TIME (Tumor Immunity in the MicroEnvironmenT) classification based on tumor CD274 (PD-L1) expression status and tumor-infiltrating lymphocytes in colorectal carcinomas

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ABSTRACT

Inhibitors targeting the PDCD1 (programmed cell death 1, PD-1) immune checkpoint pathway have revolutionized cancer treatment strategies. The TIME (Tumor Immunity in the MicroEnvironment) classification based on tumor CD274 (PD-L1 ligand 1, PD-L1) expression and tumor-infiltrating lymphocytes (TIL) has been proposed to predict response to immunotherapy. It remains to be determined clinical, pathological, and molecular features of TIME subtypes of colorectal cancer. Using 812 colon and rectal carcinoma cases from the Nurses’ Health Study and Health Professionals Follow-up Study, we examined the association of tumor characteristics and survival outcomes with four TIME subtypes (TIME 1, CD274low/TILabsent; TIME 2, CD274high/TILpresent; TIME 3, CD274low/TILpresent; and TIME 4, CD274high/TILabsent). In survival analyses, Cox proportional hazards models were adjusted for potential confounders, including microsatellite instability (MSI) status, CpG island methylator phenotype (CIMP) status, LINE-1 methylation level, and KRAS, BRAF, and PIK3CA mutation status. TIME subtypes 1, 2, 3 and 4 had 218 (27%), 117 (14%), 103 (13%), and 374 (46%) colorectal cancer cases, respectively. Compared with TIL-absent subtypes (TIME 1 and 4), TIL-present subtypes (TIME 2 and 3) were associated with high-level MSI, high-degree CIMP, BRAF mutation, and higher amounts of neoantigens (p < 0.001). TIME subtypes were not significantly associated with colorectal cancer-specific or overall survival. In conclusion, TIL-present TIME subtypes of colorectal cancer are associated with high levels of MSI and neoantigen load, supporting better responsiveness to cancer immunotherapy. Further studies examining tumor molecular alterations and additional factors in the tumor microenvironment may inform development of immunoprevention and immunotherapy strategies.

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Introduction

Immunotherapy has emerged in recent years as an attractive therapeutic modality in cancer management. In particular, immune checkpoint inhibitors that block the PD1 (programmed cell death 1, PD-1) or CD274 (PD1 ligand 1, PD-L1) protein have shown great promise in treating various malignancies with durable clinical remissions. How to effectively identify patients who would derive clinical benefits from immune checkpoint blockade therapy has therefore become a clinical question of paramount importance.

The TIME (Tumor Immunity in the MicroEnvironment) classification system has been proposed as a first-step framework to predict immunotherapeutic response based on cross-classified levels of tumor CD274 (PD-L1) expression (low vs. high) and tumor-infiltrating lymphocytes (TIL, absent vs. present). However, how this proposed scheme may correlate with tumor molecular features and clinical outcomes in general and in colorectal cancer remains to be determined. The CD274 protein expressed on cancer cells impairs T cell-mediated tumor-specific immune response by binding to PDCD1 (PD-1) on T cells. In colorectal cancer, tumor CD274 expression has been inversely correlated with FOXP3* regulatory T cells in tumor, suggesting mutually exclusive immunosuppressive mechanisms. Immune reaction plays a key role in suppressing tumor development and progression, and high-level infiltrates of lymphocytes in colorectal cancer have been associated with better clinical outcomes. Four tumor subtypes can be created based on the TIME framework, including the CD274**/TIL* subtype (TIME 1) which generally responds to immunotherapy, the CD274*/TIL* subtype (TIME 2) which suggests presence of other suppressor pathways in immune tolerance, the CD274**/TIL* subtype (TIME 3) which indicates intrinsic induction of the CD274 pathway, and the CD274*/TIL* subtype (TIME 1) which reflects immune "ignorance."

