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Original contribution

CDX2 and Muc2 immunohistochemistry as prognostic markers in stage II colon cancer☆,☆☆,★

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Summary The treatment for colorectal cancer is largely surgical followed by adjuvant chemotherapy in high-risk cases. In patients with stage II cancer, there is no clear benefit for chemotherapy, and the current tools for assessment of risk are inadequate. A recent study identified that colorectal cancer with a gene signature similar to undifferentiated colonic stem cells was associated with a worse outcome. It was later shown that loss of CDX2 detected by immunohistochemistry (IHC) alone resulted in a worse prognosis and that this could be used to predict patients who would benefit from chemotherapy. Having observed that CDX2 expression can be patchy, we elected to validate these prior results for clinical practice using whole-slide IHC. The pathology of all cases was reviewed, and 3 blocks were selected for CDX2 IHC. We also expanded the panel beyond CDX2 to assess whether other markers in the gene signature including CDX1, Muc2, GPX2, and villin could better predict outcome. Among 210 cases, CDX2 expression was diffusely lost in 11% and focally lost in 23% of cases. There was no difference in survival based on CDX2 expression, but Muc2 loss was associated with reduced survival (hazard ratio, 3.32; 95% confidence interval, 1.20 to 9.20). No significant differences in outcome were identified based on CDX1, GPX2, or villin expression. In keeping with this, assessment of The Cancer Genome Atlas gene expression data demonstrated that decreased Muc2 expression was associated with reduced overall survival. Our results with whole-slide IHC are different from the previous studies and caution against the use of CDX2 in isolation as a prognostic marker in clinical practice. We have identified that loss of Muc2 is associated with reduced survival. This supports the use of the colonic differentiation gene expression signature to identify high-risk patients but cautions against the use of any one IHC-based marker in isolation.

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1. Introduction

Treatment of colorectal cancer (CRC) is largely surgical with resection of the primary tumor and treatment with adjuvant chemotherapy in specific cases, such as stage III cancers where there is a clear survival benefit for the use of adjuvant chemotherapy [1]. However, in patients with stage II cancer, multiple clinical studies have failed to identify a significant benefit for the use of adjuvant therapy [2,3]. Most patients with stage II disease have a good prognosis with 5-year survival on the order of 75% [3]. There is a need to identify stage II patients who are at higher risk of disease recurrence, as these patients would likely benefit from adjuvant therapies. Classic high-risk features (such as T4 disease and lymphovascular or venous invasion) have failed to identify those patients who would benefit from adjuvant therapy [2]. Nevertheless, adjuvant therapy is still commonly used in clinical practice for stage II patients with perceived high risk [4].

Recently, it was demonstrated that tumors with gene expression signatures similar to undifferentiated colonic stem cells were more aggressive, and this could be used to predict relapse [5]. It was subsequently shown, using a tissue microarray–based experiment, that this could be simplified and loss of CDX2 alone could be used as a marker for the undifferentiated colonic stem cell gene signature [6]. In this study of stage II CRC, 9% of cases had loss of CDX2 expression, and this was associated with a significantly reduced disease-free survival [6]. This difference in outcome was not seen in CDX2-negative stage II patients who received chemotherapy [6], leading to the conclusion that loss of CDX2 could be used to predict patients who would respond to chemotherapy. CDX2 status was proposed as both a prognostic and predictive biomarker in CRC to identify early-stage high-risk colon cancer and predict those who would respond to chemotherapy. Publication of this role of CDX2 [7-11] was initially met with hope as a novel means to guide treatment decisions in CRC patients.

However, other groups reported that the findings could not be independently confirmed in other gene expression datasets of colon cancer [12]. Other studies have had variable findings; one showed that loss of CDX2 in metastatic disease was a negative prognostic indicator [13], whereas another study of patients with mostly advanced stage III and IV disease who received chemotherapy found that CDX2 was not an independent prognostic biomarker in CRC [14]. A gene expression study of a mixture of stage II and III cases found that CDX2 loss predicted a negative outcome only in tumors with the CMS4 molecular phenotype, a mesenchymal/stem cell phenotype [15]. Another group has generated a large tissue microarray of 613 colon cancers with multiple tumor areas arrayed to better appreciate intratumor heterogeneity but have yet to report outcome data from their study [16]. No study reported to date has been able to confirm the findings using whole-slide immunohistochemistry for CDX2 in stage II colon cancer.

CDX2 is a member of the *Caudal*-type homeobox (CDX) genes that are closely related to the *Hox* cluster and have a role in the patterning of embryos [17]. CDX2 is critical for intestinal differentiation; mice lacking CDX2 have replacement of the intestinal mucosa with gastric and esophageal type tissue [18]. Loss of CDX2 has been associated with distinct pathways of CRC tumorigenesis, for example, CpG island methylator phenotype (CIMP) [19-21]. There is a less clear association between CDX2 and microsatellite instability (MSI); although a number of studies have identified an association between CDX2 status and MSI [20-23], others have failed to identify that connection [24-26].

