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Clinical value of MLH1-negative circulating tumor cells in lung cancer patients

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Abstract

Circulating tumor cells (CTCs) serve as valuable biomarkers. However, MutL homolog 1 (MLH1)-negative CTCs and their clinical significance in lung cancer are nearly unknown.

Here, bioinformatic analysis of MLH1 expression and its clinical significance was conducted using the Oncomine, Ualcan, and Kaplan–Meier plotter websites. Size-based isolation and RNA in situ hybridization assays were used to identify CTCs and evaluate MLH1 and mesenchymal marker expression in CTCs. MLH1 was downregulated in lung cancer patients. Patients with lower MLH1 expression levels had worse prognoses. In a cohort of 32 randomly selected patients with lung cancer, the patients with poorer treatment responses had more MLH1-negative CTCs. The total CTCs, MLH1-negative CTCs and mesenchymal markers-expressing CTCs levels were negatively correlated with prognosis in the lung cancer patients.

Our data showed the clinical significance of MLH1 expression in lung cancer tissues. The characterization and numeration of CTCs based on the expression of MLH1 and mesenchymal markers may be a convenient approach for predicting treatment response and prognosis in lung cancer.

Abbreviations: CTCs = circulating tumor cells, E CTCs = epithelial CTCs, FP = first progression, H CTCs = hybrid CTCs, M CTCs = mesenchymal CTCs, M + H CTCs = mesenchymal marker-expressing CTCs, MLH1 = MutL homolog 1, MMR = mismatch repair, OS = overall survival, PFS = progression-free survival, PPS = post-progression survival, T CTCs = total CTCs.

Keywords: circulating tumor cells, lung cancer, mesenchymal markers, MutL homolog 1, prognosis

1. Introduction

Lung cancer is one of the most malignant cancers and causes the most cancer-related deaths worldwide.[1] Despite the breakthroughs in treatment strategies for lung cancer in the past decade, the overall survival (OS) of lung cancer, especially advanced lung cancer, is still unfavorable, with a 5-year survival rate of less than 15%.[2,3] Therefore, there is an urgent need to identify novel biomarkers for predicting both the treatment response and the prognosis of lung cancer patients.

MutL homolog 1 (MLH1) is a member of the mismatch repair (MMR) gene family. Previous studies have reported that MLH1 is downregulated in many cases of lung cancer, and its downregulation may be related to platinum resistance.[4–6] However, some studies have illustrated that only a small proportion of lung cancer patients lose MLH1 expression.[7] There have even been studies showing that MLH1 expression is lower in normal bronchial epithelial cells.[8,9] Furthermore, the relationship between MLH1 expression and lung cancer outcomes is controversial. Many studies suggest that the loss of MLH1 expression may lead to platinum resistance and worse outcomes.[6,10] Other studies have reported no link between MLH expression and outcome in lung cancer patients.[11–13] Therefore, we explored MLH1 expression and its clinical significance in lung cancer in a larger number of patients by summarizing data from bioinformatic websites.

Compared to tumor biopsy, the numeration and characterization of circulating tumor cells (CTCs) are considered to be a more convenient, noninvasive approach for predicting treatment response and outcome in cancer patients.[14,15] Our bioinformatic analysis has shown the clinical significance of MLH1 in lung cancer tissues. However, the clinical value of MLH1 expression in CTCs in lung cancer patients is still unknown. Our study is the first to evaluate the number and the clinical significance of MLH1-negative CTCs in patients with lung cancer.

Studies have demonstrated that in breast cancer, CTCs express epithelial and/or mesenchymal markers, and reductions in total CTCs (T CTCs) and mesenchymal CTCs (M CTCs) were

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correlated with poor outcomes.[16,17] Although some studies clarified that it was necessary to classify CTCs based on epithelial and mesenchymal markers in lung cancer, data on M CTCs and their correlation with treatment response and outcome are lacking.[18,19] In our study, we also analyzed whether CTCs expressing epithelial and/or mesenchymal markers, are correlated with the clinical characteristics, treatment response, and prognosis.

2. Methods

2.1. Bioinformatic analysis

MLH1 expression was analyzed within Oncomine and Ualcan. The relationship between survival and MLH1 expression in lung cancer patients was analyzed using the Kaplan–Meier plotter website.

2.2. Patient samples

From June 2015 to November 2016, we enrolled 32 patients who were diagnosed with lung cancer at the Cancer Center of Union Hospital in Wuhan, P.R. China. For each patient, 5 ml of peripheral blood was collected. Blood samples were collected from 16 patients before therapy and from 16 patients during treatment. Blood samples were collected for a second time from 8 patients after a period of treatment. Serial blood samples from 2 patients were analyzed. This study was approved by the Ethical Review Board of the Union Hospital in Wuhan, P.R. China, and performed according to the Declaration of Helsinki Principles. Written informed consents were obtained from the patients who were enrolled in this study.