The TIME model has been developed based on the findings in melanoma, and has been applied to other tumor types. The prevalence and clinical implications of different TIME subtypes likely vary by tumor-specific genetic aberrations and oncogenic drivers as well as other factors defining the tumor microenvironment. In colorectal cancer, it is known that high-level microsatellite instability (MSI) and resultant framshift mutations are associated with abundant immunogenic peptides ("neoantigens"), leading to increased likelihood of clinical benefits from immune checkpoint blockade. The carcinogenesis of colorectal carcinoma, however, is by itself a fairly heterogeneous process which involves stepwise accumulation of multiple genetic and epigenetic aberrations, as well as complex interactions with environmental exposures and host features. Phenotypic profiling of colorectal carcinomas that would respond to immunotherapy would therefore likely require consideration of multiple factors in association with TIME categories to precisely predict tumor immune resistance.

Using a molecular pathological epidemiology database derived from two large prospective cohort studies in the U.S., we examined the association of TIME subtypes of colorectal cancer with clinical, pathological, and molecular characteristics, and patient survival.

Results

Among 812 colorectal carcinoma cases with available data on tumor CD274 (PD-L1) expression status and TIL, TIME subtypes 1, 2, 3 and 4 had 218 (27%), 117 (14%), 103 (13%), and 374 (46%) cases, respectively. Table 1 summarizes clinical, pathological, and molecular characteristics of colorectal cancer cases according to TIME subtypes. Compared with TIL-absent subtypes (TIME 1 and 4), TIL-present subtypes (TIME 2 and 3) were statistically significantly associated with tumor location at the proximal colon, high-level MSI, high-degree CpG island methylator phenotype (CIMP), high-level long interspersed nucleotide element-1 (LINE-1) methylation, BRAF mutation, negative nuclear CTNNB1 expression, and high neoantigen load (p < 0.001 with adjusted α level of 0.003). Interestingly, TIL-present subtypes were more likely to have more poorly-differentiated tumors but lower disease stage (p < 0.003). There were no significant differences in characteristics by tumor CD274 expression status in strata of levels of TIL (p > 0.01 with adjusted α level of 0.003).

We also examined the association of TIME subtypes with colorectal cancer survival. During the median follow-up time of 12.1 years (interquartile range, 8.4–16.1 years) for all censored cases, there were 479 all-cause deaths, including 247 colorectal cancer-specific deaths. Fig. 1 shows Kaplan-Meier survival curves of colorectal cancer cases by TIME subtypes. In multivariable Cox regression analyses, TIME subtypes were not statistically significantly associated with colorectal cancer-specific or overall survival (Table 2 and Table S1). Compared with TIME subtype 1, multivariable-adjusted hazard ratios for colorectal cancer-specific mortality were 0.61 [95% confidence interval (CI), 0.37–1.01] for subtype 2, 0.76 (95% CI, 0.46–1.25) for subtype 3, and 0.93 (95% CI, 0.69–1.26) for subtype 4. We did not observe a statistically significant interaction between tumor CD274 expression status and TIL in relation to cancer-specific or overall survival (pinteraction > 0.67).

In exploratory secondary analyses, characteristics and survival of colorectal carcinoma cases were evaluated according to a modified TIME classification scheme defined by tumor CD274 expression status and intratumoral periglandular reaction. The analyses did not yield significant differences in associations (Tables S2 and S3). When modified TIME subtypes were defined by tumor CD274 expression status and density of CD3+ cells in tumor tissue (Tables S4 and S5), no significant differences across TIME subtypes in association with colorectal cancer characteristics or survival were observed.

Discussion

We conducted this study based on two large prospective cohorts to examine the association of TIME subtypes with characteristics and survival outcomes of colorectal cancer. Our findings suggest that TIL-present TIME subtypes are more likely to present with high levels of MSI, CIMP, and neoantigens, and less likely to present with LINE-1 hypomethylation and nuclear CTNNB1 expression when compared with TIL-absent subtypes. There were no significant differences in...
Table 1. Clinical, pathological, and molecular characteristics of colorectal cancer cases according to TIME (Tumor Immunity in the MicroEnvironment) subtypes based on tumor CD274 (PD-L1) expression status and tumor-infiltrating lymphocytes (TIL).

