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MicroRNA *let-7*, T cells, and patient survival in colorectal cancer

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Use of standardized official symbols: We use HUGO (Human Genome Organisation)-approved official symbols for genes and gene products, including BRAF, CACNA1G, CD3, CD8, CD274, CDKN2A, CRABP1, CTLA4, FOXP3, IGF2, KRAS, MIRLET7A1, MIRLET7A2, MIRLET7A3, MIRLET7B, MLH1, MTOR, NEUROG1, NFKB1, PDCD1, PIK3CA, PTPRC, RNU6-2, RUNX3, SOCS1, and TLR4; all of which are described at www.genenames.org. Gene names are italicized, and gene product names are non-italicized.

Abstract

Experimental evidence suggests that the *let-7* family of noncoding RNAs suppresses adaptive immune responses, contributing to immune evasion by the tumor. We hypothesized that the amount of *let-7a* and *let-7b* expression in colorectal carcinoma might be associated with limited T-lymphocyte infiltrates in the tumor microenvironment and worse clinical outcome. Utilizing the molecular pathological epidemiology resources of 795 rectal and colon cancers in two U.S.-nationwide prospective cohort studies, we measured tumor-associated *let-7a* and *let-7b* expression levels by quantitative reverse-transcription PCR, and $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, and $FOXP3^+$ cell densities by tumor tissue microarray immunohistochemistry and computer-assisted image analysis. Logistic regression analysis and Cox proportional hazards regression were used to assess associations of *let-7a* (and *let-7b*) expression (quartile predictor variables) with T-cell densities (binary outcome variables) and mortality, respectively, controlling for tumor molecular features, including microsatellite instability, CpG island methylator phenotype, LINE-1 methylation, and *KRAS*, *BRAF*, and *PIK3CA* mutations. Compared with cases in the lowest quartile of *let-7a* expression, those in the highest quartile were associated with lower densities of $CD3^+$ [multivariate odds ratio (OR), 0.40; 95% confidence interval (CI), 0.23 to 0.67; P_{trend} = 0.003] and $CD45RO^+$ cells (multivariate OR, 0.31; 95% CI, 0.17 to 0.58; P_{trend} = 0.0004), and higher colorectal cancer-specific mortality (multivariate hazard ratio, 1.82; 95% CI, 1.42 to 3.13; P_{trend} = 0.001). In contrast, *let-7b* expression was not significantly associated with T-cell density or colorectal cancer prognosis. Our data support the role of *let-7a* in suppressing antitumor immunity in colorectal cancer, and suggest *let-7a* as a potential target of immunotherapy.

Keywords

colorectum; epigenetics; molecular pathological epidemiology; noncoding RNA

INTRODUCTION

Accumulating evidence indicates that tumor molecular pathologic changes elicit immune reactions and that immune cells in the tumor microenvironment influence tumor evolution (1–3). Hence, tumor cells need to evade antitumor immune response. In colorectal cancer, the degree of infiltration of T lymphocytes, such as $CD8^+$ cytotoxic T cells and CD45RO $(PTPRC)^+$ effector memory T cells, has been associated with high-level microsatellite instability (MSI-high) (4,5) and favorable prognosis (5–7). Therapeutic antibodies against immune checkpoint molecules, including CTLA-4, PDCD1 (programmed cell death 1; PD-1), and CD274 (PDCD1 ligand 1; PD-L1), can enhance antitumor T-cell activity and improve clinical outcome in various tumor types (8). Immune checkpoint blockade has been found to be effective in the MSI-high subtype of colorectal cancer (9). MSI-high colorectal cancers generate immunogenic neoantigens that can elicit lymphocytic immune response (10), resulting in upregulation of multiple immune checkpoint pathways (11). Beyond MSI status and neoantigen load, it is of interest to identify other factors that influence immune cell infiltration in colorectal cancer.

MicroRNAs are small noncoding RNAs that epigenetically regulate diverse biological and pathological processes, including immune response and tumor progression (12). It has been

shown that synthetic oligonucleotides can be delivered into cells or tissues to modulate the function of endogenous microRNAs, and small-molecules may modify the expression or function of microRNAs (13). Through a novel delivery system that can target the tumor microenvironment, antisense oligomers have been shown to reduce tumor volumes of *miR155*-addicted lymphoma in mice, demonstrating the therapeutic potential of microRNA targeting (14). Our group has shown that tumor expression of microRNA *MIR21* is inversely associated with densities of $CD3^+$ and $CD45RO^+$ T cells in colorectal cancer tissue (15). In addition, the *let-7* microRNA family has been shown to play an important role in host immunity (16–18). Experimental evidence suggests that *let-7* suppresses TLR4-mediated immune activation during infection, and that downregulation of *let-7* expression restores immune response against pathogens (16,17). In addition, decreased *let-7* expression is associated with the self-renewal of memory T cells (18). Although tumor-associated *let-7* expression has been associated with patient survival in lung (19), ovarian (20,21), and other cancers, its prognostic association in colorectal cancer remains unclear. In human, *let-7a* and *let-7b* together account for the vast majority of *let-7* RNA molecules present in colonic epithelia (22). Therefore, we hypothesized that *let-7a* or *let-7b* expression might be associated with a lower density of T cells in colorectal cancer tissue and worse clinical outcome.

