TOWARDS A MOLECULAR CLASSIFICATION OF COLORECTAL CANCER

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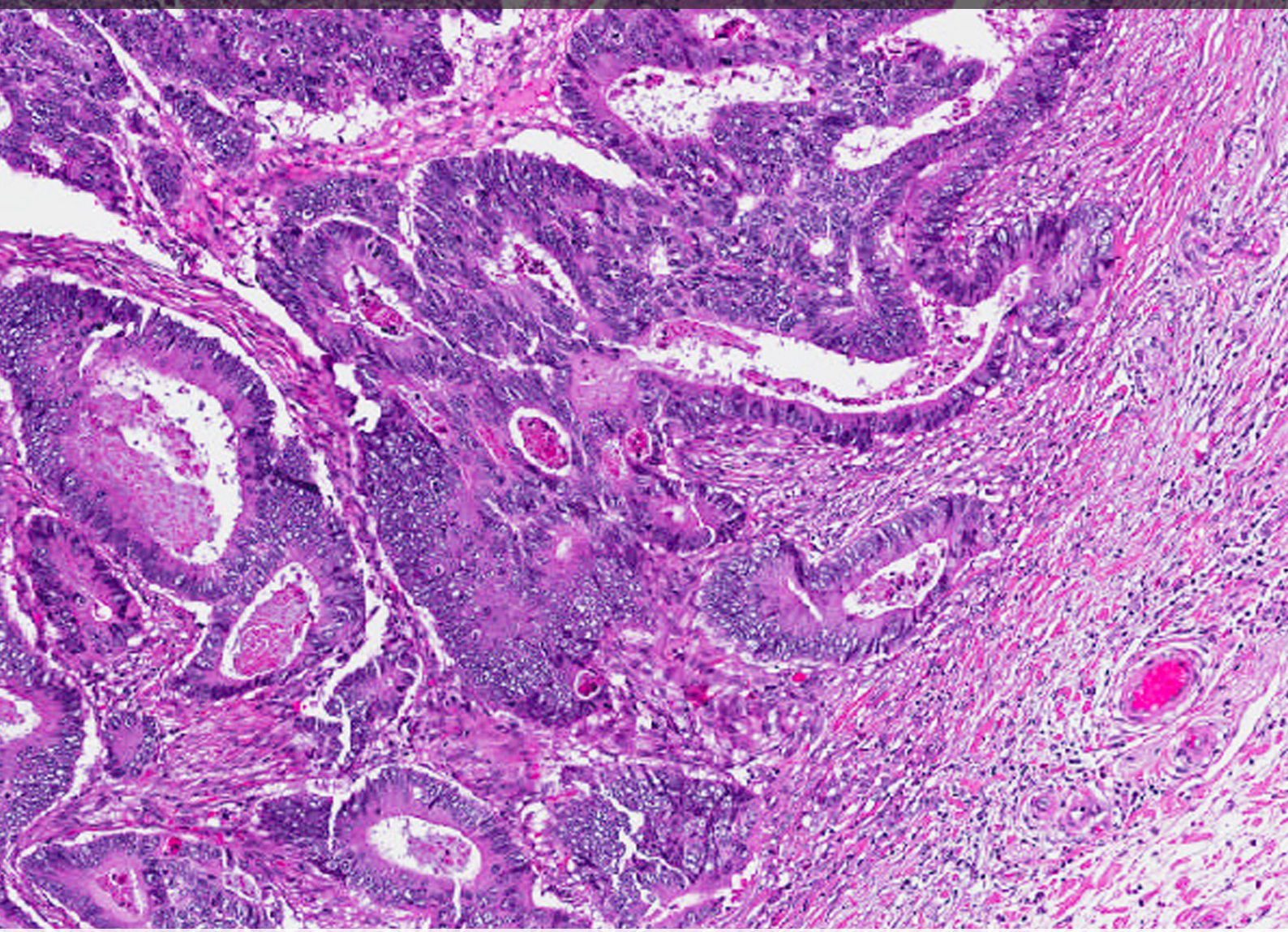

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TOWARDS A MOLECULAR CLASSIFICATION OF COLORECTAL CANCER

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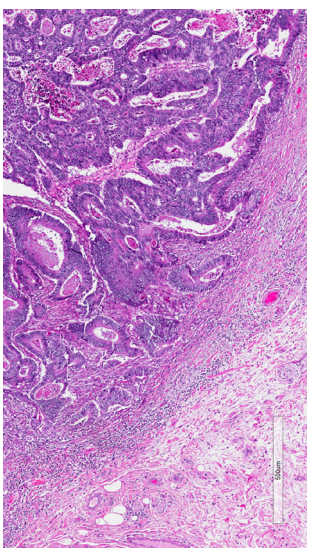

Tumor microenvironment at the invasive front of colorectal cancer (H&E).

Image by Alessandro Lugli.

In 2007, Jeremy Jass proposed a molecular classification of colorectal cancer including KRAS, BRAF, Mismatch Repair, CIMP and MGMT Status. Since then, many prognostic and predictive studies have been published on this topic.

The aim of the e-book is to summarize the knowledge in 2014 from a multidisciplinary point of view that can potentially be used as a manual by CRC researchers in every field.

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Table of Contents

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EDITORIAL
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Towards a molecular classification of colorectal cancer

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Keywords: colorectal cancer, molecular classification, Cdx2, Wnt signaling pathway, MGMT, telomere length, Ras signaling pathway, microRNA

In colorectal cancer (CRC), an internationally standardized molecular classification has not been implemented yet. Nevertheless, there are different pathogenetic aspects that could form the basis for a future molecular classification in CRC (1).

In the classic model, CRC is divided into two major pathways. The first is the chromosomal instability pathway including 85% of all CRCs. This pathway is based on the adenoma–carcinoma sequence, which is defined by consecutive mutations in the APC, KRAS, and TP53 gene and deletion of the chromosome 18q (2). An example of an autosomal dominantly inherited disease, which belongs to the chromosomal instability pathway group, is the familial adenomatous polyposis syndrome (FAP). The second, namely the microsatellite instability pathway (15% of all CRCs) is defined by a mismatch-repair deficiency, which leads to a genomic instability. The microsatellite instability status can be sporadic (12%) or hereditary (3%, Lynch syndrome-associated CRC, HNPCC). Sporadic cases can be differentiated from hereditary cases by using the Amsterdam/Revised Bethesda criteria.

In 2007, Jeremy Jass proposed a molecular classification based on clinical, morphological, and molecular parameters, which included five subgroups (3).

Group 1 (12% of all CRCs): chromosomally stable, MLH1 methylated, MSI-H, BRAF mutated, CpG island methylator phenotype (CIMP) high status; group 2 (8% of all CRCs): chromosomally stable, partially MLH1 methylated, microsatellite stable (MSS, MSI-L), BRAF mutated, CIMP high; group 3 (20% of all CRCs): chromosomally instable, MGMT methylated, MSS/MSI-L, KRAS mutated, CIMP low; group 4 (57% of all CRCs): chromosomally instable, MSS/MSI-L, CIMP negative; group 5 (3% of all CRCs): chromosomally stable, MSI-H, CIMP negative, BRAF wild type.

Following up on this last classification, in 2010, Barbara Leggett and Vicki Whitehall published a pathogenetic overview of sporadic CRC including three pathways (4).

The serrated pathway: MSI-H, CIMP high, BRAF mutated (corresponds to the Jass group 1); the alternative pathway: MSS, CIMP low, KRAS mutated (corresponds to the Jass group 3; the traditional pathway: MSS, CIMP negative, BRAT wild type, KRAS wild type (corresponds to the Jass group 4).

In 2012, the Cancer Genome Atlas Network published a promising approach for a future molecular classification based on different pathways (5).

Group 1 (wnt and TGF-beta pathway): proliferation, stem cell, and progenitor phenotype; group 2 (PIK3CA and RTK–RAS pathway): proliferation, cell survival, translation; group 3 (p53 pathway): proliferation, cell survival. This approach may elucidate promising target molecules for wnt pathway inhibitors or proteins of the PIK3CA and RTK–RAS pathway such as IGF2, IGFR, ERBB2, ERBB3, MEK, AKT, and MTOR.

Recently, a new proposal for a molecular classification of CRC was published by a research group from Oxford based on 906 stage II and III CRCs (6).

Group 1: MSI-H and/or BRAF mutated; group 2: chromosomally instable and/or TP53 mutated with KRAS and PIK3Ca wild type status; group 3: chromosomally instable, KRAS and/or PIK3CA mutated; TP53 wild type status; group 4: chromosomally stable, KRAS and/or PIK3CA mutated; TP53 wild type status; group 5: NRAS mutated; group 6: no mutations; group 7: other.

All these proposals for a CRC molecular classification have in common that some molecular features such as KRAS, BRAF, microsatellite status, and CIMP are often included. Nevertheless, it has to be kept in mind that molecular markers can be prognostic (i.e., BRAF), predictive (i.e., RAS), or both (i.e., microsatellite status). Therefore, the main aim of the research topic "Toward a molecular classification of colorectal cancer" was to include articles that focus on the role of already established (7–13) or potentially novel and promising molecular biomarkers such as telomere length (14) or microRNAs (15) and additionally to give an overview on the molecular pathology of CRC.

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Toward a molecular classification of colorectal cancer: the role of telomere length

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Telomere biology is central to the maintenance of genomic stability and telomeric dysfunction is thought to be an early stage in carcinogenesis. Reports of telomere lengths and their ascribed colorectal cancer (CRC) risks have been discordant, with both very short and very long telomeres implicated. Nevertheless, telomeres appear to play a very central role in cancer initiation. Telomere length changes also appear to impact disease burden, progression, and overall survival. This review covers contemporary views on telomere biology and CRC risk, with a brief overview of analytical methods employed in telomere measurement. We conclude with arguments in favor of including telomere assessment in the molecular profiling of CRCs.

Keywords: telomere attrition, telomerase, hTERT, colorectal cancer, cancer risk

INTRODUCTION

Telomeres are repeat TTAGGG sequences at the end of linear chromosomes, which guard against loss of genetic material during cellular replication. Due to an inherent end replication problem, chromosomes are exposed to a potential loss of genetic material, with telomeres acting as a buffer against loss of chromatin. Repeated cell cycles eventually lead to a critically shortened telomere length, signaling cellular senescence, and triggering apoptosis. This arrest in proliferation is thought to protect against malignant transformation and a failure to do so results in catastrophic genomic instability and carcinogenesis. Telomeres are thus important in managing genomic stability. This central role in genome maintenance makes telomeres key players in carcinogenesis and an attractive candidate for tumor profiling at the molecular level.

TELOMERES AND COLORECTAL CANCER

Telomere length changes have been linked to numerous cancers, including colorectal cancer (CRC). Results from studies analyzing telomere lengths in CRC have been discordant, presenting evidence that both ends of the spectrum (shorter and longer lengths) have a possible role in CRC occurrence (1–3). Moreover, reports of null association have been described (1, 4, 5). Nevertheless, studies linking telomere attrition, or shortening, to an increase in CRC risk have classically dominated literature. Telomeric dysfunction is thought to represent an early step in many epithelial cancers (6). As telomeres reach their critical length, senescent signals are sent, and cells undergo cellular arrest and apoptosis. By-passing this senescent signal and cellular arrest results in continuous replication, with progressive telomere shortening. Eventually telomeres become so short that end-to-end fusions with structural and numerical chromosomal changes, anaphase bridging, and subsequent chromosomal instability ensue (7). This so-called telomere catastrophe halts further cellular divisions (8). However, in the presence of loss of tumor suppressor function, such as an APC mutation or p53 inactivation, pre-malignant cells are able to by-pass this event through telomere maintenance mechanisms. Telomerase, a ribonucleoprotein reverse transcriptase, stabilizes the telomere lengths, protecting the altered chromosomes, and immortalizing pre-malignant cells, thus enabling cancer progression (9, 10). This telomerase upregulation occurs at the critical point in the adenoma-carcinoma transition, allowing evasion of telomeric catastrophe, and supporting malignant progression (see **Figure 1**) (3, 11). Less-commonly, telomere length may be preserved through a recombination-dependent mechanism (12).

The tumor micro-environment may also contribute to carcinogenesis. Shortened telomeres in stromal cells may participate in epithelial changes leading to cancer via autocrine or paracrine mechanisms. Thus as stromal cells undergo senescence, they exhibit a secretory phenotype that may trigger neighboring cells with shortened telomeres to by-pass the senescence signal and termination, setting the stage for chromosomal instability and malignant transformation (13). This theory, known as the senescence-associated secretory pathway, could explain the finding that shortened telomere lengths in some polyps and CRC mirrored the shortened telomeres in the surrounding tissue, suggesting that a shortened telomere length may predate malignant transformation and is not a consequence of cancer progression (14). Chronic inflammation processes in surrounding epithelial cells, as in chronic ulcerative colitis, has also been linked to an increased telomere attrition rate and malignant progression, giving credence to the theory that telomere shortening in the micro-environment may act as a nidus for malignant transformation (15).

Epidemiological studies evaluating the relationship between telomere lengths and CRC risks have produced conflicting results. Although telomere attrition is classically thought of as a risk for CRC, reports of longer telomere lengths, and a predisposition to CRC have emerged (2, 16–18). To complicate matters, some

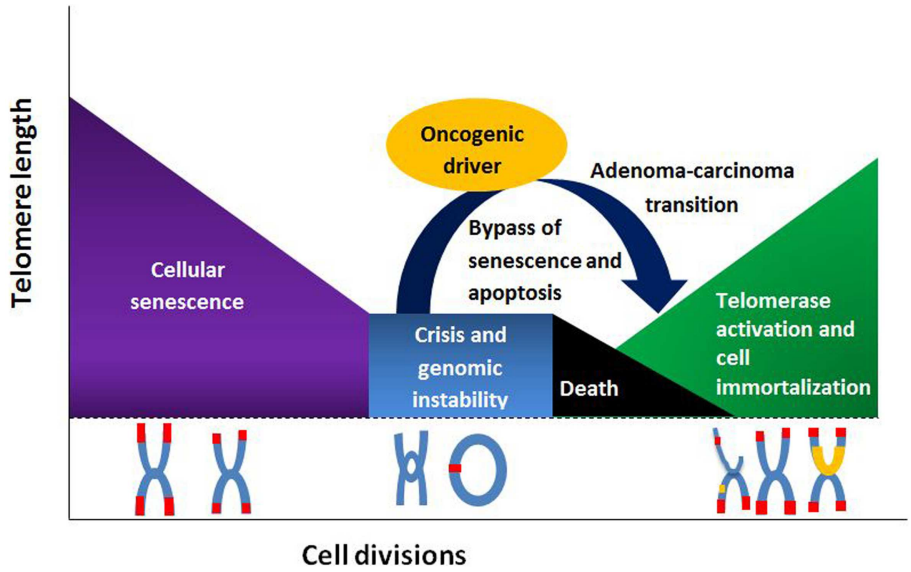

FIGURE 1 | Telomere length and its relationship to cell division, senescence, and senescence by-pass.

studies report a duality of results, with both shorter and longer telomeres associated with increased CRC risk (1, 18). Interestingly, the findings of longer telomeres and their association to CRC seem prevalent in prospective studies, while retrospective studies report, for the most part, shorter telomere lengths. Pooley et al. report an association of shortened telomere lengths and CRC risk in retrospectively collected samples, but fail to replicate their results in the prospective arm of their study (1). Given the conflicting results with respect to study timeline, the argument of reverse causality has been made. Simply put, the changes in telomere lengths, especially in the case of shorter telomeres, may represent disease progression and/or therapeutic interference. Shorter telomere lengths could therefore be a marker of disease progression rather than one of causality. Moreover, the dual findings that both extremely short and long telomeres may be associated with CRC, sometimes within the same sample set, point to the possibility of a "healthy range" of telomere lengths within which cancer risk need not be increased.

ASSESSMENT OF TELOMERE LENGTH

A multitude of analytical techniques are available for telomere length measurement, including Southern Blot, quantitative PCR (qPCR), flow cytometry with fluorescence *in situ* hybridization (flow-FISH), quantitative FISH (Q-FISH), and single or universal single telomere length analysis (STELA) (19). Southern Blot and qPCR are the two most commonly used techniques in epidemiological studies assessing peripheral blood leukocyte (PBL) telomere length. Southern Blot analysis is the gold standard for telomere length assessment, providing results, in terminal restriction fragment (TRF) units, that are deemed reproducible and that allow inter-study comparisons. However, Southern Blot analysis requires a large amount of high-quality DNA and may exaggerate telomere length by including subtelomeric DNA (20). Furthermore, the type of restriction endonuclease used may impact measurement results (20). Cancer risk association studies typically use qPCR to determine telomere lengths. qPCR uses primers to the telomeric repeats to amplify telomeres (21). The telomere to single copy gene ratio of the sample is then compared to the ratio of a reference DNA sample, yielding a relative telomere length (21). qPCR does not require a large amount of DNA but inter-laboratory differences in the reference DNA limit comparisons between studies. The estimated variability between assays is >6% for qPCR and >2% for Southern Blot (22). Telomerase activity may also be measured by way of the Telomere Repeat Amplification Protocol (TRAP), which utilizes PCR to amplify telomerase-extended primer products (23). Alternatively, telomerase activity can be measured via a direct primer extension activity assay, which has proven effective in cell assays but has as yet to be applied to tissues. This direct extension assay avoids the limitations of the TRAP assay, which can be less accurate due to non-linear amplification and due to false negative results in the presence of inhibitors of Taq DNA polymerase (24). hTERT mRNA expression using real-time PCR has also been used to characterize telomerase activity. hTERT mRNA expression is thought to provide a good correlation with telomerase activity in certain cancers, including CRC and has been suggested to have a negative prognostication value in CRC (25, 26). Given the wide range of analytical tools available, it is not unreasonable to assume that some of the discrepancies in published results may be due to measurement error and inter-laboratory variation. In a large study, Cunningham et al. find that DNA extraction methods greatly influenced telomere length readings (27). A standardized extraction and measurement method is therefore imperative to allow comparison and validation of published data.

TELOMERES AND MOLECULAR SUBCLASSIFICATION OF CRC

The lack of consensus on telomere length changes and the conferred risk for CRC could point toward the existence of distinct molecular subclasses of CRC. Telomere dysfunction may represent an alternative pathway in CRC carcinogenesis, a shift from the two classic genomic instabilities observed: chromosomal and microsatellite instability (MSI). In a study assessing telomere attrition in microsatellite stable (MSS) and chromosomally stable (CIN−) rectal cancers, Boardman et al. revealed evidence of molecular heterogeneity within MSS cancers, in regards to their CIN status and telomere maintenance mechanism (16). Of interest was the discovery of a subset of MSS and CIN-rectal cancers with the unique molecular profile of increased alternative lengthening pathway (ALT+) and longer telomere lengths, in contrast to the shortened telomeres and increased telomerase expression in the chromosomally unstable (CIN+) subgroup (16). Although ALT expression has been described in various cancers, its association to CRC is not clearly defined (28, 29). ALT offers a distinct telomere maintenance mechanism from telomerase, involving superimposed lengthening and shortenings in a recombination-dependent fashion (30). The results are long, heterogeneous telomeres, and the pathognomonic ALT associated promyelocytic leukemia bodies (APBs) (28). From this perspective, the distinct molecular profiles observed could point to the existence of a different molecular subclass of CRC. In a separate study, the authors also find an age at onset-dependent difference in PBL telomere length changes and CRC risk (31). Longer PBL telomeres appear to be a predictor of CRC in their young-onset CRC subgroup ($\leq$50 years old) while extremely short PBL telomeres are associated with CRC in older individuals. While shorter telomere lengths in the older subgroup ($\geq$50-year-olds) may partly be explained by the natural aging process and resultant chromosomal instability, the association of longer telomeres and CRC risk in younger patients may suggest genetic alterations in telomere maintenance mechanisms. While reverse causality, or the effect of disease burden and therapy, may certainly be an explanation for the longer telomere lengths observed, the result may also indicate a diverging telomere-centered mechanism, and potentially, a distinct molecular subclass of CRC with early penetrance.

Studies looking into the relationship between MSI and telomere lengths have been sparse. Telomeres may be considered a form of super microsatellite given their tandem repeat nature and it may be reasonable to assume that defects in MMR genes impact telomere length. In an experiment studying the effect of down-regulating MSH2 in fibroblast cell lines, Mendez-Bermudez et al. report a statistically significant increase in telomere attrition rate compared to control cell lines (32). However, we do not fully understand how these findings might translate into the prognosis and treatment of patients with dMMR CRC.

The impetus for adding telomere lengths to the molecular profiling of CRC lies in the possibility of tailored therapy. Differences in molecular profiles often translate into distinct clinical progression, as in the case for CIN− and CIN+ CRC. Genetic CRC may also benefit from a telomeric subclassification, as telomere length changes have been suggested as risk modifiers in mutation carriers and therefore may serve as a marker of prediction (33). The association between telomere length and disease progression has been described, both in early and more advanced cases (34, 35). Riegert-Johnson et al. report shorter PBL telomere in individuals with advanced polyps compared to age- and gender-matched polyp-free individuals, suggesting that PBL telomere be used as a biomarker for advanced adenomatous polyps (34). This biomarker for stratifying patients according to their progression risks could serve as a pre-screening step, identifying patients needing more frequent colonoscopic surveillance. Telomerase activity has also been suggested as a marker for progression, with numerous reports on the association between increased telomerase expression and malignant transformation (3, 11). Telomerase activity appears to correlate to disease stage, with Duke A and B stages expressing lower telomerase activity (36). Furthermore, telomerase activity reflected disease burden, risk of recurrence, and overall survival (11, 26, 37). Thus, quantifying telomere length and telomerase activity may serve as a useful prognostication tool.

