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Clinical significance of circulating tumor cells and their expression of cyclooxygenase-2 in patients with nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: To explore the expression of circulating tumor cells (CTCs) and cyclooxygenase-2 (COX-2) in CTCs and to assess their association with clinical parameters and treatment.

PATIENTS AND METHODS: Peripheral blood samples from 50 patients with nasopharyngeal carcinoma (NPC) were included. We applied advanced CanPatrolTM CTC enrichment technique and in situ hybridization assay to isolate, identify, and classify CTCs and COX-2 in CTCs. *Epstein-Barr* virus DNA was detected by Real Time-quantitative PCR (RT-qPCR).

RESULTS: No CTCs were identified in ten healthy volunteers (100%). Of the total patients, 48 (96%) had positive CTCs counts and 36 (72%) had positive mesenchymal CTCs counts before treatment. CTCs cells were highly expressed in different NPC stages, and the positive ratio of mesenchymal CTCs in stage IV was higher than that in other stages. The proportion of mesenchymal cells was higher expressed in metastasis patients. The expression of COX-2 was different in different types of CTCs. The positivity of COX-2 in CTCs was higher in stage IV patients than that in stage II and stage III patients. Decreased mesenchymal CTCs and that express COX-2 indicated a favorable curative effect in NPC patients. The positivity of mesenchymal CTCs and COX-2 was higher in EBV DNA positive patients compared with EBV DNA negative patients ($p<0.05$). Meanwhile, the mean number of CTCs, mesenchymal CTCs, CTCs that express COX-2, hybrid CTCs that express COX-2 and mesenchymal CTCs that express COX-2 was significantly higher in the EBV DNA positive patients than negative patients before treatment ($p<0.05$).

CONCLUSIONS: CTCs and their expression of COX-2 were correlated with NPC clinical characteristics, and have relation with Epstein-Barr virus DNA. Decreased mesenchymal CTCs and that express COX-2 indicated a favorable curative effect in NPC patients.

Key Words:
Nasopharyngeal carcinoma (NPC), Circulating tumor cells (CTCs), Cyclooxygenase-2 (COX-2), Epstein-Barr virus (EBV) DNA.

Introduction

Nasopharyngeal carcinoma (NPC) is a disease with distinct geographic and ethnic distribution[1]. The highest rates are found in Southern China, Southeast Asia, and Arctic populations[2]. The etiology of NPC is complex, including genetic, environmental factors, and a host of viral[3]. Extensive studies[4-6] have identified that *Epstein-Barr virus* (EBV) infection plays a vital role in the pathogenesis of NPC in both endemic and non-endemic areas. Although, the search for prognostic and predictive molecular and biological factors for NPC is very active, the etiology of NPC is still obscure. Thus, understanding the molecular basis of NPC is essential for developing further novel agents that are needed.

To date, circulating tumor cells (CTCs) have been reported to intimately correlate with characteristics in different kinds of cancer. Studies[7-12] in breast, colorectal prostate, and lung cancer have demonstrated that the prognostic significance of CTCs, and the variation of CTC number with therapy have showed the potential of CTCs as a predictive biomarker. CTCs were classified into three types, epithelial, mesenchymal, and hybrid. In circulation, CTCs undergo epithelial to mesenchymal transition (EMT), which have association with disease progression[13,14]. Therefore, implementing CTC analyses as a liquid biopsy might provide new insights into the complex mechanisms of NPC. Some studies[15-20] have identified the clinical signif-

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icance of CTCs in head and neck cancer. However, the number of NPC patients was too small to show clinically meaningful prognostic correlations and to define CTC role in the clinical management of these patients. The data for clinical significance of CTCs in NPC patients are limited.

Cyclooxygenase-2 (COX-2), an inducible form of COX, is the rate-limiting enzyme for the production of prostaglandins from arachidonic acid. COX-2 is rarely expressed in normal tissue, but is rapidly induced by bacterial lipopolysaccharide, cytokines, tumor promoters, and growth factors[21]. Overexpression of COX-2 can stimulate cell proliferation, antigiogensis, and invasiveness, leading to tumor growth and metastasis[22,23]. Several studies[24-27] have showed that COX-2 was correlated with the development and progression of NPC[24-27]. Recently Liao et al[28] report demonstrated that COX-2, as a potential biomarker for theranostics of NPC, played a critical role in cancer stem-like SP cells of NPC[28]. Of note, researches also unveiled that the CC-genotype of COX-2 T8473C gene polymorphism was associated with a decreased risk of NPC in Tunisian and Chinese population[29,30]. Therefore, COX-2 detection can be used as a non-invasive approach. This can be repeated during the treatment, to monitor the acquisition of novel genetic abnormalities in response to chemo-radiotherapy. However, expression of COX-2 in different types of NPC CTCs and whether COX-2 in CTCs is related to NPC clinical parameters have not been studied.