To test our hypotheses, we examined *let-7a* and *let-7b* expression in colorectal cancer tissues from two U.S.-nationwide prospective cohort studies, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS), in relation to $CD3^+$, $CD8^+$, $CD45RO^+$, and $FOXP3^+$ T-cell densities in tumor tissue, and colorectal cancer mortality.

METHODS

Study population

We utilized the databases of two U.S.-nationwide prospective cohort studies, the NHS (121,701 women followed since 1976) and the HPFS (51,529 men followed since 1986) (23,24). In both cohorts, follow-up questionnaires were sent at baseline and biennially thereafter to collect and update lifestyle and health-related information, and to identify newly diagnosed diseases. Study physicians reviewed medical records and assigned causes of death. The National Death Index was used to ascertain deaths of study participants and identify unreported fatal colorectal cancer cases. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals where participants with colorectal cancer had undergone tumor resection. Hematoxylin and eosin-stained tissue sections of all colorectal cancer cases were reviewed by a pathologist (S.O.), who was unaware of other data. Tumor differentiation was categorized as well, as moderate vs. poor (>50% vs. ≤50% glandular area). Based on the availability of follow-up data and adequate tissue specimens for analysis, 795 colorectal cancer cases (diagnosed up to 2008) were included. Written informed consent was obtained from all study participants. Tumor tissue was macro-dissected from FFPE specimens in all cases. Tissue collection and analyses were approved by the human subjects committee at the Harvard T.H. Chan School of Public Health and the Brigham and Women's Hospital (Boston, MA, USA).

Quantitative reverse-transcription PCR for *let-7a* and *let-7b*

RNA was extracted with RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Austin, TX), and cDNA was synthesized with miScript II RT Kit (Qiagen, Valencia, CA). Quantitative PCR was performed using the miScript PCR System (Qiagen) with specific miScript Primer Assays for *let-7a* (catalog number, MS00031220), *let-7b* (catalog number, MS00003122), and *RNU6-2* (catalog number MS00033740). Amplification was performed in duplicate with the StepOnePlus Real-Time PCR Systems (Applied Biosystems) under the following conditions: 15 minutes at 95°C and 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 70°C. Spearman's rank-correlation coefficient between the duplicate cycle threshold (Ct) values was 0.99 for *let-7a*, 0.97 for *let-7b*, and 0.98 for *RNU6-2*.

In our validation study, the Ct values for *let-7a*, *let-7b*, and *RNU6-2* increased linearly with serial 10-fold dilution of cDNA from the same tumor specimens ($R^2 \geq 0.98$; Supplementary Fig. S1). The interassay coefficient of variation of Ct values from the same tumor specimens in four different batches was 1.2% for *let-7a*, 1.4% for *let-7b*, and 1.0% for *RNU6-2* (Supplementary Table S1). *let-7a* and *let-7b* expression was normalized with *RNU6-2* using the $2^{-\Delta Ct}$ method (where ΔCt = "the average Ct value of *let-7a* or *let-7b*" – "the average Ct value of *RNU6-2*") (25).

Analysis of T-cell densities in tumors

Tissue microarray was constructed to assess the densities of $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, and $FOXP3^+$ T cells in tumors with immunohistochemistry techniques (5). We used an automated scanning microscope and the Ariol image analysis system (Genetix, San Jose, CA) to quantify T-cell density in tissue microarray cores as previously described (5). The median value was used to dichotomize the density of $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ T cells.

Analyses of *BRAF*, *KRAS*, and *PIK3CA* mutations and other molecular features

DNA was extracted from archival FFPE cancer tissue blocks. PCR and pyrosequencing for *BRAF* (codon 600) (26), *KRAS* (codons 12, 13, 61 and 146) (27,28), and *PIK3CA* (exons 9 and 20) (29) were performed as previously described. Microsatellite instability (MSI) status was assessed using 10 markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487) as previously (30). We defined MSI-high as the presence of instability in ≥30% of the markers, and MSI-low/microsatellite stable (MSS) as instability in <30% of the markers. Analyses of long interspersed nucleotide element-1 (LINE-1) methylation (31,32) and eight CpG island methylator phenotype (CIMP)-specific loci (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) (30) were performed.

Laser capture microdissection

To investigate *let-7* expression in tumor cells separately from stromal cells, 10 cases were selected, and paired tumor cells and tumor stromal tissues were obtained, using the Arcturus XT LCM system (Applied Biosystems, San Diego, CA). Sections were cut to 8 μm thickness, and placed on Arcturus polyethylene naphthalate membrane frame slides (catalog number, LCM0521) and allowed to dry. Based on histopathological review, tumor cells and

adjacent stromal areas were separately micro-dissected and captured onto CapSure Macro LCM Caps (catalog number, LCM0211).

