Correlation between serum circRNA and thyroid micropapillary carcinoma with cervical lymph node metastasis

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Abstract
The study aims to characterize the circular RNA (circRNA) expression profile that is functionally related with the invasiveness of papillary thyroid microcarcinoma (PTMC).
A total of 13 pairs of female patients with non-invasive PTMC or lymph node metastasis PTMC (PTMC (L)) were included and the serum RNA was obtained. CircRNA microarray was performed to identify the circRNA expression profile. Real-time PCR was used to verify circRNA expression. Bioinformatic approaches were adopted to annotate the function of differentially expressed circRNAs and construct the circRNA-miRNA-mRNA network.
In total, 400 significantly upregulated and 290 significantly downregulated circRNAs were identified in PTMC (L) compared with PTMC. Among them, 10 circRNAs were selected and validated by real-time PCR. Putative microRNAs (miRNAs) that could bind to the differentially expressed circRNAs were predicted. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses of target genes of the differentially expressed circRNAs revealed that these circRNAs may play an important role in lymph node metastasis. Finally, circRNA targeted miRNAs were predicted and a circRNA-miRNA-mRNA network was constructed for hsa_circRNA_404686.
Our results showed that several circRNAs, such as hsa_circRNA_404686, may serve as promising diagnostic marker for PTMC (L) in female.

Abbreviations: circRNA = circular RNA, miRNAs = microRNAs, ncRNA = non-coding RNA, PTMC = papillary thyroid microcarcinoma.

Keywords: circular RNA (circRNA), microarray, papillary thyroid microcarcinoma, serum

1. Introduction
Thyroid cancer is a common and frequently-occurring disease in the head and neck. In recent years, it has a markedly high incidence and has become a common dominant disease in general surgery clinics. In the past 10 to 20 years, the incidence of thyroid cancer has continued to rise, forming a worldwide “tsunami”. The incidence of thyroid cancer in China has also increased significantly, at a rate of 20.1% per year,[1] and more than 50% of newly diagnosed thyroid cancer is papillary thyroid microcarcinoma (PTMC).[2,3]

PTMC has 2 characteristics. One is high incidence, slow progression with good prognosis.[4,5] The other is lymph node metastasis, which has minimal impact on the life of the patient but is correlated with relapse.[6] The diagnosis and treatment of PTMC has become the focus of clinical studies: whether close follow-up or immediate surgery is in debate in recent years.[7] In addition, complications after surgery brings great burden to both the patients and the society. Therefore, to avoid unnecessary surgery, a simple, rapid, minimally invasive method in combination with current examination methods is needed to accurately determine the metastasis of PTMC before surgery.

Circular RNA (circRNA) is a class of single-stranded and covalently closed non-coding RNA (ncRNA) first found in plant viruses in the 1970s, which lacks 3' free cap end or 5' poly tail end.[8,9] This class of ncRNA was first considered as by-product of splicing.[10,11] CircRNA is widely expressed in mammalian cells[12] in a stable state,[13] and acts as miRNA sponge.[14] It has been reported that circRNA is abundantly expressed in red
blood cells, white blood cells, platelets, plasma, and serum. However, the biological function of circRNA is still not fully illustrated. In recent years, studies have revealed the correlation between circRNA and the occurrence and development of malignant tumors, such as bladder cancer, breast cancer, colon cancer, and liver cancer. In contrast, the role of circRNA in PTMC is still poorly understood.

In the present study, we only included female PTMC cases because the prevalence of PTMC in females is significantly higher than that in males. We first investigated the expression profile of circRNAs in patients with non-invasive PTMC or lymph node metastasis PTMC (PTMC (L)) to identify the differentially expressed circRNAs between the 2 groups. Then, we explored the circRNA/miRNA network by bioinformatics approaches.