Other markers comprising the gene expression signatures for colonic differentiation included Muc2, CDX1, GPX2, and villin. CDX1 is a related transcriptional factor to CDX2 and also has a critical role in specifying intestinal differentiation [27] and its loss functions synergistically with loss of CDX2 to promote tumorigenesis in experimental models of CRC [28]. GPX2 has an important role in reducing H_2O_2 and has been shown to promote tumor cell differentiation [29]. Villin is an actin-binding protein within the brush border of enterocytes. Muc2 is a member of the mucin family of epithelial glycoproteins that are expressed in colonic epithelium and colorectal tumors [30,31]; loss of expression has been associated with adverse prognostic features in colon cancer [32].

The current study aimed to more broadly investigate the role of CDX2 status as well as other important markers of colonic differentiation of potential prognostic value. In tissue sections, expression of CDX2 is patchy (Fig. 1) and potentially can lead to misleading results from a tissue microarray–based experiment. Therefore, to develop a means to address the known variability in expression, we designed a retrospective study looking at CDX2 expression in stage II colon cancers using multiple blocks of whole-slide immunohistochemistry. We also explored the expression of Muc2, CDX1 GPX2, and villin to assess whether these might better predict outcome in CRC.

2. Materials and methods

2.1. Patient selection and case review

Institutional research ethics board approval for retrospective review and immunohistochemical testing was obtained. Pathology archives were searched for cases of colon cancer between 2006 and 2013. Rectal cancers were excluded because the effects of neoadjuvant therapy on CDX2 immunohistochemistry were not clear. Cases with less than 3 years of follow-up were excluded. Pathology reports were reviewed to select only stage II cases. The electronic charts were then reviewed, and cases that received adjuvant chemotherapy were excluded. In total, 210 cases of colon cancer were included in the study. The pathology reports and all tumor slides were reviewed by M. J. C. and one of the other pathologists (D. K. D., J. C. W., J. P., or S. C.). Three blocks of tumor were selected for CDX2 staining in each case that included representative histology, the deepest point of invasion, and any high-grade component or area of tumor with a distinct histology.

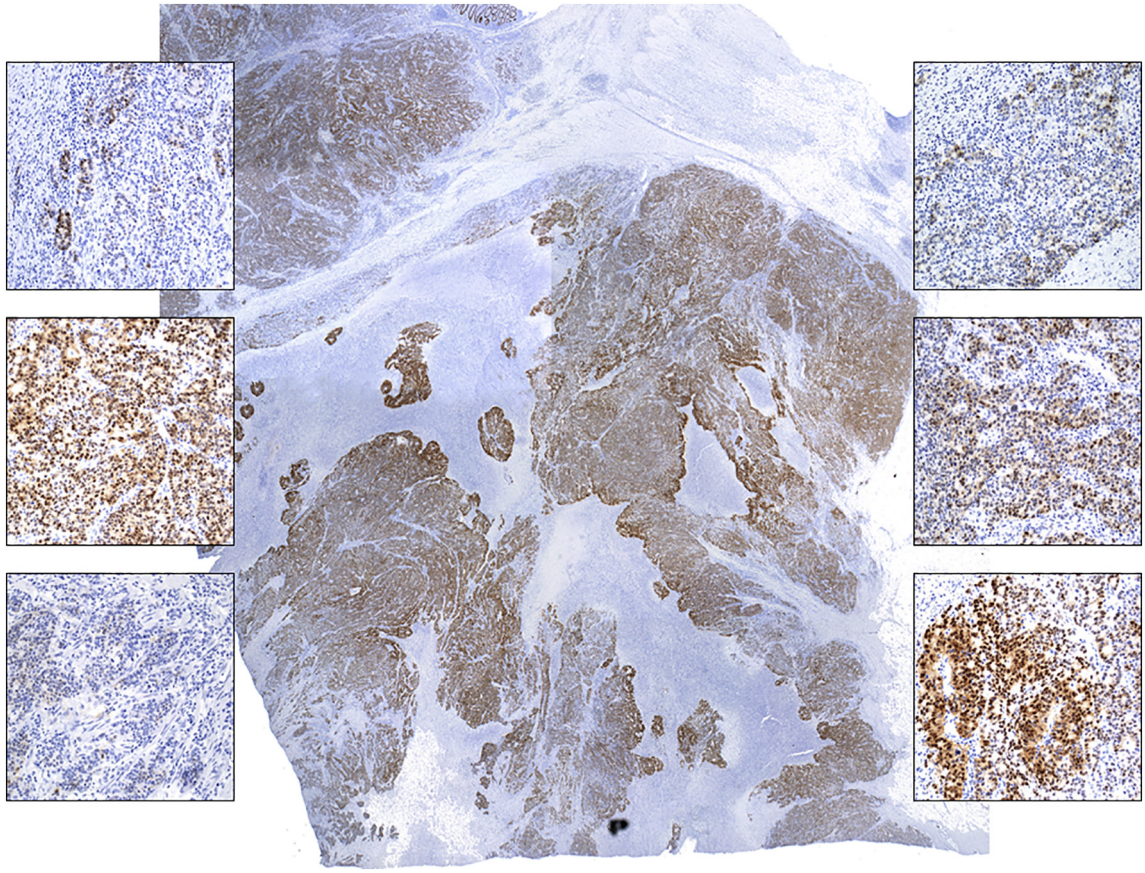

Figure 1 Low-power image of CDX2 immunohistochemistry with inset images of high-magnification (original magnification ×200) images showing distinct staining patterns observed in separate foci of the tumor.

