A combination of stromal PD-L1 and tumoral nuclear β-catenin expression as an indicator of colorectal carcinoma progression and resistance to chemoradiotherapy in locally advanced rectal carcinoma

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Supplementary Material

Supplementary Figures S1-S7
Supplementary Tables S1-S5
Figure S1. (A) A diagram and (B) pT2 (left), pT3 (middle), and pT4 (right) CRCs showing the three subclassifications of inner, middle, and outer parts of a tumor lesion on the basis of tumor size. *, **, ***, one-third of the lesions.
Figure S2. Differences in the expression of immune cell-related markers between colon and rectal carcinomas. (A) PD-L1 immunopositivity in tumor cells, (B) labeling indices (LIs) of immunopositive immune cells for the indicated markers in the outer stroma, and (C) nuclear β-catenin score in the tumor budding area in colon (Co) and rectal carcinomas (Re) without NCRT. The data shown are means ± SDs.
Figure S3. Relationship between CD3, CD4, and CD8 in CRC without NCRT. (A) Staining with hematoxylin and eosin (HE) or IHC for the indicated immune cell-related markers in CRC without NCRT. Original magnification, x100. (B) Correlations between the indicated markers in the outer stroma of CRC without NCRT.
Figure S4. Ratios of PD-L1 LIs relative to PD-1, CD4, CD8, or CD68 LIs in the inner (In), middle (Md), and outer (Out) stroma in CRC without NCRT. The data shown are means ± SDs.
Figure S5. Coexpression of immune cell-related markers in infiltrating stromal immune cells in CRC without NCRT. (A) Immunofluorescence for PD-L1, PD-1, and CD68. Infiltrating immune cells coexpressing the indicated markers are present in the outer stroma (indicated by arrows). Closed boxes are magnified in the insets. Original magnification, x100 and x400 (insets). Ca, carcinomatous lesions. (B) Correlations between the indicated markers in the stroma of CRC samples.
Figure S6. Tumoral PD-L1 and MMR expression in CRC without NCRT. Staining with hematoxylin and eosin (HE) and IHC for MMR and PD-L1 in CRC. Note that CRC case #77 (upper panels) is immunonegative for MLH1 and PMS2 in membranous PD-L1-positive tumor cells, which is in contrast to CRC case #72 which is PD-L1-negative and MMR-proficient. The closed box is magnified in the inset. Original magnification, x100 and x400 (inset).
Figure S7. Expression of immune cell-related molecules in biopsied samples of LAd-RC before NCRT. (A) Staining with hematoxylin and eosin (HE) and IHC for the indicated immune cell-related markers in pretreatment biopsied LAd-RC samples that responded poorly to NCRT (TE: G1) (upper panels), responded moderately (TE: G2) (middle panels), or responded well (TE: G3) (lower panels) in the associated resected tumors after NCRT. Closed boxes are magnified in the insets. Original magnification, x100 and x400 (insets). (B) Relationships between labeling indices (LIs) of immunopositive immune cells for the indicated markers (upper), number of immune cells immunopositive for the indicated markers per high power field (HPF) in intratumoral lesions (lower) in pretreatment-biopsied LAd-RC samples, and TE grades in the associated resected tumor tissues after NCRT. The data shown are means ± SDs.
| Antibody | Clone | Source                          | Dilution |
|----------|-------|---------------------------------|----------|
| PD-L1    | 28-8  | Abcam (Cambridge, UK)           | ×700     |
| PD1      | NAT105| Abcam                           | ×500     |
| CD3      | F7.2.38| DAKO (Copenhagen, Denmark)      | ×200     |
| CD4      | 4B12  | DAKO                            | ×100     |
| CD8      | C8/144B| DAKO                            | ×100     |
| CD68     | PG-M1 | DAKO                            | ×100     |
| β-catenin| 14/b-catenin| BD Bioscience (San Jose, CA, USA)| ×500     |
| MLH1     | ES05  | Novocastra (Newcastle, UK)      | ×100     |
| MSH2     | 25D12 | Novocastra                      | ×50      |
| MSH6     | PU29  | Novocastra                      | ×100     |
| PMS2     | M0R4G | Novocastra                      | ×100     |
|            | PD-L1 expression |            |            |
|------------|------------------|------------|------------|
|            | Carcinomatous lesion | Outer stromal lesion |            |
|            | high (≧ 5%)  | low (< 5%) | high LIs (≧ 30%) | low LIs (< 30%) |            |
| n   | n (%)  | n (%)  | n (%)  | n (%)  | P-value | n   | n (%)  | n (%)  | P-value |
| Proficient | 90  | 5 (5.6) | 85 (94.4) | 69  | 69 (80.2) | 17 (19.8) | 0.1 |
| Deficient  | 10  | 4 (40)  | 6 (60)   | 9   | 9 (100)   | 0         |      |

