Modern Pathology (2014) 27, 644–650

BRAFV600E immunohistochemistry in conjunction with mismatch repair status predicts survival in patients with colorectal cancer

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Immunohistochemistry has recently been validated for the detection of the *BRAFV600E* mutation across a range of tumor types. In colorectal carcinoma, the presence of the *BRAFV600E* mutation can be used to virtually exclude Lynch syndrome in mismatch repair-deficient tumors. In mismatch repair-proficient tumors, *BRAFV600E* mutation assessed by molecular methods has been proposed as a poor prognostic factor. We investigated whether combined *BRAFV600E* and mismatch repair status assessment by immunohistochemistry alone can be used as a prognostic marker in the routine clinical setting. We performed immunohistochemistry for BRAFV600E, MLH1, PMS2, MSH2, and MSH6 on 1426 consecutive unselected colorectal carcinomas. Ninety-one (6.4%) carcinomas were mismatch repair-proficient and *BRAFV600E* mutant, and these tumors demonstrated a significantly worse 5-year survival of 49.7% compared with mismatch repair-proficient *BRAF* wild type (74.1% of tumors, 65.4% survival), mismatch repair-deficient *BRAFV600*E mutant (12.9% of tumors, 70.1% survival), and mismatch repair-deficient *BRAF* wild type (6.6% of tumors, 73.6% survival). The poor survival was confirmed by univariate analysis ($P<0.01$) but fell away in multivariate analysis ($P=0.68$) because of the strong effect of tumor stage and age on overall survival. We conclude that in addition to its utility in screening for Lynch syndrome, reflex BRAFV600E and mismatch repair assessment by immunohistochemistry can be used as a powerful predictor of all-cause survival.
Modern Pathology (2014) **27,** 644–650; doi:10.1038/modpathol.2013.200; published online 25 October 2013

Keywords: BRAFV600E; colorectal carcinoma; microsatellite instability; mismatch repair deficiency

To be adopted in the routine clinical setting, a biomarker must both demonstrate a clear additional benefit over standard clinical, radiological, and pathological examination (usually to predict outcome or response to treatment), and also be cost effective and readily available. Although literally thousands of biomarkers have been proposed, very few have entered routine clinical practice, owing to a combination of poor efficacy, expense, and lack of availability.[1] For example, in colorectal carcinoma, the only biomarkers in widespread clinical use are *KRAS* mutation testing, which is used to predict response to anti-EGFR-targeted therapy in metastatic disease, and either microsatellite instability determination by molecular methods or immunohistochemistry for the DNA mismatch repair markers MLH1, PMS2, MSH2, and MSH6, which are essentially equally effective in triaging genetic testing for Lynch syndrome.[2] There are currently no prognostic biomarkers for colorectal carcinoma in routine clinical use.

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Received 7 August 2013; revised 25 September 2013; accepted 26 September 2013; published online 25 October 2013

The presence of *BRAF* mutation in mismatch repair-deficient tumors is commonly used to virtually exclude Lynch syndrome, and many centers now perform routine *BRAF* testing in all microsatellite-unstable tumors.[2] Recently, *BRAF*-mutant mismatch repair-proficient colorectal carcinomas have emerged as a poor prognostic phenotype[3,4] with unique features, including a poor or absent response to anti-EGFR therapy, despite being wild type for *KRAS*.[5–7] Identification of these mismatch repair-proficient *BRAF* mutant tumors may be beneficial to predict poor outcome and to guide therapy.[5–7] However, current testing, which is usually based on molecular techniques, has not been deployed universally because of the additional expense and because molecular testing is outside the routine workflow of many surgical pathology laboratories, which is based on morphology and immunohistochemistry.