2. Materials and methods

2.1. Clinical specimens and ethical statement

The study was approved by the institutional review board of Xinjiang Medical University (Approval No. 20181029–17). Informed consent was obtained from all patients. A total of 13 pairs of female patients with non-invasive PTMC or lymph node metastasis PTMC (PTMC (L)) were included in this study. They were female patients who for the first time received thyroidectomy in our department between November 2018 and December 2018. For patients with T1a nodule, the lobe of thyroid + isthmus of thyroid on the affected side was removed, and the pretracheal lymph node + paratracheal lymph node + prelaryngeal lymph node dissection was performed. When the lymph node was negative during surgery, the surgery was considered completed without further expansion of surgery scope. When the lymph node was positive during surgery, the contralateral thyroid lobe was removed as well. The average age of these PTMC patients was 47.33 ± 6.66 years old and the average age of PTMC (L) patients was 50.00 ± 9.17 years old. The metastasis of PTMC was diagnosed based on the pathological examination results of thyroid nodule. A total of 3 ml fasting blood was obtained from each patient, and then the serum was isolated. Serum from 3 pairs of patient samples were used for circRNA microarray analysis, and the remaining 10 pairs of patient samples were used for real-time PCR validation.

All the included patients were with the diameter of thyroid nodules ≤ 1 cm. To ensure that the included patients had consistent baseline data and to ensure that the results were more convincing, the factors that may affect the occurrence and development of thyroid cancer were excluded, including the age of the patient < 18 years, history of previous thyroid surgery before enrollment, history of previous head and neck radiation before enrollment, family history of thyroid malignancy, use of supplement thyroxine drugs or anti-thyroid hormone drug, and pregnancy.

2.2. CircRNA microarray analysis

CircRNA profiling was completed by Aksomics biotechnology (Shanghai, China). In brief, total RNA from serum was extracted from serum with Trizol-LS Reagent (Thermo Fisher, USA) according to the manufactures instruction. RNA was then labeled with CyDye Fluors (CF; Healthcare, USA) and hybridized using a Gene Expression Hybridization Kit (Leica) according to the manufacturers instructions. After 17 hours of hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, USA) using a Gene Expression Wash Buffer Kit (Agilent Technologies). CircRNA expression profiling and data analysis were carried out using Arraystar Human CircRNA V2.0 (Agilent).

2.3. Screening for differentially expressed circRNAs

Scanned images (in tiff format) were imported into Agilent Feature Extraction software (version 11.0.1.1) for raw data extraction. Raw data were normalized with QuanWiz algorithm, LIMMA package in R. Clustering of circRNAs was conducted by Pusamen 1.1 and Cluster 2.2 software. CircRNAs that were differentially expressed between the 2 groups were estimated by Significant Analysis of Microarrays (SAM) method of Pathway Studio software. Difference was estimated with Student t tests. CircRNAs with fold changes >1.5 and P values <0.5 were selected as significantly differentially expressed.

2.4. Gene ontology enrichment analysis

Gene ontology (GO) analyses of the circRNA parental genes were performed using DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/) software. The term/GO was drawn based on the enrichment score from the ascending order of size, showing the first 10 results of each domain.

2.5. KEGG pathway enrichment analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses of the circRNA parental genes were performed using Pathway Studio 5.0 and DAVID software. The term/pathway was drawn based on the enrichment score from the descending order of size, showing the first 11 results.

2.6. Prediction of circRNA–miRNA interactions

Arraystar Circular RNA Microarray Version 2.0 (Arraystar) was used to predict the interactions between circRNAs and miRNAs based on the predicted miRNA binding site. Five miRNA response elements with highest score were included with the differentially expressed circRNAs.

2.7. CircRNA–miRNA co-expression network

miRNA–circRNA interaction network analysis was performed using Cytoscape software. The miRNA–miRNA network was constructed on targetscan.com. Finally, the circRNA–miRNA gene was constructed.

2.8. Real-time PCR validation

We selected 5 up-regulated and 5 down-regulated differentially expressed circRNAs in PTMC compared with PTMC (L) for real-time PCR validation. RNA was extracted using GenElute™ RNB500 (Sigma-Aldrich), and the cDNA was obtained using ReverseAid First Strand cDNA Synthesis Kit (Thermo). Primers for RT-PCR were shown in Table 1. U6 snRNA was used as internal control. Quantitative real-time PCR was performed using DyNAmo Flash SYBR Green qPCR Kit (Thermo) on an ABI 7500 Real-Time PCR Systems (Thermofisher). The reaction mixture was incubated for 1 cycle at 94°C for 10 minutes, followed by 40 cycles at 94°C for 20 s, 55°C for 20 seconds, and
Table 1

