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The Prognostic Value of Ezrin Expressed on Circulating Tumor Cells in Nasopharyngeal Carcinoma

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Abstract

Background and Purpose: This study focused on exploring the clinical significance of Ezrin on Circulating Tumor Cells (CTCs) in Nasopharyngeal Carcinoma (NPC) patients.

Materials and Methods: The confirmed NPC patients were enrolled in this study, and the patients were followed-up for 6 to 28 months after therapy. CTCs from pre- and post-therapy NPC patients were captured, and Ezrin gene expressed on CTCs was detected by RNA-in situ hybridization. Simultaneously, the concentrations of Epstein-Barr (EBV) DNA and VCA-IgA in NPC patients were also detected, and the associations of Ezrin on CTCs with EBV DNA and VCA-IgA were analyzed. The correlations of CTCs count, cell types and Ezrin with the various clinical stages and metastasis of NPC were analyzed. A comparative analysis of CTCs and Ezrin levels from NPC patients at pre- and post-therapy was conducted.

Results: The proportion of biphenotypic CTCs was increased in NPC patients at the advanced stage compared to patients at the early stage. Ezrin in biphenotypic CTCs was higher than that in the epithelial and mesenchymal CTCs. Ezrin positive rates were associated with NPC stages, the advanced patients had higher Ezrin rates than the early ones. After being effectively treated, Ezrin expression of NPC patients significantly decreased. A positive correlation was observed between $Ezrin^+$ CTCs count and VCA-IgA.

Conclusion: Ezrin expression in CTCs is positively associated with NPC progress, and it decreases after effective therapy. This imply that Ezrin in CTCs may be a novel prognostic biomarker for NPC patients.

Keywords: Nasopharyngeal carcinoma; CTCs; Ezrin; Epstein-Barr virus

Introduction

Nasopharyngeal Carcinoma (NPC) is a common and malignant tumor arising from nasopharynx epithelial cells, and the occurrence of NPC is higher in Southeast Asia, Northeast India, and Arctic populations, showing the distinct geographic and ethnic distribution [1,2]. There are more than 70% of new cases in the east and Southeast Asia, especially in South China [3,4]. Currently, the etiology of NPC is still unclear, it might be involved with race differentiation, chemical carcinogens, environmental factors and smoking habits [5,6]. Epstein–Barr Virus (EBV) infection is considered as a prominent etiological factor via encoding oncoproteins and regulating various signaling pathways [7]. In particularly, EBV lytic induction therapy appears as a virus-targeted therapeutic approach, which can specifically kill EBV-infected NPC cells through synergistically exploiting chemical lytic inducers and nucleoside analogue antiviral pro-drugs [8]. Even though some clinical trials have shown the potential of several chemical compounds for reactivating EBV lytic cycle in EBV^+ lymphomas and NPC the mechanisms remain to be elaborated [9,10]. The radiotherapy and neoadjuvant/adjuvant chemotherapy are still regarded as the standard treatment strategies for advanced NPC patients but the therapeutic efficacy needs to be ameliorated [11,12]. Clinically, majority of confirmed NPC patients are in the advanced stage with severe local lymph node metastasis and even distant metastasis because of the delayed diagnosis [13]. Therefore, exploring early diagnostic techniques, especially noninvasive serum liquid biopsies, is extremely essential for improving survival rates and monitoring therapeutic efficiency in NPC patients.

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It has been found that tumor cells could be released from tumor lesions at the early stage, further entering into the circulating system. The free tumor cells are named as Circulating Tumor Cells (CTCs). CTCs, serving as the origin of distant metastasis, are responsible for various cancer progression by promoting the expression of carcinogenic genes and seeding secondary lesions [14,15]. Therefore, the analysis of CTCs count is extremely important at the early and recurrent stages, which is benefit for monitoring therapeutic efficacy, evaluating prognosis and formulating appropriate individualized treatment strategies. Meanwhile, the genetic or phenotypic biomarkers expressed on CTCs also needs to be quantitatively detected [16]. In addition, CTCs karyotyping can directly impact the sensibility of chemotherapy and radiotherapy, indicating that CTCs could function as a novel biomarker for treatment, diagnosis and follow-up of NPC patients [17]. Nevertheless, the studies about the biomarkers expressed on CTCs in the field of Head and Neck Squamous Cell Carcinoma (HNSCC) are lacking, thus, the role of CTCs in NPC patients remains to be further elucidated.

