Cell counts and clinical significance of circulating tumor cells in patients diagnosed with nasopharyngeal carcinoma

Yanjiao Mao1, Wei Yin1, Jiangtian Chen2, Qinghua Deng1, Shixiu Wu1

1Department of Radiation Oncology, Hangzhou Cancer Hospital, Hangzhou 310000, Zhejiang Province, China; 2General Office, Blood Center of Zhejiang Province, Hangzhou 310006, Zhejiang Province, China

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Abstract: Objective: Mechanisms underlying the distribution and dissemination of nasopharyngeal carcinoma (NPC) remain elusive. This study was designed to investigate the potential roles of circulating tumor cells (CTCs) in incidence and progression of NPC. Methods: A total of 45 patients, diagnosed with NPC, received 65 cycles of CTC detection. Positive rates of CTC in patients with different stages of NPC were statistically compared. Association between CTC counts and alternative clinical factors was analyzed. Variations in CTC counts were compared, before and after treatment. Moreover, correlation between MMP2 levels and CTC was investigated. Results: CTC counts were detected before, during, and after corresponding therapy. The positive rate of CTC in patients with stage II NPC was calculated as 83.3%. Rates were 100% for stage III, 90.0% for stage IV, and 100% for unknown stage. Positive rates of mesenchymal CTC were 50.0%, 85.7%, 50.0%, and 80.0% in patients with stage II, III, IV, and unknown stage NPC. Spearman’s rank correlation analysis revealed that N staging (P=0.024) and positive rates of EA/IgA (P=0.048) were significantly correlated with the number of epithelial CTCs. After corresponding treatment, total CTCs (P=0.001) and hybrid CTCs (P=0.026) counts were significantly decreased, while epithelial CTCs, mesenchymal CTCs, and mesenchymal CTCs ratios were reduced, with no statistical significance. Age was positively associated with expression levels of matrix metalloproteinase 2 (MMP2) (P=0.040). Conclusion: Present findings suggest that CTCs play potential roles in incidence and progression of NPC. However, present results should be validated by further investigation.

Keywords: Nasopharyngeal carcinoma, circulating tumor cell, mesenchymal, hybrid, correlation

Introduction

Carcinoma initially arises as organ-confined, eventually migrating to distant sites through the blood. Tumor metastasis has been considered the primary cause of cancer patient mortality. The patterns by which tumor cells escape from the original site, survive in the blood flow, and spread to distant organs [1] or relocate to the primary site of tumors [2] have remained elusive.

During the process of epithelial-mesenchimal transition (EMT), epithelial cells acquire a mesenchymal identity. This has been regarded as a basic manifestation of tumor invasiveness and aggressiveness. Tumor cells can obtain the ability to detach from the primary mass, enter blood or lymph vessels, exit from the vessels, and eventually relocate to distant sites [3]. Early spreading of tumor cells is difficult to observe, even using high-resolution imaging examinations. Circulating tumor cells (CTCs) potentially share similar features with differentiated and stem-like tumor cells and recapitulate the heterogeneous composition of primary lesions [4]. Consequently, CTC detection in the peripheral blood may offer guidance in identification of properties consistent with EMT phenotype or in unravelling the resistance of CTCs to chemo- and radio-therapy. Application of CTC detection can be utilized as a non-invasive approach, detecting the genotypes of tumor cells. This can be frequently performed throughout chemo-radiotherapy, monitoring the acquisition of novel genetic abnormalities in response...
Significance of CTCs in nasopharyngeal carcinoma

to chemo-radiotherapy. This principle is equally applicable for patients diagnosed with nasopharyngeal carcinoma, having been frequently treated with chemo-radiotherapy to achieve the purpose of organ and function sparing therapeutic options.

CTCs are rarely seen in the circulation system of the host. Identification of CTCs primarily depends upon methods for their isolation based upon epithelial cell adhesion molecules, mainly expressed by epithelial cells but absent in normal blood cells. Immunomagnetic capture methods have been adopted by treating blood specimens with antibodies to cell adhesion protein epithelial cell adhesion molecules conjugated with magnetic particles. This is followed by separation of the cells in a magnetic field. Isolated cells are stained with antibodies against alternative epithelial markers to identify and distinguish CTCs. These are capable of expressing cytokeratines, while lacking CD45 expression without contaminating white blood cells [5].

