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Differentially expressed AC077690.1, AL049874.3 and AP001037.1 lncRNAs in prostate cancer

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Abstract: Prostate cancer (PCa) is a common type of cancer worldwide. The incidence of PCa increases with age and it is the most common malignant tumor in men. Tissue biopsy and the serum prostate-specific antigen are still the standards for diagnosing suspected PCa. Long non-coding RNA (lncRNA) contributes to the progression of PCa by recruiting transcriptional regulators. We utilized high-throughput sequencing data and bioinformatics analysis to identify specifically expressed lncRNAs in PCa and filtered out three specific lncRNAs for further analysis: AC077690.1, AL049874.3 and AP001037.1. We constructed a lncRNA regulatory network and used differentially expressed mRNA interactions to predict the functions of the selected lncRNAs. Functional enrichment analysis and PCR verification of these three lncRNAs revealed that they were closely related to well-known PI3K-Akt-mTOR and the forkhead box protein (FOXO) signaling pathways involved in PCa. By understanding the related interactions between these molecules and signaling pathways, the lncRNAs could be potential candidates for therapeutic targets in PCa.

Keywords: prostate cancer; long non-coding RNA; biomarker; signaling pathway; candidate therapeutic targets

Abbreviations: prostate cancer (PCa); long non-coding RNA (lncRNA); androgen deprivation therapy (ADT); castration-resistant prostate cancer (CRPCa)

INTRODUCTION

Prostate cancer (PCa) is the fifth leading cause of male cancer deaths worldwide, with about 360,000 deaths annually. PCa is a hormone-related cancer in which steroid androgen hormones play key roles in the progression and treatment [1]. Androgen deprivation therapy (ADT) is the first line of treatment to suppress cancer growth; it functions by inhibiting male hormone testosterone production and preventing it from reaching PCa cells. ADT can lead to apoptosis of PCa, suggesting that lipid-related metabolism functions in PCa carcinogenesis and progression [2]. Castration-resistant prostate cancer (CRPCa) occurs in 10-20% of PCa patients undergoing ADT [3,4], which is an incurable stage of PCa. About 90% of CRPCa patients develop metastases mainly in the skeleton [5]. The PI3K-AKT-mTOR signaling pathway is a signal transduction pathway that has evolved into an essential regulator of catabolic and anabolic processes in CRPCa. It provides a critical connection between nutrients and growth factors for a variety of vital cellular processes, including protein synthesis, proliferation, survival, differentiation and metabolism [6]. Recent discoveries indicate that connections between the PI3K-AKT-mTOR pathway and cell signaling cascades promote cancer progression, with the imbalance leading to a failure of patients to respond to ADT [7]. The exact regulatory mechanism

between the PI3K-AKT-mTOR and FOXO signaling pathways in PCa is unclear. The FOXO subfamily of forkhead transcription factors affects cell fate. The most important pathway interacting with FOXO in different types of cancers is the PI3K-AKT pathway. This family can be tumor suppressors in a wide range of cancers [8]; thus, it is meaningful to explore the molecules active in these pathways for potentially improving the diagnosis and treatment of PCa.

LncRNAs are RNA transcripts with lengths longer than 200 bp [9]. LncRNAs rarely encode proteins, but some studies have found that the small upstream open reading frames (ORFs) in lncRNAs possess a coding potential [10]. In PCa, lncRNAs can function by recruiting transcriptional regulators serving as decoys or inhibitors, and by contributing to PCa progression [11]. PCa noncoding RNA 1 (PRNCR1) has been reported as a tumor-risk lncRNA that contains single-nucleotide polymorphisms (SNPs) associated with the risk of developing PCa [12]; it is upregulated in PCa and prostatic neoplasia cells [13]. PRNCR1 attenuates cell viability and activity of the androgen receptor (AR) when knocked down [13]. The majority of identified PCa-associated lncRNAs are overexpressed; they have oncogenic roles and promote tumor growth [13]. Prostate cancer associated transcript 19 (PCAT19) is a well-known lncRNA whose expression is associated with a poor prognosis [14]. The SNP in the promoter of the PCAT19 long isoform increases its expression, leading to cellular proliferation and migration [14]. Highly upregulated in liver cancer (HULC) was another poor prognosis-related lncRNA, the high expression of HULC promotes PCa progression by regulating epithelial-mesenchymal transition [15]. These studies revealed that lncRNAs are involved in different molecular processes involved in PCa. Different PCa tumor tissue samples and analysis methods will always have different lncRNA targets, thus the validation in additional samples is needed. PCR validation is an appropriate experimental method to prove the accuracy of the results. Although there have been many studies on the clinical value of lncRNAs in the diagnosis and prognosis of PCa, the exact function and mechanism need to be further explored.

