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ARTICLE

Mismatch repair phenotype determines the implications of tumor grade and CDX2 expression in stage II–III colon cancer

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Abstract

Mismatch repair (MMR) deficiency is an indicator of good prognosis in localized colon cancer but also associated with lack of expression of caudal-type homeobox transcription factor 2 (CDX2) and high tumor grade; markers that in isolation indicate a poor prognosis. Our study aims to identify clinically relevant prognostic subgroups by combining information about tumor grade, MMR phenotype, and CDX2 expression. Immunohistochemistry for MMR proteins and CDX2 was performed in 544 patients with colon cancer stage II–III, including a cohort from a randomized trial. In patients with proficient MMR (pMMR) and CDX2 negativity, hazard ratio (HR) for cancer death was 2.93 (95% CI 1.23–6.99, $p = 0.015$). Cancer-specific survival for pMMR/CDX2-negative cases was 35.8 months (95% CI 23.4–48.3) versus 52.1–53.5 months (95% CI 45.6–58.6, $p = 0.001$) for the remaining cases (CDX2-positive tumors or deficient MMR (dMMR)/CDX2-negative tumors). In our randomized cohort, high tumor grade was predictive of response to adjuvant fluorouracil–levamisole in pMMR patients, with a significant interaction between tumor grade and treatment ($p = 0.036$). For pMMR patients, high tumor grade was a significant marker of poor prognosis in the surgery-only group (HR 4.60 (95% CI 1.68–12.61), $p = 0.003$) but not in the group receiving chemotherapy (HR 0.66 (95% CI 0.15–3.00), $p = 0.587$). To conclude, patients with pMMR and CDX2 negativity have a very poor prognosis. Patients with pMMR and high-graded tumors have a poor prognosis but respond well to adjuvant chemotherapy. CDX2 expression and tumor grade did not impact prognosis in patients with dMMR.

Introduction

Colorectal cancer is the second most common cause of cancer death worldwide [1]. To reduce the risk of relapse after colon cancer surgery, adjuvant chemotherapy is offered to patients with stage III disease and to high-risk patients with stage II disease [2]. Still, the definition of high-risk colon cancer remains controversial [3, 4]. Biomarkers identifying patients with a high probability of relapse or patients with tumors sensitive to adjuvant chemotherapy could significantly improve treatment stratification across cancer stage [5, 6].

Microsatellite instability (MSI) is a manifestation of defective DNA mismatch repair (dMMR) and is found in 15–20% of patients with localized colon cancer [7–9]. It is caused by a germline mutation in one or more of the MMR genes, double somatic MMR gene inactivation or a somatic hypermethylation of the MLH-1 gene promoter [8–11]. The high number of mutations associated with dMMR leads to expression of cell membrane neoantigens. This often facilitates a strong anti-tumor immune response [6, 9], contributing to the low risk of relapse for dMMR patients with localized disease [7, 9]. In addition, treatment with fluorouracil (5-FU) yields no survival benefit in patients with stage II colon cancer and dMMR [12, 13]. Therefore, stage II colon cancer patients with dMMR are usually not eligible for adjuvant chemotherapy [2]. In metastatic colorectal cancer, dMMR

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predicts response to anti PD-1 immunotherapy and an studies suggest an improved prognosis for cases with concurrent BRAF-wild type [11].

However, dMMR is also associated with markers that in isolation have a negative impact on prognosis, such as high tumor grade and lack of expression of cell maturation marker caudal-type homeobox transcription factor 2 (CDX2) [14–17]. CDX2 is an emerging biomarker in colon cancer [16, 18, 19]. This nuclear transcription factor regulates proliferation and differentiation of intestinal epithelial cells and is a diagnostic biomarker for mature colon epithelial tissue [20, 21]. CDX2 immunohistochemistry (IHC) is integrated in the clinical diagnostics of gastrointestinal adenocarcinomas in cancers of unknown origin [8, 22]. In the pivotal study by Dalerba et al., lack of CDX2 expression identified a high-risk subgroup of localized colon cancer patients. The authors also proposed that CDX2-negative stage II patients benefit from adjuvant chemotherapy [16].

Histological grading is based on the degree of tumor glandular formation. High tumor grade is found in ~20% of colon adenocarcinomas and is considered a high-risk trait in stage II colon cancer and a marker of poor prognosis [2, 8, 15]. The predictive role of high tumor grade on chemotherapy effect in colon cancer is not established. Tumors with dMMR are detected in several morphological subtypes but overrepresented in mucinous adenocarcinomas, medullary carcinomas, and colon adenocarcinoma NOS (not otherwise specified) with high-grade morphology [7, 11, 17]. Central studies challenge the role of histological grading in the definition of high-risk localized colon cancer [23, 24], but the prognostic effect of high tumor grade may be confounded when not incorporating MMR phenotype in the assessment [25]. Rosty et al. propose that for colon adenocarcinoma NOS, the positive prognostic impact of dMMR might outweigh the negative impact of high tumor grade on survival [25].

