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Combined CDX2 Expression and TIL Density as a Prognostic Indicator in Adjuvant FOLFOX-Treated Stage III Colorectal Cancer.

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Abstract:

Background: Colorectal carcinomas (CRCs) with caudal-type homeobox 2 (CDX2) loss are associated with aggressive behavior but often exhibit high densities of tumor-infiltrating lymphocytes (TILs). The interplay between CDX2 loss and TIL density in CRC patient survival remains unclear.

Methods: Stage III CRC tissues were assessed for CDX2 loss by immunohistochemistry. CD8 TIL densities in intraepithelial (iTILs) and stromal areas were quantified using a machine learning-based analytic method.

Results: CDX2 loss was significantly associated with higher CD8 TIL densities in both intraepithelial and stromal areas. Both CDX2 loss and high CD8 iTIL density were independent prognostic factors for cancer-specific survival, with hazard ratios (HRs) of 2.314 (95% CI: 1.050–5.100) and 0.378 (95% CI: 0.175–0.817), respectively. CRCs with retained CDX2 expression and high CD8 iTIL density showed the best clinical outcome (HR: 0.138, 95% CI: 0.023–0.826), while those with CDX2 loss and high CD8 iTIL density exhibited the worst outcome (HR: 15.781, 95% CI: 3.939–63.230).

Conclusions: High CD8 iTIL density did not improve survival in CRCs with CDX2 loss. The combined assessment of CDX2 expression and intraepithelial CD8 TIL density is an independent prognostic marker in adjuvant chemotherapy-treated patients with stage III CRC.

Keywords: CD8 antigens, CDX2 transcription factor, Colorectal neoplasms, Prognosis, Lymphocytes, tumor-infiltrating.

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Introduction

Colorectal cancer (CRC) stands as a significant global health burden, representing one of the most commonly diagnosed malignancies and a leading cause of cancer-related mortality. The successful management of CRC hinges upon accurate staging, effective treatment strategies, and precise prognostication, particularly in stage III disease, where lymph node involvement necessitates adjuvant chemotherapy. Despite advancements in therapeutic interventions, including the widespread adoption of adjuvant FOLFOX (folinic acid, 5-fluorouracil, and oxaliplatin) chemotherapy, the clinical outcomes of stage III CRC patients remain heterogeneous. This underscores the need for robust prognostic markers to guide personalized treatment decisions and improve patient survival.

The staging system established by the American Joint Committee on Cancer (AJCC) serves as the cornerstone for clinical management, providing essential information about tumor extent and lymph node involvement. However, clinicopathological staging alone often fails to capture the intricate biological heterogeneity of CRC. Increasingly, research has focused on identifying molecular and immunological biomarkers that can refine risk stratification and predict treatment response. Among these, the transcription factor CDX2 and tumor-infiltrating lymphocytes (TILs) have emerged as promising candidates.

CDX2, a homeobox protein crucial for intestinal differentiation and maintenance, plays a pivotal role in the pathogenesis of CRC. Loss or reduced expression of CDX2 has been associated with aggressive tumor behavior, including increased invasiveness, metastasis, and poor prognosis. Notably, CDX2 expression is linked to specific molecular subtypes of CRC, further emphasizing its potential as a prognostic and predictive biomarker. In the context of adjuvant chemotherapy, the impact of CDX2 expression on treatment response remains an area of ongoing investigation.

TILs, representing the host immune response against tumor cells, have garnered considerable attention as prognostic markers in various malignancies, including CRC. The presence and density of TILs within the tumor microenvironment reflect the interplay between tumor cells and the immune system. A robust TIL infiltration, particularly of cytotoxic T lymphocytes, is generally associated with improved patient outcomes. The immune microenvironment plays a vital role in dictating the effectiveness of adjuvant therapies, thereby rendering the evaluation of TILs particularly relevant in patients undergoing FOLFOX chemotherapy.

The combined assessment of CDX2 expression and TIL density holds the potential to provide a more comprehensive and nuanced understanding of tumor biology and immune interactions. CDX2 expression potentially informs the tumours biological aggressiveness, while the TILs provides indication of host immune system responses. We hypothesis that The integrated analysis of these markers may allow improved risk stratification, potentially enabling clinicians to identify patients who are more likely to benefit from adjuvant FOLFOX, and those who might benefit from alternative or intensified therapeutic approaches.

Stage III CRC patients receiving adjuvant FOLFOX are a specific group, that need more refined prognostic tools. Adjuvant FOLFOX is a systemic chemotherapy given after the surgical removal of a cancer. Its purpose is to destroy any remaining cancer cells that may have spread but are too small to be detected. This adjuvant therapy, while effective, it still results in varying outcomes among patients.

Therefore, this retrospective cohort study aims to investigate the combined prognostic value of CDX2 expression status and TIL density in stage III CRC patients treated with adjuvant FOLFOX chemotherapy. By analyzing a cohort of patients with well-defined clinical

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characteristics, we seek to determine whether the integrated assessment of these biomarkers can enhance risk stratification and provide valuable insights into patient prognosis. Ultimately, this research strives to contribute to the development of personalized treatment strategies that optimize outcomes for stage III CRC patients.