2.3. Isolation of CTCs

Red blood cell lysis buffer was used to remove erythrocytes. The remaining cells were fixed with 4% formaldehyde for 8 minutes. CTCs were isolated and filtered by size using an epithelial tumor cells device with a calibrated membrane with 8-μm diameter pores (SurExam, Guangzhou, China). After filtration, the CTCs were fixed with 4% formaldehyde for 1 hour.

2.4. RNA in situ hybridization assay

The assays were conducted in 24-well plates. After the cells on the membrane were treated with 0.1 mg/ml protease K (Qiagen, Hilden, Germany) for 1 hour, capture probes (the sequences are shown in Table S1, http://links.lww.com/MD/D40) specific for the leukocyte biomarker CD45, the epithelial biomarkers EpCAM and CK8/18/19, and the mesenchymal biomarkers vimentin and twist were added for hybridization. Hybridization was performed at 40°C for 3 hours. The unbound probes were removed by washing with 1000 μl of wash buffer (0.1× SSC [Sigma, St. Louis]) 3 times. For signal amplification, the cells were incubated with 100 μl of preamplifier solution (30% horse serum, 1.5% sodium dodecyl sulfate, 3 mM Tris-HCl [pH 8.0] [all from Sigma], and 0.5 fmol preamplifier [the sequences are shown in Table S2, http://links.lww.com/MD/D40]) at 40°C for 30 minutes. The membranes were cooled and washed with 1 ml of wash buffer 3 times. Then the cells on the membrane were incubated with 100 μl of amplifier solution and 1 fmol amplifier (the sequences are shown in Table S2, http://links.lww.com/MD/D40) at 40°C for 30 minutes. Label probes conjugated with the fluorescent dyes (Alexa Fluor 594 for EpCAM and CK8/18/19, Alexa Fluor 488 for vimentin and twist, and Alexa Fluor 750 for CD45) were added and incubated at 40°C for 30 minutes. After washing with wash buffer, the cells were stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma) for 5 minutes. The samples were then observed with a fluorescence microscope using a 100× objective lens (Olympus BX53, Tokyo, Japan).

MLH1 was detected via the same method. The capture probe, preamplifier, and amplifier sequences are listed in Table S1, http://links.lww.com/MD/D40 and Table S2, http://links.lww.com/MD/D40. The fluorescent dye for MLH1 was Alexa Fluor 647.

CD45−DAPI+ cells expressing mesenchymal markers or epithelial markers were identified as CTCs. According to the fluorescent signals, we classified the CTCs into 3 groups: epithelial CTCs (E CTCs, only red fluorescence), hybrid CTCs (H CTCs, both red and green fluorescence), and M CTCs (only green fluorescence) (Fig. S1A, http://links.lww.com/MD/D40). CTCs expressing mesenchymal markers, including M CTCs and H CTCs, were defined as mesenchymal marker-expressing CTCs (M + H CTCs), including M CTCs and H CTCs. CTCs were classified into 4 groups according to their MLH1 expression: MLH1-negative, MLH1-low, MLH1-median, and MLH1-high CTCs (Fig. S1B, http://links.lww.com/MD/D40).

2.5. Statistical analysis

Differences in CTCs numbers between 2 groups were tested by the Mann–Whitney test. The survival analysis was tested by the log-rank (Mantel-Cox) test. All data were analyzed using SPSS v19.0 software. A *P*-value less than .05 was considered statistically significant.

3. Results

3.1. The expression and clinical significance of MLH1 expression in lung cancer patients

We searched for MLH1 data in Oncomine datasets and Ualcan. The data suggested that the expression of MLH1 was downregulated in lung cancer patients (Fig. 1A). Additional analysis in Ualcan showed no difference in MLH1 expression between normal lung tissues and adenocarcinoma (ADC), while MLH1 expression was downregulated in SCC (Fig. 1B).

Survival comparison of patients without chemo- or radiotherapy using the Kaplan–Meier plotter website showed no significant difference in OS between the low and high MLH1 groups (85.7 vs 128.8 months, $P = .25$) (Fig. 2A). However, among all patients, including those receiving chemo- or radiotherapy, OS was significantly better in the high MLH1 group than in the low MLH1 group (114 vs 54 months, $P < .001$) (Fig. 2B). Among all patients, the first progression (FP) (14 vs 35 months, $P < .001$) and post-progression survival (PPS) (13 vs 21.9 months, $P < .001$) were significantly better in the high MLH1 group (Fig. 2C and D).