Ezrin is a member of Ezrin-Radixin-Moesin (ERM) family, it is capable for promoting the combination of the actin-containing cytoskeleton with the plasma membrane molecules [18]. Ezrin has two statuses, including active and inactive conformations. Active Ezrin mainly localizes at the plasma membrane with an open form, while it dominantly resides in the cytoplasm with a dormant closed form. Inactive Ezrin can form oligomers where the folded C-terminal domain binds tightly to the FERM domain, further covering Ezrin's active sites [18,19]. Ezrin expressed on CTCs plays an essential role in cancer development and progression *via* regulating a variety of cellular activities, such as cancer cells survival, proliferation, adhesion, invasion and migration [20-22]. Increasing studies have revealed that Ezrin is highly expressed in diverse cancer types, including NPC, breast cancer, colorectal carcinoma, and gastric cancer [23-26]. In the present study, we performed RNA-ISH assay to detect the expression of Ezrin gene in CTCs. Further, the relationships of CTCs number, cell type and the expression levels of Ezrin gene with clinical stages and metastases were analyzed. The results showed that the ratio of mesenchymal CTCs in the peripheral blood of NPC correlated with distant metastases, and overexpression of Ezrin gene in CTCs promoted distant metastases. Therefore, the Ezrin gene expressed on CTCs was detected at both pre- and post-treatment in our study.

Materials and Methods

Patients and blood samples

The NPC patients were obtained from Hunan Cancer Hospital & the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University (Changsha, China) from July 2018 to November 2020, and were followed-up 6 to 28 months. The inclusion criteria were shown as follows: (1) Pathologically diagnosed NPC; (2) newly diagnosed NPC without receiving any anti-cancer treatments; (3) without prior malignancy; (4) were willful to participate in this study and provided written informed consents. Cancer stage was classified according to the International Union Against Cancer/American Joint Committee on Cancer (UICC/AJCC) TNM (Tumor Node Metastasis) staging system. Clinicopathological data were collected, including age, gender, tumor location, and clinical stage of the tumor. Table 1 summarized the characteristics of all patients. 5 ml peripheral blood sample was drawn from NPC patients, then was anticoagulated with EDTA. Blood samples were taken from the NPC patients at pre-treatment, 3, 6, and 12 months after radiotherapy and chemotherapy. All samples were analyzed using the CanPatrol system (SurExam Biotech, Guangzhou, China) [27]. The experimental protocol has been approved by the Ethics Committee of the Hunan Cancer Hospital.

Isolation of CTCs

As mentioned previously, CTCs were separated using the CanPatrol system. Peripheral blood samples (5 ml) from NPC patients were collected in Ethylenediaminetetraacetic Acid (EDTA) tubes after discarding the first 2 ml to avoid potential contamination. The blood samples were diluted 1:10 with the standard buffer, stayed at room temperature for 10 min, and filtered through an 8 μm diameter calibration membrane-based filtration system (Millipore, Billerica, MA, USA). The filtration system is consisted of a membrane-coated filtration tube (SurExam Biotech), valve-based manifold vacuum plate (SurExam Biotech), E-Z 96 vacuum manifold (Omega, Norcross, GA, USA) and vacuum pump (Auto Science, Tianjin, China). Erythrocytes were removed by the red blood cell lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA [all from Sigma, St Louis, MO, USA]) in deionized water, and then re-suspending cells with Phosphate Buffered Saline (PBS) containing 4% formaldehyde for 5 min, next transferring cells suspension to the filtration tube, which would switch on the pump valve with at least 0.08 MPa, finally, switching the manifold vacuum plate valve to perform the filtration.

Multiplex RNA-in situ hybridization (RNA-ISH) assay

Tri-color RNA-ISH assay was carried according to the procedures as previously reported using branched Deoxyribonucleic Acid (bDNA) signal amplification technology [27]. Three groups of fluorescent dyes-labeled nucleic acid probes were designed to identify and examine the expression levels of epithelial and mesenchymal genes in CTCs, including EpCAM, CK8/18/19 (epithelial biomarkers), Twist [1] and Vimentin (interstitial biomarkers), and CD45 (leukocyte biomarkers). Fluorescence signal was analyzed with an automated imaging fluorescence microscope (100x) (Olympus BX53; Olympus, Tokyo, Japan). The red and green dots of fluorescent signals represented the epithelial and mesenchymal gene expression, respectively, while the white fluorescent dots represented the CD45 gene expression (the markers of white blood cells). All sequences were synthesized by Thermo Fisher Scientific (Waltham, MA, USA).