Materials and Methods

Study Population:

Archival tissue blocks from surgical specimens of 505 stage III colorectal cancer (CRC) patients who received adjuvant FOLFOX after curative resection (R0) at Seoul National University Hospital (SNUH) between April 2005 and December 2012 were used to construct a tissue microarray (TMA). Whole-slide immunostaining for CD3 and CD8 was available for 446 of these patients. Inclusion criteria were: age ≥18 years, adenocarcinoma histology, stage III CRC, complete tumor resection (R0), and completion of at least six cycles of 5-fluorouracil plus oxaliplatin or four cycles of capecitabine plus oxaliplatin as adjuvant therapy. Exclusion criteria included: neoadjuvant chemotherapy or radiotherapy, hereditary polyposis syndromes, inflammatory bowel disease, or a history of other malignancies within 5 years. Electronic medical records were reviewed to collect demographic and clinicopathological data, including age, sex, tumor location, histological differentiation, lymphovascular invasion, perineural invasion, and AJCC/UICC stage (7th edition).

Immunohistochemistry (IHC):

One pathologist (S.Y.Y.) selected the most representative paraffin tissue block. Whole-slide IHC was performed using antibodies against CD3 (clone F7.2.38, Dako) and CD8 (clone SP57, Ventana Medical Systems). TMA blocks received 2-mm cores from two separate tumor regions. TMA sections (4 μm) were stained with primary antibodies against KRT7 (clone OV-TL 12/30, Dako), KRT20 (clone Ks20.8, Dako), and CDX2 (clone EPR2764Y, Cell Marque). Slides were scanned using an Aperio AT2 slide scanner (Leica Biosystems). KRT7 and KRT20 expression was scored based on the percentage of positively stained tumor cells, with thresholds of ≥10% for high KRT7 expression and <50% for low KRT20 expression. CDX2 expression was assessed using the H-score (3×percentage of strongly stained nuclei + 2×percentage of moderately stained nuclei + 1×percentage of weakly stained nuclei), with an H-score <20 defined as loss of expression. CD3 and CD8 IHC virtual slide files were analyzed using an automated pipeline (http://dx.doi.org/10.17504/protocols.io.yqvfvw6). After tumor area marking, the algorithm divided the area into 1-mm × 1-mm tiles and calculated median densities of intraepithelial TILs (iTILs) and stromal TILs (sTILs) (cells/mm²).

DNA Extraction, Microsatellite Instability (MSI), and Mutation Analysis:

Tumor areas with representative histology were marked on glass slides, and corresponding areas were scraped from unstained slides after deparaffinization. DNA was extracted using

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tissue lysis buffer and proteinase K. MSI status was determined by fluorescent multiplex PCR using five NCI-recommended microsatellite markers (BAT25, BAT26, D2S123, D5S346, D17S250). Tumors were classified as MSI-high (MSI-H, ≥2 unstable markers) or microsatellite-stable (MSS, ≤1 unstable marker) (n=503). BRAF V600E mutations were analyzed by real-time PCR-based allelic discrimination (n=492). KRAS mutations at codons 12 and 13 of exon 2 were determined by sequencing (n=486).

Bisulfite Modification and Methylation Analysis:

Genomic DNA was bisulfite-converted using an EZ DNA methylation kit (Zymo Research). Methylation levels of CIMP-specific markers (CACNA1G, CDKN2A (p16), CRABP1, IGF2, MLH1, NEUROG1, RUNX3, SOCS1) were assessed using the MethyLight assay (n=500). The percentage of methylated reference (PMR) was calculated, and markers with median PMR >4 were considered methylated. Tumors were classified as CIMP-high (CIMP-H, ≥5 methylated markers), CIMP-low (CIMP-L, 1–4 methylated markers), or CIMP-0 (no methylation).

Statistical Analysis:

TIL density distribution was assessed using the Shapiro-Wilk test. Non-parametric Mann-Whitney U tests were used to compare TIL densities between CDX2 loss and retention groups. Student's t-tests were used to compare age distributions. Chi-square and Kruskal-Wallis tests were used for categorical variable comparisons. Cancer-specific survival (CSS) and recurrence-free survival (RFS) were analyzed using Kaplan-Meier log-rank tests. Cox proportional hazards models were used to estimate hazard ratios, adjusted for clinicopathological factors (differentiation, venous invasion, lymphatic emboli, T category, N category, CK7 expression, KRAS mutation, CD3 sTIL, and CD8 sTIL) using a backward stepwise method. A p-value <0.05 was considered statistically significant.

Results

The mean follow-up period for the 505 patients was 68.2 months (range: 4.1–134.8 months). Demographic data are summarized in Table 1. The cohort consisted of 303 males and 202 females. Tumor subsites were: right colon (n=150), left colon (n=289), and rectum (n=66). CIMP-high (CIMP-H) and MSI-high (MSI-H) colorectal cancers (CRCs) were present in 5.4% and 5.6% of stage III CRCs, respectively. KRAS and BRAF mutations occurred in 28.8% and 3.5% of patients, respectively.

