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利用斑馬魚建立心臟衰竭研究之動物模式:專一心臟 troponin-C 蛋白表現受抑制之斑馬魚心肌功能研究

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Topic

Conditional Antisense-Knockdown of Zebrafish Cardiac Troponin C as a New Model for Dilated Cardiomyopathy

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Abstract

Background – Mutations of cardiac troponin C (cTnC) can cause dilated cardiomyopathy in humans. We attempted to generate a zebrafish transgenic line that enables us to produce the antinsense RNA strand of cTnC after induction as a new animal model for studying dilated cardiomyopathy.

Methods and Results – We constructed plasmids that the reverse tetracycline–controlled transactivator (rtTA) was driven by the cardiac myosin light chain 2 promoter. This heart-specific rtTA bound another bidirectional promoter to express the green fluorescence protein (GFP) reporter gene and to transcribe the antisense RNA of cTnC in the presence of doxycycline (Dox). After microinjection of these constructs into one-cell fertilized eggs, we cultured, screened, and generated a transgenic line of zebrafish (CA17) which enabled us to express GFP and to conditionally produce the antisense RNA of cTnC in heart after induction. When 12-h postfertilization (hpf) embryos of F2 derived from CA17 were given Dox (10 μg/ml), the expression of endogenous cTnC mRNA was not affected until 6 days postfertilization (dpf). The heart rates of the embryos in CA17 were significantly slower than those of embryos in the control T03 transgenic line (only expressing the heart-specific GFP) at 6 dpf (150±10 vs. 194±11 beats/min, $p < 0.01$) and 12 dpf (128±12 vs. 168±8

beats/min, $p < 0.01$). Moreover, the various values of cardiac chambers measured in the induced F2 embryos from CA17 were significantly greater than those of T03 embryos; while the ventricular ejection fraction of CA17 was lower than that of the T03 both at 6 dpf (44%±4% vs. 50%±3%, $p < 0.01$) and at 12 dpf (39%±5% vs. 52%±4%, $p <0.01$). In addition, asynchronized atrial and ventricular contractions were noted in a few (1.3%) F2 embryos from the induced CA17 transgenic fish. The mortality rate of F2 adult fish of the CA17 group was significantly higher (30% vs. 0%, $p < 0.001$) than that of F2 adults of the T03 group after Dox induction.

Conclusions – Using conditional expression of antisense RNA of zebrafish cTnC, we have created a new animal model with phenotypes simulating dilated cardiomyopathy.

Key Words: Zebrafish; Cardiac troponin C; Antisense RNA; Dilated cardiomyopathy

Contraction of the myocardium is regulated by the cardiac troponin complex. Calcium ions bind to the regulatory sites of cardiac troponin C (cTnC) and facilitate interactions between the actin and tropomyosin. Mutation of cTnC can cause myocardial dysfunction.[1] A cTnC mutation has been reported to cause human familial dilated cardiomyopathy.[2] Mutation analysis of the troponin complex in dilated cardiomyopathy patients may prove valuable in the early identification of individuals with an adverse prognosis and a high risk of premature death. [2] However, no animal model of dilated cardiomyopathy related to cTnC dysfunction has been reported.

The zebrafish (*Danio rerio*) is an excellent animal model for cardiac research.[3-5] It has a 2-chambered heart that is similar in many genetic ways to the 3- or 4-chambered hearts of higher vertebrates.[6-7] Transparent embryos make it possible to observe the dynamic expression of heart genes in a noninvasive manner. Embryos of zebrafish receive oxygen through diffusion. This kind of oxygenation system allows embryos to survive for up to (at least) 1 week even with cardiac defects.[8] A 1.6-kb regulatory region of the zebrafish cardiac myosin light chain 2 gene (*cmlc*2) has been demonstrated to specifically drive green fluorescent protein (GFP) expression in the heart of transgenic zebrafish.[9] We have also generated transgenic lines with

myocardium-specific expression of GFP under a tetracycline (Tet)-on conditional expression system.[10] To establish a zebrafish model that is feasible for studying dilated cardiomyopathy, we designed 2 constructs in this study. One was an upstream plasmid which contained a cmlc2 promoter to specifically drive the expression of a reverse Tet–controlled transactivator (rtTA) in the heart. The other was a downstream plasmid which contained a Tet-binding domain (TetO) and possessed a bidirectional promoter to express the GFP reporter and to transcribe cTnC-antisense RNA. Antisense knockdown has been widely applied both *in vitro* and *in vivo* studies to block the translation of endogenous mRNA.[11,12] After gene transfer, a germ line-transmitted zebrafish carrying a heart-specific Tet-On system was generated to conditionally produce the antisense RNA of the cTnC gene. Under the impediment of the translation of the endogenous cTnC gene, cardiac functional assays were carried out in this transgenic zebrafish after induction.

