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Association between BRAF expression in circulating tumor cells and the clinical feature of patients with multiple myeloma

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Multiple myeloma (MM) is the most common hematological malignancy with uncontrolled proliferation of monoclonal plasma cells. Despite treatment improvements, MM remains an incurable disease for most patients. Therefore, promising molecular markers are required for MM treatment decisions. In the present study, we explored the relationship between the BRAF expression in circulating tumor cells (CTCs) and the clinical features of patients with MM. The results showed that CTCs were associated with MM staging, and the expression of BRAF was associated with different CTCs. Moreover, the BRAF gene was correlated with patients' white blood cells, blood albumin levels, and Eastern Cooperative Oncology Group (ECOG) score. BRAF expression positively correlated with total CTCs, hybrid CTCs, and mesenchymal CTCs. Taken together, CTCs tightly correlated with the clinical stages and characteristics of MM. Our findings may provide a promising prognosis biomarker for MM treatment decisions.

Key words: multiple myeloma, circulating tumor cells, BRAF

Multiple myeloma (MM) is a hemopoietic malignancy characterized by a bone marrow infiltration of clonal plasma cells with heterogeneous involvement in many areas of the bone marrow [1]. Despite significant advances in the treatment of patients with MM, which have led to unprecedented response rates and prolonged survival, most patients eventually relapse and cannot be cured [2]. Therefore, better methods are needed to monitor the occurrence of MM changes in order to adjust clinical treatment [3]. Liquid biopsy, particularly circulating tumor cells (CTCs), has emerged as a useful tool for the diagnosis and monitoring of therapeutic responses in different tumors [4, 5]. Liquid biopsy, which is exploited for a variety of clinical applications, is becoming important in the personalized medicine of cancer [6, 7]. CTCs with various degrees of adhesion and motility are shed from primary or metastatic tumors into the bloodstream, allowing their obtainment through liquid biopsy [6, 8]. Many clinical studies have shown that CTCs are detectable in the peripheral blood (PB) of virtually all MM cases and are prognostic [9–11]. Other studies have shown that CTCs are associated with clinical stages and some blood analysis parameters [12]. Therefore, CTCs have been considered markers and the leading etiology of tumor recurrence and metastasis [13]. Epithelial-to-mesenchymal transition (EMT) has been proposed to be important for CTCs dissemination in MM [14]. MM tumor cells exhibit the downregulation of epithelial markers and upregulation of mesenchymal markers during their dissemination. The process of EMT endows tumor cells with migratory and invasive properties, promoting cancer recurrence [15, 16]. Therefore, this study focused on the relationship between CTCs expression and the disease characteristics and prognosis in patients with MM.

The protein of *BRAF* is an essential regulator of the mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK pathway is the most efficient signaling pathway in cancer, which controls malignancy and regulates apoptosis [17]. Remarkably, some of its genes are recurrently mutated in MM. Mutations affecting RAS/MAPK pathway components, such as *BRAF*, were found to be the most frequently observed pathway mutations in MM, detected in approximately 40% of patients [18]. These mutations promote myeloma survival by reducing cellular stress, thereby distancing plasma cells from the apoptotic threshold [19]. Interestingly, 4–9% of patients

with MM harbor a *BRAF* mutation at diagnosis, and the *BRAF* V600E mutation is the most common, displaying an even higher frequency of relapse (up to 18%) [20, 21]. MAPK pathway activated by *BRAF* mutation may also play an important role in the oncogenesis of MM and lead to chemoresistance [22]. *BRAF* mutation is found to be associated with the epithelial-to-mesenchymal transition in primary cutaneous melanoma [23], and *BRAF* expression in CTCs correlates with epithelial-mesenchymal transition. However, the relationship between *BRAF* and MM tumor cell dissemination during EMT is unclear, although MM CTCs and EMT play important role in tumor recurrence and metastasis.

In this study, we analyzed CTCs in 35 newly diagnosed patients with MM and investigated the relationship between *BRAF* expression in CTCs undergoing EMT and prognosis. Other clinical features in patients with MM were also elucidated. Our study provides important information to clarify the process of MM metastasis and offers a promising prognosis biomarker to improve personalized medicine for patients with MM.