There has been intense interest in anti-telomerase drugs and their potential as a targeted chemotherapeutic drug (38, 39). Targeted therapy could reduce treatment-resistance and side-effects. The combination of a telomere-centered subclassification and targeted treatment could translate into individualized medical care and better patient outcomes. Determining telomeric molecular profiles and their ascribed cancer risk also opens the possibility for chemoprevention.

CONCLUDING REMARKS

Very like the chicken or egg causality dilemma, the timeline of events involving telomeres, genomic instability, and CRC remain unclear. While distinct molecular mechanisms are conceivable, synergistic effects between different components and the interactions of alternative pathways cannot be ignored. Differences in analytic measures have been implicated in the varying results seen suggesting the need for a standardized measurement technique. The difference in results could also reflect a reverse-causation effect. Larger prospective studies are imperative to validate previously published data. Moreover, given the duality in findings, a healthy range of telomere lengths may need to be established. Nevertheless, a subclassification of CRC to include telomere status could carry significant value in predicting disease severity, progression and overall prognosis, and in directing treatment. A telomere subclassification represents an important step forward in individualized medicine and is, therefore, an important avenue to explore.

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Prognostic significance of β-catenin, E-cadherin, and SOX9 in colorectal cancer: results from a large population-representative series

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Robust biomarkers that can precisely stratify patients according to treatment needs are in great demand. The literature is inconclusive for most reported prognostic markers for colorectal cancer (CRC). Hence, adequately reported studies in large representative series are necessary to determine their clinical potential. We investigated the prognostic value of three Wnt signaling-associated proteins, β-catenin, E-cadherin, and SOX9, in a population-representative single-hospital series of 1290 Norwegian CRC patients by performing immunohistochemical analyses of each marker using the tissue microarray technology. Loss of membranous or cytosolic β-catenin and loss of cytosolic E-cadherin protein expression were significantly associated with reduced 5-year survival in 903 patients who underwent major resection (722 evaluable tissue cores) independently of standard clinicopathological high-risk parameters. Pre-specified subgroup analyses demonstrated particular effect for stage IV patients for β-catenin membrane staining ($P = 0.018$; formal interaction test $P = 0.025$). Among those who underwent complete resection (714 patients, 568 evaluable), 5-year time-to-recurrence analyses were performed, and stage II patients with loss of cytosolic E-cadherin were identified as an independent high-risk subgroup ($P = 0.020$, formal interaction test was not significant). Nuclear β-catenin and SOX9 protein, regardless of intracellular location, were not associated with prognosis. In conclusion, the protein expression level of membranous or cytosolic β-catenin and E-cadherin predicts CRC patient subgroups with inferior prognosis.

Keywords: beta-catenin, E-cadherin, SOX9 transcription factor, prognostic biomarkers, colorectal cancer, biomarker discovery, guideline adherence

INTRODUCTION

As of 2008, 1.2 million patients were diagnosed with colorectal cancer (CRC) annually, and only about half of them were alive 5 years after their initial diagnosis (1). Clinicopathological staging is the best available system to predict disease course, however, the system offers only crude estimates leading to unnecessary treatment of a large number of patients on one hand, and recurrence of disease among patients who only received surgery, on the other hand. Taken together with the fact that CRC risk increases with age and that the world's population is both growing and aging, the coming decades will put an unprecedented pressure on health care institutions worldwide (2). Therefore, the need for molecular biomarkers to guide clinical decision-makers on how to stratify patients into optimal treatment regiments has never been greater. In particular, patients with stage II CRC are not routinely offered adjuvant therapy, although about 20–30% of them experience relapse and die within 5 years after surgery (3). Also, stage III patients above 75 years of age are not routinely offered adjuvant therapy although evidence suggests a benefit from such treatment (4, 5). Prognostic biomarkers that distinguish between both high-risk and low-risk patients within these stages are highly warranted.

In the early 90s, the hereditary cancer syndrome familial adenomatous polyposis (FAP) was discovered to be directly linked to mutations within the adenomatous polyposis coli gene (APC) (6, 7). Two years later, a close interaction between APC and β-catenin was demonstrated (8, 9), and as APC mutations were found at high frequencies in colorectal adenomas and carcinomas, it was soon realized that the Wnt/β-catenin signaling pathway plays an initiating and rate-limiting role in colorectal tumorigenesis (10–12). More recently, large-scale exome-sequencing efforts have confirmed that Wnt/β-catenin signaling is deregulated in more than 90% of all CRCs (13–15).

Briefly, canonical Wnt signaling (i.e., Wnt/β-catenin signaling) is initiated when Wnt proteins are released by stromal cells and Paneth cells in the intestinal crypt, and these proteins bind to heterodimeric receptor complexes on the surface of intestinal stem cells (Frizzled/Lrp6) and their immediate descendants (16). A signal is then conveyed along a signaling cascade, which essentially inhibits degradation of cytoplasmic β-catenin. β-Catenin then soon translocates into the nucleus where it interacts with DNA-bound TCF/Lef transcription factors, causing expression of a range of genes related to proliferation and differentiation, including SOX9. In cancer cells, mutations in APC, β-catenin, or AXIN 2 cause constitutive activation of this signaling pathway, leading to excess proliferation and inhibition of differentiation of stem cell progenitors. Notably, β-catenin also serves another essential cellular function in adherens junctions by linking E-cadherin to the cytoskeleton, and recent evidence suggests that this β-catenin pool is highly stable and unrelated to its impact on Wnt signaling (16).

The prognostic potential of various components of the Wnt/β-catenin pathway in CRC has been explored in many datasets over the last decade, both on the genetic and the protein level. In particular, deregulation of APC, β-catenin, and E-cadherin has received much attention (17–19). From the very high frequency of APC mutations in sporadic CRC, at around 70–80% (20), it follows that its prognostic potential is likely limited. Indeed, few reports have documented robust clinical relevance of this biomarker, neither at the genetic level (21) nor at the protein level (22), although reports suggest that mutations affecting β-catenin-binding sites may have prognostic value (13, 23). In contrast, the literature on β-catenin and E-cadherin has been clouded by many conflicting findings due to a large number of unstandardized and underpowered studies (17, 18, 21, 24–38), and their potential as biomarkers in CRC still merits further investigation.

SOX9 is a transcription factor and a downstream target of Wnt/β-catenin signaling with possible roles in β-catenin regulation (39–42). Deregulation of SOX9 has been reported for several cancers, including CRC (24), and recent large-scale exome-sequencing efforts have revealed that SOX9 is mutated in a subset of CRCs (15). The prognostic potential of SOX9 has only been evaluated in one adequate CRC dataset, which suggested that high expression of the SOX9 protein was associated with an adverse prognosis (43).

Here, we used a tissue microarray (TMA) constructed from a large consecutive, population-representative single-hospital series of primary CRCs to explore the prognostic significance of the protein expression of β-catenin, E-cadherin, and SOX9 by immunohistochemistry. We have attempted to report the study according to the REMARK guidelines (Table S1 in Supplementary Material) (44) and primarily focused on the clinical relevance within CRC stages. We specifically sought to test the hypotheses that (i) increased nuclear β-catenin staining is associated with poor outcome, indicating active Wnt/β-catenin signaling, that (ii) loss of β-catenin and E-cadherin membrane staining is associated with poor outcome due to decoupling of adherence junctions in epithelial–mesenchymal transition (EMT), and whether (iii) differential expression of the downstream Wnt signaling target, SOX9, identifies prognostic subgroups of CRC patients.

MATERIALS AND METHODS

PATIENT MATERIAL

A population-representative consecutive series of 1290 CRC patients admitted to Oslo University Hospital – Aker (1993–2003) was analyzed. This hospital treated all CRC patients from a geographically defined catchment area, including most relapses. Of these, 929 patients underwent major resection, and DNA was extracted from corresponding formalin-fixed paraffin-embedded (FFPE) tissue from which a TMA was constructed (**Figure 1**). Major resection was defined as removal of the tumor-bearing bowel segment with the lymphovascular pedicle and mesentery. All rectal cancers were surgically removed by total mesorectal excision (TME). Resection status was defined as R0 (complete resection/no residual tumor), R1 (microscopic residual cancer at the resection margin), or R2 (macroscopic or radiological evidence of residual cancer, locally or distant). TNM-staging and histopathological grading followed the UICC/AJCC system, version 5. Comprehensive clinical data had been prospectively registered on all patients (**Table 1**). Microsatellite instability (MSI) status was previously determined for all tumors (3) (Bruun et al., unpublished). More than 95% of patients were of Caucasian ethnicity (based on name-origin). Additional clinical information has been reported elsewhere (3, 45).

The study was approved by the Regional Committee for Medical and Health Research Ethics, South-Eastern Norway (REK number 1.2005.1629) and the Norwegian Data Protection Authority, and the patients were enrolled after informed consent. The research conformed to the Declaration of Helsinki and the research biobanks have been registered according to national legislation.

IMMUNOHISTOCHEMICAL ANALYSES OF PROTEIN EXPRESSION ON TMA

Cores from FFPE tissue from 670 colonic, 233 rectal, and 26 synchronous carcinomas (one 0.6 mm diameter core per patient taken from a viable, non-necrotic tumor area) from patients treated at Oslo University Hospital – Aker (1993–2003), were organized into a TMA according to the original method described by Kononen and colleagues in 1998 (46).

The immunohistochemical analyses were done on 3–4 μm thick TMA sections on microscope slides, and were performed as previously described (47). Briefly, sections were de-paraffinized in xylene for 10 min, and then rehydrated. Antigen retrieval was performed in a microwave oven by heating the sections in plastic containers filled with Tris/EDTA-buffer (pH 9). Staining was performed according to the DAKO Envision protocol, using the reagents supplied with the K5007 kit (Dako, Glostrup, Denmark). Immunocomplexes were visualized with the chromogenic stain diaminobenzidine (DAB). Hematoxylin staining was used to visualize the nuclear compartment. A test TMA containing representative tissues from nine human organs and six types

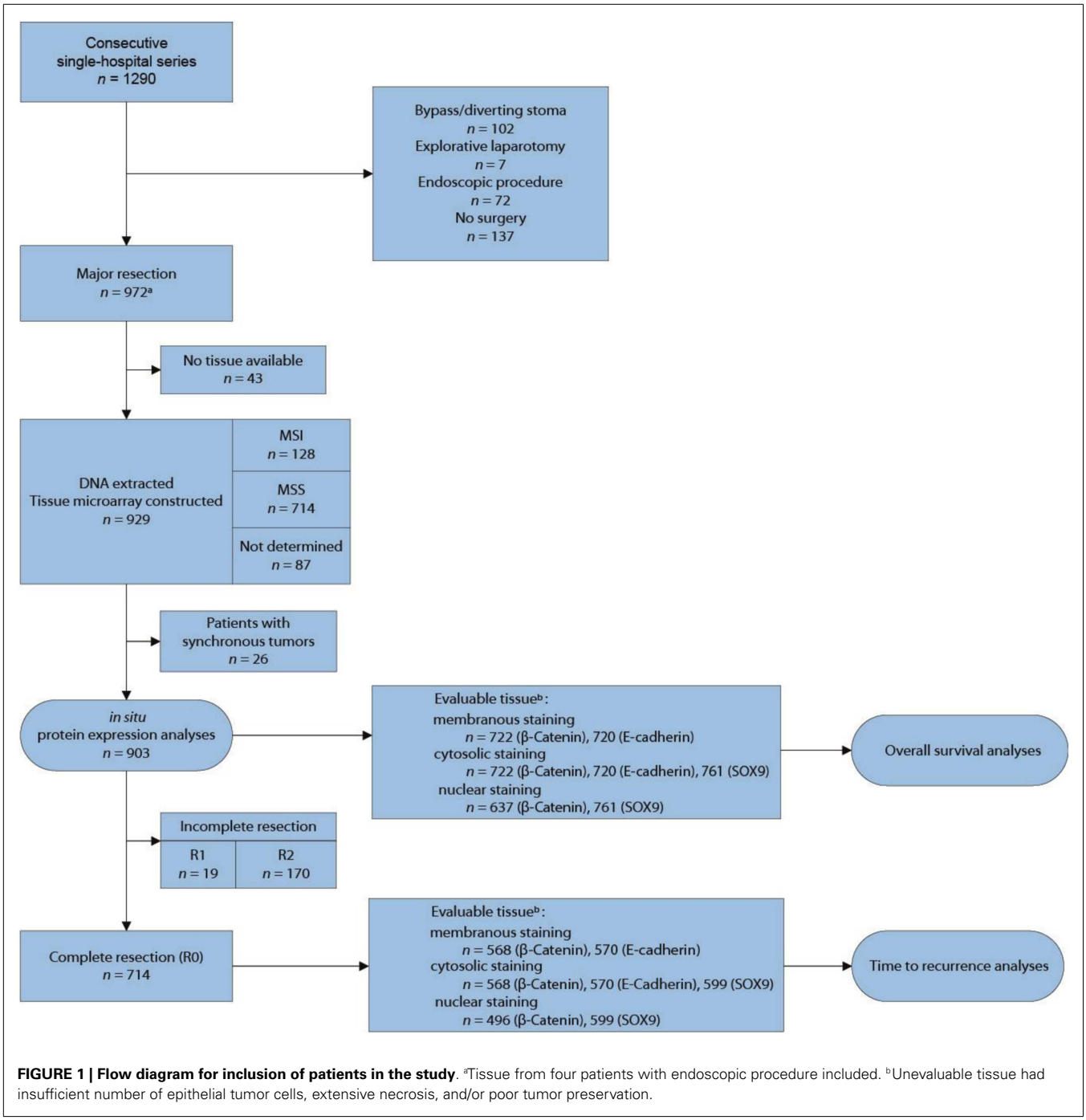

FIGURE 1 | Flow diagram for inclusion of patients in the study. [a]Tissue from four patients with endoscopic procedure included. [b]Unevaluable tissue had insufficient number of epithelial tumor cells, extensive necrosis, and/or poor tumor preservation.

of cancer was utilized to optimize staining conditions to the dynamic range of DAB by careful titration of antibodies. To evaluate non-specific secondary antibody reactions, a negative control experiment was provided by omitting the primary antibody from one slide.

For immunohistochemical analysis, mouse monoclonal anti-β-catenin (Clone 14) antibodies were obtained from BD Biosciences (San Jose, CA, USA; Catalog number 610154), recognizing a C-terminal epitope between residue 571 and residue 781 of β-catenin. Mouse monoclonal anti-E-cadherin (Clone 36) antibodies were obtained from BD Biosciences (catalog number 610181), recognizing a C-terminal epitope between residue 735 and residue 883 of E-cadherin. Rabbit polyclonal anti-SOX9 antibodies were obtained from Atlas antibodies AB (catalog number HPA001758, Stockholm, Sweden), recognizing the 117 amino acid residue C-terminal end. The antibodies were employed at dilutions of 1:800, 1:2000, and 1:500, respectively.

Table 1 | Patient characteristics for all patients included in the study.

Patient characteristic	Frequency (*n*)	Percentage (%)
Patients in the study	903	100
AGE		
Median	73	
Range	30–94	
AGE (3 GROUPS, BINNED)		
30–68	309	34.2
69–77	292	32.3
78–94	302	33.4
GENDER		
Male	429	47.5
Female	474	52.5
STAGE		
I	133	14.8
II	363	40.4
III	237	26.4
IV	165	18.4
ND	4	–
HISTOPATHOLOGIC GRADE		
G1	84	9.6
G2	674	76.8
G3	108	12.3
Mucinous[a]	12	1.4
ND[a]	25	–
TUMOR LOCATION		
Proximal colon	367	40.6
Distal colon	302	33.4
Rectum	234	25.9
RESECTION		
0	713	79.0
1	19	2.0
2	170	18.8
ND[a]	1	–
MICROSATELLITE INSTABILITY		
MSI	119	14.5
MSS	700	85.5
ND[a]	84	–

[a] *Excluded from statistical analyses. ND, no data.*

EVALUATION OF IMMUNOSTAINING

The staining of β-catenin, E-cadherin, and SOX9 was scored according to the proportion and intensity categories proposed by Allred et al. (48). The proportion score represents the estimated fraction of positive cells (0 = none, 1 = <1%, 2 = 1–10%, 3 = 11–33%, 4 = 34–66%, and 5 = 67–100%), while the intensity score represents their average staining intensity (0 = negative, 1 = weak, 2 = intermediate, and 3 = strong). The final Allred-score for each tumor is calculated by adding these two scores. Staining was evaluated and scored separately for membranous, cytoplasmic, and nuclear staining patterns. The scores were combined into strong, moderate, and weak categories. The categories were determined by the number of patients and events in each subgroup, and the ability to visually differentiate reliably between the staining scores. All analyses were done in parallel on ungrouped scores demanding that findings were valid for both ungrouped and grouped data. The scoring was performed independently by two investigators (Jarle Bruun and Matthias Kolberg), blinded to clinical data, in close collaboration with an experienced pathologist (Jahn M. Nesland). All discrepancies were resolved and reassigned on consensus of opinion.

For β-catenin the interobserver agreement, as measured by the intraclass correlation coefficients (ICC) (49) were 0.88, 0.89, and 0.85 for membranous, cytosolic, and nuclear staining, respectively; for E-cadherin 0.84 and 0.67 for membranous and cytosolic staining, respectively; for SOX9 0.93 for nuclear staining. Due to the limited ability of the Allred scoring system to differentiate proportionately between negative tumors (score 0) and weak tumors (score 4–6, mostly), these ICCs underestimate the true ICC value, especially for cytosolic staining for which tumors largely exhibited a uniform staining with proportion scores of 4 or 5. Calculations were confirmed by cross-tabular visualizations.

STATISTICAL ANALYSES

Five-year overall survival (OS) and time-to-recurrence (TTR) plots were generated using the Kaplan–Meier method in the SPSS 18.0 software (SPSS, IL, USA). TTR and OS were defined according to the guidelines given by Punt et al. (50). Briefly, TTR was defined as the time from surgery to the first event of either death from the same cancer, local recurrence, or distant metastasis. Patients were censored at death from other cancer, non-cancer death, post-operative death (<3 months), and loss to follow-up. OS was defined as the time from surgery to death from any cause, and patients were censored at loss to follow-up. No patients were lost to follow-up in the study period. The logrank test for trend was used to compare survival between ordinal groups, and Cox proportional hazards regression modeling (Wald test) was used to provide univariate and multivariate hazard-ratios (HR) and confidence intervals (CI). Age categories were created by three-tire binning to achieve sufficient statistical strength within each category. In multivariate analyses, the protein parameters with significant independent impact on patient survival were adjusted for the standard and high-risk clinicopathological variables: age, gender, tumor stage, tumor differentiation, tumor location, MSI status, and residual tumor status. Adjuvant treatment for patients with stage III colon cancer (<75 years of age) became standard treatment in 1997 and was considered in initial multivariate models. These patients were few and adjustment did not affect the models. Adjustment for pre- and post-operative radiotherapy for rectal cancer patients was also considered, but was pertinent to only a very limited number of patients and therefore not included in initial models. The proportional hazards assumptions were verified by graphical evaluation of plots of log (−log survival time) versus log time.

The correlation and survival analyses involve multiple tests and false positive findings are to be expected with a 5% significance level. However, several of the clinicopathological parameters, such as stage and differentiation, can be assumed to have

some *a priori* association with the three biomarkers, reducing the need for rigorous correction. Clinically relevant subgroup analyses were therefore pre-specified and additional subgroup analyses labeled as exploratory. Interaction tests were integrated in the Cox models to assess whether effects were different between subgroups, but must be interpreted carefully due to the low power of such tests. All *P*-values were two-sided and derived from statistical tests using SPSS, and considered statistically significant at $P < 0.05$. To correct for multiple testing in the correlation analyses, we set a significance threshold of $P < 0.001$. Correlation between expression of protein markers and standard clinicopathological variables was evaluated using Spearman's rho test.

RESULTS

TMA IMMUNOSTAINING RESULTS

Among 903 stained histospots, 722 (80%) were evaluable for β-catenin, 720 (80%) for E-cadherin, and 761 (84%) for SOX9 (**Figure 2**). The rest of the histospots were unevaluable due to insufficient number of epithelial tumor cells, extensive necrosis, poor tumor preservation, or loss of tissue on the TMA slide. Eighty-five samples with very strong cytoplasmic β-catenin staining could not be evaluated for nuclear staining, leaving 637 (71%) for this purpose. No bias of clinical data was observed for these 85 samples. Generally, the tumor exhibited various degrees of staining for all the three proteins in the compartments investigated (Figure S1 in Supplementary Material).

CORRELATION BETWEEN IMMUNOSTAINING AND WITH CLINICOPATHOLOGICAL VARIABLES

Membrane and cytosolic β-catenin staining correlated strongly with E-cadherin staining, in accordance with their common functional roles in adherens junctions (**Table 2**). Nuclear β-catenin, however, correlated only with cytosolic β-catenin, but not to β-catenin membrane staining, E-cadherin, or SOX9.

