**Observational Study**

**Hsa_circ_0002320: a novel clinical biomarker for colorectal cancer prognosis**

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**Abstract**

A great many circular RNAs (circRNAs) exist in different types of mammalian cells. Previous studies have verified that a low level of hsa_circ_0002320 is present in gastric cancer and that it might represent a good prognostic indicator. However, its value in colorectal cancer (CRC) is unclear. The aim of this research was to explore the value of hsa_circ_0002320 as a potential diagnostic biomarker for CRC prognosis.

Plasma samples, CRC tissues, and adjacent normal tissues were obtained from 50 patients with CRC, before any treatment, and 100 plasma samples were acquired from healthy individuals. Hsa_circ_0002320 levels in these samples were analyzed by reverse transcription-quantitative polymerase chain reaction. Correlations between hsa_circ_0002320, clinicopathological characteristics, and overall survival (OS) of CRC patients were also investigated. Receiver-operating characteristic (ROC) curve analysis was used to assess the value of hsa_circ_0002320 for CRC diagnosis. Finally, a bioinformatics analysis was performed to verify the effect of hsa_circ_0002320 on CRC prognosis.

Expression levels of hsa_circ_0002320 were significantly decreased in CRC plasma (P < .05). The expression level of hsa_circ_0002320 was significantly correlated with OS time (P < .05). Higher hsa_circ_0002320 reflected significantly greater OS; the HR of high hsa_circ_0002320 was 0.161 (95% CI, 0.066–0.393; P = .000). The area under the ROC curve of hsa_circ_0002320 in CRC was 0.823, which was higher than for the carcinoembryogenic antigen (area under the curve = 0.764). Kaplan-Meier analysis showed that CRC patients with low expression of hsa_circ_0002320 exhibited poorer OS times than those with high expression. Hsa_circ_0002320 could be a novel, noninvasive diagnostic blood biomarker for CRC prognosis.

**Abbreviations:** AUC = area under the curve, BP = biological processes, CC = cellular components, CEA = carcinoembryonic antigen, circRNAs = circular RNAs, CRC = colorectal cancer, CTD = comparative toxicogenomics database, EDTA = Ethylenediaminetetraacetic acid, EZH2 = enhancer of zeste homolog 2, GEPIA = Gene Expression Profiling Interactive Analysis, GO = Gene Ontology, HCC = hepatocellular carcinoma, HR = hazard ratio, KEGG = Kyoto Encyclopedia of Genes and Genomes, IncRNAs = linear noncoding RNAs, MF = molecular functions, OS = overall survival, PPI = protein-protein interaction, ROC = receiver-operating characteristic, YAP1 = Yes Associated Protein 1.

**Keywords:** bioinformatics, biomarkers, colorectal cancer, diagnosis, hsa_circ_0002320

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1. **Introduction**

Circular RNAs (circRNAs) are extremely stable, conserved across many taxonomic groups, and may become useful biomarkers for disease diagnosis.[1] They comprise a class of noncoding endogenous RNA molecules that are abundantly present in eukaryotic cells and are involved in the regulation of gene expression. circRNA is more stable than linear RNA because circRNA molecules form a closed loop structure that is not easily degraded by exonucleases.[2] Mounting evidence suggests that circRNAs play a vital role in the processes of occurrence and regulation of tumors.[3]

Colorectal cancer (CRC) is a frequently occurring malignant tumor worldwide, ranking 3rd and 4th place for incidence and mortality, respectively.[4] Despite continuous improvements in diagnosis and treatment, the survival of patients with advanced CRC remains poor.[3] Colonoscopy and subsequent pathological examinations are the criterion standard for the diagnosis of CRC. However, intestinal preparation is cumbersome and patient acceptance is low. Additionally, colonoscopy is a costly, invasive procedure that relies on high-quality standards and has potential complications.[6] Some patients even refuse the examination out of fear, which can delay the diagnosis and treatment of the disease. Therefore, new, highly effective noninvasive blood biomarkers would be of great value in the treatment of CRC.
Blood extraction is relatively simple, quick, and inexpensive, so there is an urgent need for the development of blood-based biomarkers for clinical applications.\(^7\)

To date, research has focused on the circRNA designated hsa_circ_0002320. The gene is located at chr11: 102056748-102076805, and its associated gene symbol is yes associated protein 1 (YAP1). A previous study reported that hsa_circ_0002320 was markedly decreased in tumor tissues of gastric cancer and this was related to an unfavorable prognosis.\(^6\) Here, we first determined if there was decreased expression of hsa_circ_0002320 in the plasma of patients with CRC and analyzed any correlations between hsa_circ_0002320 expression, clinicopathological features, and survival time of patients with CRC. Hsa_circ_0002320 could be a promising novel blood biomarker for CRC prognosis.

