European Journal of Cancer 172 (2022) 221–230

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Combination of CDX2 H-score quantitative analysis with CD3 AI-guided analysis identifies patients with a good prognosis only in stage III colon cancer

Valentin Derangère [a,b,c,d,1], Julie Lecuelle [a,b,c,1], Come Lepage [a,b,f,g], Oumaima Aoulad-Ben Salem [a,b,c], Ben M. Allatessem [a,b,c], Alis Ilie [c], Olivier Bouché [i], Jean-Marc Phelip [j], Mathieu Baconnier [k], Denis Pezet [l], Virginie Sebbagh [m], Eric Terrebonne [n], Gauthier Bouard [e], Valérie Jooste [a,b,o], Anne-Marie Bouvier [a,b,o], Chloé Molimard [p], Franck Monnien [p], Daniel Gonzalez [f], Karine Le Malicot [f], David Rageot [a,c], Caroline Truntzer [a,c,d,1], Fréderic Bibeau [p,1], Francois Ghiringhelli [a,b,c,d,h,*,1] For the PRODIGE 13 investigators and collaborators[2]

[a] *Centre de Recherche INSERM LNC-UMR1231, F-21000 Dijon, France*
[b] *University of Burgundy Franche-Comté, F-21000 Dijon, France*
[c] *Cancer Biology Transfer Platform, Centre Georges-François Leclerc, F-21000 Dijon, France*
[d] *Genetic and Immunology Medical Institute, Dijon, France*
[e] *Service D'Anatomie et Cytologie Pathologiques, CHU Côte de Nacre, Normandie Université, Caen, France*
[f] *Fédération Francophone de Cancérologie Digestive, Centre de Randomisation Gestion Analyse, EPICAD LNC 1231, France*
[g] *Service D'Hépato-gastroentérologie et Oncologie Digestive, CHU de Dijon, France*
[h] *Department of Medical Oncology, Centre Georges-François Leclerc, F-21000 Dijon, France*
[i] *CHU Robert Debré, Médecine Ambulatoire-Cancérologie, Reims, France*
[j] *Service D'Hépatogastro-entérologie - CHU de Saint Etienne - Hôpital Nord, Saint-Etienne, France*
[k] *Centre Hospitalier Annecy Genevois Service HGE, Pringy, France*
[l] *CHU ESTAING, Service de Chirurgie Digestive, Clermont-Ferrand, France*
[m] *Centre Hospitalier de Compiègne, Service Gastro-entérologie et Hépatologie, Compiègne, France*
[n] *Hôpital Haut Lévêque, Service d'HGE, PESSAC, France*
[o] *Digestive Cancer Registry of Burgundy, Dijon University Hospital, Dijon France*
[p] *CHU de Besançon, Service d'anatomie Pathologique, France*

Received 8 March 2022; received in revised form 19 May 2022; accepted 28 May 2022
Available online 1 July 2022

* *Corresponding author*: Centre de Recherche INSERM LNC-UMR1231, F-21000 Dijon, France,
E-mail address: fghiringhelli@cgfl.fr (F. Ghiringhelli).
[1] Contributed equally. [2] Please See the List of PRODIGE 13 Investigators and Collaborators in Supplementary Methods 3, SM3

https://doi.org/10.1016/j.ejca.2022.05.040

KEYWORDS
Colon cancer;
Prognosis;
Immuno histochemistry;
CDX2;
CD3;
Biomarkers

Abstract ***Aim:*** Stratification of colon cancer (CC) of patients with stage II and III for risk of relapse is still needed especially to drive adjuvant therapy administration. Our study evaluates the prognostic performance of two known biomarkers, CDX2 and CD3, standalone or their combined information in stage II and III CC.
Patients and methods: CDX2 and CD3 expression was evaluated in Prodige-13 study gathering 443 stage II and 398 stage III primary CC on whole slide colectomy. We developed for this study an H-score to quantify CDX2 expression and used our artificial intelligence (AI)-guided tissue analysis ColoClass to detect CD3 in tumour core and invasive margin. Association between biomarkers and relapse-free survival was investigated.
Results: Univariate analysis showed that the combined variable CD3-TC and CD3-IM was associated with prognosis in both stage II and stage III. CDX2, on the contrary, was associated with prognosis only in stage III. We subsequently associated CDX2 and combined immune parameters only in stage III. This multivariate analysis allowed us to distinguish a proportion of stage III CC harbouring a high CDX2 expression and a high immune infiltration with a particularly good prognosis compared to their counterpart.
Conclusion: This study validated the prognostic role of CDX2 and CD3 evaluated with immunohistochemistry procedures in stage III but not in stage II. This association would be conceivable in a routine pathology laboratory and could help oncologist to consider chemotherapy de-escalation for a part of stage III patients.

1. Introduction

The last data from 2020 on colorectal cancer in Europe show that it is the second most frequently occurring cancer after breast cancer and the second cause of cancer death after lung cancer [1]. The current clinical dogma encourages clinicians to treat with an adjuvant chemotherapy every patients suffering from stage III CC and patients suffering from stage II CC harbouring poor histological/clinical prognosis features such as T4, VELIPI, or occlusion/perforation [2]. Despite these approved recommendations, it is also known that on the one hand, the correct selection of stage II CC who could benefit from adjuvant chemotherapy is still to be improved using new markers such as circulating tumour DNA [3], panel gene expression [4], or immunohistochemistry (IHC) procedures [5]. On the other hand, the dogmatic chemotherapy administration for stage III could also be dubious as some patients with an especially good prognosis could benefit from surgery alone or from surgery followed by a lighter chemotherapy [6], which could subsequently relieve patients from side effects. Results published from IDEA study also underlined this potential chemotherapy administration de-escalation [7]. This it is consequently a need to develop new biomarkers to help patients' stratification in both stage and improve CC care. Obviously, these biomarkers should be developed as far as possible in order to be easily applied in a routine process.