Statistical analysis

Neither tumor-associated *let-7* expression nor log-transformed values of *let-7* expression fit a normal distribution in the Kolmogorov-Smirnov test for normality ($P \leq 0.01$). Thus, we tested our primary hypotheses using a statistical trend test across the ordinal quartiles of *let-7* expression as a continuous variable. For our first primary hypothesis that *let-7* expression might be inversely associated with immune response, we used a logistic regression model to test the association of *let-7a* or *let-7b* expression (an ordinal quartile predictor variable) with T-cell densities (a binary outcome variable). The densities of $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, and $FOXP3^+$ T cells in tumors were dichotomized at the median value. Considering multiple comparisons (using four T-cell variables for each of *let-7a* and *let-7b*), the α level was adjusted to 0.006 (= 0.05/8) by simple Bonferroni correction. To control for confounding, multivariate logistic regression model initially included age (continuous), sex, year of diagnosis (continuous), family history of colorectal cancer, tumor location (proximal colon vs. distal colon vs. rectum), MSI (high vs. MSI-low/MSS), CIMP (high vs. low/negative), *KRAS* (mutant vs. wild-type), *BRAF* (mutant vs. wild-type), *PIK3CA* (mutant vs. wild-type), and LINE-1 methylation level (continuous). A backward stepwise elimination with a threshold of $P = 0.05$ was used to select covariates in the final models. For cases missing any categorical covariate, such as family history (0.4%), tumor location (0.4%), MSI (3.9%), CIMP (8.6%), *KRAS* (2.6%), *BRAF* (2.9%), and *PIK3CA* (9.7%), we included those cases in a majority category of the covariate.

For the second primary hypothesis that *let-7* expression in colorectal cancer tissue might be associated with higher mortality, we used a Cox proportional hazards regression model to assess the association of *let-7a* or *let-7b* expression (an ordinal quartile predictor variable) with colorectal cancer mortality. Deaths from causes other than colorectal cancer were censored for cancer-specific mortality. Considering multiple comparisons (cancer-specific mortality and overall mortality for each of *let-7a* and *let-7b*), the α level was adjusted to 0.012 (= 0.05/4) by simple Bonferroni correction. To control for confounding, the multivariate Cox proportional hazards regression model initially included the same set of covariates as mentioned above, and we used the same backward stepwise elimination procedure to select covariates for the final model.

All other analyses for clinical, pathological, and molecular associations were secondary exploratory analyses, and we adjusted the two-sided α level to 0.003 (= 0.05/15) by simple Bonferroni correction for multiple comparisons. To assess associations between the ordinal categories (first to fourth quartile) of tumor-associated *let-7a* and *let-7b* expression and categorical data, the chi-square test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance assuming equal variances was performed. Because of a potential association between *let-7* and disease stage, multivariate logistic regression analysis was performed to control for confounding. We used *let-7a* or *let-7b* expression as an ordinal quartile predictor variable and disease stage as a binary outcome variable (stage I-II vs. stage III-IV), with the same set of initial covariates and the same

model construction procedure as mentioned above. We used the Wilcoxon signed rank test to compare the expression of *let-7a* or *let-7b* in colorectal cancer cells vs. tumor stromal cells, and in tumor tissues vs. adjacent mucosal tissues. We used the SAS program (Version 9.3, SAS Institute, Cary, NC) for all statistical analyses. All *P* values were two-sided.

RESULTS

Expression levels of *let-7a* and *let-7b* in colorectal cancer tissue

We measured tumor-associated *let-7a* and *let-7b* expression by quantitative reverse-transcription PCR assay on 795 colorectal cancer cases within the NHS and the HPFS. Table 1 and Supplementary Table S2 show clinical, pathological, and molecular characteristics of the 795 cases according to *let-7a* and *let-7b* expression levels, respectively. Levels of *let-7a* expression might be associated with disease stage, but statistical power was limited when we conducted a 4×4 chi-square test ($P = 0.10$). To assess a statistical trend of this association and control for confounding, we used logistic regression analysis and assessed the association of *let-7a* or *let-7b* expression as a quartile predictor variable with disease stage as a binary outcome variable (stage I–II vs. stage III–IV; Supplementary Table S3). Higher levels of *let-7a* or *let-7b* expression might be associated with stage III–IV disease (P_{trend} = 0.005 or 0.0005, respectively, with adjusted α level of 0.003).

let-7a and *let-7b* expression and T-cell density in colorectal cancer tissue

We measured the densities of T cells in colorectal cancer tissue by immunohistochemistry and image analysis. Pairwise correlations between the densities of $CD3^+$, $CD8^+$, $CD45RO^+$, and $FOXP3^+$ T cells were significant except between $FOXP3^+$ and $CD3^+$ cells and between $FOXP3^+$ and $CD8^+$ T cells (Supplementary Table S4).

Table 2 shows the distribution of colorectal cancer cases according to the densities of T cells (quartiles) and *let-7a* or *let-7b* expression (quartiles) in colorectal cancer tissue. In our primary hypothesis testing, we conducted univariate and multivariate logistic regression analyses to assess the associations of *let-7a* or *let-7b* expression as an ordinal quartile predictor variable with the density of $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ T cells in colorectal cancer tissue as a binary outcome variable (Table 3). *let-7a* expression was inversely associated with the densities of $CD3^+$ and $CD45RO^+$ T cells in multivariate analyses (multivariate P_{trend} = 0.003 and 0.0004, respectively, with adjusted α level at 0.006). Compared with cases in the lowest quartile of *let-7a* expression, those in the highest quartile were associated with lower density of $CD3^+$ T cells [multivariate OR, 0.40; 95% confidence interval (CI), 0.23 to 0.67] and $CD45RO^+$ T cells (multivariate OR, 0.31; 95% CI, 0.17 to 0.58). In contrast, *let-7b* expression was not significantly associated with any of the T-cell densities (all multivariate $P_{trend} \geq 0.04$ with adjusted α level at 0.006).

