Heat shock protein 70 (HmHsp70) from Hypsizygus marmoreus confers thermotolerance to tobacco

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Abstract

The 70-kD heat shock proteins (Hsp70s) have been proved to be important for stress tolerance and protein folding and unfolding in almost all organisms. However, the functions of Hsp70s in mushroom are not well understood. In the present study, a hsp70 gene from Hypsizygus marmoreus, hmhsp70, was cloned and transferred to tobacco (Nicotiana tabacum) to evaluate its function in thermotolerance. Sequence alignments and phylogenetic analysis revealed that HmHsp70 may be located in the mitochondria region. qPCR analysis revealed that the transcription level of hmhsp70 in H. marmoreus mycelia increased after heat shock treatment in high temperature (42 °C) compared with untreated mycelia (at 25 °C). Transgenic tobaccos expressing hmhsp70 gene showed enhanced resistance to lethal temperature compared with the wild type (WT) plants. Nearly 30% of the transgenic tobaccos survived after treated at a high temperature (50 °C and 52 °C for 4 h); however, almost all the WT tobaccos died after treated at 50 °C and no WT tobacco survived after heat shock at 52 °C. This study firstly showed the function of a hsp70 gene from H. marmoreus.

Keywords: Heat shock protein 70, Hypsizygus marmoreus, Thermotolerance, Transgenic tobacco
The role of Hsp70s in heat stress response in filamentous fungi has been studied for many years (Britton and Jacobsen 1984; Miura et al. 2006; Werner-Washburne and Craig 2002; Su and Li 2008). Mutations with hsp70 deletions reduced thermotolerance in these organisms, and the thermotolerance defect could be complemented by expression of hsp70 genes. These studies proved that Hsp70 is essential for organisms to survive a severe heat shock. Besides, thermotolerance could not only be given by the overexpression of its own hsp70 genes but also by transgenic hsp70 genes from other organisms. The high conservation of Hsp70 gives its functional properties across the species. Overexpression of herbaceous peony Hsp70, PHHSP70, confers A. thaliana high temperature tolerance (Zhao et al. 2019). Transgenic Arabidopsis expressing the hsp70 gene from T. harzianum T34 exhibited enhanced tolerance to heat, oxidative, osmotic and salt stresses (Montero-Barrientos et al. 2010). Therefore, it is a feasible way to study the function of Hsp70 in model organism.

In fungi, Hsp70 proteins perform chaperone dependent or independent functions and play a major role in various stress conditions (Tiwari et al. 2015). Fourteen genes of the Hsp70 family have been characterized in yeast and at least five of them are heat shock inducible (Boorstein and Craig 1990; Craig and Jacobsen 1984; Miura et al. 2006; Werner-Washburne and Craig 1989; Young and Craig 1993). Mutation or a function of these hsp70 genes would cause a temperature sensitive growth (Craig and Jacobsen 1984; Nelson et al. 1992). The role of Hsp70s in heat stress response in filamentous fungi has been studied for many years (Britton and Kapoor 2002; Caruso et al. 1987; Montero-Barrientos et al. 2008). Higher transcription level was observed when two pathogenic fungi, Histoplama capsulatum G222B and H. capsulatum Downs, were shocked at 34 °C or 37 °C. The strain, G222B, with higher thermostability has higher hsp70 transcription level (Caruso et al. 1987). Overexpression of hsp70 gene in Trichoderma harzianum T34 gave rise to transformants with high quantities of biomass obtained after heat shock treatment (Montero-Barrientos et al. 2008). Recently, some studies have related these proteins with heat stress resistance in edible mushrooms. The expression of hsp70 genes in Lentinula edodes and Pleurotus ostreatus were upregulated under heat stress (Fu et al. 2016; Wang et al. 2018a; Zou et al. 2018), and the Hsp70s were more highly expressed in heat-tolerance strain than heat-sensitive strain. Even so, functional studies of hsp70 genes are quite limited in mushroom compared to yeast and filamentous fungi.

