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# Cancer Stem-like Cells Derived from Chemoresistant Tumors Have a Unique Capacity to Prime Tumorigenic Myeloid Cells

Tsunaki Yamashina, Muhammad Baghdadi, Akihiro Yoneda, et al.

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ORIGINAL RESEARCH

# Low expression levels of microRNA-124-5p correlated with poor prognosis in colorectal cancer via targeting of SMC4

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**Keywords**
Colorectal cancer, EZH2, MFGE8, miR-124-5p, miR-26a, SMC4

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## Abstract

A component of polycomb repressor complex 2, enhancer of zeste homolog 2 (EZH2), plays an important role in tumor malignancy and metastasis, while milk fat globule-epidermal growth factor-factor 8 (MFGE8) plays a key role in tumor progression and prognosis. MicroRNAs (miRs) are also critically involved in various physiological and pathological processes. We here evaluated the relationship between overall survival (OS) in colorectal cancer patients and the expression of onco-miRs and miRs, which may target *EZH2* and *MFGE8*. Plasma and formalin-fixed paraffin-embedded (FFPE) samples were obtained from 71 colorectal cancer patients. The expression levels of miRs complementary to *EZH2* and *MFGE8* mRNA and cancer malignancies were evaluated. The miRs analyzed were as follows: miR-16, miR-21, miR-26a, miR-34a, miR-98, miR-101-3p, miR-101-5p, miR-124-5p (also known as miR-124*), miR-126-3p, miR-126-5p, miR-210, miR-217, and miR-630. The plasma expression levels of *MFGE8* in completely resected patients were significantly lower than those in unresectable patients. Lower miR-26a expression levels were correlated with a higher probability of OS. Higher miR-124-5p expression levels in plasma and FFPE samples were correlated with a higher probability of OS. The transfection of mimic miR-124-5p into WiDr and COLO201 cells inhibited the expression of structural maintenance of chromosomes 4 (*SMC4*) mRNA. Our results indicate that miR-124-5p may target the tumorigenesis gene, SMC4, which suggests that expression levels of miR-124-5p in plasma and FFPE samples; therefore, the expression of *MFGE8*, miR-26a, and miR-124-5p in plasma may be used as biomarkers to determine the prognosis of colorectal cancer patients.

## Introduction

Colorectal cancer remains a significant cause of mortality worldwide. Despite an earlier diagnosis and advances in available treatments, many colorectal cancers remain incurable [1, 2]. Molecular profiling will assist in the development of personalized treatment strategies [3]. Enhancer of zeste homolog 2 (EZH2), a component of the oncogene polycomb repressive complex 2, exhibits histone methyltransferase activity and induces the methylation of lysine residues in histone H3. EZH2 was previously shown to be overexpressed in cancers, and EZH2 expression levels



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correlated with aggressiveness, metastasis, and a poor prognosis [4, 5]. Milk fat globule-epidermal growth factor-factor 8 (MFGE8) plays an important role in controlling the progression of various inflammatory diseases. It is also involved in tumor progression and prognosis [6, 7].

MicroRNAs (miRs) have been shown to negatively regulate gene expression by binding to complementary sequence sites in the 3′-untranslated regions of the mRNAs of protein-coding genes, thereby degrading or blocking the translation of these mRNAs. MicroRNAs are known to play an important role in various physiological and pathological processes, such as apoptosis, cell proliferation, and differentiation, which indicates their functionality in carcinogenesis as tumor suppressor genes or oncogenes [8]. MicroRNAs have recently been detected in body fluids, such as serum, plasma, and saliva. Although initially considered to be unstable RNA molecules, circulating miRs are now known to be highly stable and readily detected in plasma. Exosomes are microvesicles with an endocytic origin that are released from various cells into the extracellular space. Exosomes have been detected in cell culture supernatants as well as body fluids, and are composed of a lipid bilayer. They contain mRNAs and miRs, which are enclosed in side exosomes and are secreted into the extracellular space [9, 10].

*EZH2* was previously shown to be suppressed by miR-101-3p (also known as miR-101) and miR-26a [11–14]. A bioinformatics, MicroRNA.org (http://www.microrna.org) is a comprehensive resource of microRNA target predictions and expression profiles. Target predictions are based on a development of the miRanda algorithm which incorporates current biological knowledge on target rules and on the use of an up-to-date compendium of mammalian microRNAs [15]. The microRNA.org predicted that *EZH2* may be targeted by miR-26a, miR-34a, miR-98, miR-101-3p, miR-217, and miR-630. Previous studies suggested that miR-16, miR-21, miR-34a, miR-101-3p, miR-124-5p (also known as miR-124*), miR-126-3p (also known as miR-126), miR-126-5p (also known as miR-126*), miR-210, miR-217, and miR-630 may be used as prognostic and diagnostic biomarkers for cancer [16–22]. MicroRNA.org also predicted that miR-124-5p may target structural maintenance of chromosomes 4 (*SMC4*). SMC4 is a core subunit of condensin I and II, which are large protein complexes, is involved in chromosome condensation, and has been associated with tumorigenesis [23]. However, the inhibitory effects of miR-124-5p on the expression of *SMC4* mRNA have not yet been elucidated in detail.

The relationship between overall survival (OS) in colorectal cancer patients and the expression of miRs, which may target *EZH2* and *MFGE8* and have been linked to cancer, was examined using plasma and FFPE samples. To investigate the involvement in survival benefit of miR-124-5p, the inhibitory effects of a possible target mRNA of miR-124-5p, *SMC4*, were evaluated.

## Materials and Methods

### Reagents

The QuantiTect Primer Assay, miScript Primer Assay, miScript Reverse Transcription Kit, and synthetic microRNA mimic and miScript SYBR Green PCR Kit were purchased from Qiagen (Valencia, CA). The real-time PCR master mix THUNDERBIRD SYBR qPCR Mix and reverse transcriptase (RT), ReverTra Ace was purchased from TOYOBO Co., Ltd. (Osaka, Japan). The High Pure RNA Isolation Kit and High Pure RNA Paraffin Kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO). The human colon adenocarcinoma cell lines, WiDr (JCRB0224) and COLO201 (JCRB0226) were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). These cell lines were tested and authenticated by the JCRB Cell Bank. Lipofectamine 2000 reagent was purchased from Life technologies (Carlsbad, CA). Synthetic SMC4 siRNA (sense: gcccaagaauguguaaacu, anti: aguuuacacauucuugggc) [23] was obtained from Bioneer Corporation (Daejeon, Republic of Korea). The PCR primers for SMC4 (sense: gagaaaattctgggaccttt, anti: tctgaatgtccttgtgttca) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense: aacagcctcaagatcatcagc, anti: ggatgatgttctggagagcc) [23] were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

### Patients and sample collection

We examined 71 patients with colorectal cancer who were recruited at Hokkaido Gastroenterology Hospital. All patients received chemotherapy according to the Japanese Society for Cancer of the Colon and Rectum Guidelines [24]. This study was approved by the Ethical Committee at the affiliations. Written informed consent was obtained from all patients. The study protocol was approved by the Institutional Review Board and conformed to the guidelines of the 2008 Declaration of Helsinki. Blood samples for medical testing purposes were collected into ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was separated from the residuum of the samples for the blood cell count inspection prior to chemotherapy. Plasma samples stored at −80°C. Formalin-fixed paraffin-embedded (FFPE) samples were obtained from tumor histology. The characteristics of the study population are shown in Table 1.

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## RNA isolation and RT-PCR

Total RNA was isolated from 200 $\mu$L of plasma and FFPE samples using the High Pure RNA Isolation Kit and High Pure RNA Paraffin Kit according to the manufacturer's instructions. Single-stranded cDNA was synthesized by RT using ReverTra Ace, and single-stranded cDNA for microRNA analysis was also synthesized by RT using the miScript Reverse Transcription Kit according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler 480 II System (Version 1.5; Roche Diagnostics GmbH, Mannheim, Germany) with TaqMan gene expression assays and the THUNDERBIRD qPCR Mix or miScript SYBR Green PCR Kit according to the manufacturer's instructions. Comparative real-time RT-PCR assays were performed for each sample in triplicate.