Prognostic assessments for patients with tumors featuring dMMR, CDX2 negativity and high-grade morphology are conflicting, despite the large overlap between these traits. To choose the optimal treatment for these patients, the impact of MMR phenotype on the prognosis of CDX2-negative and on high-grade tumors needs to be established.

Our study aims to assess the prognostic value of MMR phenotype in CDX2-negative and/or high-grade colon cancer. We hypothesize that by combining information about tumor grading, CDX2 expression and MMR phenotype, we can identify clinically relevant prognostic subgroups. This report also assesses the impact of CDX2 negativity, MMR phenotype, and tumor grading on adjuvant chemotherapy effect in our randomized controlled cohort.

Material and methods

Study cohort

The study cohort originates from two different patient materials. For material 1 (the Norwegian Gastrointestinal Cancer Group (NGICG) cohort), tissue from primary tumors was obtained from patients in a randomized controlled trial previously described by Dahl et al. [26]. In brief, 425 patients with colon or rectal cancer stage II and III were included between 1993 and 1996 and randomized to treatment with 5-FU and levamisole after surgery or to surgery only. Material 2 is a population-based cohort consisting of 374 patients undergoing complete (D3) mesocolic excision for stage I–IV colon cancer at Haraldsplass Deaconess Hospital (HDH) [27, 28]. Patients were included between January 2007 and December 2011. Thorough 5-year follow-up data and clinicopathological data were available for both materials [26, 27]. For the current study, we included cases from both materials with colon cancer stage II or III, 544 patients in total (276 patients from the NGICG cohort and 268 from the HDH cohort).

Immunohistochemistry

Tissue microarrays (TMAs) with 1 mm core diameter were constructed from formalin fixed paraffin embedded tissue in sections of 3–5 µm. CDX2 staining was performed using the diagnostic protocol on a Ventana BenchMark Ultra. After deparaffinization and rehydration, target retrieval was done with CC1 reagent (Ventana Medical systems) for 48 min. For the primary antibody (CDX2 clone EPR2764Y, Cell Marque, anti-rabbit monoclonal, 1:200 dilution), incubation time was 32 min. OptiView amplifier was used (4 min), slides were counterstained with hematoxylin, dehydrated, and mounted. Positive controls included tonsil, appendix, and pancreas. MMR protein staining for MLH-1 (1:60, DAKO, M3640, clone ES05), MSH2 (1:300, Biocare Medical, CM219B, clone FE11), MSH6 (1:50, DAKO, M3646, clone EP49), and PMS2 (1:50, DAKO, M3647, clone EP51) was performed as previously described [28]. Epitope retrieval (HIER) was done in pH9 TE-buffer and the results visualized using the MACH3 HRP-Polymer (Biocare Medical) detection kit.

Analysis of tissue microarrays

Two of the authors (KEH and KAA), blinded from other patient data, scored each tissue core for percentage of CDX2-positive tumor cells. Staining intensity was also recorded. Scoring details are included in Supplementary Material 3. The TMAs included 1–3 cores for each patient. For cases with intratumoral heterogeneity, we estimated the

average CDX2 expression. In accordance with Dalerba et al., cases were divided into four groups: a: no staining (0–5% positive cells), b: weak/scattered staining in a minority of cells (5–49% positive cells), c: moderate/strong staining in a majority of cells (50–95% positive cells), d: strong staining in all cells (95–100% positive cells). Tumors in category A and B were defined as CDX2 negative. As weak staining is a known diagnostic pitfall in CDX2 IHC [29] cases with weak staining in the majority of cells were regarded CDX2-positive (category c). This applied to one case only.

MMR protein expression was assessed by KEH, YM, NBR, and MPM. Scoring details are found in Supplementary Material 3. Loss of expression was defined as less than 5% positive tumor cells in the presence of retained expression in internal control cells (stromal cells and/or normal colon epithelium of the same tissue core), in ordinance with other studies [30]. Cases with negative staining in tumor cells and negative staining of internal control cells were omitted from analysis. There is no consensus on how to interpret equivocal MMR IHC staining patterns as this group includes cases both with and without MMR germline mutation [31]. We therefore decided to exclude cases with equivocal MMR-staining patterns. We categorized patients as MMR deficient if staining was absent for MLH-1 + PMS2, PMS2 alone, MSH2 + MSH6, or MSH6 alone.

