行政院國家科學委員會專題研究計畫 成果報告

The Role of Connexin 40 in the Pathogenesis of Atrial Fibrillation: from Gene, Cell to Bedside--心房鍵結蛋白四十基因變異型與各階段心房顫動病患之心房不穩定性、功能惡化及肺靜脈口環狀燒灼術成效之角色關係

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

（計畫名稱）

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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Background: There is evidence showing that genetic factors contribute to the pathogenesis of atrial fibrillation (Af). We investigated the association between Af and polymorphisms of the connexin 40 (Cx40) gene, which is important in the electrical coupling between atrial myocytes.

Methods: We performed an association study between two Cx40 single nucleotide polymorphisms (SNPs) (Cx40 −44 and +71 allele) and Af. We enrolled 173 patients with Af, and the control group consisted of 232 patients without Af. The luciferase assay was performed to evaluate the promoter activities of different Cx40 haplotypes in cultured atrial myocytes.

Results: We found that the two SNPs were both significantly associated with Af. In pairwise linkage disequilibrium analysis, the two SNPs were completely linked (Cx40 −44G always associated with Cx40 +71A; Cx40 −44A associated with Cx40 +71G, $P<0.001$). In haplotype analysis, we demonstrated that the frequency of Cx40 (−44A,+71G) was significantly higher in the Af group than that in the control group ($P<0.006$, odds ratio=1.514, 95% confidence interval 1.13-2.04). We also performed genotype analysis using several genetic models, finding that the recessive model showed the lowest P value ($P<0.004$) and the largest odds ratio (2.53, 95% confidence interval 1.23–5.19). In promoter activity studies using luciferase as the reporter, Cx40 (−44A,+71G) had significantly lower promoter activity than that of the Cx40 (−44G,+71A) in atrial myocytes.

Conclusions: The two SNPs in the promoter region of the Cx40 gene were significantly associated with Af. The Cx40 (−44A +71G) haplotype was associated with a higher risk for Af. This haplotype also had significantly lower promoter activity in atrial myocytes.

中文摘要

背景

心房顫動(Atrial Fibrillation, AF)屬於最常見的心律不整，常伴隨有心悸、胸痛、頭暈的症狀，且容易引起中風、心臟衰竭，及引發死亡的危險。曾有研究顯示，心房顫動與先天遺傳因素相關，而由於心臟細胞中構成間隙連接的分子，connexin40 與心肌間的電氣傳導有關，因此我們探討 connexin40 的基因多型性與心房顫動間的關係。

病患與方法

本實驗主要研究 connexin40 (Cx40 -44G>A 與 Cx40 71A>G)的單一核苷酸多型性與心房顫動的關聯性。我們收集了 173 位平均年齡為 69.6±12.6 的心房顫動病人，並收集無心房顫動的對照組進行分析。此外，並利用 repoter assay，觀察轉殖有 Cx40 (-44G, +71A)與 Cx40 (-44A, +71G)兩種不同單一核苷酸多型性之 HL-1 細胞其 luciferase 蛋白表現的差異。

結果

我們發現 connexin40 上的兩個單一核苷酸多型性 Cx40 -44G>A 與 Cx40 +71A>G 都和心房顫動有顯著的相關。單套體分析結果顯示，Cx40 上的兩個單一核苷酸多型性呈現完全連鎖的狀態。在核苷酸出現頻率的分析中，我們發現在病人的族群中，Cx40(-44A, +71G)的出現機會明顯高於對照組(p=0.006, OR=1.514, 95%信賴區間為 1.13~2.04)；在基因型的分析上，發現這兩個單一核苷酸多型性傾向以隱性模式產生影響(p=0.004, OR=2.713, 95%信賴區間為 1.34~5.50)。最後在 Cx40 多型性的功能分析上，我們利用觀察 luciferase 蛋白的表現變化，發現 Cx40 (-44A, +71G)相較於 Cx40 (-44G, +71A)其 luciferase 的表現有明顯的減少。

結論

Connexin40 上的兩個單一核苷酸多型性 Cx40 -44G>A 與 Cx40 +71A>G 確實與心房顫動相關，其中 Cx40(-44A+71G)會伴隨有較高的心房顫動的風險。而在啟動子能力分析中，我們發現 Cx40 啟動子上的兩個核苷酸多型性對 luciferase 的表現有顯著的影響。

Atrial fibrillation (Af) is the most commonly encountered tachyarrhythmia in clinical practice [1]. The prevalence of Af doubles with each advancing decade from the age of 50 [2]. Its incidence is about 5% in the population with an age greater than 65years. Compared with people in sinus rhythm, those in Af have a six-fold increase in the risk of stroke and a two-fold increase in the risk of death [3].