**Table 1.** Patient characteristics.

| Characteristics | Unresectable ($n$ = 49) | Completely resected ($n$ = 22) | $P$ |
|---|---|---|---|
| Age | | | |
| Mean (SD) | 63.0 (11.4) | 59.1 (6.8) | |
| Range | 30–83 | 47–74 | |
| Sex | | | 0.22 |
| Male | 34 | 12 | |
| Female | 15 | 10 | |
| TNM classification | | | <0.0001 |
| II | 0 | 3 | |
| III | 0 | 13 | |
| IV | 49 | 6 | |
| Primary lesion | | | 0.19 |
| Colon | 19 | 12 | |
| Rectum | 20 | 9 | |
| Other | 10 | 1 | |
| Histology | | | 0.07 |
| Tubular adenocarcinoma | 44 | 19 | |
| Mucinous adenocarcinoma | 1 | 3 | |
| Other | 4 | 0 | |
| Chemotherapy | | | 0.03 |
| mFOLFOX6 | 14 | 12 | |
| FOLFIRI | 9 | 1 | |
| IRIS | 11 | 0 | |
| XELOX | 3 | 4 | |
| Capecitabine | 5 | 1 | |
| Other | 7 | 4 | |

Statistical analysis for single comparisons was performed using the two-tailed $\chi^2$ test or Fisher's exact test (expected frequency < 5). Comparisons between two groups were performed with the Mann–Whitney $U$-test. Staging was classified according the UICC TNM classification of malignant tumors. mFOLFOX6 comprised infusional 5-fluorouracil + $l$-leucovorin + oxaliplatin, FOLFIRI comprised infusional 5-fluorouracil + $l$-leucovorin + irinotecan, IRIS consisted of S-1 (an oral prodrug of 5-fluorouracil) + irinotecan, and XELOX consisted of capecitabine + oxaliplatin.

The comparative quantification cycle threshold ($C_q$) method was used to determine the relative expression levels of the target genes. $C_q$ values were calculated with the second derivative maximum method. GAPDH and RNU6B (U6) were analyzed as a reference gene for mRNA and microRNA, respectively [25, 26]. The cycle number difference ($\Delta C_q$ = reference genes − target genes) was calculated in each replicate. Relative target gene expression values were calculated using the mean of $\Delta C_q$ from the three replicates, that is, $\mu$ ($\Delta C_q$) = $\Sigma$ ($\Delta C_q$)/3, and expressed as $2^{\mu(\Delta C_q)}$ [27].

## Cell culture and transfection assays

The human colon adenocarcinoma cell lines, WiDr and COLO201 were grown in, DMEM and RPMI1640 medium, respectively, which was supplemented with 10% fetal bovine serum, 2 mmol/L-glutamine, and 100 units/mL of penicillin at 37°C in a 5% $CO_2$ humidified atmosphere. The synthetic microRNA mimic or siRNA were transfected using the Lipofectamine 2000 transfection agent according to the manufacturer's protocol. In 96-well plates, 3 pmol of the mimic or siRNA was transfected into 1 × $10^5$ cells/ml using 0.4 $\mu$L of Lipofectamine 2000, and cells were harvested 72 h later for RT-PCR and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [28].

## Statistical analysis

Comparisons between two groups were performed with the Mann–Whitney $U$-test. Comparisons between three groups were performed with the Tukey–Kramer test. Categorical variables were analyzed with the two-tailed $\chi^2$ test or Fisher's exact test (expected frequency < 5). Survival was plotted with Kaplan–Meier curves, taking the interval from the date of colorectal cancer to death or last contact. Comparisons between each group were performed with the log-rank test. OS and progression-free survival (PFS) were evaluated using the Cox proportional hazards model. The relationship was analyzed using univariate analysis. All indicated $P$-values are two-sided. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

# Results

## The relationship between microRNA and OS in patients with unresectable colorectal cancer

The relationship between plasma RNA expression levels and survival duration was evaluated. Higher plasma miR-124-5p expression levels (more than the median value)


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were correlated with a higher probability of OS (Fig. 1). Patient characteristics are shown in Table 2. No significant difference was observed between the two groups. Expression levels of RNAs were not observed between in the two groups (data not shown). Higher FFPE miR-124-5p expression levels were also significantly correlated with a higher probability of OS (Fig. 2). Patient characteristics are shown in Table 3, and no significant differences were observed between the two groups. Plasma miR-124-5p expression levels in the high group (divided by FFPE miR-124-5p expression) were significantly higher than those in the low group (Table 4). In the present study, there was a significant correlation coefficients between plasma miR-124-5p and FFPE miR-124-5p expression levels (Fig. 3. Univariate analysis: $r = 0.451$, 95% confidence interval: 0.189 − 0.654, $P = 0.002$).

Lower plasma miR-26a expression levels were correlated with a higher probability of OS (Fig. 4). Cox proportional hazards models also estimated a significant lower hazard ratio in the plasma miR-124-5p higher expression group and plasma miR-26a lower expression group (Table 5). The FFPE miR-124-5p higher expression group was not correlated with a lower hazard ratio of OS (Table 5). No significant relationship was observed between the expression levels of *EZH2*, *MFGE8*, and other miRs, OS, or PFS. The expression levels of miR-26a and miR-124-5p did not correlated with PFS or the hazard ratio (Table 5).

**Table 2.** Patient characteristics with grouping based on microRNA-124-5p expression levels in plasma.

| Characteristics | miR-124-5p low ($n$ = 24) | miR-124-5p high ($n$ = 25) | $P$ |
|---|---|---|---|
| Age | | | |
| Mean (SD) | 62.9 (14.5) | 63.0 (7.7) | 0.62 |
| Range | 30–83 | 48–78 | |
| Sex | | | 0.69 |
| Male | 16 | 18 | |
| Female | 8 | 7 | |
| Primary lesion | | | 0.11 |
| Colon | 6 | 13 | |
| Rectum | 13 | 7 | |
| Other | 5 | 5 | |
| Histology | | | 1.00 |
| Tubular adenocarcinoma | 22 | 22 | |
| Other | 2 | 3 | |
| Chemotherapy | | | 0.54 |
| mFOLFOX6 | 5 | 9 | |
| FOLFIRI | 3 | 6 | |
| IRIS | 6 | 5 | |
| XELOX | 2 | 1 | |
| Capecitabine | 3 | 2 | |
| Other | 5 | 2 | |

Statistical analysis for single comparisons was performed using the two-tailed $\chi^2$ test or Fisher's exact test (expected frequency < 5). Comparisons between two groups were performed with the Mann–Whitney $U$-test.

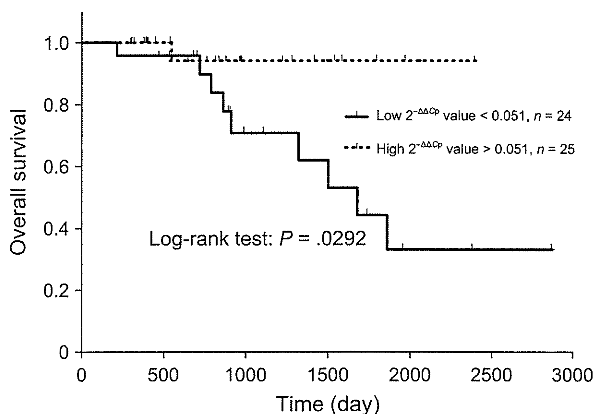


**Figure 1.** Kaplan–Meier OS curves for patients with unresectable colorectal cancer based on low and high plasma miR-124-5p expression levels. Kaplan–Meier plots showing estimates of overall survival (OS) probabilities grouped according to miR-124-5p expression levels in a completely independent set of colorectal cancer patients. The dotted line curve represents samples that expressed high levels of miR-124-5p (above median), whereas the black curve corresponds to samples that expressed low miR-124-5p levels (below median). Discontinuations of observations were indicated by spines on the lines. Comparisons between each group were performed with the log-rank test.

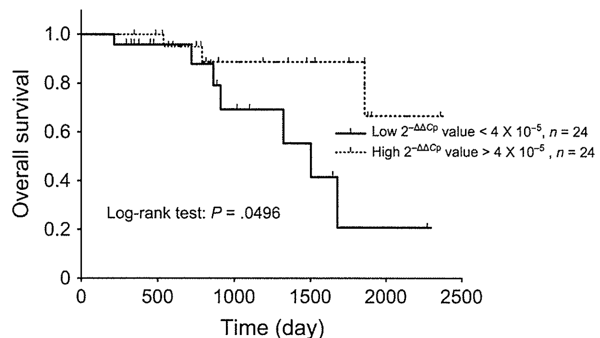


**Figure 2.** Kaplan–Meier overall survival (OS) curves for patients with unresectable colorectal cancer based on low and high miR-124-5p expression levels in formalin-fixed paraffin-embedded (FFPE) samples. FFPE samples were obtained from surgery or biopsy for histological diagnosis. Kaplan–Meier plots showing estimates of OS probabilities grouped according to miR-124-5p expression levels in a completely independent set of colorectal cancer patients. The dotted line curve represents samples that expressed high levels of miR-124-5p (above median), whereas the black line curve corresponds to samples that expressed low levels of miR-124-5p (below median). Discontinuations of observations were indicated by spines on the lines. Comparisons between each group were performed with the log-rank test.

