The Presence of Genomic Instability in Cerebrospinal Fluid in Patients with Meningeal Metastasis

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Purpose: This study aimed to explore the genomic instability in cerebrospinal fluid (CSF) in patients with meningeal metastasis (MM).

Material and Methods: We collected the blood and CSF samples of 15 MM patients and one brain parenchymal metastasis (BPM) patient. A panel of 543 cancer-related genes was conducted to analyze the status of genomic instability in CSF and plasma cell-free DNA (cfDNA) of all patients. Subsequently, nine patients underwent low-depth whole-genome sequencing (WGS) analysis to verify the existence of genomic instability, followed by genomic scoring by the application of aneuploidy scores. Diagnosis-specific graded prognostic assessment (DS-GPA) score was utilized to assess the clinical status of MM patients.

Results: There was significant difference in gene mutation between CSF cfDNA and plasma cfDNA in MM patients. Among them, 12 MM patients developed genomic instability in their CSF cfDNA, while the remaining 3 had stable genetic profile. Besides, BPM patients showed genomic stability in his CSF and paraffin-embedded tissue sections. No genomic instability was noticed in plasma cfDNA of all patients. Sensitive mutations on *EGFR*, *ERBB2*, *ALK* and *KRAS* genes and increased gene copy numbers of *MET* and *ERBB2* were detected in 10 MM patients with genomic instability, as well as the *EGFR* gene mutation in one MM case with genomic stability. Additionally, MM patients with genomic instability had lower overall survival and higher aneuploidy scores and tumor mutation burden compared with those with genomic stability. Moreover, MM patients with higher DS-GPA scores benefited from better survival.

Conclusion: Genomic instability existed in the CSF cfDNA rather than plasma cfDNA of MM patients, which might be the underlying cause of the differences in MM.

Keywords: genomic instability, cerebrospinal fluid, meningeal metastasis, panel, whole-genome sequencing, cell-free DNA

Introduction

Meningeal metastasis (MM) is a disease in which malignant tumors invade into the spinal subarachnoid space and meninges via hematogenous metastasis, cerebrospinal fluid (CSF) diffuse dissemination.¹ MM consists of dural and leptomeningeal metastases, both of which may occur simultaneously or separately, presenting as advanced malignant tumor involving the meninges. Besides, an increasing number of sites of radiographic leptomeningeal metastases is associated with a progressively shorter survival time.² In recent 10 years, the incidence of MM has increased significantly, and 4–15% of the patients with solid tumors including lymphoma, lung and breast cancers develop MM in the advanced stage.^{3,4}

Current treatments for MM include systemic or intrathecal injection chemotherapy, targeted therapy, whole-brain radiotherapy and surgery. Whole-brain radiotherapy is used to relieve symptoms caused by intracranial hypertension, hydrocephalus and partial focal lesions, but cannot achieve the goal of cure. Lee et al⁵ have confirmed the effectiveness of targeted therapy in MM patients with leptomeningeal metastases and epidermal growth factor receptor (EGFR)-positive non-small-cell lung cancer (NSCLC). Moreover, it has also been reported that treatment with high-dose EGFR-tyrosine kinase inhibitor (TKI) does not affect survival after diagnosis of leptomeningeal metastases.⁶ The particularity of MM and the existence of blood-brain barrier might explain the disparity of different researches to some extent. However, the heterogeneity between primary tumor sites and MM, as well as the complexity of multiple signaling pathways activated during drug-resistant clonal evolution, play major roles in the response of MM to targeted therapy.⁷ Therefore, it is critical to comprehensively identify genetic biomarkers associated with the whole landscape of pathway activation and interaction network in MM, which is generally caused by genomic changes in tumors. Genomic instability refers to enhanced tendency to the accumulation of genomic alterations (mutation, homozygous deletion, or amplification) during the life cycle of cells, which is also a driving force behind multistep carcinogenesis.⁸