In the previous study, we analyzed the global protein expression of H. marmoreus mycelia under optimal temperature (25 °C) and high temperature (42 °C) (Liu et al. 2014) by two-dimensional polyacrylamide gel electrophoresis. Seven highly expressed proteins, including a Hsp70 protein, were identified by MALDI-TOF-MS analysis, and these proteins were considered to provide the heat resistance to H. marmoreus. Based on these results, in this study, we cloned the Hsp70 protein coding gene, hmhsp70, from H. marmoreus and transformed tobacco with hmhsp70 gene to determine the role of HmHsp70 in heat-shock response of plants. Our data indicated that HmHsp70 could give thermotolerance to tobacco.

**Materials and methods**

**Strain and plant material and cultivation conditions**

Hypsizygus marmoreus G12 provided by Shandong Provincial Key Laboratory of Applied Mycology was used in this study. The wild type (WT) tobacco (Nicotiana tabacum var. Xanthinc) was provided by Key Lab of Plant Biotechnology in Universities of Shandong Province. Escherichia coli Trans1-T1 (Transgen, Beijing, China) was used as host for plasmid construction and propagation. Agrobacterium tumefaciens LBA4404 was used as the T-DNA donor for tobacco transformation. H. marmoreus mycelia were cultivated in potato dextrose agar (PDA: extract from 200 g potato, 20 g dextrose, 20 g agar) media in 90 mm diameter petri dishes sealed with plastic wrap. The plates were incubated in a growth chamber at 25 ± 1 °C in darkness. For subculture, mycelial plugs (8 mm diameter) were cut from the margin of old agar cultures and placed in the center of the new media. WT tobacco was germinated in 1/2 Murashige and Skoog (MS) medium in a growth chamber at 25/22 °C (day/night) under a 16/8 h (day/night) photoperiod with illumination intensity of 3000 Lx. E. coli cells were grown in Luria-Bertani (LB) broth or on LB plates supplemented with kanamycin (Kan, 50 μg/mL) when required. A. tumefaciens cells were grown in minimal media (10 mM K2HPO4, 10 mM KH2PO4, 2.5 mM NaCl, 2 mM MgSO4·7H2O, 0.7 mM CaCl2, 9 μM FeSO4·7H2O, 4 mM (NH4)2SO4, 10 mM glucose, pH 7.0).
Cloning the full-length cDNA of hmhsp70 gene

The genome of *H. marmoreus* mycelia was extracted using EZNA Fungal DNA Mini Kit (Omega Bio-Tek, USA) according to the manufacture's instruction. *H. marmoreus* mycelia were treated at 42 °C for 1 h, and then the total RNA was extracted from the treated mycelia frozen in liquid nitrogen by Trizol reagent method (RNAsio Plus, Takara, Japan) according to the manufacturer's protocol. cDNA library was prepared using RNA PCR Kit (AMV) Ver.3.0 (Takara, Japan) according to the manufacture's instruction. The DNA fragment of *hmhsp70* gene was amplified using degenerate primers De-F (5′-GCTGTHTRTYACHGTYCCAGCTTAYTTC-3′)/De-R (5′-RGCDACRGCYTCTRCNGGRTTAT-3′) with *H. marmoreus* genomic DNA as the template. The PCR product was sequenced using Sanger sequencing method in Sangon Biotech (Shanghai, China) Co. The 3′ end and 5′ end of the *hmhsp70* mRNA was confirmed using 3′ RACE and 5′ RACE methods using SMART TM RACE cDNA Amplification Kit (Clontech, CA, USA) according to the manufacture's instruction. The whole cDNA fragment of *hmhsp70* gene was amplified using primers HSPorf-F (5′-GGGGACCTTTCTCTCTATT-3′) and HSPorf-R (5′-GATTTGTGATGATGAG-3′) using the product of 5′ RACE as the template, and cloned into pMD18-T plasmid (Takara, Japan) according to the manufacture's instruction. The sequence of inserted DNA fragment was sequenced by Sanger sequence in Sangon Biotech (Shanghai, China) Co.

Construct the recombinant plasmid and plant transformation

The plant transformation vector pROK2 was used for tobacco transformation. The *hmhsp70* gene was cloned with primers ROK-hsp-F (5′-TGCTCTAGACTCTAT TCCCTACCATGTGGTC-3′), the XbaI restriction site is underlined) and ROK-hsp-R (5′-CGGGGTACCTTG CGACTAAAGATATGCT-3′), the KpnI restriction site is underlined) using cDNA as the template. The *hmhsp70* gene fragment was purified by gel purification kit (San-is underlined) using cDNA as the template. The plant transformation vector pROK2 was used for tobacco transformation. The *hmhsp70* gene was cloned into pROK2-hsp (cut with the same restriction enzymes) to produce pROK2-hyg-hsp.