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**Table 3.** Patient characteristics with grouping based on microRNA-124-5p expression levels in FFPE samples.

| Characteristics | miR-124-5p low ($n$ = 24) | miR-124-5p high ($n$ = 24) | $P$ |
|---|---|---|---|
| Age | | | |
| Mean (SD) | 63.1 (12.3) | 63.8 (9.9) | 0.82 |
| Range | 30–82 | 40–83 | |
| Sex | | | 0.35 |
| Male | 18 | 15 | |
| Female | 6 | 9 | |
| Primary lesion | | | 0.35 |
| Colon | 10 | 9 | |
| Rectum | 11 | 8 | |
| Other | 3 | 7 | |
| Histology | | | 0.35 |
| Tubular adenocarcinoma | 23 | 20 | |
| Other | 1 | 4 | |
| Chemotherapy | | | 0.74 |
| mFOLFOX6 | 8 | 5 | |
| FOLFIRI | 5 | 4 | |
| IRIS | 5 | 6 | |
| XELOX | 2 | 1 | |
| Capecitabine | 2 | 3 | |
| Other | 2 | 5 | |

Statistical analysis for single comparisons was performed using the two-tailed $\chi^2$ test or Fisher's exact test (expected frequency < 5). Comparisons between two groups were performed with the Mann–Whitney $U$-test. formalin-fixed paraffin-embedded.

**Table 4.** MicroRNA expression levels in plasma and FFPE samples, and microRNA 124-5p expression in FFPE samples.

| | Plasma microRNA ($\times 10^{-3}$) | | FFPE microRNA ($\times 10^{-5}$) | |
|---|---|---|---|---|
| | Low ($n$ = 24) | High ($n$ = 25) | Low ($n$ = 24) | High ($n$ = 24) |
| miR-16 | 363 ± 253 | 293 ± 177 | 94 ± 81 | 182 ± 153 |
| miR-21 | 85 ± 131 | 105 ± 64 | 581 ± 375 | 801 ± 597 |
| miR-26a | 432 ± 281 | 388 ± 224 | 184 ± 120 | 273 ± 201 |
| miR-34a | 119 ± 272 | 127 ± 183 | 301 ± 615 | 267 ± 239 |
| miR-98 | 36 ± 48 | 35 ± 43 | 6.4 ± 3.6 | 14.8 ± 20.8 |
| miR-101-3p | 58 ± 65 | 124 ± 138* | 86 ± 83 | 147 ± 164 |
| miR-101-5p | 65 ± 76 | 91 ± 75 | 3.3 ± 2.6 | 22.4 ± 32.9*** |
| miR-124-5p | 91 ± 152 | 158 ± 219* | 1.4 ± 1.2 | 11.8 ± 10.9*** |
| miR-126-3p | 122 ± 138 | 130 ± 124 | 551 ± 493 | 884 ± 814 |
| miR-126-5p | 207 ± 121 | 244 ± 167 | 180 ± 178 | 285 ± 291 |
| miR-210 | 394 ± 192 | 415 ± 230 | 12 ± 5 | 19 ± 14 |
| miR-217 | 20 ± 43 | 35 ± 37 | 0.21 ± 0.15 | 0.59 ± 0.52 |
| miR-630 | 94 ± 78 | 84 ± 58 | 2.6 ± 1.8 | 5.2 ± 8.7 |

The two groups was divided between median value expression of miR-124-5p in FFEP, high group corresponded to above median, low group corresponded to below median. Expression levels were calculated using the $2^{\Delta\Delta C_q)}$ $2^{-\Delta\Delta C_q}$ method. $\Delta C_q$ was defined as the mean $C_q$ value for a specific RNA in an individual sample. Each value indicates the mean ± standard deviation. Statistical analysis for single comparisons was performed using the Mann–Whitney $U$-test; FFPE, formalin-fixed paraffin-embedded.
*$P$ < 0.05; ***$P$ < 0.001.

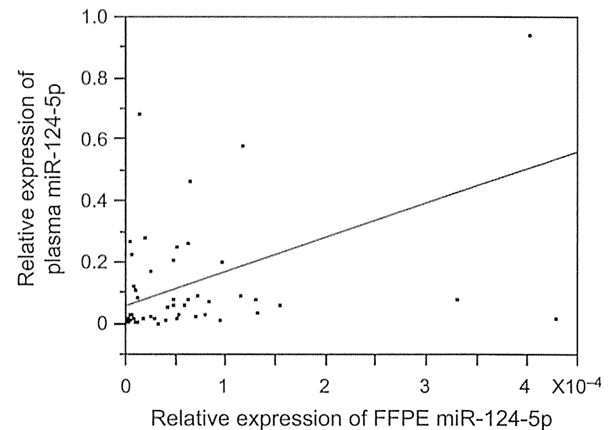


**Figure 3.** The correlation between expression levels of formalin-fixed paraffin-embedded (FFPE) miR-124-5p and plasma miR-124-5p. The relationship was analyzed by using univariate analysis, $r$ = 0.451, 95% confidence interval: 0.189 – 0.654, $P$ = 0.002. The plots from paired samples from the same patient and regression line were indicated.

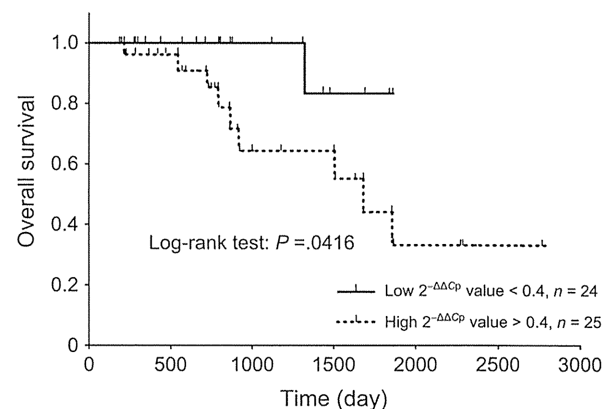


**Figure 4.** Kaplan–Meier overall survival (OS) curves for two groups defined based on low and high plasma miR-26a expression levels. Kaplan–Meier plots showing estimates of OS probabilities grouped according to miR-26a expression levels in a completely independent set of colorectal cancer patients. The dotted line curve represents samples that expressed high levels of miR-26a (above median), whereas the black line curve corresponds to samples that expressed low miR-26a levels (below median). Discontinuations of observations were indicated by spines on the lines. Comparisons between each group were performed with the log-rank test.

## Differences in plasma RNA expression levels between patients with unresectable and completely resected cancer

RNA expression levels in the plasma of unresectable and completely resected patients were evaluated. Patient characteristics are shown in Table 1. *MFGE8* expression levels in completely resected patients were significantly lower



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**Table 5.** Adjusted hazard ratios of patients with colorectal cancer in the high-expression group versus the low-expression group.

| | Hazard ratio (95% CI) | P |
|---|---|---|
| Plasma miR-124-5p | | |
| OS | 0.147 (0.008–0.789) | 0.022 |
| PFS | 0.624 (0.291–1.300) | 0.209 |
| FFPE miR-124-5p | | |
| OS | 0.281 (0.059–1.039) | 0.057 |
| PFS | 1.036 (0.490–2.252) | 0.926 |
| Plasma miR-26a | | |
| OS | 6.044 (1.097–112.5) | 0.037 |
| PFS | 1.262 (0.603–2.658) | 0.535 |

Values indicated hazard ratios and 95% confidence intervals. Statistical analysis was performed using the Cox proportional hazards model. FFPE, formalin-fixed paraffin-embedded; OS, overall survival; PFS, progression-free survival; 95% CI, 95% confidence interval.

than those in unresectable patients (Table 6). MiR-26a expression levels in completely resected patients were low, but were not significantly different from those in unresectable patients ($P = 0.08$; Table 6).

### MicroRNA-124-5p-targeted SMC4 and inhibited cell growth

The microRNA.org predicted that miR-124-5p may target *SMC4*. Transfection of the miR-124-5p mimic or siRNA was examined. Transfection of SMC4 siRNA into WiDr and COLO201 cells significantly downregulated the expression of *SMC4* mRNA. Transfection of the miR-124-5p mimic into WiDr and COLO201 cells also significantly downregulated the expression of *SMC4* mRNA (Fig. 5). Transfection of miR-124-5p mimic or SMC4 siRNA into WiDr and COLO201 cells reduced cell viability (Fig. 6).