Among strategies which can be conceivable in a routine assessment, IHC procedures are developed to help pathologists to make a diagnosis. Besides its diagnostic impact, IHC procedure can also be used for its prognostic value [8]. Thus, CDX2 (caudal-related homoeobox transcription factor 2) and CD3 are two well-known prognosis markers in CC which are easily feasible with IHC procedures as they belong to pathologists' routine portfolio [9,10]. CDX2 is a transcription factor expressed in the nuclei of gastrointestinal epithelial cells [11]. It belongs to Wnt-signalling pathway, and once activated, CDX2 leads to gastrointestinal differentiation and maintenance of epithelial lining [12]. Along with cytokeratin staining, CDX2 is mainly used to help pathologists to pinpoint a gastrointestinal origin of an undetermined origin tumour [13]. CDX2 has also been proposed to have a prognostic role since 2016 by Dalerba *et al.* where the loss of CDX2 was associated with a poor prognosis [10]. While the prognostic value for stage II CC is still discussed [14,15], loss of CDX2 was afterwards confirmed to be associated with a poor prognosis in several analysis especially in stage III or metastatic CC [16–19]. CD3 is a pan T-cell lymphocyte marker widely studied for a decade in CC by several groups [20–22] but also especially by Galon's group in CC [5,23]. The great prognostic value of CD3 on stage II and stage III led to its association with CD8, a cytotoxic T-cell marker, to a prognostic commercial test coined Immunoscore® [24] available for stage II and stage III CC in several countries. Our group also recently showed that CD3 detected with our free artificial intelligence (AI) software Coloclass could be prognostic when the counting was focussed on tumour core (TC) [25].

In the present study, we tested the rational to combine CDX2 and CD3 on two successive formalin-

fixed paraffin-embedded (FFPE) slides from the Prodige-13 cohort [26] gathering 443 stage II and 398 stage III CC to predict their relapse-free survival (RFS) over a 5-year follow-up.

2. Patients and methods

2.1. I – study design and population

The study population gathered 443 stage II and 398 stage III CC patients from the Prodige-13 study (NCT00995202) [26]. This randomised prospective multicentre study was initially designed to investigate the impact of intensive radiological monitoring *vs.* standard monitoring and carcinoembryonic antigen (CEA) monitoring *vs.* no monitoring. All use of clinical data and tissue specimen were performed with the Federation Francophone de Cancérologie Digestive (FFCD) compliance. Study approval and consent were obtained from FFCD with the following number: EudraCT 2009-A00536-51, CPP 2009/34. Patients' clinical characteristics are available in Table 1 and flow chart for biological analysis is available in supplementary methods, SM1. Endpoint for survival was RFS defined as the time between primary surgery and objective relapse of disease. For mismatch repair (MMR) proteins, relying on hMLH1, PMS2, MSH2, and MSH6 antibodies, the controls were represented by normal epithelial cells of the mucosae, lymphocytes, and stromal cells such as fibroblasts and endothelial cells. Because MMR-IHC assessment was performed on tissue-microarray, an external control was systematically added.

2.2. II – CDX2 staining and analysis

Slide staining was carried out using Autostainer 48 (Agilent) and anti-CDX2 primary antibody (clone DAK-CDX2, Agilent). Once counterstained and permanently mounted, slides were digitalised with a Nanozoomer HT2.0 (Hamamatsu) at × 20 magnification to generate a whole slide imaging (WSI) file in ndpi

Table 1
Clinical characteristics of the Prodige13 cohort patients included in the analysis.

Label	N (total)	Stage II (443)	Stage III (398)	P-value	Adjusted p-value
Sex	841				
Male		255 (57.6)	230 (57.8)	1	1
Female		188 (42.4)	168 (42.2)		
Age, years	841	67.9 (14.2)	67.5 (15.2)	0.77	0.86
Location of tumour	834				
Right colon		210 (47.4)	159 (39.9)	$<1.10^{-3}$	$<1.10^{-3}$
Left colon		232 (52.4)	183 (46)		
Rectum		0 (0)	50 (12.6)		
NA		1 (0.2)	6 (1.5)		
Number of lymph nodes	841				
N0		443 (100)	0 (0)	$<1.10^{-3}$	$<1.10^{-3}$
N1		0 (0)	289 (72.6)		
N2		0 (0)	109 (27.4)		
MSI	657				
MSS		303 (68.4)	265 (66.6)	$<1.10^{-3}$	$<1.10^{-3}$
MSI		75 (16.9)	21 (5.3)		
NA		65 (14.5)	112 (28.1)		
Tumour size	831				
T1		0 (0)	8 (2)	$<1.10^{-3}$	$<1.10^{-3}$
T2		15 (3.4)	34 (8.6)		
T3		355 (80.1)	275 (69.1)		
T4		69 (15.6)	75 (18.8)		
NA		4 (0.9)	6 (1.5)		
Grade	834				
Well		400 (90.3)	357 (89.7)	0.21	0.27
Moderately		16 (3.6)	23 (5.8)		
Poorly		23 (5.2)	15 (3.8)		
NA		4 (0.9)	3 (0.7)		
Treatment	841				
Chemotherapy		128 (28.9)	391 (98.2)	$<1.10^{-3}$	$<1.10^{-3}$
No chemotherapy		315 (71.1)	7 (1.8)		
Relapse	841				
No		345 (77.9)	274 (68.8)	0.004	0.005
Yes		98 (22.1)	124 (31.1)		
Death	841				
No		375 (84.7)	300 (75.4)	0.001	0.002
Yes		68 (15.3)	98 (24.6)		

MSS: microsatellite stable; MSI: microsatellite instable; NA: not available.

format. Using QuPath software (v2) [27], CDX2 analysis was restricted to tumour cells annotated by a pathologist. A mean of nine different areas of tumour cells, representing 1 mm^2 for each slide, was analysed for CDX2 staining. A cut-off for each subset was determined on diaminobenzidine intensity (brown staining) and automatically applied on every cell detected in annotated areas (i.e., negative, 1+, 2+, and 3+). The CDX2 H-score was then calculated with the following formula: H-score = [1*(% cells 1+) + 2*(% cells 2+) + 3*(% cells 3+)] [28]. Positive controls are shown in Supplementary Fig. 1.

2.3. III – CD3 staining and analysis using AI

Slide staining was carried out using Autostainer 48 (Agilent) and anti-CD3 primary antibody (clone F7.2.38, Agilent) post processed the same way we did for CDX2. Once digitalised, we applied our ColoClass software previously published in 2020 [25]. Briefly, the WSI was tiled with QuPath software (v1), and 127 digital parameters within each tile were extracted. A random forest model through R software was estimated to classify any tile detected on the WSI. Coloclass was finally able to automatically detect TC by collating tumour tiles when the surface was big enough and automatically determined its invasive margin (IM) by a 300 µm surrounding border of TC. The number of CD3-positive cells/mm^2 was then calculated in these specific areas, respectively, called CD3-TC and CD3-IM. Positive controls are shown in Supplementary Fig. 1.

