to extract the genomic DNA from the peripheral blood samples in order to detect epigenetic changes in the *SLC26A4* gene. The DNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The methylation of the CpG site of *SLC26A4* was measured with pyrosequencing technology combined with sodium bisulfate DNA conversion chemistry (EpiTech Bisulfite kits; Qiagen China Co., Ltd., Shanghai, China) and polymerase chain reaction (PCR) amplification (PyoMark PCR Kit; Qiagen China Co., Ltd.). The PCR protocol and sequencing primers were designed with PyroMark Assay Design software that automatically selected the appropriate CpG sites with high scores within a 70-nt fragment. The revealed sequences were as follows: PCR primers forward, 5'-TTTTTTATGTGGTATGAGAGTAT-3' and reverse 5'-CATCCCCTTACTAATCTCAA-3'; and sequence primer, 5'-TGTGGTAGGTTTTTAGAG-3'.

RNA isolation, cDNA synthesis and reverse transcription-quantitative PCR (RT-qPCR). The total RNAs were extracted from the peripheral blood using TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To synthesize the cDNA, 1 μg total RNA was used according to the instructions of the manufacturer of the GoScript™ Reverse Transcription System kit (Promega Corporation, Madison, WI, USA). To quantify the expression of the *SLC26A4* gene, RT-qPCR was performed using SYBR® Premix Ex Taq™ II (Perfect Real Time; Takara Biotechnology Co., Ltd., Dalian, China) in an Mx3005P QPCR System (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The expression of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. The primers were as follows: *SLC26A4*, forward 5'-TGGTGGCTTGCAGATTGGAT-3' and reverse 5'-AGCTGTGAGACCAGCACTTG-3'; *GAPDH*, forward 5'-AAGGTGAAGGTCGGAGTCAA-3' and reverse 5'-AATGAAGGGGTCATTGATGG-3'. The data were analyzed with the ΔC_q method (15). All experiments were performed in triplicate.

Statistical analysis. All statistical data were analyzed using SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Two-tailed Student's t-tests were applied for the comparisons of the presbycusis cases and controls.

Table I. Comparison of the *SLC26A4* methylation levels between the controls and presbycusis cases.

Characteristic	Control (mean ± SD)	Case (mean ± SD)	P-value
All	(n=104)	(n=102)	
CpG1 (%)	5.1±1.85	5.5±2.07	0.106
CpG2 (%)	15.7±3.91	16.8±4.97	0.076
CpG3 (%)	23.8±3.85	26.5±5.56	<0.0001[a]

[a]P≤0.05. *SLC26A4*, solute carrier family 26 member 4; SD, standard deviation.

Results

Methylation of the CpG3 site of SLC26A4 was significantly elevated in the patients with presbycusis. In general, the 1-kb region upstream of a transcription start site (TSS) of a given gene is considered to be an important promoter region, therefore, this region of *SLC26A4* was selected for analysis. Based on the PyroMark Assay Design software, which automatically selects the appropriate CpG sites with high scores within 70-nt fragments for primer design, the best-scoring primers harbored three CpG sites and were selected for methylation level evaluation (Fig. 1). As illustrated in Table I and Fig. 2, the methylation of the CpG3 site was significantly elevated (P<0.0001) in the presbycusis cases (26.5±5.56%) compared with the controls (23.8±3.85%). However, no association was identified between CpG1 and CpG2 with presbycusis (CpG1, P=0.106; and CpG2, P=0.076; Table I).

To further investigate the association between *SLC26A4* methylation and presbycusis, subgroup analyses were conducted according gender and age (Fig. 3). Notably, a significant difference was observed between the patients with presbycusis (28.6±5.54%) and the controls (23.4±3.92%) only among the male participants (P=0.0004; Fig. 3A), and no difference was observed among the female subgroup (P=0.063; Fig. 3A). Regarding the age subgroups, a significant difference was observed between the patients with presbycusis (27.1±5.92%) and controls (23.9±4.02%) in those ≥65 years old (P=0.0005; Fig. 3B) than in those <65 years old (P=0.03; Fig. 3B).