CTCs and COX-2 play important roles in metastasis and they are associated with the increase of the efficacy of radiotherapy and chemotherapy in NPC. In the present work, we applied advanced CanPatrol™ CTC enrichment technique and *in situ* hybridization (ISH) assay to isolate, identify, and classify CTCs and COX-2 from NPC patients[31]. We assessed the influence of treatment on the expression of CTCs and COX-2 in CTCs, and studied the relation of CTCs and COX-2 with EBV DNA.

Patients and Methods

Patient Samples

From August 2013 to August 2016, a total of 50 consecutive patients diagnosed with nasopharyngeal carcinoma by histology, without previous treatment, were included in this work. Patients demographics and clinical characteristics are listed in Table I. The mean age was 45.28±9.71 years old (range: 27-78). All patients had squamous cell carcinomas. Staging was carried out according to the tumor-node-metastasis (TNM) classification system. After discarding the first 2.5 mL of blood to avoid potential contamination with skin epithelial cells, 5.0 mL of blood were collected into heparinized tubes. At the time of the first blood sampling, 50 patients all involved. Thus, none of the patients had started definitive chemo radiation. The second time was after the end of the whole primary treatment (usually after a 2-3 months interval from the closing date of the treatment). A second blood sample was obtained from 35 patients. Otherwise, the treatment or CTC detection was not finished. Meanwhile, 10 healthy volunteers were recruited as controls. This investigation was approved by the Ethics Committee of West China Hospital, Sichuan University. Written informed consent were obtained from all participants.

Table I. Information and clinical characteristics of the patients (n=50).

	Number	%
Sex		
Female	15	30.0
Male	35	70.0
Smoking		
Yes	27	54.0
No	23	46.0
Drinking		
Yes	25	50.0
No	25	50.0
Karnofski Index		
90~100	44	88.0
80~90	5	10.0
70~80	1	2.0
Stage		
II	7	14.0
III	28	56.0
IV	15	30.0
T class		
1	5	10.0
2	21	42.0
3	15	30.0
4	9	18.0
N class		
1	14	28.0
2	33	66.0
3	3	6.0
M class		
0	46	92.0
1	4	8.0

Isolation of CTCs by Size

Blood samples were filtrated by an 8-μm-diameter pores calibrated membrane (Millipore, Billerica, MA, USA). The system consisted of a filtration tube containing the membrane (SurExam, Guangzhou, China), a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum manifold (Omega, Norcross, GA, USA), and a vacuum pump (Auto Science, Tianjin, China). Erythrocytes were removed using a red blood cell lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, all from Sigma-Aldrich, St. Louis, MO, USA) in deionized water; the remaining cells were resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) containing 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 5 min before transferred to filtration tube. After that, the pump valve was switched on to reach at least 0.08 MPa; the manifold vacuum plate valve was then switched on, and filtration began.

Tri-Color RNA In Situ Hybridization (ISH) Assay

Based on the branched DNA (bDNA) signal amplification technology[32], the RNA-ISH method was applied in this work. The protocol was performed according to the report of Wu et al[31]. A 24-well plate (Corning, Corning, NY, USA) was used to perform the assay, and the cells on the membrane were treated with a protease (Qiagen, Hilden, Germany) before hybridization with capture probes specific for the epithelial biomarkers EpCAM and CK8/18/19, the mesenchymal biomarkers vimentin and twist, and the leukocyte biomarker CD45 and COX-2 (Table II). The hybridization was performed at 42°C for 2 h and washed three times with 1,000 μl of wash buffer (0.1×SSC, Sigma-Aldrich, St. Louis, MO, USA) to remove the un-bound probes. To amplify the signal, the sample was incubated with 100 μl of preamplifier solution (30% horse serum, Sigma-Aldrich, St. Louis, MO, USA), 1.5% sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO, USA), 3 mM Tris-HCl (pH 8.0) (Sigma-Aldrich, St. Louis, MO, USA), and 0.5 fmol of preamplifier (the sequences are shown in Table III) at 42°C for 20 min. When the membranes were cooled, wash three times with 1 ml of wash buffer (0.1×SSC), and then incubate with 100 μl of amplifier solution (30% horse serum, 1.5% sodium dodecyl sulfate, 3 mM Tris-HCl (pH 8.0), and 1 fmol of amplifier (Table III). Three types of fluorescently labeled probes (Table III), which had been conjugated with the fluorescent dyes Alexa Fluor 594 (for EpCAM and CK8/18/19), Alexa Fluor 488(for vimentin and twist), and Alexa Fluor

Table II. Capture probe sequences for the EpCAM, CK8/18/19, vimentin, twist, CD45 and COX-2 genes.

