Identification of biomarkers and mechanisms of diabetic cardiomyopathy using microarray data

Hui Li*, Xiaoyan Li*, Jian Guo, Guifu Wu, Chunping Dong, Yaling Pang, Shan Gao, Yangwei Wang

Department of Endocrinology, Shaanxi Provincial People’s Hospital, Xi’an, China

Abstract
Background: The study aimed to uncover the regulation mechanisms of diabetic cardiomyopathy (DCM) and provide novel prognostic biomarkers.

Methods: The dataset GSE62203 downloaded from the Gene Expression Omnibus database was utilized in the present study. After pretreatment using the Affy package, differentially expressed genes (DEGs) were identified by the limma package, followed by functional enrichment analysis and protein–protein interaction (PPI) network analysis. Furthermore, module analysis was conducted using MCODE plug-in of Cytoscape, and functional enrichment analysis was also performed for genes in the modules.

Results: A set of 560 DEGs were screened, mainly enriched in the metabolic process and cell cycle related process. Hub nodes in the PPI network were LDHA (lactate dehydrogenase A), ALDOC (aldolase C, fructose-bisphosphate) and ABCE1 (ATP Binding Cassette Subfamily E Member 1), which were also highlighted in Module 1 or Module 2 and predominantly enriched in the processes of glycolysis and ribosome biogenesis. Additionally, LDHA were linked with ALDOC in the PPI network. Besides, activating transcription factor 4 (ATF4) was prominent in Module 3; while myosin heavy chain 6 (MYH6) was highlighted in Module 4 and was mainly involved in muscle cells related biological processes.

Conclusions: Five potential biomarkers including LDHA, ALDOC, ABCE1, ATF4 and MYH6 were identified for DCM prognosis. (Cardiol J 20xx; xX: xx–xx)

Key words: diabetic cardiomyopathy, expression profile, differential analysis, module analysis, glycolysis, ribosome biogenesis

Introduction
Type 2 diabetes mellitus (T2DM) remains a life-threatening disease worldwide with increasing incidence [1, 2]. The predominant cause of death for T2DM patients was cardiovascular disease [3]. The diabetic cardiomyopathy (DCM) has been recognized as ventricular dysfunction in the absence of hypertension and coronary artery disease, may increase the risk of developing heart failure [4]. Moreover, DCM has been defined as a primary disease progressing into a metabolic disturbance that was mainly due to the elevation of free fatty acid (FFA) and the alteration of glucose metabolism, and would change the myocardial structure and function [5, 6]. It was reported that the mortality of patients with DCM was 42%, and the ST-segment elevation myocardial infarction (STEMI) and non-STEMI mortality in diabetic patients were 72% and 67%, respectively [7]. Currently, there were no specific therapeutic interventions for this predominant complication, except a paucity of proposed drugs such as eplerenone [8]. The understanding of mechanisms on DCM progression would facilitate finding novel targets for treatment of this disease. Several mechanisms in...
charge of DCM were proposed. For instance, it was confirmed that FFA-mediated apoptosis, hypertrophy, and contractile dysfunction were the causative factors for DCM [6]. Oxidative stress was another major cause for the pathogenesis of DCM [9]. The overexpression of insulin like growth factor 1 was reported to act as an inhibitor in DCM development [10]. A more recent study elaborated molecular mechanisms that contributed to functional alterations in the diabetic heart and consequently identified several crucial advanced glycation end products (AGEs), fibrosis related genes including poly (ADP-Ribose) polymerase 1 (PARP-1), Otsuka Long Evans Tokushima fatty (OLETF) and matrix metalloproteinases 2 (MMP-2), inflammatory cytokines such as interleukin-1beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta1 (TGF-β1) and altered pathways like mitogen-activated protein kinase (MAPK signaling) and TGF-β signaling, as well as critical miRNAs (miR-143, miR-181, miR-103, miR-107 and miR-802) [11]. However, previous informative findings only partially elucidated the molecular mechanism involved in DCM, and future study for comprehensive illustrating the primary genes and the pathways for the prevention of DCM was needed.