The recombinant plasmid, pROK2-hyg-hsp, was introduced into *A. tumefaciens* LBA4404 by freeze-thaw method (Hofgen and Willmitzer 1988). Transformation of tobacco leaf disc was performed using an *Agrobacterium* mediated transformation method as described by Horsch et al. (Horsch et al. 1985; Voelker et al. 1987). Tobacco transformants were selected on MS medium supplemented with 0.5 μg/mL 6-benzylaminopurine, 400 μg/mL cefotaxime and 60 μg/mL Kan for 2 d and then they were transferred into selection MS medium plate containing 150 μg/mL Kan and 25 mg/L Hyg. Adventitious shoots were transferred to rooting medium supplemented with 0.5 μg/mL 1-naphthylacetic acid and 20 mg/L sucrose. Then, the transgenic and WT tobaccos were transferred into tissue cultivation bottles and grown in a controlled environmental growth chamber under the conditions described above.

Thermotolerance assay

Tobacco of the WT and transgenic type were used for the thermotolerance assay using a growth chamber. WT and transgenic tobacco plants were shocked at 46 °C, 48 °C, 50 °C and 52 °C for 4 h, then further incubated at 25 °C for a week. Thermotolerance was evaluated based on survival number.

Real-time quantitative PCR (qPCR) analysis of expression of *hmhsp70* gene

*H. marmoreus* mycelia on PDA plate were exposed at 42 °C for 0.5 h, 1 h, 2 h, 3 h and 4 h, respectively. Then the total RNA was isolated from mycelia immediately with Trizol reagent (RNAsio Plus, Takara), respectively. cDNA was synthesized as mentioned above. qPCR was performed using an Optical 96-well Fast Thermal Cycling Plate with the ABI 7500 real-time PCR system (Applied Biosystems, San Francisco). The reaction system was prepared using 2× SYBR Green SuperReal PreMix Plus (TianGen, Beijing, China) according to the manufacture's instruction in a total volume of 15 μL for each tube. The thermal cycle used was 95 °C for 2 min, then 40 cycles of 10 s at 95 °C, 40 s at 60 °C. The 18S rRNA gene was used as internal reference gene. To detect the expression of *hmhsp70* genes in transgenic tobaccos, total RNA was extracted from the leaf of the transgenic tobaccos. qPCR reaction was used to detect the expression of *hmhsp70* gene, and the *actin* gene was used as internal reference gene. Primers used in qPCR reactions are listed in Table 1. Relative expression levels were calculated using the relative 2^−(ΔΔCt) method, and the expression of *hmhsp70* was normalized to the expression level of the 18S rRNA or *actin* gene.
Table 1 Primers used for real-time qPCR analysis

| Primer name | Sequence (5′–3′) |
|-------------|------------------|
| Q-actin-F   | CCTGAGGTCTTTTCCAACCA |
| Q-actin-R   | GGAATCCGGCAGCTTCAATT |
| Q-18S-F     | GAGGGACTGAGAAACG |
| Q-18S-R     | ATAGACCCGAAAGGCCC |
| Q-hmhsp70-F | GCTCACTCATTTGCCGGAATA |
| Q-hmhsp70-R | CCGACAAGACGTTACCCAGC |

Data analysis

Multiple sequence alignment of protein sequences was performed by Clustal X algorithm (Larkin et al. 2007). Phylogenetic tree analysis was performed with Mega 6.0 software (Tamura et al. 2013).

Nucleotide sequence accession numbers

The 2001 bp nucleotide sequence of hmhsp70 gene has been deposited in the GenBank DNA database under accession number of GQ246176.

Results

Clone and sequence analysis of HmHsp70 from H. marmoreus

Partial sequence of hsp70 gene was amplified from cDNA library of heat treated H. marmoreus using degenerate primers. The primers were designed according to conserved sequences of heat shock gene from Achyila klebsiana (AKU02504), Candida glabrata (AY077689), Rhizopus stolonifer (AY147869). The cDNA ends of hsp70 gene were confirmed by 3′ RACE and 5′ RACE methods. A 2308 bp DNA fragment, which contains a 2001 bp coding sequence, was obtained and designated as hmhsp70 gene (GQ246176). The hmhsp70 gene contains 10 exons and 9 introns. The deduced HmHsp70 protein contains 667 amino acids and the calculated molecular weight of HmHsp70 is 72.2 kDa. This is the first hsp70 gene cloned from H. marmoreus, which may be helpful to uncover the underlying molecular mechanism of thermostolerance in H. marmoreus.