Af is a multifactorial disease. Independent risk factors include increasing age, male sex, hypertension, diabetes, smoking, valvular disease, and myocardial infarction [1]. However, some patients have Af in the absence of any known risk factor, named as lone Af. Conversely, there are patients with significant valvular diseases or left ventricular dysfunction remaining in sinus rhythm. This clinical observation may imply that predisposing genetic factors play important roles in the development of Af.

Adjacent cardiomyocytes are connected by gap junctions, which consist of a hexameric assembly of proteins known as connexins. These gap junctional channels are responsible for the electrical coupling of cardiac myocytes [4,5]. In the human heart, myocyte gap junctions may be constructed from up to three different connexin isotypes, connexin 43, connexin 40 (Cx40), and connexin 45. Cx40 is expressed mainly in the atrium and ventricular conduction system [6]. Since gap junctions provide the syncytial properties of the atrium, changes in expression and distribution of Cx40 may contribute to the pathogenesis of Af.

From this viewpoint, we investigated the association between Af and two single nucleotide polymorphisms (SNPs) in the promoter region of the Cx40 gene. Functional study of promoter activity was also performed using the luciferase assay in cultured atrial cells.

1. Methods

1.1. Study population

This study enrolled 173 consecutive patients (95 men and 78 women) who were admitted to our adult cardiology ward with Af. We excluded the patients with hyperthyroidism. The control group consisted of 232 subjects (111 men and 121 women) selected from the same ward without a history of Af. The presence of Af was determined by taking histories, serial electrocardiograms (ECG), or ambulatory ECG monitoring. Patients with palpitation without ECG documentation were excluded from this study. Among the 173 patients with Af, 37 had paroxysmal Af while 136 had chronic Af. The demographic data were collected from medical chart records. The study protocols were reviewed and approved by the Institutional Reviewing Board. All subjects agreed to participate in the study, and informed consent was obtained from each subject.

1.2. Genotyping

Genomic DNA was extracted from the buffy coat of peripheral blood samples. Polymerase chain reaction (PCR) and restriction fragment analysis were used to determine the genotype of the Cx40 gene. For the two SNPs, there was no restriction site change. Therefore, artificial restriction site was introduced with a mismatched base in the primers according to methods described by Ke et al. [7]. For the detection of Cx40 −44G > A genotype, the forward primer was CCCTCTTTTTAATCGTATCTGT*G* GC (mismatched bases are underlined), and the backward primer was GGTGGAGGGAAGAAGACTTTTAG. The PCR product was 150 base pairs in size. Digestion with HaeIII produced 126 and 24 base pair fragments if the base at the −44 position was G. For Cx40 + 71A > G, the forward primer was AGGGAAGGCGACAGATACGA, and the backward primer was CTTCTTTTCCTCCTCCCTGGA*A* TT (mismatched bases are underlined). The PCR product was 145-base pair in size. Digestion with EcoR I produced 121-base pair and 24-base pair fragments if the base at the + 71 position was G (Fig. 1).

1.3. Construction of expression variants

The proximal promoter region of the Cx40 gene from position −177 to + 98 relative to the transcription start site was amplified using PCR. Genomic DNA from an individual with the genotype −44GG/+ 71AA was used for plasmid p(−44G,+ 71A) and that from an individual with the genotype −44AA/+ 71GG for plasmid p(−44A,+ 71G). The base sequence of the forward primer was 5′-TTT*GGATCC* CTACGAGGAGGTGGAGGGAA-3′ (artificial BamH I restriction site underlined) and the reverse primer 5′-TTT*AAGCTT* CTGCTTCTTTTCCTCCTCCCT-3′ (artificial Hind III restriction site underlined). After digestion with BamH I and Hind III, the PCR products were ligated into the region between the BamH I and Hind III restriction sites of the pGL3 plasmid (Promega), which contained the gene of firefly luciferase. DNA sequencing was performed to confirm the base sequence of the insert region of the construct to ensure the absence of PCR errors.