2.4. IV – Statistical analysis

Quantitative variables are described as mean ± standard deviation (SD) or median and interquartile range (quartile 1, quartile 3, IQR), and qualitative variables as number and percentage. Clinical characteristics of patients were compared using the Chi square or Fisher's exact test for qualitative variables and the Mann–Whitney test for quantitative variables. Boxplots were drawn with median, quartiles, and Tukey's whiskers. *P*-values were adjusted using Benjamini-Hochberg FDR correction and adjusted *p*-values<0.05 were considered significant.

Univariate and multivariate Cox proportional hazards models were estimated to compute hazard ratios (HRs) with 95% confidence intervals (CIs). Survival curves were estimated using the Kaplan–Meier method and compared using log-rank tests. RFS was used for survival analysis and was calculated from the time between primary surgery and objective relapse of disease. Survivors were censored after a 5-year follow-up. All results of Cox model of main figures are summarised in supplementary methods, SM2.

For stage II and stage III patients, CDX2 H-score was dichotomised at the third quartile, while CD3IM and TC were both dichotomised at quantile 2/3. Combined immune parameters were obtained by adding TC and IM information for each patient (i.e., low = 0 and high = 1), so that CD3 combined information spanned from 0 to 2 staggered in three groups (low, intermediate, and high). For stage III patients, the final composite marker gathering CDX2 H-score and combined CD3 was obtained by adding CDX2 H-score (0–1) and combined CD3 information (0–2), thus spanned from 0 to 3 staggered in four groups. A multivariate clinical model was performed with clinical factors significantly associated with RFS in stage III (*p*-value less than 0.1 in univariate Cox model). A bootstrap strategy was performed to validate the predictive power of final composite marker. One thousand random samplings with replacement were drawn from the initial cohort. HRs were estimated on each sampling, and median and 95% CI of corresponding HRs were estimated and considered as final result.

All STROBE, REMARK, and TRI-POD recommendations were considered.

3. Results

3.1. I – Validation of CDX2 H-score quantification strategy

We used an H-score strategy to quantify CDX2 within tumour to limit a potential bias due to tissue staining heterogeneity (Fig.1A). We first observed that CDX2 H-score was not different regarding stage (median CDX2 H-score was 72.2 [IQR = 135.5] for stage II vs. 76.8 [IQR = 152.3] for stage III, p = 0.34) but was significantly higher in left-sided tumours (92.9 [IQR = 152.5] vs. 46.6 [IQR = 124.1], $p < 1.10^{-3}$), in MSS tumours (89.4 [IQR = 146.7] vs. 11.1 [IQR = 77.3], $p < 1.10^{-3}$) and in well differentiated tumours (78.4 [IQR = 145.1] vs. 25.8 [IQR = 113], $p < 1.10^{-3}$) (Fig. 1B–E). Medullary tumours, although in very small amounts (seven tumours), had a drastically lower CDX2 H-score (median CDX2 H-score was 0 [IQR = 3.96]). On the contrary, CDX2 H-score was not different toward T and N status (Supplementary Fig. 2A and B).

3.2. II – CDX2 expression impacts differentially stage II and stage III CC prognosis

We then studied the prognostic value of CDX2 H-score in stage II and stage III CC. Dichotomised CDX2 H-score was used for further analyses. All clinical characteristics describing CDX2 H-score low and CDX2 H-score high patients are reported in Table 2. In stage II, CDX2 H-score was not significantly associated with RFS (HR = 1.48 [95% CI: 0.9–2.4]; log-rank p = 0.11) (Fig. 2A) even if a slight trend was observed for a better RFS in patients harbouring a low CDX2 H-score. When