In our previous research, we sequenced six sets of RNA-seq obtained from four PCa tissues and two adjacent normal tissues from two patients [16]. Comparing sequencing data, we obtained differentially expressed lncRNAs. Two of the top ten differentially expressed lncRNAs were associated with prostate cancer, including HULC and PCAT19. In this research, we also discovered three lncRNAs that were associated with prostate cancer – they are AC077690.1, AL049874.3 and AP001037.1. After searching the lncRNA interaction database, we observed that the screened lncRNAs interact with cancer-related miRNAs and proteins such as argonaute RISC catalytic component 2 (Ago2) and autophagy-related 7 (ATG7). The downregulation of dicer and Ago2 is related to cell proliferation and apoptosis in PCa [17]. ATG7 can cooperate with the loss of phosphatase and tensin homolog (PTEN) to drive PCa [18]. The well-known miRNAs include miR-203, miR-370 and miR-216. Among them, miR-203 has an antimetastatic effect, and it affects the epithelial-to-mesenchymal transition (EMT) in PCa [19]. In the present work, we performed enrichment analysis and PCR verification and established that the three lncRNAs are enriched in PCa-related pathways, including the PI3K-AKT-mTOR and FOXO pathways.

MATERIALS AND METHODS

Patients and samples

This work was approved by the Beijing Hospital Ethics Committee (2022BJYYEC-052-01). No patients had undergone hormonal therapy prior to surgery. For sequencing samples, two pairs of PCa tissues and adjacent tissues were obtained from surgical samples. The RNA library's detailed sample information and construction were described in our previous research [16]. For quantitative real-time polymerase chain reaction(qRT-PCR), 10 pairs of PCa tissues and adjacent normal tissues with Gleason scores of 7 were obtained from the Department of Pathology of Beijing Hospital. All tissues were fixed in phosphate-buffered formalin, dehydrated with ethanol, and embedded in paraffin. The malignant status and Gleason score were obtained for these samples by histological analysis.

Quality control and mapping of sequencing data

The quality of the FASTQ data of RNA-seq was evaluated using FASTQC. Some reads were mixed with

adapters, and Trimmomatic [20] was used to filter these sequences; reads with lengths of less than 28 were dropped. The read quality was filtered through a 4-base sliding window with an average quality threshold of 15. After the reads passed the sequence quality tests, the filtered reads were mapped to the human (GRCh38.p13) genome [21] using the aligner software STAR [22] with the parameter '--chimSegmentMin 10'. For differentially expressed lncRNA, the fragments per kilobase of exon per million mapped fragments (FPKM) was calculated. The lncRNA with a fold-change>2 or a fold-change<0.5 was considered as differentially expressed.

Bioinformatics analysis of lncRNAs

All the interaction binding sites between lncRNAs and other molecules were downloaded from NPInter [23] and RISE [24]. We identified the function of the predicted miRNAs by manual literature mining. Cytoscape [25] was used to build a network between lncRNAs and other molecules. For differentially expressed mRNAs, we chose featureCounts [26] to quantify read counts for each gene. Based on the paired-end data, 'requireBothEndsMapped = TRUE' and 'isPairedEnd = TRUE' were set additionally. Then we calculated the normalized expression levels in FPKM by using the DGEList and RPKM, which was made for single-end RNA-seq where every read corresponded to a single fragment that was sequenced from edgeR [27]. The volcano plot was drawn by ggplot2 and the heatmap was drawn using the R package.

Functional enrichment analysis

The lncRNA-related mRNAs were filtered with Pearson's correlation coefficient >0.8. The circular RNA (circRNA)-related mRNA list was analyzed using the functional enrichment tool David [28]. The pathways were drawn using ggplot2 in the R language.