3.2. Patient demographics

From June 2015 to November 2016, a total of 32 patients diagnosed with lung cancer were enrolled. The patients' clinical characteristics are listed below (Table 1). At the time of analysis,

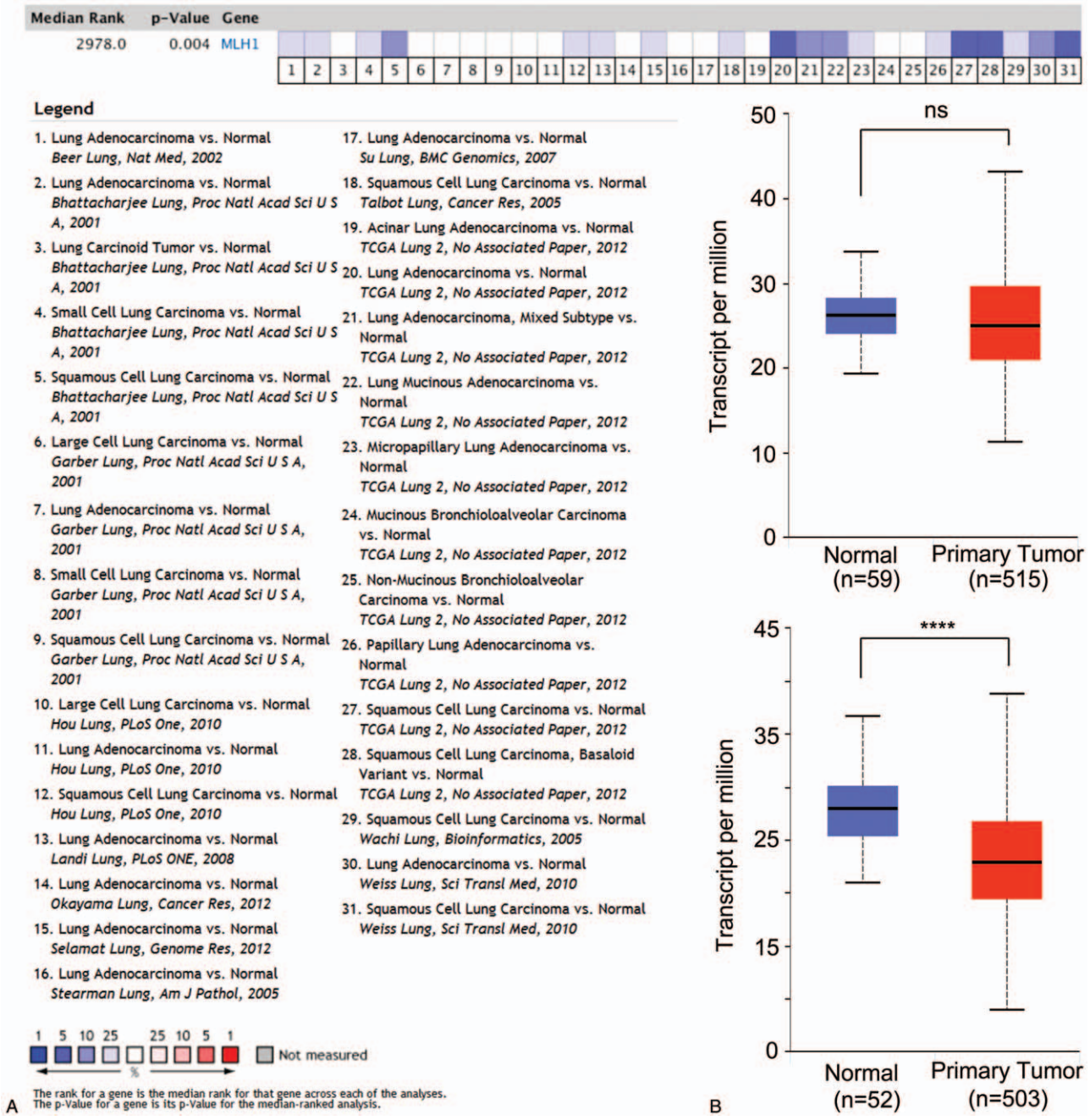

Figure 1. Bioinformatic analysis of MLH1 expression in lung cancer patients. (A) The analysis of MLH1 expression in Oncomine datasets. (B) The expression of MLH1 in ADC and SCC patients. ADC = adenocarcinoma, MLH1 = MutL homolog 1.

10 patients had died and 18 patients had progressed. The average follow-up time for the 22 patients still alive was 17.7 ± 1.6 months (range, 11.9–18.2 months).