Validation in whole tissue sections

Whole tissue sections from the HDH cohort were used to validate the CDX2 and MMR IHC (Supplementary Material 3) and to assess heterogeneity in MMR protein expression [32, 33].

Tumor grading

We recorded information about tumor grading from the original patient pathology reports. We used a two-tiered grading system (low versus high grade) in order to reduce interobserver variation [8].

Statistics

Differences in CDX2 expression and tumor grade between subgroups were tested using Pearson's chi square test and Student's *t* test. We used Cancer-specific survival (CSS) as outcome variable, defined as time from randomization until death of colon cancer or death caused by treatment complications. The effect of CDX2 expression and tumor grade on CSS in different subgroups was assessed using Kaplan–Meier curves with log rank test and the multivariate Cox proportional hazards model. We adjusted for clinicopathological variables known to be relevant in colon cancer using the enter method. All *p* values were two-sided and regarded statistically significant if <0.05. As we consider our study exploratory, we made no adjustments for multiple comparisons. Statistical analyses were performed in IBM SPSS Statistics for Windows (v25.0) and R [34].

Results

Patient characteristics, tumor grade, CDX2 expression, and MMR phenotype

The study cohort consists of 544 patients with stage II–III colon cancer (Table 1). CDX2 staining was successful for 443 patients: 23 (5.2%) had no staining, 18 (4.1%) had weak/scattered staining in a minority of cells, and 56 (12.6%) had moderate/strong staining in a majority of cells (Fig. 1). Strong staining in all cells was registered for 346 tumors (78.1%) and for the 308 (100%) corresponding samples with normal intestinal epithelium. Interobserver agreement between the four categories were 89, and 99% between positive and negative cases. CDX2 negativity (41 patients, 9.3%) was significantly associated with right sided colon cancer, dMMR and high tumor grade (Supplementary Table 1).

Validation in whole tissue sections (Supplementary Material 3) showed a satisfactory compliance between TMA and whole tissue CDX2 IHC. Discrepant results represented cases with scoring values close to the chosen cut-off of 50% positive cells.

Proficient mismatch repair (pMMR) was detected in 377 patients (69.3%) and dMMR in 105 (19.3%) patients. Out of the 479 patients (88.1%) with colon adenocarcinoma NOS, 406 patients (84.8%) had low-grade tumors and 71 patients (14.8%) had high-grade tumors. High tumor grade was associated with dMMR, CDX2 negativity and right sided colon cancer (Supplementary Table 1). The MMR phenotyping based on the whole tissue sections IHC complied with the MMR phenotyping based on the TMA IHC. However, we found two cases with different staining results; one case had heterogenous MLH-1-staining pattern in the whole tissue section and retained MLH-1 expression in the TMA. It was regarded as having retained MMR expression due to retained PMS2. Another case was regarded as retained MSH2 expression in TMA but lost expression in whole tissue section. This did not result in changes in MMR phenotype as this case had loss of expression for MSH6 in both TMA and the whole tissue section.

To illustrate the large overlap between dMMR, CDX2 negativity and high tumor grade, an Euler diagram was made for patients with colon adenocarcinoma NOS and valid staining for both MMR and CDX2 (Fig. 2).

Table 1 Patient characteristics.

	Total ($n = 544$)	NGICG cohort ($n = 276$)	HDS-cohort ($n = 268$)
Age, mean (years)	67.5	61.7	73.5
Sex			
Female	270 (49.6%)	145 (52.5%)	125 (46.6%)
Male	274 (50.4%)	131 (47.5%)	143 (53.4%)
Stage			
UJCC stage II	338 (62.1%)	174 (63.0%)	164 (61.2%)
UJCC stage III	206 (37.9%)	102 (37.0%)	104 (38.8%)
Chemotherapy[a]			
Adjuvant chemotherapy	189 (34.7%)	140 (50.7%)	49 (18.3%)
No chemotherapy	354 (65.1%)	136 (49.3%)	218 (81.3%)
Histology			
Adenocarcinoma NOS	479 (88.1%)	242 (87.7%)	237 (88.4%)
Mucinous adenocarcinoma	30 (5.5%)	30 (10.9%)	
Signet ring cell carcinoma	4 (0.07%)	4 (1.4%)	
Mucinous or signet[b]	31 (5.7%)		31 (11.6%)
Tumor grade[c]			
Low tumor grade	430 (79.0%)	220 (79.7%)	210 (78.4%)
High tumor grade	107 (19.7%)	52 (18.8%)	55 (20.5%)
MMR phenotype[d]			
dMMR	105 (19.3%)	49 (17.8%)	56 (20.9%)
pMMR	377 (69.3%)	192 (69.6%)	185 (69.0%)
CDX2 expression[e]			
CDX2 positive	402 (73.9%)	208 (75.4%)	194 (72.4%)
CDX2 negative	41 (7.5%)	24 (8.7%)	17 (6.3%)
Tumor location[f]			
Right side	325 (59.7%)	152 (55.1%)	173 (64.6%)
Left side	219 (40.3%)	124 (44.9%)	95 (35.4%)
Ki67[g]			
<40		122 (44.2%)	
>40		115 (41.7%)	

[a]Data missing from one patient.