RNA extraction and qRT-PCR

A total of 10 PCa tissues and 10 adjacent normal tissue samples were prepared. RNA was extracted from three 10-μm formalin-fixed paraffin-embedded (FFPE) sections per sample. Paraffin was removed by xylene extraction followed by washing with ethanol. RNA was isolated from the sectioned tissue blocks using the purification kit, total RNA was extracted, and RNA was subjected to DN*ase* I (Invitrogen, AM2222) treatment. The qRT-PCR was performed using the TransScript II Green One-Step qRT-PCR SuperMix kit (TransGen Biotech, China, AQ311-01) with 100 ng RNA as a template in a 20-μL reaction volume on an ABI 7500 real-time cycler (Qiagen, Germany).

PCR cycling was performed as follows: one cycle at 95°C for 10 min, 95°C for 20 s, and 40 cycles at 60°C for 45 s. The threshold cycle for a given amplification curve during RT-PCR occurs at the point where the fluorescent signal grows beyond a specified fluorescence threshold setting. The results were normalized with β-actin, and the relative RNA expression was calculated by the 2-ΔΔCt method. To evaluate the statistical significance of PCR data, the paired sample t-test was used. The AC077690.1 primer sequences were as follows: left primer 5'-TTTCTAGCCCTGCATGCTTT-3'; right primer 5'-GTGGGTGAATGTGGGGTTAG-3'. The AL049874.3 primer sequences were as follows: left primer 5'-GTCCTGTTGGTCCATTTGCT-3'; right primer 5'-TTAGCTGCCATGAATGTTGC-3'. The AP001037.1 primer sequences were as follows: left primer 5'-TGAGCATCTCCTGACACCAG-3'; right primer 5'-CCACCCATCTGTAGGGCTAA-3'.

RESULTS

Differentially expressed lncRNAs in PCa

Due to the small sample size of PCa RNA-seq data, the P value was not considered in the initial differential lncRNAs screening (Fig. 1A). Bioinformatics analysis of the sequencing data indicated that a total of 2,375 differentially expressed lncRNAs could be filtered out according to the fold change (Fig. 1B). According to this result, the top ten upregulated lncRNAs were selected for further analysis. They were LINC02376, HULC, FAM197Y7, LINC00668, AC077690.1, MIR2052HG, PCAT19, AL049874.3, AP001037.1 and FAM197Y6. The lncRNA HULC and PCAT19 are well-known lncRNAs related to PCa. AC077690.1, AL049874.3 and AP001037.1 were the first to be linked to PCa.

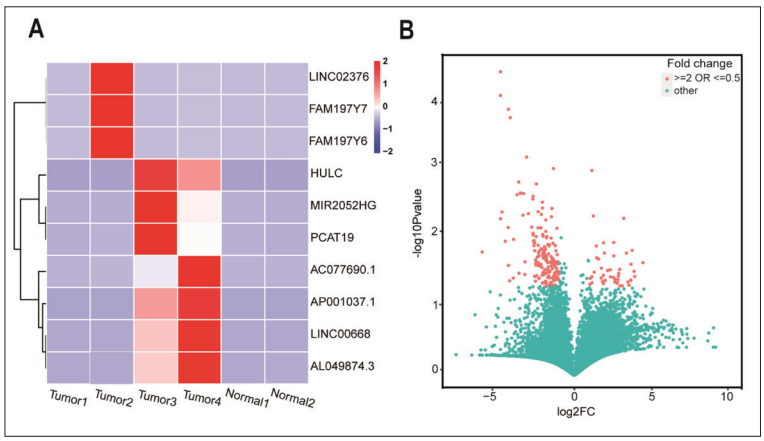

Fig. 1. A – Heatmap of the expression of differentially expressed top ten lncRNAs. The "scale=row" was used to render the graph color softer. **B** – Volcano plot of lncRNAs in PCa. The red points are differentially expressed lncRNAs in PCa with fold-change >2 or a fold-change <0.5. The paired t-test was used to obtain the P value.