2. Methods

2.1. Clinical specimens

A total of 50 patients with CRC were recruited at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between December 2014 and December 2019. A diagnosis of CRC was made by colonoscopy and pathology. Preoperative blood samples (3 mL) were collected from each of these patients, before any medical treatment was implemented. Blood samples were also obtained from 100 age- and sex-matched healthy individuals (control group). Ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant in blood sampling tubes. The blood samples were centrifuged for 10 minutes at 3000 rpm to separate the plasma. And the CRC tissues and adjacent normal tissues were also obtained from the patients. All samples were then stored at \(-80^\circ\text{C}\) until RNA isolation was performed. Additional data recorded included clinical information, such as sex, age, tumor diameter, tumor stage, and lymphatic metastasis.

No patients underwent radiotherapy or chemotherapy before their surgery. A diagnosis of CRC was made in accordance with the American Joint Commission on Cancer/Union for International Cancer Control TNM staging system (2010 seventh edition).\(^9\) The methodology of this research obeyed the criteria of the Helsinki Declaration. The research was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. Written informed content was obtained from all participants.

2.2. RNA isolation and reverse transcription

RNA in plasma specimens was isolated using TRIzol LS reagent (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA) was used to determine the RNA purity and content. The MLV first-strand kit (Life Technologies, Beijing, China) was used to synthesize cDNAs, according to the manufacturer’s protocol.

2.3. Reverse transcription-quantitative polymerase chain reaction analysis

Reverse transcription-quantitative polymerase chain reaction analysis was performed according to the SYBR Green qPCR SuperMix-UDG (Life Technologies) method. GAPDH was used as an endogenous reference to normalize circRNA expression. All primers were synthesized using Ribobio (Guangzhou, China). The sequences of the primers were as follows:

**Forward primer**, hsa_circ_0002320: 5’-ACAGATGCAGCTG-CACGCAC-3’;

**Reverse primer**, hsa_circ_0002320: 5’-TGGGTCTAGCCAAG-GAGTGG-3’;

**Forward primer**, GAPDH (control): 5’-ATCTTCCAGGACG-GAGAAGCC-3’;

**Reverse primer**, GAPDH (control): 5’-TGAGTCCTTCAAGA-TACCC-3’.

The 2\(^{-\Delta\Delta CT}\) method was used to analyze the levels of hsa_circ_0002320 and GAPDH in plasma and the CRC tissues. These values were presented as means±SD from at least 3 independent experiments.

2.4. Serological CRC-associated marker analysis

Serum carcinoembryogenic antigen (CEA) and cancer antigen 19-9 (CA19-9) were assayed using a Beckman Coulter AU5800 analyzer (Beckman Coulter, Brea, CA), according to the manufacturer’s protocol. All samples were evaluated in duplicate.

2.5. Statistical analysis

The data were expressed as percentages of the total and means±SD. Each independent experiment was repeated 3 times and average values were calculated. Any differences in plasma hsa_circ_0002320 levels between patients with CRC and healthy controls were detected using a \(t\) test.

The Pearson \(\chi^2\) test was used to explore associations between clinicopathological features and survival time for the correlation analysis. If any analytic results reached a liberal statistical threshold of \(P < .2\) for each comparison, the risk factors were forced into a multivariable linear regression model to confirm independent risk factors for the survival time. Univariate and multivariate Cox regression analysis was used to calculate the hazard ratio (HR) of each characteristic for overall survival (OS). Finally, we used the Kaplan–Meier method to explore OS. A receiver-operating characteristic (ROC) curve analysis was performed to determine the ability of CEA and hsa_circ_0002320 to predict prognoses in patients with CRC.