So far, the patient-specific induced pluripotent stem cells (iPSCs) model has been applied to mimic the DCM condition and dilated cardiomyopathy, to investigate therapeutic strategies or epigenetic regulations in these diseases [12–14]. Among them, Drawnel el al. [13] used a patient-specific induced iPSC model to exhibit metabolic disorders during the progression of DCM and finally screened several remarkable molecular drugs such as W7 (calmodulin), penitrem A (sodium and potassium channel blocker) and MCBQ (PDE5 inhibitors) for the prevention of DCM. Although several gene alterations such as the elevated MYL2, MYL4 and PLN; and the decreased NPPA, NPPB and ACTA1 were validated, the interactions among them and their functions were not interpreted, and thus lacked evidence for the prediction of potent therapeutic targets. Therefore, the expression profile GSE62203 deposited by Drawnel et al. [13] was re-analyzed to identify critical genes by extensive bioinformatical methods including differential analysis, protein–protein interaction (PPI) network and module analysis. Based on the above analyses, the aim herein was to uncover the interrelated regulation mechanisms of DCM and provide novel biomarkers for detection and prevention of DCM.

Methods

Gene expression data

A data set of the gene expression profile GSE62203 containing 4 treated samples (human iPS-derived CMs exposed to glucose, endothelin-1 and cortisol for 2 days in vitro) and 4 untreated samples (vehicle-control treated) was utilized in this study, which was deposited by Drawnel el al. [13] in the public Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) database. In the Drawnel study, CMs were derived from CDI-MRB iPSCs (cellular dynamics international [CDI]). After being cultured for 2 days with conditions of 37°C and 7% CO₂, the plating medium for the CMs was changed for maturation medium (MM) for 3 days. After 3 days, the MM was exchanged for DM (MM+ glucose, endothelin and cortisol) for treated samples or MM+ vehicle control for untreated samples for another 2 days. Thus, the DCM condition was established. The platform for the expression profile was Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix Inc., Santa Clara, California, USA).

Data preprocessing and differential analysis

The Affy package in Bioconductor (http://www.bioconductor.org/packages/release/bioc/html/affy.html) [15] was employed to perform the pretreatment. The raw data were subjected to background correction, quantile data normalization and probe summarization recruiting the robust multi-array average (RMA) algorithm [16]. After obtaining the gene expression matrix, differentially expressed genes (DEGs) between the 2 kinds of samples were selected based on a t-test using linear models for microarray data (limma, http://www.bioconductor.org/packages/release/bioc/html/limma.html) package of Bioconductor R [17]. The cut-off values for the DEGs identification were p < 0.05 and |log2 fold change| > 0.5.

Functional enrichment analysis for the DEGs

To explore the altered biological process (BP) and pathways, the DEGs were mapped into gene ontology ([GO], http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes ([KEGG], http://www.genome.jp/kegg/pathway.html) databases, using Database for Annotation, Visualization and Integration Discovery ([DAVID], http://david.abcc.ncifcrf.gov/) online tool [18] with the Modified Fisher Exact test [19]. The p-value < 0.05 and the count (number of the genes) > 2 were set as the threshold for significant BP terms and pathways.
Construction of PPI network

To further explore potential correlations from the protein level, which facilitated to illustrate the underlying molecular mechanisms, identified DEGs were mapped into the Search Tool for the Retrieval of Interacting Genes/Proteins ([STRING], http://string-db.org/) database [20]. The PPI network of protein products of the genes was established, containing pairwise interactions with required confidence (combined score) > 0.4. A protein in the network was considered as a ‘node’ and the ‘degree’ of a node referred to the interaction pair numbers of a protein. The degree was calculated for each node using connectivity degree analysis. The ‘hub’ node in the network was deemed as the node with high degrees.

Module analysis of the PPI network

Functional modules of the network was extracted using the MCODE [21] plug-in of Cytoscape software with default parameters (Degree Cutoff: 2, Node Score Cutoff: 0.2, K-Core: 2, Max. Depth: 100) for selection. Subsequently, high scored modules with substantial nodes were further screened out for enrichment analysis, as described above.