The significance CTCs in clinical practice remains largely unknown. Potential functions in early detection of cancer metastasis or evaluation of therapeutic response in patients with advanced malignant tumors have been studied in patients diagnosed with metastatic breast cancer [6], colorectal cancer [7], and hormone-refractory prostate cancer [8]. The presence of CTCs prior to chemotherapy/radiotherapy and the variation in CTC counts throughout treatment play a more vital role in predicting prognostic outcomes, compared with alternative conventional approaches. Moreover, CTC detection can be applied to analyze chemo-radiotherapy-resistant phenotypes in response to chemo-radiotherapy, delivering individualized therapeutic options in a non-invasive manner [9]. In addition, previous studies have demonstrated that [10, 11] CTC detection and monitoring can be utilized to minimize the risk of cancer metastases in early-stage breast and prostate cancer. Patients treated for both metastatic and locally advanced nasopharyngeal carcinomas are highly likely to recur even after obtaining a complete response. Detection and identification of CTCs in the blood flow can ameliorate the prognostic profile defined by tumor-node-metastasis type and other biological prognostic factors, such as positive epidermal growth factor receptor or human papilloma virus.

In this clinical trial, baseline and clinical data of patients diagnosed with different stages of nasopharyngeal carcinoma were analyzed. Counts and distribution patterns of CTCs in these patients were investigated, before and after corresponding treatment. Correlation analysis was performed concerning CTC count/types, different clinical stages, matrix metalloproteinase 2 (MMP2) gene expression levels, and clinical parameters, respectively. The current study evaluated clinical significance, offering evidence for the feasibility of CTC detection in diagnosis and treatment of nasopharyngeal carcinoma.

Materials and methods

Blood sampling procedures

A total of 45 patients, admitted to Hangzhou Cancer Hospital, from January 2016 to December 2017, were recruited for this study. Inclusion criteria: Diagnosed with nasopharyngeal carcinoma; Sufficient surgical tissue samples; Appropriate performance status (0-2); Aged ≥ 18 years old. After discarding the first 2 mL to avoid the risk of potential blood contamination, a portion of 5 mL peripheral blood samples were collected from nasopharyngeal carcinoma patients. Samples were anti-coagulated with EDTA, then collected for subsequent experimentation. Blood samples were collected at different time points, including 1-3 days prior to surgery/chemotherapy, after surgery/chemotherapy, and 3 months after discharge from corresponding treatment. Study procedures were approved by the Ethics Committee of Hangzhou Cancer Hospital. Written informed consent was obtained from all participants prior to the experiment.

CTC isolation procedures

After comparing the efficiency of the CanPatrol™ CTC-enrichment technique, before and after optimization [12], blood samples were collected within 4 hours. They were subsequently filtered by a calibrated membrane with 8-μm pores in diameter (Millipore, Billerica, MA, USA). Meeting requirements and standards, a filtration system consisting of a filtration tube containing the membrane (SurExam, Guangzhou,
Significance of CTCs in nasopharyngeal carcinoma

China), a manifold vacuum plate with valve settings (SurExam), an E-Z 96 vacuum manifold (Omega, Norcross, GA, USA), and a vacuum pump (Auto Science, Tianjin, China) was applied. Erythrocytes were eliminated by a red blood cell lysis buffer, consisting of 154 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA. Subsequently, the treated cells were transferred to a filtration tube after re-suspension in PBS buffer containing 4% formaldehyde for approximately 5 minutes. The pump valve was then switched, reaching a pressure of > 0.08 MPa. Next, the manifold vacuum plate valve was switched on, fulfilling the experimental requirements of CTC filtration.