Elastic stains were used routinely in most cases to aid in identifying venous invasion. Patient outcome was determined by manual review of the chart (by M. J. C. and R. C.) with survival calculated from the date of surgery to the date of cancer-related death or last follow-up appointment. To screen other markers of colonic differentiation, we selected a subset of 11 patients who died of CRC compared with the next historical case of a CRC patient who did not succumb to disease during the study period as a control. This subset was expanded for Muc2 to study the role of Muc2 expression in a larger cohort of cases.

2.2. Immunohistochemistry

Immunohistochemistry was performed on slides cut from paraffin-embedded blocks at 4 μm and dried at 60°C for 45 minutes and 45°C overnight. Staining was performed using the EnVision Flex System on the Autostainer Link 48 platform (Dako Santa Clara, CA). Antigen retrieval was completed in a 97°C water bath at pH 5.9 to 6.3 for 20 minutes, peroxidase block for 5 minutes, primary antibody for 20 minutes, mouse/rabbit linker for 15 minutes, horseradish peroxidase for 20 minutes, and diaminobenzidine for 10 minutes. Antibodies for CDX2 DAK-CDX2 (Dako [IR080]; ready to use) CDX2–88 (AbCam [ab157524]; 1:50 dilution), CDX1 (AbCam [ab188072]; 1:100 dilution), Muc2 (Dako [CCP58]; ready to use), GPX2 (AbCam [ab64322]; 1:1000), and villin (Dako [1D2 C3]; ready to use) were used. On slide, tissue controls were used as positive and negative controls for all cases. Immunohistochemical expression was scored as reported previously [6], with cases showing moderate to strong staining in most tumor cells scored as positive for all markers (Fig. 2).

2.3. Gene expression from The Cancer Genome Atlas database

The provisional database of colon cancer cases was accessed on August 5, 2018, through cbioportal.org, and 382 cases with RNA Seq data available were queried for cases with reduced Muc2 and CDX2 expression [33,34]. To detect cases with lower levels of Muc2 and CDX2, a cutoff of cases less than 0.4 SD from the mean was used.

3. Results

As demonstrated by the patchy staining of CDX2 in Fig. 1, it can be difficult to assess the CDX2 status of colon cancers from whole-slide immunohistochemistry. There are often

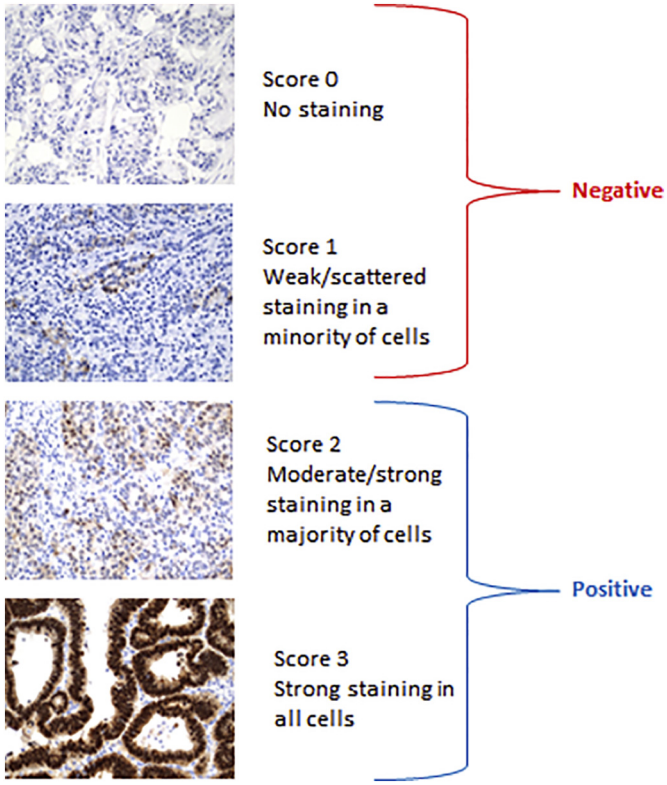

Figure 2 Semiquantitative immunohistochemistry scoring system (original magnification ×200).

focal areas in tumors that show loss of expression for CDX2 despite most of the tumor being positive. It is not clear from current studies how to score these cases. To address the variability, our approach used staining of multiple blocks for CDX2 in an effort to better understand the variability and identify a robust means to implement CDX2 status into clinical practice.

In total, we studied 210 cases from patients with an average age at diagnosis of 74 years, with an approximately equal split between male and female patients (Table). Most cases were right sided (69%). All cases were stage II lymph node negative. Most cases were T3, with 12% of cases staged as T4a or T4b. On average, 22 lymph nodes were identified. Most cases were low grade (94%). Lymphovascular invasion (small vessel invasion) was identified in 18% of cases, and venous invasion was reported in 25% of cases. In other series, venous invasion has been typically reported in the range of 30% to 40% with the routine use of elastic stains [35-37]. Our observed lower rate may reflect that our study was limited to patients with early stage disease and a small subset of our older cases predated the routine use of elastic stains for identification of venous invasion.