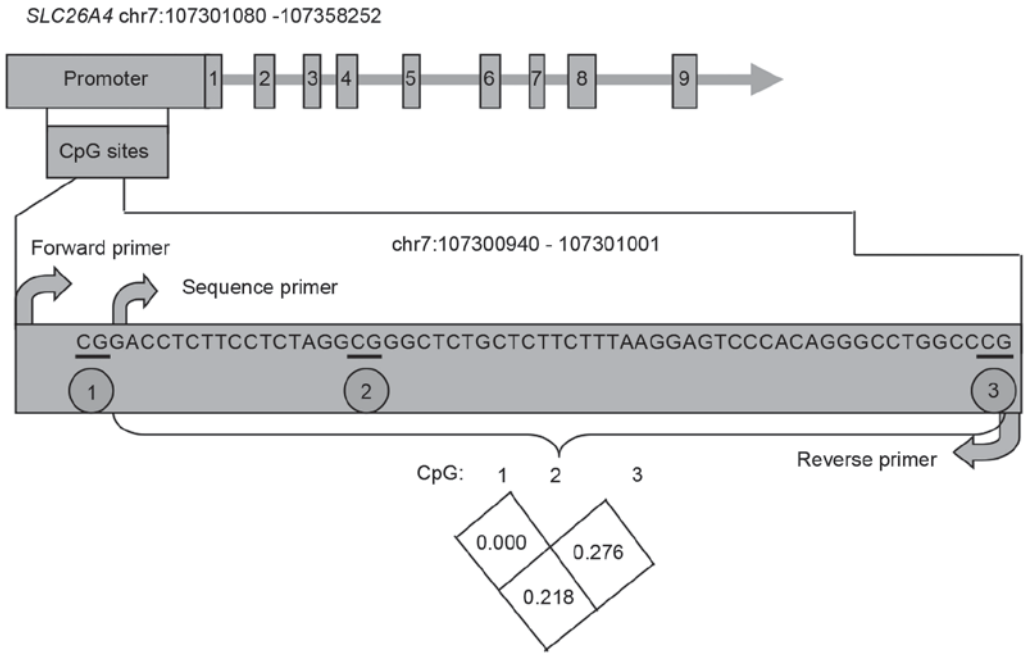


Figure 1. Correlation among the three CpG sites in the *SLC26A4* gene promoter. The sequence and gene position were obtained from the website database of the UCSC human genome browser (http://www.genome.ucsc.edu). *SLC26A4*, solute carrier family 26 member 4.

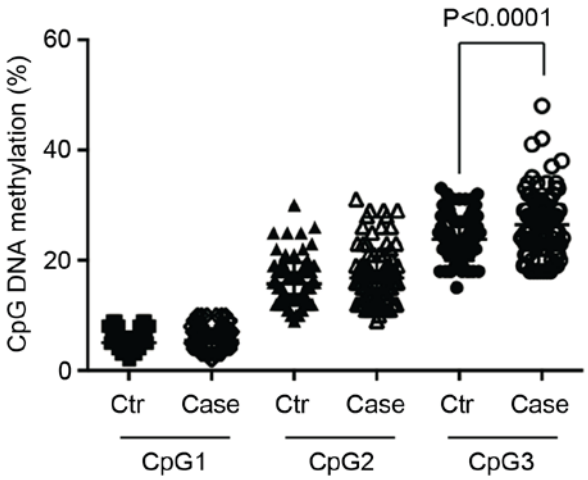


Figure 2. Increased methylation of *SLC26A4* at the CpG3 site in the presbycusis cases. The methylation levels in the promoter region of the *SLC26A4* gene were compared between the Ctr and presbycusis cases. P-values (Student's t-test) for the difference between the Ctr and case values are indicated. *SLC26A4*, solute carrier family 26 member 4; Ctr, control.