Tumor-associated *let-7a* and *let-7b* expression and colorectal cancer mortality

During a median follow-up of 13.5 years for censored cases, 445 deaths including 242 colorectal cancer-specific deaths were documented. In the second primary hypothesis test (Table 4), *let-7a* expression was positively associated with cancer-specific mortality (multivariate P_{trend} = 0.001). Multivariate hazard ratio of the highest vs. lowest quartile of

let-7a for cancer-specific mortality was 1.82 (95% CI, 1.42–3.13). We observed similar findings for the 518 cases with density of $CD45RO^+$ cells available (Table S5). In contrast, *let-7b* expression was not associated with cancer-specific mortality (multivariate P_{trend} = 0.44). In Kaplan-Meier analysis (Fig. 1), higher *let-7a* expression was associated with shorter colorectal cancer-specific survival (P = 0.003).

let-7a and *let-7b* in cancer, stromal, and adjacent nontumor cells of colonic mucosa

To determine whether *let-7* microRNAs might be expressed in colorectal cancer cells and/or cells in tumor stroma, we microdissected tumor cells and tumor stromal tissues in 10 selected cases, using a laser capture microdissection (LCM) technique. Expression *let-7a* and *let-7b* in tumor stroma were on average approximately 4 times higher than in paired tumor cells (Supplementary Fig. S2A and B, Wilcoxon signed rank test, P = 0.002 and P = 0.004 for *let-7a* and *let-7b*, respectively).

We macrodissected tumor tissue and adjacent nontumor colonic mucosa tissue in 52 colorectal cancer cases. Expression of *let-7a* and *let-7b* was generally lower in tumor tissue than in paired adjacent nontumor colonic mucosa (Supplementary Fig. S2C and D, Wilcoxon signed rank test, $P < 0.0001$ and P = 0.0003 for *let-7a* and *let-7b*, respectively).

DISCUSSION

We conducted this study to test the hypothesis that *let-7a* or *let-7b* expression might be inversely associated with T-cell densities in colorectal cancer tissue. We found the inverse association between *let-7a* expression levels and the densities of $CD3^+$ cells and $CD45RO^+$ T cells in colorectal cancer tissue. We also found that tumor-associated *let-7a* expression was positively associated with colorectal cancer-specific mortality.

The *let-7* miRNA family has been reported to regulate immune activation in various cell types. In human epithelial cells, *let-7* suppresses immune responses to pathogens by inhibition of Toll-like receptor 4 (TLR4), and downregulation of *let-7* restores immune activation (16,33). In mammalian macrophages exposed to pathogen challenge, *let-7* controls the immune response via inhibition of the NFKB1 pathway or repression of cytokine expression (17,34,35). Furthermore, the *let-7* family has been shown to regulate T-cell functions. In murine $CD4^+$ T cells receiving a T-cell receptor signal, *let-7* represses T-cell activation and facilitates T-cell anergy by targeting *Mtor* mRNA (36). In addition, *let-7a* has been reported to inhibit Th17 cell differentiation and increase regulatory T cells in a mouse hepatitis model (37), and suppression of *let-7* expression has been associated with the differentiation of central memory T cells (18). Our population-based discovery of the inverse association between expression of *let-7a* and densities of $CD3^+$ and $CD45RO^+$ cells in colorectal cancer tissues suggests that *let-7a* might inhibit the lymphocytic immune response against colorectal cancer, which is consistent with experimental evidence for immune suppressive effect of the *let-7* family.

Tumor molecular analyses are increasingly important in colorectal cancer research (38,39). In addition, ample evidence indicates the relevance of immune characterization together with tumor molecular analyses (40–43). In particular, upregulation of the immune checkpoint

ligand CD274 (PD-L1) in colorectal cancer has been associated with lower densities of $FOXP3^+$ cells and tumor microsatellite stability in 823 tumors (44) [in contrast to other much smaller studies (11,45)]. As evidence indicates influences of tumor somatic alterations on immune checkpoint pathways (46), tumor molecular alterations may be targeted to augment effects of immunotherapies. Our findings suggest that *let-7a* may be a potential therapeutic target to improve the efficacy of immunotherapy.

Previous studies have found a controversial relationship between *let-7* expression and cancer survival. Expression of *let-7a* and *let-7f* has been associated with longer survival in lung cancer (19), and *let-7b* expression with longer survival in serous ovarian cancer (20). However, *MIRLET7A3* hypomethylation, which increases *let-7a-3* expression, has been associated with shorter survival in epithelial ovarian cancers (21). To date, two studies using microarrays have investigated the prognostic value of *let-7* in colorectal cancer, but no significant prognostic association has been observed (47,48). Potential reasons for the inconsistent effects of *let-7* on cancer survival may include limited sample sizes of those studies, and differences in study populations, cancer types, and measurement methods for *let-7* family members. In the current study, we measured *let-7a and let-7b* that account for the majority of *let-7* expressed in human colonic epithelia (22) in 795 patients. As a result, we found a strong positive association of *let-7a* expression with colorectal cancer-specific mortality.

One limitation of our study is the cross-sectional design, which cannot exclude the possibility of reverse causation. It is possible that $CD45RO^+$ cells might suppress *let-7a* expression in the tumor microenvironment. Nevertheless, our hypothesis was based on various lines of experimental evidence indicating that *let-7* suppresses immune responses (16–18,33–36). Another limitation is the measurement of *let-7* expression in colorectal cancer tissues, which contain a mixture of tumor cells and stromal cells, including immune cells. Our measurement in 10 pairs of tumor cells and tumor stroma suggests higher *let-7a* expression in stromal cells, although further studies are required to validate this finding and to identify the cell types responsible for *let-7* production.