Comprehensive genomic profiling (CGP) can simultaneously study multiple genes in pathways to detect all types of carcinogenic alterations.⁹ Cell-free DNA (cfDNA) released from tumor cells is a convincing source for screening valuable tumor-specific biomarkers for cancer precision medicine. Thus, cfDNA-based CGP detection can be a feasible method to evaluate the genetic panorama of MM in the absence of qualified tissue samples. Woodhouse et al have indicated that cfDNA-based CGP can be used to detect gene mutations such as PIK3CA alterations.¹⁰ Currently, plasma cfDNA is mostly adopted to determine somatic mutations in cancer-related genes. But cfDNA derived from central nervous system tumors is more abundantly exists in CSF than in plasma.¹¹ Furthermore, Zheng et al noticed that the detection rate of driver genes in CSF cfDNA was significantly higher than that of plasma cfDNA (91.8% vs 45.5%), indicating the inconsistency of CSF cfDNA and plasma cfDNA.¹² Therefore, further studies should focus on genetic inconsistency in CSF cfDNA and plasma cfDNA in MM

patients, which might provide clues to the significant differences in efficacy of TKI in MM treatment.

Whole-genome sequencing (WGS) has been used for the detection of pathogenic genes of cancers, Mendelian genomic diseases and complex diseases from the perspectives of copy number variation (CNV), single nucleotide variants, insertion/deletion variants and structural variants, thereby finding replicable pathogenic genes.¹³ The application of WGS analysis is to overcome the gene selectivity of gene panel sequence, thus revealing the panoramic variation of genome, especially genetic instability caused by structural variations.

In this study, a panel of 543 cancer-related genes and low-depth WGS were used to perform comprehensive genomic analysis on 16 patients to compare the genetic mutation features between CSF and plasma cfDNA, thereby verifying that genomic instability might be the main cause of the MM differences between CSF and plasma cfDNA.

Materials and Methods

Patients and Samples

This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Tianjin Cancer Hospital (bc2020027). Written informed consent was obtained from patients enrolled in this study. Blood and CSF samples were obtained from 15 MM patients and one brain parenchymal metastasis (BPM) patient who were diagnosed as cancers and were treated at Tianjin Medical University Cancer Institute and Hospital from November 2018 to October 2019. The clinical characteristics of MM patients are summarized in Table 1. Ten (66.7%) MM patients were also diagnosed with lung adenocarcinoma, three with breast cancer, one with gastric cancer and one with colorectal cancer. The 66-year-old male BPM patient was diagnosed as lung adenocarcinoma (control). Diagnosis-specific graded prognostic assessment (DS-GPA) scores was utilized to assess the clinical status of MM patients.

Experimental Workflow

Briefly, the blood and CSF of 16 patients were collected, followed by the evaluation of the presence of genomic instability by Genecast panel. The Genecast panel was a 1.67 Mbp-sized panel covering the exon regions of 543 genes (Genecast, Wuxi, China), including major tumor-

Table I Basic Characteristics of Meningeal Metastasis Patients

Indices	
Age (y)	53.53±10.51
Sex (male, %)	6 (40)
Diagnosis	
Lung adenocarcinoma	10 (66.6%)
Breast cancer	3 (20%)
Gastric cancer	1 (6.7%)
Colorectal cancer	1 (6.7%)
Genomic instability	
Yes	12 (80%)
No	3 (20%)
TMB in plasma	4.174±5.4
TMB in CSF	5.498±3.55
Treatment	
Targeted therapy	12 (80%)
CSF chemotherapy	7 (46.7%)
Overall survival (month)	36.53±27.46
DS-GPA	1.7±0.676
Outcome (survived, %)	5 (33.33)

Abbreviations: TMB, tumor mutation burden; CSF, cerebrospinal fluid; DS-GPA, diagnosis-specific graded prognostic.

related genes. Paired-end sequencing was performed using Illumina HiSeq X-Ten. Notably, the single nucleotide polymorphism sites in plasma cfDNA and CSF cfDNA were analyzed with the reference of peripheral blood cells to determine the presence of genomic instability in CSF and plasma. Finally, low-depth WGS was adopted in 9 of the 16 patients to verify the existence of genomic instability.

Preparation of Plasma Cell-Free DNA

The supernatant plasma obtained from the centrifugation (10 min at 1600 g) of peripheral blood lymphocytes and plasma were transferred to 2 mL centrifuge tube for further centrifugation at 16,000 g for 10 min. The plasma cfDNA was isolated via MagMAXTM Cell-Free DNA isolation kit (Life Technologies, USA). DNA from peripheral blood lymphocytes was extracted using Tiangen Whole Blood DNA kit (Tiangen, China) in line with manufacturer's protocols. DNA concentration was quantified using Qubit dsDNA HS Assay kit or Qubit dsDNA BR Assay kit (Life Technologies, USA).