3.3. The relationship between MLH1-negative CTCs counts and clinical characteristics

Analysis of the relationship between MLH1-negative CTCs and the clinical characteristics showed that fewer MLH1-negative CTCs were found in small cell lung cancer patients than in ADC and SCC patients (6.6 vs 18.2 vs 11.7 per 5 ml; $P = .0123$). Elevated T CTCs counts were found in patients with a smoking history (15.0 vs 5.5 per 5 ml; $P = .0203$). A positive smoking history was also related to more M + H CTCs (11.5 vs 3.0 per 5 ml; $P = .0104$). Compared with older patients, patients younger than 60 years old had fewer M CTCs (0.0 vs 1.0 per 5 ml; $P = .0035$) and M + H CTCs (3.0 vs 7.0 per 5 ml; $P = .0318$). (Table 2).

3.4. Predictive significance of MLH1-negative CTCs numbers in anticancer treatments

To determine the predictive significance of MLH1-negative CTCs numbers in anticancer treatments, we analyzed the relationship

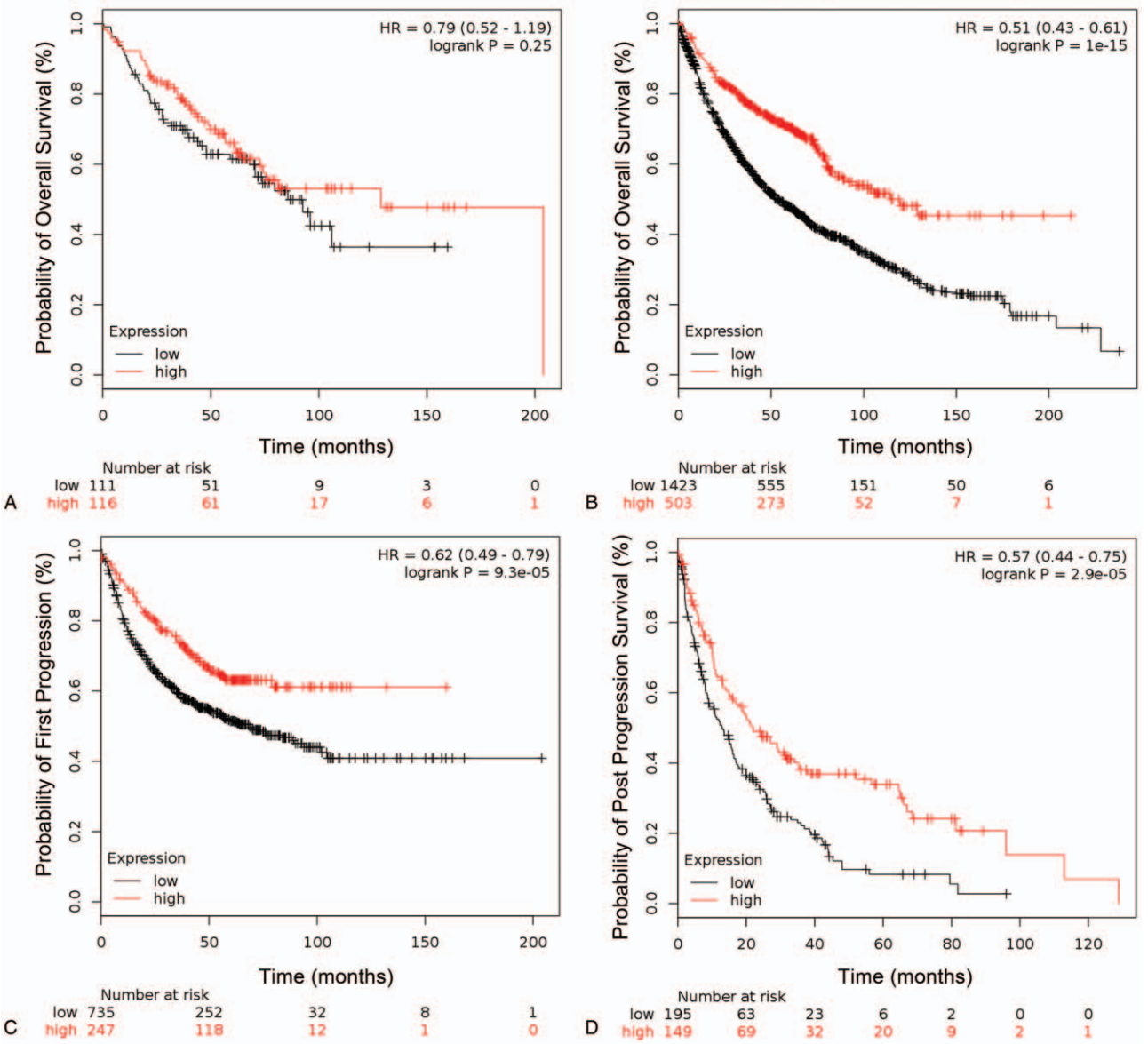

Figure 2. Survival analysis of lung cancer patients based on MLH1 expression using the Kaplan–Meier plotter website. (A) Comparison of OS in patients without chemo- or radiotherapy between the high and low MLH1 groups. (B–D) Kaplan–Meier curves for OS (B), FP (C), and PPS (D) in all patients with and without chemo- or radiotherapy. FP = first progression, MLH1 = MutL homolog 1, OS = overall survival.

between CTCs counts and treatment response. Patients with worse treatment responses had more MLH1-negative CTCs than those with better responses (8.5 vs 1.5 per 5 ml; $P = .0102$) (Fig. 3A). Although T CTCs, M CTCs, and M + H CTCs counts were elevated in patients with progression, the differences were not statistically significant (Fig. 3B). Serial evaluation of CTCs illustrated that the changes in MLH1-negative CTCs, T CTCs, and M + H CTCs counts were consistent with the treatment responses (Fig. 3C).

3.5. Predictive significance of MLH1-negative CTCs numbers in lung cancer patient survival

To determine the prognostic significance of MLH1-negative CTCs numbers in lung cancer patients, we analyzed the progression-free survival (PFS) and OS in patients with high and low CTCs counts. The Kaplan–Meier curves showed that the median PFS was significantly worse for patients with higher MLH1-negative CTCs counts than those with lower MLH1-negative CTCs counts (1.6 vs 18.2, $P = .0138$) (Fig. 4A). Although the median PFS of patients with lower T CTCs and M + H CTCs counts were still undefined, the prognosis of patients with higher T CTCs and M + H CTCs counts was worse, and the *P*-values of the log-rank test were lower than 0.05 (Fig. 4B and C). The OS of patients with lower MLH1-negative CTCs counts and lower M + H CTCs counts were also significantly better (Fig. 4D and F). Patients with lower T CTCs counts had a better OS than those with lower T CTCs counts, but the difference was not statistically significant ($P = .0735$) (Fig. 4E).