| Characteristic | All cases (n = 812) | 1 (n = 218) | 4 (n = 374) | 3 (n = 103) | 2 (n = 117) | p1 |
|---------------|---------------------|------------|------------|------------|------------|----|
| Male (HPFS)   | 357 (44%)           | 97 (44%)   | 171 (46%)  | 48 (47%)   | 41 (35%)   |    |
| Mean age ± SD (years) | 69.2 ± 9.0        | 68.3 ± 8.9 | 68.9 ± 9.3 | 71.2 ± 8.9 | 69.8 ± 8.4 | 0.038 |
| Year of diagnosis |                 |            |            |            |            | 0.37 |
| 1995 or before | 257 (32%)           | 64 (29%)   | 124 (33%)  | 31 (30%)   | 38 (32%)   |    |
| 1996 to 2000  | 271 (33%)           | 74 (34%)   | 132 (35%)  | 27 (26%)   | 38 (32%)   |    |
| 2001 to 2008  | 284 (35%)           | 80 (37%)   | 118 (32%)  | 45 (44%)   | 41 (35%)   |    |
| Tumor location |                   |            |            |            |            | < 0.001 |
| Proximal colon | 412 (51%)           | 97 (45%)   | 154 (41%)  | 75 (74%)   | 86 (74%)   |    |
| Distal colon  | 239 (30%)           | 62 (29%)   | 138 (37%)  | 15 (15%)   | 24 (21%)   |    |
| Rectum        | 157 (19%)           | 58 (27%)   | 80 (22%)   | 12 (12%)   | 7 (6.0%)   |    |
| Tumor differentiation |          |            |            |            |            | < 0.001 |
| Well to moderate | 733 (90%)        | 198 (92%)  | 361 (97%)  | 84 (82%)   | 90 (77%)   |    |
| Poor          | 77 (9.5%)           | 18 (8.3%)  | 13 (3.5%)  | 19 (18%)   | 27 (23%)   |    |
| AJCC disease stage |                 |            |            |            |            | 0.002 |
| I             | 174 (23%)           | 49 (25%)   | 75 (21%)   | 26 (27%)   | 24 (22%)   |    |
| II            | 245 (33%)           | 61 (31%)   | 97 (27%)   | 39 (41%)   | 48 (44%)   |    |
| III           | 218 (29%)           | 55 (28%)   | 112 (32%)  | 20 (21%)   | 31 (28%)   |    |
| IV            | 115 (15%)           | 29 (15%)   | 69 (20%)   | 11 (11%)   | 6 (5.5%)   |    |
| MSI status    |                   |            |            |            |            | < 0.001 |
| Non-MSI-high  | 650 (82%)           | 192 (91%)  | 341 (94%)  | 53 (52%)   | 64 (56%)   |    |
| MSI-high      | 138 (18%)           | 19 (9.0%)  | 20 (5.5%)  | 19 (18%)   | 23 (27%)   |    |
| CIMP status   |                   |            |            |            |            | < 0.001 |
| CIMP-low/negative | 611 (82%)      | 185 (92%)  | 319 (92%)  | 47 (52%)   | 60 (56%)   |    |
| CIMP-high     | 133 (18%)           | 16 (8.0%)  | 26 (7.5%)  | 43 (48%)   | 48 (44%)   |    |
| Mean LINE-1 methylation level ± SD (%) | 62.3 ± 9.7 | 61.4 ± 9.7 | 61.3 ± 9.8 | 65.1 ± 9.2 | 64.4 ± 9.2 | < 0.001 |
| KRAS mutation |                   |            |            |            |            | 0.053 |
| Wild-type     | 466 (59%)           | 129 (61%)  | 196 (55%)  | 67 (67%)   | 74 (65%)   |    |
| Mutant        | 319 (41%)           | 84 (39%)   | 163 (45%)  | 33 (33%)   | 39 (35%)   |    |
| BRAF mutation |                   |            |            |            |            | < 0.001 |
| Wild-type     | 672 (85%)           | 193 (91%)  | 333 (91%)  | 67 (66%)   | 79 (71%)   |    |
| Mutant        | 120 (15%)           | 20 (9.4%)  | 32 (8.8%)  | 35 (34%)   | 33 (29%)   |    |
| PIK3CA mutation |                |            |            |            |            | 0.38 |
| Wild-type     | 630 (85%)           | 178 (86%)  | 282 (83%)  | 84 (90%)   | 86 (83%)   |    |
| Mutant        | 111 (15%)           | 29 (14%)   | 56 (17%)   | 9 (9.7%)   | 17 (17%)   |    |
| PTGS2 (cyclooxygenase-2) expression |          |            |            |            |            | 0.010 |
| Negative      | 325 (41%)           | 84 (40%)   | 137 (38%)  | 56 (56%)   | 48 (41%)   |    |
| Positive      | 469 (59%)           | 128 (60%)  | 228 (62%)  | 44 (44%)   | 69 (59%)   |    |
| Nucleostin (beta-catenin) expression |          |            |            |            |            | < 0.001 |
| Negative      | 407 (52%)           | 99 (47%)   | 169 (47%)  | 57 (59%)   | 82 (72%)   |    |
| Positive      | 372 (48%)           | 110 (53%)  | 190 (53%)  | 40 (41%)   | 32 (28%)   |    |
| Number of neoantigens |        |            |            |            |            | < 0.001 |
| Quartile 1 (3–152) | 96 (25%)    | 33 (30%)   | 44 (28%)   | 9 (16%)    | 10 (17%)   |    |
| Quartile 2 (153–261) | 94 (25%)    | 28 (26%)   | 53 (34%)   | 4 (6.9%)   | 9 (15%)    |    |
| Quartile 3 (262–470) | 97 (25%)    | 30 (28%)   | 45 (29%)   | 11 (19%)   | 11 (18%)   |    |
| Quartile 4 (≥471) | 95 (25%)    | 18 (17%)   | 13 (8.4%)  | 34 (59%)   | 30 (50%)   |    |