Recently, four groups have demonstrated that mutation-specific immunohistochemistry using a novel commercially available mouse monoclonal antibody is a highly sensitive and specific technique to identify the *BRAFV600E* mutation in colorectal carcinomas.[2,8–10] To date, its main suggested utility has been to triage molecular testing for Lynch syndrome in mismatch repair-deficient tumors. It has been proposed that if BRAFV600E immunohistochemistry were performed universally in all tumors, it could also be used to detect the poor prognosis mismatch repair-proficient *BRAFV600E* mutant colorectal carcinomas.[2] As the antibody is now commercially available and used in many diagnostic pathology laboratories, if validated as a prognostic marker BRAFV600E immunohistochemistry assessed in conjunction with mismatch repair status could become the first prognostic biomarker for colorectal cancer deployed into routine clinical use.

In this study, we sought to validate combined BRAF and mismatch repair status as determined by immunohistochemistry as a prognostic biomarker in colorectal carcinoma.

Materials and methods

We searched the pathology database of the Royal North Shore Hospital, Sydney, Australia, for all colorectal carcinomas undergoing surgical resection during the calendar years 2004–2009. During this period, this center performed centralized pathological testing for two quaternary referral hospitals with dedicated colorectal surgery units and four community hospitals. Therefore, this patient cohort represents a true snapshot of colorectal carcinoma cases encountered in the community as a whole. Tissue microarrays containing duplicate 1 mm cores were created and immunohistochemistry for the four mismatch repair proteins (MLH1, MSH2, PMS2, and MSH6) and BRAFV600E (using clone VE1, SpringBioscience, Pleasonton, CA) were performed using previously described methods.[2]

For BRAFV600E mutation-specific immunohistochemistry, slides were stained using the Leica BondIII autostainer (Leica Microsystems, Mount Waverley, VIC, Australia) used according to the manufacturer's protocol. The slides were dewaxed in Bond Dewax solution (AR9222, Leica Microsystems) and hydrated in Bond Wash solution (AR9590, Leica Microsystems). Heat-induced epitope retrieval was performed for 60 min in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640, Leica Microsystems). Slides were then incubated with the primary antibody at a dilution of 1 in 80 for 30 min at room temperature. Antibody detection was performed using the biotin-free Bond Polymer Defined Detection System (DS9713, Leica Microsystems) according to the manufacturer's protocol. Slides were then counterstained with hematoxylin. Staining was interpreted by a single pathologist blinded to all other data. Slides were considered positive if $>20\%$ of neoplastic cells stained positively. If the tissue microarray staining was not clearly interpretable (eg, if there were no good internal controls for the mismatch repair markers), it was repeated on whole sections.

Overall survival was defined as the duration alive from time of definitive surgery. Follow-up was obtained by the examination of hospital medical records, the records from surgeons' private rooms, and archival public death notices and obituaries in the state of New South Wales, Australia. Patients were followed up until death or their last date of follow-up not >7 years after definitive surgery.

The survival for each of the four immunohistochemical phenotypes was determined as a function of cumulative survival over time (Kaplan–Meier method). Pairwise log rank test was used to determine significance in survival differences. Multivariate Cox regression was employed to explore the effect of mismatch repair/BRAF immunohistochemistry phenotype on survival, using a model that included gender, age at diagnosis, tumor anatomic location, tumor histologic grade, presence or absence of lymphovascular space invasion, peritumoral lymphocyte reaction status, and American Joint Committee on Cancer/TNM seventh edition tumor stage. A P-value <0.05 was taken to be significant. All analyses were performed using IBM SPSS Statistics version 21 on OSX.

This study was approved by the Northern Sydney Local Health District Human Research Ethics Committee under protocol 1201-035M.