Patients and methods

Patient samples. A total of 35 patients from the Third Affiliated Hospital of Southern Medical University, from January 2019 to December 2021, were recruited for this study. All patients met the International Myeloma Working Group (IMWG) diagnostic criteria [24]. The inclusion criteria were: 1) newly diagnosed MM (NDMM) with complete clinical data, 2) histopathological diagnosis of MM, and 3) signed informed consent form. Blood samples were collected before therapy. The exclusion criteria were: 1) past tumor history or diagnosis of combined tumors and 2) severe complications that may affect treatment or survival.

All the procedures in this study were examined and approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University, with approval no: 201806002. The study was carried out in line with the Declaration of Helsinki. Informed consent was obtained from all patients for the review of their medical records.

Isolation and identification of CTCs. Blood was collected from MM patients before treatment and stored at 4 °C until the isolation of cells within 2 h. All blood samples were tested using the CanPatrol™ System (SurExam Biotech, Guangzhou, People's Republic of China). The isolation and identification of blood samples were as follows. First, a red blood cell lysis buffer was used to remove erythrocytes from the blood sample, and then a filtration method was applied using an 8 μm pore diameter calibrated membrane (Millipore, Billerica, MA, USA). Second, the identification and classification of CTCs were carried out by multiplex RNA-*in situ* hybridization (RNA-ISH) assay. Four groups of nucleic acid probes were used in the assay to examine the expression levels of epithelial and mesenchymal genes. Group 1 probes contained one capture probe specific for the epithelial biomarkers cytokeratin (CK) 19, group 2 probes had two capture probes specific for mesenchymal biomarkers twist, group 3 contained the capture probe specific for the leukocyte biomarker CD45, and group 4 contained the capture probe specific for the *BRAF* gene. Probe sequences are shown in Table 1. Cells retained on the filter membrane were treated with a protease (Qiagen, Hilden, Germany) before hybridization. Then, cells were subjected to a series of hybridization reactions with different capture probes. Finally, cells were stained with 4,6-diamidino-2-phenylindole (DAPI). After DAPI nuclear staining and *in situ* hybridization of CD45, epithelial, and mesenchymal marker mRNAs, cells were observed and counted under an automatic imaging fluorescence microscope. The fluorescence number of each marker was greater or equal to 7, which was considered an effective fluorescent signal. Nuclear fuel DAPI-positive, leukocyte marker CD45-negative, mesenchymal marker- (Vimentin and Twist), or epithelial marker- (CK8, CK18, CK19, and EpCAM) positive cells were judged as CTCs. If a CTC was only positive for mesenchymal markers, it was designated "mesenchymal CTC"; if it was only positive for epithelial markers, it was designated "epithelial CTC"; and if it was positive for both mesenchymal markers and epithelial markers, it was designated "mixed CTC". The samples were analyzed with a fluorescence microscope using a 100× oil objective (Olympus BX53; Olympus, Tokyo, Japan). The red and green dots of fluorescent signal observed in the cells represent the epithelial, mesenchymal, and CD45 (the markers of white blood cells) gene expression, respectively, while blue fluorescent dots represent *BRAF* gene expression.

Statistical analysis. Data were analyzed using SPSS 25.0 software package (IBM Corp., Armonk, NY, USA). Patient data were analyzed using a t-test, chi-square test, and Wilcoxon rank-sum test. Normally distributed data

Table 1. EMT typing probe and BRAF probe sequence.

Gene	Sequence (5'-3')
CD45	TCGCAATTCTTATGCGACTCTGTCATGGAGACAGTCATGTGTATTTCCAGCTTCAACTTCCCATCAATATAGCTGGCATTTTGTG-CAGCAATGTATTTCCTACTTGAACCATCAGGCATC
CK19	AAGTCATCTGCAGCCAGACGCTGTTCCGTCTCAAACTTGGTTCTTCTTCAGGTAGGCCAGCTCAGCGTACTGATTTCCTCCTG-TAGGAAGTCATGGCGAGAAGTCATCTGCAGCCAGACG
Twist	ACAATGACATCTAGGTCTCCCTGGTAGAGGAAGTCGATGTCAACTGTTCAGACTTCTATCCCTCTTGAGAATGCATG-CATTTTCAGTGGCTGATTGGCACTTACCATGGGTCCTCAATAA
BRAF	TCGTTGCCCAAATTGATTTCGTATTTTAACCCTTGGATGTCAACTTCTCACCTGCAAACATCTACATGAGCGAGACATCCAG-TAGAATCTTGCTGGGCAAGTGTTTACTGGAAGAACCTTTACATGCTTGCTAGTCTTCTTGGAGGCATGTTTTACTGG