Methods

Experimental Fish

Zebrafish of the AB strain were cultured and maintained according to the procedures described by Westerfield.[13] The developmental stages were

determined according to criteria described by Kimmel et al.[14]

Molecular Cloning of Zebrafish cTnC (Figure 1)

The primers used to clone the cDNA sequence of zebrafish cTnC were designed on the basis of the homologous analysis of cTnC from other known vertebrates. The complete coding region sequence of zebrafish cTnC was amplified by polymerase chain reaction (PCR) using cTnC-SpeI-F (TGACTAGTATGAACGACATCTACAAAGCAGC) and cTnC-PstI-R (TGCTGCAGCTATTCCACCCCTTCATG). The PCR product was used to construct the expression plasmid after it was cut with *Spe*I and *Pst*I. cTnC polypeptides from various vertebrates were aligned using the BCM Research Laucher (available at http://searchlauncher.bcm.tmc.edu/) and a phylogenetic tree was established from the Gene Data Bank of Japan website (available at http://www.ddbj.nig.ac.jp/). The complete coding region sequence was deposited in GenBank (ascension no.: AF434188, maintained by the NCBI).

Whole-mount *in Situ* Hybridization (Figure 2)

In order to determine the tissue-specific expression pattern of the cTnC we cloned, we carried out whole-mount *in situ* hybridization using a digoxigenin-labeled RNA probe as described previously.[15]

Plasmid Construction (Figure 3)

pICML1-rT16-1b is an upstream plasmid used to produce cardiac-specific rtTA. It was derived from plasmid pICMLE-(-870/787),[9] except that the GFP cDNA was replaced by rtTA-M2.[10] The resulting pICML1-rT16-1b consisted of the regulatory region of *cmlc*2 (the -870 to –1 nt region, exon 1, intron 1, and a portion of exon 2), rtTA-M2, and inverted terminal repeats of an adeno-associated virus. Plasmid pBIEK-cTnC-antisense is a downstream plasmid used to produce antisense RNA of cTnC and to express the GFP reporter gene. It was derived from plasmid pBILE,[16] in which *Spe*I and *Pst*I were used to remove the luciferase gene, which was replaced by ligation with the opposite-direction cTnC cDNA to the rtTA-dependent bidirectional promoter, tetO (Clontech).

Gene Transfer

Fertilized eggs at the 1-cell stage were collected and microinjected with linearized constructs of pICML1-rt16-1b and pBIEK-cTnC-antisense following a previous study[17] except that a total of 15 μg/ml of each plasmid was used. This was the cTnC-antisense group (CA17). Meanwhile, we also microinjected linearized plasmids of pICML1-rt16-1b and pIBIE, in which the cTnC segment was not included, to serve as a control group (T03 group).

Transgenic Line Screening

Embryos were cultured under a 14-h light 10-h dark photoperiod at 28.5 °C. Pairs of

mature fish were kept together in tanks. The heart-specific GFP expression in F1 embryos was examined under a fluorescent microscope after induction with 10 μg/ml of Dox (Sigma) for 3 days. GFP-positive progeny were selectively bred and mated with wild-type (wt) fish when they reached adulthood.

Induction in Embryos

Heterozygotic F2 embryos derived from the transgenic germ lines were kept in water until 12 h post fertilization (hpf), at which time they were treated with 10 μg/ml of Dox. After induction, GFP expression was observed under a fluorescent microscope. Fish embryos that had heart-specific GFP expression without leaks were selected, orientated ventrally, and tilted at 45° when taking photos using an S2Pro digital camera (FinePix) with a 10-s exposure time at ISO 400 for image analysis of heart functions. Morphological changes of the heart and heart rate were also examined.