Detection of Ezrin expression level in CTCs

The expression levels of Ezrin mRNA in CTCs were also detected by RNA-ISH assay. The specific capture probe was used to capture Ezrin mRNA, followed by conjugation with the branched DNA (bDNA) signal amplification probes. Finally, the fluorescent dye-labeled probes hybridized with the bDNA sequence, then results were analyzed using a fluorescence microscope. The purple dots of fluorescent signals observed in the cells represented the Ezrin biomarker.

EBV-DNA assay

EBV-DNA assay kit was purchased from Sursan Company (Changsha, China). EBV-DNA assay was detected according to the procedure of the kit manual. Briefly, peripheral blood samples (1 ml) from NPC patients were collected in Ethylenediaminetetraacetic Acid (EDTA) tubes. 100 μL of the blood sample was extracted DNA as standard practice DNA extraction. 10 μL DNA solution was detected EBV-DNA using the fluorescence PCR provided in the kit. EBV-DNA copies were calculated based on Ct value and the calibration curve of EBV-DNA.

EBV-VCA-IgA assay

EBV-VCA-IgA assay kit was purchased from TARCINE Company (Being, China). EBV-VCA-IgA was detected according to the procedure of the kit manual. Briefly, peripheral blood samples (5 ml) from NPC patients were drawn from NPC patients in tubes for coagulation to obtain serum sample. 10 μL of serum sample was mixed with 100 μL diluents solution in 96 well plate, incubated at 37°C for 30 min. The 96 well plate were washed for four times, and then added the Enzyme-linked compound at 37°C for 20 min. The plate was washed and added the substrate solution. After being incubated at 37°C for 10 min, the absorbance of the plate was detected at 450 nm/630 nm. The EBV-VCA-IgA was calculated based on the calibration curve of EBV-VCA-IgA.

Statistical analysis

The statistical data were analyzed using SPSS 22.0 software package (IBM Corp., Chicago). The independent-samples t-test was used to analyze the correlations between mesenchymal CTC ratio and distant metastasis. The correlations of Ezrin expression with distant metastases and differences in groups were analyzed using the Chi-square test. The student t-test or ANOVA analysis was performed to quantitatively analyze the difference between 2 groups or among multiple groups. Partial correlation coefficients between the number of CTCs and other quantitative variables were adjusted according to gender and age. All data were expressed as mean ± Standard Deviation (SD). All statistical analyses were considered significant at two tails, $P<0.05$.

Results

The clinical characteristics of NPC patients

In the study, 23 NPC patients were enrolled, including 15 males and 8 females, with a median age of 50 years old (range 13 to 68 years old), and were followed up for 6 to 28 months. Their clinical characteristics are presented in Table 1. All patients were diagnosed as undifferentiated non-keratinizing NPC (the WHO type 3) with histopathology. Among these patients, 2 cases (8.7%) were in the stage I-II, and the remained 21 NPC patients (91.3%) were in the stage III-IV. Seven cases (34.4%) were in T1-2, and 16 cases (69.6%) were in T3-4 stage; 3 cases (13.0%) were in N0-1, and 20 cases (87.0%) were in N2-3 stage; 22 cases (95.7%) were in M0, and 1 case (4.3%) was in M1 stage (Table 1). The levels of serum EBV-DNA from 23 NPC patients were detected, the positive rate of EBV DNA of NPC patients was 82.6%. CTCs of all NPC patients were detected, and the results demonstrated that the positive rate of CTCs were 95.65% (22/23) (Table 1). Then, the association of the positive rate of CTCs with clinicopathological variables, including age, gender, disease stage, and EBV DNA copy, was analyzed. The data indicated that there was no significant correlation between the positive rate of CTCs and age, gender, and disease stage in NPC patients ($P>0.05$). The positive rate of CTCs in EBV-DNA positive patients (100%) was higher than that in EBV-DNA negative patients (75%) ($P<0.05$) (Table 1).