are expressed as mean ± standard deviation. For ordinal variables, the Spearman rank correlation was assessed. Due to the different orders of magnitude between different clinical indicators, the units of each clinical indicator were adjusted to allow for a better comparison. Correlations between clinical indicators, R-ISS stage, and CTCs were analyzed using ordered logistic regression models, in which sex, age, and bone marrow primitive immature plasma cell and mature plasma cell ratios were adjusted for in the multifactorial analysis. All p-values reported are two-sided, and we used a significance threshold of 0.05.

Results

Clinical characteristics. The study population included 35 patients with an average age of 61.09 ± 10.12 years, and 21 (60%) patients were female. Some (17.1%) of the patients were classified as R-ISS stage III, and there was an almost equal distribution of patients with R-ISS stage I and II disease (42.9% and 40.0%, respectively). Patient characteristics are shown in Table 2.

Expression of CTCs in MM. To study the expression of CTCs in MM, we applied the CanPatrol™ CTC-enrichment technique to isolate and analyze cells collected from patients. As shown in Figure 1, we applied different fluorescence of the epithelial biomarker CK19 and mesenchymal biomarker Twist to help us distinguish three types of CTCs. Leukocytes were established as a negative control. The results showed that we can detect various types of CTCs in patients with MM.

The expression of BRAF in CTCs with MM. Some studies had found that *BRAF* was frequently mutated in MM. Therefore in this study, we analyzed *BRAF* and CK19 expression in different types of CTCs, detecting it in different types of these cells (Figure 2). Furthermore, most of the *BRAF*

Table 2. Demographics of patients included in the study.

Clinical characteristic	All patients (n=35)	BRAF gene		p-value
		Positive (n=24)	Negative (n=11)	
Age (years)	61.09±10.12	60.46±10.72	62.45±8.99	0.596
Female	21 (60.0%)	14 (58.3%)	7 (63.6%)	1.000
R-ISS stage I	15 (42.9%)	10 (41.7%)	5 (45.5%)	1.000
II	14 (40.0%)	10 (41.7%)	4 (36.4%)	
III	6 (17.1%)	4 (16.7%)	2 (18.2%)	

Abbreviation: R-ISS-revised international staging system

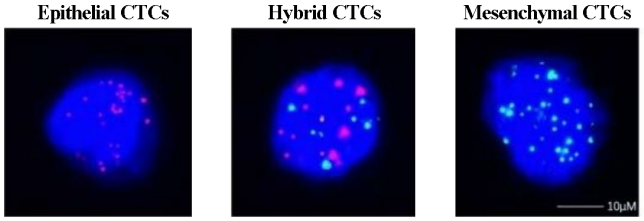

Figure 1. CK19 and Twist expression on the CTCs of patients with MM. Leukocytes were stained for CD45 (white fluorescence). CTCs were stained for CK19 (red fluorescence) and twist (green fluorescence) expression. The cells were analyzed using a 100× oil objective. Abbreviation: CTCs-circulating tumor cells