Tri-color RNA-ISH assay

According to branched deoxyribonucleic acid (bDNA) signal amplification technology, the RNA-ISH method was employed to detect the target sequence [13]. Sequences of CD45 (leukocyte biomarker), CK19 (epithelial biomarker), and twist (the mesenchymal biomarker) were used to distinguish epithelial cells and mesenchymal cells from hybrid CTCs. On the membrane of the 24-well plate, cells were treated with a protease, prior to hybridization, with a capture probe specific for CK19, twist, or CD45. After 2 hours of incubation at 42°C, cells were washed with a buffer to remove unbound probes. They were then incubated with preamplifier solution containing 30% horse serum, 1.5% sodium dodecyl sulfate, 3 mM Tris-HCl (pH=8.0), and 0.5 fmol of preamplifier at 42°C for 2 hours, amplifying the signal. Membranes were washed with 1,000 μl of wash buffer (0.1×SSC) and subsequently incubated with 100 μl of amplifier solution, consisting of 30% horse serum, 1.5% sodium dodecyl sulfate, 3 mM Tris-HCl (pH=8.0), and 2 CTCs in nasopharyngeal carcinoma. Fluorescently labeled probes, conjugated with fluorescent dyes Alexa Fluor 594 specific for CK19, Alexa Fluor 488 for twist, and Alexa Fluor 647 for CD45, were supplemented and incubated at 42°C for approximately 2 minutes. After staining with DAPI, stained cells were observed under a fluorescence microscope (Olympus BX53, Tokyo, Japan).

VCA/IgA, EA/IgA, and EBV-DNA detection

Detection analyses for VCA/IgA, EA/IgA, and EBV-DNA were performed, according to manufacturer protocol, using ELISA kits and the EBV nucleic acid quantitative detection reagent kit. Internal quality assessment of VCA/IgA and EA/IgA in each plate, including a calibrator, negative control, and positive control, was carried out. Obtained results were calculated by referring to the semi-quantitative equation: relative OD (rOD) = OD (sample)/OD (calibrator). The VCA/IgA-positive rOD value was set as 1.5. The value of EA/IgA-positive rOD was set as 0.5. Quantitative polymerase chain reaction was conducted for evaluation and assessment of EBV-DNA.

Statistical analysis

Data analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, U.S.). Statistical analyses were performed using one-way analysis of variance (ANOVA), as well as Student’s two-tailed t-test. Correlation between two variables was statistically analyzed using Spearman’s rank correlation test. P-values less than 0.05 indicate statistical significance.

Results

Baseline data

A total of 45 nasopharyngeal carcinoma patients underwent 65 cycles of CTC detection. Of these, 28 patients received CTC detection once, 14 cases received it twice, and 3 patients received it three times. All patients received CTC detection before, during, and after corresponding treatment. During CTC detection, CTC=0 indicated a negative outcome and CTC ≥ 1 indicated a positive result. Of the 28 cases receiving CTC testing before treatment, 6 patients were classified as stage II nasopharyngeal carcinoma. Based on TNM staging, 7 patients were found with stage III, 10 were found with stage IV, and 5 were found with unknown stage. The mean positive rate of CTCs for 28 patients was calculated as 92.6%, with a 100% positive rate for stage III and unknown stage groups. The average positive rate of mesenchymal CTCs was 64.3%, with the highest positive rate of 85.7% found in the grade III group. Median CTC count was 6 and the mean CTC count was 7.7. Positive rates of CTCs and mesenchymal CTCs of patients with stage II nasopharyngeal carcinoma patients were the lowest among all groups, as illustrated in Table 1.
Significance of CTCs in nasopharyngeal carcinoma

Table 1. Expression levels of CTCs in patients diagnosed with different clinical stages of nasopharyngeal carcinoma patients

<table>
<thead>
<tr>
<th>Clinical staging</th>
<th>Positive rate of CTCs (%)</th>
<th>Positive rate of mesenchymal CTCs (%)</th>
<th>Median CTCs count</th>
<th>Mean CTCs count</th>
<th>CTCs range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage II (n=6)</td>
<td>83.3%</td>
<td>50.0%</td>
<td>4</td>
<td>5.2</td>
<td>0-13</td>
</tr>
<tr>
<td>Stage III (n=7)</td>
<td>100%</td>
<td>85.7%</td>
<td>6</td>
<td>7.4</td>
<td>3-17</td>
</tr>
<tr>
<td>Stage IV (n=10)</td>
<td>90.0%</td>
<td>50.0%</td>
<td>4</td>
<td>7.9</td>
<td>0-25</td>
</tr>
<tr>
<td>Unknown (n=5)</td>
<td>100%</td>
<td>80.0%</td>
<td>10</td>
<td>10.6</td>
<td>2-19</td>
</tr>
<tr>
<td>Total</td>
<td>92.6%</td>
<td>64.3%</td>
<td>6</td>
<td>7.7</td>
<td>0-25</td>
</tr>
</tbody>
</table>