Results

DEGs between treated and untreated samples

Based on the aforementioned criteria, a cohort of 560 DEGs was identified between the treated and untreated samples, consisting of 264 up-regulated genes and 296 down-regulated genes (Supplementary material 1).

BPs and pathways altered in the treated sample

After GO and KEGG enrichment analysis, the up-regulated DEGs were mainly enriched in metabolic BP terms such as generation of precursor metabolites and energy (GO: 0006091), hexose metabolic process (GO: 0019318), monosaccharide metabolic process (GO: 0005996) and glucose metabolic process (GO: 0006006); and besides response to wounding (GO: 0009611); response to organic substance (GO: 0010033), regulation of cell proliferation (GO: 0042127); while the down-regulated DEGs were significantly enriched in the processes including positive regulation of macromolecule metabolic process (GO: 0010604), cellular response to stress (GO: 0033554), and the cell control related functions such as regulation of apoptosis (GO: 0042981), regulation of programmed cell death (GO: 0010941), cell cycle (GO: 0007049) and positive regulation of cellular biosynthetic process (GO: 0031328) (Table 1).

The over-represented pathways for the up-regulated DEGs were glycometabolism and proteometabolism related pathways including glycolysis/gluconeogenesis (hsa00010), fructose and mannose metabolism (hsa00051), pentose phosphate pathway (hsa00030), starch and sucrose metabolism (hsa00500), arginine and proline metabolism (hsa00330), cysteine and methionine metabolism (hsa00270); by contrast, the prominent ones for down-regulated DEGs were aminoacyl-tRNA biosynthesis (hsa00970) and arginine, and proline metabolism (hsa00330) (Table 2).

The PPI network of the DEGs

By mapping the DEGs into the STRING database, a PPI network was established, comprising of 317 nodes and 929 interactions. As revealed in Figure 1, the remarkable nodes with high degree (> 20) were GAPDH (degree = 49), FN1 (degree = 30), LDHA (degree = 28), ENO1 (degree = 27), PGK1 (degree = 26), ABCE1 (degree = 25), SOD2 (degree = 23), PKM (degree = 23), GOT1 (degree = 22), HK1 (degree = 22), TPI1 (degree = 21), GPI (degree = 21) and ALDOA (degree = 21).

Functional module network and the enrichment analysis for genes in the modules

According to module analysis of the PPI network, four modules with a high score (> 3) were extracted from the PPI network. There were 14 up-regulated nodes such as ALDOC, LDHA, PGK1 and TPI1 in Module 1 with a final score of 12.923; and 10 nodes including ABCE1, GAR1 and FBL in Module 2 with a final score of 8.222. The Module 3 contained five down-regulated nodes as DDIF3, ATF4, CEBPG, CEBPB and HERPUD1 and achieved a score of 4, while Module 4 consisted of 15 nodes such as CASQ2, CKMT2, IARS, CCT5, ACTA1, CKMT2 and MYH6 and had a score of 3.857 (Fig. 2).

The BP functions of the genes (which encode proteins in the modules) in the four modules were further analyzed. As presented in Table 3, the over-represented BPs for genes in Module 1 were predominantly correlated with the catabolic process of various carbohydrates such as glycolysis (GO:0006096), glucose catabolic process (GO:0006007), monosaccharide catabolic process (GO:0043635) and alcohol catabolic process (GO:0046164); while that for genes in Module 2 were mainly related to ribosome biogenesis and
and contractile dysfunction were the causative factors for DCM [6]. Oxidative stress was another major cause for the pathogenesis of DCM [9]. The overexpression of inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta 1 (TGF-β), as well as the elevated products (AGEs), fibrosis related genes including cysteinyl leukotrienes (CysLTs), and matrix metalloproteinases 2 (MMP-2), inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta 1 (TGF-β), and the elevation of precursor metabolites and energy pathways like mitogen-activated protein kinase (MAPK signaling) and pentose phosphate pathway (PPP) were critical miRNAs (miR-124, miR-181, miR-103, miR-107 and miR-802) [11]. However, previous informative findings only partially elucidated the molecular mechanism involved in DCM, and future study for comprehensive illustrating the primary genes and the pathways for the prevention of DCM will be needed.