## Discussion

*EZH2*, *MFGE8*, and miRs expression levels in plasma and FFPE samples, and OS in colorectal cancer patients were evaluated. Previous studies reported that EZH2 is a tumorigenic gene that correlates with cancer progression and a poor prognosis [4, 29]. In the present study, a correlation was not observed between OS and *EZH2* expression levels in unresectable patients. Although miR-101 was shown to negatively regulate the expression of *EZH2*, a correlation was not observed between the expression of *EZH2* and miR-101 in plasma and FFPE samples in the present study (data not shown). Plasma *EZH2* expression levels were higher in unresectable patients than completely resected patients, but this difference was not significant ($P = 0.4$). Recent studies demonstrated that MFGE8 correlated with tumor malignancy and microenvironment [6, 30, 31]. Plasma *MFGE8* expression levels were significantly higher in unresectable patients than in completely resected patients (Table 6). Previous studies reported that the expression of *MFGE8* was significantly higher in tumors than in normal tissues. Patients with primary tumors that expressed *MFGE8* had significantly shorter survival periods than those with primary tumors that did not express *MFGE8* [32]. The results of the present study suggest that *MFGE8* mRNA released into plasma from tumors can be used as a diagnostic biomarker.

There have been some reports microRNAs are released into blood from tumor cells. Skog et al. reported that tumor-derived microvesicles which contained mRNA, microRNA, and angiogenic proteins served as a means of delivering genetic information, to recipient cells in the tumor environment [22]. In the present study, there was a significant correlation coefficient between plasma miR-124-5p and FFPE miR-124-5p expression levels (Fig. 3). However, it was not clear whether miR-124-5p in plasma was derived from tumor.

Few studies have examined the function of miR-124-5p. Anwar et al. reported that the expression of miR-124-5p (miR-124*) was significantly higher in nonmethylated hepatocellular carcinoma than in methylated samples [21]. The present study is the first to examine the

**Table 6.** Expression levels of RNAs in plasma samples.

| | Unresectable ($n$ = 49) | Completely resected ($n$ = 22) | | Unresectable ($n$ = 49) | Completely resected ($n$ = 22) |
|---|---|---|---|---|---|
| miR-16 | 0.317 ± 0.202 | 0.256 ± 0.219 | miR-126-3p | 0.124 ± 0.129 | 0.104 ± 0.198 |
| miR-21 | 0.095 ± 0.101 | 0.064 ± 0.070 | miR-126-5p | 0.244 ± 0.176 | 0.161 ± 0.137 |
| miR-26a | 0.429 ± 0.264 | 0.304 ± 0.232 | miR-210 | 0.404 ± 0.208 | 0.396 ± 0.288 |
| miR-34a | 0.142 ± 0.267 | 0.242 ± 0.417 | miR-217 | 0.029 ± 0.042 | 0.036 ± 0.063 |
| miR-98 | 0.037 ± 0.045 | 0.039 ± 0.061 | miR-630 | 0.134 ± 0.313 | 0.072 ± 0.086 |
| miR-101-3p | 0.095 ± 0.113 | 0.140 ± 0.190 | *MFGE8* | 0.033 ± 0.076 | 0.014 ± 0.019* |
| miR-101-5p | 0.077 ± 0.075 | 0.144 ± 0.213 | *EZH2* | 0.021 ± 0.043 | 0.009 ± 0.015 |
| miR-124-5p | 0.123 ± 0.188 | 0.117 ± 0.174 | | | |

Expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method. $\Delta C_q$ was defined as the mean $C_q$ value for a specific RNA in an individual sample. Each value indicates the mean ± standard deviation. Statistical analysis for single comparisons was performed using the Mann–Whitney $U$-test.
*$P < 0.05$.

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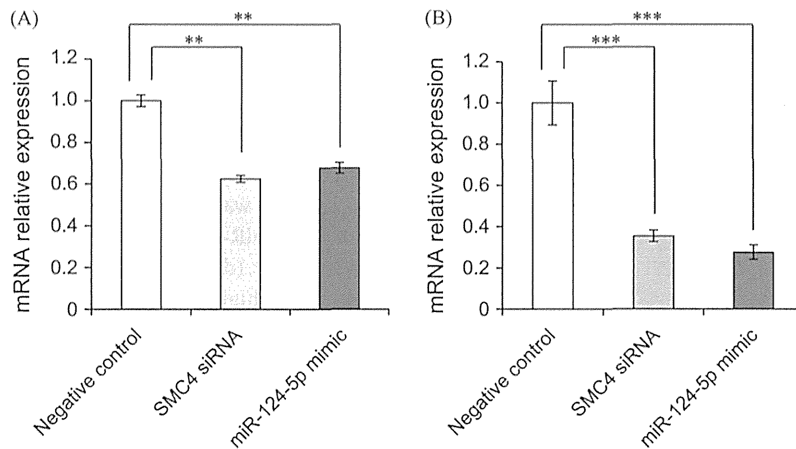


**Figure 5.** The miR-124-5p mimic inhibited *SMC4* mRNA expression in WiDr and COLO201 cells. The synthetic miR-124-5p microRNA mimic or SMC4 siRNA inhibited *SMC4* mRNA expression in WiDr (A) and COLO201 (B) cells. The synthetic microRNA mimic or siRNA were transfected using Lipofectamine 2000 transfection agent. Cells were harvested for 72 h. Values represent the relative ratio of target gene per *GAPDH*, mean ± SEM, to the control from eight independent experiments. The control group was transfected with negative control RNA (AllStars Negative Control siRNA, Qiagen). miR-124-5p: the mimic of miR-124-5p was transfected; SMC4 siRNA: synthetic SMC4 siRNA was transfected. Statistical analysis was performed using the Tukey–Kramer test; $^{**}P < 0.01$, $^{***}P < 0.001$.

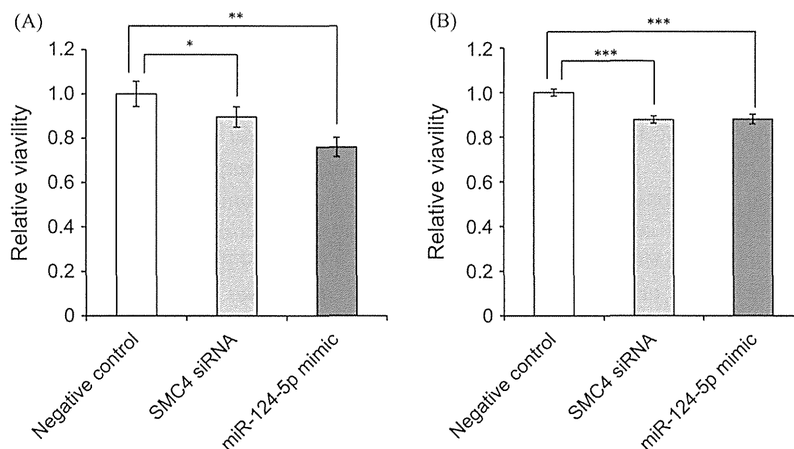


**Figure 6.** The miR-124-5p mimic inhibited cell viability in WiDr and COLO201 cells. The synthetic miR-124-5p microRNA mimic or SMC4 siRNA inhibited cell viability in WiDr (A) and COLO201 (B) cells. The synthetic microRNA mimic or siRNA were transfected using Lipofectamine 2000 transfection agent. Cells were harvested for 72 h. Cell viabilities were evaluated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which was described in detail in the method. Values represent the relative ratio of negative control treatment, mean ± SEM, to the control from eight independent experiments. The control group was transfected with negative control RNA (AllStars Negative Control siRNA, Qiagen). miR-124-5p: the mimic of miR-124-5p was transfected; SMC4 siRNA: synthetic SMC4 siRNA was transfected. Statistical analysis was performed using the Tukey–Kramer test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

relationship between the function of miR-124-5p and prognosis of patients with colorectal cancer. Wang et al. reported that the downregulation of miR-124 (miR-124-3p) correlated with a worse prognosis in patients with colorectal cancer [33]. These results confirmed the relationship between microRNA124-2, the gene of miR-124-5p, and miR-124-3p, and a worse prognosis.

The microRNA.org predicted that miR-124-5p may target *SMC4*. We examined the inhibitory effects of the miR-124-5p mimic on the expression of *SMC4* mRNA. Zhou et al. reported that the expression of *SMC4* was correlated with tumor size, de-differentiation, advanced stages, and vascular invasion of primary liver cancers, while the knockdown of *SMC4* expression reduced the


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proliferation of hepatocellular carcinoma cells [23]. Zhai et al. found that the knockdown of *SMC4* led to a chromosomal separation deficiency [34]. A previous study suggested that downregulation of *SMC4* may reduce cell proliferation. The knockdown of *SMC4* was shown to result in severe defects in chromosome assembly in HeLa Cells [35]. The present study demonstrated that miR-124-5p inhibited the tumorigenesis gene, *SMC4*, which upregulated the expression of miR-124-5p, thereby improving the OS of colorectal cancer patients.