Correlation between CTCs and clinical parameters

Clinical parameters mainly consisted of age, gender, TNM staging, T staging, N staging, tumor size, VCA/IgA, EA/IgA, and EB-DNA. Prior to corresponding treatment, correlation among CTC count, mesenchymal CTC percentages, and clinical parameters was statistically analyzed. Spearman’s rank correlation test revealed that the count of epithelial CTCs was positively correlated with N staging ($r=0.504$, $P=0.024$) and EA/IgA ($r=0.473$, $P=0.048$). Higher quantities of epithelial CTCs indicated higher N staging and higher risk of positive EA/IgA. However, CTCs count/types was not significantly associated with age, gender, tumor site, and whether VCA/IgA or EB-DNA was positive. Detailed results were demonstrated in Table 2.

Comparison of CTC counts before and after treatment

CTC counts, before treatment, were quantitatively measured 28 times. CTC detection was performed 27 times after corresponding treatment. Statistical analysis revealed that total CTC counts, as well as epithelial, hybrid, and mesenchymal CTCs and the proportion of mesenchymal CTCs, were decreased after corresponding treatment. Total CTC counts ($P=0.001$) and the count of hybrids ($P=0.026$) were significantly reduced, compared with values prior to treatment, as illustrated in Figure 1.

Correlation between MMP2 expression and clinical parameters

Clinical parameters mainly included clinical stage, T staging, N staging, tumor size, smoking habit, VCA/IgA, EA/IgA, and EB-DNA. Positive expression of MMP2 was defined as MMP2 was expressed in at least 1 CTC. Negative expression of MMP2 was defined as MMP2 not detected in any CTC. For patients with positive MMP2, the proportion of CTCs highly expressing MMP2 among CTCs with positive MMP2, exceeding 50%, was considered as high expression levels of MMP2. Percentages of CTCs lowly expressing MMP2 among CTCs with positive MMP2, exceeding lower than 50%, were regarded as low expression levels of MMP2. Expression levels of MMP2 falling into this range were considered moderate expression levels of MMP2. Spearman’s correlation analysis demonstrated that age was significantly correlated with expression levels of MMP2 ($r=0.414$, $P=0.040$). No significant association was noted between TNM staging and expression levels of MMP2 ($r=0.299$, $P=0.166$), as illustrated in Table 3. Statistical results indicate that expression levels of MMP2 were significantly upregulated during aging.

MMP2 expression in different types of CTCs

For the 52 epithelial CTCs, 28 did not express MMP2, 8 lowly expressed MMP2, 12 showed moderate expression of MMP2, and 4 CTCs highly expressed MMP2, respectively. A total of 109 hybrid CTCs were detected for MMP2 expression. Of these, 53 CTCs did not express MMP2, 33 cells lowly expressed MMP2, 20 showed moderate expression, and 2 were found with high expression levels of MMP2, respectively. For the 45 mesenchymal CTCs, 31 CTCs were found without expression, 6 were found with low expression, 7 were found with moderate expression, and 1 showed high expression levels of MMP2, respectively. See Table 4.

Discussion

Previous investigations have demonstrated that CTCs are distributed in the peripheral blood from patients diagnosed with different types of malignant tumors. Detection and quantification of CTCs have captivated widespread attention from oncologists in clinical practice. Currently utilized CTC-enrichment approaches have been performed depending on various factors, including immunocapture, CTC diameter, CTC density, and electrical charge.
Significance of CTCs in nasopharyngeal carcinoma

Table 2. Correlation analysis between CTCs and clinical parameters in nasopharyngeal carcinoma patients