Gene	Sequences (5′-3′)
EpCAM	TGGTGCTCGTTGATGAGTCA AGCCAGCTTTGAGCAAATGA AAAGCCCATCATTGTTCTGG CTCTCATCGCAGTCAGGATC TCCTTGTCTGTTCTTCTGACCTCAGAGCAGGTTATTTCAG
CK8	CGTACCTTGTCTATGAAGGA ACTTGGTCTCCAGCATCTTG CCTAAGGTTGTTGATGTAGC CTGAGGAAGTTGATCTCGTC CAGATGTGTCCGAGATCTGG TGACCTCAGCAATGATGCTG
CK18	AGAAAGGACAGGACTCAGGC GAGTGGTGAAGCTCATGCTG TCAGGTCCTCGATGATCTTG CAATCTGCAGAACGATGCGG AAGTCATCAGCAGCAAGACG CTGCAGTCGTGTGATATTGG
CK19	CTGTAGGAAGTCATGGCGAG AAGTCATCTGCAGCCAGACG CTGTTCCGTCTCAAACTTGG TTCTTCTTCAGGTAGGCCAG CTCAGCGTACTGATTTCCTC GTGAACCAGGCTTCAGCATC
Vimentin	GAGCGAGAGTGGCAGAGGAC CTTTGTCGTTGGTTAGCTGG CATATTGCTGACGTACGTCA GAGAGCCCCTAAGTTTTTAA AAGATTGCAGGGTGTTTTCG GGCCAATAGTGTCTTGGTAG
Twist	ACAATGACATCTAGGTCTCC CTGGTAGAGGAAGTCGATGT CAACTGTTCAGACTTCTATC CCTCTTGAGAATGCATGCAT TTTCAGTGGCTGATTGGCAC TTACCATGGGTCCTCAATAA
CD45	TCGCAATTCTTATGAGACTC TGTCATGGAGACAGTCATGT GTATTTCCAGCTTCAACTTC CCATCAATATAGCTGGCATT TTGTGCAGCAATGTATTTCC TACTTGAACCATCAGGCATC
COX-2	CAGCATTGTAAGTTGGTGGA AGGAGAGGTTAGAGAAGGCT TTTTACCTTTGACACCCAAG AACTGATGCGTGAAGTGCTG CTCGCTTATGATCTGTCTTG AAAAGGCGCAGTTTACGCTG TATCTTTGACTGTGGGAGGA AGCAAACCGTAGATGCTCAG

Table III. Sequences for the bDNA signal amplification probes.

	Function (copies)	Sequences (5'-3')	Complement
bDNA probes for EpCAM and CK8/18/19	capture probe tail (1) preamplifier repeat (5) amplifier repeat (5)	CTACAAACAAACAATATT CGCAGCCTCAGCC CCCAGACCCTACC	preamplifier leader (1) amplifier leader (1) label probe (1)
bDNA probes for vimentin and twist	capture probe tail (1) preamplifier repeat (5) amplifier repeat (5)	CTTCTCAATAACTAACAT GACGGTCGGCGTT GTCACCGCTCCAC	preamplifier leader (1) amplifier leader (1) label probe (1)
bDNA probes for CD45	capture probe tail (1) preamplifier repeat (5) amplifier repeat (5)	GTAAAAAGAAAGGTATAA AATTATACATCTC GAAATGAATGAAT	preamplifier leader (1) amplifier leader (1) label probe (1)
bDNA probes for COX-2	capture probe tail (1) preamplifier repeat (5) amplifier repeat (5)	CTTTATACCTTTCTTTCA GCGCGCTGTAGGG AGGCGAGGGGAGA	preamplifier leader (1) amplifier leader (1) label probe (1)

The sequences labeled 'leader' appear once in the indicated construct, while sequences labeled 'repeat' appear the indicated number of times. The tail on the capture probe is a single sequence.

647 (for CD45 or COX-2), were added and incubated at 42°C for 20 min. After washing with 0.1×SSC, the cells were stained with 4', 6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA) for 5 min and analyzed with a fluorescence microscope using a 100x oil objective (Olympus, Tokyo, Japan).

Detection of Epstein-Barr virus (EBV) DNA

Plasma DNA was extracted with a QIAamp DNA Blood MinniKit (Qiagen, Hilden, Germany). The concentration of EBV DNA in plasma was measured using a Real Time quantitative PCR assay of the BamHI-W region of the EBV genome. The forward and reverse primers sequences were 5'-CCCAACACTCCACCACACC-3' and 5'-TCTTAGGAGCTGTCCGAGGG-3', respectively. A dual fluorescence labelled oligomer, 5' (FAM) CACACACTACACACACCCACCCGTCTC (TAMRA) 3' served as a probe. The real-time quantitative PCR assay and reaction procedures were as previous study[33]. Samples were considered to have zero copied if they have an undetectable EBV signal after processing under our real time quantitative PCR conditions (40 cycles).

Statistical Analysis

The R software from the "The Comprehensive R Archive Network" was used to perform statistical processing. Differences between groups were evaluated using analysis of covariance. The correlation between the different variables was evaluated with the Fisher exact probability test or with Chi-square test. The significant level was 0.05 (two-tailed).