All the tested biomarkers in all subcellular locations were more highly expressed in MSS tumors than in MSI, except for β-catenin membrane and nuclear SOX9 staining, but these showed the same trend (**Table 3**). Similar, but weaker associations were found for left-sided tumors (including rectum) as compared to right-sided tumors (**Table 3**). Histopathologic grade was also positively correlated with expression of all biomarkers. The correlation was weaker for β-catenin membrane and nuclear staining, and SOX9 nuclear staining, but these showed the same trend.

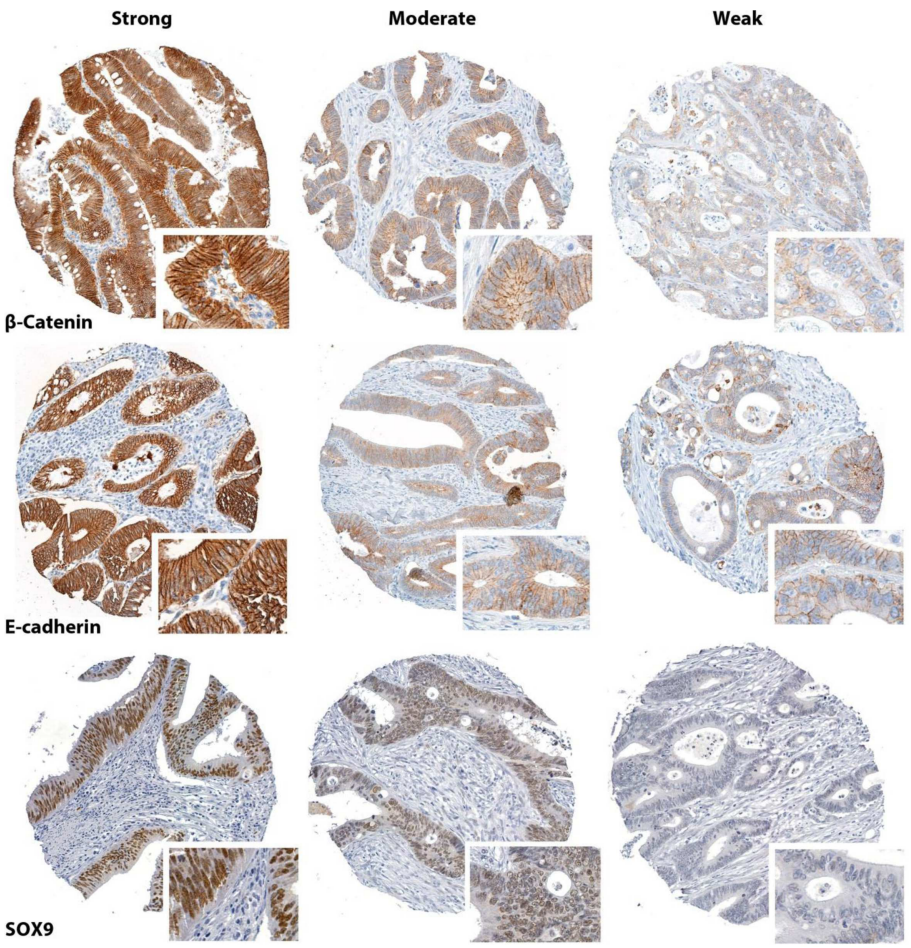

FIGURE 2 | Representative immunostaining patterns for P-catenin, E-cadherin, and SOX9 (core diameter 0.6 mm).

Table 2 | Correlation between staining of studied biomarkers.

Marker	β-Catenin (*P, r, n*)			E-cadherin (*P, r, n*)		SOX9 (*P, r, n*)	
	Cytosol	**Membrane**	**Nucleus**	**Cytosol**	**Membrane**	**Cytosol**	**Nucleus**
β-CATENIN							
Cytosol	1	6.6×10^{-25}	1.2×10^{-49}	9.7×10^{-26}	5.3×10^{-22}	2.0×10^{-10}	1.3×10^{-5}
		0.37	0.54	0.38	0.35	0.24	0.16
		722	637	702	702	700	700
Membrane		1	0.0079	3.0×10^{-32}	2.5×10^{-63}	0.36	0.22
			−0.11	0.43	0.58	0.034	0.046
			637	702	702	702	702
Nucleus			1	0.17	0.011	0.17	0.037
				0.055	0.1	0.055	0.084
				618	618	616	616
E-CADHERIN							
Cytosol				1	3.1×10^{-86}	0.57	0.42
					0.65	0.022	−0.03
					720	694	694
Membrane					1	0.48	0.064
						0.027	0.07
						694	694
SOX9							
Cytosol						1	3.1×10^{-70}
							0.58
							761
Nucleus							1

P-values and correlation coefficients (r) from Spearman's rho test. The correlations are calculated using ungrouped Allred staining scores.

There were no significant correlations with any of the clinical parameters age, gender, or tumor stage.

SURVIVAL ANALYSIS

Univariate and multivariate OS analysis of all patients and TTR analysis of patients with complete resection were carried out in order to assess the prognostic potential of each of the three biomarkers.

LOSS OF β-CATENIN INDEPENDENTLY PREDICTS POOR OUTCOME

Univariate analyses showed that decreased membranous staining of β-catenin was significantly associated with a worse prognosis (**Figure 3**; **Tables 4** and **5**).

A multivariate Cox regression model including standard clinicopathological variables demonstrated that β-catenin membranous staining was an independent prognostic marker using OS as an endpoint (**Table 4**), but not using TTR as an endpoint (**Table 5**).

Stratification according to stage demonstrated valid significance for OS only within stage IV ($P = 3.7 \times 10^{-7}$, $n = 134$ with 125 events, **Table 6**), supported by formal interaction tests (integrated in the Cox-model) assessing the probability of subgroup effects ($P = 0.018$ for β-catenin and $P = 0.025$ for the interaction test between β-catenin and stage). Further exploratory subgroup analyses of β-catenin membrane staining by standard clinicopathological variables showed that the initial association to survival was also particularly significant for females (OS, Females, $P = 5.2 \times 10^{-4}$, $n = 380$; Males, $P = 0.18$, $n = 342$; TTR, Females, $P = 0.047$, $n = 292$; Males, $P = 0.78$, $n = 276$). However, interaction tests were not significant.

Decreased cytosolic β-catenin staining was significantly associated with a worse prognosis in both OS (**Table 4**) and TTR univariate analyses (**Figure 3**; **Table 5**).

Multivariate analysis (OS) demonstrated that cytosolic β-catenin expression was an independent prognostic marker (**Table 4**). Multivariate TTR analysis exhibited a similar trend, but was not statistically significant at the conventional 5% level (**Table 5**).

Exploratory subgroup analyses (OS) showed a particular age-related effect for younger patients (<69 years of age, $P = 0.0010$, $n = 246$; 69–77 years of age, $P = 0.62$, $n = 227$, 78–96 years of age, $P = 0.26$, $n = 249$). This finding was supported by significant interaction tests ($P = 0.0025$ for β-catenin and $P = 0.038$ for the interaction test). The strata had unfortunately too few patients and events to perform an adequate TTR analysis.

Nuclear β-catenin staining was not associated with prognosis alone or stratified according to standard clinicopathological variables (**Figure 3**; **Tables 4** and **5**).

LOSS OF E-CADHERIN INDEPENDENTLY PREDICTS POOR OUTCOME

In univariate analyses, there was no significant association between membranous E-cadherin staining and prognosis, but the KM-plots and logrank tests suggest that loss of membranous E-cadherin is associated with a worse prognosis (**Figure 4**; **Tables 4** and **5**).

Table 3 | Correlation between studied biomarkers and patient characteristics.

Patient characteristic	β-Catenin (*P, r, n*)			E-cadherin (*P, r, n*)		SOX9 (*P, r, n*)	
	Cytosol	Membrane	Nucleus	Cytosol	Membrane	Cytosol	Nucleus
Age	0.60–0.020	0.83	0.81	0.45	0.57	0.097	0.033
	722	0.0078	0.01	−0.028	0.021	0.06	0.077
		722	637	720	720	761	761
GENDER							
Female = 1 Male = 2	0.036	0.4	0.025	0.072	0.028	0.85	0.43
	0.078	0.031	0.089	0.067	0.082	−0.0069	−0.029
	722	722	637	720	720	761	761
Stage	0.081	0.043	0.3	0.013	0.088	0.035	0.74
	−0.065	−0.076	0.041	−0.093	−0.064	−0.077	−0.012
	718	718	633	716	716	757	757
HISTOPATHOLOGIC GRADE							
G1, G2, G3	2.4×10^{-6}	0.014	0.14	8.0×10^{-6}	2.9×10^{-5}	7.1×10^{-5}	0.057
	0.18	0.093	0.059	0.17	0.16	0.15	0.07
	694	694	612	691	691	731	731
TUMOR LOCATION							
Right = 1	2.8×10^{-9}	0.17	1.2×10^{-6}	3.8×10^{-7}	2.2×10^{-6}	0.13	0.11
Left = 2	0.22	0.051	0.19	0.19	0.18	0.055	−0.059
Rectum = 3	722	722	637	720	720	761	761
RESECTION							
R0, R1, R2	0.028	0.2	0.48	0.045	0.35	0.027	0.93
	−0.082	−0.047	0.028	−0.075	−0.035	−0.08	−0.0032
	721	721	636	719	719	760	760
MSI status	3.6×10^{-21}	0.012	1.1×10^{-12}	2.6×10^{-9}	1.5×10^{-9}	1.8×10^{-6}	0.091
MSI = 1	0.36	0.097	0.29	0.23	0.23	0.18	0.064
MSS = 2	661	661	584	657	657	693	693

P-values and correlation coefficients (r) from Spearman's rho test. Correlations are calculated using ungrouped Allred staining scores.

However, there was a significant association between loss of cytosolic E-cadherin and a worse prognosis, both employing OS (**Table 4**) and TTR (**Table 5**) as an endpoint (**Figure 4**).

When standard clinicopathological variables were adjusted for by Cox modeling, cytosolic E-cadherin staining was still an independent prognostic biomarker in TTR analysis (**Table 5**), but not in OS analysis (**Table 4**). Stratification by stage suggested that the effect was limited to stage II [$P = 0.046$, $n = 299$ with 111 events (OS) and $P = 0.033$, $n = 279$ with 74 events (TTR) (**Table 6**)]. However, interaction tests were not significant. Further exploratory subgroup analyses did not pinpoint other effects.

SOX9 IS NOT ASSOCIATED WITH PROGNOSIS

Neither nuclear nor cytosolic staining of SOX9 protein was associated with prognosis, neither unstratified nor stratified on subgroups (**Figure 5**; **Tables 4** and **5**).

DISCUSSION

In the present study, we took advantage of the high-throughput capabilities of the TMA technology to evaluate the prognostic value of β-catenin, E-cadherin, and SOX9 protein expression in a large consecutive population-representative series of primary CRCs. We found that loss of β-catenin or E-cadherin protein expression in tumors was associated with worse disease outcome. This result fits well with a large body of evidence which demonstrates that β-catenin and E-cadherin are often down-regulated in cancer (17, 18, 51–54), reflecting the invasive properties of cancer cells and follows logically from a malignant cancer cell's need to detach from neighboring cells through decoupling of adherens junctions and activation of Wnt/β-catenin signaling in order to invade neighboring tissue (55). SOX9, on the other hand, was shown to not carry any prognostic information.

In normal colonic epithelia, both β-catenin and E-cadherin are predominantly expressed at the cell membrane (22, 25, 26, 56, 57), and many previous studies have analyzed the prognostic value of their altered expression in tumors, but the reported results are highly divergent (18, 19). While some studies have reported worse outcome for patients with low expression of β-catenin in the nucleus and other compartments (27–30), others have reported no difference in prognosis (26, 31, 43), and yet others have found that strong expression is associated with poor outcome (22, 32–34). The literature on E-cadherin is also marked by conflicting results, where some groups have reported no prognostic effect of altered E-cadherin expression (35, 36), whereas others have reported that patients with low E-cadherin expression have a poor prognosis (37, 38, 58).

Likely reasons for these discrepant results may be that the large majority of the published studies were carried out retrospectively

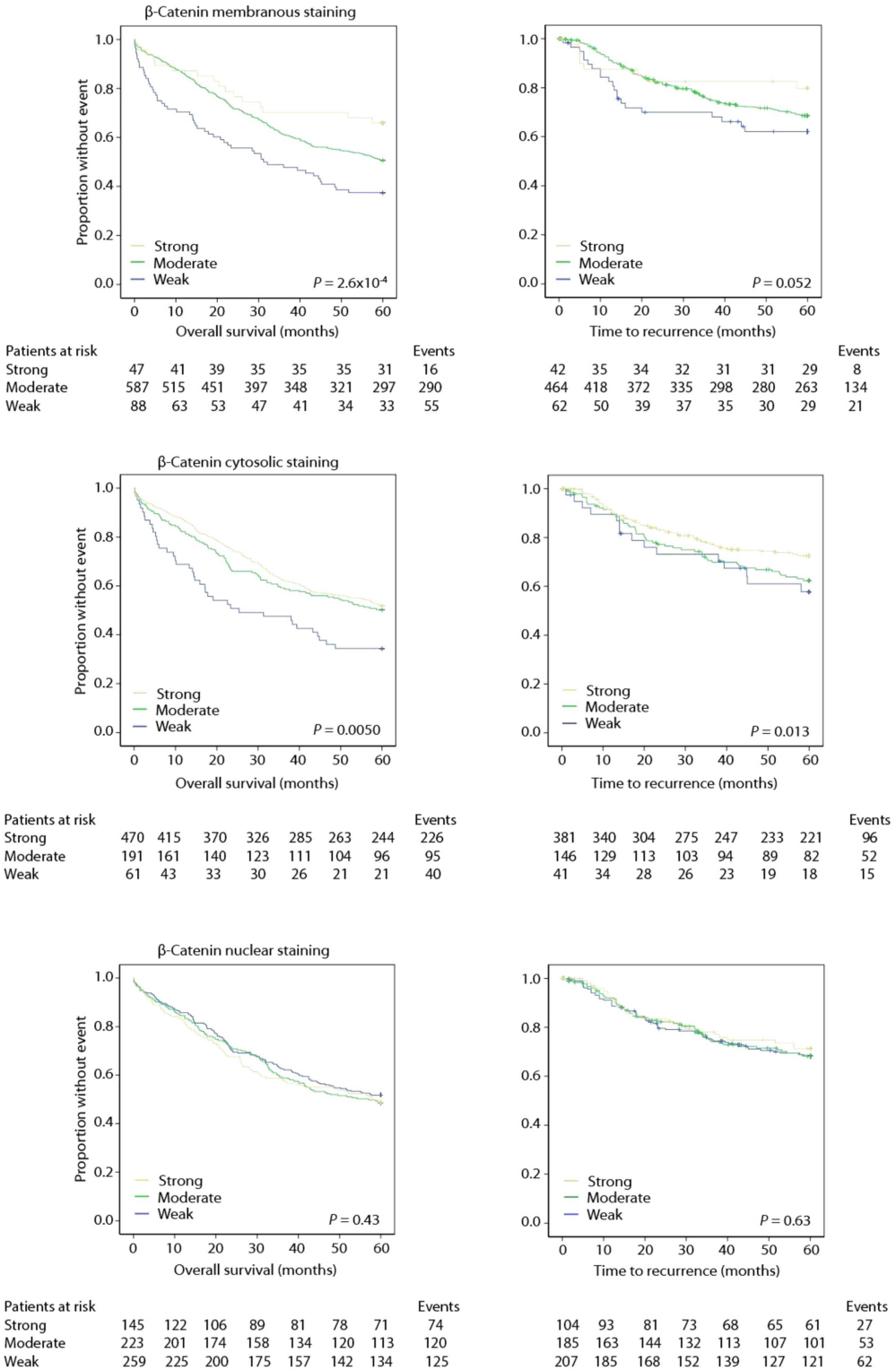

FIGURE 3 | Weak staining of β-catenin predicts poor outcome.

in small series with different patient inclusion criteria, and the analytical approaches employed vary greatly, particularly in reference to clinical endpoints, primary antibodies, immunohistochemical scoring systems, cutoff thresholds, and reporting of statistical methodology. The selection, quality, and representativeness of a patient series may also bias the results.

Table 4 | Univariate and multivariate modeling by Cox regression (Wald test), overall survival (OS).

Variable	Univariate						Multivariate				
	P[a]	HR	95%	*P*	*n*	Events	HR	95% CI	*P*	*n*	Events
Age[b]	–	1.027	1.019–1.036	5.35×10^{-10}	903	449	1.036	1.027–1.046	2.0×10^{-13}		
GENDER											
Female		1					1				
Male	0.4	0.92	0.77–1.11	0.4	903	449	1.1	0.88–1.33	0.48		
STAGE											
I		1					1				
II		1.89	1.27–2.80				1.61	1.05–2.46			
III		3.06	2.06–4.57				2.83	1.85–4.33			
IV	1.6×10^{-56}	11.5	7.77–17.2	1.7×10^{-63}	898	446	3.91	2.22–6.89	1.6×10^{-8}		
HISTOPATHOLOGIC GRADE											
G1		1					1				
G2		1.49	1.03–2.16				1.21	0.81–1.80			
G3	1.6×10^{-7}	2.8	1.84–4.27	2.0×10^{-7}	866	433	2.33	1.46–3.71	1.3×10^{-5}		
TUMOR LOCATION											
Proximal colon		1					1				
Distal colon		1.01	0.82–1.25				0.95	0.75–1.21			
Rectum	0.0061	0.69	0.54–0.88	0.0048	903	449	0.9	0.068–1.20	0.76		
RESECTION											
0		1					1				
1		2.03	1.11–3.71				1.7	0.84–3.45			
2	6.2×10^{-85}	6.17	5.03–7.56	2.88×10^{-67}	902	448	3.47	2.31–5.21	8.4×10^{-9}		
MICROSATELITE STATUS											
MSI		1					1				
MSS	0.039	1.37	1.01–1.84	0.04	819	412	1.69	1.18–2.42	0.0043	787	396
β-CATENIN MEMBRANE STAINING[c]											
Weak		1					1				
Moderate		0.64	0.48–0.85				0.61	0.45–0.83			
Strong	2.6×10^{-4}	0.41	0.23–0.71	0.0012	722	361	0.54	0.29–0.99	0.0065	637	321
β-CATENIN CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.61	0.42–0.89				0.6	0.40–0.89			
Strong	0.005	0.56	0.40–0.79	0.0038	722	361	0.64	0.44–0.93	0.033	637	321
β-CATENIN NUCLEAR STAINING[c]											
Weak		1					1				
Moderate		1.09	0.85–1.40				0.99	0.75–1.30			
Strong	0.43	1.11	0.83–1.48	0.7	637	319	0.97	0.71–1.34	0.99	562	282
E-CADHERIN MEMBRANE STAINING[c]											
Weak		1					1				
Moderate		0.97	0.70–1.36				0.93	0.63–1.36			
Strong	0.1	0.82	0.60–1.12	0.23	720	355	0.79	0.54–1.15	0.3	633	315
E-CADHERIN CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.8	0.64–0.99				0.93	0.73–1.18			
Strong	0.0014	0.69	0.48–1.00	0.045	720	355	0.85	0.57–1.28	0.69	633	315
SOX9 CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.87	0.70–1.08				1.06	0.83–1.34			
Strong	0.5	0.99	0.70–1.39	0.4	761	383	1.04	0.72–1.51	0.89	668	339

(Continued)

Table 4 | Continued

Variable	Univariate						Multivariate				
	P[a]	HR	95%	*P*	*n*	Events	HR	95% CI	*P*	*n*	Events
SOX9 NUCLEAR STAINING[c]											
Weak		1					1				
Moderate		0.98	0.76–1.27				1.13	0.85–1.50			
Strong	0.82	1.03	0.77–1.36	0.93	761	383	0.99	0.72–1.36	0.53	668	339

[a] *Logrank test.*

[b] *Age is implemented as a continuous variable.*

[c] *Adjusted for age, gender, stage, grade, location, resection, and microsatellite status.*

The patients in the present study were consecutively enrolled from a geographically defined catchment area, and all relevant clinical data were prospectively registered. Repeated quality controls have been performed for the hospital records to ensure high quality of these data. Furthermore, completeness of the series was verified against the Cancer Registry of Norway where all cancer diagnoses in Norway are recorded. Hence, the series can be considered to be population-representative and of a size that allows for stratification and subgroup analyses.

Recently, a meta-analysis assessed the prognostic significance of β-catenin protein expression in CRC and concluded that nuclear expression was significantly associated with a poor prognosis, while cytoplasmic expression was not associated to prognosis (59). However, when we repeated the meta-analysis using their input data, we found a significant publication bias, and when we adjusted for this using a trim-and-fill approach (60), we found that there was no prognostic effect of nuclear β-catenin expression (Figure S2 in Supplementary Material), which is in agreement with our finding. Furthermore, two other large studies that did not find any effect of nuclear β-catenin were not included in the meta-analysis (22, 30). An overview of all the main findings in studies having sample sizes above 200 is summarized in Table S1 in Supplementary Material.

Even though the corrected meta-analysis suggests that there is no prognostic value in assessing nuclear β-catenin expression, one cannot exclude an undefined functional and prognostic relationship among membranous, cytosolic, and nuclear β-catenin protein expression. Furthermore, the phosphorylation status of β-catenin has also been shown to carry prognostic information (26). More quantitative tools are needed to determine these relationships exactly. It may also be functionally relevant to investigate the expression of β-catenin at the tumor invasion front where it has been shown to play an important role in the process of EMT (55, 61–63).