Our study has several strengths. First, this study utilizes our molecular pathological epidemiology (49,50) database of 795 colorectal cancer cases in two U.S. nationwide prospective cohorts, which integrates epidemiological exposures, clinicopathological features, tumor molecular markers, and immune reaction status. This enabled us to rigorously test the association of *let-7* expression with T-cell density and mortality, controlling for potential confounders. Second, we used robust methods, including validated PCR assays for microRNA expression and objective quantification of T-cell densities by image analysis. Third, our tumor specimens were obtained from many hospitals throughout the U.S., which improves the generalizability of our findings.

In conclusion, higher *let-7a* expression was associated with lower densities of $CD3^+$ and $CD45RO^+$ cells in colorectal cancer tissue and with higher colorectal cancer-specific mortality. Our findings support a possible role of *let-7a* in downregulating T cell–mediated immunity in colorectal cancer. Further prospective studies are needed to validate these findings from the current hypothesis-generating study. Upon validation, these population-

based data may have implications for expanding the benefit of immune therapies through targeting microRNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cDNA	complementary DNA
CI	confidence interval
CIMP	CpG island methylator phenotype
Ct	cycle threshold
FFPE	formalin-fixed paraffin-embedded
HPFS	Health Professionals Follow-up Study
LCM	laser capture microdissection
LINE-1	long interspersed nucleotide element-1
MSI	microsatellite instability
MSS	microsatellite stable
NHS	Nurses' Health Study
OR	odds ratio
PCR	polymerase chain reaction
SD	standard deviation

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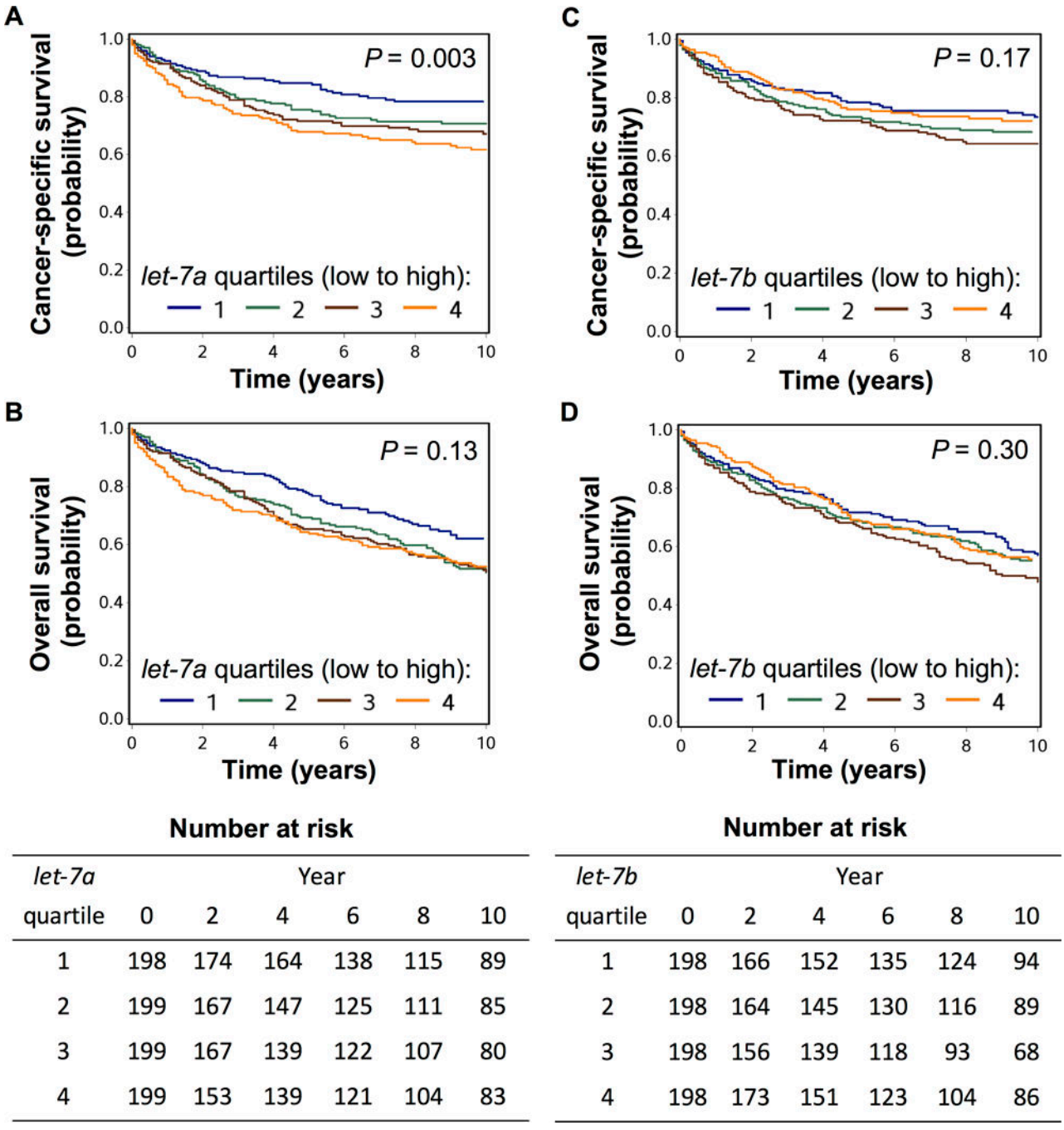

Number at risk

let-7a quartile	Year 0	2	4	6	8	10
1	198	174	164	138	115	89
2	199	167	147	125	111	85
3	199	167	139	122	107	80
4	199	153	139	121	104	83

Number at risk

let-7b quartile	Year 0	2	4	6	8	10
1	198	166	152	135	124	94
2	198	164	145	130	116	89
3	198	156	139	118	93	68
4	198	173	151	123	104	86

Figure 1.