[b]For the Haraldsplass cohort, signet ring cell carcinoma or mucinous adenocarcinoma have the same annotation.

[c]Data missing from 7 patients (1.3%).

[d]Results missing from 62 patients (11.4%).

[e]Results missing from 101 patients (18.6%).

[f]Right side: Ascending and transverse colon. Left side: Descending and sigmoid colon.

[g]Data available for the NGICG cohort only, data missing from 39 patients [47].

CDX2 expression status could not be determined in 101 (18.6%) cases due to technical issues, e.g., few tumor cells in tissue cores, tissue core detached from slide during staining or necrosis in tissue core. MMR phenotype could not be determined in 62 cases (11.4%) due to the mentioned technical issues, equivocal staining patterns or absence of staining in normal tissue used as internal positive control. Our goodness of fit analyses showed no significantly different distribution of clinicopathological variables between cases with successful CDX2- or MMR staining and the original study cohort (Supplementary Table 2).

Patients with pMMR and CDX2 negativity have a very poor prognosis

In Kaplan–Meier analyses with log rank test, there was no statistically significant difference in CSS between patients with dMMR and pMMR ($p = 0.333$, Supplementary Fig. 1A).

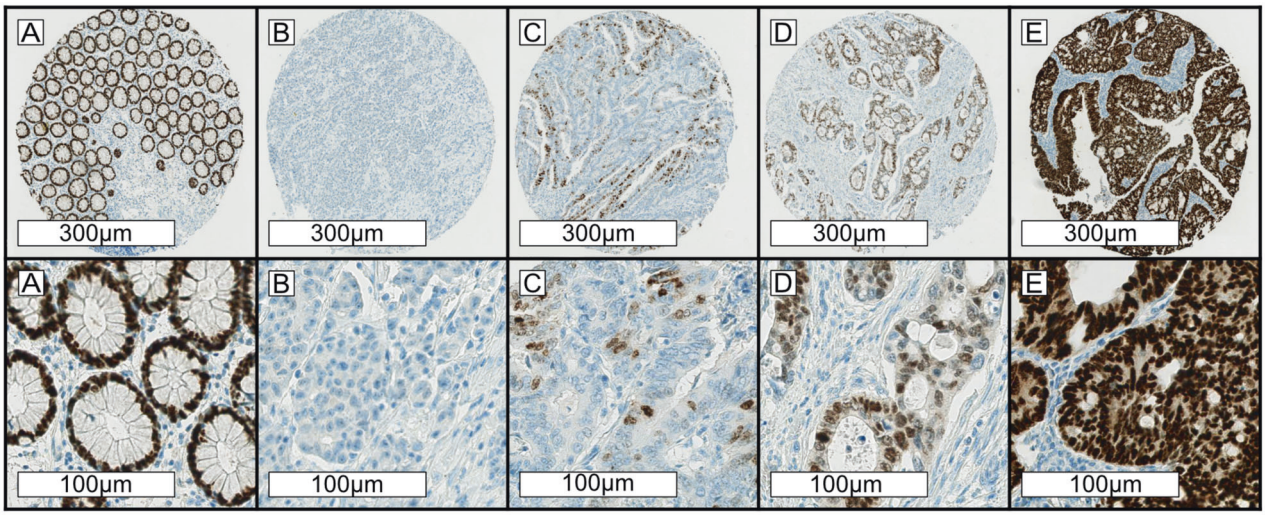

Fig. 1 Immunohistochemical staining for CDX2. a Normal colon. **b** Colon tumor, no staining. **c** Colon tumor, weak/scattered staining in a minority of cells. **d** Colon tumor, moderate/strong staining in a majority of cells. **e** Colon tumor, strong staining in all cells.