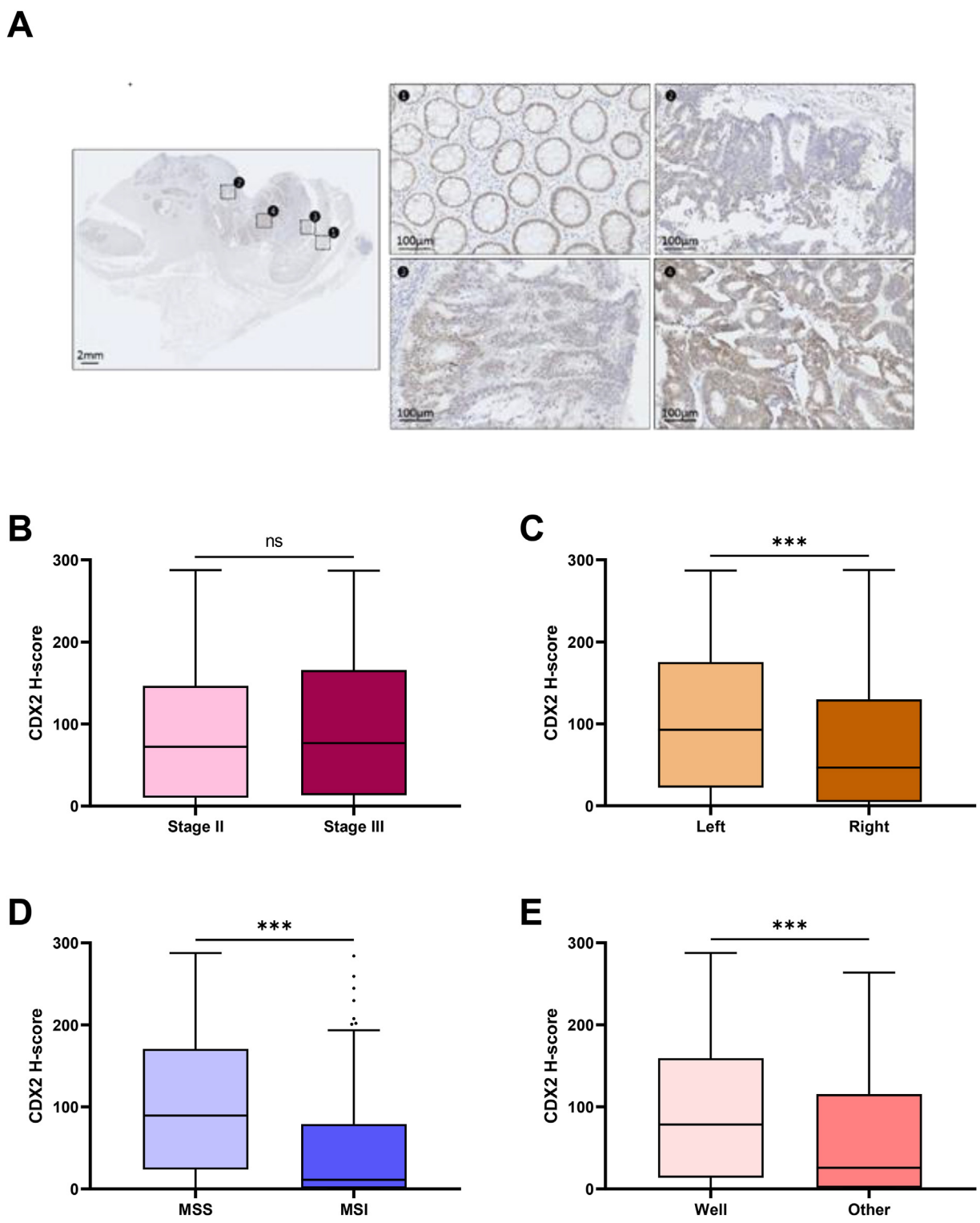

Fig. 1. **Validation of CDX2 H-score quantification strategy.** Representative potential CDX2 heterogeneity staining on a whole slide image (A); boxplot displaying CDX2 H-score evaluation depending on stage (B), sidedness (C), MSS/MSI status (D), and histology grade (E).

our analysis was focussed on MSS tumours, this trend was partially concealed (Supplementary Fig. 3A). On the contrary, high CDX2 H-score was associated with a good RFS (HR = 0.62 [95% CI: 0.37–0.99]; log-rank p = 0.05) in stage III CC (Fig. 2B). The MSS subgroup analysis confirmed this result (HR = 0.49 [95% CI: 0.3–0.9]; log-rank p = 0.02) (Fig. 2C). As for MSI-subgroup analysis, CDX2 H-score was not associated with RFS in both stage II and stage III CC (Supplementary Fig. 3B and C).

Table 2
Clinical characteristics of the Prodige13 cohort patients dichotomised by CDX2 H-score and stage.

Label	Stage II				Stage III			
	$CDX2^{Low}$ 332 (75)	$CDX2^{High}$ 111 (25)	P-value	Adjusted p-value	$CDX2^{Low}$ 298 (75)	$CDX2^{High}$ 100 (25)	P-value	Adjusted p-value
Sex								
Male	188 (56.6)	67 (60.4)	0.56	0.72	168 (56.4)	62 (62)	0.38	0.69
Female	144 (43.4)	44 (39.6)			130 (43.6)	38 (38)		
Age, years	67.9 (14.1)	67.5 (15)	0.91	0.91	67.6 (15.6)	67.3 (15.6)	0.49	0.71
Location of tumour								
Right colon	168 (50.6)	42 (37.8)	0.02	0.08	130 (43.6)	29 (29)	0.006	0.05
Left colon	163 (49.1)	69 (62.2)			124 (41.6)	59 (59)		
Rectum	0 (0)	0 (0)			41 (13.8)	9 (9)		
NA	1 (0.3)	0 (0)			3 (1)	3 (3)		
MSI								
MSS	214 (64.4)	89 (80.2)	$<1.10^{-3}$	0.006	195 (65.4)	70 (70)	0.63	0.71
MSI	68 (20.5)	7 (6.3)			17 (5.7)	4 (4)		
NA	50 (15.1)	15 (13.5)			86 (28.8)	16 (16)		
Tumour size								
T1	0 (0)	0 (0)	0.03	0.08	6 (2)	2 (2)	0.57	0.71
T2	14 (4.2)	1 (0.9)			22 (7.4)	12 (12)		
T3	256 (77.1)	99 (89.2)			208 (69.8)	67 (67)		
T4	58 (17.5)	11 (9.9)			57 (19.1)	18 (18)		
NA	4 (1.2)	0 (0)			5 (1.7)	1 (1)		
Grade								
Well	293 (88.3)	107 (96.4)	0.07	0.15	266 (89.3)	91 (91)	0.08	0.23
Moderately	15 (4.5)	1 (0.9)			21 (7)	2 (2)		
Poorly	20 (6)	3 (2.7)			9 (3)	6 (6)		
NA	4 (1.2)	0 (0)			2 (0.7)	1 (1)		
Treatment								
Chemotherapy	101 (30.4)	27 (24.3)	0.27	0.4	293 (98.3)	98 (98)	1	1
No chemotherapy	231 (69.6)	83 (75.7)			5 (1.7)	2 (2)		
Relapse								
No	264 (79.5)	81 (73)	0.19	0.34	199 (66.8)	75 (75)	0.16	0.35
Yes	68 (20.5)	30 (27)			99 (33.2)	25 (25)		
Death								
No	282 (85)	93 (83.8)	0.89	0.91	217 (72.8)	83 (83)	0.06	0.23
Yes	50 (15)	18 (16.2)			81 (27.2)	17 (17)		

MSS: microsatellite stable; MSI: microsatellite instable; NA: not available.