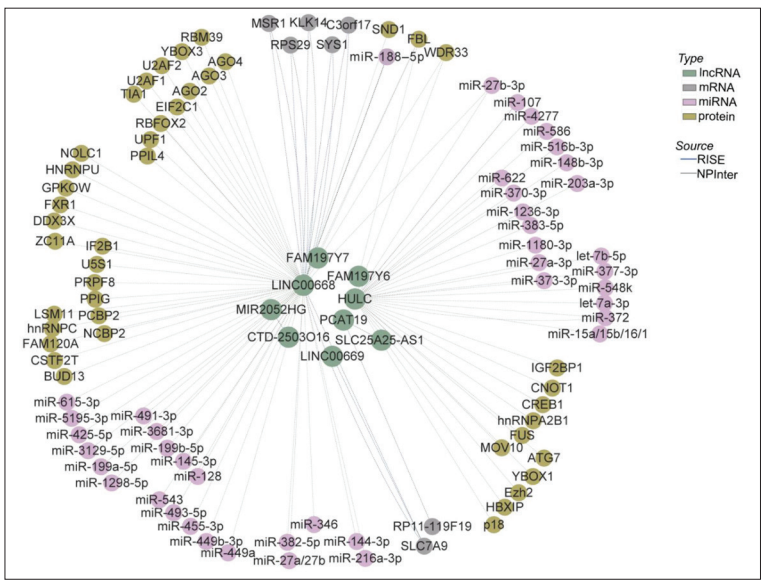

Fig. 2. The network between lncRNAs and their interactions from NPInter and RISE. All interactions were obtained and extracted from LINC02376, HULC, FAM197Y7, LINC00668, AC077690.1, MIR2052HG, PCAT19, AL049874.3, AP001037.1 and FAM197Y6 to build the network.

Relationships between differentially expressed lncRNAs

To verify whether the differentially expressed lncRNAs were associated with PCa, we extracted the records of the top ten lncRNAs in NPInter [23] and RISE [24] and constructed a lncRNA regulatory network (Fig. 2). Among the top ten lncRNAs, six lncRNAs had interactions with target miRNAs, proteins and mRNAs in these two databases, including HULC, FAM197Y7, LINC00668, MIR2052HG, PCAT19 and FAM197Y6. Except for HULC and PCAT19, the other four lncRNAs (FAM197Y7, LINC00668, MIR2052HG and FAM197Y6) were not reported in the literature before. The proteins interacting with lncRNAs included many well-known tumor-related proteins such as Ago2, ATG7 and the RNA processing protein 'fused in sarcoma' (FUS), which proved that these differentially expressed lncRNAs were indeed related to the tumor.

Pathways of newly discovered PCa-related lncRNAs

We further utilized functional enrichment analysis to explore the functions of the three newly discovered PCa lncRNAs, which include AC077690.1, AL049874.3 and AP001037.1. We calculated the Pearson correlation coefficient (PCA) to obtain the mRNAs associated with all three lncRNAs, all of which were involved in known PCa pathways such as the FOXO signaling pathway, the AMP-activated protein kinase (AMPK) signaling pathway, the epidermal growth factor receptor (ErbB) signaling pathway, the mammalian target of rapamycin (mTOR) signaling pathway, the regulation of epithelial to mesenchymal transition (EMT) and the PCa signaling pathway (Fig. 3A).

Then we separately analyzed the signaling pathways involved in the three lncRNA-related mRNAs and found that the three lncRNAs were all associated with the PI3K-AKT signaling pathway, the FOXO signaling pathway and the regulation of apoptotic signaling pathways (Fig. 3B, C, D, respectively). Meanwhile, the Wnt signaling pathway, the ERBB2 signaling pathway and the regulation protein kinase B signaling pathway were involved in the downstream pathway of AC077690.1, AL049874.3 and AP001037.1, respectively.