Results

Expression of CTCs and Association with Clinical Characteristics

Three types of CTCs were showed in Figure 1. No CTCs were identified in ten healthy volunteers (100%). Of all patients, 48 (96%) had positive

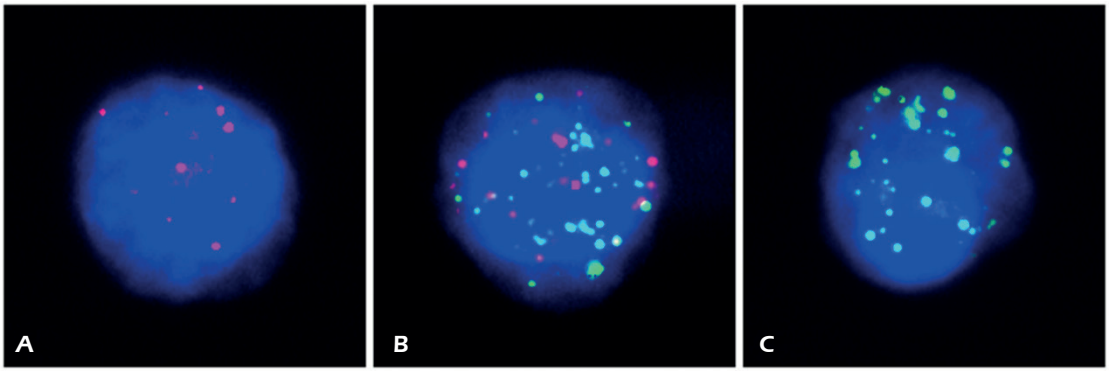

Figure 1. CTCs detected in a blood sample from a NPC patient. ***A***, Epithelial CTCs. ***B***, Hybrid CTCs. ***C***, Mesenchymal CTCs).

Table IV. Expression of CTCs before treatment and association with clinical characteristics (N=50).

	n	Patient number		Cells number			
		CTC+ (%)	M+ (%)	CTCs cells	mean	M^+ cells (%)	M^+ cells mean
Stage							
II	7	7 (100.0)	5 (71.4)	75	10.7	12 (16.0)	1.7
III	27	26 (96.3)	18 (66.7)	365	13.52	81 (22.2)	3.0
IV	16	15 (93.7)	13 (81.3)	212	13.25	42 (19.8)	2.6
Metastasis							
No	46	45 (97.8)	34 (73.9)	623	13.5	126 (20.2)	2.7
Yes	4	3 (75.0)	2 (50.0)	29	7.3	9 (31.0)	2.2

CTC^+, CTCs positivity. M^+, mesenchymal CTCs positivity. M^+ cells, mesenchymal CTCs cells.

CTCs counts and 36 (72%) had positive mesenchymal CTCs counts before treatment (Table IV). Meanwhile, positive ratios of mesenchymal CTCs were 71.4%, 66.7% and 81.3% in stage II, stage III and stage IV groups, respectively. CTCs cells were highly expressed in different NPC stages, and the positive ratio of mesenchymal CTCs in stage IV was higher than that in other stages. Of the total CTCs cells, the proportion of mesenchymal cells was 16.0%, 22.2% and 19.8% in stage II, stage III and stage IV. The proportion of mesenchymal cells was higher expressed in metastasis patients.

Variation of CTCs and Correlation with Response to the Treatment

A second blood sample for CTCs analysis was obtained from 35 patients after one cycle treatment. Of the 28 patients who responded to therapy, 60.7% showed a decrease in CTCs number (Table V). While the patients with progressive disease showed an increased or unchanged number of mesenchymal CTCs in the post-treatment sample. Compared with patients without recurrence, only 16.7% of relapse patients had a decreased number of mesenchymal CTCs. Further, as shown in Table VI, the difference values (CTCs number before treatment subtract the number of CTCs after treatment) of total CTCs, epithelial CTCs, mesenchymal and hybrid CTCs were all larger in completely respond patients than other NPC patients. This was also observed in patients without recurrence, and the difference value was larger than relapse patients.

Correlation Between CTCs and EBV DNA

Table VII shows the relation between CTCs and EBV DNA of NPC. We found that the mesenchymal CTCs positivity was higher in EBV DNA positive patients compared with EBV DNA negative patients. The mean number of CTCs and mesenchymal CTCs was significantly higher in the EBV DNA positive patients than negative patients before treatment ($p<0.05$).

Table V. Correlation between CTCs number changes and clinical response during the treatment in patients (n=35).