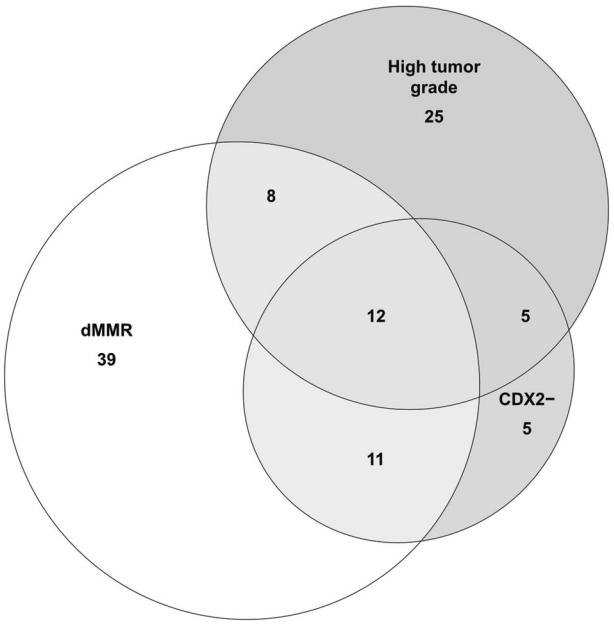

Fig. 2 Euler diagram showing the overlap between number of patients with deficient mismatch repair, CDX2 negativity, and high tumor grade. Analysis done for cases with colon adenocarcinoma NOS histology and valid staining for both CDX2 and MMR.

Patients with CDX2-negative tumors had a significantly shorter 5-year CSS than patients with CDX2-positive tumors (47.8 (95% confidence interval (CI) 41.7–53.9) versus 53.6 (95% CI 52.1–55.1) months, $p = 0.024$) (Supplementary Fig. 1B). CDX2 negativity was not an independent adverse prognostic marker in multivariate Cox regression analyses when analyzing the whole study cohort (hazard ratio (HR) 1.70, 95% CI 0.83–3.49, $p = 0.145$) (Table 2).

When considering MMR phenotype and histology in addition to CDX2 status, we identified subgroups with high risk of death from colon cancer. CDX2 negativity was a strong marker of poor prognosis in our multivariate models in patients with pMMR only, (HR 2.93, 95% CI 1.23–6.99, $p = 0.015$), patients with colon adenocarcinoma NOS histology (HR 3.03, 95% CI 1.34–6.88, $p = 0.008$) and patients with colon adenocarcinoma NOS + pMMR (HR 5.18, 95% CI 2.25–11.90, $p < 0.001$) (Table 2), with HRs surpassing UJCC stage III versus II in the latter group. Patients with pMMR and CDX2 negativity had a 5-year mean CSS of 35.8 months (95% CI 23.4–48.3) compared to 52.1–53.5 months (95% CI 45.6–58.6) ($p = 0.001$) for patients with CDX2 positive tumors or dMMR/CDX2-negative tumors (Fig. 3). CDX2 expression did not affect CSS in the dMMR subgroup ($p = 0.501$). The predictive value of CDX2 expression was analyzed in our randomized cohort (NGICG). CDX2 negativity did not predict effect of adjuvant chemotherapy in our uni- or multivariate models, but due to lack of statistical power of the CDX2-negative subgroup this analysis is considered explorative.

MMR phenotype directs the influence of high tumor grade on prognosis and effect of adjuvant chemotherapy for patients with adenocarcinoma NOS

In this section, the predictive and prognostic impact of tumor grade will be discussed for patients with adenocarcinoma NOS only to eliminate bias introduced by cases with other colon cancer histology.

Overall, patients with high tumor grade had a worse 5-year CSS than patients with low tumor grade (48.6 months

Table 2 Multivariate cox regression analysis for cancer specific survival.

	All, $n = 544$		Adenocarcinoma NOS only, $n = 479$		pMMR only, $n = 377$		Adenocarcinoma NOS + pMMR, $n = 345$	
	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value
CDX2 negative versus positive	1.70 (0.83–3.49)	0.145	3.03 (1.34–6.88)	0.008	2.93 (1.23–6.99)	0.015	5.18 (2.25–11.90)	<0.001
Tumor stage[a]	4.45 (2.66–7.44)	<0.001	3.99 (2.34–6.82)	<0.001	2.91 (1.72–4.94)	<0.001	3.11 (1.79–5.39)	<0.001
Right versus left side	1.04 (0.65–1.66)	0.876	0.97 (0.59–1.60)	0.915	1.04 (0.64–1.68)	0.891	1.00 (0.60–1.66)	0.995
Cohort[b]	1.85 (1.16–2.96)	0.009	1.98 (1.18–3.31)	0.009	1.79 (1.06–3.03)	0.030	2.00 (1.14–3.52)	0.016
Tumor grade[c]	2.10 (1.22–3.62)	0.007	2.05 (1.14–3.68)	0.016	2.44 (1.32–4.51)	0.004	2.49 (1.33–4.66)	0.004
Treatment[d]	1.60 (1.01–2.53)	0.044	1.60 (0.97–2.63)	0.064	1.63 (0.99–2.70)	0.056	1.68 (0.98–2.85)	0.057
pMMR versus dMMR	1.13 (0.56–2.28)	0.729	1.82 (0.75–4.45)	0.188				
Histology[e]	1.07 (0.55–2.11)	0.839			0.71 (0.29–1.74)	0.456		

[a]Stage III versus stage II.
[b]NGICG versus Haraldsplass.
[c]High versus low grade.
[d]Surgery only versus adjuvant chemotherapy.
[e]Other histology versus adenocarcinoma NOS.