A BLAST search in GenBank database was performed and revealed that HmHsp70 of H. marmoreus shared high homology with HSPs in other basidiomycetes fungi such as Pleurotus ostreatus, Hypholoma sublateritium and Psilocybe cyanescens. Three Hsp70 family signatures were found in HmHsp70 sequence. The first signature (IDLGTTNS) was found at positions 39–46 in the N-terminal section, and the others were located at positions 227–240 (VYDLGGGTFDISIL) and positions 368–380 (VILVGGMTRVPRV) in the central part of HmHsp70 (Fig. 1). To investigate the evolutionary relationship of HmHsp70 and other Hsp70 proteins, a neighbor-joining tree was constructed after the alignment of Hsp70 proteins from different organisms: HmHsp70, ten Hsp70 proteins with highest sequence identity to HmHsp70 in UniProt database, and Hsp70 proteins with highest sequence identity to HmHsp70 from human, plant, bacteria, nematoda, tobacco, maize, Arabidopsis, Saccharomyces, fission yeast, Aspergillus, Penicillium, Beauveria and Ustilago, respectively (Fig. 2). HmHsp70 is associated with Hsp70 proteins from basidiomycetes fungi, and exhibits the highest sequence identity (88.7%) with Hsp70 from P. ostreatus PC15. It could be speculated that Hsp70s from basidiomycetes fungi evolved from the same ancestral HSP protein. Furthermore, the Hsp70 proteins from plants, animal, yeast, filamentous fungi and bacteria are clustered, respectively. The results indicated that Hsp70 proteins are highly conserved and could be used as molecular scale in biological classification.

Expression of hmhsp70 gene in H. marmoreus

The expression pattern of hmhsp70 gene in H. marmoreus was investigated by qPCR analysis. H. marmoreus mycelia were exposed to a high temperature of 42 °C for 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h, respectively. After heat stress treatment, total RNA was isolated from mycelia. cDNA library was synthesized from RNA and used as the template for qPCR analysis. The expression of hmhsp70 was significantly increased after heat shock at 42 °C (Fig. 3). The expression level was rapidly triggered and peaked at 0.5 h with a 1.48-fold increase (Fig. 3). After 0.5 h, the expression of hmhsp70 gradually decreased. The expression level of hmhsp70 still remained at a significant high level at 4 h compared to untreated mycelia. The results of qPCR confirmed the involvement of hmhsp70 in heat shock response in H. marmoreus.

Transformation and constitutive expression of hmhsp70 gene in tobacco

To clarify the role of hmhsp70 gene in thermostolerance, we introduced hmhsp70 gene into tobacco. Tobacco is an ideal platform to test the effect of stress response proteins not only from plants but also from other species (Sanmiya et al. 2004; Zhu et al. 2018). The full length of hmhsp70 gene was inserted into plasmid pROK2 under the control of CaMV 35S promoter, and the recombinant plasmid was transformed into WT tobaccos by Agrobacterium mediated transformation. Twelve independent transgenic tobaccos (T0) were generated. Transformation of hmhsp70 gene in these plants was confirmed by PCR using the genomic DNA from transgenic tobaccos as the templates. A clear band at about 2000 bp for transgenic tobacco was selected as positive transformants, while the negative WT tobacco control did not show any band. In addition, qPCR analysis was used to detect the expression...
Fig. 1 Alignments of the deduced amino acid sequences of HmHsp70 and 10 relative Hsp70 proteins from basidiomycetes fungi. Hsp70 family signature sequences are indicated with bars above the sequences.
level of \textit{hmhsp70} in transgenic tobaccos. qPCR results indicated that \textit{hmhsp70} gene was introduced into the transgenic tobacco and successfully expressed in transgenic tobaccos (Fig. 4). Three transgenic tobacco lines, T-hsp70-8, T-hsp70-12 and T-hsp70-15, with high \textit{hmhsp70} expression level were selected for the following experiments.