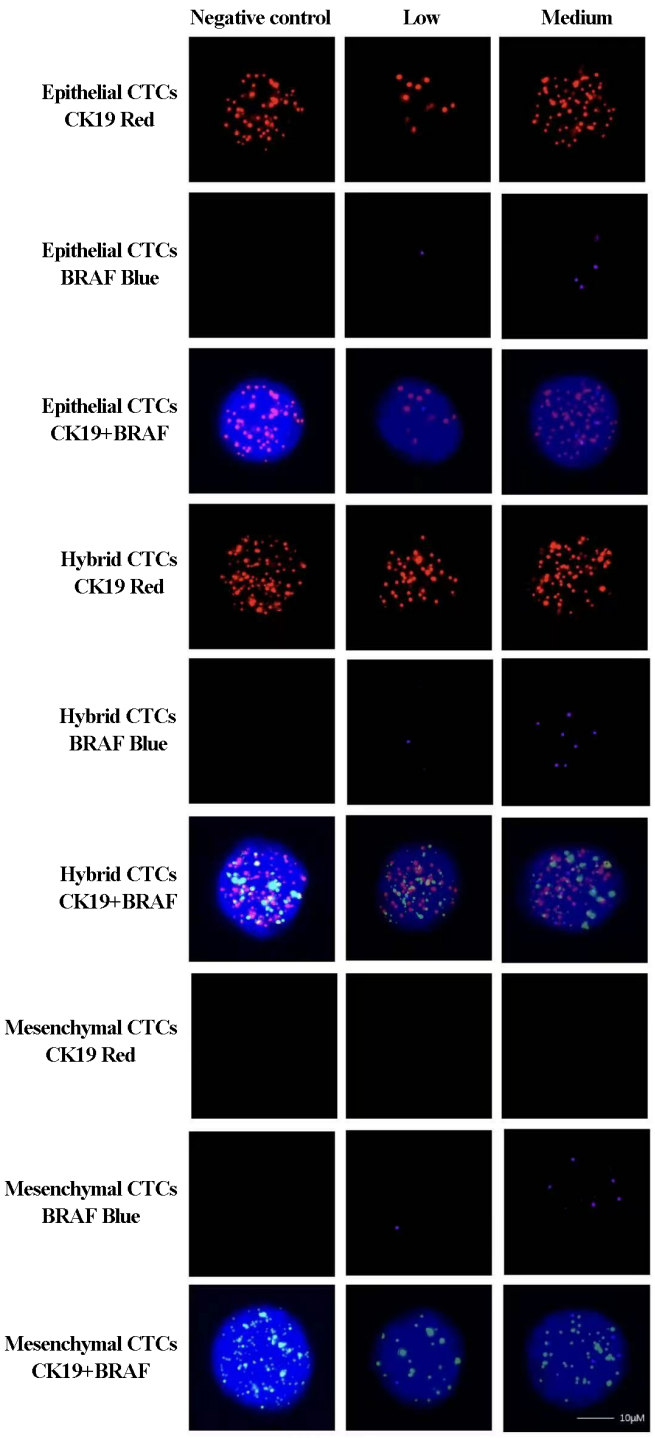

Figure 2. *BRAF* and CK19 expression in different types of CTCs. Expression in different types of CTCs stained for BRAF (blue fluorescence) and CK19 (red fluorescence) biomarkers. The cells were analyzed using a 100× oil objective. Abbreviation: CTCs-circulating tumor cells

Table 3. Correlation between CTCs and R-ISS stage.

	R-ISS stage					
	Univariate			Multivariate		
	β	95% CI	p-value	β	95% CI	p-value
Total CTCs	0.037	−0.014, 0.088	0.154	0.032	−0.026, 0.090	0.281
Epithelial CTCs	−0.777	−1.570, 0.016	0.055	−0.736	−1.595, 0.122	0.093
Epithelial CTCs ≤1	/			/		
Epithelial CTCs >1	−2.297	−4.589, −0.005	**0.049***	−2.109	−4.491, 0.274	0.083
Hybrid CTCs	0.041	−0.016, 0.099	0.160	0.036	−0.030, 0.101	0.285
Mesenchymal CTCs	0.101	−0.220, 0.423	0.537	0.112	−0.218, 0.442	0.506
Epithelial CTC ratio	−2.612	−5.530, 0.306	0.079	−2.894	−6.018, 0.230	0.069
Epithelial CTC ratio ≤0.25	/			/		
Epithelial CTC ratio >0.25	−2.557	−4.402, −0.711	**0.007****	−2.611	−4.702, −0.521	**0.014***
Hybrid CTC ratio	1.728	−0.407, 3.863	0.113	1.440	−1.371, 4.251	0.315
Mesenchymal CTC ratio	0.200	−2.248, 2.648	0.873	1.850	−1.360, 5.060	0.259

Notes: * and ** represent those differences which were considered statistically significant with $p<0.05$ and $p<0.01$, respectively; Epithelial CTC ratio-number of Epithelial CTCs/number of total CTCs; Hybrid CTC ratio-number of Hybrid CTCs/number of total CTCs; Mesenchymal CTC ratio, number of mesenchymal CTCs/number of total CTCs. Abbreviations: R-ISS-revised international staging system; CTCs-circulating tumor cells

Table 4. Correlation between CTCs and MM clinical characteristics.