Table 1

Patient demographics and clinical characteristics.

Characteristic		N	Proportion (%)
Age	<60	16	50.0
	>=60	16	50.0
Gender	Male	22	68.8
	Female	10	31.3
Smoking history	Yes	12	37.5
	No	20	62.5
Histology	ADC	16	50.0
	SCC	7	21.9
	SCLC	5	15.6
	Unknown	4	12.5
EGFR mutation	19+	4	12.5
	21+	6	18.8
	–	7	21.9
	Unknown	15	46.9
TNM stage	Early	4	12.5
	III	7	21.9
	IV	20	62.5
	Unknown	1	3.1

ADC = adenocarcinoma, EGFR = epidermal growth factor receptor, SCLC = small cell lung cancer.

4. Discussion

The MMR system recognizes and corrects DNA mismatches generated during DNA replication and recombination.[20] An MMR deficiency may increase mutations and result in microsatellite instability and carcinogenesis.[21,22] Defective MLH1 has been reported in many cancers.[23,24] Xinarianos found that 58.6% of non-small cell lung cancer specimens had reduced MLH1 expression.[5] A similar observation was reported in Wang's study.[4] However, some studies have clarified that the majority of lung cancer patients have normal MLH1 expression.[7,25] Meanwhile, other studies have found increased MLH1 expression in lung cancer cell lines and epidermal growth factor receptor (EGFR)-mutated lung cancer patients.[26,27] In addition, previous studies have enrolled only a small number of patients. The data of 1177 normal and 1856 lung cancer samples from bioinformatic websites were summarized in our study. The clinical value of MLH1 expression in lung cancer also remains controversial. Previous research has shown that MLH1 expression loss may be responsible for platinum resistance and worse prognosis in lung cancer.[6,10,28,29] In contrast, the loss of MLH1 expression was associated with significantly improved survival compared to normal MLH1 expression in Mario Scartozzia's study.[25] However, Cooper and his group found that MLH1 expression had no relationship with lung cancer patient prognosis.[11] We summarized data from the bioinformatic website Kaplan–Meier plotter, which included more than a thousand lung cancer patients with or without chemo- or radiotherapy. Among all patients, including those with or without chemo- or radiotherapy, OS, FP, and PPS were significantly better in patients with higher MLH1 expression. No difference was observed among patients without chemo- or radiotherapy. These findings suggest that MLH1 can predict prognosis in lung cancer patients, especially those receiving chemo- or radiotherapy.