Kaplan-Meier curve for cancer-specific survival and overall survival classified by quartiles of *let-7a* (A, B) or *let-7b* expression (C, D) in colorectal cancer tissue. Expression level increases from quartile 1 to quartile 4. Statistical analyses were performed using log rank test.

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Table 1

Clinical, pathological, and molecular characteristics according to *let-7a* expression in 795 colorectal cancer tissues

Characteristic[a]	Total (795)	*let-7a* expression				*P*[b]
		Q1 (198)	Q2 (199)	Q3 (199)	Q4 (199)	
Age						
Mean ± SD (year)	68.6 ± 8.7	69.3 ± 8.5	69.1 ± 8.9	67.7 ± 8.6	68.4 ± 8.7	0.26
Sex						0.49
Men	348 (44%)	86 (43%)	94 (47%)	89 (45%)	79 (40%)	
Women	447 (56%)	112 (57%)	105 (53%)	110 (55%)	120 (60%)	
BMI (kg/m2)						0.30
<25	317 (40%)	78 (40%)	87 (44%)	83 (42%)	69 (35%)	
25–30	315 (40%)	75 (38%)	81 (41%)	78 (39%)	81 (41%)	
>30	161 (20%)	44 (22%)	31 (16%)	37 (19%)	49 (25%)	
Year of diagnosis						0.01
Prior to 1998	374 (47%)	85 (43%)	113 (57%)	91 (46%)	85 (43%)	
1998 to 2008	421 (53%)	113 (57%)	86 (43%)	108 (54%)	114 (57%)	
Family history of colorectal cancer						0.82
Absent	631 (80%)	155 (79%)	156 (78%)	157 (80%)	163 (82%)	
Present	161 (20%)	42 (21%)	43 (22%)	40 (20%)	36 (18%)	
Tumor location						0.38
Cecum	139 (17%)	35 (18%)	40 (20%)	33 (17%)	31 (16%)	
Ascending to transverse colon	251 (32%)	62 (31%)	51 (26%)	72 (36%)	66 (33%)	
Splenic flexure to sigmoid	223 (28%)	53 (27%)	58 (29%)	48 (24%)	64 (32%)	
Rectosigmoid and rectum	179 (23%)	47 (24%)	50 (25%)	45 (23%)	37 (19%)	
No. of negative lymph nodes						0.42
Median (interquartile range)	7 (0–12)	7.5 (0–14)	7 (2–11)	6 (0–12)	7 (0–12)	
Disease stage						0.10
I	183 (24%)	52 (28%)	52 (28%)	41 (22%)	38 (20%)	
II	242 (32%)	70 (37%)	58 (31%)	60 (32%)	54 (29%)	
III	221 (30%)	48 (26%)	54 (29%)	61 (33%)	58 (31%)	
IV	104 (14%)	18 (10%)	24 (13%)	25 (13%)	37 (20%)	

Characteristic[a]	Total (795)	*let-7a* expression				*p*[b]
		Q1 (198)	Q2 (199)	Q3 (199)	Q4 (199)	
Tumor differentiation						0.65
Well to moderate	721 (91%)	183 (93%)	179 (90%)	181 (91%)	178 (89%)	
Poor	73 (9%)	14 (7%)	20 (10%)	18 (9%)	21 (11%)	
MSI status						0.37
MSI-low/MSS	648 (85%)	162 (86%)	164 (84%)	155 (82%)	167 (88%)	
MSI-high	116 (15%)	27 (14%)	31 (16%)	35 (18%)	23 (12%)	
CIMP status						0.34
Low/negative	595 (82%)	144 (84%)	154 (81%)	148 (80%)	149 (82%)	
High	132 (18%)	28(16%)	35 (19%)	36 (20%)	33 (18%)	
BRAF mutation						0.04
(−)	650 (84%)	166 (88%)	173 (88%)	155 (80%)	156 (81%)	
(+)	122 (16%)	23 (12%)	23 (12%)	38 (20%)	38 (19%)	
KRAS mutation						0.56
(−)	459 (60%)	114 (60%)	109 (56%)	121 (63%)	115 (60%)	
(+)	308 (40%)	76 (40%)	85 (44%)	70 (37%)	77 (40%)	
PIK3CA mutation						0.52
(−)	595 (83%)	155 (86%)	143 (80%)	152 (83%)	145 (82%)	
(+)	123 (17%)	25 (14%)	35 (20%)	31 (17%)	32 (18%)	
LINE-1 methylation level						0.63
Mean ± SD (%)	62.2 ± 9.3	62.0 ± 8.0	61.7 ± 9.6	62.1 ± 9.5	62.9 ± 10.0	

[a]Percentage indicates the proportion of cases with a specific clinical, pathological, or tumor molecular feature in patients with each expression level of *let-7a*. There were cases that had missing values for any of the characteristics except for age, sex and year of diagnosis.