All data analysis was conducted using SPSS software 25.0 (IBM, Armonk, NY) and GraphPad Prism software 8.0 (GraphPad Prism Software Inc, San Diego, CA). A \(P\) value <0.05 was considered statistically significant.

2.6. Similar genes to YAP1 predicted (hsa_circ_0002320) and construction of a protein–protein interaction network

Gene Expression Profiling Interactive Analysis (GEPIA)\(^{10}\) was performed to predict genes similar to hsa_circ_0002320. The datasets (“TCGA Tumor” and “TCGA Normal”) were selected. In the “TCGA Tumor,” the “COAD Tumor” and “READ Tumor” were selected. And in the “TCGA Normal,” the “COAD Normal” and “READ Normal” were selected. The “YAP1”, which represented the “Hsa_circ_0002320”, was selected as gene.

The STRING\(^{11}\) (Search Tool for the Retrieval of Interacting Genes) tool was used to construct a PPI network, which was visualized using Cytoscape (version 2.8).\(^{12}\) Basic settings are first, meaning of network edges is evidence (line color indicates the type of interaction evidence); second, active interaction sources include...
Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence; third, minimum required interaction score is low confidence (0.150); fourth, max number of interactors to show in the 1st shell is none/query proteins only, and max number of interactors to show in the 2nd shell is none. Advanced Settings are network display mode is interactive svg (network is a scalable vector graphic [SVG]; interactive).

2.7. Enrichment analysis for all similar genes using the online DAVID tool

An online tool, DAVID[13] (version 6.8, Maryland), was applied to carry out the functional annotation of the gene list. Gene Ontology (GO)[14] mainly includes biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The Kyoto Encyclopedia of Genes and Genomes (KEGG)[15] is a comprehensive database of genomic, chemical, and systemic functional information. DAVID was used to analyze both GO and KEGG.

2.8. Identification of YAP1 (hsa_circ_0002320) associated with CRC and the effect of its expression on OS via the Kaplan–Meier plotter

The comparative toxicogenomics database (CTD)[16] was searched to identify integrated chemical–disease, chemical–gene, and gene–disease interactions and to predict novel associations and generate expanded networks. The relationships between YAP1 (hsa_circ_0002320) products and CRC were analyzed using the CTD. The effect of hub gene expression on OS was analyzed using the Kaplan–Meier plotter.

3. Results

3.1. The lower expression level of hsa_circ_0002320 in CRC patients

We examined hsa_circ_0002320 expression levels in the plasma of the 2 groups; we found that plasma hsa_circ_0002320 expression levels in patients with CRC were lower than the expression levels in the control group (P < .05; Fig. 1A). And we examined the Hsa_circ_0002320 expression of CRC tissues and the adjacent normal tissues, and found a significant difference in Hsa_circ_0002320 expression. The hsa_circ_0002320 expression levels in CRC tissues were lower than the expression levels in the normal tissues (P < .05; Fig. 1B).

3.2. Associations between characteristics and survival time of patients with CRC. based on the $\chi^2$ test

Among the individuals analyzed, the levels of carcinoembryonic antigen (CEA) ($P = .040$) and hsa_circ_0002320 ($P = .000$)
were significantly correlated with the survival time of patients with CRC. However, no significant associations were found between other characteristics and survival time (Table 1).

### 3.3. Spearman correlation test and multiple linear regression analysis for all characteristics

To confirm whether the potential characteristics of hsa_circ_0002320 played an important role in relation to survival time, a further correlation analysis was performed. Spearman correlation coefficient showed that survival time was significantly correlated with the levels of CEA ($r = 0.290, P = .041$) and hsa_circ_0002320 expression levels ($r = 0.655, P = .000$) (Table 2). The natural logarithmic survival time remained correlated with hsa_circ_0002320 expression levels ($b = 20.676, P = .000$) when all other variables were held at a fixed value in the multivariate linear regression model. However, survival time was not significantly correlated with any other characteristics in the multivariate linear regression model (Table 3).