Abbreviations: AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; NHS, Nurses’ Health Study; SD, standard deviation; TIME, tumor immunity in the microenvironment.

*Percentage indicates the proportion of cases with a specific clinical, pathological, or molecular characteristic in all cases or in strata of TIME subtypes.

To compare characteristics between subgroups, we used the chi-square test for categorical variables, and an analysis of variance for continuous variables. We adjusted two-sided α level to 0.003 (≈ 0.05/17) based on Bonferroni correction for multiple hypothesis testing.
colorectal cancer-specific or overall survival by TIME subtypes, which may be supported by our previous findings of the lack of a prognostic role of tumor CD274 (PD-L1) expression status in colorectal cancer. No statistically significant interaction was noted between tumor CD274 expression and TIL in relation to patient survival.

TIL has been demonstrated in multiple tumor types to reflect local immune effector response, and, along with regulatory T cells, has generally been associated with improved survival in colorectal carcinomas. Tumor CD274 (PD-L1) expression was previously shown to inversely associate with FOXP3+ regulatory T cells, but not with CD3+ pan-T cells or CD8+ cytotoxic T cells in colorectal cancer, suggesting the potential influence of CD274-expressing cells on the tumor microenvironment via immune regulation. The TIME classification framework based on tumor CD274 expression status and TIL was hence proposed as a pragmatic approach to predict the immune resistance of tumors. Evidence suggests that the CD274 protein can be selectively expressed on tumor cells in response to proinflammatory cytokine IFNG (interferon-gamma) released from activated antigen-specific CD8+ T cells, rather than through oncogenic pathways. In TIME 2 (CD274high/TILpresent) tumors containing abundant TIL, the CD274-PDCD1 pathway is adaptively activated as negative feedback to help cancer cells evade immune attack; therefore, these tumors are believed to represent the group that would largely benefit from immune checkpoint blockade therapy. TIME 3 (CD274low/TILpresent) tumors, while harboring high-level TIL, utilize tumor suppressive pathways other than the CD274-PDCD1 axis, and hence, might better respond to non-CD274 immune checkpoint inhibitors. TIME 1 and 4 subtypes lack intrinsic TIL and may therefore require complementary therapeutic strategies to recruit lymphocytes into local tumors to increase efficacy of immune checkpoint inhibitors, such as via combination CTLA4 blockade or radiotherapy to induce T cell response. Application of the TIME classification needs to be interpreted in the context of other variables defining the tumor microenvironment for precise disease management and response prediction in the era of precision medicine. In this study, we sought to evaluate the implications of TIME subtypes on colorectal cancer in association with patient survival as well as tumor histopathologic and molecular features.