Induction in Adult Fish

The 6-month-old adult fish derived from the homozygote of transgenic lines of the cTnC-antisense line (CA17) and the control line (T03) were selected to carry out Dox induction. Ten fish from each group were raised in a tank and treated with 10 μg/ml Dox. In order to prevent Dox from being photodegraded, we covered the tank with tinfoil. The fish were fed with artemia shrimp and the

water in the tank was replaced daily. After the induction, we observed the fluorescent signal in fish under fluorescent microscope for 3 weeks.

Extraction of Zebrafish Embryonic mRNA

The heterozygous F2 embryos derived from the CA17 and T03 groups were treated with Dox (10 μg/ml) when they were at the 12-hpf stage. Another negative control group consisted of those embryos derived from the CA17 transgenic line without Dox treatment. Five embryos at 6 dpf from each of these 3 groups were selected, and their mRNAs were extracted for further analysis.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

In order to detect the endogenous cTnC transcription and the antisense RNA of cTnC transcription after induction, we collected 6-dpf F2 embryos derived from the CA17 and T03 lines. Their total RNA was extracted. All of the RT-PCR procedures followed those of a previous study[18] except that (1) in the first-strand cDNA synthesis, we used the Oligo-dT primer to detect the endogenous the cTnC transcription and used TnCA-RT1F primer (CGACATCTACAAAGCAGCGGC- AG; Fig. 1C) to detect the cTnC-antisense strand transcription; and (2) in the following PCR amplification, we used primers TnCA-RT2R (GCCAGAACCCT- ACCCCTGAAGAGC) and

TnCA-RT2F (GTCTCCGTCCCTCATCAGTTCCTC) (Figure 1C).

Video File Capture and Processing

The lens adaptor of a stereomicroscope was connected to a DV camera. The LCD monitor of the camera was turned on and a connected to the computer with Ulead video studio 7.0 system. The image was captured for 10 s and recorded as an AVI file in the computer. Then the AVI file was converted to bmp files sequentially according to a previously described method.[19] One bmp file contained 1 frame of cardiac motion. The cardiac motion was viewed frame by frame to define the systolic and diastolic phases. An area with the most-even fluorescent distribution was selected, and the region of the fluorescent heart to be analyzed was marked. The ventricular volume was calculated using the formula for a prolate spheroid:[20] Volume = $4/3 \times \pi \times a \times b^2$ where a and b are the long and short radii of the ventricle, respectively. The cardiac ejection fraction was calculated by the following formula: (cardiac diastolic volume – cardiac systolic volume) ÷ cardiac diastolic volume.

Statistical Analysis

All data are expressed as the mean±standard deviation (SD). Statistical differences among groups were obtained using the Student's *t*-test. It was considered statistically significant at $p < 0.01$.

Results

Primary Structure of Zebrafish cTnC

The complete coding sequence of zebrafish cTnC we cloned was 486 bp (Figure 1c), which encoded a 161-amino acid polypeptide (Figure 1a). The zebrafish cTnC polypeptide shared 90% identities with those of a mammal, chicken, and *Xenopus* (Figure 1), in which 4 E-F hand calcium-binding domains were highly conserved. On the basis of the phylogenetic tree, the zebrafish cTnC was clustered into the cardiac/slow skeletal muscle group.

Expression of cTnC is Heart-Specific

As shown in the *in situ* hybridization, the transcripts of cTnC we cloned were heart-specific (Figure 2). The cTnC transcripts were first detectable in 17-hpf embryos, and cTnC was expressed bilaterally in heart precursory cells behind the notochord (Figure 2a). Transcription of the cTnC gene was detected only in the heart-tube of 24-hpf embryos (Figure 2b), in the whole heart of 48-hpf embryos (Figure 2c), and in the mature heart of 72-hpf embryos (Figure 2d). The intensity of the fluorescent signal expressed in the ventricle was much stronger than that in the atrium. The reason was there is only 1 layer of cardiomyocytes in the atrium whereas there are 2 to 3 layers of cardiomyocytes in the ventricle.