| Primer name | Sequence (5’-3’) |
|-------------|-----------------|
| hsa_circRNA_000121-RT-F | ATTCTGCTTACCCATCCTGT |
| hsa_circRNA_000121-RT-R | TCCCGGTCCGAGCTCAGGT |
| hsa_circRNA_000466-RT-F | TCAAAGGAGAAGAGGCTGGCC |
| hsa_circRNA_000466-RT-R | AGACTGACAGTGATGACGTCG |
| hsa_circRNA_001059-RT-F | TCCCTACGACGGTTCCTCA |
| hsa_circRNA_001059-RT-R | AAGGGGAAAGTCAGCTGGTA |
| hsa_circRNA_001729-RT-F | AACATCCATTCTGCTCAAGT |
| hsa_circRNA_001729-RT-R | CCCTGAAGGCGGGTTCTC |
| hsa_circRNA_004183-RT-F | TGTCCTACAGGCTTACCTCA |
| hsa_circRNA_004183-RT-R | GTCAGGTTTCACACCTGGA |
| hsa_circRNA_051239-RT-F | TCCATTCCACGAGGTTCTCA |
| hsa_circRNA_051239-RT-R | GTCCTTCACGAGGTTCTCA |
| hsa_circRNA_102051-RT-F | CGCATCTACCGCAGCAGAC |
| hsa_circRNA_102051-RT-R | AGACAGACAGCGACACAAAG |
| hsa_circRNA_102116-RT-F | TGTCCTACAGGCTTACCTCA |
| hsa_circRNA_102116-RT-R | GTCCTACAGGCTTACCTCA |
| hsa_circRNA_102820-RT-F | CCGGAGAGGCTTACCTCA |
| hsa_circRNA_102820-RT-R | TGGCTTACAGGCTTACCTCA |
| hsa_circRNA_103615-RT-F | TGGCTTACAGGCTTACCTCA |
| hsa_circRNA_103615-RT-R | TGGCTTACAGGCTTACCTCA |
| hsa_circRNA_104866-RT-F | CCGGAGAGGCTTACCTCA |
| hsa_circRNA_104866-RT-R | TGGCTTACAGGCTTACCTCA |
| hsa_circRNA_105571-RT-F | AGGGCTTACAGGCTTACCTCA |
| hsa_circRNA_105571-RT-R | TGGCTTACAGGCTTACCTCA |
| U6_human-RT-F | TGGCTTACAGGCTTACCTCA |
| U6_human-RT-R | TGGCTTACAGGCTTACCTCA |

3.3. GO enrichment and KEGG analysis of the differentially expressed circRNAs

CircRNA can regulate the expression of its parental gene in 3 ways.[13,20,21] so we performed Gene ontology (GO) and KEGG analysis of their parental genes to determine the functions of differently expressed circRNAs between PTMC and PTMC (L) patients. GO covers 3 terms: cellular component (CC), molecular function (MF), and biological process (BP), and the enrichment score reflects the degree of enrichment for the analyzed GO term in certain disease. Here the top ten enriched GO terms for the differentially expressed circRNAs in PTMC (L) patients were shown in Figure 3A. The following items were enriched in BP: homophilic cell adhesion via plasma membrane adhesion molecules, cell-cell adhesion via plasma-membrane adhesion molecules, regulation of Ras protein signal transduction, Ras protein signal transduction, regulation of small GTPase mediated signal transduction, ARF protein signal transduction, regulation of ARF protein signal transduction, small GTPase mediated signal transduction, cell-cell adhesion, cell adhesion. The following items were enriched in CC: plasma membrane part, intrinsic component of plasma membrane, membrane, membrane part, integral component of plasma, membrane, plasma membrane, cell periphery, membrane, bounding membrane of organelle, intrinsic component of membrane, endomembrane system. The following items were enriched in MF: ion binding, anion binding, ARF guanyl-nucleotide exchange factor activity, calmodulin binding, phospholipid binding, monovalent inorganic cation transmembrane, transporter activity, potassium ion transmembrane transporter activity, calcium ion binding, inorganic cation transmembrane transporter activity, potassium: proton antiporter activity. KEGG analysis was performed to analyze gene function and related pathways. The top 11 KEGG pathways were identified for the differentially expressed circRNAs (Fig. 3B). The main pathway included Other types of Glycan biosynthesis, Salivary secretion, Endocytosis, Gastric acid secretion, Calcium signaling pathway, Adrenergic signaling in cardiomyocytes, Insulin secretion, Gs/cAMP signaling pathway, Inflammatory mediator regulation of TRK channels, and Hedgehog signaling pathway.