SLC26A4 transcription was markedly decreased in the patients with presbycusis with elevated SLC26A4 methylation levels. RT-qPCR analyses were performed to investigate whether the evaluated methylation affected the transcription level of *SLC26A4* using the total RNAs extracted from the peripheral blood samples of 93 controls and 80 patients with presbycusis. As illustrated in Fig. 4, a significant difference in the *SLC26A4* transcription levels between the cases and controls was observed (P=0.02).

CpG3 methylation in SLC26A4 predicted the risk of presbycusis in the males. As illustrated in Fig. 5, we compared the differences between the presbycusis cases and controls based

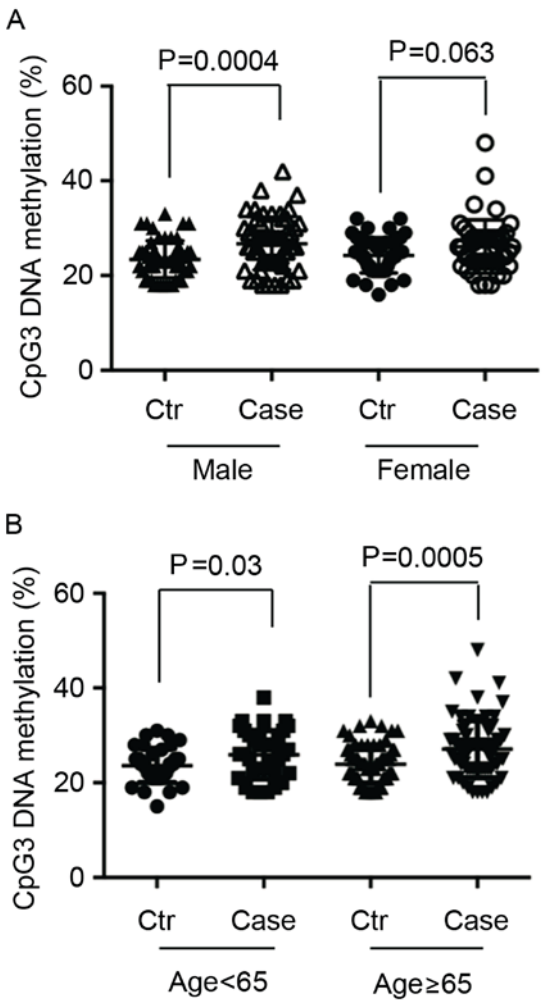


Figure 3. Comparisons of *SLC26A4* CpG3 site methylation levels according to the (A) gender and (B) age subgroups. The P-values (Student's t-test) for the differences the between Ctr and case samples are indicated. *SLC26A4*, solute carrier family 26 member 4; Ctr, control.