The present study showed that lower miR-26a expression levels correlated with a higher probability of OS (Fig. 4). Qian et al. recently reported that miR-26a promoted tumor growth and angiogenesis in glioma [36]. The overexpression of miR-26a was shown to increase the proliferation of cholangiocarcinoma cells and colony formation in vitro [37]. These findings suggested that the upregulation of miR-26a may promote tumor growth and malignancy. The present study demonstrated that higher miR-26a expression levels were associated with a lower probability of OS, which indicated that miR-26a in plasma may be used as a biomarker to determine the prognosis of colorectal cancer patients.

In conclusion, the results of the present study demonstrated that *MFGE8* expression levels were significantly lower in completely resected patients than in unresectable patients. Furthermore, higher miR-26a expression levels, and lower miR-124-5p expression levels were associated with a lower probability of OS; therefore the expression of *MFGE8*, miR-26a and miR-124-5p in plasma may be used as biomarkers to determine the prognosis of colorectal cancer patients.

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## Conflict of Interest

None declared.

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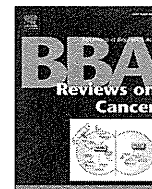

Review

# Tumor-associated macrophages as an emerging target against tumors: Creating a new path from bench to bedside

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ABSTRACT

Tumor-associated macrophages are a critical component of tumor microenvironments, which affect tumor growth, tumor angiogenesis, immune suppression, metastasis and chemoresistance. There is emerging evidence that many anticancer modalities currently used in the clinic have unique and distinct properties that modulate the recruitment, polarization and tumorigenic activities of macrophages in the tumor microenvironments. Educated tumor-associated macrophages significantly impact the clinical efficacies of and resistance to these anticancer modalities. Moreover, the development of drugs targeting tumor-associated macrophages, especially c-Fms kinase inhibitors and humanized antibodies targeting colony-stimulating factor-1 receptor, are in early clinical stages and show promising benefit for cancer patients. These experimental and clinical findings prompted us to further evaluate the potential targets that exhibit tumorigenic and immunosuppressive potential in a manner specific for tumor associated macrophages.

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## 1. Overview: phenotypic and functional characteristics of tumor-associated macrophages

Emerging evidence has revealed that tumor-infiltrating macrophages play a critical role in regulating tumor growth, progression and anticancer drug responses [1,2]. Although macrophages serve as a first-line of defense against pathogens and environmental insults through release of anti-microbe mediators such as proinflammatory cytokines, they also play an important role in fine-tuning inflammatory responses that are associated with tissue repair and remodeling processes [3]. The complexity of tissue environments may render macrophages, which already possess functional diversification and plasticity, able to acquire pro- and anti-inflammatory properties.

Tumor cells possess a high degree of genetic heterogeneity and form a complex "society", termed a "tumor microenvironment". Within the tumor microenvironment, various non-transformed cells such as fibroblasts, endothelial cells, and inflammatory cells as well as extracellular matrix components are densely packed and in communication with tumor cells and each other. Thus, the phenotypic and functional

*Abbreviations:* TAM, Tumor-associated macrophages; HIF, Hypoxia-inducible factor; miR, microRNA; MDSC, myeloid-derived suppressor cells; EGFR, epidermal growth factor receptor; FLT, fms-like tyrosine kinase; CSF-1, colony-stimulating factor-1.

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diversity of macrophages may be further expanded in the context of heterogeneous tumor microenvironments [4].

Accumulating evidence has revealed that macrophages differentiate predominantly into two major subsets depending on tissue microenvironments and/or inflammatory status; these subsets are referred to as pro-inflammatory M1-type or anti-inflammatory M2-type macrophages [5,6]. M1-type macrophages are differentiated via multiple transcription factors such as IRF-1, Stat1 and nuclear factor-kappaB (NF-κB), and amplify inflammatory responses [5,6]. M2-type macrophages are important in the regulation of tissue remodeling, repair, and antifungal immunity under physiological conditions. This subset is regulated through various transcription factors including IRF-4, Stat-6, PPAR-γ, TRIB1, and chromatin modifiers including the histone demethylase Jmjd3 [2,5].

Tumor-associated macrophages (TAM) have been defined as macrophages infiltrating tumor tissues or other tumor-enriched microenvironments (pleural or peritoneal effusions, etc.). TAM originate from bone marrow precursors, as well as circulating and splenic monocytes [7]. $CCR2^{+}Ly6C^{high}$ inflammatory monocytes are the main precursors of TAM recruited into tumor tissues by CCL2 [8,9]. Within tumor microenvironments, monocytes are induced to differentiate into "pro-tumor" macrophages through networks comprised of multiple soluble mediators, such as M-CSF, GM-CSF, and immunosuppressive cytokines like IL-4, IL-10 and TGF-β, etc. [10–12]. There is abundant evidence that TAM are able to polarize into immunosuppressive M2 macrophages upon exposure to M2 macrophage-differentiation factors produced by tumor microenvironments [12–16]. Furthermore, lactic acid released from tumor cells undergoing aerobic glycolysis drives differentiation of macrophages expressing high levels of VEGF-A and arginase-I, which contribute to supporting tumor growth [17]. On the other hand, it has been recognized that TAM are composed of heterogeneous subpopulations that possess high plasticity and flexibility enabling them to adapt to different tumor microenvironments [18,19]. Indeed, Li et al. proposed that TAM obtained from MMTV-PyMT breast tumor models displayed unique phenotypes characterized by a $CD11b^{low}$MHC class $II^{+}VCAM^{+}$ population and a genetic profile not shared with either M1 or M2 macrophages [20]. The divergent properties of TAM may reflect their interactions with tumor cells with heterogeneous oncogenic profiles, ultimately impacting tumorigenicity and responses to anticancer therapies.

In this review, we provide a general overview of the pro-tumor activities and the clinical impact of TAM, and the current status of TAM-targeting strategies in pre-clinical and clinical studies.

## 2. Mechanisms regulating pro-tumor properties of macrophages: new insights from the bench

Tumor microenvironments generate distinct sets of soluble factors that contribute to the recruitment of macrophage precursors and differentiation of pro-tumor and immunosuppressive macrophages. The interaction of angiotensin-II and S1P1, which occurs preferentially in tumors, amplifies the expansion of hematopoietic stem cells, which serve as the source of macrophage precursors infiltrating tumor tissues [21]. CCL2 serves as a major chemokine in promoting the infiltration of $CCR2^{+}Ly6C^{high}$ inflammatory monocytes into tumor tissues [8]. One of the mechanisms whereby CCL2 is highly induced in tumors results from commensal microbes and their inflammatory derivatives. These contribute to CCL2 induction in tumor tissues through prostaglandin E2 and TNFα/TNF receptor-mediated inflammatory cascades, which trigger the infiltration of $CCR2^{+}$ inflammatory monocytes into tumors [22]. IL-4 and IL-13, which are produced at high levels by tumor tissues, also contribute to the recruitment of $F4/80^{+}CD11b^{high}$ M2 macrophages into inflammatory colonic mucosa and promote the production of IL-6 and TGF-β. These latter factors trigger pro-tumorigenic phenotypes, through Myd88-dependent signaling cascades [23,24].

TAM also have the capacity to produce high levels of CCL18 and GM-CSF, which serve as critical regulators of the differentiation of pro-metastatic and immunosuppressive macrophages, respectively [25,26]. CCL18 and GM-CSF produced by TAM augment the pro-metastatic and immunosuppressive potential of breast tumor cells by inducing genetic programs that are associated with mesenchymal transition [9,27].

Phagocytic systems serve as important safeguards against inflammation and the disruption of tissue homeostasis. However, these systems are also manipulated by tumors to evade antitumor surveillance. The phagocytic receptor TIM-4 also mediates immune tolerance by activating autophagy and targeting the presentation of tumor-associated antigens by TAM. CD47 receptor, on the other hand, transduces "don't eat me" signals that protect tumor cells from engulfment by macrophages [28–30].

A critical role in the initiation of tumor cell metastasis involves the protease-mediated proteolytic activities of the tumor-associated matrix. In particular, the cathepsin protease family is upregulated in macrophages infiltrating murine and human breast tumor tissues after chemotherapy and contributes to the suppression of chemotherapy-mediated cytotoxicity [31,32]. In addition, 15-lipoxygenase-2 pathways and Wnt5a-mediated β-catenin signals serve as additional signaling components that support the immunosuppressive functions of TAM [33,34].