Results

A total of 1426 colorectal carcinomas were assessed in the tissue microarray. The clinical and pathological features are presented in Table 1. Briefly, the mean age was 74 years (range 17–100 years) and

Table 1 Clinical and pathological characteristics of 1426 consecutive colorectal cancer patients (2004–2009)

Variable	*Count (%) unless otherwise stated*	*Single variable* P-*value*[a]	*Univariate analysis HR (95% CI),* P-*value*	*Multivariate analysis HR (95% CI),* P-*value*
Gender		0.13		
Female	743 (52.1)		1.00	1.00
Male	683 (47.9)		0.82 (0.67–1.01), 0.07	1.24 (0.94–1.65), 0.13
Age at diagnosis, median (range)	74 (17–100)		1.03 (1.02–1.04), <0.01	1.04 (1.03–1.05), <0.01
Anatomic location		<0.01		
Rectum	363 (25.4)		1.00	1.00
Cecum	312 (21.9)		1.36 (1.00–1.85), 0.05	1.08 (0.72–1.62), 0.71
Ascending colon	219 (15.3)		1.22 (0.85–1.75), 0.29	1.00 (0.62–1.62), 0.99
Transverse colon	168 (11.8)		1.95 (1.37–2.77), <0.01	1.17 (0.72–1.90), 0.54
Descending colon	51 (3.6)		1.65 (0.96–2.83), 0.07	0.88 (0.42–1.86), 0.74
Sigmoid colon	302 (21.2)		1.14 (0.83–1.58), 0.42	1.02 (0.67–1.55), 0.93
Histologic grade		<0.01		
Low	826 (57.9)		1.00	1.00
High	214 (15.0)		1.87 (1.42–2.47), <0.01	1.27 (0.87–1.84), 0.21
Lymphovascular space invasion		0.02		
Absent	538 (37.7)		1.00	1.00
Present	459 (32.2)		2.26 (1.72–2.95), <0.01	1.57 (1.13–2.19), <0.01
Peritumoral lymphocyte reaction		<0.01		
Absent	45 (3.2)		1.00	1.00
Present	999 (70.1)		2.21 (0.91–5.37), 0.08	1.90 (0.74–4.89), 0.18
Overall stage AJCC/TNM 7th edn		<0.01		
I	235 (16.5)		1.00	1.00
IIA	415 (29.1)		2.00 (1.30–3.17), <0.01	2.21 (1.20–4.11), 0.01
IIB	85 (6.0)		2.44 (1.34–4.42), <0.01	2.66 (1.20–5.89), 0.02
IIC	15 (1.1)		9.66 (4.17–22.40), <0.01	11.93 (4.68–30.41), <0.01
IIIA	64 (4.5)		1.10 (0.50–2.43), 0.82	0.94 (0.27–3.33), 0.93
IIIB	376 (26.3)		3.05 (1.97–4.72), <0.01	2.75 (1.49–5.08), <0.01
IIIC	174 (12.2)		6.48 (4.12–10.17), <0.01	5.85 (3.11–11.02), <0.01
IVA	32 (2.2)		8.06 (4.24–15.31), <0.01	11.76 (4.88–28.32), <0.01
IVB	30 (2.1)		14.10 (7.47–26.64), <0.01	15.86 (6.71–37.48), <0.01
Mismatch repair IHC status		<0.01		
Proficient	1148 (80.5)		1.00	
Deficient	278 (19.5)		0.74 (0.55–0.99), 0.04	
BRAFV600E immunohistochemistry status		<0.01		
Wild type	1151 (80.7)		1.00	
Mutant	275 (19.3)		1.14 (0.88–1.49), 0.32	
Immunhoistochemistry phenotypes		<0.01		
Mismatch repair-proficient/ BRAF wild type	1057 (74.1)		1.00	1.00
Mismatch repair-deficient/ BRAFV600E mutant	184 (12.9)		0.84 (0.60–1.19), 0.32	0.57 (0.35–0.93), 0.03
Mismatch repair-deficient/ BRAF wild type	94 (6.6)		0.66 (0.40–1.08), 0.10	0.65 (0.34–1.27), 0.21
Mismatch repair-proficient/ BRAFV600E mutant	91 (6.4)		1.79 (1.24–2.60), <0.01	1.10 (0.69–1.76), 0.68

[a]Reports on the significance of differences between two or more categories within each variable as a one sample non-parametric binomial or χ^2-test.