<table>
<thead>
<tr>
<th>Spearman’s rho</th>
<th>Total CTCs</th>
<th>Epithelial CTCs</th>
<th>Hybrid CTCs</th>
<th>Mesenchymal CTCs</th>
<th>Percentage of mesenchymal CTCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T staging (n=20)</td>
<td>r 0.131</td>
<td>0.138</td>
<td>0.138</td>
<td>-0.049</td>
<td>-0.103</td>
</tr>
<tr>
<td></td>
<td>P 0.212</td>
<td>0.188</td>
<td>0.190</td>
<td>0.649</td>
<td>0.330</td>
</tr>
<tr>
<td>N staging (n=20)</td>
<td>r 0.111</td>
<td>0.504</td>
<td>0.171</td>
<td>-0.050</td>
<td>-0.084</td>
</tr>
<tr>
<td></td>
<td>P 0.291</td>
<td>0.024</td>
<td>0.104</td>
<td>0.635</td>
<td>0.428</td>
</tr>
<tr>
<td>TNM staging (n=23)</td>
<td>r 0.093</td>
<td>0.122</td>
<td>0.013</td>
<td>0.045</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>P 0.376</td>
<td>0.246</td>
<td>0.901</td>
<td>0.669</td>
<td>0.933</td>
</tr>
<tr>
<td>EA/IgA (n=18)</td>
<td>r 0.220</td>
<td>0.473</td>
<td>0.017</td>
<td>0.051</td>
<td>-0.125</td>
</tr>
<tr>
<td></td>
<td>P 0.378</td>
<td>0.048</td>
<td>0.860</td>
<td>0.626</td>
<td>0.512</td>
</tr>
</tbody>
</table>

Table 3. Correlation analysis between age, TNM staging, and MMP2 expression levels

<table>
<thead>
<tr>
<th>Spearman’s rho</th>
<th>MMP2 status (positive/negative)</th>
<th>MMP2 expression (high/moderate/low)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (n=25)</td>
<td>r 0.436</td>
<td>0.414</td>
</tr>
<tr>
<td></td>
<td>P 0.091</td>
<td>0.040</td>
</tr>
<tr>
<td>TNM staging (n=23)</td>
<td>r 0.251</td>
<td>0.299</td>
</tr>
<tr>
<td></td>
<td>P 0.247</td>
<td>0.166</td>
</tr>
</tbody>
</table>

Table 4. Distribution patterns of MMP2 in epithelial, hybrid, and mesenchymal CTCs

<table>
<thead>
<tr>
<th>CTCs type</th>
<th>MMP2 expression</th>
<th>None</th>
<th>Low expression</th>
<th>Moderate expression</th>
<th>High expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial CTCs</td>
<td>28</td>
<td>8</td>
<td>12</td>
<td>4</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Hybrid CTCs</td>
<td>53</td>
<td>33</td>
<td>20</td>
<td>3</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Mesenchymal CTCs</td>
<td>31</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

[14]. However, each of these available technologies has a certain degree of flaws, including low purity, poor recovery rates, and high levels of time consumption. [15-19]. A novel technique of CanPatrol™ CTC enrichment has yielded high efficiency in isolating and characterizing CTCs [20]. Compared with alternative approaches, CanPatrol™ CTC-enrichment technique has multiple advantages in terms of high recovery efficiency, isolation of CTCs from circulating tumor microemboli, antibody-independent capture, and identification and classification of CTCs. In this present investigation, this technique was applied to accomplish the isolation and analysis of CTCs in nasopharyngeal carcinoma patients, exploring distribution patterns and clinical significance of CTCs concerning incidence and progression of nasopharyngeal carcinoma.
Previous studies have demonstrated that CTCs are closely and intimately correlated with clinical features of patients diagnosed with certain types of cancers [21, 22]. In the year of 2007, according to the American Society of Clinical Oncology (ASCO), CTCs were defined as a hallmark of multiple types of cancers. CTCs have been proven to detect lesions occurring during early stages in patients without malignant tumors [23]. Consequently, CTCs act as one of the proper parameters in the early diagnosis of certain types of malignant tumors. In this investigation, the clinical significance of CTCs in the diagnosis of nasopharyngeal carcinoma was thoroughly explored. Findings in this study show that the total positive ratio of CTCs was extremely high, exceeding 90% in patients suffering from different stages of nasopharyngeal carcinoma. Interestingly, the positive ratio of mesenchymal CTCs was the highest in patients diagnosed with stage II nasopharyngeal carcinoma, in contrast with previous findings [24]. Such irregular distribution patterns were probably catered to the special requirements of development and metastasis of nasopharyngeal carcinoma. During circulation, CTCs gradually proceed the epithelial-to-mesenchymal transition. This is closely correlated with cancer progression. In this study, total CTCs, hybrid CTCs, mesenchymal CTCs, and positive ratios of mesenchymal CTCs were not significantly correlated with clinical stage of nasopharyngeal carcinoma. However, epithelial CTCs were significantly associated with N staging of nasopharyngeal carcinoma. This phenomenon suggests that the transition of CTCs is a complicated process, constantly proceeded. Subsequent experiments are urgently required to unravel the underlying mechanisms of this event.