3.4. Analysis of the circRNA/miRNA interaction

Functionally, circRNAs can interact with miRNAs and work as miRNA sponge,[22–24] regulating the development of certain disease by modulating the level of disease-related miRNAs. Therefore, interactions between circRNAs and their targeting miRNAs were theoretically predicted by conserved seed-matching sequence using the Arraystar software. We selected 10 most differentially expressed circRNAs and 5 highly matched miRNAs with miRNA response elements were predicted according to the complementary miRNA matching sequence (Table 3). For instance, the downregulated hsa_circRNA_404686 in PTMC (L) patient was predicted to inhibit the expression level of hsa-miR-1249–5p, hsa-miR-3916, hsa-miR-6760–5p, hsa-miR-214–3p and hsa-miR-4739, which may further regulate the expression of miRNA-targeted miRNAs (Fig. 4A). Prediction results of the hsa_circRNA_404686 formed a circRNA-miRNA-miRNA interaction network (Fig. 4B).

3.5. Real-time PCR validation of differentially expressed circRNAs

To verify microarray expression data, expression of 10 circRNAs (hsa_circRNA_000121, hsa_circRNA_000466, hsa_circRNA_
Figure 1. CircRNA microarray expression in serum between PTMC and PTMC (L) patients. (A) The typical results of circRNA microarray. Each bright spot represents a circRNA gene. (B) Normalized data were plotted as a Box plot to determine the overall characteristics of distribution.

Figure 2. Identification of differentially expressed circRNAs between PTMC and PTMC (L) patients. (A) The heatmap depicts an intuitive graphical representation of gene expression in different samples after hierarchical clustering of all circRNAs. (B) Scatter plots of chip data assessing overall distribution of the 2 sets of data. The X and Y axes values in the scatter-plot are the normalized signal values of the samples (log2 scaled). Portions beyond the lines indicate differentially expressed circRNA (Fold change > 1.5 and P-values < .5). (C) Volcano plots were constructed using the fold change and P-values. The horizontal line corresponds to a 1.5-fold upregulation and a 1.5-fold down-regulation, and the vertical line represents P-values < .5. The red point on the plot represents the differentially expressed circRNAs with statistical significance.
001059, hsa_circRNA_051239, hsa_circRNA_102116, hsa_circRNA_001729, hsa_circRNA_004183, hsa_circRNA_102051, hsa_circRNA_404686 and hsa_circRNA_405571) with either upregulated or downregulated expression in PTMC (L) patients compared with PTMC patients was detected via real-time PCR in serum from 3 pair of patients (Fig. 5A). These 10 circRNAs were significantly differentially expressed between PTMC (L) and PTMC patients in qRT-PCR and showed consistent results with microarray data (Table 4). Further validation of hsa_circRNA_404686 in an enlarged set of 7 patient pairs obtained similar results (Fig. 5B).

### 4. Discussion

CircRNA is protected from exonuclease by its own unique closed, circular structure, which makes circRNA more conservative and stable than linear RNA. CircRNA also has cell specificity, tissue specificity and time specificity. Xu et al.[25] screened the circRNA expression profile between colorectal cancer with no metastasis and that with liver metastasis and showed that several circRNAs were differentially expressed. After validation, circRNA0001178 and circRNA0000826 were considered as potential biomarkers of colorectal cancer with liver metastasis. Ju et al.[26] found that