Most cases had diffuse strong complete expression of CDX2, as shown in Fig. 3. However, 11% had complete loss and 23% showed at least focal loss of CDX2 expression. Focal loss was defined as at least one high-power field meeting the criteria for loss of CDX2 expression. In many of the CDX2-negative cases, there were distinct areas of the tumor that were

Table Case demographics

Patient characteristics	
Age (y), mean ± SD	74 ± 11
Sex, n (%)	
Male	102 (49)
Female	108 (51)
Location, n (%)	
Right sided	146 (69)
Left sided	64 (31)
T stage, n (%)	
T3	184 (88)
T4a	18 (8)
T4b	8 (4)
Lymph nodes	
Average (range)	22 (4-110)
Number with <12 (%)	17 (8)
Grade, n (%)	
Low	198 (94)
High	12 (6)
Lymphovascular invasion, n (%)	
Positive	38 (18)
Negative	164 (78)
Indeterminate	8 (4)
Venous invasion, n (%)	
Positive	52 (25)
Negative	154 (73)
Indeterminate	4 (2)

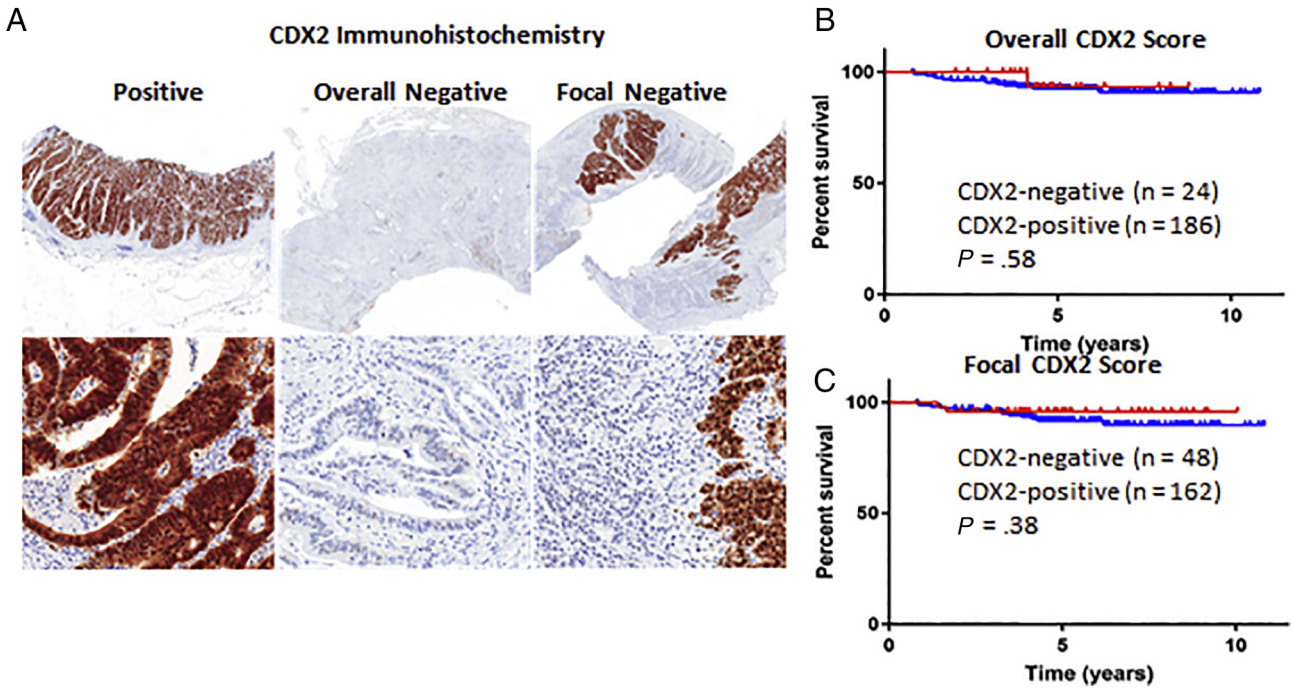

Figure 3 A, Representative CDX2 immunohistochemistry (high-power magnification ×200). Kaplan-Meier survival curves based on overall CDX2 score (B) and focal CDX2 score (C).

negative for CDX2 juxtaposed with areas of the tumor that maintained expression of CDX2. CDX2 expression was variable across the 3 slides examined, with 23 of 210 cases having variability in the overall score and 86 of 210 cases with variability in the focal score across the 3 stained slides.

The cancer-specific survival for our study population was favorable, with 7% of cases dying of CRC, which is in keeping with a stage II colon cancer population with no adjuvant treatment. In contrast to the previous study [6], we did not identify a difference in the cancer-specific survival for either cases with