1.4. Transient transfection of the HL-1 cell

Cx40 promotor-luciferase constructs p(−44G,+ 71A) and p(−44A,+ 71G) were transiently transfected into HL-1 cells (cell line derived from mouse atrial cardiomyocyte [8]) cultured in 96-well plates using a transient liposome cotransfection protocol (Lipofetamine2000; Invitrogen) with 0.25 μg of pGL3 plasmid and 0.01 μg of pRL-TK control vector for Renilla luciferase expression as an internal control to normalize efficiency of transfection. For each construct, the experiments were performed in triplicate. Six hours after transfection, the medium was changed to a fresh complete medium with the same volume per well. After another 48 h, we measured the luciferase activity by Dual-Glo™ Luciferase Assay System (Promega).

1.5. Statistical analysis

The χ^2 test was used for comparison of dichotomous variables for between-group data and among three different genotypes. Comparison of the numeric variables between case and control groups was performed using the Student's unpaired *t* -test, and numeric variables among the three Cx40 genotypes were compared using one-way analysis of variance.

Allele frequencies were calculated from the genotypes of the subjects. Differences in allele frequencies and genotype distributions between Af and control groups were compared using the χ^2 test or the Fisher's exact test. Hardy–Weinberg equilibrium (HWE) was assessed by the χ^2 test. The genotype–phenotype correlations were tested with additive, dominant, and recessive models by logistic regression analyses. Odd ratios (ORs) and their 95% confidence intervals (CIs) were calculated. Multivariate analysis was performed to correct for diabetes using the SPSS 11.0 program. Haplotypes analysis was performed for the two polymorphisms to determine whether there were any specific haplotypes that were associated with Af, and tests of linkage disequilibrium (LD) known as D' and r^2 were calculated by PowerMarker 3.23 [9]. High values of LD were defined as $r^2 > 1/3$ as suggested by Ardlie et al. or as $D' > 0.7$ as suggested by Gabriel et al. [10,11]. Data are expressed as mean ± standard deviation, and a P value of < 0.05 was considered statistically significant.

2. Results

2.1. Characteristics of study subjects

The characteristics of the study subjects are shown in Table 1. The age, gender and percentage of patients with hypertension, coronary artery disease and left ventricular dysfunction were not significantly different between the two groups. However, the control group had a significantly higher proportion of diabetes mellitus.

2.2. Pairwise LD analyses and HWE tests for the two SNPs

With regard to both groups as well as to the total population, the D' and the r^2 values were approximately 1.0 ($P < 0.001$). Actually, the two SNPs were completely linked. The Cx40 − 44G was always associated with Cx40 + 71A, while Cx40 − 44A was associated with Cx40 + 71G. Therefore, they were put together for further analysis. The Cx40 genotype distribution in patients did not significantly deviate from the HW distribution predicted with the allele frequencies ($P = 0.23$). Tests of HWE were also performed for the two loci among the Af and control groups separately. The Cx40 genotype distributions did not significantly deviate from the HW distribution in both the Af and control groups ($P = 0.89$; $P = 0.06$).

2.3. Comparison of the Cx40 haplotype frequency between the two groups

The haplotype frequency of Cx40 (− 44A,+ 71G) and Cx40 (− 44G,+ 71A) was compared between the Af and control group. The Af group had a significantly higher Cx40 (− 44A,+ 71G) haplotype frequency than the control group (37.6% vs 28.4%, $P < 0.006$). The OR of the Cx40 (− 44A,+ 71G) haplotype for Af was 1.51 (95% CI 1.13–2.04). Because the two groups were significantly different in the incidence of diabetes, we performed the same analysis corrected for diabetes. The OR was 1.49, and the 95% CI was 1.10–2.02 after adjusting for diabetes ($P < 0.009$).