Potential benefit from combining the markers β-catenin, E-cadherin, and SOX9 was explored, but this did not improve stratification of patients, likely due to the high correlation between β-catenin and E-cadherin and the lack of prognostic information carried by SOX9.

Notably, we found that MSI tumors exhibit a significantly lower expression of β-catenin, E-cadherin, and SOX9 protein than MSS tumors. Similarly, right-sided tumors showed lower protein expression compared to left-sided and rectal tumors, although this is likely dependent on the higher level of MSI tumors on the right side. Similar correlations have been reported by other groups (22, 30, 31, 64, 65). The low expression of β-catenin, E-cadherin, and SOX9 in MSI tumors suggests that the Wnt/β-catenin pathway may be less important in these tumors. On the other hand, both MSI and MSS tumors are dependent on active Wnt/β-catenin signaling. Recent studies have suggested that tumorigenic Wnt/β-catenin signaling may be subject to dose- and tissue-dependent regulation through the existence of different *APC* genotypes in right- and left-sided tumors (66). *APC*-mutations reflecting differential inactivation have been documented in independent tumor series (67–69), supporting the "just-right"-hypothesis that different thresholds exist for optimal tumorigenic Wnt/β-catenin signaling (66, 70), which may explain the observed differences in staining between MSI and MSS tumors. Moreover, it has been reported that small absolute changes (but rather relevant fold-changes) in β-catenin levels may have significant effect on Wnt/β-catenin signaling (71). Hence, a lower expression of these proteins may not necessarily indicate that the functional effects are different from in tumors with a higher absolute protein expression.

There was also a positive correlation between the three biomarkers and the histopathologic grade of the tumor. This is in accordance with the assumption that tumors with lower differentiation grade have a more mesenchymal phenotype with down-regulated levels of adherens junctions and presumably lower activity of particular components of the Wnt/β-catenin signaling pathway. We also note that the nuclear staining of SOX9 does not correlate significantly to MSI and histopathologic grade, which may suggest different biological roles for SOX9 in these two compartments.

In our dataset, SOX9 did not carry any prognostic information contrasting with the finding by Lü et al., which reported that strong SOX9 protein expression was an independent adverse prognostic biomarker in a Chinese patient population of 188 primary CRCs with complete resection (43). Selection bias and preanalytical variability may partly explain the lack of accordance as their samples were retrieved from three different hospitals and also constitute a considerably smaller series in total. Significant population effects may also exist due to genetic differences between populations.

Limitations to our study are primarily related to the previously mentioned shortcomings of protein analyses by

Table 5 | Univariate and multivariate modeling by Cox regression (Wald test), time to recurrence (TTR).

Variable	Univariate						Multivariate[a]				
	***P*[a]**	**HR**	**95%**	***P***	***n***	**Events**	**HR**	**95% CI**	***P***	***n***	**Events**
Age[b]	–	1.017	1.005–1.029	0.0067	693	206	1.023	1.009–1.037	9.4×10^{-4}		
GENDER											
Female		1					1				
Male	0.96	1.007	0.77–1.32	0.96	693	206	1.09	0.80–1.47	0.6		
STAGE											
I		1					1				
II		2.68	1.55–4.63				2.38	1.35–4.18			
III		4.97	2.88–8.58				4.75	2.72–8.30			
IV	2.3×10^{-45}	9.56	4.29–21.3	5.1×10^{-11}	689	205	8.02	3.5–18.2	3.1×10^{-10}		
HISTOPATHOLOGIC GRADE											
G1		1					1				
G2		1.34	0.81–2.21				1.14	0.68–1.93			
G3		2.47	1.37–4.47	0.0024	666	200	2.29	1.21–4.31	0.0042		
TUMOR LOCATION											
Proximal colon		1					1				
Distal colon		1.02	0.74–1.41				0.99	0.68–1.43			
Rectum	0.82	1.04	0.74–1.45	0.98	693	206	1.27	0.86–1.88	0.37		
MICROSATELITE STATUS											
MSI		1					1				
MSS	0.2	1.31	0.86–1.97	0.21	627	194	1.63	0.99–2.71	0.057	603	187
β-CATENIN MEMBRANE STAINING[c]											
Weak		1					1				
Moderate		0.72	0.45–1.14				0.84	0.50–1.41			
Strong	0.052	0.47	0.21–1.05	0.16	553	163	0.68	0.29–1.60	0.67	489	149
β-CATENIN CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.88	0.49–1.56				0.72	0.39–1.35			
Strong	0.013	0.61	0.35–1.05	0.043	553	163	0.55	0.30–1.01	0.1	489	149
β-CATENIN NUCLEAR STAINING[c]											
Weak		1					1				
Moderate		0.99	0.69–1.43				0.91	0.61–1.36			
Strong	0.63	0.89	0.56–1.39	0.86	485	142	0.86	0.53–1.41	0.82	429	129
E-CATENIN MEMBRANE STAINING[c]											
Weak		1					1				
Moderate		1.11	0.68–1.81				0.93	0.53–1.65			
Strong	0.1	0.79	0.49–1.26	0.12	555	164	0.79	0.45–1.39	0.55	489	150
E-CATENIN CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.65	0.47–0.89				0.67	0.47–0.95			
Strong	9.3×10^{-4}	0.46	0.26–0.83	0.004	555	164	0.48	0.25–0.92	0.02	489	150
SOX9 CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.9	0.65–1.24				1.04	0.72–1.49			
Strong	0.87	1.05	0.63–1.75	0.74	583	168	1.09	0.63–1.90	0.95	512	153
SOX9 NUCLEAR STAINING[c]											
Weak		1					1				
Moderate		1.06	0.72–1.55				1.03	0.68–1.56			
Strong	0.52	0.88	0.56–1.37	0.61	583	168	0.79	0.48–1.29	0.41	512	153

[a] *Logrank test.*

[b] *Age is implemented as a continuous variable.*

[c] *Adjusted for age, gender, stage, grade, location, and microsatellite status.*

Table 6 | Univariate and multivariate modeling of subgroup analyses by Cox regression (Wald test).

Variable	Univariate						Multivariate[a]				
	***P*[a]**	**HR**	**95%**	***P***	***n***	**Events**	**HR**	**95% CI**	***P***	***n***	**Events**
β-CATENIN MEMBRANE STAINING[b]											
Weak		1					1				
Moderate		8.6	2.86–25.8				3.2	0.97–10.5			
Strong	3.7×10^{-7}	2	0.73–5.41	5.7×10^{-8}	134	125	0.92	0.32–2.67	6.7×10^{-5}	120	112
E-CADHERIN CYTOSOLIC STAINING[c]											
Weak		1					1				
Moderate		0.63	0.39–1.03				0.55	0.32–0.93			
Strong	0.033	0.51	0.21–1.19	0.091	273	74	0.36	0.14–0.94	0.024	239	64

[a] *Logrank test for trend.*

[b] *Subgroup analysis for tumor stage IV using overall survival as endpoint, adjusted for age, gender, grade, location, resection, and microsatellite status.*

[c] *Subgroup analysis for tumor stage II using time to recurrence as endpoint, adjusted for age, gender, grade, location, and microsatellite status.*

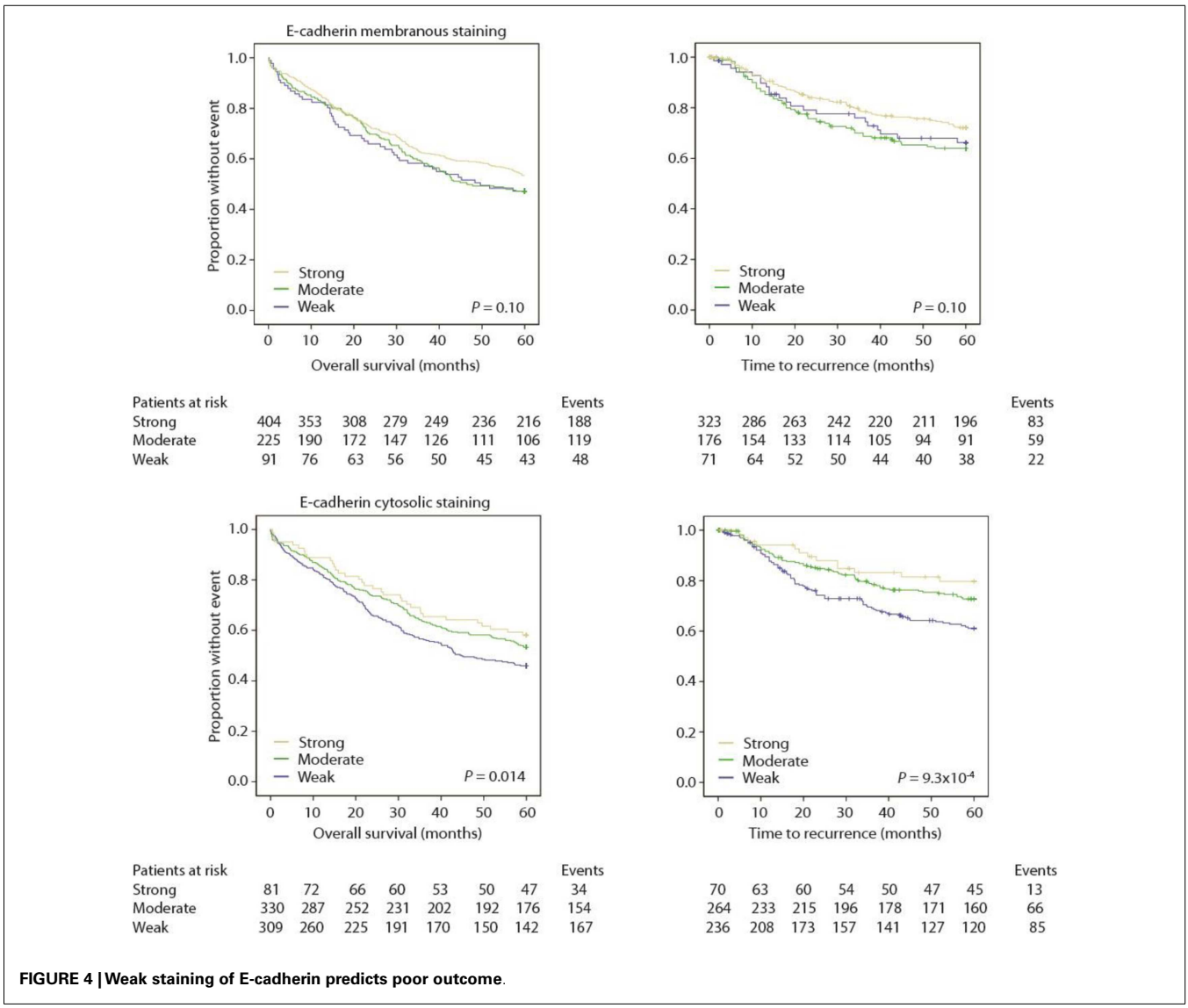

FIGURE 4 | Weak staining of E-cadherin predicts poor outcome.

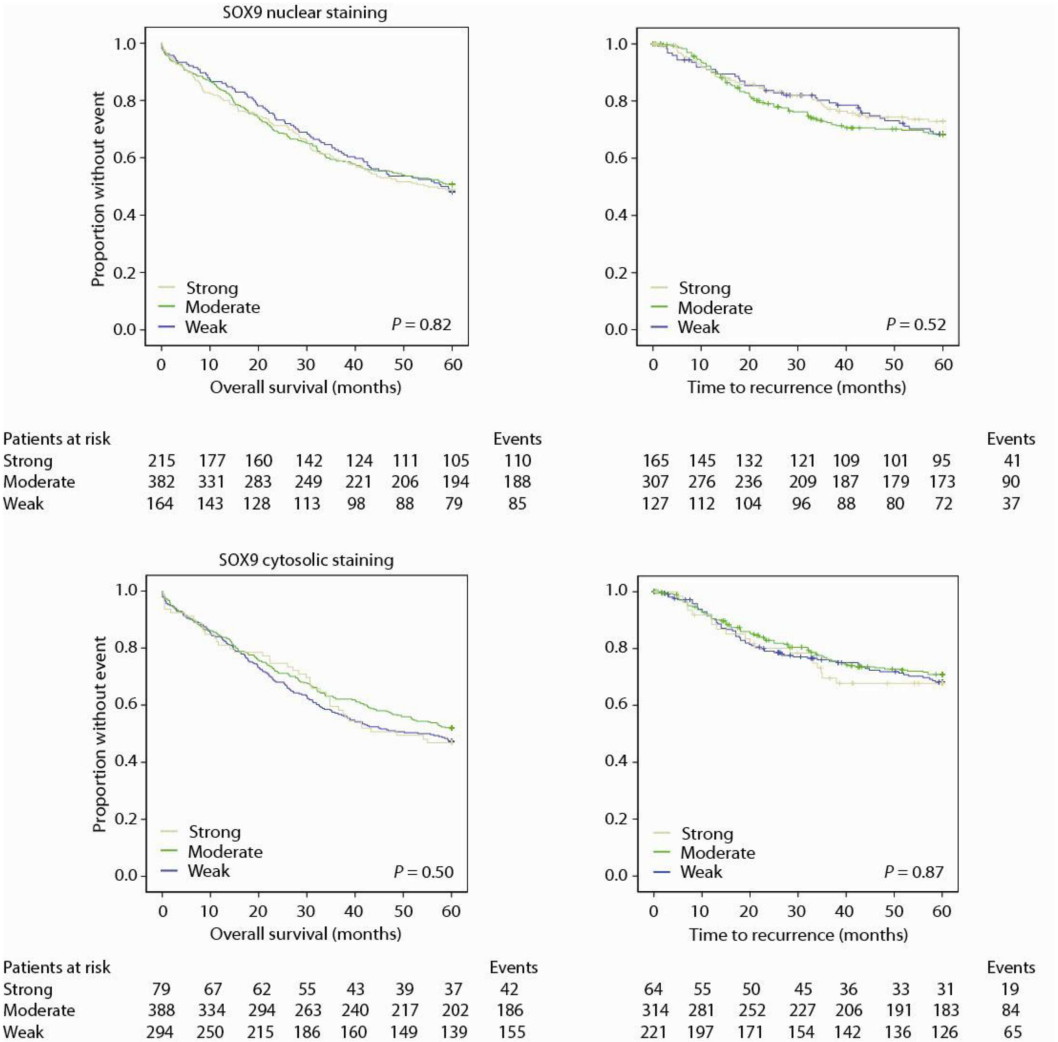

FIGURE 5 | SOX9 does not predict disease outcome.

immunohistochemistry being subject to preanalytical variability, tumor heterogeneity, and subjective scoring systems limiting its reproducibility. The two former are to a certain degree compensated for by using a large sample series, while the latter can only be assessed properly by independent validations. We are also aware that stage migration, due to more patients being classified to higher stages while diagnostic methods have become more sensitive, may bias our assessments of stage specific survival (45).

TNM-stage classification is well-established to assess CRC prognosis. Survival varies considerably between stages, at about 90% for localized disease compared to about 10% for metastatic disease (72), although prognosis may vary significantly within stages. Adjuvant chemotherapy is offered as standard treatment to high-risk stage II patients and stage III patients below 75 years of age. However, up to one-third of stage II patients relapse (3) and elderly patients seem to benefit from adjuvant therapy (4, 5), justifying a need for prognostic biomarkers that can identify high- and low-risk patients in these subgroups. Recently, MSI was introduced into clinical guidelines as a marker for improved prognosis that also likely predicts lack of response to 5-FU monotherapy (73) (www.nccn.org). In our study, strong versus weak β-catenin membrane expression showed the clearest stratification of patients into poor and good prognostic groups (**Table 4**). Unfortunately, subgroup analysis by stage demonstrated that this effect was evident neither in stage II nor in stage III, but rather in stage IV, suggesting that stage IV patients with low β-catenin membrane expression may benefit from a more *or* less intensive treatment depending on their health condition. Cytosolic E-cadherin on the other hand, might have some prognostic value for stratification of stage II patients. Independent validations are warranted to confirm these results. If validated, we believe β-catenin and E-cadherin may serve as valuable biomarkers in a panel of biomarkers. They are not likely to separate high- and low-risk patient groups with sufficient precision for a clinical test as sole biomarkers.

In summary, nuclear β-catenin protein expression lacks prognostic value for CRC, while decreased expression of both membranous and cytosolic E-cadherin and β-catenin are associated with worse outcome among primary CRC patients, having potential to serve as biomarkers in stage II and IV CRC, respectively.

AUTHOR CONTRIBUTIONS

Jarle Bruun participated in the study design, performed IHC experiments and scoring, interpreted all results, performed all statistics, and drafted the manuscript. Matthias Kolberg participated in IHC experiments and in the statistical analyses and did an independent scoring of results. Arild Nesbakken collected the patient samples and provided the clinical data. Jahn M. Nesland performed quality control of the IHC analysis and carried out independent scoring of a subset of the data. Aud Svindland performed morphological identification of qualified tumor areas for TMA construction. Ragnhild A. Lothe conceived and coordinated the study, was responsible for the study design, and participated in discussion of results and in the drafting of the manuscript. All authors participated in manuscript writing and in scientific discussions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2014.00118/abstract

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PIK3CA in colorectal cancer

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PIK3CA, the catalytic subunit of PI3K, is mutated in many different tumors, including colorectal cancer (CRC). Mutations of PIK3CA have been reported in 10–20% of CRC, about 80% of mutations found in two hot spots in exon 9 and exon 20. In RAS wild-type CRC, PIK3CA mutations have been associated with a worse clinical outcome and with a negative prediction of a response to targeted therapy by anti-EGFR monoclonal antibodies. However, these findings have not been confirmed in all studies and subsequent more detailed analysis has revealed that these effects may be restricted to mutations in Exon 20. Finally, mutations in PIK3CA may be the long sought biomarker for successful adjuvant therapy with aspirin in patients with CRC. Therefore, PIK3CA mutations appear to be a promising predictive biomarker; however, further data are needed to conclusively define the impact of somatic mutations in the PIK3CA gene for the management of patients with CRC.

Keywords: biomarker, colorectal cancer, PIK3CA, prognosis, prediction, aspirin, adjuvant therapy, EGFR

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy worldwide, affecting more than 1.2 million patients and leads to over 6,000,000 deaths every year (1). Since the seminal paper by Kinzler and Vogelstein in 1996, it is known that the development of CRC is based on an accumulation of hereditary and somatic genetic alterations ultimately leading to the malignant phenotype (2). The development of CRC through adenomatous precursor lesions has further led to secondary prevention strategy of colonoscopy screening. In addition, a large body of work has finally shown that CRC is not a single disease but a heterogeneous group of neoplasms with a different genetic and epigenetic background. A number of molecular classifications have been suggested and the presence of at least three pathways are generally accepted today, including the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI), and the epigenetic CpG island methylator phenotype (CIMP) (3). Despite this increasing body of knowledge, the therapeutic options in patients with advanced, metastatic disease remains rather restricted, and the prognosis poor. The introduction of new targeted therapeutics, namely the development of antibodies against the epidermal growth factor receptor (EGFR), has raised new hope for successful treating of advanced CRC (4). However, only a subgroup of patients, especially those with a KRAS wild-type tumor, profit from the anti-EGFR therapy (5). Unfortunately, KRAS wild-type is not sufficient to predict clinical response and mutations in other effectors of the KRAS or KRAS related pathway have been anticipated to be predictive for anti-EGFR response. A promising candidate for this prediction is PIK3CA, which may not only be predictive for targeted therapy by anti-EGFR antibodies but also turned out to be probably a positive biomarker for the neoadjuvant use of aspirin.

BASIC BIOLOGY OF PI3K AND PIK3CA

The phosphatidylinositol-3-kinase (PI3K) belongs to a family of heterodimeric lipid kinases consisting of a regulatory and a catalytic subunit, phosphorylating phosphatidylinositol, an important cell membrane element and second messenger involved in cell signaling (6). Activated by various receptor tyrosine kinases, EGFR, human EGFR 2 (HER2), insulin growth factor (IGF-1R), and platelet derived growth factor (PDGFR), the PI3K promotes and regulates various cellular processes, including proliferation, survival, apoptosis, migration, and metabolism (**Figure 1**). PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), the catalytic p110-alpha subunit of PI3K, has been described to be commonly mutated in various cancer, including glioblastoma, gastric, breast, ovary, lung, and CRC (7, 8). More than 80% of mutations detected in PIK3CA were reported in two hotspots, the helicase domain of exon 9 (codon 542 and 545) and the kinase domain in exon 20 (codon 1047) (8). Downstream effectors of the PI3K pathway include AKT (protein kinase B), a serine–threonine kinase, directly activated by PI3K and the mTOR (mammalian target of rapamycin), another serine–threonine kinase leading to an increased translation of various mRNAs encoding cell cycle regulators, including MYC and cyclin D1 and a potential target of therapeutic inhibition (9). In the PI3K/AKT/mTOR pathway, the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a direct antagonist and mutation or loss of PTEN expression has shown to be correlated with a poor outcome in CRC (10, 11).