The expression of Ezrin gene in CTCs

To analyze the expression of Ezrin gene in CTCs, we firstly obtained 411 CTCs using the CanPatrol system, then RNA-ISH was applied to investigate the expression of Ezrin in CTCs of 23 NPC patients. The data showed that Ezrin$^+$ CTCs were observed in 19 patients (82.6%); the positive rate of Ezrin in all CTCs was 29.7% (160/411); furthermore, the expression rate of Ezrin in different types of CTCs was analyzed, 13.51% (5/37) in epithelial CTCs, 43.75% (147/336) in biphenotypic CTCs and 28.57% (8/28) in mesenchymal CTCs (Table 2). Significantly, the expression of Ezrin in biphenotypic CTCs were stronger than those in the epithelial or mesenchymal CTCs (Figure 1, 2). At the same time, we analyzed the association of Ezrin expression with NPC stages, the results showed that Ezrin$^+$ cells in N3 stage were higher than that in N2 and N1, N2 stage Ezrin$^+$ cells were higher than N1 stage (Figure 3A, 3C); Ezrin$^+$ cells in clinical stage III were higher than that in stage II and I, II stage Ezrin$^+$ cells were higher than I stage (Figure 3B, 3D and Table 3). These data indicated that the expression level of Ezrin was positively correlated with the distant metastases.

Table 1: Clinical features and CTCs of 23 patients with nasopharyngeal carcinoma.

		CTCs		CTC positive rate (%)	P-value
Variables	All	Positive	Negative		
Total number	23	22	1	95.65	
Age (years)					
<50	11	10	1	90.9	>0.05
≥ 50	12	12	0	100	
Gender					
Male	15	14	1	93.3	>0.05
Female	8	8	0	100	
Pathological types					
WHO II	6	6	0	100	>0.05
WHO III	17	16	1	94.1	
Primary tumor					
T1-2	7	7	0	100	>0.05
T3-4	16	15	1	93.8	
Lymph nodes					
N0-1	3	3	0	100	>0.05
N2-3	20	19	1	95	
Metastasis					
M0	22	21	1	95	>0.05
M1	1	1	0	100	
Tumor stage					
I-II	2	2	0	100	>0.05
III-IV	21	20	1	95	
EBV					
Negative	4	3	1	75	<0.05
Positive	19	19	0	100	

Note: NPC: Nasopharyngeal Carcinoma; EBV: Epstein–Barr Virus; CTCs: Circulating Tumor Cells

Table 2: Expressions of the Ezrin gene in different types of CTCs.

Types of CTCs	Counts	Positive	Negative	Positive rate (%)
Epithelial	37	5	32	13.51
Hybrid	336	147	189	43.75
Mesenchymal	28	8	20	28.57
Total	401	160	241	39.9

Note: CTCs: Circulating Tumor Cells. Hybrid, Biophenotpic epithelial/mesenchymal. Epithelial *vs.* Mesenchymal $P<0.05$; Epithelial or Mesenchymal *vs.* Hybrid, $P<0.05$

Correlation between CTCs and EBV in NPC patients

EBV is a key pathogenetic factor for NPC. Circulating EBV DNA

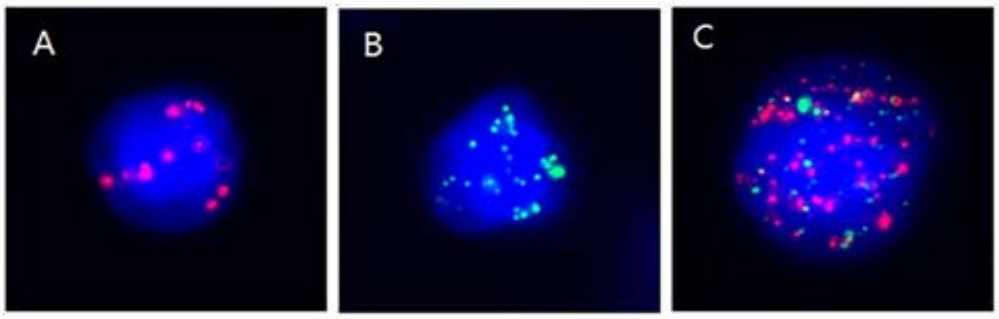

Figure 1: Fluorescence microscopy images of three subgroups of CTCs isolated from NPC patients based on RNA-ISH staining of epithelial (red dots) and mesenchymal (green dots) markers. A) Epithelial CTCs; B) Mesenchymal CTCs; C) Epithelial/mesenchymal CTCs.