52.1% were females. Most patients (79.2%) presented with stage 2 or 3 disease. One thousand one hundred and forty-eight (80.5%) were mismatch repair-proficient, comprising 1057 (74.1%) mismatch repair-proficient *BRAF* wild type and 91 (6.4%) mismatch repair-proficient/*BRAFV600E* mutant. One hundred and eighty-four cases (12.9%) were mismatch repair-deficient/*BRAFV600E* mutant and 94 (6.6%) mismatch repair-deficient/*BRAF* wild type.

During follow-up, (mean 5.29 years, 75th centile 3.21 years), 353 patients died. Survival curves correlating immunohistochemical staining pattern and survival by both Kaplan–Meier and Cox regression methods are presented in Figure 1 along with photomicrographs of representative staining patterns. The 5-year survivals progressively deteriorated from mismatch repair-deficient/*BRAF* wild type (73.6%) to mismatch repair-deficient/*BRAFV600E* mutant (70.1%), to mismatch repair-proficient/*BRAF* wild type (65.4%) to mismatch repair-proficient/*BRAFV600E* mutant (49.7%). Pairwise comparisons with mismatch repair-proficient/*BRAF* wild-type

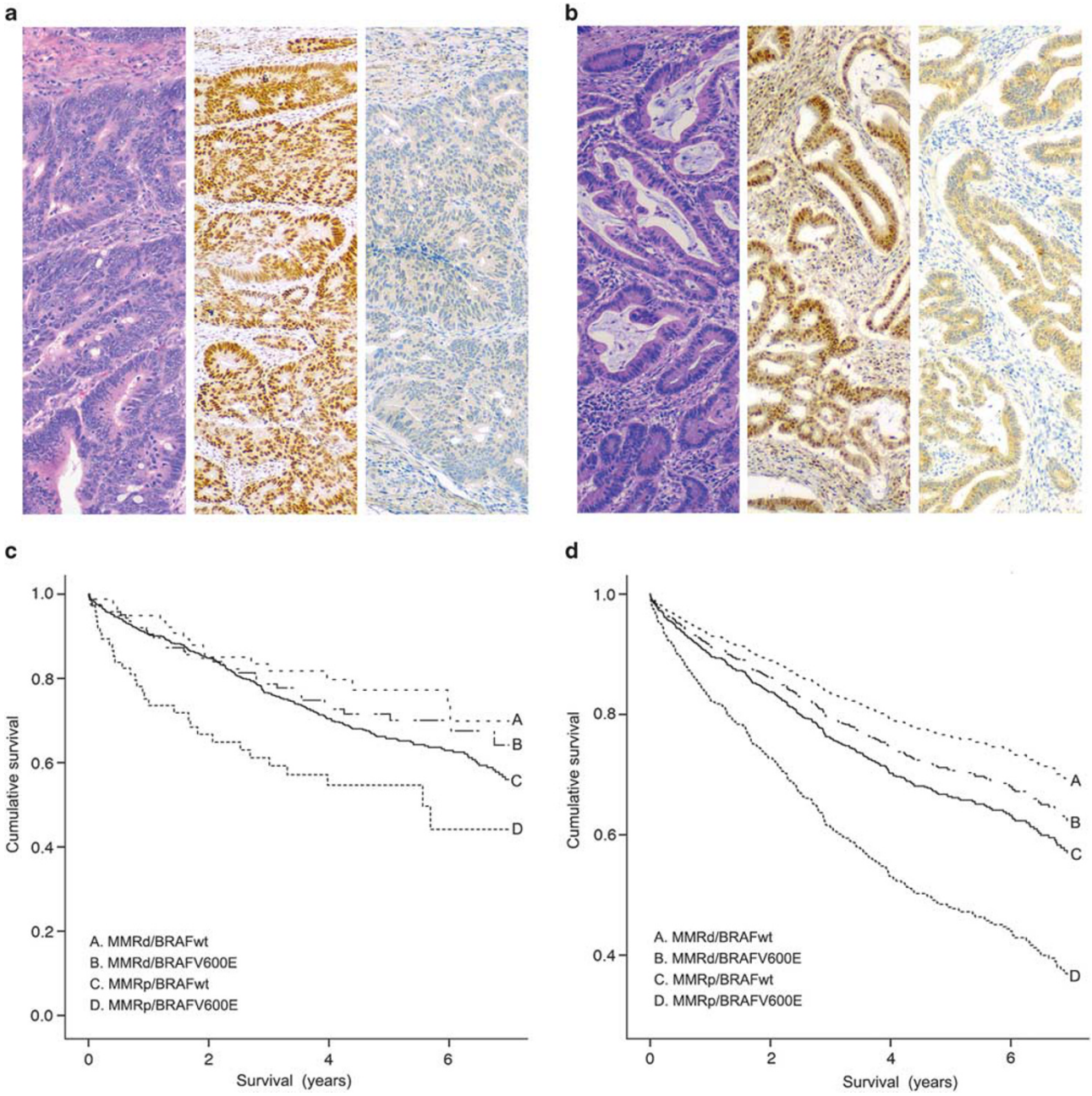

Figure 1 (**a**,**b**) Representative photomicrographs serially stained for hematoxylin and eosin, PMS2 and BRAFV600E of (**a**) DNA mismatch repair-proficient/BRAF wild-type colorectal carcinoma and (**b**) DNA mismatch repair-proficient/BRAFV600E mutant tumor (original magnifications, ×400). (**c**) Kaplan–Meier survival functions of the four immunohistochemistry phenotypes. (**d**) Univariate Cox regression survival function of the four immunohistochemistry phenoytpes.