FREQUENCY AND PROGNOSTIC IMPACT OF PIK3CA MUTATION IN COLORECTAL CANCER

Roughly, PIK3CA mutation has been reported to be present in 10–20% of CRC. Variation in the frequency has been observed in large population based studies compared to clinical studies (12). In addition, as expected, the technique used to evaluate the PIK3CA mutation has a direct impact on the frequency of mutation observed and using pyrosequencing, generally considered a more sensitive assay, shows higher incidence of PIK3CA mutations (15–18%) (13–15) compared to Sanger sequencing (11–12%) (16–18). Mutations are more commonly found in exon 9 compared to

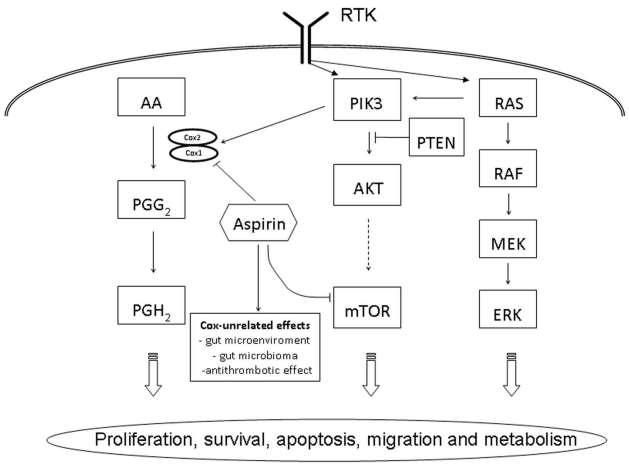

FIGURE 1 | Schematic overview of PIK3CA related cell signaling pathways in colorectal cancer. AA, arachidonic acid; AKT, protein kinase B; ERK, extracellular signal-regulated kinase, MEK, MAP kinase kinase; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PGG_2, prostaglandin G2; PGH_2, prostaglandin H2; PI3K, phosphatidylinositol-3-kinase; RAF, rapidly accelerated fibrosarcoma; RAS, rat sarcoma; RTK, receptor tyrosine kinase.

exon 20, usually in a ratio of 3:1 to 5:1 and few tumors (<5%) harbor both mutations (19–21). Interestingly, detailed analysis have shown a gradual decrease of PIK3CA mutation from the proximal (cecum/colon ascendens) to the distal (sigma/rectum) site of colon from 21–25% down to 8–9%, respectively (19, 20). Furthermore, PIK3CA mutated CRC has been associated with a mucinous histological phenotype (15, 19, 20). In contrast to other molecular markers, namely BRAF and KRAS, which are mutually exclusive, PIK3CA mutations have shown in the majority of studies to be significantly associated with KRAS mutation and the loss of MGMT (O6-methylguanine-DNA methyltransferase) expression (15, 19, 20). Less consistent or no correlations where reported for CIMP-H, MSI-H, and BRAF mutations (19, 20, 22). In an early study by Ogino and co-workers analyzing patients with curatively resected CRC stage I–III, mutations in PIK3CA were detected in 18% and reported to be associated with a significant worse outcome. This effect, however, was restricted to tumors with wild-type KRAS (14). Similar results were reported in other patient collectives naïve to anti-EGFR therapy with a frequency of PI3KCA mutation ranging from 12 to 13%; but again, this correlation with poor outcome was restricted to KRAS wild-type tumors (16, 23). Further studies, taking in consideration a broader range of molecular markers, did not confirm these previous data for PIK3CA, being an independent prognostic marker for CRC. Mouradov and co-worker analyzed the disease free survival (DFS) in 822 patients with CRC stage II/III and correlated this data with MSI, CIN, and a number of molecular biomarkers, including PIK3CA (18). In this study, only CIN and MSI were associated with DFS but with none of the molecular biomarkers, including PIK3CA (18). Similarly, in a Scandinavian evaluation, analyzing two study cohorts of 611 patients, PIK3CA did not show any prognostic impact (24). In this study, however, the molecular analysis of PIK3CA was restricted to exon 20, leading to the expected low frequency of 2.2% PIK3CA mutated tumors. The rationale behind this approach is that some studies have suggested that the poor prognosis of PIK3CA is restricted to exon 20 mutations and that the analysis of exon 9 may produce false positive results due to presence of pseudogenes (25, 26). Indeed, in breast cancer, the prognostic impact of PIK3CA has been similarly reported to be restricted to exon 20 (27, 28). This concept is supported by *in vitro* studies showing that mutations in the helical (exon 9) and kinase (exon 20) domain use different and independent mechanisms for cell transformation (29). In addition, the effect of PIK3CA mutation is RAS dependent in the helical but not the kinase domain, which may explain the stronger association of KRAS mutation with exon 9 mutations of PIK3C (19, 22). Taken together, mutation of PIK3CA in CRC may have a slight prognostic impact in anti-EGFR naïve patients; the extent, if present, of this impact, however, especially in respect to different mutations, remains to be clarified.

PIK3CA AS PREDICTIVE MARKER IN ANTI-EGFR THERAPY

Despite the fact that CRC can curably be treated at early stages, advanced tumors, namely metastatic cancer are associated with a high mortality rate and a 5-year survival of below 10% (30). The introduction of a targeted therapy using monoclonal anti-EGFR antibody, namely panitumumab and cetuximab, in combination chemotherapy or as a single agent, has added a further promising treatment option (4, 31). However, only a subgroup of patients, usually <10% in unselected patients, profit of anti-EGFR antibody treatment (5, 32). Several clinical trials have shown that RAS mutations are the most important negative predictive factor in CRC, primarily mutations in exon 1 and 2 of RAS, but, as recently been shown, also of exon 3 and 4 of KRAS and NRAS, respectively (32, 33). However, even in wild-type RAS tumors, 50–60% of patients do not profit from an anti-EGFR therapy. Based on the well-established pathway of the EGFR receptor, other downstream elements of the direct or associated signaling pathway, including BRAF/MEK/ERK and PIK3/PTEN/AKT/mTOR have been analyzed as potential biomarker (**Figure 1**). In a first study, analyzing 110 patients with CRC, Sartore-Bianchi and co-workers reported a significant resistance to EGFR-targeted therapy in the 13.6% of PIK3CA mutated cancers (34). The predictive value of PIK3CA mutation in RAS wild-type CRC was supported subsequent by additional studies (35, 36). Interestingly, however, in a study by Prenen and co-workers analyzing 200 chemorefractory patients treated with cetuximab, PIK3CA mutation, detected in 11.5% of tumors, was no predictor of anti-EGFR response (37). Further detailed studies, analyzing PIK3CA mutation of exon 9 and exon 20 separately, may possibly give the explanation for the discrepancy of the predictive value of PIK3CA as a biomarker for anti-EGFR response. In a carefully performed, retrospective study including 743 CRC, de Roock and co-workers describe in KRAS wild-type tumors a significant association of objective response, overall survival, and progression free survival in exon 20 but not in exon 9 mutated tumors (22). As expected, the incidence of exon 20 mutation in PIK3CA was low, i.e., 3.0%, however, the mutation analysis added another 1.3% improvement of anti-EGFR response prediction, similar to the improvement of prediction by testing NRAS (i.e., 1.5%) (22).

PIK3CA AS BIOMARKER FOR ADJUVANT ASPIRIN THERAPY

Based on several observational studies as well as randomized trials, it has been long considered that aspirin is efficient in preventing colorectal adenomas and cancers (38, 39). This anti-tumor effect is thought to be driven by the inhibition of cyclooxygenases [COX-2, officially called HGNC:9605 or PTGS2 (prostaglandin-endoperoxide synthase 2)], interacting with the arachidonic acid metabolite pathway, however, the detailed mechanism of action is not completely understood [reviewed in Ref. (40)]. This anti-tumor effect has reported to be restricted to patients with cancers showing an over expression of COX-2 demonstrated by immunohistochemistry (41). However, as 60–85% of CRCs has been reported to over express COX-2 (42), immunohistochemistry is considered a less reliable predictive marker for adjuvant aspirin therapy. Due to its side effects, namely gastrointestinal irritation and bleeding, wide spread and unselected chemoprevention by aspirin is not recommended. In addition, more specific COX-2 inhibitors, such as rofecoxib or celecoxib, had to be withdrawn from the market due to their cardiovascular side effects. Therefore, the recent study by Liau and co-workers, reporting an improved survival of CRC patients using regular aspirin in tumors harboring a PIK3CA mutation, has created a lot of interest (13). Using data of two large prospective studies, the Nurses' Health study and the Health Professionals Follow-up Study, the authors were able to follow 964 patients for a median follow-up time of 153 months. PIK3CA mutations were detected in 16.7% of tumors, and in the patients with mutated cancer, the regular use of aspirin was associated with a reduction of tumor specific and over all mortality of 82 and 46%, respectively. The precise molecular and biological mechanisms of aspirin to the PIK3/AKT/mTOR pathway have to be clarified in detail (**Figure 1**). *In vitro* studies indicate that the PIK3CA induces the expression of COX-2 (43). In addition, COX unrelated effects, namely the change of the microenvironment and subsequently the intestinal microbioma in the gut as well as the anti-thrombotic effect of aspirin, which may be relevant in the development of metastasis, have to be considered (44, 45). An additional clinical study by Domingo and co-workers, again retrospectively analyzing a large prospective study cohort of 896 patients with CRC, the patients of the VICTOR trial, has confirmed the predictive value of PIK3CA mutation for taking aspirin in an adjuvant setting (46). Interestingly, however, this effect could not be observed in patients taken the specific COX-2 inhibitor rofecoxib. These data indicate that the predictive value of PIK3CA mutation goes beyond COX-2 inhibition and underlies the importance of a COX-independent effect of aspirin in the prevention of cancer development and spread. Despite the promising results reported by these two studies, there is some caveat to express: based on the stratification necessary, i.e., patients with PIK3CA mutated tumors and taking regularly aspirin, the numbers of patients is relatively small in both studies (60 and 45 patients, respectively) and prospective, large scale clinical trials are needed to confirm this data, highly relevant for the future management of patients with CRC.

In conclusion, somatic mutations of PIK3CA are present in 10–20% of CRC, basically confined to exon 9 and exon 20 (or more precisely, these are the exons usually analyzed). The majority of studies are based on pooled data in respect to the exon mutated, but experimental as well as epidemiological evidence point in the direction that mutation in exon 20 but not in exon 9 may be biologically relevant. So far, mutation of PIK3CA as a single prognostic marker seems to have, if some, a minor effect of the overall prognosis of CRC but a small and distinct predictive impact for anti-EGFR therapy in RAS wild-type tumors. In addition, there is strong evidence of the predictive value of PIK3CA mutations for adjuvant therapy using aspirin, however, further data are needed to definitively define the impact of, especially exon specific, PIK3CA mutation in the management of patients with CRC.

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Functions and regulation of the PTEN gene in colorectal cancer

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Phosphatase and TENsin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor gene located at chromosome 10q23.31, encoding for a 403-amino acid protein that possesses both lipid and protein phosphatase activities. The main function of PTEN is to block the PI3K pathway by dephosphorylating phosphatidylinositol (PI) 3,4,5-triphosphate to PI-4,5-bisphosphate thus counteracting PI3K function. PTEN inactivation is a frequent event in many cancer types and can occur through various genetic alterations including point mutations, large chromosomal deletions, and epigenetic mechanisms. In colorectal cancer (CRC) PTEN is altered through mixed genetic/epigenetic mechanisms (typically: mutations and promoter hypermethylation or 10q23 LOH and promoter hypermethylation), which lead to the biallelic inactivation of the protein in 20–30% of cases. The role of PTEN as a prognostic and predictive factor in CRC has been addressed by relatively few works. This review is focused on the report and on the discussion of the studies investigating these aspects. Overall, at the moment, there are conflicting results and, therefore it has not been clarified whether PTEN might play a prognostic role in CRC. The same is valid also for the predictive role, leading to the fact that PTEN evaluation cannot be used in routinely diagnosis for the early identification of patients who might be addressed to the treatment with EGFR-targeted therapies, at odds with other genetic alterations belonging to EGFR-downstream pathways. The reason of discordant results may be attributable to several issues: (1) the size of the analyzed cohort, (2) patients inclusion criteria, (3) the methods of assessing PTEN alteration. In particular, there are no standardized methods to evaluate this marker, especially for immunohistochemistry, a technique suffering of intra and inter-observer variability due to the semi-quantitative character of such an analysis. In conclusion, much work, especially in large and homogeneous cohorts of cases from different laboratories, has to be done before the establishment of PTEN as prognostic or predictive marker in CRC.

Keywords: PTEN, colorectal cancer, mutation, immunohistochemistry, prognosis, predictive, EGFR-targeted therapies

INTRODUCTION

Phosphatase and TENsin homolog deleted on chromosome 10 (*PTEN*), known also as mutated in multiple advanced cancer 1 (*MMAC1*), is a tumor suppressor gene located at chromosome 10q23.31 and encodes for a 403-amino acid protein that possesses both lipid and protein phosphatase activities. The crystal structure of PTEN revealed two major functional domains (a phosphatase domain and a C2 domain) and three structural regions [a short N-terminal phosphatidylinositol (PI)-4,5-bisphosphate (PIP2) binding domain and a C-terminal tail containing PEST sequences and a PDZ-interaction motif] (**Figure 1**) (1). The PTEN protein is principally involved in the homeostatic maintenance of PI3K/Akt signaling originating from EGFR activation (or activation of other tyrosine kinase receptors or G-protein-coupled receptors) (**Figure 2**). Its typical function consists of the dephosphorylation of the lipid-signaling second messenger PI 3,4,5-triphosphate (PIP3), a lipid product of the PI-3-kinase (PI3K) (2), thereby directly antagonizing the PI3K function and blocking therefore the activation of downstream signaling events, including PDK1 (akt) and akt/mammalian target of rapamycin (mTOR). The opposite biochemical reaction is catalyzed by PI3Ks, which are associated with cell growth and cell survival (**Figure 2**). Thus PTEN, which counteracts PI3Ks activity, is involved in inhibition of cell cycle progression, induction of cell death, modulation of arrest signal, and stimulation of angiogenesis (3).

The lipid phosphatase activity of PTEN is the best-characterized physiological function contributing to the tumor suppressor function of PTEN. As no other redundant and/or compensatory family members have been found, PTEN is the only known lipid phosphatase counteracting the PI3K pathway. It is not surprising that loss of PTEN function, resulting therefore in increased PIP3 and persistent activation of PI3K effectors, has an important impact on multiple aspects of cancer development such as cell proliferation, apoptosis resistance, angiogenesis, metabolism regulation, genomic instability, stem cell self-renewal, cellular senescence, and cell migration and metastasis (4, 5).

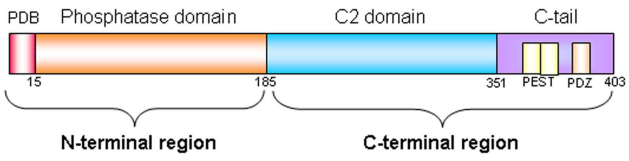

FIGURE 1 | PTEN protein structure. PTEN is composed of 403-amino acids and contains: a N-terminal region of 185 aminoacids (1–185) composed by a PIP2-binding domain (PBD) and by a phosphatase domain of 218 aminoacid (186–403), and a C-terminal region composed by a C2 domain and by a C-terminal tail containing two PEST (proline, glutamic acid, serine, threonine) sequences, and a PDZ-interaction motif at the end.

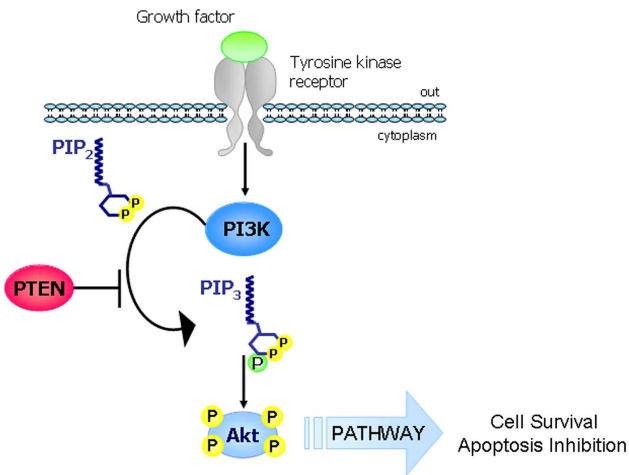

FIGURE 2 | The PI3K-PTEN-Akt pathway. The main function of PTEN consists in the regulation of the PI3K/Akt/mTOR. In response to extracellular stimuli (e.g., presence of insulin, growth factors, chemokines), PI3K is activated by tyrosine kinase receptors or G-protein-coupled receptors and it phosphorylates PIP2 to generate PIP3 which in turn phosphorylates and activates Akt. PTEN is a lipid phosphatase that antagonizes the action of PI3K by dephosphorylating PIP3 to generate PIP2 (thus blocking the PI3K signaling cascade).

In its inactive state, PTEN is phosphorylated on a cluster of serine and threonine residues located on its C-terminal tail, leading to a closed PTEN state and maintaining PTEN protein in a stable conformation. When PTEN is being activated, dephosphorylation of its C-terminal tail opens its phosphatase domain, thereby increasing PTEN activity. Meanwhile, the open state of PTEN is more susceptible to ubiquitin-mediated proteasomal degradation (4, 6): therefore, this mechanism is a negative feed-back leading to decreasing and switching off the effect of PTEN, in absence of specific stimuli.

The functionality of PTEN is also regulated by subcellular localization. PTEN is well characterized as a cytosolic protein that is recruited to the membrane by interacting with a number of membrane-anchored proteins, via its C-terminal PDZ domain and PIP2-binding domain (7). In addition, PTEN mono-ubiquitination controls PTEN nuclear entry. In some tumors, the subcellular localization of PTEN protein seems to mediate its activity (8). The absence of PTEN has been reported to be associated with more aggressive diseases and with high degree of neoplastic transformation, suggesting an important nuclear function for PTEN in tumor suppression (9, 10).

A number of factors have been shown to transcriptionally regulate *PTEN* mRNA [reviewed by Song et al. (5)], including peroxisome proliferation-activated receptor γ (PPARγ), early growth-response protein 1 (EGR1), and p53. *PTEN* mRNA is also post-transcriptionally regulated by *PTEN*-targeting microRNAs such as miR19 and miR21 and is now emerging that also *PTEN* pseudogene (*PTENP1*) may be able to regulate PTEN expression (5).

PTEN loss of function occurs in a wide spectrum of human cancers through various genetic alterations including point mutations (missense and nonsense mutations), large chromosomal deletions (homozygous/heterozygous deletion, frameshift, inframe deletion, and truncation), and epigenetic mechanisms as hypermethylation of the *PTEN* promoter region. In addition, PTEN could be inactivated by other non-structural alterations affecting transcript stability, protein stability, and differential subcellular compartmentalization (4, 5, 8).

Despite its serine, threonine and tyrosine phosphatase activity, the lipid phosphatase function of PTEN has been shown to be the major driving force in tumor suppression. In fact the G129E mutation, observed in cancer specimens and abrogating the lipid phosphatase activity but maintaining its protein phosphatase activity, leads to PTEN tumor suppressor function inactivation *in vitro* (11–13).

Loss of heterozygosity at 10q23 occurs frequently in many sporadic tumors at advanced stage; for example, approximately 70% glioblastoma and 60% advanced prostate cancer are characterized by loss of that region. Somatic mutation in the second allele of *PTEN*, which results in biallelic inactivation, occurs in 25–40% of glioblastomas.

Somatic mutations of *PTEN* have been identified as the main mechanism of inactivation in many tumor types, particularly those of the endometrium, brain, skin, and prostate. The tumor suppressor function of PTEN is usually abrogated following mutations occurring in its phosphatase domain (encoded by exon 5): typically, the C124S mutation (that abrogates both lipidic and protein phosphatase activity) and the G129E mutation (that abrogates only lipid phosphatase activity) (4, 14). Although the N-terminal phosphatase domain is principally responsible for PTEN physiological activity, approximately 40% of *PTEN* tumorigenic mutations may occur in the C-terminal C2 domain (corresponding to exons 6, 7, and 8) and in the tail sequence (corresponding to exon 9), encoding for tyrosine kinase phosphorylation sites important for maintaining PTEN function and protein stability (3, 4, 8, 15). In endometrial carcinoma, glioblastoma, and lymphoma, cancer-specific mutations have been found also in the PIP2-binding region, thus highlighting the importance of this motif for the functionality of PTEN protein (16, 17). In addition to missense mutations, a number of nonsense and frameshift mutations have been described leading to truncated PTEN proteins lacking the C-terminal tail and the PDZ-interaction motif, important domains for PTEN protein stability and recruitment to the membrane, without which PTEN is biochemically inactive (5, 8).

However, in sporadic tumors, loss of heterozygosity of *PTEN* occurs at a much higher frequency than biallelic inactivation. It remains unclear whether haploinsufficiency of PTEN provides a selective growth advantage in tumors lacking a second hit in the remaining *PTEN* allele. Evidence for a role of PTEN haploinsufficiency was demonstrated in a mouse model of prostate cancer in which the dosage of PTEN was inversely correlated to the severity of tumor phenotype (18).

Finally, PTEN can be altered also in inherited syndromes. That is the case of the Cowden disease whose patients tend to develop breast, thyroid, and skin tumors. In these types of tumor PTEN exerts its role in the initiation and in the progression of cancer (3, 12, 19).

PTEN IN COLORECTAL CANCER

In CRC PTEN is altered through a mixed genetic/epigenetic mechanism (typically: mutations and promoter hypermethylation or 10q23 LOH and promoter hypermethylation), which leads to the biallelic inactivation of the protein in 20–30% of cases.