| Probe ID     | P value       | FDR    | FC (abs)     | circRNA          |
|--------------|---------------|--------|--------------|------------------|
| ASERP3000003 | 0.0290681531  | 0.36788406 | 1.5402557    | hsa_circRNA_007418 |
| ASERP3000048 | 0.02921545688 | 0.36788406 | 1.8673173    | hsa_circRNA_101633 |
| ASERP3000059 | 0.02468094999 | 0.36788406 | 2.008026     | hsa_circRNA_102820 |
| ASERP3000073 | 0.04165591557 | 0.36788406 | 2.094792     | hsa_circRNA_402810 |
| ASERP3000099 | 0.04097526003 | 0.36788406 | 1.5126155    | hsa_circRNA_047135 |
| ASERP3000083 | 0.03074536954 | 0.36788406 | 0.639477     | hsa_circRNA_102642 |
| ASERP3000373 | 0.04141095334 | 0.36788406 | 0.641032     | hsa_circRNA_102691 |
| ASERP3000763 | 0.0399880825  | 0.36788406 | 0.634402     | hsa_circRNA_039830 |
| ASERP3000892 | 0.02425451212 | 0.36788406 | 0.625043     | hsa_circRNA_038615 |
| ASERP3000916 | 0.02510964515 | 0.36788406 | 0.649859     | hsa_circRNA_058902 |

P value: calculated by student t test; FC (abs): absolute fold changes between PTMC and PTMC(L) groups.
circRNA = circular RNA.

001059, hsa_circRNA_051239, hsa_circRNA_102116, hsa_circRNA_001729, hsa_circRNA_004183, hsa_circRNA_102051, hsa_circRNA_404686 and hsa_circRNA_405571 with either upregulated or downregulated expression in PTMC (L) patients compared with PTMC patients was detected via real-time PCR in serum from 3 pair of patients (Fig. 5A). These 10 circRNAs were significantly differentially expressed between PTMC (L) and PTMC patients in qRT-PCR and showed consistent results with microarray data (Table 4). Further validation of hsa_circRNA_404686 in an enlarged set of 7 patient pairs obtained similar results (Fig. 5B).
circ0067531 and circ0000853 were among the differentially expressed circRNAs of oral mucosal cancer and that they may regulate the occurrence and development of oral mucosal cancer by participating in MAPK and NF-κB signaling pathways. Wang et al.[27] reported that circ0008305 may be used as a biomarker for tumor metastasis in patients with advanced non-small cell lung cancer. The above studies suggest that circRNAs play an important role in the occurrence, development, invasion and metastasis of malignant tumors, making them potential markers for tumor metastasis. However, these studies all used invasive methods to detect circRNAs in tissues, which showed less significance for preoperative diagnosis.

In this study, we analyzed the circRNA expression profile in the serum from 3 pairs of female patients with non-invasive PTMC or PTMC (L). CircRNA microarray identified 690 differentially expressed circRNAs between the 2 groups, which may be involved in the lymph node metastasis of PTMC. We also selected 5 upregulated circRNA (circRNA000121, circRNA051239, circRNA001059, circRNA102116, circRNA000466) and 5 downregulated circRNA (circRNA001729, circRNA000121, circRNA051239, circRNA0008305, circRNA004183, circRNA102051, circRNA405571) in PTMC (L) based on their fold change and P value for qRT-PCR analysis and obtained consistent results as microarray. Next, GO and KEGG analysis was used to investigate the biological functions or pathway enriched among the upregulated parental genes in PTMC (L).

Since circRNAs typically function as miRNA sponge, we predicted the top 5 miRNA binding sites in the 6 validated circRNAs to construct the circRNA-miRNA-mRNA network. The results indicate that interaction of circRNA000121 and miR-146b-3p may be closely related with the metastasis of PTMC. In a prospective study in 237 patients undergoing total thyroidectomy plus central lymph node dissection by Patricia et al, both BRAFV600E gene mutation and miR-146b-3p could be used as
independent risk factor for lymph node metastasis in central region. This study indicate that in patients with prophylactic central lymph node dissection, preoperative miR-146b-3p level could predict central lymph node metastasis. In a study on papillary thyroid carcinoma (PTC) with cervical lymph node metastasis, Yu et al found that miR-146b-5p and miR-146b-3p upregulation was associated with PTC metastasis. By inhibiting the expression of NF2, miRNA-146b-3p more significantly enhanced cell invasion and metastasis than miR-146b-5p, suggesting the potential diagnostic and therapeutic value of miRNA-146b-3p in PTC metastasis.

We thus speculate that circRNA000121/ miRNA-146b-3p/NF2 may be an important pathway in the metastasis and invasiveness of PTC and PTMC.

