Evaluation of *KRAS*, *NRAS* and *BRAF* mutational status and microsatellite instability in early colorectal carcinomas invading the *submucosa* (pT1): towards an in-house molecular prognostication for pathologists?

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ABSTRACT

Aim We aimed to study the prognostic value of *KRAS*, *NRAS*, *BRAF* mutations and microsatellite stable (MSS)/instable (MSI) in the field of colorectal cancer invading the *submucosa* (ie, pT1 colorectal cancer (CRC)).

Methods We led a case-control study in tumour samples from 60 patients with pT1 CRC with (20 cases) and without (40 cases) metastatic evolution (5 years of follow-up) which were analysed for *KRAS*, *NRAS*, *BRAF* mutations (Idylla testing and next generation sequencing, NGS) and MSS/MSI status (Idylla testing and expression of mismatch repair (MMR) proteins using immunohistochemistry).

Results *KRAS* mutations were encountered in 11/20 (55%) cases and 21/40 (52.5%) controls (OR=1.11 (0.38 to 3.25), p=0.8548), *NRAS* mutations in 1/20 (5%) cases and 3/40 (7.5%) controls (OR=3.08 (0.62 to 15.39), p=0.1698) and *BRAF* mutations in 3/20 (15%) cases and 6/40 (15%) controls (OR=1.00 (0.22 to 4.5), p=1.00). A MSI status was diagnosed in 3/20 (15%) cases and 5/40 (12.5%) controls (OR=1.2353 (0.26 to 5.79), p=0.7885). Beyond the absence of significant association between the metastatic evolution and any of the studied molecular parameters, we observed a very good agreement between methods analysing *KRAS, NRAS* and *BRAF* mutations (Kappa value of 0.849 (0.748 to 0.95) between Idylla and NGS) and MSS/MSI (Idylla)—proficient MMR/deficient MMR (immunohistochemistry) status (Kappa value of 1.00).

Conclusion Although being feasible using the fully automated Idylla method as well as NGS, the molecular testing of *KRAS, NRAS, BRAF* and MSS/MSI status does not seem useful for prognostic purpose in the field of pT1 CRC.

INTRODUCTION

Early colorectal cancers (CRC) are defined as invasive adenocarcinomas invading into the *submucosa* but not beyond and are classified as pT1 tumours in the American Joint Committee on Cancer/Union for International Cancer Control tumour node metastasis system.[1 2] They account for 0.75%–5.6% of colorectal polyps removed in general diagnostic colonoscopy practice and cause nodal metastases in 6%–16% of patients.[3–10] Surgical resection with the dissection of regional lymph nodes has long remained the standard treatment for early CRC but, over the past 15 years, consensus guidelines has emerged encouraging a sole endoscopic treatment in patients with low risk of CRC recurrence and metastatic evolution.[8–14] Indeed, the diagnosis and management of early CRC require expertise of the endoscopists in charge of their resections and of the gastrointestinal pathologists in charge of their histopathological diagnosis. On the basis of several histopathological prognostic markers established to distinguish between low-risk and high-risk early CRC, a multidisciplinary decision is necessary to weight up the risks of patient's morbidity and mortality versus the risk of potential lymph node involvement, local recurrence and distant metastasis and to propose or not a complementary colorectal surgery with lymph node dissection.

Unfortunately, the histopathological parameters that weight the therapeutic decision suffer of imperfect interobserver reproducibility and diagnosis variations could have damageable consequences in terms of therapeutic decision.[15–17] Moreover, extranodal recurrences are more difficult to predict and diagnose earlier than locoregional metastases on the basis of histopathological prognostic criteria.[18] Several molecular parameters are known to be of prognostic relevance and are now integrated in daily-practice diagnostic guidelines for treatment choices in patients with stages II to IV CRC (ie, pT3-pT4 invasive CRC without metastasis or any pT score associated with nodal of distant metastasis).[19–21] Nevertheless, little is known about the utility of these biomarkers for the therapeutic decision in patients with endoscopy-removed early CRC with controversial values in the risk assessment of CRC recurrence and metastatic evolution in the literature.[22–27]

In this work, we intended to perform a case-control study comparing the frequency of *KRAS*, *NRAS* and *BRAF* mutations as well as of microsatellite stability/instability (MSS/MSI) between pT1

CRC without metastatic evolution and pT1 CRC with metastatic evolution. Beyond the molecular status itself, we also considered and compared different molecular methods. On the one hand, next generation sequencing (NGS) and mismatch repair (MMR) proteins immunohistochemistry (IHC) as reference and highly sensitive methods to diagnose *RAS-BRAF* mutations and proficient MMR (pMMR)/deficient MMR (dMMR) status, respectively. On the other hand, the fully-automated and Real-Time PCR Idylla platform now permits to analyse *KRAS*, *NRAS*, *BRAF* mutations and also MSS/MSI inside the pathology laboratories, even those without any experience in molecular methods, which could be an advantage of this method for daily prognostication of CRCs.