PTEN expression and mutational rate was reported to be lower in left-sided (distal) CRC in comparison to right-sided (proximal) cancers (20–22). This finding may be related to different genetic mechanisms underlying the tumorigenesis of proximal and distal sporadic CRCs. Cancers arising in right colon are usually characterized by microsatellite instability (MSI), whereas those arising in the distal colon and in the rectum are very often characterized by chromosomal instability (CIN). Therefore, it can be argued that PTEN alterations may be linked to MSI and to the mechanisms leading to MSI (including high frequency of promoter hypermethylation, the main mechanism of mismatch repair genes silencing, whose absence of function is directly responsible of MSI). Consistent with this hypothesis, Day and colleagues found that *PTEN* mutations, identified in about 6% out of 744 stage I-IV CRC, were associated with mucinous histology, MSI, CpG island methylator phenotype, and *BRAF* mutations (22). Furthemore, other reports demonstrated a direct association between *PTEN* mutations and MSI, suggesting that the *PTEN* gene is a target of genomic instability in MSI colorectal tumorigenesis (23–25). In particular, Zhou and colleagues found that among 11 HNPCC CRC, 32 MSI sporadic cancer, and 39 microsatellite stable tumors, *PTEN* somatic mutations were found in 18, 13, and 0% of cases respectively, and PTEN loss of expression (evaluated by IHC) in 31, 41, and 17%, respectively. The majority of somatic mutations occur in the two 6(A) coding mononucleotide tracts, suggesting an etiological role of the deficient mismatch repair system (25). Moreover, it was also reported that *PTEN* promoter hypermethylation is a frequent event in sporadic CRC with MSI and may represent an important epigenetic mechanism of PTEN inactivation in this setting (26).

Overall, although another study did not confirm this association (because gene mutations and LOH were found in about 20 and 17% of sporadic CRC respectively, all but one of which were microsatellite stable) (27), we can assume that PTEN alterations and MSI are correlated.

In addition to PTEN level, the PI3K pathway can be altered following mutations in genes encoding for PI3K proteins, typically in *PIK3CA* gene. Therefore, it has been proposed that PTEN alterations and *PIK3CA* mutations may be mutually exclusive. However, this concept has not been deeply demonstrated as few studies investigating this topic showed conflicting results. There is in fact a clear evidence that mutations in multiple components of the PI3K pathway are not necessarily redundant. Although activating mutations in *PI3K* and loss of PTEN function both enhance PI3K signaling, these alterations seem not to cover equivalent functions. For example, in endometrial cancer, mutations in *PTEN* and *PIK3CA* both occur frequently and often concomitantly within the same tumor, indicating a potential additive or synergistic effect (28–30).

As for the other genetic alterations mainly occurring in CRC, it has been demonstrated that loss of PTEN expression measured by IHC co-occurs with *KRAS* and *BRAF* mutations and with EGFR polysomy (31), whereas *PTEN* and *TP53* mutations seem to be mutually exclusive (27).

PROGNOSTIC ROLE OF PTEN

The role of PTEN as a prognostic factor in CRC has been addressed by relatively few works.

Although accumulating evidence has strongly suggested that PTEN is a crucial factor in various central processes of cancer development, and although in several tumor types (e.g., non-small-cell lung cancer, prostate and breast cancer) PTEN protein status has been correlated with poor prognosis, the association between PTEN expression and clinical parameters in CRC is still controversial. The studies reporting the clinical impact of PTEN alterations on patient outcome in CRC are here summarized. Several of these studies suggest an association between loss of PTEN protein expression with advanced disease, liver metastasis, and poor patient survival, whereas other works do not find such an association (**Tables 1** and **2**).

One of the first paper reporting an association between PTEN alteration and tumor aggressiveness was published in 2001 and examined *PTEN* somatic mutations in a series of 36 sporadic CRC. The authors found that *PTEN* gene mutations were detected only in patients with locally advanced or metastatic CRC (32).

The majority of the next studies have been performed by analyzing PTEN protein expression by IHC assay, the most effective way to assess the loss of PTEN function by any mechanism (LOH, somatic mutation, or promoter epigenetic silencing). In fact, it has been reported that all tumors with *PTEN* gene alterations (mutation and/or deletion) showed a reduction or absence of PTEN expression evaluated by IHC, and this finding was correlated with advanced stage of disease (33). This association was confirmed by Sawai and colleagues, who demonstrated that PTEN loss was significantly correlated with local recurrence, advanced TNM stage ($p < 0.01$), lymph node metastasis ($p < 0.05$) and with lower 5-year survival rate ($p = 0.012$), indicating a link between PTEN deregulation and CRC aggressive phenotype (34). A positive association of PTEN expression with histological grade and distant metastasis was also demonstrated by Lin and colleagues (35). Similarly, Li and co-workers, by examining nuclear PTEN protein expression on tissue microarray in 327 CRC, found that low level of PTEN protein expression was positively correlated with tumor size, depth of invasion, lymphatic invasion, lymph node metastasis, and higher tumor staging ($p < 0.05$).

Table 1 | List of papers finding a positive correlation between PTEN loss and prognosis.

Author	No.	Type of tissue	Method	% PTEN alteration
Dicuonzo et al. (32)	36	Frozen CRC	Sequencing	17% mutations
Nassif et al. (33)	41	Frozen normal tissue and CRC	Sequencing, LOH, IHC	19% mutations 17% LOH 70% reduction or loss of expression (IHC) (cytoplasm and nuclear staining)
Sawai et al. (34)	69 with liver metastasis; 70 without liver metastasis	FFPE CRC and liver metastasis	IHC	75.4% weak expression (cytoplasm and nuclear staining)
Lin et al. (35)	139	FFPE TMA CRC	IHC	7% weak or loss expression (cytoplasm staining)
Li et al. (36)	327	FFPE TMA CRC	Sequencing, IHC	29% weak or loss of expression (PTEN immunoreactivity localized in the nucleus)
Jang et al. (37)	482	FFPE TMA CRC	IHC	50% loss of expression
Jin et al. (38)	68	FFPE CRC	IHC	67.6% loss of expression (cytoplasm and nuclear staining)
Atreya et al. (39)	56	FFPE mCRC	IHC	12.3% loss of expression (cytoplasm and nuclear staining)
Bohn et al. (40)	307	FFPE TMA CRC	FISH	8.8% gene loss

No.: number of patients; CRC: colorectal cancer; FFPE: formalin-fixed paraffin embedded; FISH: fluorescent in situ hybridization; IHC: immunohistochemistry; LOH: loss of heterozygosity; mCRC: metastatic colorectal cancer; TMA: tissue microarray.

Table 2 | List of papers finding no correlation between PTEN loss and prognosis.

Author	No.	Type of tissue	Method	% PTEN alteration
Colakoglu et al. (21)	76	FFPE CRC	IHC	5% loss of expression; 67% weakly moderate positive expression (cytoplasm staining)
Eklöf et al. (41)	197 and 414*	FFPE CRC	IHC	12.5 and 14% loss of expression (cytoplasm staining)
Price et al. (42)	302	FFPE advanced CRC	Taqman copy number assay	38.7% loss
Day et al. (22)	1093	FFPE stage I-IV CRC	Sequencing	5.8% mutations

**Separate cohort; No.: number of patients; CRC: colorectal cancer; FFPE: formalin-fixed paraffin embedded; IHC: immunohistochemistry.*

In addition, univariate and multivariate analysis indicated that patients characterized by PTEN loss of protein expression had a shorter survival than patients with a normal expression of PTEN (36).

Another study performed on 482 CRC revealed that PTEN protein expression (evaluated again on a tissue microarray) was associated with poor overall survival (OS) and disease-free survival ($p = 0.03$ and $p = 0.046$, respectively), although in multivariate analysis, a significant difference was observed only in patients with stage II of disease (37).

Jin and colleagues by evaluating the prognostic value of PTEN, STAT3, and VEGF-C protein expression by IHC in 68 cases of CRC, showed that PTEN expression was correlated with pathological grade, but not with tumor size, lymph node metastasis, or clinical stage. Moreover the 3- and 5-years survival rates of patients normally expressing PTEN were significantly higher than those of patients with a PTEN-negative tumor (38).

In a very recent study conducted on 56 patients affected by a metastatic disease, PTEN protein expression was analyzed by an optimized PTEN IHC assay recently developed and it was found that the median OS of patients whose tumors did not express PTEN was 9 months, compared to 49 months for patients with a normal expression of PTEN [HR = 6.25, 95% confidence intervals (CI), $p = 0.0023$]. The association of absence of PTEN expression with increased risk of death remained significant in multivariate analysis (Hazard Ratio, HR = 6.31, 95% CI, $p = 0.0023$) (39).

Finally, the positive correlation between worse prognosis and PTEN alteration was also found after the analysis of genetic lesions. Through the evaluation of *PTEN* deletion and gene rearrangements by FISH on 307 CRC, the authors confirmed an association

between *PTEN* alteration with reduced patient survival in univariate and multivariate analyses in rectal cancer ($p = 0.012$, HR 2.675; 95% CI) but not in colon cancer (40).

On the contrary with respect to the results obtained by the studies reported above, Colakoglu and colleagues, by investigating 76 CRC patients, found no correlation between PTEN immunohistochemical status and patient survival, tumor grade, TNM stage, lymphatic invasion, and liver metastasis (21), although they found a significant association between PTEN loss and local recurrence. Another study investigating the prognostic role of *KRAS*, *BRAF*, *PIK3CA* mutations, and PTEN expression in two separate CRC cohorts of 197 and 414 patients respectively, observed absence of correlation between PTEN status and prognosis by analyzing each molecular marker separately (41). The prognostic value of PTEN was also explored through the evaluation of *PTEN* gene copy number alteration (CNA) assessed by a Taqman assay by Price and colleagues in a cohort of 302 patients with advanced CRC enrolled in the AGITG MAX trial, a randomized Phase III trial of capecitabine ± bevacizumab or mitomycin C. The authors did not find any correlation between *PTEN* status and progression free survival (PFS) or OS in multivariate analysis (42). The absence of association with prognosis in stage II and III CRC was also supported by the work of Day and colleagues who analyzed *PTEN* mutations in a large cohort of sporadic CRC (22).

In conclusion, at the moment there are no clinical data clearly supporting the notion of PTEN alteration as a prognostic factor in CRC.

PREDICTIVE ROLE OF PTEN IN EGFR-TARGETED THERAPIES RESPONSE

In addition to the evaluation of the prognostic role of PTEN, several studies have investigated its predictive role in the field of targeted therapies. Since in breast cancer patients it has been demonstrated that PTEN loss of expression confers resistance to trastuzumab (a monoclonal antibody, MoAb, against Her-2, a tyrosine kinase receptor belonging to the Her family, as EGFR) (43), recent reports have investigated whether PTEN alterations may affect responsiveness of mCRC patients to anti-EGFR MoAbs cetuximab and panitumumab (**Table 3**). These studies have primarily used IHC to assess expression at protein level, and some have shown a correlation between PTEN expression and clinical response. A preliminary work on a retrospective series of patients reported that loss of PTEN expression, observed in 40% of primary tumor in mCRC patients, was significantly associated with non-responsiveness to cetuximab (44). The authors found that no patients with PTEN loss of expression in tumor tissue responded to a combination of irinotecan and cetuximab, whereas 10 out of 16 (63%) patients with intact PTEN expression experienced a partial response to these therapies. *In vitro* studies have confirmed this evidence by showing that *PIK3CA* mutations or PTEN loss may predict the efficacy of cetuximab administration in colon cancer cell lines (45). The role of PTEN in predicting resistance to anti-EGFR MoAbs was confirmed by Sartore-Bianchi and colleagues, who found that loss of PTEN protein was associated with lack of response to cetuximab and panitumumab ($p = 0.001$) in a cohort of 81 tumor specimens. Loss of PTEN expression was also

Table 3 | List of papers investigating the predictive role of PTEN in CRC treated with EGFR-targeted therapies cetuximab or panitumumab.

Author	No.	Type of tissue	Method	% PTEN alteration and clinical response
Frattini et al. (44)	27	FFPE mCRC	IHC	100% PTEN-negative patients were NR ($p < 0.001$)
Sartore-Bianchi et al. (46)	81	FFPE mCRC	IHC	97% PTEN-negative patients were NR ($p = 0.001$)
Perrone et al. (47)	32	FFPE mCRC	Sequencing, FISH	All patients with a decreased *PTEN* gene copy number or with PTEN mutation were NR
Razis et al. (48)	72	FFPE mCRC	IHC and FISH	PTEN gene deletion detected only by FISH associated with no response
Loupakis et al. (49)	102	FFPE mCRC (primary and metastatic lesion)	IHC	95% PTEN-negative patients were NR. Association with clinical response found only in the metastatic lesion
Negri et al. (50)	50	FFPE mCRC (primary and metastatic lesion)	Immunofluorescence	100% PTEN-negative patients were NR ($p < 0.05$). Association with clinical response found only in the metastatic lesion
Tol et al. (51)	559	FFPE mCRC	IHC	Loss of PTEN expression observed in 42% but not associated with response
Ulivi et al. (52)	67	FFPE mCRC	IHC	Loss of PTEN expression observed in 60% but not associated with response
Laurent-Puig et al. (53)	162	FFPE mCRC	IHC	Loss of PTEN expression observed in 19% but not associated with response

No: number of patients; CRC: colorectal cancer; FFPE: formalin-fixed paraffin embedded; FISH: fluorescent in situ hybridization; IHC: immunohistochemistry; mCRC: metastatic colorectal cancer; NR: non-responder to anti-EGFR therapies.

associated with shorter PFS and worse OS (46). Supporting these data, another study showed that inactivation of PTEN protein by gene mutation or deletion (detected by FISH) was responsible of cetuximab resistance (47). Razis and colleagues, did not find any association between PTEN protein expression as evaluated by IHC with clinical outcomes, although the lack of *PTEN* gene amplification evaluated by FISH was associated with a better response rate and longer time to progression (48).

A substantial but not complete confirmation of these data has been reported by Loupakis and colleagues which demonstrated that loss of PTEN expression was not associated with resistance to cetuximab plus irinotecan in the primary tumor ($n = 96$), but was associated with lack of response in the metastatic lesion (the analysis was performed in 59 cases). In the PTEN-positive group, 12 out of 33 (36%) patients benefited from the therapy whereas only 1 patient out of 22 (5%) cases with a PTEN-negative profile responded to EGFR-targeted drugs ($p = 0.007$). Moreover, patients with PTEN-positive metastases and *KRAS* wild-type gene sequence had longer PFS compared with other patients (49). According to these data, Negri and colleagues evaluated PTEN expression by immunofluorescence both in primary and metastatic sites in CRC patients treated with cetuximab and they found that the loss of PTEN expression in metastatic sites was negatively associated with response (50).

On the contrary with respect to the previous works, other studies failed to demonstrate a correlation between loss of PTEN expression and response to anti-EGFR MoAbs. In a large cohort of 559 mCRC patients treated with chemotherapy and bevacizumab with or without cetuximab (phase III CAIRO2 study), the authors did not find any correlation between PTEN loss evaluated by IHC and response to treatment with cetuximab, neither individually nor in combination with other markers (51). This result was confirmed by Ulivi et al. by the analysis of 67 mCRC patients receiving cetuximab (52). Finally, the investigation of 162 samples by Laurent-Puig and colleagues reported the PTEN null expression rate of 19.9% with an association of poorer OS in the *KRAS* wild-type population ($p = 0.013$) but not with tumor response or PFS, thus suggesting the PTEN loss of expression as a prognostic rather than a predictive role (53).

CONCLUSION

According to the reported results, the role played by PTEN as a prognostic or predictive marker in CRC is still a matter of debate. Discordant results have been reported and this fact could be attributable to several issues: (1) the size of the analyzed cohort, (2) patients inclusion criteria, (3) the methods of assessing PTEN alteration. For the latter point, it should be noted that the majority of studies have evaluated PTEN alteration by IHC, the easier and cheaper method to be used. However, these studies showed highly discordant results, with PTEN loss ranging from 5% up to 66% (40). Reasons for this variability might include inherent issues with IHC. Interpreting PTEN data can be challenging, because immunohistochemistry can produce variable results. The lack of standardized methods and the variability of tissue handling may bias the PTEN expression analysis by IHC. In addition, IHC is afflicted by intra and inter-observer variability due to the semi-quantitative character of such an analysis. A standard, universally accepted PTEN testing and scoring system for PTEN IHC evaluation, has yet to be established. Assessment of PTEN expression is further complicated by potential discordance between the expression of PTEN in the primary and in the metastatic tissue. Concordance rates vary from 47 to 98% between primary and metastatic lesions (39, 49, 54–56). These differences may impair the prediction of anti-EGFR therapies outcome. Loupakis and colleagues reported in fact that PTEN loss was predictive of cetuximab resistance only by evaluating the metastatic lesion (49). Sangale and co-workers however, has recently developed an optimized PTEN IHC assay developed through a rigorous testing of antibody specificity and selectivity using samples with known molecular alterations in PTEN, paired with reproducible method of interpretation. The Authors found a 98% of concordance of PTEN expression between primary and metastatic tumors (57). Another issue that has recently emerged is the intracellular localization of PTEN protein. Some researchers demonstrated that PTEN is localized both in the cytoplasm and into the nucleus and shuttles between these two compartments can be influenced by a variety of mechanisms. Accumulating genetic, pathologic and biochemical evidence suggests that the localization of PTEN either in the nucleus or cytoplasm may affect the proliferation of tumor cells (58–60). Another point that could affect the establishment of the prognostic and predictive value of PTEN is haploinsufficiency, determined when only one allele is altered, as it remains unclear whether this condition could provide a selective growth advantage in tumors lacking a second hit in the remaining *PTEN* allele.

To clarify the problems concerning PTEN evaluation, it would be necessary a comparison of the results obtained by analyzing the several PTEN alterations through different methodologies (FISH, promoter methylation, LOH, and immunohistochemistry performed with different antibodies) both on cancer cell lines with a well known PTEN status and on a large series of patients in order to better establish IHC evaluation criteria. An international inter-laboratory reproducibility ring study [as that performed for EGFR FISH analysis in metastatic colorectal cancer (CRC) patients] (61) is missing and has to be performed in order to ascertain the difficulties and the discrepancies in PTEN evaluations in different laboratories.

In conclusion, much work, especially in large and homogeneous cohorts of cases from different laboratories, has to be done before the establishment of PTEN as prognostic or predictive marker in CRC. On the contrary, in other tumor types (such as breast cancer), this role is clearer.

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Toward a molecular classification of colorectal cancer: the role of BRAF

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Different genetic aberrations of *BRAF* have been reported in various malignancies. BRAF is member of the RAS/RAF/MEK/ERK pathway and constitutive activity of this pathway can lead to increased cellular growth, invasion, and metastasis. The most common activating *BRAF* mutation in colorectal cancer is the V600E mutation, which is present in 5–15% of all tumors, and up to 80% of tumors with high microsatellite instability (MSI) harbor this mutation. *BRAF* mutation is associated with proximal location, higher age, female gender, MSI-H, high grade, and mucinous histology, and is a marker of poor prognosis in colorectal cancer. The role of *BRAF* mutation as a predictive marker in respect of EGFR targeted treatments is controversial. *BRAF* V600 selective inhibitors have been approved for the treatment of V600 mutation positive metastatic melanoma, but the response rates in colorectal cancer are poor. This might be due to innate resistance mechanisms of colorectal cancers against the treatment solely targeting BRAF. To overcome resistance the combination of treatments, simultaneous inhibition of BRAF and MEK or PI3K/mTOR, might emerge as a successful therapeutic concept.

Keywords: BRAF, colorectal cancer, Lynch syndrome, microsatellite instability, V600E, V600K, vemurafenib, dabrafenib

INTRODUCTION

BRAF (v-raf murine sarcoma viral oncogene homolog B1) is a serine/threonine protein kinase of the RAF family. RAF proteins are kinases in RAS/RAF/MEK/ERK pathway. ARAF and CRAF are other family members of the RAF family, however, BRAF displays the best binding to RAS and has the highest phosphorylating activity (1). The RAS/RAF/MEK/ERK pathway usually responds to growth factors and cytokines. However, aberrant signaling of this pathway, for example by constantly active kinases can result in abnormal cellular growth, invasion, and metastasis (2).

BRAF is mutated at a high frequency in several cancers, although also amplification of the protein and aberrant splicing variants have been reported as well (1). The *BRAF* V600E mutation, deriving from a point mutation of the DNA (1799T → A) is the most common *BRAF* mutation and accounts for around 90% (3). *BRAF* V600E mutation is most prominent in melanoma (40–60%), papillary thyroid carcinoma (45%), low grade serous ovarian carcinoma (35%), and in colorectal adenocarcinoma (5–15%) (4). Other *BRAF* mutations include V600K and V600D/R, accounting for 16–29% and 3% of all *BRAF* mutations in melanoma, respectively (5, 6). Another activating *BRAF* mutation that is almost exclusively found in pilocytic astrocytomas is the *KIAA1549-BRAF* fusion, found in 66–100% of these tumors (7, 8).

Colorectal cancer development and progression can be divided into two separate pathways: chromosomal instability pathway and microsatellite instability (MSI) pathway. In roughly 75% of the cases, colorectal cancer develops through chromosomal instability pathway, and these tumors can harbor *APC* mutations (>90%), *KRAS* mutations (50%), *TP53* mutations (70%), and allelic loss of 18q (80%) (9). MSI pathway covers approximately 15% of sporadic colorectal cancers and almost all Lynch syndrome (LS) cases. In cancers developing through the MSI pathway the DNA mismatch repair (MMR) function is dysfunctional, which leads to insertions and/or deletions of nucleotide repeats in the DNA (9). Remaining tumors belong to CpG island methylator pathway (CIMP) and Serrated Adenoma Pathway, and approximately one third of CIMP tumors are MSI-H while most of the serrated tumors have a deficient *MLH1* gene due to promoter methylation.