### 3.4. High expression of hsa_circ_0002320 in patients with CRC was correlated with improved OS

Table 4 presents the univariate HR and 95% confidence intervals (95% CI) for OS of individuals. The HR for OS was 0.173 (95% CI, 0.080–0.376, $P = .000$) in the group with high hsa_circ_0002320 expression levels compared with that in the group with low hsa_circ_0002320. However, there were no characteristics that were significantly disadvantageous for OS (Table 4). To effectively control the influence of confounding factors, all risk factors were simultaneously incorporated into a multivariate Cox regression model, which can also predict the most independent risk characteristic. Table 5 shows the results of multivariate Cox proportional regression analysis. The higher the expression level of the hsa_circ_0002320, the significantly greater the advantage for OS; the HR of high hsa_circ_0002320 was $0.161$ (95% CI, $0.066$–$0.393; P = .000$) (Table 5). Figure 1B shows the Kaplan–Meier OS curves. Higher expression levels of hsa_circ_0002320 in the plasma was predictive of a longer OS ($P < .001$, Fig. 1C).

Furthermore, the CRC patients with high expression of hsa_circ_0002320 in the CRC tissues had a better OS than those with low expression ($P < .001$, Figure 1D).

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**Table 1**

| Characteristics | Survival time |
|-----------------|--------------|
|                 | Short (%) | Long (%) | $P$  |
| Sex Male | 27 | 17 (34.0%) | 10 (20.0%) | .441 |
| Female | 23 | 12 (24.0%) | 11 (22.0%) | .793 |
| Age $< 60$ | 18 | 10 (20.0%) | 8 (16.0%) | .242 |
| $\geq 60$ | 32 | 19 (38.0%) | 13 (26.0%) | .58 |
| Tumor diameter $< 5$ cm | 31 | 16 (32.0%) | 15 (30.0%) | .68 |
| $\geq 5$ cm | 19 | 13 (26.0%) | 6 (12.0%) | .85 |
| Tumor stage I–II | 16 | 10 (20.0%) | 6 (12.0%) | .438 |
| III–IV | 34 | 19 (38.0%) | 15 (30.0%) | .000* |
| Lymphatic metastasis No | 35 | 20 (40.0%) | 15 (30.0%) | .040* |
| Yes | 15 | 9 (18.0%) | 6 (12.0%) | .448 |
| CEA Negative | 32 | 22 (44.0%) | 10 (20.0%) | .000* |
| Positive | 18 | 7 (14.0%) | 11 (22.0%) | .851 |
| CA19-9 Negative | 43 | 24 (48.0%) | 19 (38.0%) | .000* |
| Positive | 7 | 5 (10.0%) | 2 (4.0%) | .000* |
| hsa_circ_0002320 Low | 24 | 22 (44.0%) | 2 (4.0%) | .000* |
| High | 26 | 7 (14.0%) | 19 (38.0%) | .000* |

Pearson $\chi^2$ test was used. CEA = carcinoembryonic antigen, CRC = colorectal cancer.

* $P < .05$.

**Table 2**

| Characteristics | Survival time | $r$  | $P$  |
|-----------------|--------------|------|------|
| Sex Male | 27 | 0.109 | .451 |
| Female | 23 | -0.037 | .798 |
| Age $< 60$ | 18 | -0.165 | .251 |
| $\geq 60$ | 32 | 0.063 | .666 |
| Tumor diameter $< 5$ cm | 31 | -0.027 | .855 |
| $\geq 5$ cm | 19 | 0.290 | .041* |
| Tumor stage I–II | 16 | 0.110 | .448 |
| III–IV | 34 | 0.655 | .000* |

Spearman correlation test was used.

* $P < .05$.

**Table 3**

| Characteristics | Survival time | $b$  | $P$  | VIF |
|-----------------|--------------|------|------|-----|
| Sex Male | 27 | 0.593 | .866 | 1.136 |
| Female | 23 | -3.796 | .299 | 1.121 |
| Age $< 60$ | 18 | -0.859 | .624 | 1.290 |
| $\geq 60$ | 32 | 5.275 | .167 | 1.142 |
| Tumor diameter $< 5$ cm | 31 | 1.773 | .660 | 1.256 |
| $\geq 5$ cm | 19 | 3.508 | .348 | 1.237 |
| Tumor stage I–II | 16 | 2.068 | .685 | 1.157 |
| III–IV | 34 | 20.676 | .000* | 1.267 |

$\beta = $ parameter estimate, VIF = variance inflation factor.