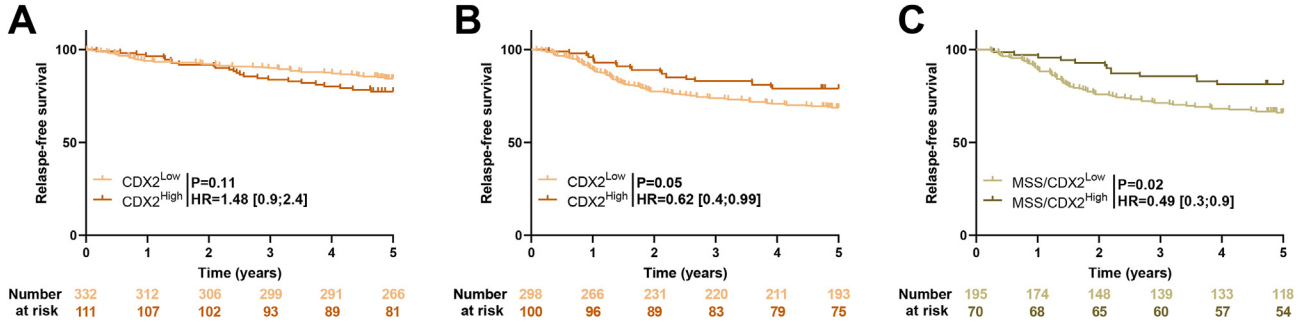

Fig. 2. **CDX2 expression impacts differentially stage II and stage III CC prognosis.** Kaplan–Meier stratification for CDX2 H-score high and low in stage II CC (A), in stage III CC (B) and in MSS stage III CC (C). Log-rank test; HR = hazard ratio, 95% CI between square brackets.

3.3. III – AI-automated CD3 quantification is prognostic in stage II and in stage III CC

To evaluate the pan-lymphocyte marker CD3 on whole slide, we used the AI software ColoClass previously generated as mentioned in the Methods part. Thanks to the use of ColoClass, we were able to automatically detect accurately TC and its IM the same way as a pathologist (Supplementary Fig. 4A and B). We dichotomised the CD3 variable in TC (CD3-TC) and in IM (CD3-IM) (Supplementary Fig. 4C–F). For stage II CC, CD3-TC and CD3-IM were both significantly associated with a good RFS (respectively HR = 0.56 [95% CI: 0.3–0.9]; log-rank p = 0.03; HR = 0.56 [95%

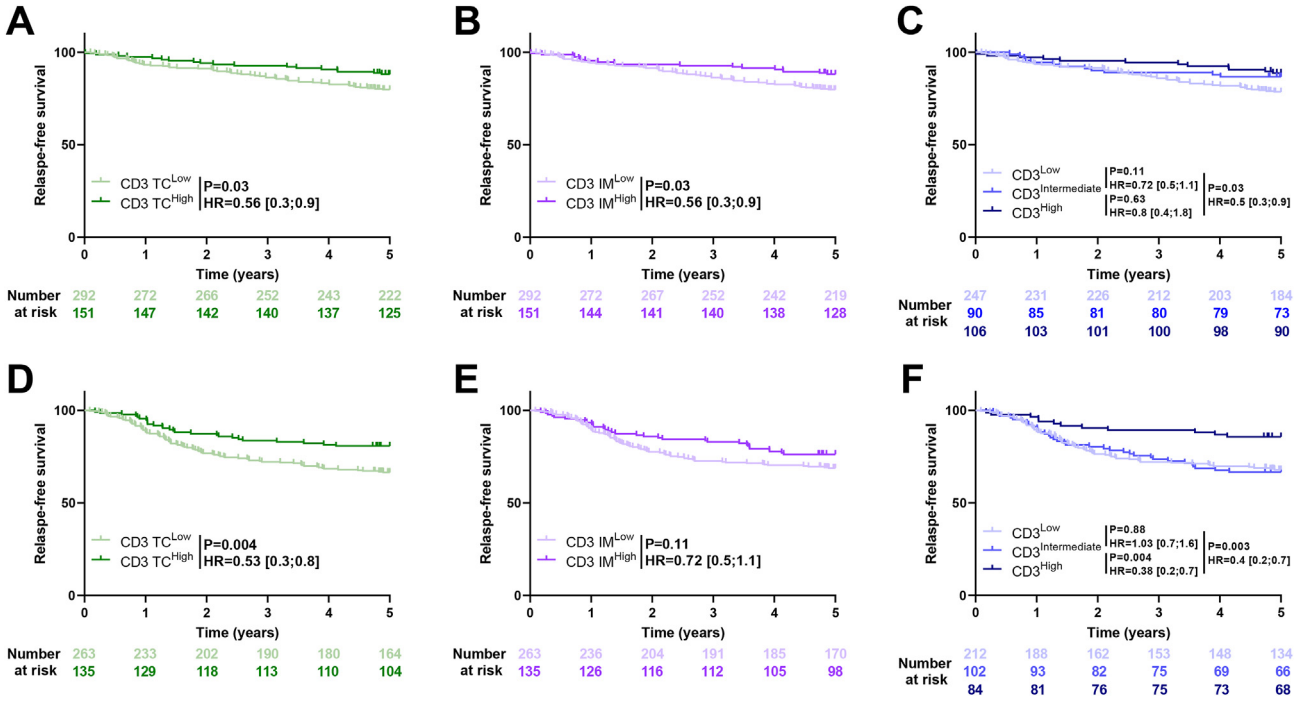

Fig. 3. **AI-automated CD3 quantification is prognostic in stage II and stage III CC.** Kaplan-Meier stratification for CD3 high and low for IM (A), TC (B) and combined information (C) in stage II CC; for IM (D), TC (E) and combined information (F) in stage III CC. Log-rank test; HR = Hazard Ratio, 95% CI between square brackets.

CI: 0.3–0.9]; log-rank p = 0.03) (Fig. 3A and B). When using both CD3-IM and CD3-TC information to estimate prognosis, patients with high infiltration had a significant better RFS compared to their low counterparts (HR = 0.5 [95% CI: 0.3–0.9]; log-rank p = 0.03) (Fig. 3C). While the combination of immune markers was still significantly associated with RFS in MSS tumours, no association was observed in MSI tumours (Supplementary Fig. 5A and B).

For stage III CC, a high immune infiltrate within the TC was clearly associated with a good RFS (HR = 0.53 [95% CI: 0.3–0.8]; log-rank p = 0.004), while a high CD3-IM had a trend for being correlated with a good RFS (HR = 0.72 [95% CI: 0.5–1.1]; log-rank p = 0.11 (Fig. 3D and E). Combination of IM-CD3 and TC-CD3 showed that patients with high infiltration had a significant better RFS compared to intermediate and low counterparts (respectively HR = 0.38 [95% CI: 0,2–0,7]; log-rank p = 0.004; HR = 0,4 [95% CI: 0,2–0,7]; log-rank p = 0.003) (Fig. 3F). MSS subgroup analysis in stage III CC for combined immune markers showed that RFS was still significantly associated with RFS (Supplementary Fig. 5C). Despite the trend, this observation was not confirmed in MSI subgroup (Supplementary Fig. 5D).