MATERIAL AND METHODS

Cases selection

The cases studied in this work were patients with pT1 CRC diagnosed from 1 January 2009 to 31 December 2013 in the area of Finistère (France, population 899 870 in 2011) who have presented a locoregional recurrence and/or metastatic evolution during the 5 years following their initial treatment. Controls with no recurrence (two controls included per case) from the same database were selected to match with the cases studied about sex, age and colorectal location. Histopathology slides have been reviewed by two pathologists (FB and LD) to reach a consensus about risk of recurrence parameters (ie, depth of submucosal invasion, tumour differentiation, vascular invasion, tumour budding, deep and lateral margins). According to the JSCCR guidelines, patients with pT1 CRCs were retrospectively classified as having 'low risk' tumours if all the following criteria were present: R0 margins, low grade (ie, grade one or 2) tumour differentiation, not signet-ring cell or mucinous adenocarcinoma, no vascular invasion, an invasion depth <1000 µm and a low-grade tumour budding (ie, grade 1). Any tumour lacking any of these criteria was classified as a 'high-risk' tumour).[19]

All cases were registered in the digestive cancer registry database of Finistère (Brest, France) that gathers information about patient's demographics, tumour characteristics, clinical and pathological staging, treatment modalities, recurrence and survival. The quality and exhaustiveness of the registry are certified every 4 years by an audit of the French National Committee of Registries. Formalin-fixed paraffin embedded (FFPE) tumour samples of pT1 CRC were collected from pathology laboratories' archives on the basis of initial pathology reports and slides review. As a retrospective and non-interventional one, the present study based on the data from the digestive cancer registry did not require informed consent of the patients and it was conducted in accordance with our national and institutional guidelines (French law text 'loi Jardé n°2012–300'). All samples were included in a registered tumour tissue collection.

Determination of the dMMR/pMMR status by immunohistochemistry

MLH1 (clone M1,prediluted, Roche Diagnostics, Meylan, France), MSH2 (clone FE11, Calbiochem, 1:50), MSH6 (clone 44, Roche Diagnostics, prediluted) and PMS2 (clone EPR3947, Roche Diagnostics, prediluted) IHC tests were performed analysing the expression of MMR proteins (Benchmark-Ventana Ultra automation, ultraView DAV revelation kit, Roche Diagnostics, Meylan, France). Nuclear loss of expression of one or more proteins by tumour cells with preserved expression in non-tumour cells was interpreted as a positive IHC test (ie, dMMR; otherwise it was said negative that is, pMMR).

KRAS, NRAS and BRAF analyses using next generation sequencing

As previously described, a single primer pool customised panel leading to the selection of 42 amplicons (ranging from 125 to 175 bp) targeting six genes (*EGFR, KRAS, NRAS, BRAF, cKIT* and *PDGFRa; EGFR, cKIT* and *PDGFRa* results being not taken into account in the present work) was designed by using the Ion AmpliSeq Designer suite V.5.3.1 with hg19 as reference genome.[28 29] The amplicons design covering 5.2 kb of genomic DNA was optimised for the simultaneous analysis of 16 samples with the 510 chip (Thermofisher, Foster City, California, USA) using Ion S5 System based on Ion Torrent Technology (Thermofisher). In relation to the extraction modalities after a visual inspection by a fully qualified pathologist, a neoplastic area with more than 5% of neoplastic cells was selected. Cells were scraped by using a sterile scalpel from the slides and DNA extraction was performed with QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK), following the manufacturer's instructions, resuspending the DNA in 30 µL of RNAsi/DNAsi free water (Ambion, Thermofisher, USA). To evaluate the quantity (ng/µL) of extracted DNA, 2 µL of resuspended DNA for each sample was analysed by using the Qubit Fluorometer (Thermofisher). SiRe panel NGS library preparation and sequencing analysis starting from 15 µL of genomic DNA, by using SiRe panel, libraries were prepared and purified on the Ion Chef automatic platform (Thermofisher), and eight samples were added per run. Libraries generation were carried out on Ion Code plates and amplified using Ion AmpliSeq DL8 Kit (Thermofisher). Then, under the thermal conditions defined by the manufacturer, we used 26 cycles for amplification and six cycles for library reamplification after barcoding. Purified and combined libraries from two Ion Chef runs, derived from 16 patients, were diluted to 70 pM. The pooled libraries were reloaded into the Ion Chef instrument, and templates were prepared by using the Ion 510, Ion 520 and Ion 530 Kit—Chef (Thermofisher). Finally, templates were loaded into the 510 chip and sequenced on Ion S5 System. In any single case, signal processing and base calling were carried out using the default base-caller parameters on Torrent Suite (V.5.0.2), and coverage analysis was performed using SiRe-designed bed files with coverage plug-in (V.5.0.2.0). The bioinformatics pipeline was based on the SiRe Variant caller plug-in (V.5.0.2.1) parameters enabled for automatic variant calls; the threshold parameters were specifically optimised for tissue based diagnostic. Only variants with >20X allele coverage and a quality score >20 within an amplicon that covered at least 500X alleles were called.