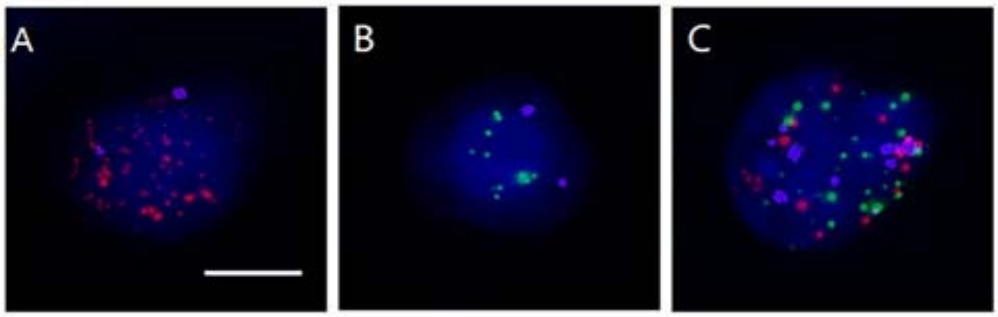

Figure 2: Fluorescence microscopy images of Ezrin expression in three subgroups of CTCs isolated from NPC patients based on RNA-ISH staining of epithelial (red dots), mesenchymal (green dots), and Ezrin (purple dots) markers. A) Epithelial CTCs with Ezrin expression; B) mesenchymal CTCs with Ezrin expression; C) Epithelial/mesenchymal CTCs with Ezrin expression. Scale bar, 5 μm.

Table 3: Ezrin expressions in CTCs from the NPC patients at stage II to stage IV.

Tumor stage	CTC counts	High expression	Medium expression	Low expression	No expression
II	29	0	0	2	27
III	108	0	3	42	63
IV	274	0	11	102	161
Total	411	0	14	146	251

Table 4: Association between CTCs and EBV activation in NPC patients.

	CTCs			Ezrin-positive CTCs		
EBV parameters	**Negative**	**Positive**	**P value[b]**	**Negative**	**Positive**	**P value[b]**
EBV DNA[a]	2.59	3.41	0.312	2.91	3.47	0.201
VCA-IgA[c]	0.95	1.6	0.568	0.55	1.79	0.045
EA-IgA[c]	0.82	2.23	0.439	0.88	2.44	0.102

Note: [a]: the raw data of EBV DNA load was log-transformed to be a normal distribution; [b]: comparisons were performed using one-way ANOVA; [c]: the counting Unit of VCA-IgA and EA-IgA were 'S/CO'

load, VCA-IgA and EA-IgA are the most common indicators of NPC. Therefore, the relationship of CTCs with EBV status was analyzed. The results showed that Ezrin$^+$ CTCs was positively associated with higher EBV VCA-IgA levels in all NPC patients (P=0.045) (Table 4). Moreover, the relativities of CTCs count *vs.* VCA-IgA and Ezrin$^+$ CTCs counts *vs* VCA-IgA were analyzed, the data showed that there was a positive correlation between CTCs counts and VCA-IgA (R=0.469, P=0.032), as well as between Ezrin$^+$ CTCs counts and VCA-IgA (R=0.569, P=0.007) in all NPC patients.

Ezrin expression in CTCs from NPC patients at pre-therapy and post-therapy

The association of Ezrin expression in CTCs with NPC patients' clinical characteristics was analyzed, the results showed that there was no significant correlation between the Ezrin expression in CTCs and patients' clinical characteristics such as age, gender, and disease stage (Table 5). All NPC patients have received radiotherapy combined with chemotherapy, and were followed up for 6 to 28 months. The CTCs were comparatively analyzed in pre-therapy and post-therapy NPC patients, the results showed that the number of CTCs was not significantly different between pre-therapy and post-therapy (P>0.05, Table 6, Figure 4). Furthermore, the association of Ezrin expression with NPC therapy was observed, Ezrin expression was detected in CTCs from pre-therapy and post-therapy NPC patients. The data from the followed-up cases showed that the positive rate of Ezrin was 43.1% (113/262) in pre-treatment patients, while the positive ratio of

Table 5: Clinical features of NPC patients with Ezrin⁺ CTCs.