Inducible GFP Expression in the Germ Line-Transmitting Zebrafish

The 12-hpf embryos derived from F1 of the transgenic cTnC-antisense line (CA17) and control T03 were collected and treated with 10 ug/ml Dox. The CA17 line expressed both the GFP and the antisense mRNA of cTnC, while the T03 line expressed the GFP only. Without Dox treatment, there was no green fluorescent signal shown in the fish. However, after induction, the green fluorescent signal began to appear specifically in the heart about 12 h after treatment (Fig 4a).

Transcription of cTnC Antisense RNA in Transgenic Fish

As shown in Figure 4b, cTnC mRNA was produced by the transgenic lines of both CA17 and T03 after induction by Dox; whereas the antisense RNA transcribed from the cTnC gene was clearly shown in embryos derived from CA17 transgenic fish after Dox induction. Although a very weak signal of antisense RNA was shown prior to induction, the green fluorescent signal was not detected. On the contrary, the antisense RNA of cTnC was not produced in embryos derived from the T03 control transgenic line. As long as cTnC antisense RNA was expressed in CA17 embryos, the expression of endogenous cTnC mRNA remained the same.

Heart Rates of CA17 and T03 Transgenic Zebrafish at 6 and 12 dpf

At 3 dpf, heart rates of the CA17 group were slightly faster than those of the T03 group (Figure 5). With further growth, the situation gradually reversed. The heart rates of CA17 were significantly slower than those of the T03 at 6 (150±10 vs. 194±11 beats/min, $p < 0.01$) and 12 dpf (128±12 vs. 168±8 beats/min, $p < 0.01$).

Ventricular Assessments of CA17 and T03 Zebrafish at 6 and 12 dpf

End-diastolic and end-systolic diameters were significantly different between the T03 and CA17 groups at both 6 and 12 dpf (Figure 6, Tables 1 and 2). The ventricular ejection fraction of CA17 was lower than that of T03 transgenic fish (44%±4% vs. 50%±3%; $p < 0.01$). At 12 dpf, the ventricular ejection fraction of CA17 was even lower than that of T03 (39%±5% vs. 52%±4%; $p < 0.01$). Measurements from the hearts of the T03 transgenic fish were calculated to obtain the arithmetic mean and standard deviation. The range of mean ± 2 standard deviations was defined as the normal range. Percentages of the upper outlier for cardiac chambers and lower outlier for the ejection fraction and heart rates in the CA17 group are presented in Table 3.

Atrial and Ventricular Asynchrony in CA17 Zebrafish

At 6 dpf, atrial and ventricular contractions were synchronized in F2 embryos from the CA17 group after induction. However, 1.3% of F2 embryos from CA17

transgenic fish developed asynchronized atrial and ventricular contractions at 12 dpf (Figure 7 and supplemental video file).

Slurred Movement and Excess Mortality in cTnC-Antisense Transgenic Adult Fish after Dox Induction

After the Dox has been continuously administrated to 6-month-old fish derived from homozygotic parents, we found that the movement of fish from CA17 was slower than that of T03 group. Furthermore, 30% (3/10) of fish from the CA17 group had died by day 13; whereas no mortality had occurred in the T03 group.

Discussion

cTnC and cardiac troponin T mutations have been reported to cause human familial dilated cardiomyopathy.[2] The clinical manifestations include heart failure and sudden death.[2] Cardiac troponin T variants produced by aberrant splicing of multiple exons in animals have been related to high instances of dilated cardiomyopathy. [21] However, there has been no report of a transgenic animal model for cTnC dysfunction. On the other hand, cTnC is the target protein of a novel calcium sensitizer (levosimendan).[22] This drug has proven to be a well-tolerated and effective treatment for patients with severe decompensated heart failure.[22] Therefore, the role of cTnC dysfunction in

heart failure is worth studying.

The zebrafish heart contains 4 components (sinus venosus, atrium, ventricle, and bulbus arteriosus). Although it has a prototypic vertebrate heart, the zebrafish has been used to study bradycardia, heart valve formation and cardiac regeneration.[23-29] By fusing the Tet repressor and activating the transcription domain of virion protein 16 of the herpes simplex virus, Tet-controlled transactivator (tTA) is generated.[30] A Tet-On system is further developed by mutagenesis, substituting 4 amino acids in tTA to generate rtTA which requires Tet for binding to the Tet operon and subsequent activation of the downstream gene.[31] For pharmacokinetic reasons, controlling genes by means of rtTA is particularly advantageous in the study of transgenic animals. We have successfully developed such a Tet-On system in zebrafish that enables the transgene to specifically be expressed in the heart after induction.[10] This technique also makes it feasible for later onset of abnormal gene expression in adult transgenic fish. Therefore, this methodology extends the utilization of zebrafish as a model organism from congenital cardiac disorders to acquired heart diseases.