Fully-automated cartridge-based Idylla molecular analyses

Idylla testing was performed on the same block used for IHC and NGS genetics testing. The proportion of tumour cells was established on each sample by a pathologist on a dedicated 3-µm-thick haematoxylin-eosin-saffron stained tissue slide. Serial 10 µm tissue sections were produced for molecular analyses. The tumour zones were macroscopically circled to allow macrodissection of tumour tissue for molecular analyses if required to reach a tumour surface between 25 and 300 mm^2 containing at least 20% of tumour cells following the manufacturer's instructions about the three cartridges Idylla MSI Assay, Idylla NRAS-BRAF Mutation Test and Idylla KRAS Mutation Test (Biocartis). Tumour areas were macrodissected and then transferred in each Idylla cartridge itself inserted in the instrument. Inside the cartridge, the sample was homogenised and cells lysed using a combination of high-intensity-focused ultrasound,

enzymatic/chemical digestion and heat. The nucleic acids were liberated and ready for subsequent PCR amplification. The PCR was real time and used a fluorophore-based detection system. After 120 min (*NRAS-BRAF* and *KRAS* tests) to 150 min run (MSI assay), all steps were automatically performed inside the cartridges and final reports were directly available on the system after an automatic on-board post-PCR curve analysis. These final reports contained the final result in terms of 'MSS' or 'MSI' or the name of the mutation(s) of *KRAS*, *NRAS* or/and *BRAF* if any detected by the different cartridges as detailed in a previous review.[30]

Statistical analyses

Statistical analyses were performed using MedCalc Statistical Software V.13.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). OR calculation was used to compare cases and controls groups about the histopathological criteria in terms of 'high risk' versus 'low risk' criteria, oncogenic mutations (detected by any or the two Idylla or/and NGS methods) and MSI/MSS status. Spearman's rank correlation test was used to study the correlation between histopathological criteria and molecular results. The level of significance was set at $p<0.05$. The Kappa statistic test was used to quantify the intermethod agreement for *KRAS, NRAS, BRAF* (ie, NGS versus Idylla tests) and MSI/MSS-dMMR/pMMR analyses (ie, Idylla versus IHC tests, respectively). The values of Kappa strength agreements were interpreted as follows: <0.20 poor, 0.21–0.40 fair, 0.41–0.6 moderate, 0.61–0.80 good and 0.81–1.00 very good agreement.

RESULTS

Cases included and histoprognostic parameters

Cases and controls consisted, respectively, in 20 cases (16 with locoregional nodal metastases and 4 with distant metastases without nodal ones) and 40 controls (ie, without any metastatic evolution) and were 28 men and 32 women with a mean age of 70.5 years (range: 45–90 years) with tumours involving the right colon (12 cases), the transversal colon (2 cases) or the left colon (46 cases). More details and ORs about the histoprognostic parameters among cases and controls groups (ie, margins, tumour differentiation, vascular invasion, invasion depth and tumour budding) are provided in table 1.