2.4. Genotype analysis

For genotype analysis, three different models were applied. The rare allele Cx40 (− 44A,+ 71G) was assumed to have dominant, recessive and additive effects in these models. The Cx40 polymorphisms were significantly associated with Af in all models, and the ORs were greater than 1.0 in all models. Among the three models, the recessive model had the smallest *P* value and the largest OR (2.71, 95% CI 1.34–5.50, $P < 0.004$). We also performed multivariate analysis to correct for diabetes. The results were similar after the correction. The ORs were greater than 1.5 in all three models. The smallest *P* value and the largest OR (2.53, 95% CI 1.23–5.19, $P < 0.004$) was found in the recessive model after adjusting for diabetes (Table 2).

2.5. Transcriptional activity of Cx40 promoter haplotypes

The effect of Cx40 polymorphisms on the promoter activity was evaluated using luciferase assay. We performed a parallel transient transfection to assay the transcriptional activity of the two different constructs in cultured atrial cells (HL-1 cell lines). The luciferase activity of the plasmid with the rare haplotype p(− 44A,+ 71G) was significantly lower than that of the more common haplotype p(− 44G,+ 71A) ($P < 0.05$) (Fig. 2).

3. Discussion

In the present study, we investigated the association between Af and Cx40 SNPs with a case-control study design. We demonstrated that Cx40 (− 44A,+ 71G) was associated with an increased risk of Af. However, a causal relationship cannot be demonstrated in such a study design. Therefore, a functional study was performed. We showed that the promoter activity of the Cx40 (− 44A,+ 71G) was significantly lower than that of Cx40 (− 44G,+ 71A) when expressed in a cultured atrial cell line. The results supported the hypothesis that Cx40 gene variations with functional changes might be associated with Af.

Functional changes of Cx40 have been reported in transgenic animals, acquired Af models as well as in human subjects. Atrial conduction velocity was reduced by 30% in Cx40 knock-out mice when compared with the wild type [12]. Cx40 deficiency resulted in increased atrial vulnerability and might have contributed to arrhythmogenesis in the atrial myocardium in the mouse [13]. In animal models of Af, alterations in Cx40 distribution have been observed [14,15]. Therefore, changes in the amount or distribution of atrial Cx40 protein might form a cellular substrate favoring Af.

Association studies have been used to study the genetic factors associated with complex genetic diseases. There are several association studies regarding Af without Medelian heredity. Yamashita et al. reported that the angiotensin-converting enzyme insertion/deletion polymorphism was not associated with Af in a Japanese population [16]. Lai et al. reported that the ORs for Af in patients with minK 38G allele were higher in a Chinese population [17]. Tsai et al. reported the association between angiotensinogen haplotypes and Af [18]. The relationship between Cx40 SNPs and Af has also been demonstrated before. Firouzi et al. performed invasive electrophysiological studies in patients with the Cx40 − 44AA genotype. They found that patients with such a genotype were associated with increased spatial dispersion of refractoriness in the

atria [7]. An association between the − 44A allele and idiopathic Af was also demonstrated in a small group of patients ($n = 14$) [19]. In the present study, we demonstrated that the Cx40 − 44G > A and Cx40 + 71A > G polymorphisms were associated with Af in a large group of common Af ($n = 173$). The Af group had a significantly higher Cx40 (− 44A,+ 71G) genotype frequency than the control group. Compared with other genotypes, the risk of developing Af increased 2.7-fold for the patients with the Cx40 (− 44A,+ 71G) genotype ($P < 0.004$). Our results provided evidence to support the involvement of Cx40 polymorphisms with Af.