n, number of cases
**Table S3.** Correlation between IHC marker expression and clinicopathological factors in the outer stroma in CRC without NCRT

|               | PD-1 | CD4  | CD8  | CD68 |
|---------------|------|------|------|------|
|               | high | low  | high | low  | high | low  | high | low  |
|               | (≥ 8 %) | (< 8 %) | (≥ 34 %) | (< 34 %) | (≥ 41 %) | (< 41 %) | (≥ 27 %) | (< 27 %) |
| n            | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| Age (years)  |      |      |      |      |      |      |      |      |
| ≥ 68         | 51   | 47(50.0) | 4(4.3) | 42   | 33(41.3) | 9(11.3) | 0.3 |
| <68          | 43   | 37(39.4) | 6(6.4) | 38   | 33(41.3) | 5(6.3) | 0.3 |
| Gender       |      |      |      |      |      |      |      |      |
| Male         | 57   | 17 (18.1) | 40(42.6) | 0.1 |
| Female       | 37   | 16 (17) | 21(22.3) | 30   | 18 (22.5) | 12(15) | 0.4 |
| Location     |      |      |      |      |      |      |      |      |
| left side    | 39   | 17(18.1) | 22(23.4) | 0.1 |
| right side   | 55   | 16 (17.0) | 39(41.5) | 0.4 |
| Histology    |      |      |      |      |      |      |      |      |
| well/mod     | 79   | 28 (33.3) | 51(60.7) | 0.8 |
| muc/pap      | 5    | 2 (2.3) | 3 (3.6) | 0.03 |
| Clinical stage |     |      |      |      |      |      |      |      |
| I            | 16   | 4 (4.3) | 12 (12.7) | 15   | 11(13.8) | 4 (5) | 0.3 |
| II           | 35   | 16 (17) | 19 (20.2) | 31   | 14(17.5) | 17 (21.3) | 0.3 |
| IIIa         | 25   | 7 (7.4) | 18 (19.1) | 22   | 11 (13.8) | 11 (13.8) | 0.3 |
| IIIb         | 18   | 6 (6.4) | 12 (12.8) | 12   | 7 (8.8) | 5 (6.3) | 0.3 |

P-values for comparisons:
- Age: 0.3, 0.7, 0.2, 0.8
- Gender: 0.1, 0.4
- Location: 0.1, 0.4
- Histology: 0.8
- Clinical stage: 0.7, 0.7

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|                | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
|----------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| LN meta        |          |          |          |          |          |          |          |          |          |          |
| Positive       | 43 (13.8)| 13 (13.8)| 30 (31.9)| 12       | 16 (20.3)| 22 (23.4)| 0.9      | 34 (21.3)| 18 (22)  | 16 (19.5)|          |
| Negative       | 51 (21.3)| 20 (21.3)| 31 (32.9)| 22 (26.3)| 0.3      | 16 (20.3)| 22 (23.4)| 0.8      | 48 (26.8)| 26 (31.7)|          |
| Ly infiltration|          |          |          |          |          |          |          |          |          |          |
| Positive       | 68 (24.4)| 51 (24.4)| 45 (47.9)| 34 (35)  | 0.6      | 34 (35)  | 32 (39)  | 0.3      | 29 (35.4)| 10 (12.2)| 0.7      |
| Negative       | 26 (10.6)| 10 (10.6)| 16 (17)  | 12 (15)  | 9 (11.3) | 16 (17)  | 0.7      | 11 (13.4)| 16 (17)  | 10 (12.2)|          |
| V infiltration |          |          |          |          |          |          |          |          |          |          |
| Positive       | 79 (27.7)| 53 (27.7)| 53 (56.4)| 36 (45)  | 0.3      | 36 (45)  | 39 (48)  | 0.9      | 35 (42.7)| 10 (12.2)| 0.2      |
| Negative       | 15 (7.4)| 7 (7.4)  | 8 (8.5)  | 7 (8.8)  | 0.7      | 7 (8.8)  | 8 (8.5)  | 0.9      | 15 (7.4) | 10 (12.2)|          |