Variables	CTCs Counts	Ezrin⁺ CTCs counts	Ezrin⁺ epithelial CTCs	Ezrin⁺ hybrid CTCs	Ezrin⁺ mesenchymal CTCs
		M (IQR)	M (IQR)	M (IQR)	M (IQR)
Sex					
Male	92	5 (8)	0 (1)	4 (7)	0 (0)
Female	68	6 (15)	0 (0)	5.5 (15)	0 (1)
p		0.649	0.443	0.697	0.823
Age (years)					
<50	73	6.0 (8)	0 (0)	5 (7)	0 (0)
≥50	87	5 (9)	0 (1)	3.5 (8)	0 (2)
p		0.951	0.699	0.71	0.135
Pathological types					
WHO II	65	8 (18)	0.5 (1)	7 (19)	0 (1)
WHO III	95	5 (7)	0 (0)	4 (7)	0 (1)
P value		0.244	0.056	0.307	0.808
T stage					
T1	21	2 (-)	0 (-)	2 (-)	0 (0)
T2	39	6.5 (21)	0 (1)	6 (21)	0 (0)
T3	61	6 (3)	0 (0)	4.5 (5)	0 (1)
T4	39	5.5 (13)	0 (1)	4.5 (11)	0 (1)
P value		0.966	0.687	0.955	0.314
N stage					
N1	2	0 (-)	0 (0)	0 (-)	0 (0)
N2	52	4.5 (7)	0 (1)	3.5 (7)	0 (0)
N3	106	7.5 (11)	0 (0)	7.5 (13)	0 (1)
P value		0.014*	0.549	0.024*	0.21
M stage					
M0	125	4.5 (7)	0 (1)	3 (7)	0 (0)
M1	35	11 (-)	0 (-)	9 (-)	0 (-)
P value		0.076	0.514	0.076	0.698
Tumor stage					
II	2	0 (-)	0 (0)	0 (-)	0 (0)
III	45	4 (5)	0 (1)	3 (4)	0 (1)
IV	113	7 (12)	0 (1)	7 (13)	0 (1)
P value	0.011	0.027*	0.603	0.044*	0.572

Note: M: Median; IQR: Interquartile Range; Ezrin⁺ CTCs: Ezrin positive CTCs; *: P<0.05

Table 6: Comparison of CTCs counts from NPC patients at pre- and post-treatment.

	Pre-treatment	Post-treatment		
Types of CTCs	**M (IQR)**	**M (IQR)**	**Z**	**P**
Total CTCs	20 (24)	24 (29)	-0.623	0.533
Hybrid and mesenchymal CTC	20 (28)	20 (24)	-1.068	0.289
Total Ezrin⁺ CTCs	10 (11)	6 (9)	-1.79	0.074
Hybrid and mesenchymal Ezrin⁺ CTCs	9 (11)	5 (9)	-2.355	0.019*

Note: M: Median; IQR: Interquartile Range; Ezrin⁺ CTCs: Ezrin positive CTCs; *, *P*<0.05

Ezrin was 38.21% (107/280) in post-treatment NPC patients. Ezrin⁺ mesenchymal CTCs was less in post-therapy NPC patients than that in pre-therapy (P<0.05, Figure 5, Table 6). This suggested that Ezrin expression in CTCs was inhibited when NPC patients were effectively treated.

Discussion

NPC early diagnosis was essential for enhancing treatment efficiency, improving prognosis and increasing the survival rates. Currently, NPC diagnostic techniques and individualized

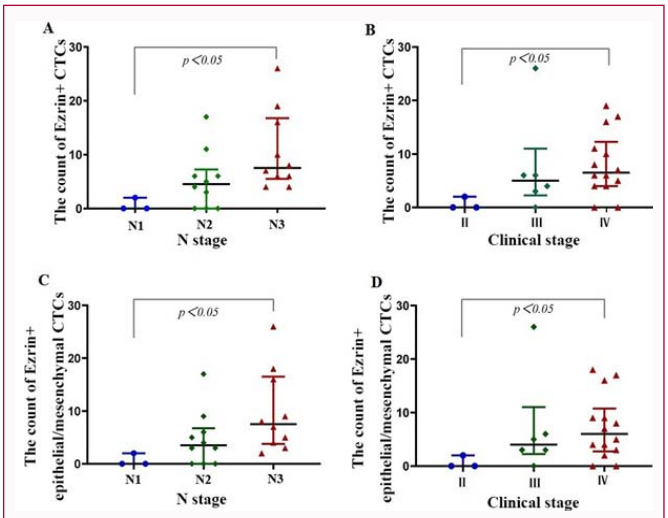

Figure 3: Ezrin expression in CTCs from NPC patients at various stages. A) An analysis on the association of Ezrin expression with N stages; B) An analysis on the association of Ezrin expression with clinical stages; C) The association of Ezrin+ Epithelial and mesenchymal CTCs with N stages. D) The association of Ezrin$^+$ Epithelial and mesenchymal CTCs with clinical stages (P<0.05). +: positive.