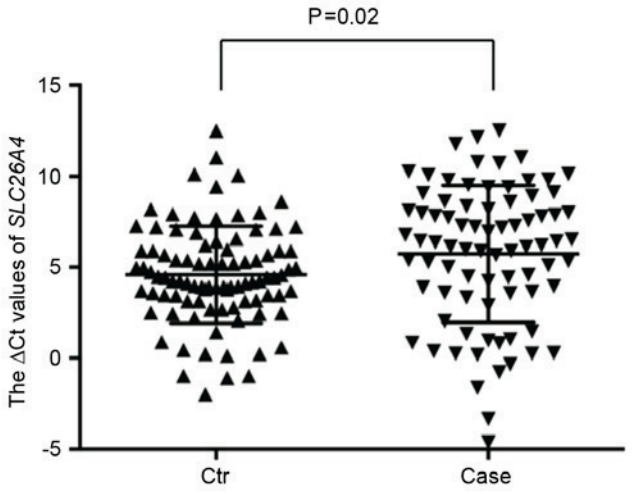
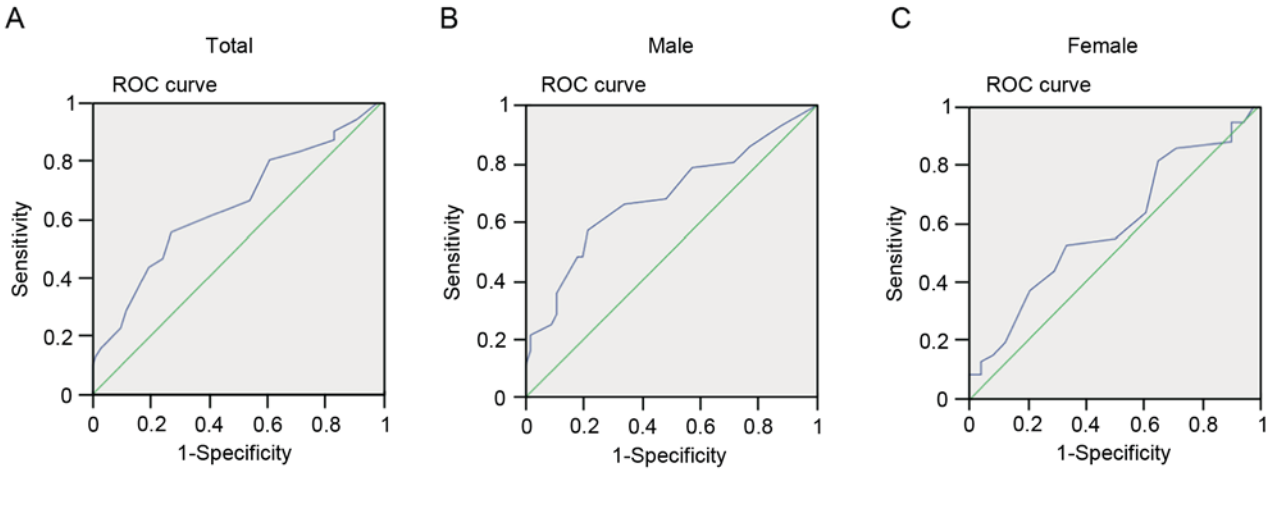


Figure 4. Comparison of the *SLC26A4* transcription levels of the controls and presbycusis cases. RT-qPCR was used to examine the *SLC26A4* mRNA levels in the blood cells. The P-values (Student's t-test) for the differences between the Ctr and case samples are indicated. Greater than three independent experiments were performed. *SLC26A4*, solute carrier family 26 member 4; Ctr, control.

on the cut-off value (25.5%) were obtained from the ROC curve. The areas under the ROC curves (AUCs) reached 0.643 [95% confidence interval (CI) from 0.567-0.718; P=0.0004; Fig. 5A], and the further subgroup analyses demonstrated a male-dependent effect of *SLC26A4* methylation (AUC=0.684; 95% CI=0.584-0.784; P=0.001; Fig. 5B) and no correlation among the female participants (AUC=0.570; 95% CI=0.470-0.702; P=0.150; Fig. 5C). These results suggest that the CpG3 methylation of *SLC26A4* predicted the risk of presbycusis among the male participants.

Discussion

In addition to being one of the most important epigenetic alterations, the methylation of DNA CpG sites is closely associated with environmental factors (6). Numerous studies have verified the contribution of CpG site methylations to various disorders. In the present study, 102 presbycusis cases and 104 controls were analyzed to test the association between the DNA methylation level of the *SLC26A4* gene and the risk of presbycusis. In the presbycusis cases, it was observed that the CpG3 methylation of *SLC26A4* in the peripheral blood was significantly elevated. Additionally, the male gender was positively correlated with the CpG3 methylation level. Widespread CpG island methylation has been previously identified to cause the silencing of the tumor suppressor gene in colorectal cancer (16), and in cases of hepatocellular carcinoma, the normal tissues exhibit lower CpG island methylation levels than those of the tumor tissue, and the promoters in the tumor tissues possesses higher CpG island



Area under the curve

Test result variable (s)