Cerebrospinal Fluid DNA Extraction

The supernatant obtained from the centrifugation (3000 g for 15 min) of CSF was transferred to 5 mL centrifuge tube, followed by centrifugation at 16,000 g for 10 min. MagMAXTM Cell-Free DNA isolation kit and Dynabeads Myone Silane isolation kit were adopted to extract CSF supernatant cfDNA and DNA from CSF precipitation, respectively. The concentration of DNA was quantified via Qubit dsDNA HS Assay kit or Qubit dsDNA BR Assay kit.

Library Preparation and Next-Generation Sequencing (NGS)

Genomic DNA were sheared into 150–200 bp fragments via Covaris M220 focused-ultrasonicator (Covaris, USA), and then KAPA HTP Library Preparation Kit (Illumina Platform) (KAPA Biosystems, USA) was utilized to establish fragmented DNA library. DNA library was captured by designed NimbleGen SeqCap EZ Library (Roche, USA) containing typical cancer-related genes, and then subjected to Novaseq 6000 for paired-end sequencing. Sequencing depth, DNA concentration and library input quantity were 471–9770, 0.02–79.4 ng/μl and 1–401 ng.

Sequencing Data Analysis

The generated raw sequencing reads were filtered via Trimmomatic for adapter trimming and quality filtering, and were then aligned to human genome reference HG19 using Burrows-Wheeler Aligner (BWA). The resulted SAM files were sorted and converted to BAM files using NovoSort. Subsequently, we adopted Genome Analysis Toolkit (GATK v3.7) for local realignment of potential small insertions or deletions (indels), as well as base recalibration for further mutation calling process with removal of duplicated reads. VarDict and FreeBayes were used for determination of SNV and small indels and complex mutations, respectively. Paired tumor-normal sample calling was processed during the mutation-calling. DNA translocation analysis was conducted using FusionMap (v8.0.2.32) for filtering out individual germline mutation.

Low-depth WGS at 1× depth (Illumina Novaseq) analysis was performed in 9 of the 16 patients. Then, IchorCNA software was utilized to analyze the CNV to find a set of replicable pathogenic genes.

Calculation of Aneuploidy Score

We calculated aneuploidy scores of the nine patients undergoing low-depth WGS to assess the association between aneuploidy and genomic instability as previously described.¹⁴ Firstly, copy number calls per sample, including somatic copy-number alterations (SCNAs) of all sizes, were generated. Then, arm-level alterations within these SCNAs were distinguished. Finally, aneuploidy score (number of altered arms) for each patient was calculated as the sum total of altered arms.

Statistical Analysis

Statistical analysis was constructed by GraphPad Prism 7.0 software (San Diego, USA). Comparison between groups were performed using *t*-test. $p < 0.05$ was considered as statistically significant.

Results

Genomic Instability Was Found in CSF cfDNA Rather Than Plasma cfDNA

High disparity of gene mutations including CNV, indels, fusion, splice and nonsense in CSF were observed among these MM patients, indicating potential existence of genomic instability in several samples (Figure 1). Subsequently, a panel of 543 cancer-related genes was used to distinguish whether there was genomic instability in CSF. As shown in Figure 2, genomic instability was noticed in their CSF in 12 of 15 MM patients, while genomic stability in the remaining 3 MM patients. In addition, the CSF supernatant of the BPM patient also showed genomic stability. Notably, no genomic instability was noticed in the plasma cfDNA of all patients, indicating clonal evolution.

Figure 1 The blood and cerebrospinal fluid of 15 meningeal metastasis patients and 1 brain parenchymal metastasis patient were detected by a panel of 543 cancer-related genes. Heatmap displayed gene mutations in CSF and blood of these patients.

Figure 2 (A–L) A panel analysis of 543 cancer-related genes in blood and CSF in 12 patients with genomic instability. **(M–O)** A panel analysis of 543 cancer-related genes in blood and CSF in three patients with genomic stability. **(P)** A panel analysis of 543 cancer-related genes in blood and CSF in a brain parenchymal metastasis patient. **Abbreviations:** BC, white blood cells; CF, plasma; CSF, cerebrospinal fluid; CSFCD, cerebrospinal fluid precipitation; FFPE, formalin-fixed, paraffin-embedded tissues.