The qRT-PCR validation of newly discovered PCa-related lncRNAs

We then verified the expression levels of the three lncRNAs in 10 instances of PCa tissues and normal

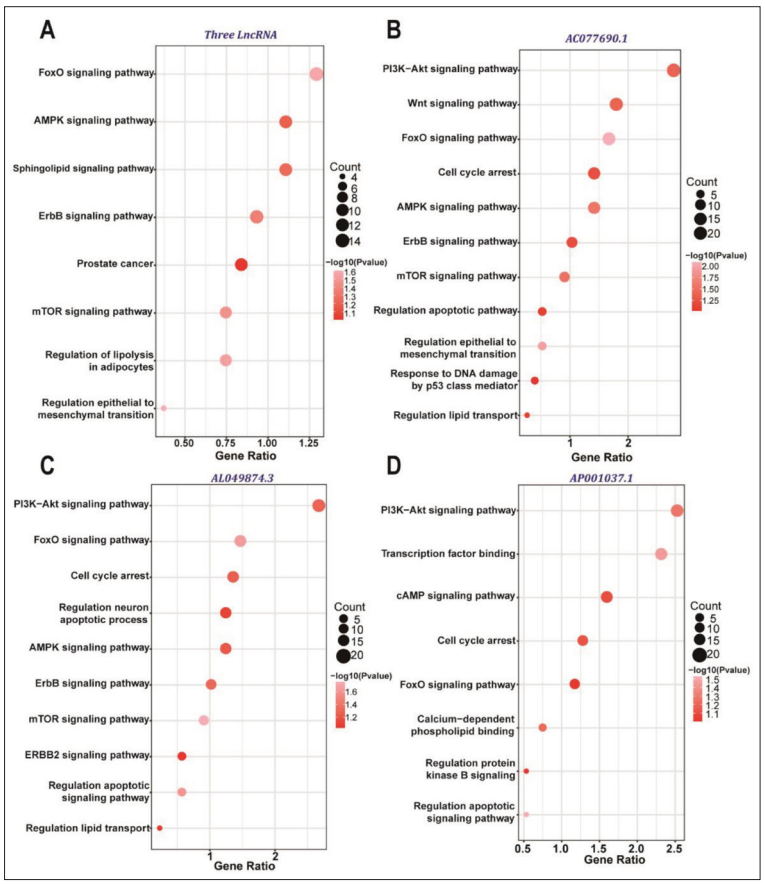

Fig. 3. Three specific lncRNA-related pathways. **A** – The three lncRNA-related pathways. Only differentially expressed mRNAs with Pearson's correlation coefficient higher than 0.8 were considered. **B** – The AC077690.1 related pathways. **C** – The AL049874.3 related pathways. **D** – The AP001037.1 related pathways.

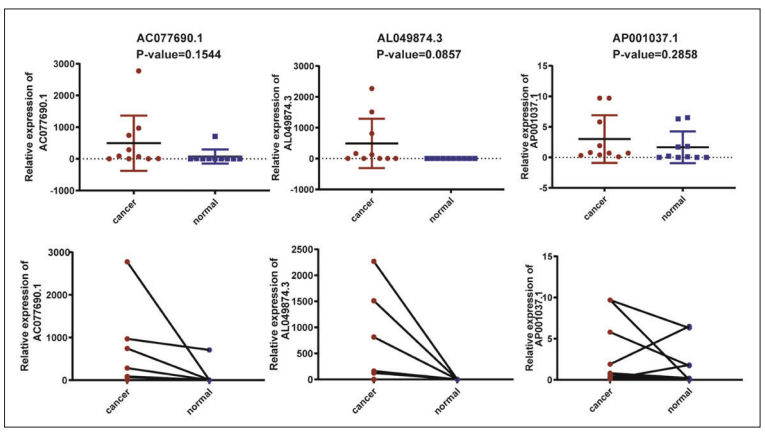

Fig. 4. qRT-PCR validation for PCa tissues and adjacent normal tissues in 10 samples obtained from patients diagnosed with PCa. Only three lncRNAs were significantly validated.

tissues by qRT-PCR (Fig. 4). The results showed that these three lncRNAs were highly expressed in PCa. Due to the small sample size, the P value was not significant, but the overall upward trend was obvious. Combined with the results of RNA-seq expression of lncRNAs, the expression of these lncRNAs in tumor tissues was indeed higher than in normal tissues.