Table 1. Biological processes significantly affected by the DEGs in treated samples.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Count</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>Positive regulation of macromolecular metabolic process</td>
<td>28</td>
<td>1.37E-13</td>
</tr>
<tr>
<td>BP</td>
<td>Positive regulation of cellular response to stress</td>
<td>21</td>
<td>1.97E-04</td>
</tr>
<tr>
<td>BP</td>
<td>Regulation of programmed cell death</td>
<td>20</td>
<td>2.32E-02</td>
</tr>
<tr>
<td>BP</td>
<td>Regulation of cell death</td>
<td>20</td>
<td>2.54E-02</td>
</tr>
<tr>
<td>BP</td>
<td>Positive regulation of biosynthetic process</td>
<td>19</td>
<td>1.17E-02</td>
</tr>
<tr>
<td>BP</td>
<td>Regulation of cellular biosynthetic process</td>
<td>19</td>
<td>3.14E-02</td>
</tr>
<tr>
<td>BP</td>
<td>Positive regulation of macromolecular metabolic process</td>
<td>18</td>
<td>6.09E-06</td>
</tr>
<tr>
<td>BP</td>
<td>RNA processing</td>
<td>18</td>
<td>2.41E-03</td>
</tr>
<tr>
<td>BP</td>
<td>Positive regulation of cellular biosynthetic process</td>
<td>18</td>
<td>2.05E-02</td>
</tr>
</tbody>
</table>

So far, the patient-specific induced iPSC model to exhibit metabolic disorders during the progression of DCM and finally screened several therapeutic molecular drugs such as W7 (calmodulin), penitrem A (neuroblastoma cell line blocker) and MCBQ (PDE5 inhibitors) for the prevention of DCM. The informative findings only partially elucidated the molecular mechanism involved in DCM, and the decrease of miR-20A by them and their functions were not interpreted, and thus lacked evidence for the prediction of potent therapeutic targets. Therefore, the expression profile GSE62203 deposited by Drawnel et al. [15] was re-analyzed to identify critical genes by extensive bioinformatic analysis including differential expression, protein–protein interaction (PPI) network and module analysis, and pathway analyses, the aim is to provide novel biomarkers for detection and prevention of DCM.
Figure 1. Protein–protein interaction network of differentially expressed genes in iPS-derived cardiomyocytes treated by glucose, endothelin-1 and cortisol. Circles represent protein products of differentially expressed genes, and red denotes up-regulated, green denotes down-regulated; color depth indicates the significance of differential expressed genes.

Figure 2. Modules of the protein–protein interaction network. A. Module 1; B. Module 2; C. Module 3; D. Module 4. Circles represent protein products of differentially expressed genes, and red denotes up-regulated genes, green denotes down-regulated genes, as well as diamonds stand for hub nodes; color depth indicates the significance of differentially expressed genes.
confirmed that PFA-mediated apoptosis, hypertrophy, and contractile dysfunction were the causative factors of DCM [6]. Oxidative stress was another major cause for DCM [9]. The overexpression of specific genes, as reported to act as inhibitors in DCM development, has been identified [10]. A more recent study elaborated the cellular mechanisms that contributed to functional alterations in the diaphragm and consequently identified several crucial advanced glycation end products (AGEs), hormones related genes including poly (ADP-Ribose) polymerase 1 (PARP-1), Otsuka Long Evans Tokushima fatty (OLETF) and matrix metalloproteinases 2 (MMP-2), inflammatory cytokines such as interleukin-1beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β1) and some other pathways like mitogen-activated protein kinase (MAPK signaling) and TGF-β signaling, as well as critical miRNAs (miR-143, miR-181, miR-103, miR-107 and miR-802) [11]. However, previous informative findings only partially elucidated the molecular mechanisms involved in the pathogenesis of DCM, and further study for comprehensive illustration of key genes and the pathways for the prevention of DCM was needed. So far, the patient-specific induced pluripotent stem cells (iPSCs) for DCM have been used for understanding the DCM condition and the role of focused genes to investigate therapeutic strategies and genetic regulations in these diseases [12–14]. Among them, Drawnel et al. [13] used patient-specific induced iPSC model to exhibit metabolic disorders during the progression of DCM and finally screened several remarkable molecular drugs such as W7 (calmodulin), penitrem A (sodium and potassium channel blocker) and MCBQ (PDE5 inhibitors) for the prevention of DCM. Although several gene alterations such as the expression of actomyosin organization and PLN; and the overexpression of AGEs and metalloproteinase 2 (MMP-2) were previously identified as the crucial roles of protein translation and their pathways were not interpreted, and thus lacked evidence for the potential therapeutic targets. Therefore, the expression profile GSE62203 deposited by Drawnel et al. [13] was re-analyzed to identify critical genes with the metabolic process involved in the cellular biosynthesis such as positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (GO:0045935), positive
regulation of nitrogen compound metabolic process (GO:0051173), positive regulation of cellular biosynthetic process (GO:0031328); and besides the cell control related BPs including regulation of apoptosis (GO:0042981) and regulation of programmed cell death (GO:0043067); whereas the prominent BPs for the genes in the Module 4 were involved in the processes relating to muscle cells such as muscle contraction (GO:0006936), muscle system process (GO:0003012) and muscle cell development (GO:0055001).