Integrated analyses of tumor, immunity, and microenvironment including the microbiota are important. While the TIME model does not correlate significantly with survival outcomes, our findings highlight a distinct molecular profile correlated with TIL-present TIME subtypes regardless of tumor CD274 expression status. TIL-present TIME subtypes were shown to be associated with presence of BRAF mutation, as well as high levels of MSI and neoantigens, which have been among the most well-documented predictive factors for tumor CD274 expression and/or response to immune checkpoint inhibitors regardless of primary organ site. Of note, a smaller proportion of CD274high/TILpresent subtype showed positive nuclear CTNNB1 (beta-catenin) expression when compared to CD274low/TILpresent subtype, although both subtypes were associated with high-level MSI. Nuclear expression or accumulation of CTNNB1 has been associated with more aggressive cancer behavior and suppression of anti-tumor immune response. Its presence in a greater proportion of CD274low/TILpresent TIME subtype could reflect potential mutations in the WNT signaling pathway or tumor-intrinsic mediators.
Abbreviations: CI, confidence interval; HR, hazard ratio; TIL, tumor-infiltrating lymphocytes; TIME, tumor immunity in the microenvironment.

The multivariable Cox regression model initially included sex, age at diagnosis, year of diagnosis, family history of colorectal cancer, prediagnosis body mass index, tumor location, tumor differentiation, disease stage, microsatellite instability status, CpG island methylator phenotype-specific promoter status, long interspersed nucleotide element-1 methylation level, KRAS mutation, BRAF mutation, PIK3CA mutation, PTGS2 (cyclooxygenase-2) expression, and nuclear CTNNB1 (beta-catenin) expression. A backward elimination with a threshold p of 0.05 was used to select variables for the final models. The variables which remained in the final models were shown in Table S1.

Our findings would inform further studies to elucidate the associations between TIME subtypes and other parameters within the tumor microenvironment to better tailor combination immunotherapies. Additional analyses with other local immune effector cells (e.g., memory and regulatory T cells, tumor-associated macrophages), as well as their densities and spatial distribution in relation to tumor invasive fronts, would enrich the TIME model in predicting tumor immune deficits and resistance. Sequencing-based assessment of intratumoral genetic heterogeneity of tumors at primary and metastatic sites in relation to CD274 expression and TIL would also facilitate understanding of the tumor microenvironment and its implications on therapeutic options. Other factors that could influence tumor recruitment and extravasation of immune effector cells to tumor sites, such as the gut microbiome, tumor vasculature, and related expression of adhesion molecules, would also warrant further investigations to correlate with TIME subtypes and their prognosis.

Our study has notable strengths including the use of a molecular pathological epidemiology database derived from two U.S. prospective cohort studies with long duration of follow-up. Integrated data on tumor molecular characteristics and pathological findings allowed us to comprehensively characterize TIME subtypes of colorectal cancer. Of note, our study population was derived from a large number of cases from hospitals located throughout the U.S., contributing to increased generalizability of our findings.

Limitations should be considered in our study. While we cannot entirely exclude the possibility of potential unmeasured or residual confounding in survival analyses, we collected detailed data on a comprehensive panel of colorectal cancer characteristics and evaluated them by multivariable models to control for potential confounding. Our study was also limited in information on cancer treatments. This lack of data, however, was unlikely to differ substantially by tumor CD274 expression or TIL levels as such information would not have been available for decision-making in management upfront. Our study was also based on relatively selected populations as most participants were non-Hispanic health professionals; therefore, our findings would need to be validated in independent cohorts. In addition, due to cellular structural changes caused by tissue processing and lack of standardized antibodies for CD274, assessment of tumor CD274 expression status and TIL in tissue samples could pose challenges that might have resulted in potential misclassifications. Intratumoral spatial heterogeneity and inter-observer variability in evaluating tumor CD274 expression and TIL may be relevant to update subtype designation in guiding immunotherapeutic treatments.