	Patient number	CTCs		Mesenchymal CTCs		
		pos-more	pos-less	pos-more	pos-noc	pos-less
Response evaluation						
CR	28	11 (39.3)	17 (60.7)	6 (21.4)	10 (35.7)	12 (42.8)
PR/SD	5	2 (40.0)	3 (60.0)	0 (0.0)	2 (40.0)	3 (60.0)
PD	2	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)
Relapse						
No	29	12 (41.4)	17 (58.6)	5 (17.2)	10 (34.5)	14 (48.3)
Yes	6	2 (33.3)	4 (66.7)	2 (33.3)	3 (50.0)	1 (16.7)

*pos-less: if the number decreased during the treatment. pos-more: if the number grew during the treatment. pos-noc: if the number unchanged after treatment.

Table VI. Correlation between difference value of CTCs number and clinical response during the treatment in cells level (n=35).

	Patient number	Difference value of CTCs (mean±SD)			
		CTCs	E CTCs	H CTCs	M CTCs
Response evaluation					
CR	28	4.04±14.97	0.25±3.36	3.00±11.02	0.79±3.63
PR/SD/PD	7	0.14±9.96	-0.57±1.51	0.29±9.69	0.43±0.98
Relapse					
No	29	3.62±14.95	0.14±3.36	2.62±11.12	0.86±3.54
Yes	6	1.50±9.44	-0.17±1.17	1.67±9.14	0.00±1.10

Difference value of CTCs that express COX-2 refer to the value of CTCs number that express COX-2 before treatment subtract the number of CTCs that express COX-2 after treatment. E CTCs, Epithelial CTC. H CTCs, Hybrid CTCs M CTCs, Mesenchymal CTCs.

Expression of COX-2 in CTCs and Association with Clinical Characteristics

With Tri-color RNA in situ hybridization, we use blue fluorescence as indicator of COX-2 in CTCs (Figure 2). In CTCs, COX-2 positive rate was 36.2% (Table VIII). The expression of COX-2 was different in different types of CTCs. Hybrid CTCs have the highest positive ratio in three types of CTCs. Positive ratios of COX-2 in epithelial, hybrid and mesenchymal CTCs were 32.2%, 37.8% and 32.6%. Epithelial CTCs (36.8%) was lower than that of hybrid (65.3%) and mesenchymal CTCs (77.3%) in the percentage of low COX-2 expression. However, the percentage of medium and high COX-2 expression in epithelial CTCs was higher than that of hybrid CTCs and mesenchymal CTCs. The relation between COX-2 and clinical parameters of NPC was analyzed (Table IX). We found that the positivity of COX-2 in CTCs was higher in stage IV patients than that in stage II and stage III patients. COX-2 was highly expressed in metastasis patients, even if not statistically significant.

Correlation Between COX-2 and Response to the Treatment

Table X shows the correlation between COX-2 expression changes and clinical response during the treatment of 35 patients. The proportion of patients who showed an increase number of mesenchymal CTCs in the post-treatment sample was 25.0%, 20.0% and 50% in complete response, partial response and progressive patients. None of relapse patients had a decreased number of mesenchymal CTCs that express COX-2, compared with 17.2% patients without recurrence. In cells level, as shown in Table XI, the difference values (CTCs number before treatment subtract the number of CTCs after treatment) of total CTCs, epithelial CTCs, mesenchymal and hybrid CTCs were all higher in completely respond patients than other NPC patients. Apart from difference value of mesenchymal CTC that express COX-2, the other difference values in relapse patients were also larger than that in patients without recurrence.

Correlation Between COX-2 and EBV DNA

The relation between COX-2 in CTCs and EBV DNA of NPC was shown in Table XII. We found that the COX-2 positivity was higher in EBV DNA positive patients compared with EBV DNA negative patients ($p<0.05$). Meanwhile, the mean number of CTCs, hybrid CTCs and mesenchymal CTCs that express COX-2 was signifi-

Table VII. Correlation between CTCs and EBV DNA.

EBV DNA	n	Patient number		Cells number			
		CTC^+ (%)	M^+ (%)	CTCs cells	CTCs mean	M^+ cells (%)	M cells mean
Pre-treatment							
Negative	26	26 (100.0)	17 (66.7)	261	6.77±6.01*	45 (17.2)	1.23±1.31*
Positive	22	21 (95.5)	18 (81.8)	391	17.77±15.38*	90 (23.0)	4.09±5.00*
Post-treatment							
Negative	30	27 (90.0)	15 (50.0)	240	8.00±6.44	41 (17.1)	1.37±2.08
Positive	5	4 (80.0)	3 (60.0)	11	2.20+1.92	4 (36.4)	0.80±0.84

*$p<0.05$. CTC^+, CTCs positivity. M+, mesenchymal CTCs positivity. M^+ cells, mesenchymal CTCs cells. M cells mean, mean of mesenchymal CTCs number.