Discussion

The DCM is defined as ventricular dysfunction that occurs in diabetic patients [22] and the iPSC model was applied to detect the metabolic alterations and screen potential genes and molecular drugs [13, 23]. In the present study, the expression profile GSE62203 was utilized to conduct a series of bioinformatic analyses and as a result, identify a cohort of 560 DEGs between treated and untreated samples. The hub nodes in the PPI network were LDHA, ALDOC and ABCE1, which were also highlighted in Module 1 or Module 2 and predominantly enriched in the glycolysis and ribosome biogenesis. Besides, ATF4 was prominent in Module 3; while MYH6 was highlighted in Module 4 which was mainly involved in muscle cells related BPs.

The LDHA (lactate dehydrogenase A) is one of the subunits of LDH which play significant roles in the final step of anaerobic glycolysis by interconversion of pyruvate and lactate using NADH/NAD+ as a co-substrate to allow continuous energy production [24]. It was reported that overexpression of LDHA activity may influence normal glucose metabolism and insulin secretion in the islet beta-cell type, and also result in insulin secretory defects in some forms of T2DM [25, 26]. In addition, the overexpression of LDHA activity might increase the lactate level and lactate–pyruvate interconversion rates in diabetes patients [27]. Similarly, the increased level of LDH was observed in the diabetic group, while luteolin exerted a protective effect against DCM by reducing the content of LDH in serum [28]. Hypoxia-inducible factor (HIF)-1α, was a crucial transcription factor in brain ischemic pre-conditioning [29] and the expression of HIF-1α was decreased by a diabetic environment [30]. Partial deficiency of HIF-1α was proposed to increase the risk of DCM, and interestingly, LDHA was one of the target genes of HIF-1 that is involved in glucose metabolism and was upregulated in the HIF-1α heterozygous-null mutants [31]. In the present study, LDHA was the striking node in both PPI network and Module 1, and significantly enriched in glycolysis, giving potent evidence that LDHA might emerge as a central regulator in the progression of DCM via disturbing the glycolysis process.

ALDOC (aldolase C, fructose-bisphosphate) encodes a member of the class I fructose-bisphosphate aldolase family gene, which acts as a catalyst that catalyzes the reversible aldol cleavage of fructose-1,6-bisphosphate and fructose 1-phosphate to dihydroxyacetone phosphate and either glyceraldehyde-3-phosphate or glyceraldehyde, respectively in the glycolysis process [32]. Increased glucose was one hallmark of diabetes mellitus (DM) and ALDOC was one of the enzymes that promoted glycolysis and was induced by the elevated glucose [33]. Then, the up-regulated ALDOC was a positive correlation with the increase of FFA in plasma which might impair insulin secretion to develop T2DM [34, 35]. Additionally, ALDOC was up-regulated in the heart tissue in a rodent model of myocardial I/R injury [36]. Though no direct evidence existed that ALDOC and LDHA were interplayed with regard to diabetes or cardiomyopathy, it was indicated that ALDOC and LDHA were both up-regulated in a cervical cancer cell line of paclitaxel-resistant HeLa sublines [37]. On the other hand, DM was tightly related to the risk of various cancers including cervical cancer [38]. Notably, ALDOC and LDHA were both linked to HIF-1, which was associated with the risk of DCM as mentioned above [31]. These findings collectively suggested that the interacted ALDOC and LDHA might be involved in the regulation of the glycolysis process during DCM progression, as predicted by the current module analysis and enrichment analysis. However, more validations are needed to confirm the regulatory relationship between the two genes.