**HmHsp70 confers thermotolerance to tobacco**

The growth of transgenic tobaccos and WT tobaccos has no difference under normal temperature (25 °C) (Fig. 5). In order to confirm the ability of \textit{hmhsp70} overexpression tobaccos to heat, WT and transgenic tobaccos were treated at different temperature 46 °C, 48 °C, 50 °C, 52 °C for 4 h. Then the temperature was changed back to 25 °C and the heat treated tobaccos grew for 7 days. Ten tobaccos of each group were treated at one time, and the experiments repeated three times. The survival number of the heat treated tobaccos was recorded and compared. All the WT and transgenic tobaccos survived after heat shock at 46 °C, and no significant different survival number was observed after treated at 48 °C (Fig. 5a). The survival numbers of transgenic tobaccos are significantly higher than WT tobaccos after heat shock at 50 °C and 52 °C (Fig. 5a). Most of the WT tobaccos are died after treated at 50 °C and all WT tobaccos died after treated at 52 °C; however, 2 to 6 of the transgenic tobaccos survived. Old leaves are more sensitive to heat shock and new leaves grow from the stalk (Fig. 5b). The results indicated that transgenic tobaccos showed more heat tolerance than WT tobaccos in high lethal temperature.

**Discussions**

Previous studies on \textit{H. marmoreus} have mainly focused on cultivation, nutrition and chemical compounds (Liu et al. 2014; Mleczek et al. 2018; Qiu et al. 2013), but little is known about stress resistance of \textit{H. marmoreus}. Heat shock is an important adverse environmental stress
that influences the growth and development of mushrooms. Therefore, understanding of physiological alterations in response to heat stress and the corresponding mechanisms involved is essential for the breeding of heat-resistance *H. marmoreus* strains (Liu et al. 2019).

Although, the heat shock response has been studied in considerable detail in yeast and plant (McAlister and Fink 1980; Richter et al. 2010; Song et al. 2012), the mechanism of heat shock response in basidiomycetes remains elusive. Mushrooms evolved various strategies to response and alleviate heat shock stress, including HSPs synthesis, trehalose accumulation, and reactive oxygen species scavenging (Chen et al. 2017; Liu et al. 2016, 2018, 2019; Wang et al. 2017). In this study, we focused on a HSPs gene involved in heat shock response in *H. marmoreus*. A new *hsp70* gene was isolated from *H. marmoreus* and characterized. HmHsp70 exhibited conserved motifs with reported Hsp70 proteins from other organisms (Clerico et al. 2019; Kiang and Tsokos 1998), indicating those motifs are important for maintaining the function of HmHsp70. Among all the Hsp70 proteins in *Saccharomyces cerevisiae*, HmHsp70 showed the highest sequence identity with SSC1. SSC1 is located in the mitochondria in *S. cerevisiae* (Craig and Jacobsen 1984; Craig et al. 1987). Besides, HmHsp70 does not contain the typical cytosolic compartment sequence, (GP (T/K) (V/I) EE (V/M) D), suggesting that *hmhsp70* is located in the mitochondria region.

HSPs, function as molecular chaperones, play critical roles in stress response to adverse environment (Kiang and Tsokos 1998; Kurahashi et al. 2014). In our study, the expression of *hmhsp70* increased under heat shock indicating that *hmhsp70* is involved in heat shock response in *H. marmoreus*. The result is consistent with the expression of Hsp70 genes in other mushrooms (Chen et al. 2017; Wang et al. 2018a; Zhang et al. 2016; Zou et al. 2018). The silencing of Hsp40 gene, *LeDnaJ*, in *L. edodes* defected its resistance to heat stress (Wang et al. 2018b), while the over-expression of *LeDnaJ* gene in *L. edodes* S606 conferred the strain better tolerance to heat stress (Wang et al. 2018a). Overexpression of an Hsp100 gene, *PsHsp100*, from *Pleurotus sajor-caju* complemented a thermotolerance defect in *hsp104* mutant *S. cerevisiae* (Lee et al. 2006). The expression increase of *hmHsp70* is not as high as *hsp70* genes in the cytosolic region (Wang et al. 2016). The same result was also reported in *S. cerevisiae*, that the increase of *SSC1* transcripts ranged from 1.5- to 4.0- fold after heat shock for 0.5 h (Craig et al. 1987). To investigate the role of *hmhsp70*, we introduced this gene into tobacco. In this study, the seedling plants were used as the experimental materials. The *hmhsp70* overexpression transgenic tobaccos exhibited higher heat tolerance in lethal temperature compared to WT lines. The results indicated that overexpression of *hmhsp70* gene in tobacco conferred enhanced tolerance to heat stress. Nwaka et al. (1996) reported that *SSC1* is necessary for recovery from heat shock in *S. cerevisiae*. In another study, increase expression of *SSC1* partially suppressed the cold sensitive growth defect of the *SSH1* mutant (Schilke et al. 1996). All the reports combined with our results indicate that *hmhsp70* may necessary for the heat tolerance and recovery in *H. marmoreus*.