3.4. IV – Combination of CDX2 and CD3 improves prognosis stratification in stage III CC

As CDX2 and CD3 combined marker were prognostic only in stage III, we then focussed our intention on this group of patients. We observed no correlation between CDX2 H-score and immune variables for stage III patients (Supplementary Fig. 6A). We therefore generated a composite variable using both dichotomised CDX2-H score and combined CD3 TC and IM (Supplementary Fig. 6B). Results of our composite variable on stage III CC stratified continuously patients with especially patients with a good prognosis with a 5-year RFS of 90% (HR = 0.3 [95% CI: 0.1–0.9]; log-rank p = 0.04) (Fig. 4A). Using Cox univariate model, our composite variable was the most relevant to identify patients with a good RFS in Prodige-13 cohort and was robust through the whole cohort using a bootstrap strategy (HR = 0.25 [95% CI: 0.08–0.8]; p = 0.02) (Fig. 4B). Interestingly upon a multivariate model associating clinical features and the composite variable (Supplementary Method 2), our composite variable was the strongest prognostic marker associated with a good RFS along an internal validation using a bootstrap strategy (HR = 0.22 [95% CI: 0.05–0.9]; p = 0.04) (Fig. 4C).

4. Discussion

In the present study, we evaluated the prognosis value of two variables CDX2 and CD3 in a large and well-described cohort gathering both stage II and stage III CC called Prodige-13. When focussing on CDX2 analysis, our quantification strategy using an H-score was first validated as the quantified variable behaved as expected towards histological and clinical variables [29]. Using this kind of alternative strategies to quantify

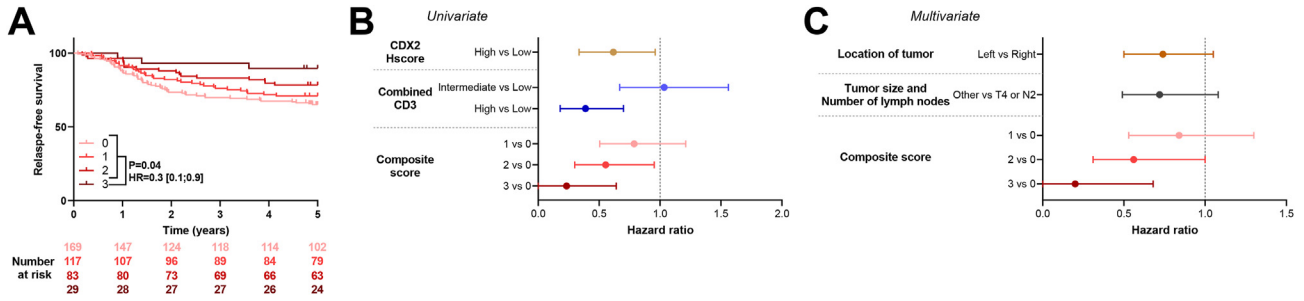

Fig. 4. **Combination of CDX2 and CD3 improves prognosis stratification in stage III CC.** Kaplan–Meier stratification for composite variable using CDX2 and CD3 combined information from the lowest to the highest (0–3) for stage III patients (A); barplots of validation through Prodige-13 using bootstrap strategy of the different univariate models (B) and multivariate model (C). Log-rank test; HR = hazard ratio, 95% CI between square brackets.

CDX2 has recently been proposed by other authors, either with immunofluorescence techniques [18] or with tandem mass spectrometry-based proteomics [19]. These quantitative strategies compared to visual estimation might be more considered as they might bring sharper information and take into account potential expression heterogeneity through tumour specimen. We confirmed here some previous works showing that high CDX2 H-score was associated with a good RFS for stage III. However, we did not observe a significant association despite a slight trend for CDX2 H-score being associated with a poor prognosis for stage II CC. As den Uil also reported [19], this result for stage II could be in part explained by MSI tumours which were over represented in low CDX2 H-score subgroup in our dataset and which are known to have a better prognosis [30–32]. It is moreover still unclear whether CDX2 has a major impact on stage II prognosis and literature.

The immune parameters especially evaluated with T-cell lymphocytes are well known to be prognostic in stage II and stage III CC. Galon with Immunoscore® [5] but also other works clearly described the importance of T lymphocytes within TC and IM of CC [20–22]. We recently made our contribution to this topic by adding an automated AI-based software called ColoClass to detect CD3+ lymphocytes within TC and IM [25]. This automated quantified evaluation on a WSI is important to alleviate pathologists' ROI selection bias and also save time in IHC analysis. In the present study, we obviously confirmed the major impact of CD3 in both TC and IM location using our AI software ColoClass. We confirmed here our previous data that especially CD3-TC has a prevailing prognostic value for stage III CC. For stage II CC, CD3-IM evaluation and CD3-TC standalone were both associated with a good prognosis. By combining information from TC and IM, CD3 staining clearly showed a great value to stratify stage II but also in stage III tumours.

As CDX H-score and immune parameters were not correlated, we finally combined those two variables to test whether this strategy could help patients' prognosis stratification. We restricted our analysis on stage III as both CDX2 and combined IM and TC-CD3 had a prognostic value only in stage III. Almost 8% of patients harbouring a high CDX2 H-score and a high combined immune parameters emerged from other with a clear better prognosis. Recently published data [29] also tried to combine several IHC parameters, including immune parameters like CD3 but also CD8, PD-L1, and HLA-G, with CDX2 especially on bad prognosis CC tumours (*i.e* T3 and T4) staggered from stage II to stage IV. By combining these information, the authors published a model which selected 6% from 188 patients with a better prognosis regardless of stage. This model, like our study, supported the rational to combine immune markers with CDX2 to determine CC patients' prognosis.

It is to remind that it is an unmet need for oncologists to accurately select patients who could benefit from adjuvant therapy for stage II CC. In our dataset, unfortunately our strategy to combine CDX2 H-score and immune information for these patients failed to have an interest as the prognostic value was only supported by immune parameters. For stage III CC, the current clinical dogma is to treat all patients with adjuvant chemotherapy and patients share the same follow-up. As a consequence, the purpose would be to find patients with biomarkers related to prognosis of whom a lighter adjuvant chemotherapy or a lighter follow-up could be considered. The present combination presented here with CDX2 H-score, and CD3 AI-guided analyses could in part find these patients of interest for stage III. Obviously, our work had limitation as it was a retrospective work and the results observed here, despite an internal validation with a bootstrap strategy, were not externally validated yet. It should consequently be interpreted with caution and would need further investigations on other larger cohorts before being clinically considered.