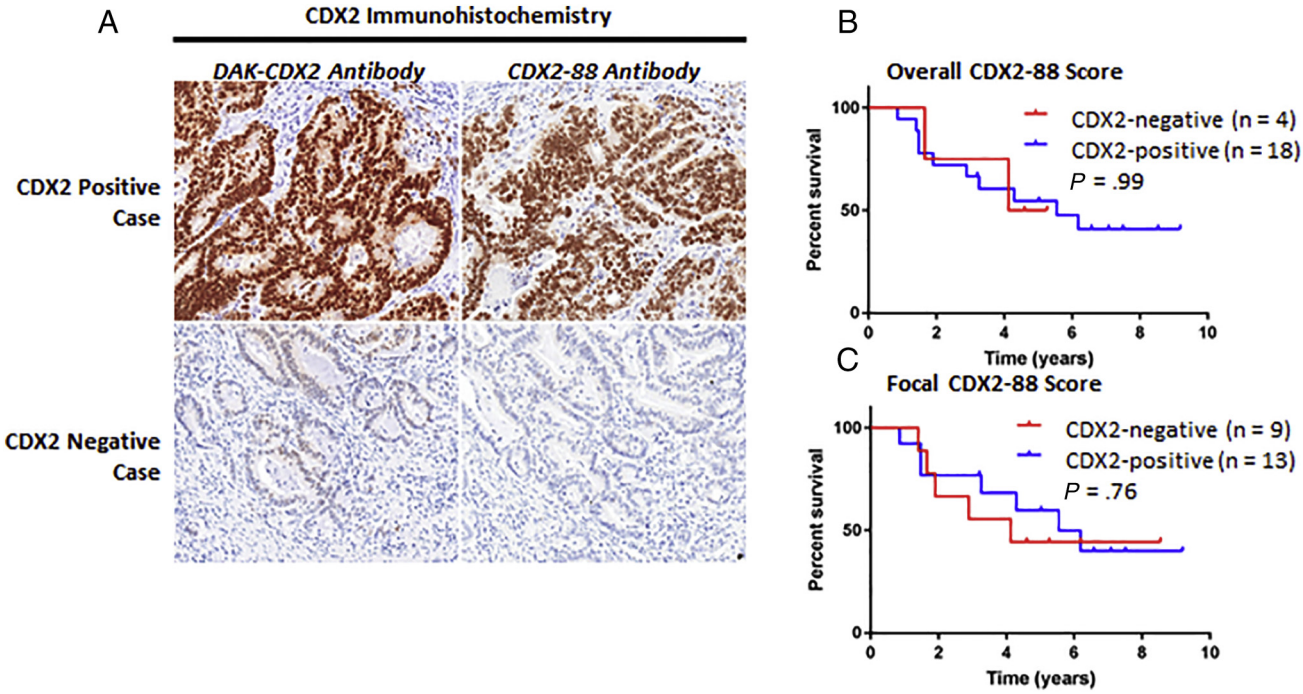

Figure 4 A, Representative CDX2 immunohistochemistry (original magnification ×200) using the DAK-CDX2 and CDX2-88 antibodies. Kaplan-Meier survival curves based on overall CDX2-88 score (B) and focal CDX2-88 score (C).

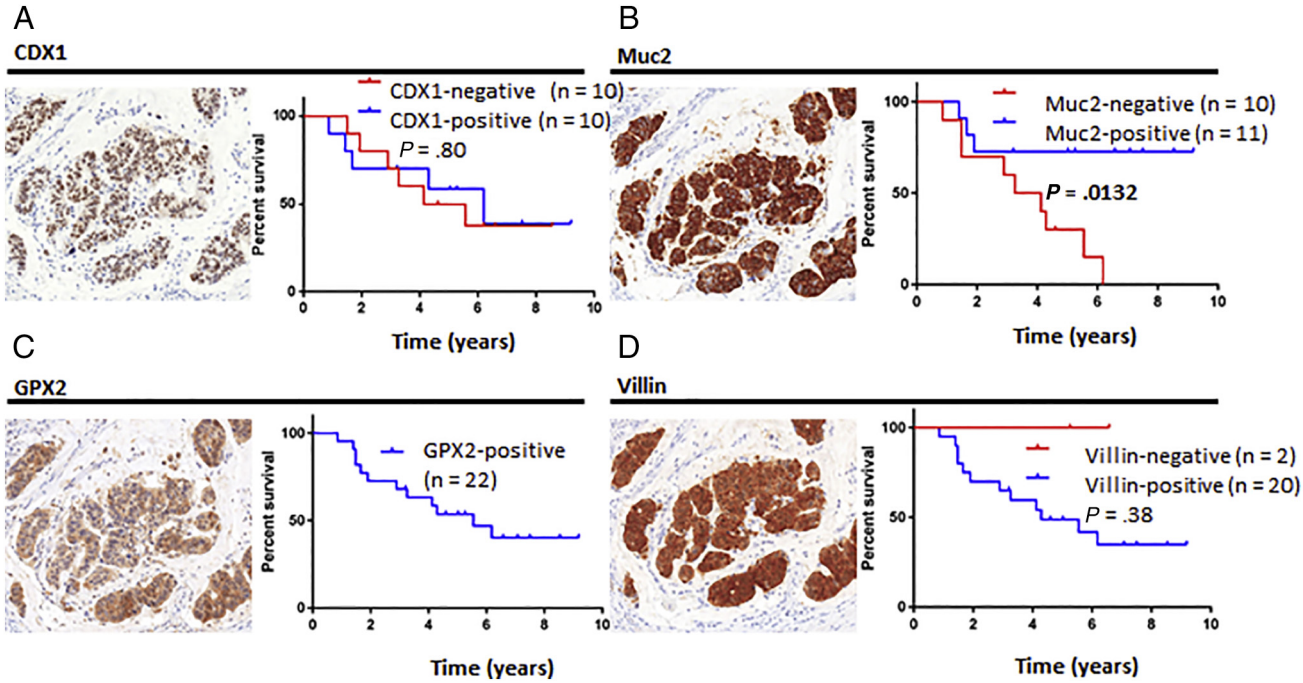

Figure 5 Representative immunohistochemistry (original magnification ×200) and Kaplan-Meier survival curves for CDX1 (A), Muc2 (B), GPX2 (C), and villin (D).