Characteristics	CTCs and CTCs ratio (R; p-value)						
	Total CTCs	Epithelial CTCs	Hybrid CTCs	Mesenchymal CTCs	Epithelial CTC ratio	Hybrid CTC ratio	Mesenchymal CTC ratio
CRP (mg/l)	0.075; 0.669	−0.382; **0.024***	0.096; 0.583	0.148; 0.396	−0.450; **0.014***	0.227; 0.236	0.137; 0.477
PCT (*10^-2 μg/l)	0.179; 0.302	−0.089; 0.613	0.182; 0.295	0.162; 0.353	−0.230; 0.230	0.079; 0.683	0.127; 0.511
Ca2+ (mmol/l)	0.025; 0.887	−0.273; 0.113	0.066; 0.707	0.095; 0.589	−0.261; 0.172	0.247; 0.196	−0.036; 0.853
Cr (*10 μmol/l)	−0.093; 0.596	−0.256; 0.138	−0.028; 0.873	−0.148; 0.396	−0.106; 0.584	−0.070; 0.719	0.154; 0.424
GFR (*10 ml/min)	0.315; 0.065	0.072; 0.681	0.301; 0.078	0.210; 0.227	−0.133; 0.492	−0.044; 0.820	0.191; 0.321
LDH (*10^2 μg/l)	0.220; 0.205	0.022; 0.902	0.082; 0.639	0.194; 0.264	−0.058; 0.767	0.156; 0.420	−0.025; 0.897
ALB (*10 g/l)	−0.114; 0.516	0.444; **0.008****	−0.254; 0.140	0.039; 0.826	0.464; **0.011***	0.039; 0.842	−0.513; **0.004****
SF (*10^2 ng/ml)	0.263; 0.127	−0.024; 0.891	0.291; 0.090	0.174; 0.318	−0.152; 0.430	0.029; 0.882	0.302; 0.112
VitaB12 (*10^2 pg/ml)	−0.046; 0.793	0.224; 0.196	−0.139; 0.424	0.116; 0.508	0.283; 0.137	0.211; 0.272	−0.281; 0.140
β2-MG (μg/ml)	0.125; 0.474	−0.218; 0.209	0.182; 0.295	−0.016; 0.929	−0.229; 0.232	−0.092; 0.636	0.354; 0.060
WBC (*10^9 /l)	−0.036; 0.836	−0.184; 0.289	−0.047; 0.788	−0.139; 0.424	−0.081; 0.676	−0.046; 0.813	0.084; 0.666
neutrophil (*10^9 /l)	0.018; 0.919	0.061; 0.729	−0.069; 0.692	−0.032; 0.854	0.134; 0.489	−0.037; 0.849	−0.103; 0.596
Hb (*10^9 /l)	−0.101; 0.564	0.111; 0.525	−0.146; 0.401	−0.042; 0.811	0.226; 0.238	0.010; 0.960	−0.187; 0.332
PLT (*10^9 /l)	0.038; 0.828	−0.024; 0.889	0.078; 0.657	−0.027; 0.877	−0.055; 0.778	−0.214; 0.264	0.156; 0.419

Notes: * and ** represent those differences which were considered statistically significant with $p<0.05$, $p<0.01$, respectively; Epithelial CTC ratio-number of Epithelial CTCs/number of total CTCs; Hybrid CTC ratio-number of Hybrid CTCs/number of total CTCs; Mesenchymal CTC ratio-number of mesenchymal CTCs/number of total CTCs. Abbreviations: CTCs-Circulating tumor cells; R-Spearman's correlation coefficient; CRP-C-reactive protein; PCT-Procalcitonin; Ca^{2+}-Calcium ions; Cr-Serum creatinine; GFR-Glomerular filtration rate; LDH-Lactate dehydrogenase; ALB-Serum albumin; SF-Serum ferritin; β2-MG-β2-microglobulin; WBC-White blood cell; Hb-Hemoglobin; PLT-Platelets

comparison, due to differences in the orders of magnitude of the different blood markers. Both epithelial CTCs and the epithelial CTC ratio showed a dramatic negative correlation with the level of blood C-reactive protein (CRP) (r=−0.382, p=0.024). Furthermore, epithelial CTCs and the epithelial CTC ratio were significantly positively correlated with blood serum albumin (ALB) levels in patients with MM (r=0.444, p=0.008; r=0.464, p=0.011). In contrast, the mesenchymal CTCs ratio a showed negative correlation with the blood ALB (r=−0.513, p=0.004) levels in patients with MM (Table 4).