DETECTION OF *BRAF* MUTATION IN COLORECTAL CANCER

Until recently the detection of *BRAF* mutations was performed with Sanger sequencing or PCR-based assays. These methods require representative amount of malignant cells and extraction of the DNA. For specimens with a low content of tumor tissue, the DNA based protocols thus might not be sensitive enough to detect the *BRAF* mutations. A recent report compared the detection of *BRAF* mutations between two next generation sequencing (NGS) technologies and Sanger sequencing/q-PCR and found NGS to be reliable in detecting *BRAF* mutations and other standard-of-care mutations (10). Immunohistochemical (IHC) detection of BRAF V600E with a mutation specific antibody (clone VE1) was first described in metastatic melanoma and papillary thyroid carcinoma (11), and the antibody is currently commercially available (**Figures 1A,B**). The advantage of IHC lies in the minimal amount of the needed tissue and the availability of this technique in most pathological laboratories. Colorectal cancer has been analyzed with the BRAF V600E mutation specific antibody and most studies find high sensitivities and specificities (98.8–100%) in comparison

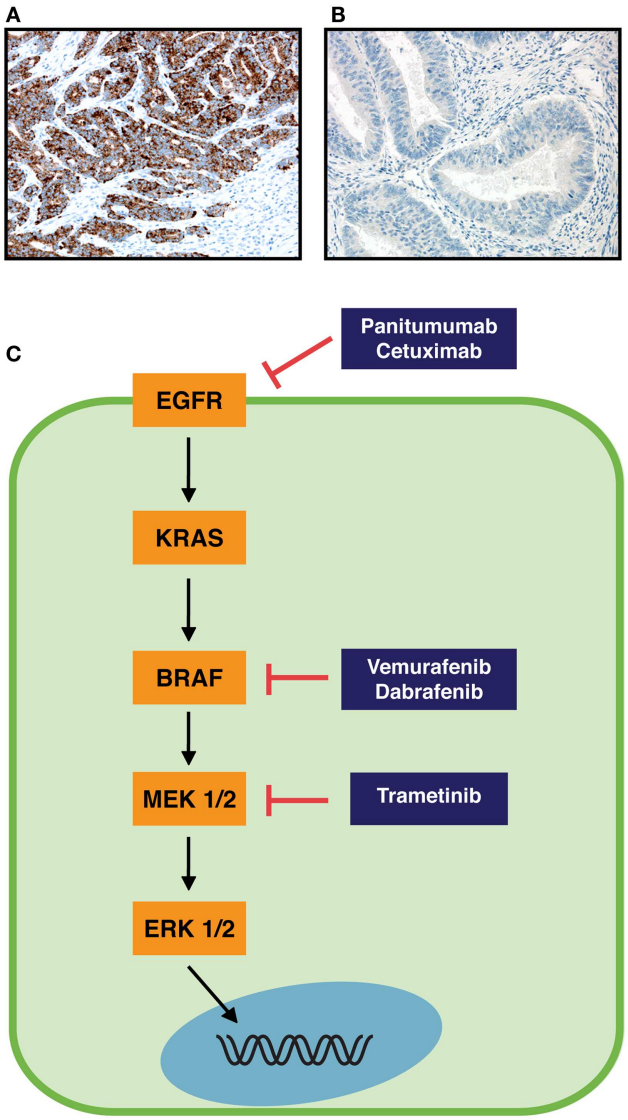

FIGURE 1 | RAS-RAF pathway and immunohistochemical staining of colorectal cancer specimens with BRAF V600E mutation specific monoclonal antibody. **(A)** Strong immunopositivity in cancer cells with a BRAF V600E mutation. **(B)** No staining of cancer cells in a specimen without BRAF V600E mutation. Original magnifications are 200×. **(C)** Schematic RAS-RAF pathway (orange boxes) and inhibitors of components of this pathway (blue boxes). Arrows indicate an activation process, and blocked arrows an inhibition process.

with PCR-based methods or sequencing (12–16). In one study however, the sensitivity and specificity were only 71 and 74%, respectively (17). The choice of the positive control tissue and the amplification protocol seem to be crucial in successful detection of BRAF V600E mutation by IHC (16).

OCCURRENCE OF *BRAF* MUTATION IN COLORECTAL CANCER

The frequency of *BRAF* V600E mutation differs in tumors with high MSI (MSI-H) compared to tumors that are microsatellite-stable (MSS). Whereas *BRAF* V600E mutation frequencies below 10% are reported for MSS tumors (3, 15, 16, 18), they range from 13 to 78% in MSI-H tumors, including cases with germ line mutation for one of the MMR genes (12, 15, 16, 18). In our consecutive colorectal cancer material *BRAF* V600E mutation was found in 78% of MSI-H and 8% of MSS tumors ($p < 0.0001$) (16). A recent study reported *BRAF* V600E mutation in 100% of sessile serrated adenomas/polyps, 94% of traditional serrated adenoma, and in 62% of micro vesicular hyperplastic polyps (19). *BRAF* V600E mutation in microvesicular hyperplastic polyps might indicate the polyps that have a higher risk for progression to adenomas/adenocarcinomas (19). The *BRAF* V600K mutation seems to be a rare event in colorectal cancer, at least in MSI-H tumors (16).

SIGNIFICANCE OF BRAF MUTATION IN COLORECTAL CANCER

CONNECTION TO CLINICOPATHOLOGICAL PARAMETERS

BRAF V600E mutations are associated with several clinicopathological parameters and the ones most often reported are: proximal location, higher age, female gender, MSI-H, high grade, and mucinous histology (16, 20–26). Whereas in most studies colorectal cancers are classified into proximal and distal location, Yamauchi et al. described a gradual linear increase of *BRAF* mutation, MSI-H, and high CpG island methylator phenotype frequency from rectum to ascending colon (27). The frequencies of all three factors were lower in cecum than in ascending colon, indicating that cecal cancers are a unique subtype (27).

High microsatellite instability is associated with a higher number of harvested lymph nodes (28, 29), and a recent study reported that *BRAF* V600E mutation was associated with a lower node harvest in the MSI-H group in colon cancer (30). The lymph node count is a predictor of long-term survival in colorectal cancer. Rather than just reflecting the quality of care, the lymph node count might be associated with several factors such as tumor location, tumor and host genetics, and immune interaction (30).

PROGNOSTIC ROLE

BRAF V600E mutation is associated with reduced survival (overall survival, disease-free survival, or cancer-specific survival) especially in MSS tumors (**Table 1**) (18, 21, 23, 24, 26, 31, 32). Its role in MSI-H tumors is not so clearly defined; while some studies attribute MSI-H tumors with excellent survival regardless of *BRAF* status (18), *BRAF* V600E mutation decreased overall survival independent of MSI status in another report (25). In addition, *BRAF* V600E mutation was associated with poor prognosis in all groups of advanced colorectal cancer (33) and was an independent prognostic factor for overall survival and cancer-specific survival in a pooled stage II/III cohort (22). In a couple of studies, no prognostic role was found to be associated with *BRAF* mutation (**Table 1**) (34, 35). Finally, in a meta-analysis that included 26 colorectal cancer studies, *BRAF* mutation was found to increase the risk of mortality (HR = 2.25, 95% CI: 1.82–2.83) (36).

PREDICTIVE ROLE

It has been suggested that in order for metastatic colorectal cancer patients to receive a response for treatment with monoclonal antibodies targeting EGFR (panitumumab and cetuximab, **Figure 1C**), the *BRAF* gene needs to be present as wild-type (37, 38). Yuan et al. recently concluded in a meta-analysis that *BRAF*

Table 1 | *BRAF* mutation as prognostic factor in colorectal cancer.

BRAF mutation as prognostic factor	Tested for *BRAF* mutation (*BRAF* mutated)	Comments	Reference
Independent	911 (87)	Stage II-IV, microsatellite-stable tumors, age, stage, tumor site, and CpG island methylator phenotype adjusted, reduced OS, HR = 3.06, 95% CI: 2.06–4.54; (1.0 reference *BRAF* wt)	Samowitz et al. (18)
Independent	1307 (103)	Stage II/III, reduced OS, HR = 1.78, 95% CI: 1.15–2.76; (1.0 reference *BRAF* wt)	Roth et al. (21)
Independent	297 (59)	Stage II/III, reduced OS, HR = 0.45, 95% CI: 0.25–0.8, and reduced cancer-specific survival, HR = 0.47, 95% CI: 0.22–0.99; (1.0 reference *BRAF* mut)	Farina-Sarasqueta et al. (22)
Independent	506 (75)	Stage III, reduced OS, HR = 1.66; 95% CI: 1.05–2.63; (1.0 reference *BRAF* wt)	Ogino et al. (23)
Independent	475 (56)	Stage I-III, reduced OS, HR = 1.79, 95% CI: 1.05–3.05; (1.0 reference *BRAF* wt)	Kalady et al. (25)
Independent	196 (35)	Stage I-IV, reduced cancer-specific survival, HR = 2.00, 95% CI: 1.16–3.43; (1.0 reference *BRAF* wt)	Eklöf et al. (26)
Independent	1253 (182)	Stage I-IV, higher cancer-specific mortality in microsatellite-stable tumors, HR = 1.60, 95% CI: 1.12–2.28; (1.0 reference MSS/*BRAF* wt)	Lochhead et al. (32)
Non-independent	711 (56)	Advanced CRC, reduced OS, HR = 1.82; 95% CI: 1.36–2.43; (1.0 reference *BRAF* wt)	Richman et al. (33)
Non-independent	181 (20)	Stage I-IV, proficient DNA mismatch repair, stage-adjusted reduced OS and DSF, HR = 6.63, 95% CI: 2.60–16.94 and HR = 6.08, 95% CI: 2.11–17.56; (1.0 reference *KRAS/BRAF* wt)	Pai et al. (24)
Non-independent	243 (18)	Metastatic CRC, reduced PSF, HR = 2.39, 95% CI: 1.36–4.21; (1.0 reference *KRAS/BRAF* wt)	Peeters et al. (31)
No prognostic significance	490 (77)	Stage II/III, no effect on DFS, HR = 1.0, 95% CI: 0.6–1.6; no effect on OS, HR = 1.2, 95% CI: 0.8–1.8; (1.0 reference *BRAF* wt)	French et al. (34)
No prognostic significance	822 (10%)	Stage II/III, no effect on DFS, HR = 1.07, 95% CI: 0.66–1.73; (1.0 reference *BRAF* wt)	Mouradov et al. (35)

CRC, colorectal cancer; DSF, disease-free survival; mut, mutant; OS, overall survival; PFS, progression-free survival; wt, wild-type.

mutation is a predictive biomarker and indicates poor prognosis when metastatic colorectal cancer patients are treated with monoclonal antibodies against EGFR (39). In contrast to these results, a recent guideline does not recommend testing for *BRAF* mutations in colorectal cancer patients before anti-EGFR treatment (40). Garcia-Alfonso et al. (40) conclude that *BRAF* mutation is not predictive for anti-EGFR treatment in randomized trials. For patients (*KRAS* wild-type metastatic colorectal tumors) treated with chemotherapy/bevacizumab with or without cetuximab in the phase III CAIRO2 study, *BRAF* mutation was correlated to a shorter progression-free survival and overall survival, in both treatment arms (41). Similarly, *BRAF* mutation was not predictive for treatment with cetuximab, but was a marker of poor prognosis in metastatic colorectal cancer patients (*KRAS* wild-type) that were randomly assigned to treatment with FOLFIRI (irinotecan, fluorouracil, leucovorin) with or without cetuximab in the CRYSTAL study (42). The pooled analysis of the CRYSTAL and OPUS trials on metastatic colorectal cancer showed that *BRAF* mutation was not predictive for treatment with cetuximab in *KRAS* wild-type patients, but indicated poor prognosis (43). Finally, in a retrospective analysis of the PRIME study, *BRAF* mutation was not predictive for overall or progression-free survival in *KRAS* wild-type patients treated with FOLFOX4 (oxaliplatin, fluorouracil, leucovorin) with or without panitumumab (44).

As for treatment with standard chemotherapy agents (fluorouracil with irinotecan or oxaliplatin), *BRAF* V600E mutation was not predictive (33). Similarly, *BRAF* mutation was not predictive for fluorouracil-based therapy in mostly stage II colorectal cancer (45). A non-significant trend for better survival with fluorouracil/leucovorin + irinotecan (vs. fluorouracil/leucovorin alone) was detected in colorectal cancer stage III patients with *BRAF* V600E mutation (23).

ROLE IN IDENTIFYING LS PATIENTS

Lynch syndrome is a hereditary form of colorectal cancer that accounts for 1–3% of all CRC cases. It is the most common form of hereditary CRC and is caused by a germ line mutation of one of the MMR genes (46). As not all LS patients fulfill the Amsterdam II criteria or revised Bethesda guidelines, not all of them are detected in the routine clinical setting (47, 48). *BRAF* is usually present as wild-type in LS patients, and only 1.4% of the LS patients carry a *BRAF* V600E mutation (49). In sporadic colorectal cancer the

BRAF V600E mutation rate ranges from 5 to 15% (4), and in the MSI-H group of consecutive primary colorectal cancers the *BRAF* V600E mutation rate reached 78% (16). This has led to the suggestion that the detection of *BRAF* V600E mutation might be a useful additional tool in finding LS patients, and several recent studies have used *BRAF* V600E IHC to implement this step (12, 15, 16).

BRAF INHIBITORS IN TREATMENT OF CANCER

The first RAF inhibitor, sorafenib, was not effective in clinical use for metastatic melanoma, as it did not improve median overall survival in randomized, double-blind, placebo-controlled phase III studies, when given in combination with paclitaxel and carboplatin as second-line treatment or to chemotherapy-naïve patients (50–52). The reason for the disappointing results with sorafenib in melanoma might be that this multi-targeted tyrosine kinase inhibitor has a higher affinity for isoforms other than BRAF and targets several other pathways as well (50, 53). However, in advanced hepatocellular carcinoma, the median survival time was increased by nearly 3 month in patients treated with sorafenib, in a phase III, double-blind, placebo-controlled trial (54). Vemurafenib (PLX4032) and dabrafenib (GSK2118436) are approved for treatment of unresectable or metastatic melanoma (Food and Drug Administration) and vemurafenib is also approved by the European commission/European Medicines Agency. Both selectively inhibit the *BRAF* V600 mutated form of BRAF, inhibit phosphorylation of ERK, and have high clinical response rates in melanoma patients (**Figure 1C**) (50, 53). Whereas patients with *BRAF* V600 mutated melanomas had a clear survival benefit when treated with BRAF inhibitors, the response rate in metastatic colorectal carcinoma (harboring *BRAF* V600E mutation) was poor, since only one patient (1/19) displayed a partial response and 4 out of 19 patients a minor response (55, 56). It has been noted already in xenografts from BRAF V600E mutant colorectal cancer cell lines that tumor growth inhibition was most efficient when vemurafenib was combined with EGRF or Akt inhibitors and/or chemotherapeutic agents (57).

RESISTANCE TO BRAF INHIBITION IN MELANOMA AND COLORECTAL CANCER

BRAF V600E mutant melanomas initially have a good response rate. However, most of them acquire a drug resistance after 6–7 months, and roughly 10% have tumor progression at earlier stages (53, 55). *BRAF* V600E mutated colorectal cancer on the contrary, seems to display an innate resistance to inhibition with BRAF inhibitors, which was also demonstrated in colorectal cancer cell lines (55, 58, 59). The mechanisms of resistance can be grouped according to their dependence on ERK signaling (60). ERK-dependent resistance mechanisms can occur via activating *MEK1* mutations (61), activating *NRAS* mutations (62), *COT* overexpression (63), elevated CRAS activity (64), *BRAF* V600E alternative splicing or amplification (65). ERK-independent mechanisms include the PI3K pathway (66), overexpression of PDGFRβ (62), IGF1R activation (67), and hepatocyte growth factor (59). Importantly, Romano et al. report that different mechanisms of resistance can occur in the same patient at different metastatic locations (68).

In *BRAF* V600E mutant colorectal cancer cells the amplification of the *BRAF* gene was identified as mechanism of resistance to MEK and BRAF inhibition (69). Two studies detected the critical role of EGFR in *BRAF* V600E mutant colorectal cancer cells that did not respond to BRAF inhibition (58, 70). Corocan et al. reported that *BRAF* V600E mutant colorectal cancer cell lines harbored more phospho-EGRF than melanomas with the same mutation, and reactivated MAPK signaling via EGFR (58). Prahallad et al. described a rapid feedback activation of EGFR (via CDC25C inhibition) upon RAF inhibition, and EGFR was highly expressed *BRAF* V600E mutant colorectal cancer cells as compared to *BRAF* V600E mutant melanoma cells (70).

OVERCOMING OF RESISTANCE AND COMBINATION TREATMENTS

To overcome resistances upon treatment with a BRAF inhibitor, targeting novel downstream kinases of the pathway or combination of therapies might be helpful. As for melanoma treatment, the combination of vemurafenib with the HDM2 inhibitor nutlin-3 (leading to p53 restoration), has shown synergistic effect on inducing apoptosis and suppressing tumor growth in melanoma cell lines and xenografts (71). Novel combinatorial treatment options include BRAF inhibition simultaneously with PI3K/mTOR as shown in colorectal cell lines and animal models (72–74). Coffee et al. used the *Apc-Braf* mouse model (mice bearing a *BrafV600E* allele) and showed that concomitant inhibition of PI3K/mTOR and BRAF resulted in tumor regression due to induction of apoptosis and decrease in proliferation (73). Also Rad et al. reported the potent growth inhibitory effect of combined BRAF/PI3K inhibition on xenografts of *BRAF* mutant mouse and human colorectal cancer cell lines (74). Furthermore, MEK inhibition alone caused regression of xenografted and orthotopically transplanted tumors, and reduced proliferation in tumors of $Braf^{LSL\text{-}V637E/+}$ mice (orthologous to human *BRAF* V600E mutation) (74). A combined inhibition of BRAF (dabrafenib 150 mg) and MEK1/2 (trametinib, 1 or 2 mg) was performed in metastatic melanoma patients with *BRAF* V600E mutation, in a open-label phase II study with randomly assigned patients. Both median progression-free survival (9.4 vs. 5.8 months) and complete/partial response (76 vs. 54%) were significantly improved in the combination group (150 + 2 mg) vs. dabrafenib immunotherapy (75). Both dabrafenib and trametinib, were recently (May 2013) approved by the Food and Drug Administration for treatment of metastatic/inoperable melanoma (**Figure 1C**).

CONCLUSION

BRAF V600E mutation is a marker of poor prognosis in colorectal cancer. Detection of this mutation can also be used to identify LS patients. Targeted treatment of *BRAF* V600E mutation is in use in advanced melanoma. However, the response is short-lived in melanoma patients, due to the development of acquired resistance. In colorectal cancer targeted treatment of mutated *BRAF* is not feasible due to the innate resistance. New insights into possible resistance mechanisms were reported recently, and combinatorial treatment options might impact therapy of tumors carrying a *BRAF* mutation.

AUTHORS' NOTE

After acceptance of this review, a novel study reported the combined use of BRAF V600E and MMR immunohistochemistry as a prognostic tool in colorectal cancer (Toon CW, Chou A, DeSilva

K, Chan J, Patterson J, Clarkson A, et al. BRAFV600E immunohistochemistry in conjunction with mismatch repair status predicts survival in patients with colorectal cancer. Modern Pathol (2013) Oct 25. doi:10.1038/modpathol.2013.200). The authors restricted their analysis to only immunohistochemistry of BRAF V600E and MMR status on 1426 consecutive colorectal cancer cases, and found that MSS/BRAF V600E mutant tumor status was a marker for poor prognosis in univariate analysis when compared to MSS/BRAF wild type tumors (HR = 1.79, 95% CI: 1.24–2.60). Immunohistochemical screening for BRAF V600E mutation and MMR gene expression thus can facilitate the detection of Lynch syndrome patients and can also identify subgroups with a poor prognosis.

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Toward a molecular classification of colorectal cancer: the role of microsatellite instability status

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Microsatellite instability (MSI) is the molecular hallmark of DNA mismatch repair deficiency. Since its initial description in colorectal cancer (CRC) in 1993 and its association with Lynch syndrome, the most common inherited cancer predisposition world-wide, accumulating evidence suggests that MSI status may also be of concrete prognostic and predictive value in the management of sporadic CRC. This mini review aims at providing a concise survey of the molecular basis and the multifaceted role(s) of MSI status in today's clinical practice.

Keywords: microsatellite instability, mismatch repair, Lynch syndrome, hereditary non-polyposis colon cancer, colorectal cancer, therapy, prognosis

INTRODUCTION

Twenty years ago, in 1993, in search for molecular clues to the pathogenesis of colorectal cancer (CRC) several research groups had made an intriguing observation: widespread somatic alterations at short, repetitive DNA sequences, referred to as replication error (RER+) phenotype or, more specifically, microsatellite instability (MSI) (1–3). They detected this novel form of genomic instability in about 10–15% of sporadic, predominantly proximally located, colorectal carcinomas as well as in most (>90%) of those from patients with the most common inherited cancer predisposition, Lynch syndrome (LS), also referred to as Hereditary Non-Polyposis Colon Cancer (HNPCC). The discovery allowed to link the hereditary disorder to a defect in a key DNA metabolic pathway, the mismatch repair (MMR) system, which had previously been known to cause MSI in *Saccharomyces cerevisiae*, and eventually opened up a new field in cancer research (4).