colorectal carcinomas as the baseline group in univariate Cox regression modeling revealed overlapping 5-year survival figures for all tumor groups, except mismatch repair-proficient/*BRAFV600E* mutant tumors, which showed a statistically significant worse outcome—hazard ratio of death 1.79 (95% CI = 1.24–2.60), $P<0.01$. In multivariate analysis, the poor prognosis of mismatch repair-proficient/*BRAFV600E* mutant tumors was negated (hazard ratio of 1.10 (95% CI = 0.69–1.76), $P=0.68$) by the dominant effect of stage and age on overall survival. However, the better prognosis of mismatch repair-deficient/*BRAFV600E* mutant tumors became significant (hazard ratio of 0.57 (95% CI = 0.35–093), $P=0.03$) when compared with the baseline group of mismatch repair-proficient/*BRAF* wild-type tumors (Table 1).

Discussion

The use of molecular markers to predict outcome in colorectal carcinoma, particularly mismatch repair

deficiency/microsatellite instability and *BRAF* mutation, has been an area of active research. Briefly, most studies have indicated that mismatch repair deficiency is associated with a good outcome.[3,11–17] In contrast, the presence of *BRAF* mutation is usually found to be a marker of poor prognosis.[3,18–24] Although some studies have found that *BRAF* mutation does not predict outcome in an unselected population,[25] this discrepancy appears to be because of the strong association between *BRAF* mutation (a poor prognostic factor) with mismatch repair deficiency (a good prognostic factor) through the somatic hypermethylation pathway.

Therefore, recently several groups have used the combination of MMR and *BRAF* mutation status as determined by molecular means to predict outcome in colorectal cancer. Using this approach, mismatch repair-deficient/*BRAF* wild-type colorectal carcinomas have been consistently found to have a good prognosis,[4,20,26,27] whereas mismatch repair-proficient/*BRAFV600E* mutant colorectal carcinomas have emerged as a poor prognostic group in most[3,4,12,27,28] but not all studies.[26]