Area	Std.Errorra	Asymptotic Sig.b
0.643	0.039	0.0004

Asymptotic 95% Confidence interval	
Lower bound	Upper bound
0.567	0.718

Area under the curve

Test result variable (s)

Area	Std.Errorra	Asymptotic Sig.b
0.684	0.051	0.001

Asymptotic 95% Confidence interval	
Lower bound	Upper bound
0.584	0.784

Area under the curve

Test result variable (s)

Area	Std.Errorra	Asymptotic Sig.b
0.586	0.059	0.150

Asymptotic 95% Confidence interval	
Lower bound	Upper bound
0.470	0.702

Figure 5. ROC curves for the *SLC26A4* methylation levels in the (A) total, (B) male and (C) female samples. The CpG3 methylation levels of *SLC26A4* were used for the analyses, and the areas under the curve are indicated in the lower tables. ROC, receiver operating characteristic; *SLC26A4*, solute carrier family 26 member 4; Ctr, control.

methylation levels (17). In addition, the majority of cholangiocarcinomas (91%) exhibit aberrant methylation in at least one locus, which suggests that CpG island methylation is a common event (18). Specific mutation loci in *SLC26A4,* including IVS7-2A>G (c.919-2A>G) and p.H723R (c.2168A>G), are recognized as risk factors for non-syndromic hearing loss in Asian populations, in particular in Chinese populations (5). The results of the current study indicated that CpG3 methylation of the *SLC26A4* gene was significantly increased in the male presbycusis patients compared with the male controls. In addition, the area under the ROC curve reached 0.684, which indicates the importance of the CpG methylation of *SLC26A4*. Methylation at this site can result in moderate to severe hearing loss in a manner similar to mutations at specific loci (19), thus could serve as a potential diagnostic biomarker.

Pendrin is the protein encoded by the *SLC26A4* gene and is known to mediate $Cl^-/HCO3^-$ exchange in the inner ear and to serve a role in the maintenance of the higher pH of the cochlear endolymph compared with the perilymph. Abnormal pendrin expression could disrupt the pH balance and lead to the acidification and enlargement of the membranous labyrinth, which would directly affect the cochlea and cause deafness (7-9,20). As indicated in a previous study (21), a novel c.-103T→C mutation in the key transcriptional regulatory element of the *SLC26A4* promoter can interfere with the binding of forkhead box I1 (FOXI1), which is a transcriptional activator of the *SLC26A4* gene, and can completely abolish FOXI1-mediated transcriptional activation (22). However, inactivating *SLC26A4* mutations that cause profound deafness can also be involved in the etiology of moderate to severe hearing loss, which indicates that the severity of hearing loss cannot be predicted by analyzing the type of mutation in all cases (19). In the current study, the significant difference in *SLC26A4* transcription between the presbycusis cases and controls strongly suggested a correlation between elevated CpG site methylation in *SLC26A4* and presbycusis.

However, there are several limitations to the present study that need to be taken into account. Firstly, due to a limitation of the PyroMark Assay Design software, which automatically selects the appropriate CpG sites with high scores within a 70-nt fragment for primer design, the best-scoring primers harbored only three CpG sites that may not fully represent the overall contribution of *SLC26A4* to presbycusis. Further analysis of the far upstream region and gene body sequence may be necessary to reduce the deviation. In addition, the current study involved only a moderate number of samples. Although positive results were detected, future studies with larger pools of individuals are required to provide a more reliable conclusion, particularly in terms of those with high levels of CpG site methylation. Due to the fact that ear tissues were not obtained, the *SLC26A4* DNA methylation and gene transcription levels were tested only in the peripheral blood. Additional comprehensive studies are required to test the concordances of *SLC26A4* methylation and gene transcription levels between ear tissues and peripheral blood.

In conclusion, the present study indicated that the evaluation of *SLC26A4* CpG site methylation reflected an increased risk of presbycusis among the male participants. This association may aid in the clarification of the molecular mechanisms that underlie the pathogenesis of presbycusis, and provide a potential clinical diagnostic marker.

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