In summary, our findings suggest distinctive pathologic and molecular characteristics of colorectal cancer associated with subtypes defined by the TIME classification. Consistent with prior literature, our data support the role of TIL as an
important effector in tumor-immune interactions. Our findings would likely inform future studies to better understand tumor-immune microenvironment of colorectal carcinomas in the era of immunotherapy.

Patients and methods

Study population

We utilized two prospective cohort studies in the U.S., the Nurses’ Health Study (NHS, 121,701 women aged 30–55 years followed since 1976) and the Health Professionals Follow-up Study (HPFS, 51,529 men aged 40–75 years followed since 1986).56 Participants are followed with biennial questionnaires on lifestyle factors and newly-diagnosed diseases including colorectal cancer. The response rate has been more than 90% for each follow-up questionnaire in both cohorts. In both studies, the National Death Index was used to ascertain deaths of participants and to identify unreported lethal colorectal cancer cases. Study physicians, who were blinded to exposure data, reviewed medical records of identified colorectal cancer cases to confirm the disease diagnosis and to collect data on tumor clinical characteristics including tumor size, anatomical location, and disease stage.

Among participants diagnosed with colorectal cancer until 2012, we analyzed 812 cases with available data on tumor CD274 (PD-L1) expression status and TIL in tissue samples. We included both colon and rectal carcinomas based on the colorectal continuum model.57,58 Participants with a history of inflammatory bowel disease or cancer (except for non-melanoma skin cancer) were excluded from this study. Participants were followed until death or the end of follow-up (30 June 2014 for the NHS; and 1 January 2014 for the HPFS), whichever came first.

Informed consent was obtained from all participants at enrollment. This study was approved by the institutional review boards at Harvard T.H. Chan School of Public Health and Brigham and Women’s Hospital (Boston, MA, USA).

Histopathologic evaluation of colorectal cancer

Formalin-fixed paraffin-embedded tumor tissue blocks were collected from hospitals throughout the U.S. where colorectal cancer patients had undergone surgical resection. Hematoxylin and eosin-stained tissue sections were examined by a pathologist (S.O.) who was blinded to other data. Lymphocytic reaction to tumor was histopathologically evaluated, as previously described.17 TIL was defined as lymphocytes on top of cancer cells (Fig. S1). Intratumoral periglandular reaction was defined as lymphocytic reaction in intratumoral stroma. Each lymphocytic reaction pattern was graded as negative/low, intermediate, or high. Lymphocytic reaction patterns in a subset of cases were independently reviewed by a second pathologist (J.N.G.) with a good inter-observer correlation, as previously described.17 In the present study, TIL was categorized into absent (negative/low) vs. present (intermediate to high), and intratumoral periglandular reaction was categorized into low (negative/low to intermediate) vs. high.

Immunohistochemical evaluation

We constructed tissue microarrays of colorectal cancer cases with sufficient tissue materials, including up to four tumor cores approximately 600 μm in diameter from each case in one tissue microarray block.59 Immunohistochemical study for CD274 (PD-L1) was performed using an anti-CD274 antibody (dilution, 1:50; eBioscience, San Diego, CA, USA; Fig. S2). As previously described,13,51 a single pathologist (Y.M.) scored overall tumor CD274 expression level as an ordinal scale of 0–4 by summing cytoplasmic intensity score [absent (0), weak (1), moderate (2), or strong (3)] and membrane expression score [absent (0) or present (1; if distinct membrane staining above cytoplasmic expression level existed)]. When the staining intensity was different across tumor cores in the same case, predominant staining pattern in tumor cells was recorded. CD274 expression in selected tumors (n = 148) was independently examined by a second pathologist (A.d.S.), and the concordance between the two observers was reasonable with a weighted κ of 0.65 (95% CI, 0.57–0.73).13 We categorized CD274 levels as low (scale of 0 to 1) vs. high (scale of 2 to 4), as consistent with our previous study.51