Although traditional biopsy is the gold standard for diagnosing lung cancer, liquid biopsy, including CTCs and circulating tumor DNA, is attracting increasing attention because of its convenience, noninvasion, and ability to reflect heterogeneity. The

Table 2

Relationships between CTCs and clinical characteristics.

Characteristic	N	T CTCs	M CTCs	M + H CTCs	MLH1 − CTCs
Age					
<60	16	6.5	0	3.0	2.0
>=60	16	11.0	1.0	7.0	2.5
P-value		.1158	.0035*	.0318*	.9773
Gender					
Male	22	9.0	1.0	7.0	2.0
Female	10	5.0	0	3.0	2.0
P-value		.1205	.1318	.0553	.6189
Smoking history					
Yes	12	15.0	1.0	11.5	2.0
No	20	5.5	0	3.0	2.5
P-value		.0203*	.2177	.0104*	.7928
Histology					
ADC	16	12.4	1.188	8.125	6.688
SCC	7	11	1.3	6.2857	3.3
SCLC	5	7.6	1.8	5.2	0.6
P-value		.8294	.4312	.9272	.0123*
EGFR mutation					
19+	4	8	0	3.5	3.75
21+	6	13	1.5	8	9.333
–	7	13	1.4	10	5.4
P-value		.7159	.3270	.5386	.4058
TNM stage					
Early	4	5.5	0.5	3.75	4
III	7	9.8571	1	6.1429	2.7143
IV	20	11.15	1.5	7.75	4.4
P-value		.4624	.8935	.5477	.8207

ADC = adenocarcinoma, CTCs = circulating tumor cells, EGFR = epidermal growth factor receptor, H CTCs = hybrid CTCs, M CTCs = mesenchymal CTCs, M + H CTCs = mesenchymal marker-expressing CTCs, MLH1 = MutL homolog, SCLC = small cell lung cancer, T CTCs = total CTCs.