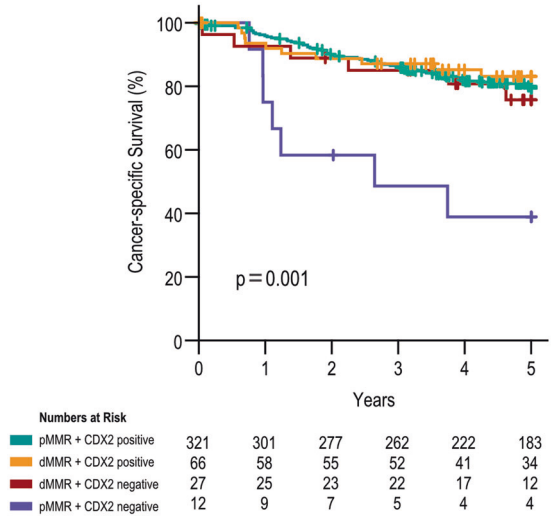

Fig. 3 Prognostic impact of CDX2 expression status illustrated by Kaplan–Meier curves for the whole study cohort stratified by mismatch repair (MMR) phenotype. p value calculated for tumors with pMMR and CDX2 negativity versus the other groups.

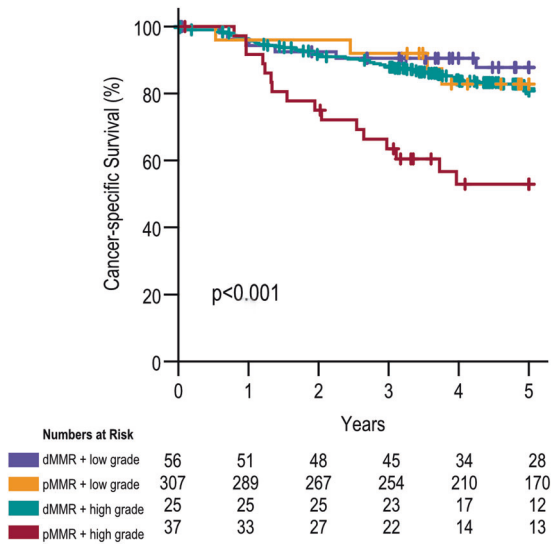

Fig. 4 Prognostic impact of tumor grade illustrated by Kaplan–Meier curves stratified by mismatch repair (MMR) phenotype. Analysis done for patients with colon adenocarcinoma NOS histology from the whole study cohort. p value calculated for tumors with pMMR and high tumor grade versus the other groups.

(95% CI 44.4–52.8) versus 54.6 months (95% CI 53.2–56.0) $p = 0.002$). (Supplementary Fig. 1C). When separating patients into categories based on grade and MMR phenotype, the group with pMMR and high-grade tumors had a poor prognosis compared to the other groups (CSS 43.7 months (95% CI 37.3–50.0) versus 54.2–55.4 months (95% CI 50.3–60.0), $p < 0.001$) (Fig. 4).

In multivariate Cox regression analyses, high tumor grade was an independent negative prognostic factor in colon adenocarcinoma NOS (pMMR and dMMR) but with