Idylla and NGS KRAS, NRAS and BRAF analyses and intermethods comparisons

Among the 60 tumour samples, valid Idylla analyses were obtained for 59 (98.3%) samples using Idylla KRAS Mutation Tests, 58 (96.7%) samples using Idylla NRAS-BRAF Mutation Tests and 60 (100%) samples using the Idylla MSI Assay. NGS analyses were contributive for 51 (85%) samples with a failure of analyses for 9 (15%) samples. Only one sample was non-contributive with both Idylla and NGS testing (case #21). Using Idylla, *KRAS, NRAS* and *BRAF* mutations were encountered in 32 (53.3%), 4 (6.7%) and 7 (11.7%) samples, respectively. NGS analyses concluded in *KRAS*, *NRAS* and *BRAF* mutations in 23 (38.3%), 3 (5%) and 6 (10%) samples, respectively. Two samples contained multiple mutations: one sample with *NRASA146T* and *BRAFV600E* mutations using Idylla and NGS testing (case #56) and one sample with *KRAS* codon 13, *NRAS* codon 12 and *BRAF* codon 600 mutations detected by the Idylla system but not with NGS analyses (case #42). Among the nine samples with non-contributive NGS analyses, Idylla testing resulted in valid analyses permitting the detection of nine *KRAS* mutations, one *NRAS* mutation and three *BRAF* mutations.

Discrepant mutational results were obtained between Idylla and NGS for five cases: one case with *BRAF* mutations not included in the panel of Idylla NRAS-BRAF Mutation Test (*BRAF p.G466E* in case #57), two cases with mutations detected by the Idylla KRAS Mutation Test but not using NGS (*KRAS p.G13D* mutation in case #1 and *KRAS p.G12A* in case #23), one case with a *KRAS p.A146P/T/V* detected by Idylla but not by NGS and a *BRAF p.G469R* mutation not included in the panel of Idylla NRAS-BRAF Mutation Test but detected by NGS (case #9) and the case #42 with three *KRAS p.G13D*, *NRAS p.G12A/V* and *BRAF p.V600E/D* mutations detected using Idylla but not using NGS. This resulted in a Kappa value of 0.849 (0.748 to 0.95) reflecting a very good agreement between NGS and Idylla analyses among samples with contributive analyses (Kappa values of 0.844 (0.699 to 0.989) for *KRAS* testing, 0.847 (0.553 to 1.0) for *NRAS* testing and 0.695 (0.37 to 1.0) for *BRAF* testing separately).

Results and comparison of methods for MSI/MSS-dMMR/pMMR status analyses

All samples reached contributive results using the Idylla MSI Assay and a MSI status was concluded for 8 (13.3%) samples. Among the 60 samples also analysed using MLH1, PMS2, MSH2 and MSH6 IHC, the loss of expression of at least one of the MMR proteins (ie, a dMMR status) was diagnosed in the eight MSI samples (see table 2 for details and figure 1 for one example of molecular results provided by the Idylla platform with case #13). This resulted in a Kappa value of 1.00 reflecting a very good agreement between the two methods. Of note, two MSI samples were initially considered difficult to interpret using MMR proteins IHC because of a greatly diminished but not completely negative expression of PMS2 (case #43) and MSH6 (case #5) and, for these two samples, Idylla testing has permitted to definitely conclude in the loss of expression of the two proteins in a concordant context of MSI/MMR deficiency.

Table 1 Summary of 'high-risk' histoprognostic parameters among cases and controls

'High-risk' pT1 colorectal cancer criteria	Cases (n=20)	Controls (n=40)	ORs, p values
Deep margin R1/NA	18 (90%)	29 (72.5%)	3.41 (0.68–17.21), p=0.1368
Lateral margin R1/NA	17 (85%)	29 (72.5%)	2.15 (0.52–8.8), p=0.2875
Invasion depth >1000 µm	18 (90%)	32 (80%)	2.25 (0.43–11.76), p=0.3365
Poor tumour differentiation	4 (20%)	1 (2.5%)	9.75 (1.01–94.12), p=0.049*
Vascular invasion	8 (40%)	1 (2.5%)	26 (2.95–229.37), p=0.0034*
High grade tumour budding	7 (35%)	3 (7.5%)	6.64 (1.49–29.55), p=0.0129*
Tumours with any criterion(a) of 'high risk' of recurrence	20 (100%)	32 (80%)	10.72 (0.59–195.92), p=0.1095

*p<0.05

Table 2 Details of molecular results among cases with metastatic evolution and controls without metastatic evolution