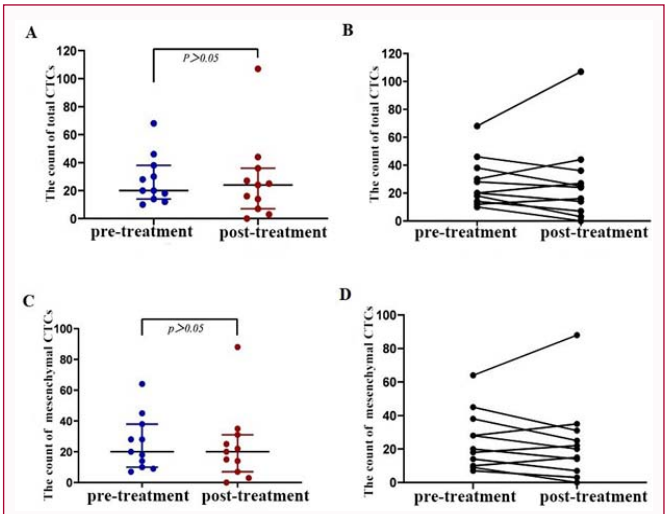

Figure 4: Total CTCs from NPC patients at pre-therapy and post-therapy. A) A comparative analysis on CTC median from NPC patients at pre-therapy and post-therapy. B, A change of CTCs from NPC patients at pre-therapy and post-therapy. C, A comparative analysis on mesenchymal CTC from NPC patients at pre-therapy and post-therapy. D, A change of mesenchymal CTCs from NPC patients at pre-therapy and post-therapy (P > 0.05).

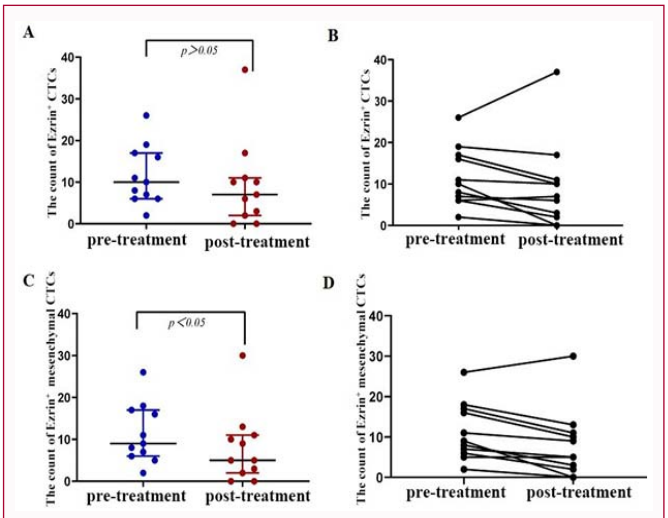

Figure 5: Ezrin$^+$ CTCs from NPC patients at pre-therapy and post-therapy. A) A comparative analysis on Ezrin CTC median from NPC patients at pre-therapy and post-therapy. B) A change of Ezrin CTCs from NPC patients at pre-therapy and post-therapy. C) A comparative analysis on Ezrin mesenchymal CTC from NPC patients at pre-therapy and post-therapy. D) A change of Ezrin mesenchymal CTCs from NPC patients at pre-therapy and post-therapy (P<0.05). +: Positive.

chemoradiotherapy strategies have made great progress, but the therapeutic efficiency remains to be poor. More sensitive and rapid diagnostic methods are urgently to be explored, especially in the field of peripheral serology and genetics. CTCs are emerging "liquid biopsy" that can express various diagnostic, therapeutic and prognostic biomarkers in solid carcinomas, including liver, esophageal and breast cancers [28-30]. The isolation and detection of CTCs rely on the surface molecules expressed on CTCs or physical characteristics of CTCs [31]. A prospective study has shown that higher CTCs count was significantly associated with worse Disease-Free Survival (DFS) in HNSCC patients, but the CTCs count was not correlated with patient survival or cancer recurrence [32,33]. However, some CTCs might not participate in cancer progression due to the highly heterogeneousness [16]. Therefore, it is of significance to explore the subsets of CTCs with aggressive features in clinical.