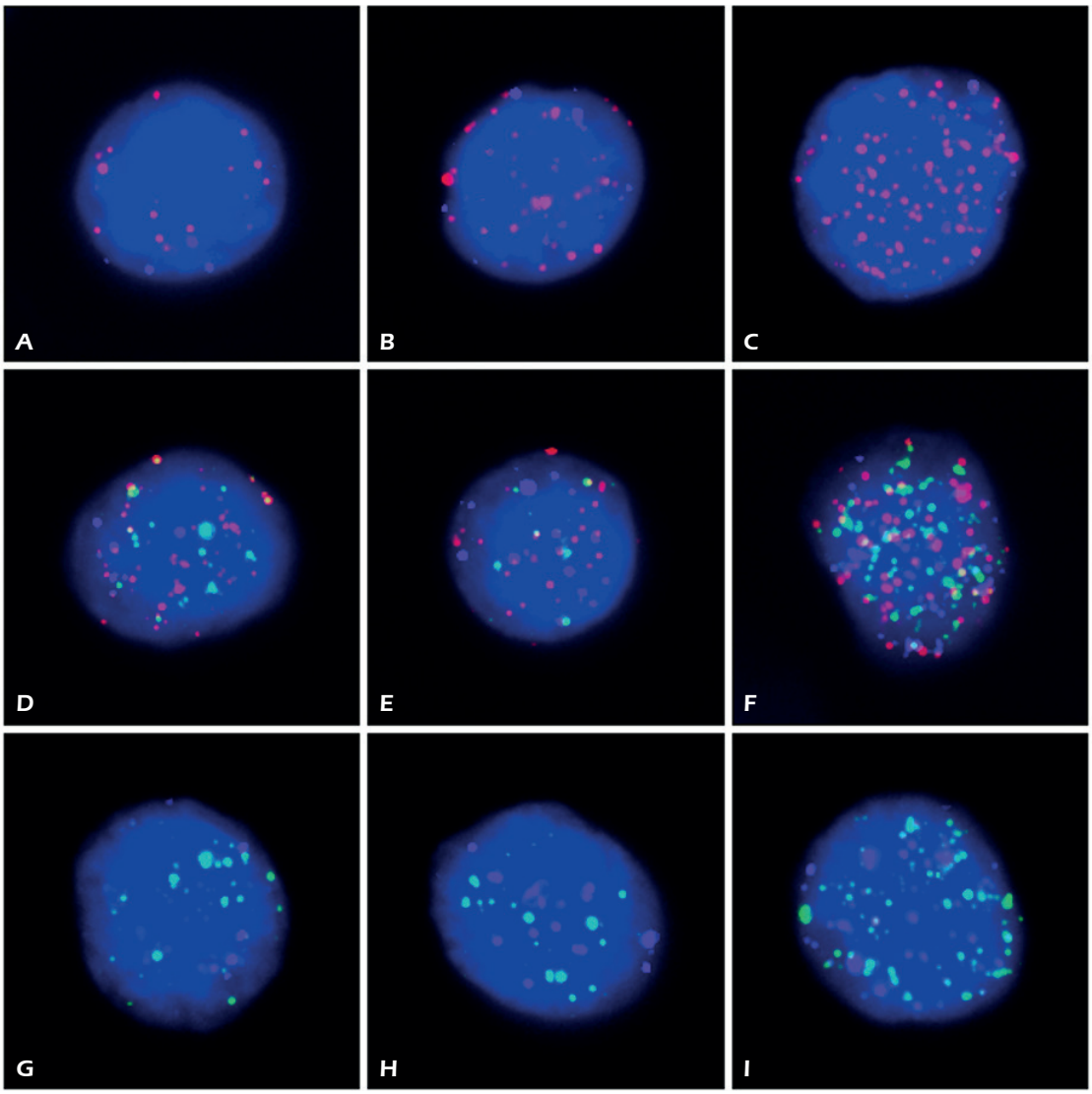

Figure 2. COX-2 expression in different types of CTCs. ***A***, Low expression in epithelial CTCs. ***B***, Medium expression in epithelial CTCs. ***C***, High expression in epithelial CTCs. ***D***, Low expression in hybrid CTCs. ***E***, Medium expression in hybrid CTCs. ***F***, High expression in hybrid CTCs. ***G***, Low expression in mesenchymal CTCs. ***H***, Medium expression in mesenchymal CTCs. ***I***, High expression in mesenchymal CTCs).

cantly higher in the EBV DNA positive patients than negative patients before treatment ($p<0.05$). But this relation disappeared after treatment.