Recent evidence has also validated the roles of several transcription factors in regulating the recruitment and differentiation of macrophages in tumor tissues. TAM promote the transcriptional activities of hypoxia-inducible factor-1α (HIF-1α), which serves as an upstream regulator of arginase-I and VEGF-A. HIF-1α-mediates the induction of arginase-I and VEGF-A, which suppress the antitumor responses of cytotoxic T lymphocytes (CTL) and support tumor angiogenesis, respectively [35]. I-κB kinase-α (IKKα), which modulates NF-κB pathways through phosphorylation and degradation of the I-κBα protein, has recently emerged as a critical node in the control of inflammation and carcinogenesis [36, 37]. Recent studies have revealed that IKKα serves as a key transcription factor suppressing the recruitment of antitumor M1 macrophages into tumor tissues, whereas the NF-κB p50 element in macrophages inhibits polarization into the M1 phenotype [38,39]. Thus, multiple transcriptional and soluble networks, within the heterogeneous tumor microenvironments may be critical elements in controlling the recruitment of pro- and anti-tumor macrophages into tumor tissues.

MicroRNA-mediated regulation of TAM has emerged as one of the hallmarks of TAM-associated tumorigenicity [40]. For example, the miR-511-3p is preferentially expressed on mannose receptor $CD206^{+}$ TAM and serves as a pivotal regulator of their tumorigenic actions by targeting the 3′ UTR of multiple genes including rho-dependent kinase-2 (ROCK2) [41]. Moreover, CUE domain-containing protein CUEDC2 promotes tumoricidal macrophage differentiation by triggering pro-inflammatory cytokine expression on monocytes. In addition, IL-4-mediated upregulation of miR-324-5p down-regulates CUEDC2 expression on TAM [42]. The miR-126/miR-126* complex directly inhibits SCF-1α mRNA expression. SCF-1α is an upstream regulator of CCL2 in breast cancer cells and the impaired SDF-1α-CCL2 axis leads to suppression of $CCR2^{+}$ monocyte recruitment into tumor tissues and inhibits tumor metastasis [43]. The miR-142-3p represses gp130 and the LAP*isoform of C/EBPβ, which are critical for generating pro-tumor M2 macrophages through regulation of TGF-β signals [44,45]. Other microRNA families, including miR-19a-3p, miR-17 and miR-30e, etc. are also involved in the regulation of TAM differentiation and function by the targeting of various oncogenic and angiogenic factors [39]. Thus, tumor microenvironments adopt multiple strategies to counter the microRNA-mediated inhibition of macrophage recruitment and differentiation.

Taken together, the above data demonstrate that microRNAs regulated by tumor microenvironments play a critical role in supporting the tumorigenic and immunoregulatory activities of macrophages (Fig. 1).

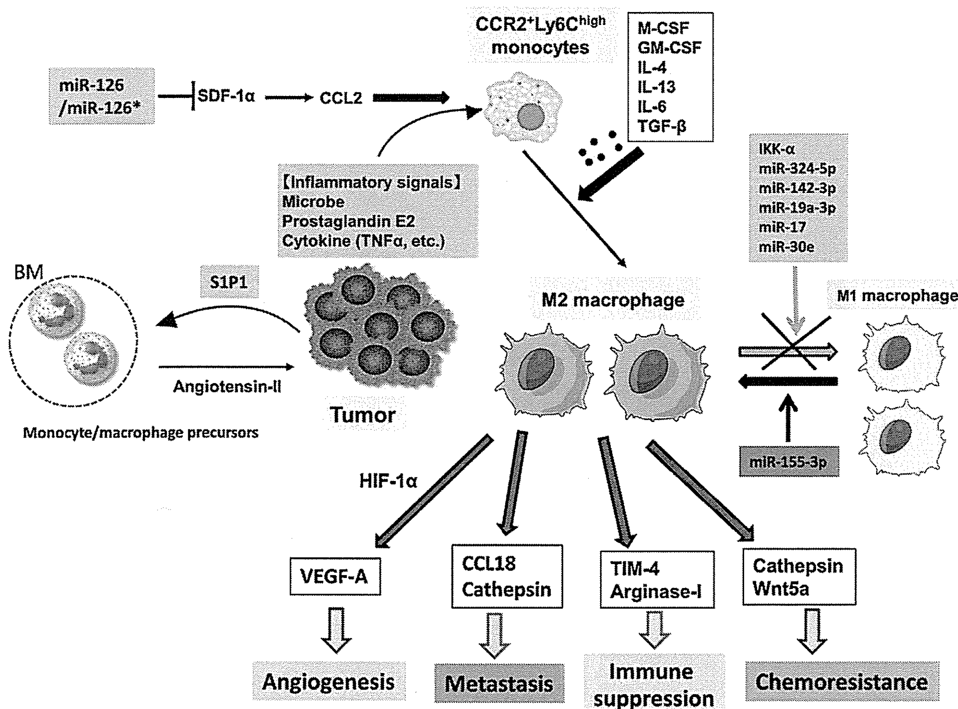


**Fig. 1.** Pathways regulating recruitment, differentiation and pro-tumor activities of TAM. Tumor microenvironments directly recruit monocytic precursors in bone marrow through S1P1 and angiotensin-II interaction in some cases. However, circulating and splenic CCR2$^+$Ly6C$^{high}$ inflammatory monocytes serve as a major reservoir of tumor-infiltrating macrophages, which are recruited into tumor tissues by tumor-derived CCL2 and SCF-1 whose expression is controlled by inflammatory mediators and the miR-126/miR-126* pair. The monocyte precursors are differentiated into M2 macrophages by soluble mediators (M-CSF, GM-CSF, IL-4, IL-13, etc.) delivered from tumor microenvironments. The tumor-mediated regulation of microRNA expression profiles also plays a critical role in directing macrophages toward M2 polarization. Tumor-associated macrophages then exert diverse arrays of protumorigenic activities through various effector molecules: VEGF-A mediates tumor angiogenesis, CCL18 and cathepsin support invasive and metastatic potentials, TIM-4 and arginase-I contribute to immunosuppression and cathepsin and Wnt5a suppress therapeutic responses to chemotherapy. Strategies targeting these pathways may offer new opportunities to reverse the pro-tumor activities of macrophages.

## 3. Clinical evidence of TAM-mediated tumor progression

Emerging evidence has revealed the importance of TAM derived from patient materials in predicting poor prognosis in many hematologic and solid tumors [46]. In solid tumors, TAM are detected mainly as a stromal component within the invasive front along with cancer-associated fibroblasts. In hematologic tumors, including gliomas and lymphomas, TAM serve as the main component of the tumor microenvironment and the tumor infiltration of CD68$^+$ macrophages is a sign of poor prognosis in patients with Hodgkin lymphoma [47] (Fig. 2). CD163 and CD204 are markers for the pro-tumor M2 phenotype in studies using human materials. Based on statistical analysis using clinical data related to survival rates or survival times, high numbers of CD163- or CD204-positive cells within the TAM infiltration closely correlate with tumor progression and a worse clinical prognosis [6,48].

In some malignant tumors, the number of infiltrating TAM is associated with the Ki-67 labeling index, which reflects tumor cell proliferation [49]. In in vitro studies using glioma and lymphoma cell lines, the proliferation of tumor cells was induced significantly by direct contact with macrophages [49,50]. TNF-α, I-309, GRO-α, IL-6, and C5a in addition to EGF, bFGF, and PDGF secreted from activated macrophages contribute to tumor cell proliferation [50]. CD163 and CD204 are scavenger receptors, and both antigens are specifically expressed on monocytes and macrophages. The ligands of CD163 are hemoglobin-haptoglobin complex and bacterial components [6]. CD163 activation and phosphorylation of the cytoplasmic portion of CD163 are linked to the activation of casein kinase II and protein kinase C [51]. CD204 recognizes various negatively charged macromolecules and is involved in cell adhesion and host defense [6]. CD204 suppresses the inflammatory responses of macrophages by competitively binding TLR4-ligands [6]. Although further studies are necessary to delineate scavenger receptor-mediate macrophage activation, one possibility is that scavenger receptors might be involved in cell–cell interactions between macrophages and tumor cells.

Some studies using human surgical specimens have demonstrated that the number of TAM also positively correlates with angiogenesis and the number of regulatory T cells present [52]. TAM produce VEGF, IL-8, bFGF, thymidine phosphorylate and MMPs, which are associated with various tumorigenic potentiators, such as angiogenesis, tumor inflammation and metastasis. TAM also produce various immunosuppressive factors, including $PGE_2$, IDO and IL-10 and contribute to immunosuppression [6]. TAM may also be involved in the maintenance of tumor cell stemness and are associated with resistance to chemotherapy [53,54]. An in vitro study using tumor cell lines and primary macrophages showed that co-culture with macrophages induces Stat3 activation in co-cultured tumor cells [6]. Since Stat3 serves as a critical regulator of cancer stem cell functions, TAM may influence the maintenance of stem-like states and chemoresistance via Stat3 activation, which is induced by cell–cell interactions between tumor cells and TAM.