EMT, as a multistep and complicated process, has an important influence on cancer cells metastasis and dissemination, and CTCs with EMT phenotype are confirmed to promote cancer invasion and metastasis [34]. Moreover, synergistic detection of CTCs and EMT status might be a good tool for predicting therapeutic response [34]. In our study, we observed three types of CTCs in 22 of 23 NPC patients (95.65%) through detecting the expression of EpCAM, CKs, vimentin and twist, reflecting that the characteristic of NPC was prone to distant metastasis. The proportion of biphenotypic CTCs in each positive NPC sample was higher at the late stage than that at the early stage, indicating that mesenchymal transformed CTCs (biphenotypic and mesenchymal CTCs) may play an important role in accelerating NPC cells metastasis.

Ezrin is highly expressed in metastatic cancers with involvement in filopodia formation as well as actin cytoskeleton alteration [35,36]. Growing number of studies have reported that overexpressed Ezrin is a key factor for cancer cells invasion and metastasis via modulating various molecular signaling pathways, like HGF/Met autocrine or non-autocrine and phosphorylated protein kinase B (Akt) signaling [37,38]. Moreover, increased Ezrin phosphorylation at Thr567 and Tyr447 dominantly facilitates cancer cells mobility [36]. In agreement with this, inhibiting Ezrin phosphorylation would effectively reduce the formation of filamentous pseudopods, further suppressing NPC cells exercise capacity, invasiveness and metastasis [39]. Similarly, knockdown of Ezrin can down-regulate Matrix Metalloproteinase (MMP2) expression through modulating Phosphatidylinositol 3-Kinase (PI3K)/pAkt signaling pathway, then blocking the migration and invasion of NPC cells [23]. Therefore, Ezrin may function as a potential therapeutic target for NPC patients [23]. In this study, data indicated that the expression level of Ezrin was remarkably different between stage II and stage IV. Although Ezrin was lowly expressed in human NPC specimens, the expression level in stage IV was higher than that in stage III, indicating that the highly expression of Ezrin positively stimulated the distant metastases (Table 4). Thereby, we

observed a tightly relationship between the expression of Ezrin in CTCs and NPC distant metastases, and Ezrin expressed on CTCs could act as a significant biomarker to unveil aggressive features, monitor prognosis and further predict treatment efficiency.

EBV activation is a common and frequent event in NPC carcinogenesis, and circulating EBV DNA load, VCA-IgA and EA-IgA serve as the most common indicators of EBV activation [40]. Thus, we analyzed the relationship of CTCs with EBV activation in NPC, results showed that the number of $Ezrin^+$ CTCs was associated with high EBV VCA-IgA level in all NPC patients. CTCs count and $Ezrin^+$ CTCs count were positively correlated with the level of EBV DNA load and VCA-IgA in NPC patients. To observe the changes of CTCs, count and Ezrin expression and assess the response to main treatment methods, we detected the number of CTCs twice in NPC patients, pre-treatment and at 1 week after chemoradiotherapy.

The study examined CTCs in 22 of 23 NPC patients (95.7%), and observed $Ezrin^+$ CTCs in 19 of 23 NPC patients (82.6%). But there may be some potential bias in the results because of the limited sample size of this study and the heterogeneity of NPC treatment methods. Although, Ezrin was not highly expressed in NPC patients, Ezrin expression in the stage IV was higher than that in the stage III, indicating that the up-regulated Ezrin might promote the distant metastases of NPC. Therefore, the prognostic value of Ezrin in NPC patients is worthy of profound exploring.

Conclusion

This study illuminates a method for isolating and characterizing CTCs from NPC patients with the assistance of physical and biological methods. Furthermore, our findings demonstrate that there is a positive correlation between $Ezrin^+$ CTCs count and EBV activation, and the expression level of Ezrin in CTCs is also associated with distant metastases of NPC. However, some limitations in this study should be focused on: Firstly, the sample size is not enough; secondly, the dynamic changes of CTCs are not comprehensive. Further studies could be performed to investigate the correlation of the rate of EMT-like CTCs and the expression level of Ezrin with disease-free survival and overall survival of NPC.

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