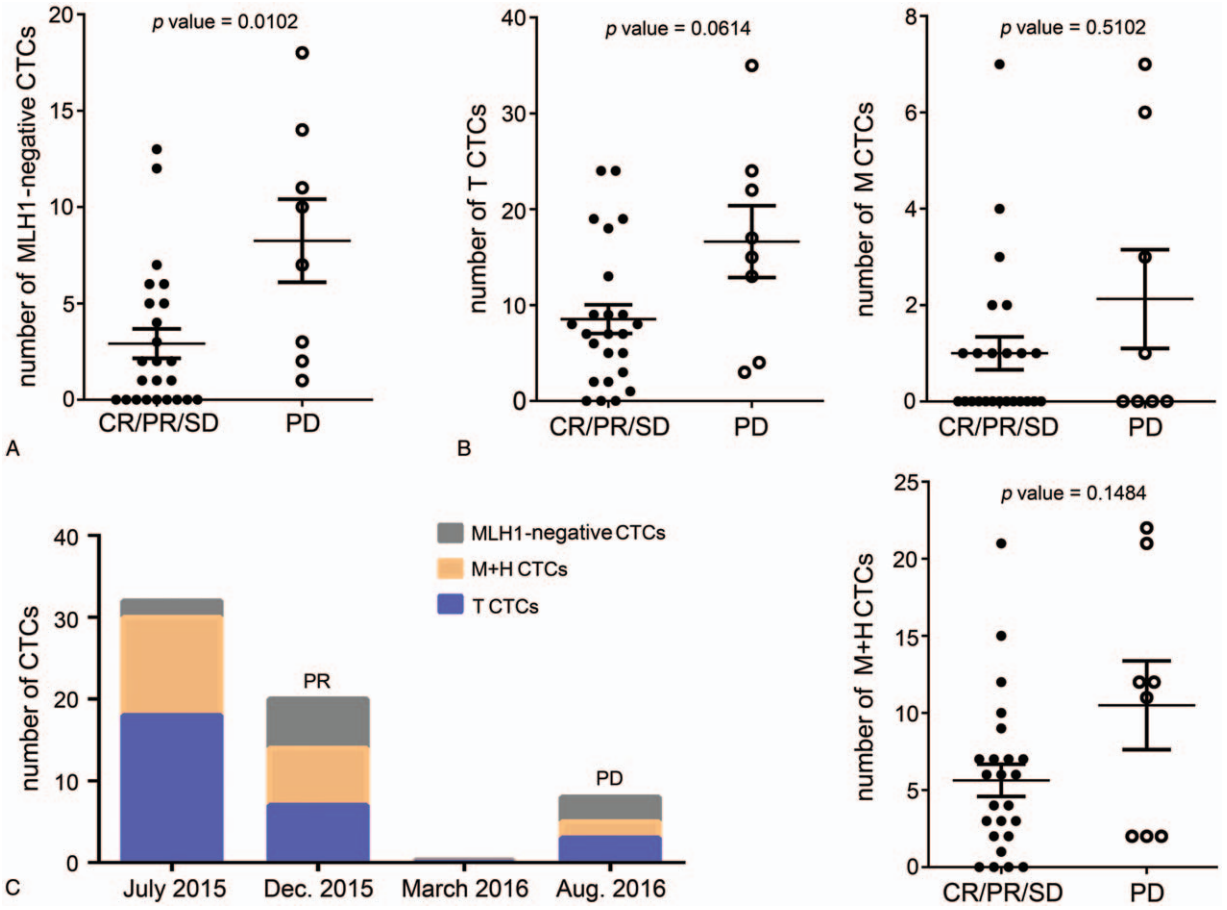

Figure 3. The relationship between MLH1-negative CTCs counts and treatment responses in lung cancer patients. (A) MLH1-negative CTCs in patients with progression (PD) and without progression (CR/PR/SD). (B) The association between T CTCs, M CTCs, and M + H CTCs numbers, and treatment responses. (C) Serial observation of MLH1-negative CTCs, T CTCs, M + H CTCs numbers, and treatment responses in 1 patient. CTCs = circulating tumor cells, H CTCs = hybrid CTCs, M CTCs = mesenchymal CTCs, M + H CTCs = mesenchymal marker-expressing CTCs, MLH1 = MutL homolog, T CTCs = total CTCs.

clinical value of biomarkers, including EGFR mutations and others in CTCs, has been reported.[30,31] Previous studies on MLH1 have focused on only cancer tissues. According to our bioinformatic analysis, MLH1 downregulation in lung cancer was negatively correlated with prognosis. Therefore, we hypothesized that MLH1 expression may also have clinical value at the CTCs level. To the best of our knowledge, this is the first study to explore MLH1-negative CTCs. Our results show that MLH1-negative CTCs are more common in ADC and SCC patients, which is consistent with previous work on MLH1 expression based on specimens.[11,32,33] Although there was no relationship between M CTCs or M + H CTCs and treatment response, the increased number of MLH1-negative CTCs predicted a poor response to treatment. This result indicated that MLH1-negative CTCs could better predict treatment responses in lung cancer.

The characterization and classification of CTCs based on epithelial–mesenchymal transition markers have been reported to be necessary in many studies.[18,19] In Wu's research, more M CTCs were observed in patients with advanced cancer.[34] However, this study did not analyze the prognostic significance. Another study on breast cancer showed that reductions in T CTCs and M CTCs were related to better treatment responses. In addition, mesenchymal markers were more common in the CTCs cluster, which was proven to be associated with metastasis and progression.[17] However, data on lung cancer are lacking, especially regarding patient prognosis. Our results showed no relationships between the number of M CTCs and stage or treatment response. One reason may be that we enrolled only 32 patients in our study. In addition, some patients had experienced different treatment regimens at the time of blood drawing. Although there was no significant prognostic value in the M CTCs level, the levels of M + H CTCs were negatively correlated with OS and PFS. Simple survival analysis based on T CTCs showed no significant difference in OS. Therefore, the characterization and numeration of CTCs according to mesenchymal marker expression may better predict patient survival than T CTCs. In addition, classifying CTCs into E CTCs and M + H CTCs may be more appropriate for predicting prognosis than classifying CTCs into 3 classes: E CTCs, H CTCs, and M CTCs. CTCs expressing mesenchymal markers, but not those expressing only mesenchymal markers, may have an impact on tumor metastasis and progression.

However, further research involving more patients is needed. The relationship between baseline CTCs and clinical characteristics should be analyzed, along with the relationship between their dynamic changes and treatment response. Furthermore, tumors with MLH1 hypermethylation, which regulates MLH1