## 4. Anticancer strategies influencing the tumorigenic activities of macrophages

Although tumor cells are the major targets for most anticancer therapeutics, compelling evidence exists that tumor microenvironments play a critical role in regulating responses to anticancer therapies. In particular, TAM serve as the main players for impeding the therapeutic efficacy of various anticancer agents, including cytotoxic chemotherapy, radiotherapy and molecular targeting therapies [55,56].

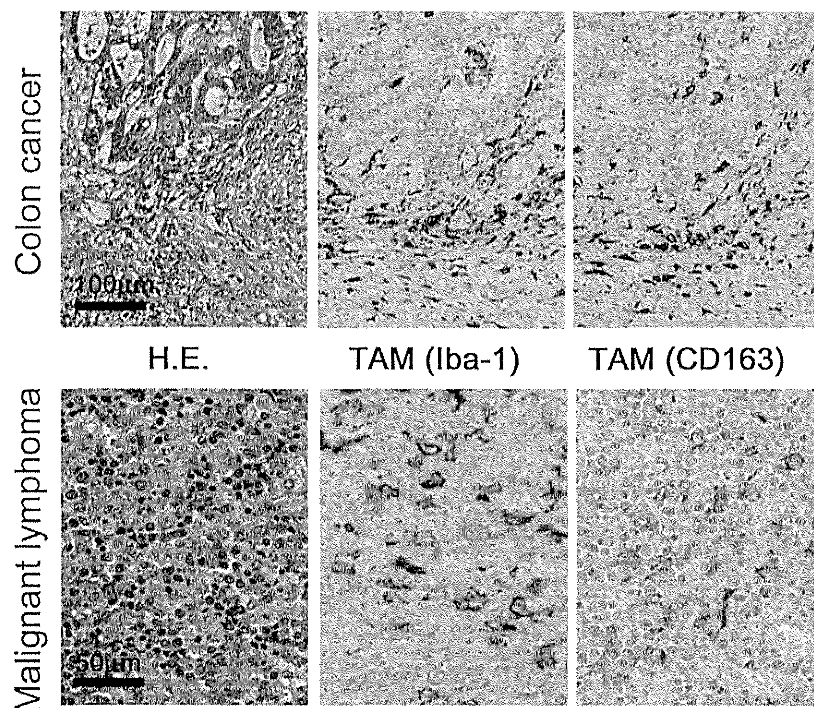


**Fig. 2.** Distribution of TAM in human malignant tumors. Macrophages are mainly detected in the cancer invasive front in colon tumor tissue. In malignant lymphoma, TAM and lymphoma cells are mixed within tissues. Iba-1 and CD163 are used as markers for total TAM or the pro-tumor M2 phenotypes, respectively.

Certain anticancer treatments elicit therapeutic responses by manipulating the infiltration and numbers of macrophages in tumor tissues. Various types of anticancer therapies, such as cisplatin, paclitaxel, and ionizing radiation, trigger recruitment of macrophages into tumors by inducing CSF1 and IL-34 expression [57]. Allavena et al. demonstrated that trabectidin (ET-743), an anticancer agent approved for late-stage soft-tissue sarcoma, exerts antitumor responses by depleting monocytes and macrophages. In addition, certain sets of cytotoxic agents, such as 5-FU, taxane and docetaxel, are proposed to have unique properties of selectively depleting M2 macrophages and myeloid-derived suppressor cells (MDSC), while assisting the survival and increasing the intra-tumor numbers of M1 macrophages [58–60]. These findings suggest that distinct classes of anticancer agents regulate recruitment and optimize the quantity of TAM through divergent processes, although the molecular mechanisms of chemotherapy-mediated regulation of TAM remain largely unclear.

Ample evidence exists that subsets of anticancer drugs have distinct properties driving macrophages toward anti-tumor subsets within the tumor microenvironments. Low-dose irradiation elicits antitumor responses at least in part through the recruitment and differentiation of inducible nitric oxide synthase (iNOS) $^{+}$ M1 macrophages in tumors, which is critical in stimulating infiltration and activation of tumor-specific T lymphocytes [61]. Moreover, antigen-targeting antibodies manifest modulating effects on the functional properties of TAM. For example, the numbers of TAM provide a better prognostic value in patients with non-Hodgkin lymphoma who receive the anti-CD20 mAb rituximab and chemotherapy [62]. Moreover, treatment with anti-CD40 agonistic mAb augmented the antitumor activities of gemcitabine by eliciting the infiltration of M1$^{+}$ macrophage into tumor tissues and enhancing antitumor immune responses in patients with pancreatic cancer [63,64]. Thus, the antitumor machineries exploited by several immunotherapies and by irradiation might rely on the polarization of tumoricidal macrophages in tumor microenvironments. Some chemotherapeutic agents, such as oxaliplatin and doxorubicin, exploit the process of "immunogenic cell death" (ICD) for tumor cells, leading to the release of inflammatory mediators recognized by pattern-recognition receptors and activating antigen-presenting cells [65,66]. Indeed, clinical responses to anthracyclin and radiotherapy are significantly impaired in patients with advanced breast cancer who possess a loss-of-function allele of TLR-4, as TLR-4 is critical in exploiting ICD-meditated antitumor immunity [67]. Thus, it is likely that the increased immunogenicity mediated by ICD-inducing drugs may have an impact on restraining the tumorigenic status of TAM.

On the other hand, recent results indicate that several anticancer agents generate pro-tumor macrophages. The KIT oncogene inhibitor Imatinib has the characteristic property of driving polarization of M2 macrophages via the C/EBP transcription factor induced by apoptotic tumor cells [68]. A recent randomized phase III clinical trial (CAIRO2 study) demonstrated that the addition of cetuximab to chemotherapy had a detrimental effect on overall survival compared to chemotherapy alone in patients with metastatic colorectal cancer [69]. Furthermore, the frequency of CD163$^{+}$ M2 macrophages is increased in tumor tissues in patients with advanced colorectal carcinomas after treatment with cetuximab mAb, which targets epidermal growth factor receptor (EGFR) [70]; this finding provides a potential mechanism whereby EGFR inhibitors reduce the clinical efficacy of chemotherapy. This suggests that cetuximab may antagonize the EGFR-mediated differentiation of antitumor macrophages, which negatively impacts the clinical courses of patients with colorectal cancers.

The mTOR pathway also positively regulates M2 macrophage polarization and the mTOR inhibitor rapamycin causes monocytes to develop into IL-12$^{high}$IL10$^{low}$ M1 macrophages, which exhibit antitumor immunity and anti-angiogenic activities in murine tumor models [71]. In addition, TAM contribute to negative regulation of the antitumor efficacy of the multi-kinase inhibitor sorafenib and anti-VEGF-A mAb by interfering with their anti-angiogenic actions. In contrast, others propose that sorafenib triggers pro-inflammatory TAM and activates NK cell-mediated antitumor responses [72,73]. These results suggest that the status of different tumor environments may exert distinct influences on the tumorigenic and immunogenic properties of TAM modified by multi-kinase inhibitors.

In summary, different types of anticancer therapies have unique propensities for directing monocytes and macrophages into different phenotypic and functional subsets in tumor microenvironments (Fig. 3). Moreover, the comprehensive analysis of TAM in patients receiving anticancer therapies may offer useful information for selecting appropriate anticancer agents that preferentially activate the antitumor properties of TAM. A deep understanding of the macrophage-modulating effects of each anticancer therapy will optimize the combinations of drugs according to TAM-modulating effects, which may be a rational strategy with which to improve clinical efficacy.