DISCUSSION

Many studies have shown that lncRNAs can be used as potential biomarkers and therapeutic targets for PCa [35-37]. In view of the diversity of the pathological types and the complex pathogenesis of prostate cancer, different mining methods can always identify new lncRNAs. Differentially expressed lncRNAs must be identified and verified in many samples. In the present study, which was based on the sequencing data of PCa tissues and adjacent normal tissues reported in our previous study [16], we identified several differentially expressed lncRNAs. Among the top 10 abnormally high-expressed lncRNAs, we focused on three PCa-related lncRNAs, namely AC077690.1, AL049874.3 and AP001037.1. The high expression levels of these three lncRNAs in PCa tumor tissues were confirmed by qRT-PCR in another 10 PCa samples. According to the functional enrichment analysis of these three lncRNAs, it can be concluded that they were all involved in PCa-related signaling pathways. We believe that the newly discovered lncRNAs provide potential new targets for diagnosing and treating PCa.

After obtaining the differentially expressed lncRNAs, we used the interaction network to evaluate whether the top-ranked lncRNAs were related to PCa. By mining the miRNAs, mRNAs and proteins that interacted with these lncRNAs,

we found that some well-known PCa-related proteins were included in the interaction network, including ATG7, AGO2 and fibrillarin (FBL). In addition, some other molecules were also related to PCa. For example, among the molecules interacting with LINC00668, miR-216 inhibits the malignant progression of small-cell lung cancer by regulating the B-cell lymphoma 2 (Bcl2) family proteins [29]; BUD13 participates in apoptosis, autophagy and aerobic glycolysis of PCa cells by interacting with miR-653 and CircSERPINA3 [30]; both lncRNAs, FAM197Y6 and FAM197Y7, are associated with FBL. FBL is an essential nucleolar protein in hepatocellular carcinoma cells, and its high expression is significantly correlated with tumor diameter [31]. In addition, the HULC-related protein IGF2BP1 is an oncofetal mRNA-binding protein, which regulates gene expression in cancer [32]. This evidence suggests that the role of the lncRNAs we screened in cancer, especially in PCa, cannot be ignored. Therefore, we focused our research on these newly discovered lncRNAs.

By calculating the Pearson correlation coefficient of mRNAs related to lncRNAs, functional enrichment analysis was carried out by using the corresponding mRNAs. We found that the lncRNAs AC077690.1, AL049874.3 and AP001037.1-related mRNAs were all involved in apoptosis signaling pathways. Normal prostate development and maintenance depend on androgen and the androgen receptor (AR), while apoptosis of prostate epithelial cells is regulated by the AR signaling pathway, which plays an important role in the occurrence and development of PCa [33]. In addition, targeting the apoptosis signaling pathway is also an important direction of clinical treatment, and there have been a number of clinical trials targeting PCa anti-apoptosis signals. Therefore, these three newly discovered lncRNAs can be used as potential drug targets.

AC077690.1, downstream of the WNT signaling pathway, plays an important role in maintaining the homeostasis of the tumor microenvironment in PCa. The secretion of WNT protein by tumor stroma leads to treatment resistance and promotes disease progression [34]. Thus, AC077690.1 could be used as a candidate target for drug development. ErbB-2 targeting drugs such as erlotinib and gefitinib are effective in the treatment of solid tumors, suggesting that the ERBB2 signaling pathway-related AL049874.3 can also serve as candidate therapeutic targets. Similarly, AP001037.1, whose downstream target genes are enriched in the cyclic adenosine monophosphate (cAMP) signaling pathway, has the potential to be used in tumor therapy.

To sum up, lncRNAs have a great potential to become candidate targets for cancer therapy. They are widely distributed and can be easily detected in the saliva, serum, plasma, urine and cancer tissues [38]. Although there are currently no drugs targeting lncRNAs in cancer treatment, the obstacles to the development of lncRNA-targeting therapeutics should be precisely explored. Our study has identified potential candidates for this area of work, and it is essential to study the precise characteristics of lncRNAs to produce efficient and safe drugs.

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Author contributions: WZ and FS conceived the study. LLZ and YQZ designed the detailed analysis pipeline. LLZ and FS performed the bioinformatics analysis. HXL, XKT, GYS, SYX and LYW performed the experiments. HXL and FS wrote the manuscript, and LLZ and LXZ participated in revising the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors have never submitted the manuscript, in whole or in part, to other journals.

Data availability: The expression data of this manuscript have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Li%20et%20al_8115_Data%20Report.xlsx
The raw sequencing data that in this study are available on request from the corresponding author.

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