* $P < .05$. 
3.5. hsa_circ_0002320 Could more sensitively and specifically predict CRC than CEA based on an ROC curve

We constructed ROC curves to identify accurate thresholds for CEA and hsa_circ_0002320 in predicting CRC. CEA was most associated with CRC (AUC [area under the curve] for CRC, 0.764; 95% CI, 0.680–0.848; \( P = .000 \)) (Fig. 2A). The hsa_circ_0002320 level was most closely correlated with CRC (AUC, 0.823; 95% CI, 0.097–0.258; \( P = .000 \)) (Fig. 2B).

3.6. The PPI network of similar genes predicted by hsa_circ_0002320 and the enrichment analysis

There was a close association among the similar genes, which was manifested in the PPI network. The number of nodes is 38.

Table 4

| Characteristics | OS | HR | 95% CI | P    |
|-----------------|----|----|--------|------|
| Sex             |    |    |        |      |
| Male            | 27 | 1  | 0.588–2.082 | .754 |
| Female          | 23 | 1  |        |      |
| Age             |    |    |        |      |
| <60             | 18 | 1  |        | .201 |
| ≥60             | 32 | 1.579 | 0.783–3.184 |      |
| Tumor diameter  |    |    |        |      |
| <5 cm           | 31 | 1  |        | .861 |
| ≥5 cm           | 19 | 1.061 | 0.547–2.056 |      |
| Tumor stage     |    |    |        |      |
| I–II            | 16 | 1  |        | .881 |
| III–IV          | 34 | 1.055 | 0.523–2.127 |      |
| Lymphatic metastasis | |    |        |      |
| No              | 35 | 1  |        | .480 |
| Yes             | 15 | 0.771 | 0.375–1.586 |      |
| CEA             |    |    |        |      |
| Negative        | 32 | 1  | 0.717–1.586 | .072 |
| Positive        | 18 | 0.535 | 0.270–1.058 |      |
| CA19-9          |    |    |        |      |
| Negative        | 43 | 1  |        | .956 |
| Positive        | 7  | 0.974 | 0.379–2.500 |      |
| hsa_circ_0002320 |    |    |        |      |
| Low             | 24 | 1  | 0.080–0.376 | .000 |
| High            | 26 | 0.173 |        |      |

95% CI=95% confidence interval, HR=hazard ratio.

Table 5

| Characteristics | OS | HR | 95% CI | P    |
|-----------------|----|----|--------|------|
| Sex             |    |    |        |      |
| Male            | 1.235 | 0.593–2.574 | .573 |
| Female          | 1.447 | 0.629–3.326 | .385 |
| Age             |    |    |        |      |
| <60             | 0.992 | 0.463–2.123 | .083 |
| ≥60             | 0.816 | 0.346–1.927 | .643 |
| Tumor diameter  |    |    |        |      |
| <5 cm           | 0.460 | 0.180–1.179 | .106 |
| ≥5 cm           | 0.689 | 0.324–1.467 | .334 |
| Lymphatic metastasis | |    |        |      |
| No              | 1.628 | 0.562–4.716 | .369 |
| Yes             | 0.161 | 0.066–0.393 | .000 |

95% CI=95% confidence interval, HR=hazard ratio, OS=overall survival.

3.7. Identification of YAP1 (hsa_circ_0002320) associated with CRC

The CTD database showed that YAP1 (hsa_circ_0002320) targeted CRC; the data are shown in Figure 5A. Kaplan–Meier analysis showed that CRC patients with low expression levels of YAP1 (hsa_circ_0002320) had poorer OS times than those with high YAP1 expression levels (\( P < .05 \); Fig. 5B).
circMMP9 is overexpressed in osteosarcoma tissue. The overexpression of circMMP9 delays apoptosis of osteosarcoma cells and is associated with advanced tumors and poor prognosis.\(^{25}\) Hsa_circ_0008450 levels are remarkably elevated in hepatocellular carcinoma (HCC); the knockout of hsa_circ_0008450 inhibited the migration, proliferation, and invasion of HCC cells. Our GO analysis results showed that variations in DEGs linked with BP were mainly enriched in protein ubiquitination. We speculated that this might regulate the cell cycle, cell proliferation, and apoptosis by affecting the expression of ubiquitin proteasome.