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**Fig. 3** Expression profile of *hmhsp70* under heat stress. Mycelia of *H. marmoreus* were treated at 42 °C for 0, 0.5, 1, 2, 3 and 4 h. The 18S rRNA gene was used as an internal control. Each value is the mean from three parallel replicates ±SD. One-tailed t test, performed by SPSS 18.0 (Chicago, Illinois), was used to calculate statistical significance. Different letters indicate a significant difference at P < 0.05

**Fig. 4** Expression of *hmhsp70* gene in transgenic tobaccos. The 2−ΔΔCt values normalized to actin gene were averaged for each investigated transgenic tobaccos. The expression level of T-hsp70-11 was considered to be the reference expression level.
HSPs play a vital role in heat stress response in edible mushroom, therefore, they have received a wide range of attention. Zhang et al. demonstrated that heat stress induces a significantly increased cytosolic Ca$^{2+}$ level in *G. lucidum*. Cytosolic Ca$^{2+}$ participates in heat shock signal transduction and the increased intracellular calcium triggers the accumulation of HSPs (Zhang et al. 2016). Liu et al. (2018) reported that heat stress induced a significant increase in the cytosolic reactive oxygen species concentration, which also participate in the regulation of HSP expression. The expressed HSPs function as molecular chaperones in blocking protein aggregation, dissolving the denatured proteins and helping damaged proteins to fold. Wang et al. reported that HSPs could regulate the IAA biosynthesis, and IAA accumulation could enhance thermotolerance of *L. edodes* (Wang et al. 2018a). These studies demonstrated that the metabolic network of HSPs synthesis and functions under heat stress are complicated. The diversity of HSPs is one of the reasons for this complexity. Werner-Washburne reported eight Hsp70 homologues in yeast, of which six are localized to the cytosolic region and two are located in mitochondria or endoplasmic reticulum (Werner-Washburne and Craig 1989) regions, respectively. Human also contains at least eight Hsp70 family proteins, which distribute in different regions the same as those in yeast (Daugaard et al. 2007). We search the Hsp70 proteins in the genome of *H. marmoreus* using BLAST software. Eight Hsp70s proteins were found in the genome of *H. marmoreus*. Other Hsp70 proteins in *H. marmoreus* may also participate in heat shock response, and the heat shock induced Hsp70 synthesis may have different sources. The mechanism of Hsp70 proteins involved in heat shock response in edible mushroom requires further study. This will provide a basis for improving the thermotolerance ability by genetic manipulation in the near future.

**Abbreviations**
Hsp70s: 70-kD heat shock proteins; WT: wild type; HSPs: heat shock proteins; PDA: potato dextrose agar; MS: Murashige and Skoog; LB: Luria Bertani; Kan: kanamycin; Hyg: hygromycin; qPCR: real-time quantitative PCR.

**Authors' contributions**
LX, LG and JG performed the experiments. LX, JG and HY analyzed the data. LX and LG provided materials. LX and HY conceived the project and wrote the manuscript. All authors read and approved the final manuscript.

**Funding**
This study was supported by the 13th Five-year National Key Research and Development Plan (Project 2018YFD0400200), Shandong Modern Agricultural Technology System Edible Fungus Innovation Team (SDAIT-11-011-02) and Mushroom Breeding Foundation (660-2418103).

**Availability of data and materials**
The dataset supporting the conclusions of this article is included within the article. All data are fully available without restriction.

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no conflict of interest.

**Received** 28 November 2019  **Accepted** 6 January 2020  **Published online** 18 January 2020

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