complete loss or focal loss of CDX2 (Fig. 3). We were adequately powered to detect a similar difference to the effect size seen in the 2 cohorts reported previously [6] with our 210 cases. All cases had at least 3 years of follow-up with an average follow-up of 5.3 years to detect possible late recurrences.

We used a more sensitive antibody for the detection of CDX2 than the antibody used in the previous study that used CDX2–88 [6]. The CDX2–88 antibody has been shown to be the least sensitive for CDX2 expression in a comparison of 5 commercially available CDX2 antibodies [38]. To ensure

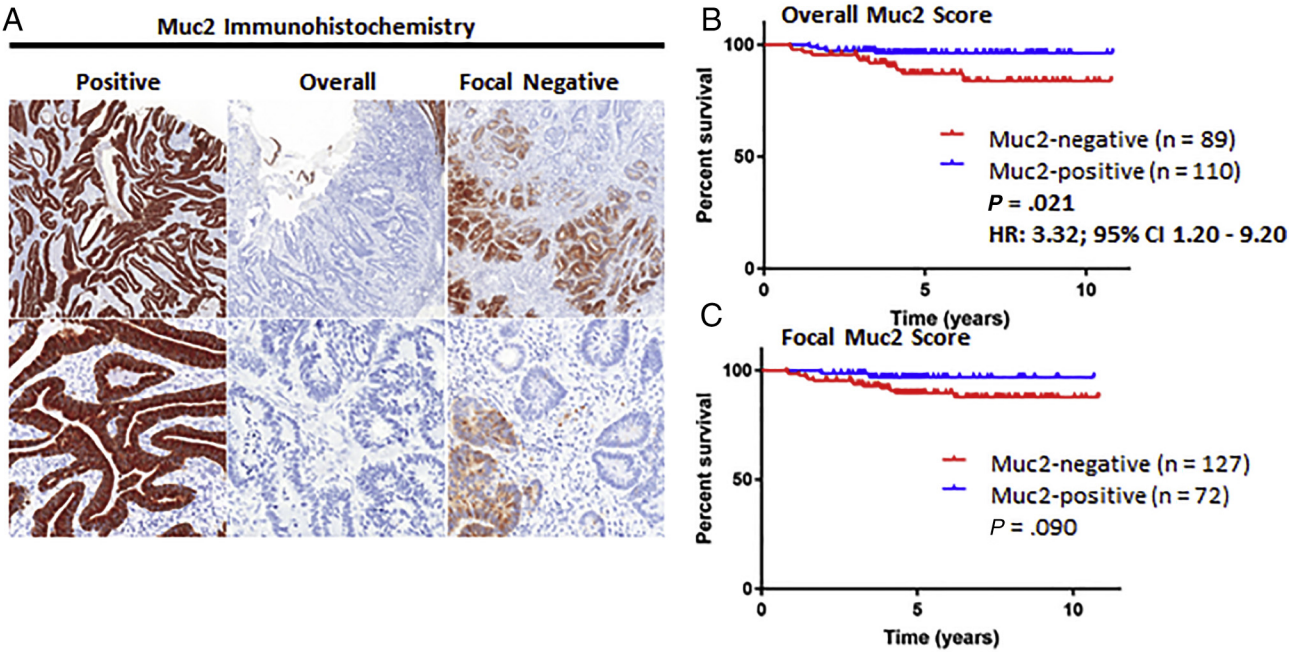

Figure 6 A, Representative Muc2 immunohistochemistry (original magnification ×200). Kaplan-Meier survival curves based on overall Muc2 score (B) and focal Muc2 score (C).