## 5. Clinical development of antitumor therapies targeting TAM

Emerging evidence has validated the concept that inhibition of key signaling pathways critical for the survival and functioning of TAM could elicit potent antitumor activities in preclinical tumor models and cancer patients. In particular, c-Fms kinase serves as an indispensable node controlling the survival and differentiation of TAM [4]. The bcr-abl and c-kit kinase inhibitors (imatinib, dasatinib, etc.) and the multi-kinase inhibitor sunitinib represent clinically approved anticancer agents that also target c-Fms kinase and are proposed to modulate the tumorigenic and immunosuppressive functions of TAM [74–76]. PLX3397 (Plexxikon) was originally developed as a selective FLT3 inhibitor for hematological malignancies but it functions as a multi-kinase inhibitor targeting CSF1 receptor-associated kinases and c-KIT [77,55]. Treatment with PLX3397 elicited clinical benefit for patients with pigmented villonodular synovitis (PVNS), which is characterized by high levels of CSF1R-expressing tumor cells [78]. The main antitumor actions of PLX3397 result from the reduced survival of tumor-infiltrating M2 macrophages and increased infiltration of antitumor CTL in patients with advanced breast cancers [55]. Furthermore, PLX3397 augmented the therapeutic efficacies of various anticancer therapies such as rapamycin, imatinib and adoptive T cell transfer [79–81]. These preclinical and clinical manifestations of CSF1 receptor-targeting therapy further validate the importance of TAM in the regulation of tumorigenicity and resistance to anticancer drugs. However, there are concerns that imatinib and other multi-kinase inhibitors may be deficient in maintaining the survival and activities of antitumor macrophages, since some molecular targeting therapies might be beneficial by antagonizing tumorigenic subsets while maintaining tumoricidal and immunogenic subsets of TAM [70]. Thus, it is critical to develop drugs specifically targeting key signaling nodes or downstream effectors for tumorigenic macrophages. In this regard, recent development of the selective c-Fms kinase inhibitor, BLZ945 (Novartis), may provide a proof-of-concept that strategies specifically targeting TAM are feasible options for treating cancer patients. BLZ945 is a selective CSF-1R inhibitor with an IC50 of 1 nM and is over 1000-fold more selective against the closest receptor tyrosine kinase homologs, c-Kit and Platelet-Derived Growth Factor Receptor (PDGFR). The preclinical studies have shown that BLZ945 treatment produced potent antitumor activities in various types of malignancies, including proneural glioblastoma models, MMTV-PyMT breast carcinoma and K14-HPV-16 cervical carcinoma models [82]. BLZ945 had little effect in promoting TAM survival, but converted the tumorigenic macrophages with M2 phenotypes into GM-$CSF^{+}IFN\text{-}\gamma^{+}$ macrophages [83]. The BLZ945-primed macrophages create an immunogenic antitumor milieu as manifested by increased activation of tumor-specific $CD8^{+}T$ cells and their infiltration into tumor tissues [83].

Preclinical animal studies have demonstrated the antitumor and anti-metastatic activities of an anti-CSF1 receptor mAb against

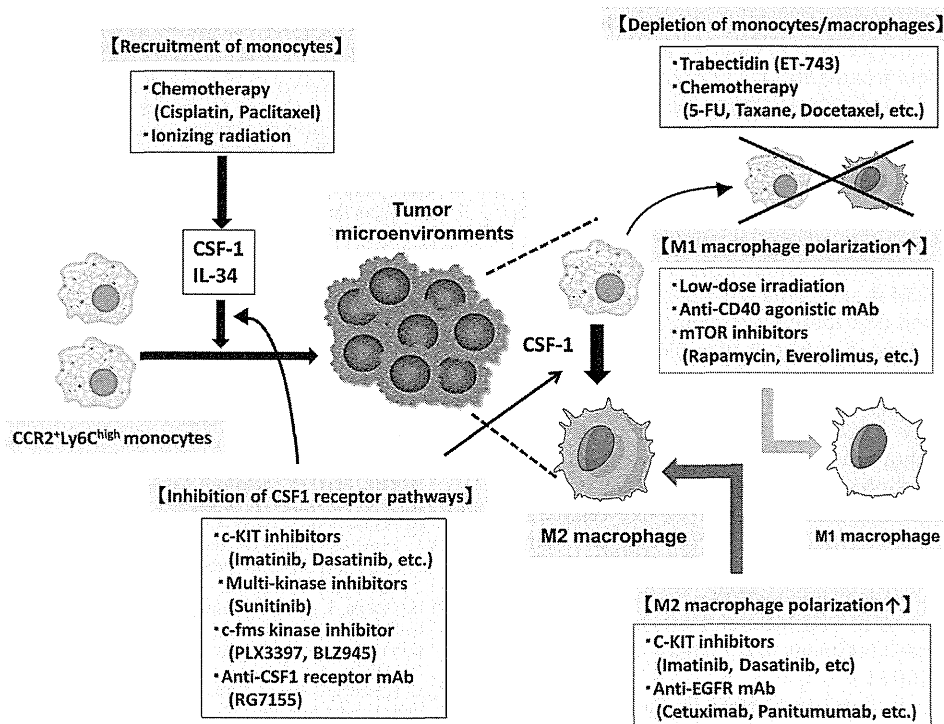


**Fig. 3.** Anticancer strategies influencing the tumorigenic and immunosuppressive activities of tumor-associated macrophages. Chemotherapeutic agents impact the tumorigenic activities of macrophages by regulating recruitment of monocytes into tumor tissues (cisplatin and paclitaxel) and controlling the numbers of TAM (5-FU, taxane, docetaxel). Irradiation also regulates the monocyte infiltration of tumors by promoting CSF-1 and IL-34 expression. Anticancer modalities have a peculiar effect on the polarization of M1/M2 macrophages in tumor microenvironments. Low-dose irradiation, anti-CD40 mAb, and mTOR inhibitors trigger antitumor responses in part through the differentiation of M1 type proinflammatory macrophages from TAM, whereas c-KIT inhibitors and anti-EGFR mAbs support the polarization of M2 type immunosuppressive subsets. The CSF1 receptor-mediated pathway serves as a key player for generating tumorigenic macrophages, and recent clinical studies have revealed the therapeutic efficacy of CSF1 receptor inhibitors against various human cancers, stimulating further development of TAM-targeting strategies.

subcutaneous EL4 lymphoma models and MMTV-PyMT breast tumor models [84]. In line with this potential clinical utility, the RG7155 antibody has been developed as a humanized anti-human CSF1 receptor mAb that specifically inhibits dimerization of human and cynomolgus CSF1 receptors (Roche). RG7155 specifically depletes CSF1 receptor $^{+}$CD163$^{+}$ M2 macrophages and augments T cell-mediated antitumor immune responses in tumors of various cancer patients. More importantly, RG7155 elicits potent antitumor immunity and offers a durable clinical benefit for patients with diffuse-type giant cell tumors [85].

Given these findings, many pharmaceutical companies are now focusing on the development of therapeutic tools targeting TAM, and early clinical studies have validated the clinical utilities of these drugs, in particular CSF1 receptor inhibitors, against various types of human malignancies. These clinical observations will encourage further studies on the subject of the application of CSF1 receptor inhibitors for diverse types of malignancies. In addition, it is critical to pursue more suitable combination strategies to improve clinical outcomes in the future.

## 6. Future perspectives: creating a path for the development of TAM-specific drugs

The recent clinical success of CSF1 receptor inhibitors for human malignancies should stimulate the development of TAM-targeting strategies in the future. However, inhibition of the CSF1 receptor may cause severe adverse events, such as opportunistic infections and delayed tissue repair, since CSF1 and IL-34 are indispensable for macrophages in maintaining normal homeostasis and defending against pathogens [86,87]. In this regard, it is necessary to focus the development of drugs on the targeting of molecules expressed specifically on TAM. These "TAM-specific therapies" may be a suitable option for further increasing the specificity and reducing the toxicity of macrophage-targeted drugs. As shown above, there are multiple sets of molecules that target the recruitment of inflammatory monocytes and/or polarization to M2 macrophages in tumor microenvironments. For example, inhibitors of angiotensin-II, CCL2, IL-13 and prostaglandin E2 may be useful for impeding the generation and recruitment of CCR2$^{+}$ monocytes into tumor tissues [6,20–22]. Furthermore, the therapeutic strategies targeting IL-6, TGF-β and cancer-associated metabolites such as lactic acid, may reverse the M2 differentiation of TAM [88,89]. A CD47 blocking mAb exhibits strong antitumor responses by promoting phagocytosis of viable tumor cells by macrophages, whereas inhibition of TIM-4 augments antitumor immunity by preventing TAM-mediated degradation of tumor-associated antigens [28–30]. Drugs targeting distinct sets of miRNA, such as miR-115-3p and miR-324-5p, which have regulatory roles for the protumor and immune regulatory activities of TAM, should serve as next-generation agents for remodeling the functional propensities of TAM to create antitumor environments [38]. In addition, the clinical efficacies of TAM-targeting therapies should be improved by optimizing appropriate combinations with conventional anticancer agents, which have alternative properties that modulate the phenotypic and functional status of macrophages. In turn, conventional anticancer agents may augment the clinical efficacy of TAM-targeting therapies, since suitable combinations of chemotherapy and antibody-based regimens may increase the immunogenic potentials of TAM [90,91].

Altogether, there are a wealth of opportunities for the development of new types of anticancer agents whose major activities are focused on the modulation of macrophages in tumor microenvironments. A more detailed analysis and deeper understanding of molecular machineries whereby tumor microenvironments regulate the functional plasticity of TAM should provide useful insights into the development of new therapeutic approaches for specifically targeting the tumorigenic and immunosuppressive subtypes of TAM in the future.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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