This combined mismatch repair/*BRAF* prognostic algorithm was tested recently by Lochhead *et al*[29] who used molecular techniques to determine microsatellite instability and *BRAF* mutation status in 1253 colorectal carcinoma patients. Compared with the majority of tumors that were mismatch repair-proficient/*BRAF* wild type, mismatch repair-proficient/*BRAF* mutant colorectal carcinomas demonstrated a poor prognosis (hazard ratio of colon cancer-specific mortality 1.6 (95% CI = 0.12–2.28)). Mismatch repair-deficient/*BRAF* mutant colorectal carcinomas demonstrated a good prognosis with a hazard ratio of 0.48 (95% CI = 0.27–0.87) and mismatch repair-deficient/*BRAF* wild type demonstrated a very good prognosis with a hazard ratio of 0.25 (95% CI = 0.12–0.52).

The results of our study, although based on all cause rather than cancer-specific survival, are essentially confirmatory of Lochhead *et al*,[29] with the significant advantage that we did not use any molecular techniques, only immunohistochemistry—an approach that is readily available in virtually any diagnostic surgical pathology laboratory. In centers where universal Lynch syndrome screening is already undertaken with mismatch repair deficiency immunohistochemistry for MLH1, PMS2, MSH2, and MSH6, it would simply be a matter of performing immunohistochemistry for five markers rather than four, with estimated additional disposable costs of <US$10 and minimal extra labor costs.[2]

We note that the very poor prognosis of mismatch repair-proficient/*BRAF* mutant tumors found in univariate analysis ($P<0.01$) fell away in multivariate analysis because of the dominant effect of stage and age on overall survival. However, given the ease with which *BRAF* status can be determined in conjunction with mismatch repair deficiency in the routine clinical setting, and the established indication for combined mismatch repair deficiency and *BRAF* testing to triage formal genetic testing for Lynch syndrome,[2] our study makes an argument for its potential use as a prognostic marker in all colorectal cancers.

To date, four of the five independent studies investigating BRAFV600E mutation-specific immunohistochemistry in colorectal carcinoma have determined that it is highly sensitive and specific for the presence of the *BRAFV600E* mutation with only one study suggesting limited utility.[2,8–10,30] It would be reasonable to conclude that local factors such as tissue processing techniques and staining methods can affect the performance of the antibody, but most laboratories including our own[2] have found it to be a robust and reliable marker. However, we do caution that introduction of BRAF immunohistochemistry should only be performed with the appropriate quality control measures, including the use of controls and the validation of the accuracy of the testing in individual laboratories.

Previous studies of BRAFV600E mutation-specific immunohistochemistry in colorectal carcinoma have concentrated on its utility in triaging formal genetic testing for Lynch syndrome in microsatellite-unstable tumors.[2,8–10] Other studies have concentrated on the prognostic predictive power of combined *BRAF* and mismatch repair deficiency assessment when determined by molecular means.[3,4,20,26–29] This is the first study to demonstrate that BRAF determination by immunohistochemistry alone, when interpreted in conjunction with mismatch repair deficiency status, also identifies subgroups of colorectal carcinomas with different overall survivals—most significantly the poor prognostic group of mismatch repair-proficient/*BRAFV600E* mutant tumors.

In summary, our results suggest that the addition of BRAFV600E immunohistochemistry to mismatch repair deficiency immunohistochemistry identifies distinct prognostic subgroups of colorectal carcinomas, including the poor prognostic group of mismatch repair-proficient/*BRAFV600E* mutant tumors. If our results are confirmed in other large independent cohorts, a strong argument could be made to perform routine BRAFV600E immunohistochemistry at the same time as mismatch repair deficiency assessment on all colorectal carcinomas, not just to facilitate universal screening for Lynch syndrome in mismatch repair-deficient tumors but also to identify the poor prognosis mismatch repair-proficient/*BRAFV600E* mutant group.

Acknowledgments

We thank Ms Nicole Watson for her assistance with the initial stages of the data assembly. This study was approved by the Northern Sydney Local Health

District (NSLHD) Human Research Ethics Committee (HREC) under protocol 1201-035M.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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