Moreover, total CTC counts, epithelial, hybrid, and mesenchymal CTCs, and the proportion of mesenchymal CTCs were decreased after corresponding treatment, especially total CTC counts. Counts of hybrid and mesenchymal CTCs were significantly declined, compared with values prior to treatment, suggesting that therapeutic options yield clinical efficacy by downregulating total CTCs counts and subgroups of CTCs. Of the various risk factors, no factors were detected to be strongly associated with progression, local recurrence, poor prognosis, metastasis, and risk of death in nasopharyngeal carcinoma, in accord with previous findings [25]. This discrepancy is probably due to the limited sample size of the current investigation.

Previous investigations have demonstrated that EBV is involved in incidence and development of nasopharyngeal carcinoma. Expression of EBV gene and protein can enhance the risk of nasopharyngeal carcinoma patients suffering from higher incidence, malignant growth, and poor immune systems [26, 27]. In this present investigation, EBV expression slightly differed in nasopharyngeal carcinoma patients with mesenchymal, hybrid, and epithelial types of CTCs. Hybrid CTCs were not significantly associated with expression of EA/IgA. In terms of mesenchymal CTCs, EBV expression showed no association. However, epithelial CTCs were significantly correlated with expression levels of EA/IgA, suggesting that EBV might play a role in the distribution patterns of CTCs in the epithelial layer.

MMP2 has been proven to be correlated with cancer metastasis. Thus, whether MMP2 plays a role in CTCs was investigated, as well as whether it is associated with risk of NPC. First, correlation between MMP2 expression status and levels and other clinical parameters was analyzed. Results demonstrated that expression levels of MMP2 (low/moderate/high) were significantly correlated with the age of nasopharyngeal carcinoma patients, suggesting that expression of MMP2 is upregulated over the process of aging of nasopharyngeal carcinoma patients. However, status and levels of MMP2 expression were not significantly associated with clinical staging (N, TNM, and T staging) of nasopharyngeal carcinoma patients. In addition, expression patterns of MMP2 in hybrid, mesenchymal, and epithelial CTCs significantly differed. The positive ratio of MMP2 in hybrid CTCs was the highest, up to 51.2%. It was higher than the 46.3% in epithelial CTCs and the 31.1% in mesenchymal CTCs. Interestingly, the percentage of low MMP2-expressed CTCs was the highest in hybrid CTCs. However, the moderate MMP9-expressed proportion was the highest in epithelial CTCs. Furthermore, the highest percentage of high MMP2-expressed CTCs was detected in epithelial CTCs (7.7%), compared with 2.8% in hybrid CTCs and 2.2% in mesenchymal CTCs. However, discrepancies in terms of distribution patterns of MMP2 in hybrid, epithelial, and mesenchymal
CTCs should be explained by subsequent research.

Conclusion

Taken together, positive rates of CTCs in nasopharyngeal carcinoma patients are alarmingly high. N staging and positive rates of EA/IgA are significantly correlated with the number of epithelial CTCs. Age is positively associated with expression levels of MMP2.

Disclosure of conflict of interest

None.

Address correspondence to: Shixiu Wu, Department of Radiation Oncology, Hangzhou Cancer Hospital, 34 Yanguan Lane, Zhongshan Nan Lu, Shangcheng District, Hangzhou 310000, Zhejiang Province, China. E-mail: wushixiu2018@aliyun.com

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Significance of CTCs in nasopharyngeal carcinoma