Discussion

It has been increasingly accepted that CTCs were intimately related with clinical characteristics of some cancers[11,12,20]. In the present study, CanPatrol™ CTC-enrichment technique was used to fulfil isolation and analysis of CTCs and COX-2 in CTCs in NPC patients. We found that no CTCs were identified in ten healthy volunteers (100%). Of all patients, 96% had positive CTCs counts and 72% had positive mesenchymal CTCs counts before treatment. Meanwhile, positive ratios of mesenchymal CTCs were 71.4%, 66.7%, and 81.3% in stage II, stage III, and stage IV groups, respectively. CTCs cells were highly expressed in different NPC stages and the positive ratio of mesenchymal CTCs in stage IV was higher than that in other stages. Of the total CTCs cells, the proportion of mesenchymal cells were 16.0%, 22.2%, and 19.8% in stage II, stage III and stage IV. The proportion of mesenchymal cells was higher expressed in metastasis patients. Previous studies have demonstrated that while in circulation, CTCs would gradually

Table VIII. Correlation between CTCs and COX-2 expression.

	Cell number	COX-2 negative number (%)	COX-2 positive number (%)	Expression level		
				Low expression number (%)	Medium expression number (%)	High expression number (%)
Total CTCs	652	416 (63.8)	236 (36.2)	154 (65.3)	68 (28.8)	14 (5.9)
Epithelial CTCs	59	40 (67.8)	19 (32.2)	7 (36.8)	10 (52.6)	2 (10.5)
Hybrid CTCs	458	285 (62.2)	173 (37.8)	113 (65.3)	48 (27.7)	12 (6.9)
Mesenchymal CTCs	135	91 (67.4)	44 (32.6)	34 (77.3)	10 (22.7)	0 (0.0)

Table IX. Correlation between COX-2 and clinical characteristics.

	Patient number					Cells number	
	No. (%)	COX-2$^+$ (%)	M COX-2$^+$ (%)	E COX-2$^+$ (%)	H COX-2$^+$ (%)	COX-2$^+$ (%)	M COX-2$^+$
Stage							
II	7	5 (71.4)	1 (14.3)	1 (14.3)	4 (57.1)	24 (32.0)	2 (16.7)
III	27	29 (70.4)	3 (11.1)	8 (29.6)	19 (70.4)	149 (40.8)	28 (34.6)
IV	16	13 (81.3)	2 (12.5)	4 (25.0)	12 (75.0)	63 (29.7)	14 (33.3)
Metastasis							
No	46	34 (73.9)	5 (10.9)	12 (26.1)	33 (71.7)	229 (36.8)	41 (32.5)
Yes	4	3 (75.0)	1 (25.0)	1 (25.0)	2 (50.0)	7 (24.1)	3 (33.3)

COX-2$^+$, COX-2 positivity. M COX-2$^+$, mesenchymal CTCs that express COX-2. E COX-2$^+$, epithelial CTCs that express COX-2. H COX-2$^+$, hybrid CTCs that express COX-2.

develop epithelial-to-mesenchymal transition that promotes cancer development[14]. With the stage increasing, the positivity of mesenchymal CTCs increased. This phenomenon was probably due to migratory and invasive abilities of mesenchymal CTCs. Further studies were needed to elucidate the potential mechanism.

CTCs features in pre- and post-treatment blood sample from 35 cases were compared. Of the 28 patients who responded to therapy, 60.7% showed a decrease in CTCs number. While the patients with progressive disease showed an increased or unchanged number of mesenchymal CTCs in the post-treatment sample. After treatment, the patients showed disease progression, along with increased mesenchymal CTCs. Compared with patients without recurrence, only 16.7% of relapse patients had a decreased number of mesenchymal CTCs. Further, the difference values (CTCs number before treatment subtract the number of CTCs after treatment) of total CTCs, epithelial CTCs, mesenchymal and hybrid CTCs were all larger in

Table X. Correlation between COX-2 expression changes and clinical response during the treatment in patients (n=35).

	Patient Number	CTCs express COX-2			Mesenchymal CTCs express COX-2		
		pos-more	pos-noc	pos-less	pos-more	pos-noc	pos-less
Response evaluation							
CR	28	15 (53.6)	3 (10.7)	10 (35.7)	7 (25.0)	18 (64.3)	3 (10.7)
PR/SD	5	2 (40.0)	1 (20.0)	2 (40.0)	1 (20.0)	2 (40.0)	2 (40.0)
PD	2	1 (50.0)	0 (0.0)	1 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)
Relapse							
No	29	15 (51.7)	4 (13.8)	10 (34.5)	7 (24.1)	17 (58.6)	5 (17.2)
Yes	6	3 (50.3)	0 (0.0)	3 (50.0)	2 (33.3)	4 (66.7)	0 (0.0)

*pos-less: if the number decreased during the treatment. pos-more: if the number grew during the treatment. pos-noc: if the number unchanged after treatment.

Table XI. Correlation between difference value of CTCs that express COX-2 and clinical response during the treatment in cells level (n=35).