To further understand the link between CTCs and the Eastern Cooperative Oncology Group (ECOG) score of MM, we analyzed the correlation between various types of circulating tumor cells, their ratio, and the ECOG score using an ordered logistic regression model, in which the multifactorial analysis was adjusted for sex, age, fluorescence *in situ* hybridization (FISH), bone marrow primitive immature plasma cells, and mature plasma cell ratio. Data from the model with $0.05<p\leq1$ were analyzed in strata to further determine correlations. We found that in the univariate logistic regression analysis, total CTCs (p=0.045), total CTCs ≥10 (p=0.008), and hybrid CTCs ≥10 (p=0.011) were significantly positively correlated with the ECOG score in MM patient. And in the multivariate logistic regression analysis,

total CTCs ≥10 (p=0.029) and hybrid CTCs ≥10 (p=0.041) also showed a significant positive correlation with the ECOG score (Table 5).

Correlation between BRAF and CTCs. To reveal the relationship between *BRAF* and CTCs, we analyzed the correlation between *BRAF* gene expression levels and CTCs, as well as their ratios, using a linear regression model in which sex, age, fluorescence *in situ* hybridization (FISH), and bone marrow primitive immature plasma cells, and mature plasma cell ratios were adjusted for in a multifactorial analysis. We found that total CTCs, hybrid CTCs, and mesenchymal CTCs were significantly positively correlated with *BRAF* expression. However, the epithelial CTCs ratio was negatively correlated with *BRAF* expression (Table 6).

Correlation between BRAF and MM clinical characteristics. Correlations between *BRAF* gene expression levels and hematological markers were analyzed using a linear regression model that adjusted for sex, age, fluorescence *in situ* hybridization (FISH), and bone marrow primitive immature plasma cell, and mature plasma cell ratios in a multifactorial analysis, to understand the relationship between *BRAF* and the clinical characteristics of MM. *BRAF* showed a dramatic negative correlation with the level of blood white blood cells (WBC) (p=0.034) and neutrophils (p=0.034) (Table 7). We also found that low *BRAF* expression is significantly positively associated with the ECOG score (p=0.023) (Table 8).

Discussion

Previous studies have shown that CTCs are closely related to the characteristics of some cancers, such as breast cancer, pancreatic cancer, and nasopharyngeal cancers [24–26]. In 2007, the American Society of Clinical Oncology defined CTCs as markers of cancer [27]. We explored the clinical significance of circulating tumor cells in MM and detected CTCs in patients with MM (Figure 1). Furthermore, epithelial CTCs and the epithelial CTC ratio were significantly and negatively correlated with the R-ISS stage (Table 3). This distribution may be due to the EMT of CTCs. EMT plays a significant role in cancers by causing the active intravasation of CTCs from tumor sites and increasing the metastatic capability of CTCs in circulation [28–30]. CTCs with EMT have a survival advantage in blood circulation. EMT can promote the development of tumors by downregulating the expression of epithelial markers such as E-cadherin and CPS1, upregulating the expression of interstitial markers such as vimentin, fibronectin, and N-cadherin, and inducing the expression of cytokines and transcription factors [31, 32]. Further experiments are needed to elucidate their underlying mechanisms.

As our results suggested that CTCs may play a role in the clinical staging of MM, we next explored the relationship between CTCs and MM clinical characteristics. We

Table 5. Correlation between CTCs and ECOG score.

	ECOG Score					
	Univariate			Multivariate		
	β	95% CI	p-value	β	95% CI	p-value
Total CTCs	0.101	0.002, 0.199	**0.045***	0.101	−0.012, 0.214	0.080
Total CTCs <10	/					
≥10	2.195	0.577, 3.813	**0.008****	2.026	0.212, 3.841	**0.029***
Epithelial CTCs	−0.063	−0.596, 0.470	0.817	0.107	−0.469, 0.683	0.715
Hybrid CTCs	0.113	−0.002, 0.228	0.054	0.100	−0.031, 0.232	0.135
Hybrid CTCs <10	/					
≥10	2.184	0.498, 3.870	**0.011***	1.910	0.074, 3.746	**0.041***
Mesenchymal CTCs	0.122	−0.188, 0.433	0.441	0.200	−0.130, 0.530	0.235
Epithelial CTCs ratio	−1.244	−3.516, 1.028	0.283	−0.311	−2.775, 2.153	0.805
Mesenchymal CTCs ratio	−0.588	−2.948, 1.773	0.626	0.292	−2.394, 2.977	0.831
Hybrid CTCs ratio	1.362	−0.619, 3.343	0.178	0.049	−2.423, 2.520	0.969