The ABCE1 encoded ATP Binding Cassette Subfamily E Member 1 which belongs to a family member of the ATP-binding cassette (ABC) transporters and is primarily known as RNase L inhibitor (RLI) [39]. Zeng et al. [40] had indicated that RNase L activation was responsible for type I diabetes, and it was also suggested that the increased expression of RNase L or down-regulated of its inhibitor (RLI) might enhance the insulin response in muscle cells of obese people [41]. Additionally, further studies demonstrated that the mutation of ABCB and gene polymorphisms of ABCG8 and ABCG5 have been linked to T2DM.

7

Hui Li et al., Biomarkers of diabetic cardiomyopathy
had interplayed with DCM, and it still needs further confirmation via extensive bioinformatical methods including gene expression profile GSE62203 deposited by Drawnel et al. [7, 12]. Among them, the cardiac muscle myosin MYH6 was diminished under the hypertrophic stress (DM-treated with CMs) [13] and was considered a cardiac marker by fluorescent analysis in an iPSC model of dilated cardiomyopathy. Cell Stem Cell. 2014; 9(3): 810–821, doi: 10.1016/j.stem.2015.04.020.

None declared

Conflict of interest: None declared

References


charge of DCM were proposed. For instance, it was confirmed that TRAIL-mediated apoptosis, hypertrophy, and contractile dysfunction were the causative factors for DCM [6]. Oxidative stress was another polymorphisms at type 2 diabetes mellitus in the Turkish population for the pathogenesis of DCM [9]. The overexpression of insulin-like growth factor 1 was reported to act as an inhibitor in DCM development [10]. A more recent study elaborated molecular mechanisms that contributed to functional and structural alterations in the diabetic heart and consequently identified several crucial advanced glycation end products (AGEs), fibrosis related genes including poly (ADP-Ribose) polymerase 1 (PARP-1), Otsuka Long Evans Tokushima fatty (OLETF) and matrix metalloproteinases 2 (MMP-2), inflammatory cytokines such as Interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta1 (TGF-β1) and altered pathways like mitogen-activated protein kinase (MAPK signaling) and TGF-β signaling, as well as critical miRNAs (miR-143, miR-181, miR-103, miR-107 and miR-802) [11]. However, previous informative findings only partially elucidated the molecular mechanism involved in DCM, and future study for comprehensive illustrating the primary genes and the pathways for the prevention of DCM was needed.

So far, the patient-specific induced pluripotent stem cells (iPSCs) model has been applied to mimic the DCM condition and dilated cardiomyopathy, to investigate therapeutic strategies or epigenetic regulations in these diseases [12–14]. Among them, Drawnel et al. [13] used a patient-specific induced iPSC model to exhibit metabolic disorders during the progression of DCM and finally screened several remarkable molecular drugs such as W7 (calmodulin), penitrem A (sodium and potassium channel blocker) and MCBQ (PDE5 inhibitors) for the prevention of DCM. Although several gene alterations such as the elevated MYL2, MYL4 and PLN; and the decreased NPPA, NPPB and ACTA1 were validated, the interactions among them and their functions were not interpreted, and thus lacked evidence for the prediction of potent therapeutic targets. Therefore, the expression profile GSE62203 deposited by Drawnel et al. [13] was re-analyzed to identify critical genes by extensive bioinformatical methods including differential analysis, protein–protein interaction (PPI) network and module analysis. Based on the above analyses, the aim herein was to uncover the interrelated regulation mechanisms of DCM and provide novel biomarkers for detection and prevention of DCM.