3.1. Functional analysis of Cx40 gene polymorphisms

In vitro functional assay showed that the transcriptional activity of the Cx40 (− 44A,+ 71G) construct was significantly lower than that of the Cx40 (− 44G,+ 71A) construct in HL-1 mouse atrial cardiomyocyte ($P < 0.05$). This finding is consistent with a recent study by Groenewegen et al., who also showed reduced transcriptional activity of the Cx40 (− 44A,+ 71G) haplotype in rat arterial smooth muscular cells [20]. This suggests that the Cx40 (− 44A,+ 71G) haplotype might be associated with a reduced amount of Cx40 mRNA. Gap-junctional remodeling in the heart may lead to abnormal electrical coupling and is, therefore, arrhythmogenic [21]. Lack of the atrial gap junction protein Cx40 has been reported as resulting in increased atrial vulnerability and propensity to arrhythmias in mice [13,22]. There are also reports in humans showing downregulation of Cx40 in human atrial tissue in patients with Af [23,24, 5,26]. Based on these studies and our results, it is possible that the Cx40 (− 44A,+ 71G) in the promoter region of the Cx40 gene results in increased Af susceptibility by reducing Cx40 expression.

3.2. Limitations

First, all study subjects were selected from a Taiwanese population. Applicability to other ethnic group is uncertain and warrants further study. Secondly, the functional assay was performed on atrial cells from mice instead of humans. The differences in the two species may result in differences in the expression levels of the Cx40 gene.

4. Conclusion

There is growing evidence suggesting that genetic factors are important in the pathogenesis Af. Patients with the Cx40 (− 44A,+ 71G) haplotype are more susceptible to the development of Af. We hope such genetic studies might elucidate the underlying mechanism of Af and contribute to the development of novel therapeutic modalities for Af.

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Table 1: The baseline characteristics of the subjects in the two groups

	Af (*n* = 173)	Control (*n* = 232)	*P* value
Gender M/F	95/78	111/121	0.160
Age (years)	69.6 ± 12.6	70.6 ± 12.4	0.414
Hypertension	57.80% (100/173)	62.50% (145/232)	0.339
DM	18.50% (32/173)	37.07% (86/232)	< 0.001
Smoking	29.4% (51/173)	30.1 (70/232)	0.880
CAD	23.1% (40/173)	19.8 (46/232)	0.423
LVEF < 55%	14.4% (15/173)	9.9% (23/232)	0.671
VHD	29.4% (51/173)	30.2% (70/232)	0.88
SSS	8.7% (15/173)	10.3% (24/232)	0.57
HCM	1.7% (3/173)	3.4% (8/232)	0.39
DCM	5.2% (9/173)	4.3% (10/232)	0.77

Af = atrial fibrillation; CAD = coronary artery disease; DCM = dilated cardiomyopathy; DM = diabetes mellitus; F = female; HCM = hypertrophic cardiomyopathy; LVEF = left ventricular ejection fraction; M = male; SSS = sick sinus syndrome; VHD = valvular heart disease.

Table 2: Distribution of genotypes and alleles in patients with Af and control

	Af (*n* = 173)	Control (*n* = 232)	No adjustment		Adjustment for DM	
			P value	OR (95% CI).	*P* value	OR (95% CI)
Allele frequency analysis						
Cx40 – 44A/+ 71G	130	132	< 0.006□	1.51 (1.13 – 2.04)	< 0.009□	1.49 (1.10 – 2.02)
Cx40 – 44G/+ 71A	216	332				
Genotype analysis						
Dominant model						
Cx40 – 44AA + AG/+ 71GG + GA	106	119	< 0.046	1.50 (1.01 – 2.24)	< 0.051	1.50 (1.00 – 2.25)
Cx40 – 44GG/+ 71AA	67	113				
Recessive model						
Cx40 – 44AA/+ 71GG	24	13	< 0.004□	2.71 (1.34 – 5.50)	< 0.004□	2.53 (1.23 – 5.19)
Cx40 - 44AG + GG/+ 71GA + AA	149	219				
Additive model						
Cx40 – 44AA/+ 71GG	24	13	< 0.005□	1.56 (1.14 – 2.13)	< 0.008□	1.54 (1.12 – 2.11)
Cx40 – 44AG/+ 71GA	82	106				
Cx40 – 44GG/+ 71AA	67	113				

Af = atrial fibrillation; CI = confidence interval; Cx = connexin; DM = diabetes mellitus; OR = odds ratio.