As previously described,59,60 immunohistochemical analyses for PTGS2 (cyclooxygenase-2) and nuclear CTNNB1 (beta-catenin) expression were performed using an anti-PTGS2 antibody (dilution, 1:300; Cayman Chemical, Ann Arbor, MI, USA) and anti-CTNNB1 antibody (dilution, 1:400; BD Transduction Laboratories, Franklin Lakes, NJ, USA), respectively. We measured densities (cells/mm²) of CD3+ cells in colorectal cancer tissue, based on immunohistochemistry using an anti-CD3 antibody (dilution, 1:250; Dako Cytomation, Carpinteria, CA, USA) and image analysis using an automated scanning microscope and the Ariol image analysis system (Genetix, San Jose, CA, USA), as previously described.18

Evaluation of tumor molecular characteristics

DNA was extracted from colorectal cancer tissue in archival formalin-fixed paraffin-embedded tissue sections using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). MSI status was analyzed using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487). MSI-high was defined as presence of instability in ≥ 30% of the markers, and non-MSI-high as instability in < 30% of the markers, as previously described.61 Using bisulfite-treated DNA, methylation status of eight CIMP-specific promoters (CACNA1G, CDKN2 A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1) and LINE-1 was determined, as previously described.40,41 CIMP-high was defined as ≥ 6 methylated promoters of eight promoters, and CIMP-low/negative as 0–5 methylated promoters, as previously described.62 Polymerase chain reaction and pyrosequencing were performed for KRAS (codons 12, 13, 61, and 146),63 BRAF (codon 600),64 and PIK3CA (exons 9 and 20).64 Neoantigen load, the number of immunogenic peptides, was predicted by using a neoantigen prediction pipeline for
somatic mutations based on whole-exome sequencing and identifying peptides that bind to personal HLA molecules with high affinity (< 500 nM), as previously described. Using NetMHCpan (version 2.4), we predicted the binding affinities of all possible 9- and 10-mer mutant peptides to the corresponding HLA alleles inferred by the POLYSOLVER algorithm.

Definitions of TIME (Tumor Immunity in the MicroEnvironment) subtypes

TIME subtypes of colorectal carcinoma were assessed as the primary outcome in cross-sectional analyses with tumor characteristics, and as the primary exposure in survival analyses with colorectal cancer survival outcomes. As described previously, TIME subtypes were defined based on tumor CD274 (PD-L1) expression status (low vs. high) and TIL (absent vs. present): TIME 1, CD274 expression-low and TIL-absent; TIME 2, CD274 expression-high and TIL-present; TIME 3, CD274 expression-low and TIL-present; and TIME 4, CD274 expression-high and TIL-absent.

Exploratory secondary analyses with modified TIME classification schemes were also performed with assessment of intratumoral periglandular reaction (low vs. high), as well as density of CD3+ cells (dichotomized by median value), instead of TIL as markers for tumor immune status.

Statistical analyses

All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA), and all p values were two-sided.

We used the chi-square test for categorical variables, and analysis of variance for continuous variables to compare tumor characteristic across TIME subtypes. The α level was set at 0.003 (= 0.05/17) with Bonferroni correction to adjust for multiple hypothesis testing. In subgroup analyses, results were interpreted cautiously in addition to the use of adjusted α level of 0.003.

Cumulative survival probabilities were estimated using the Kaplan-Meier method and compared using the log-rank test. Deaths from other causes were dealt as censored. Univariable estimates were calculated using the Kaplan-Meier method and compared using the log-rank test. All cases with missing data on any of the covariates did not yield substantial differences in our results (data not shown). Statistical interaction between tumor CD274 expression status (low vs. high) and TIL (absent vs. present) was evaluated using the Wald test on the cross-product. The assumption of proportional hazards was validated using a time-varying covariate in the Wald test on the cross-product. The level of 0.05 were used to determine the majority category of a given categorical covariate to limit the degrees of freedom of the models: family history of colorectal cancer (1.1%), prediagnosis body mass index (0.7%), tumor location (0.5%), tumor differentiation (0.3%), MSI status (3.0%), CIMP status (8.4%), KRAS mutation (3.3%), BRAF mutation (2.5%), PIK3CA mutation (8.7%), PTGS2 expression (2.2%), and nuclear CTNNB1 expression (4.1%). For cases with missing data on LINE-1 methylation level (2.8%), a separate indicator variable was used. The other authors declare that they have no conflicts of interest.
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