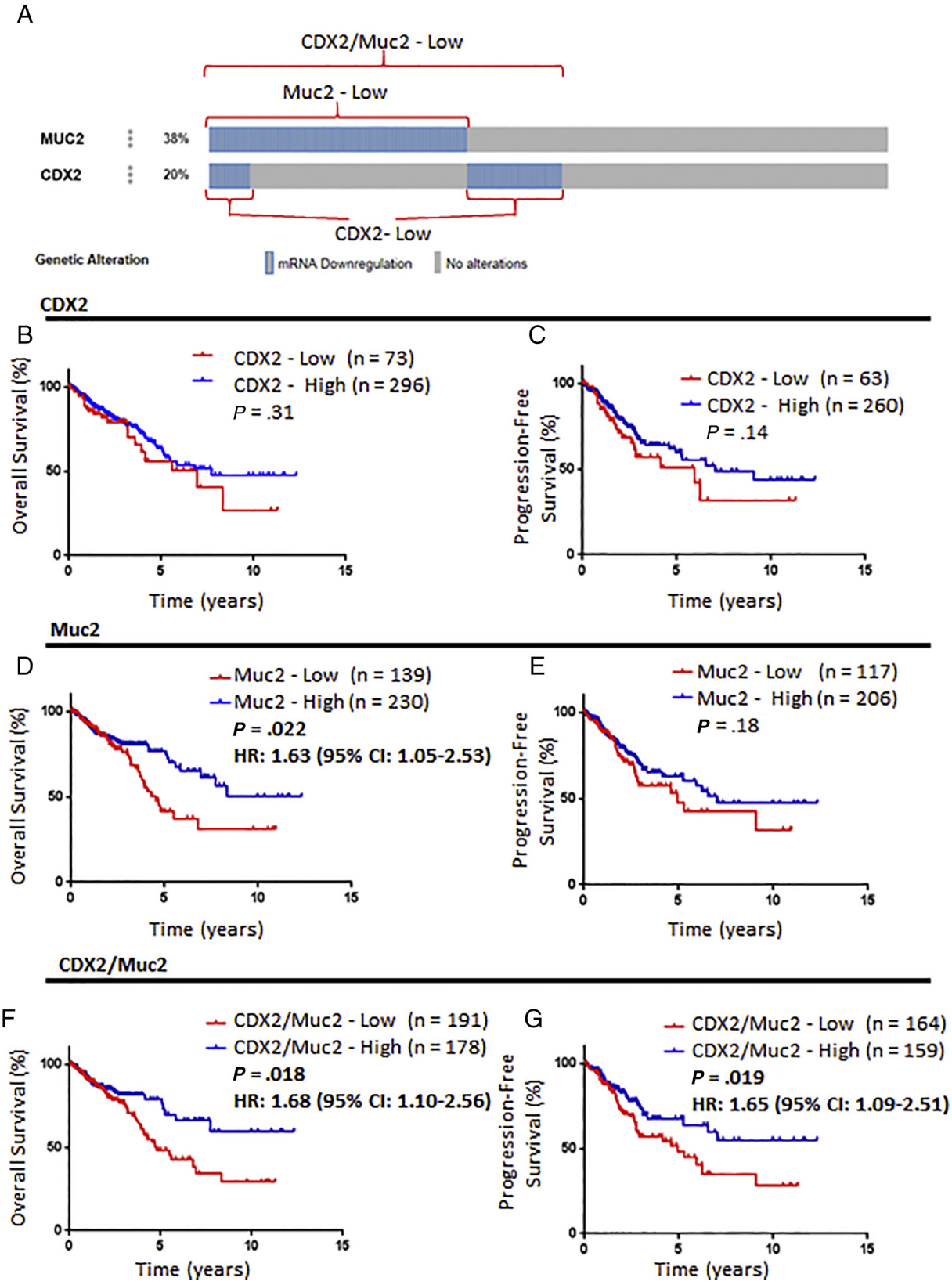

Figure 7 A, Prevalence of cases with low expression of CDX2 and Muc2 in the TCGA provisional database accessed August 2018. B-G, Kaplan-Meier curves for overall survival (B, D, and F) and progression-free survival (C, E, and G) for CDX2 (B and C), Muc2 (D and E), and combined CDX2 and Muc2 (F and G).

that the difference did not relate to the choice of antibody, we selected a subset of 22 cases that had a recurrence with matched control cases that did not have a recurrence. We found similar results with the CDX2-88 antibody used in previous studies with our DAK-CDX2 antibody, with slightly decreased expression noted for the CDX2-88 antibody in comparison to the DAK-CDX2 antibody. As shown in Fig. 4B and C, no difference in outcome was identified for cases stratified based on CDX2 expression assessed by the CDX2-88 antibody.

It has been shown that gene expression signatures can be used to identify colon cancers with a so-called dedifferentiated or stem cell–like gene expression pattern to identify cases that might have a negative outcome [5,6]. We reviewed the gene expression data from these studies and found other markers that could be assessed using immunohistochemistry. We evaluated the same 22 cases for expression of CDX1, Muc2, GPX2, and villin as potential markers to identify cases with a negative outcome. CDX1 was expressed in half of the cases and overall showed a weaker, patchier staining than CDX2. In a similar manner to CDX2, it was not associated with a difference in outcome (Fig. 5A). Villin was expressed in 20 of 22 cases and did not predict outcome (Fig. 5D). GPX2 was diffusely expressed in all 22 cases (Fig. 5C). In contrast, we found that Muc2 expression was lost in 10 of 21 cases and the Muc2-negative cases had a significant decrease in cancer-specific survival (Fig. 5B).

Given the observed difference in survival identified based on Muc2 status in our screening cohort of cases, we then expanded this to our larger cohort of cases to assess if Muc2 might be used to identify early-stage colon cancers with a poor outcome. As is shown in Fig. 6, Muc2 expression was completely lost in 42% of cases and focally lost in 60% of cases using the same scoring criteria used for CDX2. We found that overall loss but not focal loss of Muc2 was associated with a significant decrease in cancer-specific survival (hazard ratio, 3.32; 95% confidence interval, 1.20-9.20).