5. Conclusion

Despite improvement of colon cancer care, the discovery of new biomarkers or combination of known biomarkers related to patients' prognosis is a crucial need for oncologist to improve decision of chemotherapy

administration and follow-up. Our study, with two simple IHC procedures, that is, CDX2 and CD3, make emerge almost 10% of stage III CC with a very good prognosis. If confirmed, these results could be of a major interest to select stage III patients who could benefit from a potential therapy de-escalation in future clinical trials.

Funding

VD had a funding from French Anti-cancer Research Association ARC "Projet Fondation ARC" tracked with number 20191209484.

Author statement

Valentin Derangère: writing – original draft, software, conceptualization, investigation, funding acquisition, and visualisation. Julie Lecuelle: software, formal analysis, methodology, and visualisation. Oumaima Aoulad-Ben Salem: software and formal analysis. Come Lepage: supervision and project administration. Ben Mbairo Allatessem: software and formal analysis. Alis Ilie: investigation. Daniel Gonzalez: data curation and project administration. Karine Le Malicot: data curation and formal analysis. David Rageot: investigation. Olivier Bouché: resources. Jean-Marc Phelip: resources. Mathieu Baconnier: resources. Denis Pezet: resources. Virginie Sebbagh: resources. Eric Terrebonne: resources. Gauthier Bouard: resources. Valérie Jooste: data curation and formal analysis. Anne-Marie Bouvier: data curation and formal analysis. Chloé Molimar: data curation and resources. Franck Monnien: data curation and resources. Caroline Truntzer: methodology, validation, supervision, and writing – review and editing. Fréderic Bibeau: conceptualization, supervision, and project administration. Francois Ghiringhelli: conceptualization, supervision, writing – review and editing, funding aquisition.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank ARC Fundation for fundings (grant #20191209484).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2022.05.040.

References

[1] European Cancer Information System Available online: https://ecis.jrc.ec.europa.eu/ (accessed on 25 February 2022).
[2] Taieb J, Karoui M, Basile D. How I treat stage II colon cancer patients. ESMO Open 2021;6:100184. https://doi.org/10.1016/j.esmoop.2021.100184.
[3] Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, Silliman N, Tacey M, Wong H-L, Christie M, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8:346ra92. https://doi.org/10.1126/scitranslmed.aaf6219.
[4] Niedzwiecki D, Frankel WL, Venook AP, Ye X, Friedman PN, Goldberg RM, Mayer RJ, Colacchio TA, Mulligan JM, Davison TS, et al. Association between results of a gene expression signature assay and recurrence-free interval in patients with stage II colon cancer in cancer and leukemia group B 9581 (alliance). J Clin Oncol 2016;34:3047–53. https://doi.org/10.1200/JCO.2015.65.4699.
[5] Pagès F, Mlecnik B, Marliot F, Bindea G, Ou F-S, Bifulco C, Lugli A, Zlobec I, Rau TT, Berger MD, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. Lancet 2018;391:2128–39. https://doi.org/10.1016/S0140-6736(18)30789-X.
[6] Auclin E, Zaanan A, Vernerey D, Douard R, Gallois C, Laurent-Puig P, Bonnetain F, Taieb J. Subgroups and prognostication in stage III colon cancer: future perspectives for adjuvant therapy. Ann Oncol 2017;28:958–68. https://doi.org/10.1093/annonc/mdx030.
[7] André T, Vernerey D, Mineur L, Bennouna J, Desrame J, Faroux R, Fratte S, Hug de Larauze M, Paget-Bailly S, Chibaudel B, et al. Three versus 6 Months of oxaliplatin-based adjuvant chemotherapy for patients with stage III colon cancer: disease-free survival results from a randomized, open-label, international duration evaluation of adjuvant (IDEA) France, phase III trial. J Clin Oncol 2018;36:1469–77. https://doi.org/10.1200/JCO.2017.76.0355.
[8] Bărbălan A, Nicolaescu AC, Măgăran AV, Mercuţ R, Bălăşoiu M, Băncescu G, Şerbănescu MS, Lazăr OF, Săftoiu A. Immunohistochemistry predictive markers for primary colorectal cancer tumors: where are we and where are we going? Rom J Morphol Embryol 2018;59:29–42.
[9] Dahlin AM, Henriksson ML, Van Guelpen B, Stenling R, Oberg A, Rutegård J, Palmqvist R. Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. Mod Pathol 2011;24:671–82. https://doi.org/10.1038/modpathol.2010.234.
[10] Dalerba P, Sahoo D, Paik S, Guo X, Yothers G, Song N, Wilcox-Fogel N, Forgó E, Rajendran PS, Miranda SP, et al. CDX2 as a prognostic biomarker in stage II and stage III colon cancer. N Engl J Med 2016;374:211–22. https://doi.org/10.1056/NEJMoa1506597.
[11] Drummond F, Putt W, Fox M, Edwards YH. Cloning and chromosome assignment of the human CDX2 gene. Ann Hum Genet 1997;61:393–400. https://doi.org/10.1046/j.1469-1809.1997.6150393.x.
[12] Silberg DG, Swain GP, Suh ER, Traber PG. Cdx1 and Cdx2 expression during intestinal development. Gastroenterology 2000;119:961–71. https://doi.org/10.1053/gast.2000.18142.
[13] Bayrak R, Haltas H, Yenidunya S. The value of CDX2 and cytokeratins 7 and 20 expression in differentiating colorectal adenocarcinomas from extraintestinal gastrointestinal adenocarcinomas: cytokeratin 7-/20+ phenotype is more specific than CDX2 antibody. Diagn Pathol 2012;7:9. https://doi.org/10.1186/1746-1596-7-9.
[14] Ribeirinho-Soares S, Pádua D, Amaral AL, Valentini E, Azevedo D, Marques C, Barros R, Macedo F, Mesquita P,

Almeida R. Prognostic significance of MUC2, CDX2 and SOX2 in stage II colorectal cancer patients. BMC Cancer 2021;21:359. https://doi.org/10.1186/s12885-021-08070-6.