Case number	Patient with metastatic evolution	Idylla KRAS mutation test	Idylla NRAS-BRAF mutation test		Idylla MSI assay (valid/instables microsatellites sequences)	MLH1, PMS2, MSH2 and MSH6 IHC	NGS analyses (% of mutated alleles)		
			NRAS	*BRAF*			*KRAS*	*NRAS*	*BRAF*
#1	Yes	*G13D*	–	–	MSS (0/7)	–	–	–	–
#2	Yes	*G12D*	NC	NC	MSS (0/7)	–	NC	NC	NC
#3	Yes	*G12C*	–	–	MSS (0/7)	–	*G12C* (2.1%)	–	–
#4	Yes	*G12C*	–	–	MSS (0/7)	–	*G12C* (58.3%)	–	
#5	Yes	*G12D*	–	–	MSI (3/7: *DIDO1*, *MRE11*, *RYR3*)	Isolated loss of MSH6 *	*G12D* (22.5%)	–	–
#6	Yes	*G12V*	–	–	MSS (0/7)	–	*G12V* (40.8%)	–	–
#7	Yes	*G12V*	–	–	MSS (0/7)	–	*G12V* (33.3%)	–	–
#8	Yes	*G12V*	–	–	MSS (0/7)	–	*G12V* (23.5%)	–	–
#9	Yes	*A146P/T/V*	–	–	MSS (0/7)	–	–	–	*G469R* (11%)
#10	Yes	*G12C*	–	–	MSS (0/7)	–	NC	NC	NC
#11	Yes	*A146P/T/V*	–	–	MSS (0/7)	–	*A146T* (52.8%)	–	–
#12	Yes	–	–	*V600E/D*	MSI (5/7: *ACVR2*, *BTBD7*, *DIDO1*, *MRE11*, *SULF2*)	Loss of MLH1, PMS2 and MSH6	–	–	*V600E* (22.5%)
#13	Yes	–	–	*V600E/D*	MSI (5/7: *ACVR2*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*)	Loss of MLH1 and PMS2	NC	NC	NC
#14	Yes	–	–	–	MSS (0/7)	–	–	–	–
#15	Yes	–	–	–	MSS (0/7)	–	–	–	–
#16	Yes	–	–	–	MSS (0/7)	–	–	–	–
#17	Yes	–	–	–	MSS (0/7)	–	–	–	–
#18	Yes	–	*Q61K*	–	MSS (0/7)	–	–	*Q61K* (54.5%)	–
#19	Yes	–	–	–	MSS (0/7)	–	–	–	–
#20	Yes	–	–	–	MSS (0/7)	–	–	–	–
#21	No	NC	NC	NC	MSS (0/7)	–	NC	NC	NC
#22	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (42.3%)	–	–
#23	No	*G12A*	–	–	MSS (0/7)	–	–	–	–
#24	No	*G12C*	–	–	MSS (0/7)	–	*G12C* (55.6%)	–	–
#25	No	*G12D*	–	–	MSS (0/7)	–	NC	NC	NC
#26	No	*G12D*	–	–	MSS (0/7)	–	*G12D* (43.6%)	–	–
#27	No	*G12D*	–	–	MSS (0/7)	–	*G12D* (42.1%)	–	–
#28	No	*G12D*	–	–	MSS (0/7)	–	*G12D* (32%)	–	–
#29	No	*G12D*	–	–	MSS (0/7)	–	*G12D* (8.4%)	–	–
#30	No	*G12D*	–	–	MSS (0/7)	–	*G12D* (18.8%)	–	–
#31	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (29.3%)	–	–
#32	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (49%)	–	–
#33	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (1.16%)	–	–
#34	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (51.5%)	–	–
#35	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (48.6%)	–	–
#36	No	*G13D*	–	–	MSI (6/7: *ACVR2*, *BTBD7*, *DIDO1*, *RYR3*, *SEC31A*, *SULF2*)	Isolated loss of MSH6	*G13D* (11%)	–	–
#37	No	*G13D*	–	–	MSS (0/7)	–	NC	NC	NC
#38	No	*Q61H*	–	–	MSS (0/7)	–	*Q61H* (30.3%)	–	–
#39	No	*G12C*	–	–	MSS (0/7)	–	NC	NC	NC
#40	No	*G12V*	–	–	MSS (0/7)	–	*G12V* (41.6%)	–	–
#41	No	*G13D*	–	–	MSS (0/7)	–	*G13D* (41%)	–	–
#42	No	*G13D*	*G12A/V*	*V600E/D*	MSS (0/7)	–	–	–	–
#43	No	–	–	*V600E/D*	MSI (2/7: *MRE11*, *SULF2*)	Isolated loss of PMS2 *	–	–	*V600E* (64.2%)
#44	No	–	–	–	MSI (4/7: *ACVR2*, *DIDO1*, *MRE11*, *RYR3*)	Loss of MLH1 and PMS2	–	–	–
#45	No	–	–	*V600E/D*	MSI (5/7: *ACVR2*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*)	Loss of MLH1 and PMS2	NC	NC	NC
#46	No	–	–	*V600E/D*	MSI (7/7: *ACVR2*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*, *SEC31A*, *SULF2*)	Loss of MSH2, MSH6 and MLH1	–	–	*V600E* (4%)
#47	No	–	–	–	MSS (0/7)	–	–	–	–