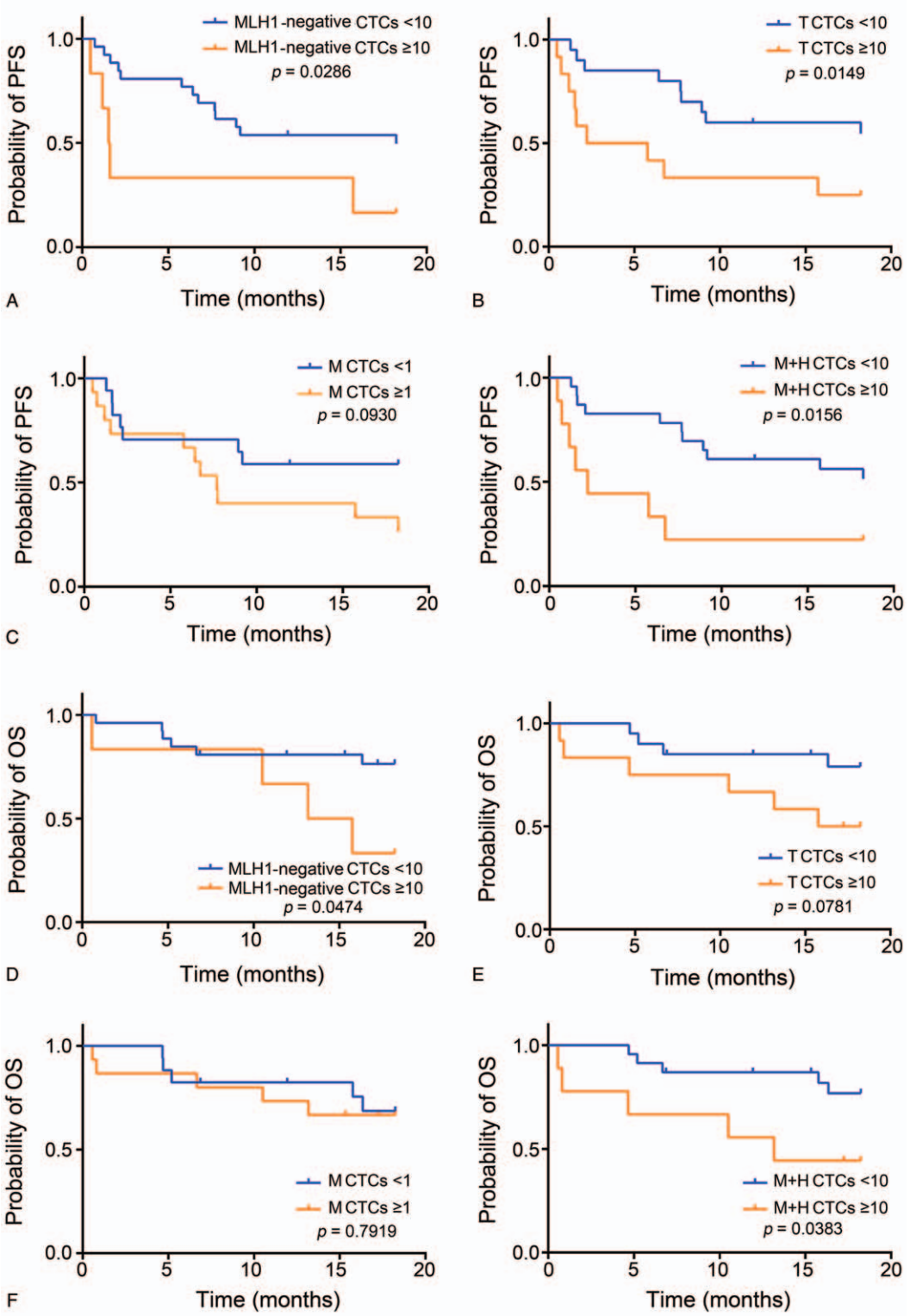

Figure 4. The relationship between MLH1-negative CTCs counts and the prognosis of lung cancer patients. (A) Kaplan–Meier curves for the PFS of patients with high and low levels of MLH1-negative CTCs. (B–C) PFS comparison between groups with high or low levels of T CTCs (B), M CTCs and M + H CTCs (C). (D) Comparison of OS between patients with high or low levels of MLH1-negative CTCs. (E and F) Kaplan–Meier curves for the OS of patients with high and low levels of T CTCs (E), M CTCs and M + H CTCs (F). CTCs = circulating tumor cells, H CTCs = hybrid CTCs, M CTCs = mesenchymal CTCs, M + H CTCs = mesenchymal marker-expressing CTCs, MLH1 = MutL homolog, OS = overall survival, PFS = progression-free survival, T CTCs = total CTCs.

expression, have been shown to have higher programmed cell death-ligand 1 (PD-L1) levels.[35] This suggests the predictive value of MLH1 in immunotherapy. Therefore, further exploration can be conducted by enrolling patients who accept anti-PD-L1 immunotherapy.

5. Conclusions

In summary, we found a reduction in MLH1 expression and a correlation with prognosis in lung cancer patients. We reported for the first time the MLH1-negative CTCs and their clinical value in lung cancer. Our study provides evidence for the analysis of MLH1 and mesenchymal markers in CTCs as predictive and prognostic biomarkers in lung cancer.

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