Relationships Between CDX2 Loss and Clinicopathological Features:

CDX2 loss was observed in 12.5% of stage III CRCs (Figure 1). CDX2 loss was significantly associated with younger age at diagnosis (56.1 vs. 59.8 years, p=0.003), right-sided tumor location, high-grade histological differentiation, CIMP-H status, MSI-H status, BRAF

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mutations, decreased KRT20 expression, and KRT7 expression (Table 1). CRCs with CDX2 loss exhibited significantly higher densities of both intraepithelial CD8 tumor-infiltrating lymphocytes (CD8 iTILs) and stromal CD8 tumor-infiltrating lymphocytes (CD8 sTILs) compared to CRCs with retained CDX2 expression (Figure 2). The difference was more pronounced for CD8 iTILs. While CD3 iTIL and sTIL densities tended to be higher in CDX2 loss CRCs, the difference did not reach statistical significance (Figure 2).

Association of CDX2 Loss with Clinical Outcome:

Univariate survival analysis revealed that CDX2 loss was significantly associated with shortened cancer-specific survival (CSS) but not recurrence-free survival (RFS) (Figure 3A, B). Several clinicopathological parameters, including tumor differentiation, T category, N category, lymphatic emboli, venous invasion, KRAS mutation, KRT7 expression, CD3 sTILs, CD8 iTILs (Figure 3C, D), and CD8 sTILs, were significantly associated with CSS in univariate analysis (Table 2).

Combined CDX2 Expression and CD8 iTIL Density as a Prognostic Parameter:

The combination of CDX2 expression and CD8 iTIL density created four subgroups of CRCs. Kaplan-Meier analysis of CSS showed that the subgroup with retained CDX2 expression and high CD8 iTIL density had the best clinical outcome. Subgroups with CDX2 loss, regardless of CD8 iTIL status, had worse outcomes than the CDX2-retained, high-iTIL subgroup (Figure 4). No significant survival difference was observed between CDX2 loss subgroups with high vs. low CD8 iTIL density ($p=0.384$ for CSS, $p=0.501$ for RFS). Multivariate analysis identified the combined CDX2 expression and CD8 iTIL density as an independent prognostic parameter.

Review of Literature:

In this study, we investigated whether CDX2 loss was associated with shortened survival, altered tumor-infiltrating lymphocyte (TIL) density, and whether there was an interplay between CDX2 expression and TIL density in stage III colorectal cancer (CRC) patients treated with adjuvant FOLFOX. Our findings demonstrated that CDX2 loss was a poor prognostic factor, accompanied by increased CD8 intraepithelial TILs (iTILs) and stromal TILs (sTILs), and that the combined CDX2 expression and CD8 iTIL density was an independent prognostic parameter.

We observed that CDX2 loss CRCs exhibited higher CD8 iTIL and sTIL densities compared to those with retained CDX2 expression. However, the prognostic implications of CDX2 loss and high CD8 iTIL density were conflicting, as CDX2 loss was associated with worse survival while high CD8 iTIL density correlated with better survival.

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Consistent with Derangere et al. [24], we found that CRCs with retained CDX2 expression and high CD8 iTIL density had the best clinical outcome. However, in contrast to their study, which reported the worst prognosis in CRCs with low CDX2 expression and low CD3 TIL density, we observed the worst outcome in CRCs with CDX2 loss and high CD8 iTIL density. This discrepancy may be attributed to differences in cutoff values for CDX2 expression and TIL density. Derangere et al. classified a larger proportion of tumors as low CDX2 expressors and low CD3 TIL density, while we observed CDX2 loss in a smaller subset and used a different cutoff for CD8 iTIL density.

Given the established association between high CD8 iTIL density and favorable prognosis, we anticipated that CDX2 loss with low CD8 iTIL density would result in the worst survival. However, CD8 iTIL density did not significantly impact survival in CDX2 loss CRCs. This unexpected finding suggests that CD8 iTILs might be ineffective against CDX2 loss tumors. While the underlying mechanisms remain unclear, spatial transcriptomics at the single-cell level may provide insights. Our observation challenges the conventional belief of a direct correlation between high CD8 TIL numbers and improved survival [25], as exemplified in renal cell carcinoma [26-28] and pancreatic cancer [28]. CDX2 loss may indicate a distinct tumor biology where the prognostic role of CD8 TILs differs.

We found that CDX2 loss was associated with increased CD8 iTIL and sTIL infiltration. However, this association may be confounded by the known association between CDX2 loss, CIMP-high (CIMP-H), and MSI-high (MSI-H), all of which are linked to increased TILs. To address this, we analyzed CIMP-low/0 and microsatellite-stable (MSS) CRCs, demonstrating that CDX2 loss was independently associated with increased CD8 iTIL density.

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