Genomic Variations Detected in CSF cfDNA of MM Patients

From Table 2, increased gene copy numbers of mesenchymal-epithelial transition (MET) and epidermal growth factor receptor 2 (ERBB2) were noticed in genomic instability group (n=3). Besides, EGFR, ERBB2, anaplastic lymphoma kinase (ALK), and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations were detected in 10 cases of genomic instability group and 1 of genomic stability group ($p=0.0033$). Among them, ERBB2 and EGFR had more gene mutations and were prone to instability. Moreover, the mean tumor mutation burden in both plasma and CSF of the genomic instability group was higher than that of the genomic stability group (4.365 vs 3.408 for plasma; 6.115 vs 3.03 for CSF) (Table 1). These data revealed the existence of two different molecular types of MM, namely genomic instability and genomic stability, and the genomic instability group had higher mutation burden and more mutations.

Copy Number Profiling in CSF cfDNA

WGS is a gold standard for determining the instability of genome. To further verify the genomic instability in these patients, CSF were collected from 9 of the 16 patients, followed by the performance of low-depth WGS analysis. Consistent with the results of 543-gene panel, we found 6 MM cases of genomic instability (Figure 3) and 2 MM cases of genomic stability in CSF cfDNA (Figure 4). Additionally, the CSF cfDNA of BPM patients showed genomic stability. No genomic instability was noticed in their plasma cfDNA.

Then we calculated an aneuploidy score that reflected the correlation between genomic instability and aneuploidy of the nine patients. As shown in Table 3, the aneuploidy score of MM patients ranged from 0 to 29, and the aneuploidy score of two MM patients with genomic stability were both 0. By contrast, MM patients with genomic instability had higher aneuploidy score in CSF supernatant than in plasma. The aneuploidy scores of CSF supernatant and plasma in the BPM case were 2 and 0, respectively. Collectively, MM patients with genomic instability were more likely to have aneuploidy events.

Table 2 Genomic Variation in Cerebrospinal Fluid of the 15 Meningeal Metastasis Patients

Genomic Variation	Genomic Instability (n)	Genomic Stability (n)	Exon	Frequency (%) / Gene Copy Number (n) in CSF-SQ
ERBB2 p. V659E	1	–	17	26.8%
Increased copy number of ERBB2	1	–	–	n=35.03
EGFR p. T790M	1	–	19	25.82%
EGFR p. L858R	1	–	21	42.62%
EGFR p. G719A	–	1	18	3.55%
EGFR exon 20 insertion			20	
EGFR p. A767_V769dupASV	1	–		37.17%
ERBB2 p.Y772_A775dupYVMA	1	–		72.41%
EGFR exon 19 deletion			19	
EGFR p.E746_A750delELREA	3	–		26.09%
				80.65%
				9.81%
Increased copy number of MET	2	–	–	n=2.65
				n=3.1
KRAS p. G13R	1	–	2	19.32%
ALK p. L1196M	1	–	23	13.87%

Notes: N referred number of cases. Autosomal gene copy number n=2; n<1.5 mean reduced copy number; n> 2.5 mean increased copy number. (–): no data.

Abbreviation: CSF-SQ, cerebrospinal fluid supernatant.

Figure 3 (A–F) Copy number variation in blood and CSF in six meningeal metastasis patients with genomic instability. The log₂ ratio value is plotted on the y-axis; the x-axis represents chromosomes. Red indicates copy number gain, green indicates copy number loss, and blue indicates no change.

Abbreviations: CF, plasma; CSF-SQ, cerebrospinal fluid supernatant.

The Correlation Between Genetic Subtype and Clinical-Pathological Features

The mean age of the 15 MM participants was 53.53 years. Mean overall survival of MM patients with genomic instability was lower than MM patients with genomic stability (31.6 months vs 56 months, $p=0.7405$)

(Figure 5). A higher DS-GPA score (1.5–2.5) was associated with a longer median survival in both genomic instability and genomic stability groups.