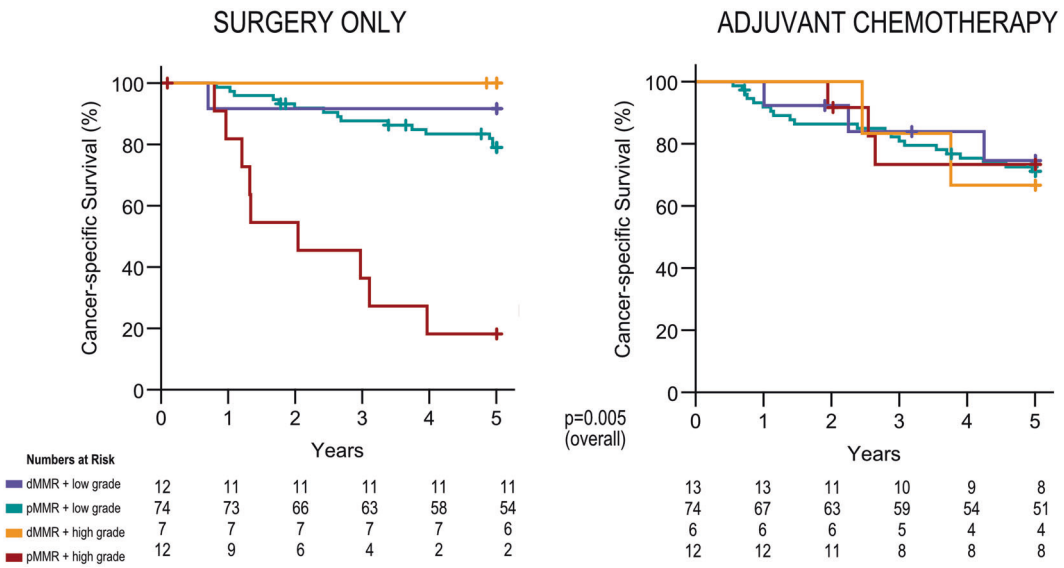

Fig. 5 Predictive impact of tumor grade illustrated by separate Kaplan–Meier curves for patients randomized to surgery only versus adjuvant chemotherapy with fluorouracil–levamisole after surgery. Results stratified by mismatch repair (MMR) phenotype and tumor grade. Analyses performed on patients with colon adenocarcinoma NOS histology and in the randomized cohort only.

a higher HR for the colon adenocarcinoma NOS and pMMR subgroup (Table 2).

To avoid patient selection bias, the predictive effect of tumor grade was assessed in our randomized cohort only. In the group randomized to receive surgery only, there was a large difference in mean 5-year CSS between pMMR patients with high-grade tumors versus pMMR patients with low-grade tumors (CSS 30.2 months (95% CI 19.5–41.0) versus 54.6 months (95% CI 51.6–57.6)). In contrast, in the group randomized to adjuvant chemotherapy, there was no statistically significant difference in CSS between these groups (Fig. 5). As tumor grade did not affect benefit of chemotherapy in the dMMR group ($p = 0.947$), a split multivariate Cox regression model with treatment × tumor grade interaction was used as a predictive model for pMMR patients (Table 3). There was a significant interaction between tumor grade and treatment ($p = 0.036$). High tumor grade was a significant marker of poor prognosis in the surgery-only group (HR 4.60 (95% CI 1.68–12.6), $p = 0.003$) but not in the group receiving chemotherapy (HR 0.66 (95% CI 0.15–3.00), $p = 0.587$). The results from our Kaplan–Meier curves and multivariate models indicate that although high tumor grade is a marker of poor prognosis in pMMR patients, these tumors respond well to adjuvant 5-FU-based chemotherapy.

Table 3 Split multivariate cox regression analysis for cancer specific survival with treatment*tumor grade interaction variable. Analysis done in the NGICG cohort for patients with adenocarcinoma NOS and pMMR ($n = 173$).

	Hazard ratio (95% CI)	p value
CDX2 expression (low versus high)	2.56 (1.50–4.37)	0.001
Tumor stage (III versus II)	4.04 (1.90–8.56)	<0.001
Ki67 (<40% versus >40%)	2.17 (1.05–4.50)	0.038
Treatment (adjuvant chemotherapy versus surgery only)	1.65 (0.79–3.45)	0.184
Interaction treatment by tumor differentiation		0.036
High versus low tumor grade		
In patients receiving adjuvant chemotherapy	0.66 (0.15–3.00)	0.587
In patients treated with surgery only	4.60 (1.68–12.6)	0.003

Discussion

In this study, we have assessed the prognostic and predictive impact of CDX2 expression and tumor grade in the context of MMR phenotype. We observe that patients with tumors that are both pMMR and CDX2 negative have a particularly poor prognosis. Our study also shows that pMMR combined with high-grade morphology indicates a particularly poor prognosis in colon adenocarcinoma NOS treated with surgery alone. Still, this group of patients respond very well to adjuvant 5-FU-based chemotherapy.

The frequency of CDX2 negativity in our material (9.3%) is in line with the reported rate (4–15%) in localized colon cancer [14, 16, 17, 35–39]. Loss of CDX2 expression may be a result of epigenetic silencing by CpG island promoter hypermethylation [19, 38, 40, 41], a mechanism also known to cause dMMR by silencing of MLH-1 [9, 40]. This might explain the large overlap between CDX2

negativity and dMMR. This observation is supported by previous studies: 45–81% of CDX2-negative cases have dMMR, and 25–41% of dMMR cases are CDX2 negative [14, 17, 36, 37, 39, 42].