Continued

Table 2 Continued

Case number	Patient with metastatic evolution	Idylla KRAS mutation test	Idylla NRAS-BRAF mutation test		Idylla MSI assay (valid/instables microsatellites sequences)	MLH1, PMS2, MSH2 and MSH6 IHC	NGS analyses (% of mutated alleles)		
			NRAS	*BRAF*			*KRAS*	*NRAS*	*BRAF*
#48	No	–	–	–	MSS (0/7)	–	–	–	–
#49	No	–	*Q61K*	–	MSS (0/7)	–	–	*Q61K* (22.3%)	–
#50	No	–	–	–	MSS (0/7)	–	–	–	–
#51	No	–	–	–	MSS (0/7)	–	NC	NC	NC
#52	No	–	–	–	MSS (0/7)	–	–	–	–
#53	No	–	–	–	MSS (0/7)	–	–	–	–
#54	No	–	–	–	MSS (0/7)	–	–	–	–
#55	No	–	–	–	MSS (0/7)	–	–	–	–
#56	No	–	*A146T/V*	*V600E/D*	MSS (0/7)	–	–	*A146T* (4.6%)	*V600E* (15.7%)
#57	No	–	–	–	MSS (0/7)	–	–	–	*G466E* (17.8%)
#58	No	–	–	–	MSS (0/7)	–	–	–	–
#59	No	–	–	–	MSS (0/7)	–	–	–	–
#60	No	–	–	–	MSS (0/7)	–	–	–	–

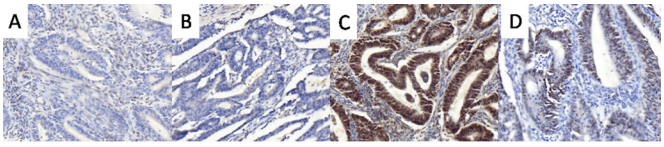
*Doubtful IHC result during initial interpretation
–, negative result; IHC, immunohistochemistry; MSI, microsatellite instability; MSS, microsatellites stability; NC, non-contributive analysis; NGS, next generation sequencing.

Correlation between histoprognostic and molecular parameters and comparisons between cases and controls

Taking into account both NGS and Idylla results, *KRAS* mutations were encountered in 11/20 (55%) cases and 21/40 (52.5%) controls (OR=1.11 (0.38 to 3.25), p=0.8548), *NRAS* mutations in 1/20 (5%) cases and 3/40 (7.5%) controls (OR=3.08 (0.62 to 15.39), p=0.1698) and *BRAF* mutations in 3/20 (15%) cases and 6/40 (15%) controls (OR=1.00 (0.22 to 4.5), p=1.00). A MSI status was diagnosed in 3/20 (15%) cases and 5/40 (12.5%) controls (OR=1.2353 (0.26 to 5.79), p=0.7885).

A *BRAF* codon 600 mutation was detected in 5/8 (62.5%) MSI tumours (vs 1/52, 1.9% of MSS tumours, OR=85 (7.39 to 977.64), p=0.0004). There was no significant association between MSS/MSI status and *BRAFnon-V600*, *KRAS* or *NRAS* mutations (detailed data not shown). See table 2 for detailed mutations.

Rank correlation analyses revealed only a significant correlation between *KRAS* mutations and the tumour differentiation with less frequent *KRAS* mutations among tumours with poor differentiation (0/5 tumours, p=0.0104). There was no other significant correlation between any *KRAS*, *NRAS* or *BRAF* mutation or MSI/MSS statuses and any of the histoprognostic parameters mentioned above.