Of the 12 MM patients with genomic instability in CSF, 2 had breast cancer, 1 had gastric cancer and the other 9 all suffered from lung adenocarcinoma. Of the three MM participants with genomic stability in CSF,

Figure 4 (A and B) Copy number variation in blood and CSF in two meningeal metastasis patients with genomic stability. **(C)** Copy number variation in blood and CSF in brain parenchymal metastasis case with genomic stability. The log₂ ratio value is plotted on the y-axis; the x-axis represents chromosomes. Red indicates copy number gain, green indicates copy number loss, and blue indicates no change.

Abbreviations: CF, plasma; CSF-SQ, cerebrospinal fluid supernatant.

only one had lung adenocarcinoma and the other two had other cancers, including breast and colorectal cancers (Table 1). Taken together, genomic instability was more

likely to occur in CSF in patients with lung adenocarcinoma than in patients with other cancers.

Discussion

The spread of tumor cells into CSF is a devastating complication of various solid tumors, such as lung cancer, breast cancer and melanoma. Due to the difficulty in sampling MM, plasma and CSF cfDNA detections were recommended to analyze the gene variation characteristics of MM.¹⁵ However, plasma tumor-derived cfDNA in the central nervous system is rarely detected because the blood-brain barrier prevents cfDNA from entering the blood circulation.¹⁶ At present, growing studies have explored the sensitivity of CSF cfDNA to the prediction of actionable mutation carrying MM to targeted therapy.¹² However, there were still some patients who were resistant to TKI therapy despite carrying drug-sensitive mutations. A clinical study performed by Zhou et al¹⁷ suggested that of 417 patients treated with EGFR-TKI therapy, 194 developed new metastatic lesion. These above results imply the clonal evolution of MM, which requires more detailed molecular typing of MM.

The most notable finding of this study was that 12 of 15 MM patients developed genomic instability in CSF, while the remaining 3 had genomic stability. Besides, due to the ethical

Table 3 Aneuploidy Score of Patients with Genomic Instability or Genomic Stability

Patient No.	Aneuploidy Score of Genomic Instability		Aneuploidy Score of Genomic Stability	
	CSF-SQ	CF	CSF-SQ	CF
2	15	0	–	–
3	–	–	0	0
4	28	0	–	–
5	23	22	–	–
6	28	29	–	–
7	–	–	0	0
8	26	14	–	–
9	25	0	–	–
16	–	–	2	0

Abbreviations: CSF-SQ, cerebrospinal fluid supernatant; CF, plasma.

Figure 5 Overall survival analysis between meningeal metastasis patients with genomic instability and genomic stability.

difficulties in obtaining CSF samples from patients without MM, one BPM patient was selected as a control in this study. The panel of 543 cancer-related genes and low-depth WGS were performed on the plasma cfDNA, CSF supernatant and paraffin-embedded tissue sections of this patient, and results showed no genomic instability. MM patients with genomic instability exhibited higher mean tumor mutation burden than that of MM patients with genomic stability. These findings suggested high disparity of CNV profiling and actionable mutation burden in CSF of MM patients. Angelika and Nakao et al have pointed out the high chromosomal instability of colorectal cancer with brain metastases.^{18,19} Genetic instability is the result of chromosome segregation errors during mitosis,²⁰ which triggers the activation of cGMP-AMP synthase-stimulator of interferon genes (cGAS-STING) cytosolic DNA-sensing pathway and downstream nuclear factor- κ B (NF- κ B) signaling.^{21,22} A number of researches have shown that cells with high genetic instability facilitates migration and invasion, and the upregulated mRNA expression of NF- κ B and hPTTG1 in tumors with high genetic instability genes is closely connected with shorter disease-free survival in lung and breast cancers.^{23,24}

MM is usually a late event in malignant tumors, with poor prognosis and short survival rate. Likewise, the mean overall survival for MM patients in this study was 36.53 months, among which the mean overall survival of patients with genomic instability was lower than patients with genomic stability. Therefore, clinicians need to choose the most appropriate drug therapy for MM patients in such a short survival period and minimize drug-related adverse reactions, thereby improving the quality of life of patients. In these patients with genomic instability, sensitive mutations and comparable gene amplification of actionable genes were determined though these patients displayed significantly resistant to TKI target therapy.