In our study, CDX2 negativity was an adverse prognostic marker for patients with pMMR tumors. Few other studies have differentiated between pMMR and dMMR tumors when assessing the effect of CDX2 negativity on prognosis and we propose that not stratifying may understate the prognostic impact of CDX2 negativity in previous studies [14, 43]. Pilati et al. studied the relation between CDX2 negativity and the consensus molecular subtype (CMS) classification. CDX2 negativity was an adverse prognostic marker in the CMS4 group and for cases with MSS but not in the CMS1 group or for cases with MSI [35]. Other studies also support our findings; Lugli et al. demonstrated an association between CDX2 negativity and advanced tumor stage in cases with pMMR but not for cases with dMMR. Yet, they were unable to demonstrate any prognostic value for CDX2 negativity in multivariate analyses, this may be caused by not including MMR phenotype in their multivariate models [22]. Ryan et al. studied dMMR tumors only and conclude that CDX2 negativity is not a prognostic marker in this group [44]. Slik et al. demonstrate a higher frequency of death from CRC for patients with CDX2 negativity and MSS compared to patients with CDX2 negativity and MSI.

In contrast to our results, Ma et al. report a negative prognostic impact of SATB2 and CDX2 in patients with dMMR but not pMMR [42]. However, independent prognostic value was only reported for the combined SATB2/CDX2 marker, not for CDX2 negativity alone. Kim et al. studied 109 patients with MSI-H colorectal cancer. The combination of CDX2 negativity and CK 20 loss indicated a poor prognosis, but CDX2 negativity alone was not an independent prognostic marker in multivariate analyses [40].

In our study, the prognostic value of CDX2 expression was analyzed in the joined patient material (NGICG and HDH). The two groups have similar clinicopathological data including fraction of dMMR, high tumor grade and CDX2 negativity. The indications for adjuvant chemotherapy changed between the time of including patients in these two different studies and this probably explains the statistically significant difference in survival between the two hospitals. We have adjusted for differences in survival between the cohorts in the multivariate model (variable "hospital", Table 2). The number of CDX2-negative cases is in line with other studies but the number of cases with aberrant CDX2 expression is low. This calls for caution when interpreting our findings and validation in larger patient cohorts.

Despite the high incidence of dMMR tumors in localized colon cancer with high-grade morphology [7, 17], high tumor grade is regarded a negative prognostic factor [2]. Rosty et. al found that the negative prognostic impact of high tumor grade was restricted to MSS tumors and proposed that the grading of colon adenocarcinoma should be made in accordance with MSI status [25]. In our study, patients with pMMR and high-grade morphology had a very poor prognosis, but high-grade morphology did not influence the prognosis of dMMR patients, supporting these findings. The eUpdate of the ESMO guidelines also emphasizes the importance of assessing established high-risk factors in the context of MMR/MSI phenotype. Here, MSS, and either T4 or more than one validated risk factor (number of examined lymph nodes <12, primary tumor perforation or occlusion, high tumor grade), constitute the newly defined "very high risk" group in colon cancer stage II [4].

Liu et al. reported that stage IIA colon cancer patients with poorly differentiated or undifferentiated tumors benefit from adjuvant chemotherapy [45], in accordance with our results for stage II–III pMMR patients. However, they assessed high-grade adenocarcinomas together with tumors with other histology (undifferentiated tumors) and did not stratify by MSI/MMR phenotype.

The randomized design of the NGICG study offers unique opportunities to study markers that predict effect of adjuvant chemotherapy. We therefore assessed predictive markers in this material only. In our study, a predictive effect of high tumor grade was demonstrated when analyzing stage II and III colon cancer together, but due to the limited number of events in stage II colon cancer in the randomized cohort, our study was underpowered to show this effect in the colon cancer II subgroup alone. The adjuvant chemotherapy in the randomized study consisted of 5-FU-levamisol. Today, 5-FU or capecitabine in combination with oxaliplatin is the standard regimen for adjuvant chemotherapy in stage III CRC in Europe, but as most of the survival effect of the XELOX/FLOX/FOLFOX treatment can be attributed to the fluorouracil component [46], we expect our results to be applicable to these combinations as well.

This study emphasizes the importance of assessing CDX2 expression and tumor grade in the context of MMR phenotype. We propose that CDX2, together with MSI or MMR phenotype, should be implemented in the routine diagnostics of colon cancer stage II–III. Patients with pMMR and CDX2 negativity represent a high-risk group irrespective of cancer stage. We therefore suggest that adjuvant chemotherapy is considered for pMMR CDX2-negative stage II patients.

Our study is, to our knowledge, the first study to assess the predictive impact of high tumor grade on effect of adjuvant chemotherapy in pMMR patients in a randomized trial. We demonstrate that although patients with pMMR

and high tumor grade have an inferior prognosis after surgery alone, they seem to respond very well to adjuvant chemotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics The Regional Committee for Medical Research Ethics of Western Norway and the Data Inspectorate for National Registries approved the study protocols. All patients signed informed consents.

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