Although Muc2 is one of the downstream transcriptional targets of CDX2, 42% of cases had discordant overall expression of CDX2 and Muc2 with loss of expression of one but not both genes. Data from The Cancer Genome Atlas (TCGA) database of colon cancer cases showed reduced Muc2 gene expression in 38% of cases and reduced CDX2 expression in 20% of cases (Fig. 7A). However, there is little overlap between these cases; most cases with reduced Muc2 expression showed no decrease in CDX2 expression. Separating cases based on CDX2 expression alone in the TCGA cohort did not show a difference in overall survival or progression-free survival (Fig. 7B and C). In contrast, if Muc2 gene expression status is used, a significant difference in overall survival is present and the progression-free survival shows a trend toward improved survival in cases that express Muc2 (Fig. 7D and E). In cases with loss of CDX2 and/or Muc2, there is a significant difference in both overall and progression-free survival (Fig. 7F and G).

4. Discussion

Given the need for an improved means to identify high-risk early-stage CRC patients who would benefit from chemotherapy, some studies have suggested that CDX2 status may provide a means to identify these patients [6]. However, as our study shows, CDX2 expression status is difficult to implement into clinical practice as a reliable predictive biomarker. We found that CDX2 expression is variable within a section as well as between different sections of the same tumor. The decreased CDX2 expression is thought to be mediated by variable methylation of the CDX2 gene and has been associated with CIMP in CRC [19-21]. Furthermore, the lack of well-characterized mutations, deletions, or gene arrangements precludes the use of sequencing or fluorescence in situ hybridization–based assays to confirm equivocal cases. Taken together, this makes CDX2 immunohistochemistry a potentially unreliable biomarker for the prognostication of patients with CRC.

We focused our study on stage II patients who did not receive adjuvant chemotherapy, as this group had the largest difference in survival in the previous studies [6] and represents the population where there is an unclear role for chemotherapy. All cases were reviewed retrospectively, as this would be a challenging question to answer in a prospective manner given the low rate of recurrence and long follow-up times required. Ideally, the question of CDX2 as a predictive marker would be addressed in a randomized prospective trial, but this is beyond the scope of this and previous studies. In our cohort, there was an increased number of right-sided colon cancers, which may have selected for an increased number of MSI-related cancers. There are mixed results regarding the association between MSI status and CDX2 expression [20-26], but it seems clear that CDX2 loss is associated with the CIMP methylator phenotype [19-21] that is often seen in right-sided colon cancers. The MSI status is unknown for most of our cases, as they predated routine immunohistochemistry testing for MMR proteins at our institution. Given the fact that this question has been extensively studied in the literature with varying results and we have not identified an association between CDX2 and outcome, linking CDX2 and MSI status in our cohort would likely be uninformative.

We did not identify a difference in survival based on CDX2 immunohistochemical expression status as has been reported previously [6]. Other groups have also reported variable findings in terms of CDX2 expression and outcome in colon cancer [12-15]. The reason for the discordant results is not clear. To exclude an antibody factor, we repeated immunohistochemistry with the same antibody used in another study [6] in a subset of our cases (Fig. 4). We found a similar number of CDX2-negative cases in our cohort (11% versus 9%) to the previous study [6]. The rate of CDX2 loss in CRC has been variably reported in the literature from 4% to 29% [6,19,39-41]. The negative effect of CDX2 loss has been suggested to be limited to a specific subgroup of colon cancers with a mesenchymal gene expression signature [15]. One possible

explanation for the divergent results may be a larger number of cases with this mesenchymal gene signature in some patient cohorts.

Our study was powered to detect a difference based on the outcome reported in the previous 2016 study [6]. A very recent study has addressed this question using a large cohort of patients with 1157 stage II colon cancer cases and has found a statistically significant but less dramatic effect on outcome with a hazard ratio of 1.54 [42]. Using this effect size, our study would be underpowered to detect a difference in outcome based on CDX2 status in stage II colon cancer. However, in our cohort, Muc2 loss was associated with significantly reduced cancer-specific survival with a hazard ratio of 3.32. This suggests that other markers alone or in combination with CDX2 may better predict early-stage colon cancer with a poor prognosis that may benefit from additional therapy.

Predictive or prognostic biomarkers that test for expression of a gene that is regulated at the level of transcriptional control can be challenging, as they can often result in patchy variable expression detected on immunohistochemistry and do not allow for testing for genetic changes in equivocal cases. To address this challenge, we studied a panel of 6 immunohistochemistry markers involved in colonic differentiation. We found that CDX2 status in isolation may have limited utility in routine practice to assess for risk in colon cancer. However, Muc2 was identified as a potential marker for prognostication in CRC in our cohort and in gene expression data obtained from the TCGA; the combination of both CDX2 and Muc2 gene expression status was able to identify patients with a worse overall survival and increased rate of disease recurrence from the TCGA database.

A reproducible but small effect of CDX2 status has been identified in stage II colon cancer patients [6,15,42]; however, our work suggests that Muc2 may be a more effective means of prognostication. Instead of focusing on the expression of one marker by immunohistochemistry, it may be more accurate and reproducible to assess gene expression signatures to identify cases of CRC with a more undifferentiated phenotype as reported initially [5]. As next-generation sequencing and RNA-based testing become more prevalent in routine surgical pathology, there may be opportunities to capitalize on this technology to design further trials revising and better defining our prognostic and predictive tools in CRC.

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