E Idylla™ MSI Assay

Sample MSI Status	MSI-H
ACVR2A	Mutation detected
MSI Score	1.00
BTBD7	Mutation detected
MSI Score	0.99
DIDO1	Mutation detected
MSI Score	0.99
MRE11	Mutation detected
MSI Score	1.00
RYR3	Mutation detected
MSI Score	1.00
SEC31A	No mutation detected
MSI Score	0.05
SULF2	No mutation detected
MSI Score	0.08

F Idylla™ KRAS Mutation Test

KRAS GENOTYPE	NO MUTATION DETECTED IN KRAS CODON 12,13,59,61,117,146

G Idylla™ NRAS-BRAF Mutation Test

NRAS GENOTYPE	NO MUTATION DETECTED IN NRAS CODON 12,13,59,61,117,146
BRAF GENOTYPE	MUTATION DETECTED IN BRAF CODON 600
Mutation	V600E/D
Protein	p.Val600Glu / p.Val600Asp
Nucleotide Change	c.1799T>A ; c.1799_1800delinsAA / c.1799_1800delinsAC
CQ OF NRAS CONTROL	37.3

Figure 1 Example of molecular results in a sample of pT1 colorectal cancer (case #13). Immunohistochemistry concluded in a loss of expression of MLH1 (A) and PMS2 (B) with a preserved expression of MSH2 (C) and MSH6 (D) in cancer cells (×20 magnification, diaminobenzidine revelation, haematoxylin counterstaining). This was concordant with the MSI result of the Idylla MSI Assay with 5/7 instable microsatellites sequences (E). Idylla KRAS Mutation Test (F) and Idylla NRAS-BRAF Mutation Test (G) concluded in no *KRAS* or *NRAS* mutation but diagnosed a *BRAFV600E/D* mutation (E–G: screenshots of Idylla automatically generated written reports). MSI, microsatellite instability.

DISCUSSION

The value of *KRAS*, *NRAS* and *BRAF* mutations and MSS/MSI status in the risk assessment of CRC recurrence and metastatic evolution in pT1 CRC remains controversial. Besides some studies considering together pT1 and pT2 as stage I CRC or regrouping pT1 to pT3 CRC as 'small adenocarcinomas', studies dedicated specifically to pT1 CRC and focusing on molecular biomarkers are rare.[22–27]

In a subset of 48 pT1 CRC tumours including 22 cases with nodal metastases and 26 cases without nodal metastases, Pai *et al* have found no statistically significant difference for molecular alterations among 50 genes including *KRAS*, *NRAS* and *BRAF* as well as for MSI/MSS status between cases with or without metastatic evolution.[22] This is in accordance with our results. Nevertheless, at the opposite, among 28 cases of stage I rectal cancers, Sideris *et al* found that a *KRAS* mutation was associated with distant recurrence of disease (no *BRAF* mutation or MSI tumour in this file of rectal tumours).[25] Also focusing on rectal cancers, in the study led by Leong *et al* including 32 stage I rectal cancer, neither *KRAS* mutation nor MSS/MSI status was associated with advanced disease in univariate analyses.[26]

In a file of 103 pT1 CRC, Nosho *et al* detected *KRAS* and *BRAF* mutations in 33% and 1.9% of cases, respectively, and

found using multiple logistic regression analyses that these mutations were independent risk factors for venous invasion in pT1 CRC and implicated in lymphatic invasion and lymph node metastasis (*NRAS* and MSS/MSI statuses were not investigated in their study).[23] The frequencies of *KRAS* and *BRAF* mutations were higher in our study (53.3% and 11.7%, respectively) than in the study by Nosho *et al* and were consistent with the frequencies encountered in our daily practice analysing tumour samples of patients with advanced CRC for prognostic and theranostic purpose. Beyond a correlation between *KRAS* status and tumour differentiation, we did not encounter any other significant correlation between the mutational status and the histoprognostic parameters associated with an increased risk of tumour recurrence as the vascular invasion mentioned by Nosho *et al*. Caution is nevertheless required when comparing epidemiological data from different parts of the world because cancer epidemiology including molecular subtypes can highly vary from one geographic area to another.

Studying the prognostic value of *KRAS* mutation and microsatellite instability in stage I-IV CRC including 88 stage I (ie, pT1 and also pT2) CRC, Nash *et al* have found trends (but no significant difference) of worse disease-specific survival in MSS tumours compared with MSI (70 cases vs 12 cases, respectively, p=0.17) and in *KRAS* mutated versus *KRAS* wild-type stage I CRC (32 cases vs 56 cases, respectively, p=0.07). Among 532 CRC, they also found no significant difference between stages I, II, III and IV CRC for the prevalence of *KRAS* mutations (between 34% and 40%) whereas MSI was more common in early-stage CRC (I: 15%, II: 31%, III: 10%, IV 2%, p=0.0001; no data about *NRAS* and *BRAF*).[24] Our data did not permit to perform survival analyses in our study but the prognostic data reported by Nash *et al* are effectively in accordance with the reported poor prognostic value of *RAS*-mutations in patients with advanced CRC whereas MSI CRC has in a general manner a better prognosis than MSS ones. Beyond the frequency of *KRAS* mutations in early vs advanced CRC discussed above, the frequency of MSI tumours among our case series of pT1 CRC (13.3%) was concordant with the data of Nash *et al* and with the frequency of MSI cancers among more advanced CRC analysed in our daily practice.