	Patient number	Difference value of CTCs that express COX-2 (mean±SD)			
		CTCs	E CTCs	H CTCs	M CTCs
Response evaluation					
CR	28	-0.07±5.13	-0.18±1.91	0.07±3.79	-0.04±1.48
PR/SD/PD	7	-2.43±6.83	-0.29±0.95	-1.86±6.17	-0.29±1.38
Relapse					
No	29	-0.55±5.94	-0.24±1.90	-0.41±4.66	0.10±1.47
Yes	6	-0.50±2.59	-0.00±0.63	0.17±2.40	-0.67±1.21

Difference value of CTCs that express COX-2 refer to the value of CTCs number that express COX-2 before treatment subtract the number of CTCs that express COX-2 after treatment. E CTCs, Epithelial CTC. H CTCs, Hybrid CTCs M CTCs, Mesenchymal CTC.

completely respond patients than other NPC patients. This was also observed in patients without recurrence, the difference value was larger than relapse patients. Some cancers have been reported the reduction of the number of CTCs during treatment with better clinical response and survival[34]. The presence of CTCs before treatment and a change in their number during treatment have a higher predictive/prognostic value than that of the conventional methods.

As far as we know, this is the first study to report the clinical significance of COX-2 in CTCs in patients with NPC. The results demonstrate that the expression of COX-2 is different in different types of CTCs. Hybrid CTCs have the highest positive ratio in three types of CTCs. We found that the positivity of COX-2 in CTCs was higher in stage IV patients than that in stage II and stage III patients. COX-2 was highly expressed in metastasis patients, even if not statistically significant. The correlation between COX-2 expression changes and clinical response during the treatment in 35 patients was explored. The proportion of patients who showed an increase number of mesenchymal CTCs in the post-treatment sample was 25.0%, 20.0% and 50% in completely response, partial response and progressive patients. None of relapse patients had a decreased number of mesenchymal CTCs that express COX-2, compared with 17.2% patients without recurrence. COX-2 could possibly be used to monitor – not invasively – the acquisition of a chemo-radio resistant phenotype in response to chemo-radiotherapy and to individualize the treatments, and they can be used to estimate the risk of metastases in early stage cancer.

It is believed that EBV has a vital role in the occurrence of NPC, and the expression of EBV risk patients for higher incidence, progressive of NPC[4,35]. We found that mesenchymal CTCs positivity and COX-2 positivity were higher in EBV DNA positive patients compared with EBV DNA

Table XII. Correlation between COX-2 and EBV DNA.

EBV DNA	Patient number			Cells number that express COX-2			
	No.	COX-2$^+$ (%)	M COX-2$^+$ (%)	CTCs mean	E cell mean	H cell mean	M cell mean
Pre-treatment							
Negative	26	16 (61.5)*	2 (7.7)	2.27±3.31*	0.23±0.51	1.81±2.64*	0.23±0.51*
Positive	22	20 (90.5)*	4 (18.2)	7.14±8.86*	0.55±1.01	5.14±7.95*	1.45±2.11*
Post-treatment							
Negative	30	24 (80.0)	11 (36.7)	4.13±4.08	0.63±1.54	2.87±3.52	0.63±0.96
Positive	5	4 (80.0)	1 (20.0)	1.60+1.14	0.40+0.89	1.00+0.71	0.20±0.45

*$p<0.05$. COX-2$^+$, COX-2 positivity. M COX-2$^+$, mesenchymal CTCs that express COX-2. E cell mean, mean value of epithelial CTCs that express COX-2. H cell mean, mean value of hybrid CTCs that express COX-2.M cell mean, mean value of mesenchymal CTCs that express COX-2.

negative patients. Meanwhile, the mean number of CTCs, hybrid CTCs and mesenchymal CTCs that express COX-2 was significantly higher in the EBV DNA positive patients than negative patients before treatment. But this relation disappeared after treatment. These results indicated that COX-2 and CTCs have a relation with EBVDNA, which may be associated with the occurrence of NPC. The mechanism was still vague.

This report discovered that CTCs and COX-2 in CTCs were associated with clinical characteristics and EBV DNA. The influence of treatment on the expression of CTCs and COX-2 in CTCs was assessed. One limitation of this study is that this is a single-institution study and all the patients come from southern China. The lack of information on other subgroup patients may have an impact on the strength of the association. Because of the single-institution study and observational nature of our study, the small number of cases prevented us from a more detailed analysis. Future studies with large sample size and long follow-up will be performed. The clinical importance of CTCs and COX-2 as potential biomarkers of therapeutic resistance and as a potential drug target in NPC warrants further investigation. However, this study provides the initial exploration demonstrating the association between CTCs, COX-2 and EBV DNA, treatment. The data may be of value for clinical assessment and treatment of NPC.

Conclusions

We found that CTCs and their expression of COX-2 were correlated with NPC clinical characteristics, and have relation with *Epstein-Barr* virus DNA. Decreased mesenchymal CTCs and that express COX-2 indicated a favorable curative effect in NPC patients. As potential biomarkers for NPC, CTCs and COX-2 provide valuable clinical information for assessing disease and treatment. These findings may have extensive clinical implications in the diagnosis and therapy of NPC.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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