In this study, cTnC antisense mRNA was only noted in CA17 fish induced by Dox. T03 transgenic fish could not produce cTnC antisense mRNA. The

ventricular size and the prevalence of ventricular enlargement had increased significantly in the CA 17 group at 6 and 12 dpf. The ventricular ejection fraction of the CA17 group was also significantly lower than that of the T03 group at 6 and 12 dpf. On the other hand, relative bradycardia was noted despite the impaired contractility in the CA17 group. Chronotropic incompetence was another major finding in the cTnC antisense zebrafish. Even 1.3% of CA17 transgenic fish manifested asynchronized atrial and ventricular contractions. Atrioventricular conduction block can lead to sudden death which has been reported for troponin related dilated cardiomyopathy in humans.[2] According to Sedmera et al., trabecular bands form direct myocardial continuity between the atrioventricular canal and the apex of the ventricle in zebrafish.[32] Therefore, cTnC may play a role in these trabecular bands.

It is interesting to observe the activities of adult fish derived from the homozygotes of the CA17 transgenic line after continuous treatment with Dox. The movement of cardiomyopathy-induced fish was slower, and 30% of them experienced early death; whereas fish from the T03 control group lived normally. It seemed that the low cardiac output failed to supply adequate perfusion to skeletal muscles, resulting in a slurring of body movements. The high mortality of fish from the CA17 line after induction may have been due to

either a power failure or conduction abnormality of the hearts. These phenomena are frequently observed in human patients with heart failure.

Recently, the zebrafish has become an important vertebrate model organism, because this species can be used to study human diseases, physiology, and pharmacology.[33] The cTnC antisense zebrafish in this study offers a new investigative tool for human heart failure.

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Table 1. Cardiac measurements of F2 embryos at 6 dpf from transgenic fish after continuously Dox induction from 12 hpf.

	T03 (*N* = 15)	CA17 (*N* = 26)	*p* value
Diastolic			
Long diameter (um)	159±5	164±14	0.106
Short diameter (um)	99±5	109±6	< 0.01
Systolic			
Long diameter (um)	130±6	132±13	0.24
Short diameter (um)	87±4	90±4	0.02
End-diastolic volume (um^3)	870,226±122,525	1,027,030±157,561	0.13
End-systolic volume (um^3)	491,800±45,258	568,976±837,191	< 0.01
Ventricular ejection fraction (%)	50±3	44±4	< 0.01
Heart rate (beats/min)	194±11	150±10	< 0.01

T03, control transgenic line which expressed the GFP after induction; CA17, transgenic line which expressed the GFP and transcribed the antisense strand of cTnC after induction.

Table 2. Cardiac measurements of F2 embryos at 12 dpf from transgenic fish after continuous Dox induction from 12 hpf.

	T03 (*N* = 15)	CA17 (*N* = 20)	*p* value
Diastolic			
Long diameter (um)	225±8	252±16	< 0.01
Short diameter (um)	125±9	146±18	< 0.01
Systolic			
Long diameter (um)	196±8	211±19	< 0.01
Short diameter (um)	110±4	124±14	< 0.01
End-diastolic volume (um^3)	1,831,044±245,255	2,870,476±846,137	< 0.01
End-systolic volume (um^3)	1,303,185±155,464	1,738,336±472,814	< 0.01
Ventricular ejection fraction (%)	52±4	39±5	< 0.01
Heart rate (beats/min)	168±8	128±12	< 0.01

T03, control transgenic line which expressed the GFP after induction; CA17, transgenic line which expressed the GFP and transcribed the antisense strand of cTnC after induction.

Table 3. Prevalence of abnormal cardiac measurements in F2 embryos derived from the cTnC-antisense transgenic line (CA17) of zebrafish after induction by Dox.