Beyond the controversial prognostic value of molecular alterations in pT1 CRC, another reason to study *KRAS*, *NRAS*, *BRAF* and MSI/MSS status in early CRC may consist in the fact that, in comparison with more advanced and larger tumours, the small size of some pT1 CRC may represent a challenge for tumour dissection and subsequent molecular analysis. We hypothesise that sample-related issues combined with different methods for genotyping pT1 CRC samples may have contributed to the contradictory results obtained in previous works investigating for the prognostic value of molecular alterations in pT1 CRC. Given this hypothesis, we have chosen to perform a method evaluation study together with the prognostic dedicated one in our series of pT1 CRC. In this manner, we have evaluated the performances of the Idylla platform which has been already reported of particular interest in the analysis of CRC samples.[28 31–34] Our study has confirmed the very good agreement between Idylla tests and NGS results (*KRAS*, *NRAS* and *BRAF* status) and between Idylla MSI Assay and MMR proteins IHC (MSI/MSS-dMMR/pMMR testing) and it has also highlighted the high rate of contributive molecular analyses using Idylla in comparison with NGS analyses in our case series. The high proportion of non contributive results using NGS in our study may be explained by a poor quality of DNA extracted from archival FFPE samples in our series. Nevertheless, performing Idylla tests has permitted to obtain contributive analyses in NGS-non contributive samples and also to diagnose some *KRAS*, *NRAS* and *BRAF* mutations in these samples. The two molecular methods applied to the same pT1 CRC samples have also resulted in some discrepant results (five samples) supporting our hypothesis that technical issues could at least partially contribute to the different and sometimes contradictory conclusions of previous works dedicated to molecular analysis of pT1 CRC.

CONCLUSION

At present, there is no obvious prognostic gain in performing molecular analyses in pT1CRC. Maybe any additional biomarker will better help to determine the prognosis of these tumours in the future and will merit completing the histoprognostic parameters recommended in the current guidelines for reporting pathological examination of pT1 CRC. If one day such a molecular parameter would become relevant in this field, it would be obviously interesting that its analysis could be performed in the same workflow as the histopathological examination of the tumour by a pathologist. Platforms as the Idylla one would be especially interesting in this field. Beyond the *RAS-RAF* status, MSI/MSS testing could nevertheless have to be performed in some pT1 CRC, not for theranostic purpose, but if the clinical data are evocative for hereditary non-polyposis CRC (so-called Lynch) syndrome. As the Idylla MSI Assay appeared very performing in our study as in previous works and as this test is easy to implement in any pathology laboratory as other Idylla ones, it could represent an interesting tool to perform rapid in-house molecular testing ancillary to MMR protein IHC.[34] Further studies, if feasible in larger series, will be required to better define how and when to test for molecular alterations in early CRC taking into account the new opportunities provided by the development of new testing methods applicable in pathology laboratories.

Take home messages

- The prognostic value of molecular status in pT1 colorectal cancer remains debated.
- We performed a case-control study in 60 pT1 colorectal cancer cases with (20) and without (40) metastatic evolution.
- Neither *KRAS, NRAS, BRAF* nor MSI/MSS status was significantly associated with metastatic evolution.
- *KRAS, NRAS, BRAF* and MSI/MSS-dMMR/pMMR statuses showed good agreement between Idylla tests, NGS and immunohistochemistry analyses.

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Contributors AU, GT, LD, MC, JBN and MR conceived and designed the study. FB, LD, LS and AB collected samples and pathological data and reviewed slides. AB, CDL, EV, FC, GT and LD performed molecular and immunohistochemistry analyses. AU, AB, GT and CDL analysed data, wrote, edited and reviewed the manuscript. MC, FB and BB provided clinical follow-up data. AU, GT, AB, GT, CDL and PM wrote and reviewed the manuscript. All authors gave final approval for publication.

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Ethics approval Data were registered in the digestive cancer registry of Finistere database certified by the French National Committee of Registries and the present

study was conducted in accordance with the Declaration of Helsinki and after approval by our institutional review board with tumour samples registered in a tumour tissue collection (CHRU Brest, CPP n° DC—2008–214).

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