[15] Cecchini MJ, Walsh JC, Parfitt J, Chakrabarti S, Correa RJ, MacKenzie MJ, Driman DK. CDX2 and Muc2 immunohistochemistry as prognostic markers in stage II colon cancer. Hum Pathol 2019;90:70–9. https://doi.org/10.1016/j.humpath.2019.05.005.

[16] Zhang BY, Jones JC, Briggler AM, Hubbard JM, Kipp BR, Sargent DJ, Dixon JG, Grothey A. Lack of caudal-type homeobox transcription factor 2 expression as a prognostic biomarker in metastatic colorectal cancer. Clin Colorectal Cancer 2017;16:124–8. https://doi.org/10.1016/j.clcc.2016.09.003.

[17] Aasebø K, Dragomir A, Sundström M, Mezheyeuski A, Edqvist P-H, Eide GE, Ponten F, Pfeiffer P, Glimelius B, Sorbye HCDX2. A prognostic marker in metastatic colorectal cancer defining a better BRAF mutated and a worse KRAS mutated subgroup. Front Oncol 2020;10:8. https://doi.org/10.3389/fonc.2020.00008.

[18] Lopes N, Bergsland CH, Bjørnslett M, Pellinen T, Svindland A, Nesbakken A, Almeida R, Lothe RA, David L, Bruun J. Digital image analysis of multiplex fluorescence IHC in colorectal cancer recognizes the prognostic value of CDX2 and its negative correlation with SOX2. Lab Invest 2020;100:120–34. https://doi.org/10.1038/s41374-019-0336-4.

[19] den Uil SH, de Wit M, Slebos RJC, Delis-van Diemen PM, Sanders J, Piersma SR, Pham TV, Coupé VMH, Bril H, Stockmann HBAC, et al. Quantitative analysis of CDX2 protein expression improves its clinical utility as a prognostic biomarker in stage II and III colon cancer. Eur J Cancer 2021;144:91–100. https://doi.org/10.1016/j.ejca.2020.10.029.

[20] Sinicrope FA, Rego RL, Ansell SM, Knutson KL, Foster NR, Sargent DJ. Intraepithelial effector (CD3+)/Regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma. Gastroenterology 2009;137:1270–9. https://doi.org/10.1053/j.gastro.2009.06.053.

[21] Eriksen AC, Sørensen FB, Lindebjerg J, Hager H, dePont Christensen R, Kjær-Frifeldt S, Hansen TF. The prognostic value of tumor-infiltrating lymphocytes in stage II colon cancer. A nationwide population-based study. Transl Oncol 2018;11:979–87. https://doi.org/10.1016/j.tranon.2018.03.008.

[22] Ko YS, Pyo J-S. Clinicopathological significance and prognostic role of tumor-infiltrating lymphocytes in colorectal cancer. Int J Biol Markers 2019;34:132–8. https://doi.org/10.1177/1724600818817320.

[23] Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4. https://doi.org/10.1126/science.1129139.

[24] Marliot F, Chen X, Kirilovsky A, Sbarrato T, El Sissy C, Batista L, Van den Eynde M, Haicheur-Adjouri N, Anitei M-G, Musina A-M, et al. Analytical validation of the Immunoscore and its associated prognostic value in patients with colon cancer. J Immunother Cancer 2020;8:e000272. https://doi.org/10.1136/jitc-2019-000272.

[25] Reichling C, Taieb J, Derangere V, Klopfenstein Q, Le Malicot K, Gornet J-M, Becheur H, Fein F, Cojocarasu O, Kaminsky MC, et al. Artificial intelligence-guided tissue analysis combined with immune infiltrate assessment predicts stage III colon cancer outcomes in PETACC08 study. Gut 2020;69:681–90. https://doi.org/10.1136/gutjnl-2019-319292.

[26] Lepage C, Phelip J-M, Cany L, Faroux R, Manfredi S, Ain J-F, Pezet D, Baconnier M, Deguiral P, Terrebone E, et al. Effect of 5 Years of imaging and CEA follow-up to detect recurrence of colorectal cancer: the FFCD PRODIGE 13 randomised phase III trial. Dig Liver Dis 2015;47:529–31. https://doi.org/10.1016/j.dld.2015.03.021.

[27] Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, et al. QuPath: open source software for digital pathology image analysis. Sci Rep 2017;7:16878. https://doi.org/10.1038/s41598-017-17204-5.

[28] Goulding H, Pinder S, Cannon P, Pearson D, Nicholson R, Snead D, Bell J, Elston CW, Robertson JF, Blamey RW. A new immunohistochemical antibody for the assessment of estrogen receptor status on routine formalin-fixed tissue samples. Hum Pathol 1995;26:291–4. https://doi.org/10.1016/0046-8177(95)90060-8.

[29] Bennedsen ALB, Cai L, Hasselager RP, Özcan AA, Mohamed KB, Eriksen JO, Eiholm S, Bzorek M, Fiehn A-MK, Hviid TVF, et al. An exploration of immunohistochemistry-based prognostic markers in patients undergoing curative resections for colon cancer. BMC Cancer 2022;22:62. https://doi.org/10.1186/s12885-022-09169-0.

[30] Tejpar S, Bosman F, Delorenzi M, Fiocca R, Yan P, Klingbiel D, Dietrich D, Van Cutsem E, Labianca R, Roth A. Microsatellite instability (MSI) in stage II and III colon cancer treated with 5FU-lv or 5FU-lv and irinotecan (PETACC 3-EORTC 40993-SAKK 60/00 trial). J Clin Orthod 2009;27. 4001–4001. https://doi.org/10.1200/jco.2009.27.15_suppl.4001.

[31] Sinicrope FA, Mahoney MR, Smyrk TC, Thibodeau SN, Warren RS, Bertagnolli MM, Nelson GD, Goldberg RM, Sargent DJ, Alberts SR. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. J Clin Oncol 2013;31:3664–72. https://doi.org/10.1200/JCO.2013.48.9591.

[32] Hutchins G, Southward K, Handley K, Magill L, Beaumont C, Stahlschmidt J, Richman S, Chambers P, Seymour M, Kerr D, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol 2011;29:1261–70. https://doi.org/10.1200/JCO.2010.30.1366.