CA17 (*N* = 26, 6 dpf)		CA17 (*N* = 20, 12 dpf)	
	n (%)		*n* (%)
Diastolic		Diastolic	
Abnormally long diameter (> 169 µm)	7 (26.9%)	Abnormally long diameter (> 241 µm)	14 (70.0%)
Abnormally short diameter (> 110 µm)	14 (53.8%)	Abnormally short diameter (> 142 µm)	10 (50.0%)
Systolic		Systolic	
Abnormally long diameter (> 141 µm)	5 (19.2%)	Abnormally long diameter (> 211 µm)	9 (45.0%)
Abnormally short diameter (> 94 µm)	1 (3.8%)	Abnormally short diameter (> 118 µm)	13 (65.0%)
Abnormally end-diastolic volume (> 115276 μm^3)	4 (15.4%)	Abnormally end-diastolic volume (> 2321554 μm^3)	12 (60.0%)
Abnormally end-systolic	10 (38.5%)	Abnormally end-systolic	10 (50.0%)

volume (> 581424 μm^3)		volume (> 1614112 μm^3)	
Abnormally ventricular ejection fraction (< 44%)	12 (46.2%)	Abnormally ventricular ejection fraction (< 45%)	18 (90%)
Abnormally heart rate (< 173 beats/min)	26 (100%)	Abnormally heart rate (< 153 beats/min)	20 (100%)

Legends

Fig. 1. Molecular cloning of cardiac troponin C (cTnC). In vertebrates, the amino acid sequences of cTnC are highly conserved from fish to mammal. The overall identities are exceed 90% not only in the 4 calcium-binding domains but also in the entire fragment. The nucleotide sequences of others species were obtained form the NCBI EST database (a). In the phylogenic tree, zebrafish troponin C was clustered in the ss/cTnC group (b). The full coding region sequence is shown in (c).

Fig. 2. Expression pattern of the zebrafish cardiac troponin C (cTnC) gene. Images of 18 hpf (A) and 24 hpf (B) are in the dorsal view; the image at 48 hpf (C) is in the ventral view; and image at 72 hpf (D) is left-side up. The expression of cTnC was restricted to the heart field from fusion of the 2 bilateral cells (at 18 hpf) to the mature heart (at 72 hpf).

Fig. 3. Construction map of antisense knockdown of cardiac troponin C (cTnC). After adding Dox, the transactivator, rtTA-M2, was driven by the *cmlc*2 promoter combined with Dox after 16 hpf. This complex was capable of binding the bidirectional promoter Tet-O and simultaneously drove the

expression of the reporter green fluorescent protein (GFP) and the transcription of cTnC antisense RNA.

Fig. 4. Detection of the expression of green fluorescent protein (GFP) and the transcription of cardiac troponin C (cTnC) antisense RNA. The 12-hpf embryos derived form the cTnC-antisense transgenic line CA17 were collected and incubated in 10 μg/ml Dox. Green fluorescence was observed in the heart at 48 hpf (A). The reverse-transcription polymerase chain reaction was used to detect the transcription of endogenous cTnC mRNA and the transcription of cTnC antisense RNA (B). Embryos were treated with Dox after 12 hpf, and the total RNA was extracted when these embryos reached 6 dpf. Antisense cTnC was strongly detected in the CA17 group after induction. Although the antisense RNA of the cTnC gene was slightly detected in the non-induced group, the green fluorescent signal was not detected. Expression of the endogenous cTnC gene was almost the same in the T03 and CA17 groups. This suggests that the antisense of cTnC did not interfere in the expression of the endogenous cTnC gene. Arrow indicates the position of the heart.

Fig. 5. Heart rates at different stages in the T03 and CA 17 groups. Cardiomyopathy in F2 embryos was induced with 10 μg/ml Dox at 12 hpf. The

heart rates of embryos derived from the CA17 group were significant slower than those of embryos from theT03 group at 6 and 12 dpf. ∗ $p < 0.01$.

Fig. 6. Changes in ventricular morphology. The end-systolic and end-diastolic ventricular dimensions of the F2 embryos of the CA17 group were larger than those of the T03 group.

Fig. 7. Asynchronized contractions of the atrium and ventricle were observed in some F2 embryos derived from the CA17 group.