Notes: * and ** represent those differences which were considered statistically significant with p<0.05 and p<0.01, respectively; Epithelial CTC ratio-number of Epithelial CTCs/number of total CTCs; Hybrid CTC ratio-number of Hybrid CTCs/number of total CTCs; Mesenchymal CTC ratio-number of mesenchymal CTCs/number of total CTCs. Abbreviation: ECOG-Eastern Cooperative Oncology Group

Table 6. Correlation between BRAF and CTCs.

	CTCs and CTCs ratio (β; 95%CI; p-value)						
	Total CTCs	Epithelial CTCs	Hybrid CTCs	Mesenchymal CTCs	Epithelial CTCs ratio	Hybrid CTCs ratio	Mesenchymal CTCs ratio
BRAF	6.613 (2.372, 10.854); **0.003****	0.025 (−0.354, 0.405); 0.892	5.683 (1.298, 10.068); **0.013***	0.905 (0.324, 1.486); **0.004****	−0.120 (−0.240, −0.001); **0.049***	0.078 (−0.048, 0.205); 0.213	0.042 (−0.077, 0.161); 0.470

Notes: * and ** represent those differences which were considered statistically significant with p<0.05 and p<0.01 respectively; Epithelial CTC ratio-number of Epithelial CTCs/number of total CTCs; Hybrid CTC ratio-number of Hybrid CTCs/number of total CTCs; Mesenchymal CTC ratio-number of mesenchymal CTCs/number of total CTCs. Abbreviation: CTCs-circulating tumor cells

found that epithelial CTCs and the epithelial CTCs ratio were significantly negatively correlated with the blood CRP levels in patients with MM (Table 4). Previous studies have shown that patients with high serum CRP concentrations have a later stage of disease and a higher rate of lymph node metastasis [33, 34]. Moreover, patients with high serum CRP concentrations before treatment have a poor prognosis [35, 36]. A large number of studies have reported that CRP is related to an early diagnosis, differentiation of benign and malignant tumors, pathological stage, lymph node metastasis, histological characteristics, and prognosis [37, 38]. We found that the inflammatory mediators such as CRP were negatively correlated with the positive rate of epithelial CTCs and the epithelial CTC ratio, likely because CRP was involved in the EMT process of CTCs, thus promoting the proliferation of tumor cells. Additionally, epithelial CTCs and the epithelial CTCs ratio had a negative correlation with the level of blood ALB (Table 4). Research shows that malnutrition and an increased turnover of albumin by tumors synergistically lower ALB levels, as observed in cancer patients [39]. ALB comprises about 55% of the total serum protein, and cancer-associated hypoalbuminemia is associated with a variety of systemic changes in response to tumors. Hypoalbuminemia is indicative of increased catabolism related to an inflammatory systemic response, suppression of albumin synthesis, and an increased vascular permeability followed by a shift of albumin from the intravascular sector towards the interstitium [40]. This suggests that CTCs can suppress ALB synthesis, thus promoting cancer development. We also found that the more total CTCs and hybrid CTCs were detected in a MM patient, the higher the ECOG score in that same patient, which may indicate a worse prognosis (Table 5). This phenomenon suggested that CTCs may be predictors of tumor prognosis.