Fig. 1: Representative results of agarose gel electrophoresis of polymerase chain reaction and restriction digestion for determination of Cx40 − 44A > G (up) and Cx40 + 71G > A (down)

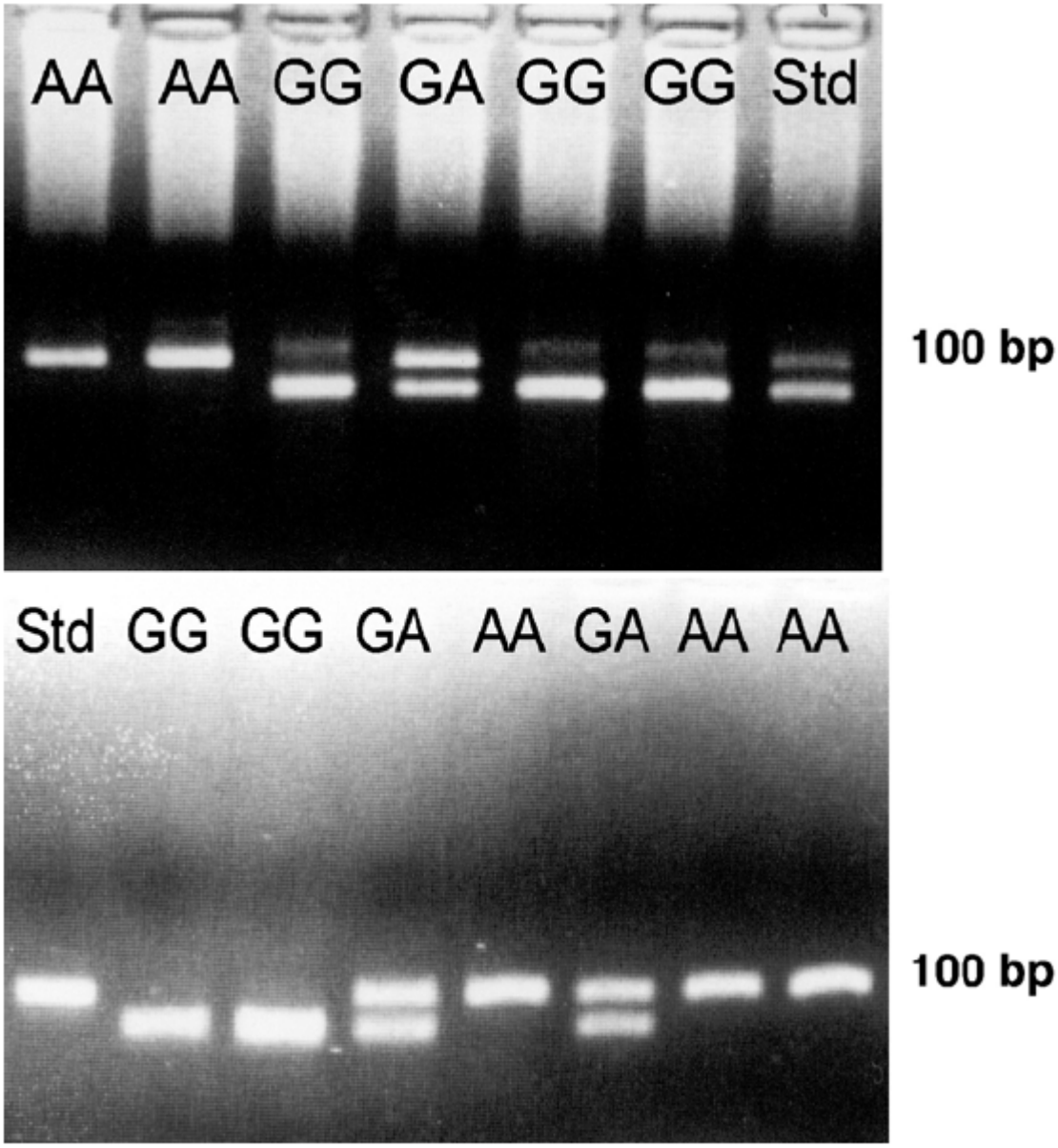

Fig. 2: Relative transcriptional activities of the two different haplotype reporter constructs in HL-1 cells. The activity of the major haplotype Cx40 (− 44G, + 71A) was set at 100, and the relative activity of other plasmids was calculated. The pGL3 represented the blank vector, which did not contain Cx40 promoter sequence