well/mod, well/moderately differentiated; muc/pap, mucinous/papillary
n, number of cases; LN meta, lymph node metastasis; Ly, lymph vessel; V, venous vessel
Cut-off values for each category were defined as (mean - standard deviation) values
Clinical stage refers to the criteria of the Japanese Classification of Colorectal, Appendiceal and Anal Carcinoma
Table S4. Correlation between TE grade and BD score in LAd-RC after NCRT

| BD score | TE grade | 1 (n) | 2 (n) | P-value |
|----------|----------|-------|-------|---------|
| 1        | n        | n (%) | n (%) | 0.004   |
| 2        | 11       | 7 (14)| 4 (8) |         |
| 3        | 11       | 11 (22)| 0     |         |

n, number of cases
**Table S5.** Univariate analysis for OS and PFS in LAd-RC after NCRT

| Variables               | Cut-off | Log rank c2 | P-value | Unfavorable factor |
|-------------------------|---------|-------------|---------|--------------------|
| **Overall survival**    |         |             |         |                    |
| PD-L1 (stroma)          | 1       | 3.9         | 0.06    |                    |
| PD-1 (stroma)           | 1       | 0.038       | 0.84    |                    |
| CD4 (stroma)            | 1       | 0.008       | 0.93    |                    |
| CD8 (stroma)            | 3       | 0.004       | 0.95    |                    |
| CD68 (stroma)           | 1       | 0.56        | 0.45    |                    |
| MMR status              | prof/def| 0.0004      | 0.98    |                    |
| Age                     | 63/64   | 2.95        | 0.33    |                    |
| Gender                  | Male/Female | 0.45       | 0.5     |                    |
| Pathological stage      | < II >/ III | 6.05      | 0.018   | > III              |
| LN metastasis           | −/+     | 7.5         | 0.008   | +                  |
| Ly involvement          | −/+     | 0.47        | 0.49    |                    |
| Venous involvement      | −/+     | 1.67        | 0.2     |                    |
| BD score                | BD:1/BD:2,3 | 2.16      | 0.15    |                    |
| **Progression-free survival** |         |             |         |                    |
| PD-L1 (stroma)          | 1       | 2.19        | 0.15    |                    |
| PD-1 (stroma)           | 1       | 0.31        | 0.58    |                    |
| CD4 (stroma)            | 1       | 0.001       | 0.98    |                    |
| CD8 (stroma)            | 3       | 0.048       | 0.83    |                    |
| CD68 (stroma)           | 1       | 0.67        | 0.4     |                    |
| MMR status              | prof/def| 0.09        | 0.77    |                    |
| Age                     | 63/64   | 0.023       | 0.88    |                    |
| Gender                  | Male/Female | 1.24     | 0.27    |                    |
| Pathological stage      | < II >/ III | 10.9       | 0.001   | > III              |
| LN metastasis           | −/+     | 13.6        | 0.0005  | +                  |
| Ly involvement          | −/+     | 2.15        | 0.15    |                    |
| Venous involvement      | −/+     | 3.47        | 0.07    |                    |
| BD score                | BD:1/BD:2,3 | 1.73      | 0.19    |                    |

LN, lymph node; Ly, lymph vessel; prof/def, proficient/deficient; BD, budding
Cut-off values for each category were defined as (mean - standard deviation) values
Clinical stage refers to the criteria of the Japanese Classification of Colorectal, Appendiceal and Anal Carcinoma