Moreover, several other differentially expressed circRNAs were predicted to target critical miRNAs in tumor metastasis. For example, miR-574-5p is a miRNA that may bind to circRNA051239. Wang et al found that the PTCS3-miR-574-5p-SCAI-Wnt/β-catenin pathway mediated the proliferation and migration of PTC-1 cells, which is closely related to the treatment and prognosis of PTC.

Another case is circRNA001729, which was downregulated in the serum of PTMC (L) patients, and miR-363-3p was a predicted interactor of circRNA001729. Liu et al found that miR-363-3p inhibited tumor growth in TPC-1 cells by suppressing the expression of PIK3CA and further inhibiting the PIK3CA/Akt signaling pathway. Mechanically, miR-363 specifically targets the CACNA1C gene in the Ca²⁺ signaling pathway and the DUSP10 gene in the MAPK signaling pathway. It can be speculated that circRNA001729 may play a role in the process of cervical lymph node metastasis of PTMC through targeting miR-363.

According to our results, circRNA 404686 could be targeted by miR-214. Lin et al has reported that miR-214 could negative regulate the expression of PSMd10 by targeting its 3' UTR, and the knockdown of PSMd10 inhibited the colony formation, migration and invasion of PTC cells, possibly by suppressing GSK3β/β-catenin and AKT signaling. They also showed that expression of miR-214 and PSMd10 were negatively correlated, indicating that miR-214 is a potential target for treating PTC.

circRNA000466, an upregulated circRNA in PTMC (L) patients, was predicted to target miR-888-5p, miR-372-5p, miR-607, miR-500a-5p, and miR-485-5p. Zhang et al found that miRNA-485-5p was involved in the proliferation and metastasis of PTC, and FOXD2-AS1 acted as a competing endogenous RNA (ceRNA) in PTC via a “sponge”-like effect on

### Table 4

| CircRNA          | Regulation | RT-PCR Fold change | P value | Microarray Fold change | P value |
|------------------|------------|--------------------|---------|------------------------|---------|
| hsa_circRNA_000121 | Up         | 3.24               | <.01    | 3.36                   | <.01    |
| hsa_circRNA_000466 | Up         | 3.12               | <.01    | 3.40                   | <.02    |
| hsa_circRNA_001059 | Up         | 2.92               | <.01    | 7.02                   | <.03    |
| hsa_circRNA_051239 | Up         | 2.44               | <.01    | 4.23                   | <.01    |
| hsa_circRNA_102116 | Up         | 2.93               | <.01    | 5.26                   | <.03    |
| hsa_circRNA_001729 | Down       | 2.94               | <.01    | 40.53                  | <.01    |
| hsa_circRNA_004183 | Down       | 1.75               | <.01    | 31.15                  | <.01    |
| hsa_circRNA_102051 | Down       | 2.44               | <.01    | 26.33                  | <.01    |
| hsa_circRNA_404686 | Down       | 3.84               | <.01    | 39.57                  | <.01    |
| hsa_circRNA_405571 | Down       | 2.04               | <.01    | 23.66                  | <.01    |

*P value: calculated by student t test; Fold change: the fold change for PTMC (L) / PTMC.
miR-485-5p, thereby enhancing KLK7 expression. Therefore, we validated the expression of circRNA404686 in an enlarged cohort of samples. Consistently, it was significantly increased in PTMC (L) patients compared with non-invasive PTMC, indicating its critical role in the invasion and metastasis of PTMC.

This study has some limitations. First, the sample size was relatively small. Second, this study only included female PTMC cases, which may cause gender bias and limit the generalization of the study findings. Further studies are warranted.

5. Conclusions
In conclusion, our study demonstrated that several circRNAs could be used as circulating biomarkers for the lymph node metastasis of female PTMC, providing reference for their treatment options. However, the results of this study still need to be validated in large cohort of patients.

Author contributions
Li Zhang and Zongbao Li conceived and designed the study. Chao Bai, Wenwen Yang, Li Zhang, Zongbao Li, Ye Tian, Zhenwei Yang, Liang Wang and Wennian Wu performed experiments and collected the data. Chao Bai, Li Zhang and Zongbao Li analyzed the data and interpreted the data. Chao Bai wrote the paper. All authors approved the final version of the manuscript.

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