[b]To assess associations between the ordinal categories (first to fourth quartile) of *let-7a* expression and categorical data, the chi-square test was performed. To compare mean age and mean LINE-1 methylation levels, the analysis of variance was performed. We adjusted two-sided α level to 0.003 (= 0.05/15) by simple Bonferroni correction.

BMI, body mass index; Q1 to Q4, quartile 1 (lowest) to quartile 4 (highest); SD, standard deviation.

Table 2

Distribution of cases according to *let-7a* and *let-7b* expression and T-cell densities in colorectal cancer tissue

T-cell density	Total No.	*let-7a* expression					*let-7b* expression				
		Q1	Q2	Q3	Q4	P_{trend}[a]	Q1	Q2	Q3	Q4	P_{trend}[a]
$CD3^+$ cell density						0.004					0.18
Q1	116 (23%)	19 (16%)	33 (24%)	27 (21%)	37 (28%)		33 (26%)	21 (16%)	31 (23%)	30 (25%)	
Q2	121 (23%)	24 (20%)	32 (24%)	26 (20%)	39 (30%)		37 (30%)	33 (25%)	23 (17%)	28 (23%)	
Q3	144 (28%)	41 (34%)	33 (24%)	36 (28%)	34 (26%)		29 (23%)	43 (33%)	38 (29%)	34 (28%)	
Q4	133 (26%)	35 (29%)	37 (27%)	40 (31%)	21 (16%)		26 (21%)	34 (26%)	42 (31%)	30 (24%)	
$CD8^+$ cell density						0.010					0.008
Q1	130 (26%)	30 (25%)	27 (21%)	32 (25%)	41 (32%)		40 (32%)	35 (27%)	32 (25%)	23 (19%)	
Q2	129 (25%)	26 (22%)	31 (24%)	34 (27%)	38 (29%)		31 (25%)	38 (30%)	30 (23%)	29 (24%)	
Q3	127 (25%)	34 (28%)	41 (32%)	27 (21%)	25 (19%)		26 (21%)	29 (23%)	34 (26%)	37 (30%)	
Q4	120 (24%)	30 (25%)	31 (24%)	34 (27%)	25 (19%)		27 (22%)	26 (20%)	34 (26%)	33 (27%)	
$CD45RO^+$ cell density						0.001					0.05
Q1	133 (26%)	20 (17%)	34 (25%)	29 (22%)	50 (38%)		28 (22%)	31 (23%)	39 (29%)	34 (28%)	
Q2	131 (25%)	22 (19%)	38 (28%)	33 (25%)	38 (29%)		32 (26%)	36 (27%)	27 (20%)	36 (30%)	
Q3	126 (24%)	33 (28%)	33 (24%)	37 (28%)	23 (17%)		34 (27%)	27 (20%)	31 (23%)	34 (28%)	
Q4	128 (25%)	43 (36%)	30 (22%)	34 (26%)	21 (16%)		32 (25%)	39 (30%)	39 (28%)	17 (14%)	
$FOXP3^+$ cell density						0.88					0.40
Q1	126 (26%)	29 (26%)	32 (26%)	30 (24%)	35 (27%)		31 (26%)	32 (26%)	30 (24%)	32 (27%)	
Q2	130 (26%)	30 (27%)	33 (26%)	36 (29%)	31 (24%)		32 (27%)	30 (24%)	29 (23%)	38 (32%)	
Q3	121 (25%)	29 (26%)	28 (22%)	31 (25%)	33 (25%)		30 (25%)	29 (23%)	32 (25%)	30 (26%)	
Q4	114 (23%)	25 (22%)	32 (26%)	26 (21%)	31 (24%)		27 (22%)	34 (27%)	35 (28%)	18 (15%)	

[a] P_{trend} value was calculated by the linear trend test across the ordinal (first to fourth quartile) categories of *let-7a* or *let-7b* expression as a continuous variable in the univariate ordinal logistic regression model for the density of $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ cells (an ordinal quartile outcome variable). We adjusted two-sided α level to 0.006 (= 0.05/8) by simple Bonferroni correction.

Q1 to Q4, quartile 1 (lowest) to quartile 4 (highest).