Most of these actionable genes, including EGFR, ERBB2, MET, KRAS and ALK, have been implicated in the development of MM, and some hotspot mutations such as phosphatidylinositol-3-kinase catalytic subunit (PIK3CA) p.H1047R, PIK3CA p.E545K, ERBB2 p.V842I and tumor protein 53 (TP53) p.Y234H were also observed exclusively in CSF cfDNA, which implied that the mutation of actionable genes promoted the occurrence of MM. Consistently, Riess et al²⁵ have indicated that EGFR-mutated lung adenocarcinoma is more prone to MM in terms of biological behavior, without gender difference, among which the mutation rate of EGFR was the highest, followed by ERBB2.

EGFR-TKIs exhibit a significant efficacy in treating NSCLC with sensitive EGFR mutations. Several researches have clarified that NSCLC patients harboring EGFR exon 19 deletion or exon 21 L858R can benefit from icotinib and osimertinib.^{26,27} In this study, 38.82% of MM patients bearing EGFR mutations in the 18–21 exon region, among which exon 19 deletion and L858R accounted for 62.5% of all EGFR mutations. The lung adenocarcinoma patients harboring sensitive EGFR mutations benefit from EGFR-TKI drugs.²⁸ Furthermore, osimertinib has been reported to be effective in patients with EGFR-mutated MM.²⁹ However, in this study, genomic instability patients with actionable mutation rarely benefited from EGFR-TKI target therapy, such as EGFR p. L858R and EGFR p.E746_A750delELREA patients who received targeted therapy with icotinib and osimertinib but progressed rapidly. Notably, several patients with ERBB2 or EGFR mutations improved after CSF chemotherapy combined with target therapy, resulting in a significant increase in overall survival. These data indicated that MM patients with genomic instability might lead to poor efficacy of target therapy, while combined CSF chemotherapy might improve clinical outcome.

To evaluate the clinical prognostic disparity between the genetic stable and unstable patients, GPA scoring system was used composing several indicators including age, Karnofsky performance score, brain metastasis number and the presence or absence of extracranial metastasis, which was established by Sperduto et al³⁰ for predicting the prognosis of brain metastases. Antoni and Noel believed that GPA is a predictive indicator that is easy to quantitatively analyze and use.³¹ In the current study, we found that the MM patients with genomic instability had lower overall survival and higher aneuploidy scores compared with those with genomic stability. Furthermore, MM patients with higher DS-GPA scores benefited from better clinical outcome in both genomic stability and genomic instability groups. ERBB2 amplification in breast

cancer is associated with poor overall survival and tumor recurrence time.³² ERBB2 and MET activate downstream signaling pathways, such as mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, through gene amplification and overexpression in breast, lung and gastric cancers.³³ Our results further suggested that an increased gene copy numbers of ERBB2 and MET in patients with genomic instability might result in worse outcomes, and even death ($n=2.65$ for MET; $n=35.03$ for ERBB2).

Interestingly, another finding of this study was that unlike MM, there was no genomic instability in brain dural metastasis of lung adenocarcinoma, revealing a potential existence of tumor heterogeneity. Given the limited sample size and the lack of histological and serum data of MM patients in this study, it is necessary to confirm the presence of CSF genomic instability in lung adenocarcinoma MM patients and to discuss the absence of genomic instability in brain dural metastasis of lung adenocarcinoma in a larger cohort. Besides, we hypothesized that the combination of TKI and CSF chemotherapy is more suitable for people with genomic instability MM, because genomic instability has a poor effect on TKI treatment, which also needs further verification.

Conclusion

Genomic instability presents in the CSF cfDNA rather than plasma cfDNA of MM patients. In addition, genomic instability might be the potential cause of the difference in MM, which partly explains the source of drug resistance in MM patients bearing sensitive mutations even if specific target gene mutations are present in blood and CSF. Therefore, a more appropriate cytotoxic chemotherapy regimen is recommended for these patients. In summary, MM patients should be sub-grouped according to the status of genetic instability, which is of far-reaching significance for treatments and clinical trials of MM in the future.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author (Peng Wang) on reasonable request.

Ethics Approval and Informed Consent

This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Tianjin Cancer

Hospital (bc2020027). Written informed consent was obtained from patients enrolled in this study.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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