Hsa_circ_0008450 promoted levels of the enhancer of zeste homolog 2 (EZH2) protein.\(^{26}\) These findings indicate that circRNAs may be involved in tumorigenesis and tumor development. However, the diagnostic value of circRNAs in malignant tumors requires further research. We first determined the potential clinical diagnostic value of hsa_circ_0002320 in CRC. We found that the levels of hsa_circ_0002320 were remarkably closely correlated with survival time (\(P < .05\)).

CEA and CA199 are blood biomarkers widely used for the clinical diagnosis of CRC. The sensitivity and specificity of CEA and CA199 are 0.53 and 0.86, and 0.47 and 0.92, respectively.\(^{27}\) The sensitivity of current clinical biomarkers for CRC is poor, suggesting that they have very limited application in diagnosis.\(^{28}\) Therefore, there is a clinical need for an improved tumor biomarker for the diagnosis of CRC. In contrast, hsa_circ_0002320 has high sensitivity and specificity for the diagnosis of CRC. Hsa_circ_0008450 has the characteristics of high sensitivity and specificity, and could therefore be used as a blood cancer biomarker to improve the clinical diagnosis of CRC.

Our results demonstrated that the relationship between hsa_circ_0002320 in plasma and clinicopathological features was inconsistent. Abnormally expressed circRNAs may reflect changes in the condition-specific transcriptome of blood cells, or their direct release from diseased tissues.\(^{29}\) CircRNAs have been detected in exosomes secreted by colon cancer cell lines and vascular smooth muscle cells, suggesting that circRNAs secreted by exosomes are a common feature of many cells.\(^{30}\) These findings suggest that the mechanisms by which cell-free RNA circulates in the blood are complex.

And through the results of PPI and enrichment analysis, this research found that there existed closed interaction among the genes similar with the hsa_circ_0002320, and these genes were mainly enriched in ubiquitination. Ubiquitination refers to the process in which ubiquitin molecules, under the action of a series of special enzymes, classify the proteins in the cell, select the target protein molecules from them, and specifically modify the target proteins.\(^{31}\) Ubiquitination plays an important role in protein localization, metabolism, function, regulation, and degradation.\(^{32}\) At the same time, it is involved in the regulation of cell cycle, proliferation, apoptosis, differentiation, metastasis, gene expression, transcriptional regulation, signaling, inflammation, and immunity. Ubiquitination is closely related to the pathogenesis of tumors.\(^{33}\) Therefore, the value of the PPI and enrichment analysis is that the results could suggest that the hsa_circ_0002320 might influence the occurrence and development of CRC via the ubiquitination.

However, the present study had some limitations. The sample size of this experiment was not large; multiple centers and large
samples are needed to further verify the experimental results. Significant methodological limitations in the analysis of plasma circRNAs remain, mainly due to the low concentration of these molecules. We require further study to understand the mechanisms underlying the occurrence and development of CRC.

5. Conclusions

We concluded that the levels of hsa_circ_0002320 were significantly correlated with OS time in patients with CRC. Furthermore, for the first time, we have identified a potentially valuable clinical application for hsa_circ_0002320, that is, determining CRC prognosis. This biomarker showed a high sensitivity and specificity in CRC diagnosis. After the pathological diagnosis, it would be better to examine the Hsa_circ_0002320 expression in CRC tissues and the adjacent normal tissues to determine the prognosis. The detection of plasma hsa_circ_0002320 could improve diagnostic accuracy. Hsa_circ_0002320 could be a highly efficiently diagnostic biomarker for CRC prognosis.

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Author contributions

All authors read and approved the final manuscript. Conceptualization, Methodology: Bing-hui Li. Data curation, Formal analysis, Investigation: Mei Han. Project administration, Resources, Software: Peng Kong.
Validation, Visualization: Bin Xu.
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