Table 3

let-7a and *let-7b* expression and T-cell densities in colorectal cancer tissue

let-7a expression	Univariate OR (95% CI)	Multivariate OR (95% CI)	*let-7b* expression	Univariate OR (95% CI)	Multivariate OR (95% CI)
Model of CD3$^+$ cell density as a binary outcome (n = 514)[a]					
Q1	1 (reference)	1 (reference)	Q1	1 (reference)	1 (reference)
Q2	0.61 (0.37–1.01)	0.62 (0.37–1.03)	Q2	1.82 (1.11–2.98)	1.75 (1.06–2.88)
Q3	0.81 (0.49–1.36)	0.82 (0.49–1.39)	Q3	1.89 (1.15–3.09)	1.67 (1.01–2.78)
Q4	0.41 (0.25–0.68)	0.40 (0.23–0.67)	Q4	1.40 (0.85–2.32)	1.30 (0.78–2.18)
P_{trend}[b]	0.004	0.003	P_{trend}[b]	0.18	0.40
Model of CD8$^+$ cell density as a binary outcome (n = 506)[a]					
Q1	1 (reference)	1 (reference)	Q1	1 (reference)	1 (reference)
Q2	1.08 (0.66–1.79)	1.05 (0.63–1.76)	Q2	1.01 (0.61–1.66)	1.03 (0.61–1.72)
Q3	0.81 (0.49–1.33)	0.77 (0.46–1.29)	Q3	1.47 (0.90–2.41)	1.47 (0.88–2.47)
Q4	0.55 (0.34–0.92)	0.54 (0.32–0.91)	Q4	1.80 (1.09–2.99)	1.59 (0.94–2.69)
P_{trend}[b]	0.010	0.008	P_{trend}[b]	0.008	0.04
Model of CD45RO$^+$ cell density as a binary outcome (n = 518)[a]					
Q1	1 (reference)	1 (reference)	Q1	1 (reference)	1 (reference)
Q2	0.50 (0.29–0.87)	0.50 (0.28–0.88)	Q2	1.22 (0.71–2.11)	1.34 (0.76–2.36)
Q3	0.60 (0.35–1.03)	0.52 (0.30–0.92)	Q3	1.18 (0.68–2.04)	1.32 (0.75–2.31)
Q4	0.33 (0.18–0.60)	0.31 (0.17–0.58)	Q4	0.48 (0.25–0.92)	0.52 (0.27–1.00)
P_{trend}[b]	0.001	0.0004	P_{trend}[b]	0.05	0.09
Model of FOXP3$^+$ cell density as a binary outcome (n = 491)[a]					
Q1	1 (reference)	1 (reference)	Q1	1 (reference)	1 (reference)
Q2	1.01 (0.61–1.68)	1.07 (0.64–1.81)	Q2	1.12 (0.68–1.85)	1.09 (0.65–1.83)
Q3	0.94 (0.57–1.57)	0.89 (0.53–1.51)	Q3	1.26 (0.76–2.07)	1.17 (0.70–1.96)
Q4	1.06 (0.64–1.76)	1.00 (0.60–1.68)	Q4	0.76 (0.45–1.27)	0.77 (0.46–1.32)
P_{trend}[b]	0.88	0.84	P_{trend}[b]	0.40	0.43

[a]The multivariate logistic regression model initially included age, sex, year of diagnosis, family history of colorectal cancer in parent or sibling, tumor location, microsatellite instability, CpG island methylator phenotype, KRAS, BRAF, and PIK3CA mutations, and LINE-1 methylation level. A backward stepwise elimination with a threshold of $P < 0.05$ was used to select variables in the final models.

[b] P_{trend} was calculated by the linear trend test across the ordinal categories (first to fourth quartile) of *let-7a* or *let-7b* expression as a continuous variable in the logistic regression model for the density of $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ cells as a binary outcome variable. We adjusted two-sided α level to 0.006 (= 0.05/8) by simple Bonferroni correction.

CI, confidence interval; Q1 to Q4, quartile 1 (lowest) to quartile 4 (highest).

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Table 4

let-7a and *let-7b* expression in colorectal cancer tissue and survival

		Cancer-specific mortality			Overall mortality		
***let-7* expression**	**No. of cases**	**No. of events**	**Univariate HR (95% CI)**	**Multivariate HR[a] (95% CI)**	**No. of events**	**Univariate HR (95% CI)**	**Multivariate HR[a] (95% CI)**
let-7a (*n* = 795)							
Q1	198	43	1 (reference)	1 (reference)	96	1 (reference)	1 (reference)
Q2	199	57	1.38 (0.93–2.05)	1.38 (0.93–2.05)	120	1.28 (0.98–1.68)	1.34 (1.02–1.75)
Q3	199	66	1.63 (1.13–2.45)	1.66 (1.48–3.25)	115	1.31 (1.00–1.71)	1.41 (1.08–1.86)
Q4	199	76	1.97 (1.25–2.66)	1.82 (1.42–3.13)	114	1.35 (1.03–1.77)	1.39 (1.06–1.84)
P_{trend}[b]			0.0002	0.001		0.04	0.02
let-7b (*n* = 792)							
Q1	198	52	1 (reference)	1 (reference)	109	1 (reference)	1 (reference)
Q2	198	63	1.26 (0.87–1.82)	1.30 (0.90–1.88)	114	1.31 (0.87–1.47)	1.17 (0.90–1.52)
Q3	198	70	1.45 (1.01–2.08)	1.53 (1.07–2.20)	114	1.08 (0.96–1.63)	1.26 (0.97–1.64)
Q4	198	56	1.08 (0.74–1.58)	1.11 (0.76–1.62)	105	1.01 (0.77–1.32)	1.00 (0.77–1.32)
P_{trend}[b]			0.53	0.44		0.76	0.84

[a]The multivariate Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer in parent or sibling, microsatellite instability, CpG island methylator phenotype, KRAS, BRAF, and PIK3CA mutations, and LINE-1 methylation level. A backward stepwise elimination with a threshold of $P < 0.05$ was used to select variables in the final models.

[b]P_{trend} was calculated by the linear trend test across the ordinal categories (first to fourth quartile) of *let-7a* or *let-7b* expression as a continuous variable in the Cox regression model for cancer-specific mortality and overall mortality. We adjusted two-sided α level to 0.012 (= 0.05/4) by simple Bonferroni correction.

CI, confidence interval; HR, hazard ratio; Q1 to Q4, quartile 1 (lowest) to quartile 4 (highest).