BRAF is a serine/threonine kinase with a key role in the mitogen-activated protein kinase (MAPK) pathway, which regulates cell proliferation, differentiation, and apoptosis. *BRAF* gene mutation occurs in many malignant tumors and is closely related to their occurrence and development, including those of papillary thyroid carcinoma, melanoma, and hairy cell leukemia [41–43]. In our results, total CTCs, hybrid CTCs, and mesenchymal CTCs were positively correlated with the *BRAF* gene expression. Moreover, the epithelial CTCs ratio was negatively correlated with the *BRAF* expression (Table 6). These were likely due to *BRAF* gene expression playing a role in MM development promotion by CTCs. Therefore, further research is warranted to explore its mechanisms in the future. We also found that the *BRAF* gene was negatively correlated with the level of blood WBC and neutrophils (Table 7). Neutrophils, which are the most abundant circulating leukocytes in humans, are the first line of defense against bacterial and fungal infections [44]. In cancer, pro-or anti-tumor properties have been attributed to tumor-associated neutrophils (TAN) [45]. Additionally, neutrophils can modulate the adaptive immune responses at the inflammation site through interaction with antigen-presenting cells and lymphocytes [46]. Numerous studies have shown that inflammation is correlated with cancer pathogenesis [47]. Cancer-related inflammation, a hallmark of tumor biology, involves both stromal and inflammatory cells in the tumor microenvironment (TME) [48, 49]. As neutrophils play an important role in the TME, they are potential targets to enhance the efficacy of immunotherapy [44]. We believe this situation may be due to the *BRAF* gene affecting the tumor microenvironment and inhibiting neutrophil produc-

Table 7. Correlation between BRAF and MM clinical characteristics.

Characteristic	BRAF gene		
	β	95% CI	p-value
CRP (mg/l)	−2.421	−11.064, 6.221	0.571
PCT (*10^-2 μg/6)	10.058	−12.073, 32.190	0.360
Ca^{2} (mmol/l)	0.051	−0.012, 0.114	0.107
Cr (*10 μmol/l)	1.670	−4.510, 7.849	0.584
GFR (*10 ml/min)	−0.004	−0.723, 0.716	0.992
LDH (*10^2μ/l)	0.169	−0.676, 1.014	0.685
ALB (*10g/l)	0.034	−0.174, 0.242	0.741
SF (*10^2 ng/ml)	1.499	−0.345, 3.343	0.107
VitaB12 (*10^2 pg/ml)	−5.610	−13.484, 2.265	0.156
β2-MG (ug/ml)	0.945	−1.260, 3.151	0.387
WBC (*10^9/l)	−0.841	−1.616, 0.067	**0.034***
Neutrophil (*10^9 /l)	−9.074	−17.401, −0.746	**0.034***
Hb (*10^9 /l)	−0.258	−1.027, 0.512	0.498
PLT (*10^9 /l)	−0.097	−0.387, 0.192	0.497

Notes: *represent those differences which were considered statistically significant at p<0.05. Abbreviations: CRP-C-reactive protein; PCT-Procalcitonin; Ca^{2+}-Calcium ions; Cr-Serum creatinine; GFR-Glomerular filtration rate; LDH-Lactate dehydrogenase; ALB-Serum albumin; SF-Serum ferritin; β2-MG-β2-microglobulin; WBC-White blood cell; Hb-Hemoglobin; PLT-Platelets

Table 8. Correlation between BRAF and ECOG score.

	ECOG Score					
	Univariate			Multivariate		
	β	95% CI	p-value	β	95% CI	p-value
BRAF expression negative	/			/		
Low	1.723	−0.083, 3.528	0.062	2.282	0.319, 4.244	**0.023***
Medium	−0.186	−1.741, 1.370	0.815	0.187	−1.485, 1.859	0.826
High	1.591	−0.201, 3.383	0.082	1.270	−0.650, 3.190	0.195

Note: *represent those differences which were considered statistically significant with p<0.05. Abbreviation: ECOG-Eastern Cooperative Oncology Group

tion, thereby promoting inflammatory cell production, and thus the cancer progression. However, the role of neutrophils in MM biology remains unclear, and further studies are required to explore their mechanism in the future. We also found that the less low *BRAF* expression detected in patients with MM, the lower the ECOG score obtained in the same patient, which may imply a better prognosis (Table 8) and suggests that *BRAF* expression may be a predictor of tumor prognosis. Overall, circulating tumor cells were tightly correlated with the clinical stages and characteristics of MM, as well as with the ECOG score. Furthermore, *BRAF* expressions were associated with different CTC subsets.

In conclusion, this study further reinforces the value of CTC levels as prognostic markers in myeloma. Additionally, CTCs can interact with inflammatory mediators and promote cancer progression, in which the *BRAF* gene is also involved. This finding provides an idea for the mechanism of EMT transformation of CTCs leading to poor prognosis and makes it possible to develop and implement targeted treatments.

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