THE MOLECULAR BASIS OF MSI

Microsatellites, also referred to as short tandem repeats (STRs), consist of a few to several thousands of tandemly repeated motifs made up of one (mono) up to six (hexa) nucleotides and are thought to account for approximately 3% of the human genome (5). Given their number and the fact that they are scattered throughout the genome, their (hyper)variability in length coupled with a high degree of heterozygosity made them ideal polymorphic markers for genome mapping, population genetics, and genetic linkage studies as well as indispensable tools in forensics and transplantation medicine ("DNA fingerprint").

Due to their repetitive sequence structure, it is not surprising that microsatellites exhibit a particularly high mutation rate compared to non-repetitive, unique DNA stretches. During replication of the nuclear genome DNA polymerases, due to slippage, often fail to correctly duplicate such microsatellite tracts. The resulting insertion/deletion loops can consequently lead to insertions or deletions of repeats, thereby altering the length of the respective microsatellite (replication error, RER). In eukaryotes these errors are corrected by the DNA MMR system: its heterodimers MSH2/MSH6 and MSH2/MSH3 detect the replication error (licensing step) and recruit the MLH1/PMS2 complex through which degradation of the mutated stretch and resynthesis are initiated (4). As originally observed in yeast, defects in MMR consequently result in the genome-wide accumulation of mutations at microsatellite loci, MSI, which has also been referred to as a "mutator phenotype" (6).

ASSESSMENT OF MSI

Since MSI analysis of colorectal tumors provided a straightforward, though indirect, means to identify LS patients great attention was given to the development of selection criteria and microsatellite markers to be used for testing. In 1996 and 2002 the National Cancer Institute in Bethesda, MD, held workshops in which the Bethesda guidelines for the identification of LS patients were defined (7, 8). MSI testing is recommended in patients who meet one of the following criteria: (a) diagnosed with CRC before age 50 years, (b) synchronous or metachronous CRC or other LS-related tumors, (c) CRC with typical MSI-high morphology and diagnosed before age 60 years, (d) CRC in one or more first-degree relatives with CRC or other LS-related tumors, one being diagnosed before age 50 years, and (e) CRC with two or more relatives with CRC or other LS-related tumors, regardless of age. The revised Bethesda guidelines thus incorporate personal as well as family history and pay attention to the fact that LS actually comprises a spectrum of different tumor types (endometrial, gastric, etc.).

To assess the presence of MSI in a given tumor the NCI workshop recommended to analyze a panel of five microsatellites including two mono- (BAT25, BAT26) and three dinucleotide markers (D2S123, D5S346, and D17S250); depending

on the mutational pattern, a secondary panel including additional mononucleotide (e.g., BAT40) and/or complex microsatellites (e.g., MYCL) should be tested (8). Alternatively, a panel of five quasimonomorphic mononucleotide repeats can be used which display even better sensitivity and which may obviate the need for normal tissue for comparison (9). Depending on the number of microsatellite markers displaying novel alleles, MSI can subsequently be rated as MSI-high (MSI-H, >2 out of 5 markers), MSI-low (MSI-L, 1 out of 5), or microsatellite stable (MSS, 0 out of 5).

The molecular dissection of CRCs into MSI-H and MSS tumors allowed to delineate two major, virtually exclusive pathways of genetic instability: chromosomal instability, which results in aneuploidy and is present in about 85% of CRC, and MSI. Whether MSI-L CRC constitute a pathogenetic class of their own is still a matter of debate: with regard to clinical, biological, and morphological parameters they closely resemble those of MSS CRC. Since the analytical sensitivity to detect MSI-L is dependent on the number of microsatellite markers analyzed, result interpretation, and comparison between different studies investigating MSI-L and MSS tumors are heavily compromised; furthermore, extensive genotyping efforts have failed to demonstrate fundamental differences (10, 11). Given these unresolved issues, a molecular subdivision into MSI-L and MSS CRC does currently not seem appropriate.

ROLE OF MSI IN LYNCH SYNDROME

The discovery of MSI in the majority of LS-related CRC led by analogy to a similar biochemical defect in yeast to the identification of the underlying MMR germ line mutations in MLH1, MSH2, MSH6, and PMS2. Heterozygous carriers of a MMR gene alteration are at a greatly increased lifetime risk to develop cancers of the LS tumor spectrum, mainly CRC (25–70%) and endometrial cancer (30–70%) (12). Knowledge of the underlying germ line mutation not only allows life-saving intensive-cancer surveillance but also gives asymptomatic family members the opportunity to clarify their carrier status; due to autosomal dominant inheritance offspring of a LS patient has an *a priori* chance of 50% of having inherited the pathogenic MMR mutation.

In contrast to their sporadic MSS CRC LS patients' tumors typically have a comparatively favorable prognosis and absence of distant organ metastasis; together with the observation that LS cancers are accompanied by an intense immune response with dense lymphocytic infiltrates points to a possible protective effect by the immune system (13).

The revised Bethesda guidelines are probably the most commonly applied criteria to identify individuals with LS, yet many physicians dealing with familial CRC have the impression that LS *per se* remains underdiagnosed (14). An expert group therefore recently suggested to screen all individuals with CRC or endometrial cancer below age 70 by immunohistochemistry or MSI, both of which having similar clinical sensitivity and specificity. In order to discriminate between a hereditary (about 2–3% of all CRC) or a sporadic event (about 15%), tumors with immunohistochemical loss of MLH1 should then be further investigated for MLH1 promoter hypermethylation and targeted BRAF (V600E hotspot mutation) testing to decide on further (germ line) genetic testing. With high-throughput sequencing techniques entering routine genetic testing it is likely that these diagnostic screening algorithms will considerably change in the foreseeable future (15).

ROLE OF MSI IN SPORADIC CANCER

Since their initial description it became evident that the 15% of sporadic MSI-H CRCs exhibit a distinct clinico-pathological profile, which they largely share with LS-related CRC and which distinguishes them from their MSS counterparts (**Figure 1**). Already in the seminal work by Thibodeau et al. (2) MSI-H CRC were predominantly located in the proximal colon and associated with increased patient survival and prognosis (2). Most of them were later found to exhibit loss of MLH1 protein expression which could be attributed to epigenetic silencing of the respective promoter, later also referred to as "CpG island methylator phenotype" since it often occurs in the context of global hypermethylation (16, 17). Regarding their molecular-histopathological profile MSI-H CRC display a diploid state, tend to be poorly differentiated, mucinous, and show prominent lymphocytic infiltration (15).

A recent meta-analysis by Guastadisegni et al. who pooled data from 31 studies reporting survival in 12782 patients confirmed the initial observations between MSI-H status and a more favorable prognosis: patients with stages I-IV MSI-H CRC appeared to have a statistically significantly better outcome in terms of overall survival, disease-specific as well as disease-free survival (18). Moreover, results from a recent Norwegian study have shown that MSI status had an independent positive prognostic impact on stage II CRC patients after complete resection (19). How these findings and the inclusion of additional molecular markers may eventually impact on routine clinical and surgical practice, however, remains to be seen.

Guastadisegni et al. also investigated the effect of standard, 5-fluoro-uracil (5-FU) based chemotherapy. In the context of MSI and an underlying (hereditary or sporadic) defect in MMR the use of 5-FU in MSI-H CRC merits particular attention: as demonstrated in numerous *in vitro* studies, inactivation of the MMR system can result in resistance, or rather tolerance (i.e., failure

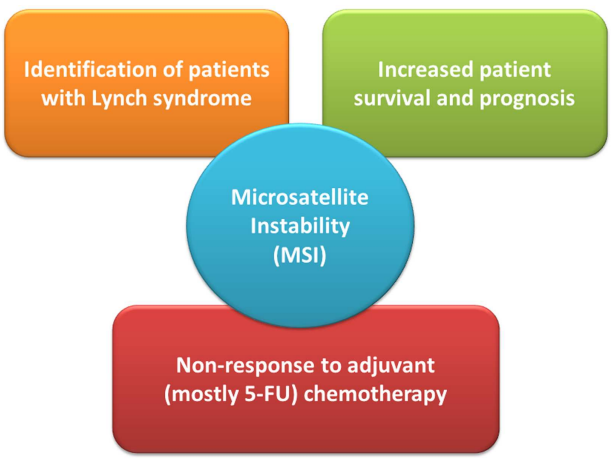

FIGURE 1 | The role of microsatellite instability (MSI) status in clinical management of colorectal cancer patients. 5-FU denotes 5-fluoro-uracil.

to induce cell-cycle arrest), to 5-FU treatment (4, 20). In line with these observations the meta-analysis found a clear significant beneficial effect of 5-FU therapy in patients with MSS CRC only. Further studies by Des Guetz et al. (meta-analysis) and Sargent et al. provided comparable findings in that relapse-free survival was similar for treated and untreated MSI-H patients demonstrating MSI-H status as a predictive factor of non-response to adjuvant, mostly 5-FU-based chemotherapy (21, 22). The high variability in treatment response observed in the MSI-H CRC group may actually reflect the (in) efficiency of other DNA repair systems, like base-excision repair, to repair/tolerate chemotherapy-induced DNA lesions. Overall, current data advocate CRC patient stratification by determining the tumor's MMR status, either by testing for MSI or immunohistochemical analysis of MMR proteins, in order to decide on adjuvant chemotherapy on an individual basis.

CONCLUDING REMARKS

As exemplified by the rapidly growing list of cancer genomes analyzed by means of ever more complete as well as cost-effective high-throughput ("next generation") sequencing methods, a novel era of truly personalized medicine seems to be at hand (23). It is the hope of the author that, despite the inherent difficulties and (data) challenges when trying to get a deeper understanding of biological systems as complex as cancer, the novel "omics" and systems biology approaches will not only allow more comprehensive insights into tumor biology but eventually result in individual patient (tumor)-tailored treatment and, last not least, enable true cancer prevention.

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MicroRNA-135b and its circuitry networks as potential therapeutic targets in colon cancer

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The study of regulatory non-coding RNAs has deepened our understanding of cancer on the molecular and clinical front. MicroRNAs, which encompass over 1000 short, endogenous nucleic acids, include important candidates that drive carcinogenesis. In this discussion, we review the literature on miR-135b, purporting its role as an oncogene in multiple cancers, and we highlight STAT3 inhibition as a potential therapeutic strategy mediated by miR-135b and demonstrate particular clinical relevance to colon cancer.

MicroRNA-135b (miR-135b) OVEREXPRESSION AND ONCOGENIC BEHAVIOR

miR-135b levels are elevated in a variety of cancers including breast, non-small cell lung cancer (NSCLC), prostate, and colon (1–3). Microarray analysis and quantitative reverse transcription-PCR (qRT-PCR) studies in NSCLC have demonstrated that miR-135b upregulation is far more robust in highly invasive lines compared to the less invasive. Overexpression of miR-135b conferred an increased tumorigenic ability to the relatively benign CL1-0 cells, resulting in more than a fourfold greater tumor burden in xenograft mouse models. *In vivo*, stable expression of a miR-135b antagonist decreased the number of metastatic tumor nodules in mice injected with highly invasive CL1-5-F4 cells shown to have high levels of miR-135b (4).

Clinically, high levels of miR-135b in lung cancer specimens significantly correlated with decreased overall survival (4). The investigators demonstrated that miR-135b suppresses key components of the Hippo pathway, a serine-threonine kinase pathway studied extensively in *Drosophila melanogaster* that plays a role in inhibition of overgrowth, regulation of cell division, and apoptosis. Mammalian orthologs within the Hippo pathways include LATS2 and LZTS1, which were identified as targets of miR-135b. Downregulation of LATS2 and LZTS1 and consequent nuclear localization of the transcriptional activator TAZ in patients with high miR-135b levels correlated with poor overall survival (4). Clinically, deregulation of this pathway also appears to be an important driver of hepatocellular and cholangiocarcinoma, although, to our knowledge, its regulation has not been linked to miR-135b in the setting of liver disease (5).

Microarray analysis and qRT-PCR also demonstrate that miR-135b levels are more than twofold higher in head and neck squamous cell carcinoma (HNSCC) in an orthotopic mouse model compared to controls (6). Parallel to the findings in lung cancer, *in vitro* studies with HNSCC also demonstrate that miR-135b overexpression confers an invasive phenotype – cells expressing miR-135b mimic displayed increased colony formation, cell migration, and proliferation. miR-135b mimics also induced increased tube formation of human umbilical vein endothelial cells, providing *in vitro* support for its role is promoting angiogenesis.

Investigators subsequently identified upregulation of hypoxia inducible factor 1 alpha (HIF-1α) and vascular endothelial growth factor A (VEGFA), which is a downstream target of HIF-1α, as a consequence of miR-135b overexpression. *In vitro* data suggests that miR-135b decreases available factor inhibiting HIF (FIH), which is also known as HIF-1α subunit inhibitor (HIFIAN). The *Tgfbr1/Pten* 2c knockout mice serving as the orthotopic HNSCC model in this study demonstrated decreased levels of *Fih* mRNA and protein in tongue squamous cell carcinoma compared to surrounding tongue epithelium and compared to control mice. These findings correlated with elevated levels of miR-135b and *Hif-1α* mRNA. When HNSCC cell lines were transfected with a miR-135b mimic, a corresponding increase in protein levels of HIF-1α and decrease in FIH was elicited.

Immunofluorescence and Western blot analysis demonstrated parallel correlation between elevated miR-135b levels and decreased FIH signal in the *Tgfbr1/Pten* 2cKO mice. Nuclear localization of HIF-1α and VEGFA signals were significantly increased in mice with elevated miR-135b levels. Finally, microvessel density of HNSCC in *Tgfrb1* knockout mice was over fourfold greater compared to control *Pten* knockout HNSCC, providing *in vivo* data consistent with miR-135b promotion of angiogenesis.

Studies in colon cancer also demonstrate a distinct increase in expression of miR-135b in both adenomas and carcinomas compared to normal epithelium (1, 7, 8). Its role in driving adenoma to carcinoma progression, however, has not been established. Nonetheless, the progressive increase in miR-135b expression from normal tissue to polyp to carcinoma, suggests that miR-135b deregulation is an early event that is amplified with increasing dysplasia. Nagel et al. support this hypothesis by demonstrating adenomatous polyposis coli (APC) to be an important target of miR-135b (7). On a genomic level, it is known that biallelic mutations in the *APC* gene are the primary initiating events in the adenoma to carcinoma sequence

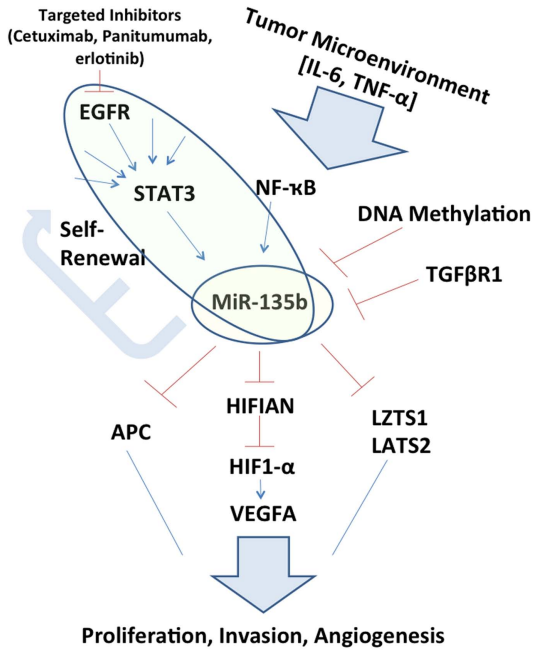

FIGURE 1 | Schematic representation of miR-135b circuitry network. Studying the network surrounding miR-135b may elucidate links between otherwise far reaching pathways, such as EGFR/STAT3 signaling to Hippo (LZTS1, LATS2) and inhibition of growth. miR-135b silencing *in vivo* may itself be therapeutic and understanding its function may uncover additional therapeutic targets.

through deregulation of the β-catenin pathway. These mutations typically result from premature stop codons in the *APC* transcript and are detected in >70% of colon cancers. In cases where hypomorphic mutations are present, miR-135b activation may function as "*third hit*" to indeed suppress any residual tumor suppression (β-catenin regulating) activity. miR-135b expression is shown to be over 10-fold elevated in carcinoma compared to normal colon epithelium and clearly associated with decreased *APC* expression irrespective of chromosomal mutations. This tight association is supported by confirmation of a miR-135b binding site at the *APC* 3′ untranslated region and suggests that miR-135b upregulation leads to a more penetrant effect of APC derangement (7).

REGULATION OF miR-135b EXPRESSION

miR-135b is encoded in the first intron of *LEMD1* gene, a cancer/testis antigen shown to be aberrantly expressed in colon cancers (9). miR-135b is located on 1q32.1 which shows DNA copy number gain in CRC progression (7). Lung cancer lines demonstrate alteration of *LEMD1* and miR-135b expression when treated with a demethylating agent, suggesting a mode of epigenetic control in that cell population. Furthermore, investigators predicted that the transcription factor NF-κB has a potential binding site on the miR-135b promoter region (**Figure 1**). After demethylation, TNFα, a known potentiator of NF-κB transcription, indeed increased expression of miR-135b, suggesting a potential link between inflammatory signals and miR-135b transcription (4).

Interestingly, in the HNSCC study, miR-135b overexpression appeared to be induced from a knockdown of transforming growth factor beta receptor 1 (*TGFBR1*) (6). Clinically, mutations in *TGFBR1* are associated with an increased risk of developing colon cancer (10).

In lymphoma cells, *LEMD1* and miR-135b expression were demonstrated to be under STAT3 regulation (11). Signal transducer and activator of transcription (STAT) proteins are a family of cytoplasmic transcription factors that mediate external cytokine signaling and growth factor stimulation to transcription of genes involved in proliferation, differentiation, cell survival, development, and inflammation (12, 13). While there are several isoforms, constitutive activation of STAT3 has been identified to activate anti-apoptotic pathways and proliferative behaviors in a variety of cancers (12, 14).

If miR-135b expression is pervasively regulated by STAT3 in a variety of cancer types, it may perform a key exchange between the tumor microenvironment to inhibition of tumor suppressors. This extracellular ligand stimulation may also explain differences that may be observed in the role of miR-135b between one organ system and another, owing to the variable tumor microenvironments (**Figure 1**). This potential signaling cascade also elucidates the mechanism behind therapeutic benefits of STAT3 inhibition (13).

STAT3 AND ANTI-EGFR THERAPY

miR-135b may also represent a mechanism by which a network of tumor suppressors is downregulated despite targeted therapy against the cancer cells. The most apparent example would be the upstream activation of STAT3 via epidermal growth factor receptor regulation (EGFR). Multiple anti-EGFR drugs are utilized for treatment of HNSCC, bladder cancer, NSCLC, and colon cancer. The patients that qualify for this class of medications represent a selected group, and some continue to experience disease progression during treatment.

There is evidence that amongst HNSCC and NSCLC cells that are resistant to anti-EGFR therapy, STAT3 remains persistently phosphorylated in its active state (15, 16). The presence of phosphorylated STAT3 suggests a mode of activation that bypasses the EGFR signaling cascade (15). Concurrently, aggressive efforts are geared toward developing clinically available STAT3 inhibitors to restore the desired therapeutic effect or even replace anti-EGFR therapy (13, 16).

In treatment of colon cancer, anti-EGFR therapy is generally reserved for cases without *KRAS* mutations (17). What remains to be understood is the role of phosphorylated STAT3 as a predictor of response to anti-EGFR therapy. In a recent retrospective study, investigators examined levels of phosphorylated STAT3 amongst patients receiving anti-EGFR therapy. Multivariate analysis demonstrated that absence of phosphorylated STAT3 was associated with improved overall survival and increase time to progression of the disease (18). This finding suggests that if phosphorylated STAT3 is truly a marker of poor outcome, mir-135b

also has the potential to be explored as a biomarker and driver of resistance to anti-EGFR therapy.

Persistent STAT3 activation also has a specific association with cell proliferation and tumor growth in colon cancer. In a study of 32 tumor specimens, 29 demonstrated STAT3 binding to DNA via electrophoretic mobility shift assay (EMSA). Immunohistochemistry of colon cancer biopsies confirmed presence of phosphorylated STAT3 in the nuclei of dedifferentiated epithelium but not in surrounding normal colonic crypts (14). When colon cancer cell lines were transfected with constitutively active STAT3, cell proliferation was increased, whereas transformation of the cells with a dominant negative construct of STAT resulted in decreased proliferation.

This study also points to a potential limitation of *in vitro* models in studying the role of phosphorylated STAT3, and presumably, miR-135b. In multiple colon cancer lines, investigators confirmed the abundance of STAT3 expression by Western Blot, similar to analysis of tumor specimens. However, when STAT3-DNA binding was assessed via EMSA, none of the cell lines demonstrated constitutive binding. Upon treating the cells with interleukin-6 (IL-6), a known activator of the STAT3 pathway, constitutive DNA binding was successfully demonstrated. Similarly, when the cell lines were injected subcutaneously to produce xenograft tumors where they were presumably exposed to *in vivo* paracrine and autocrine signaling, EMSA experiments confirmed abundance of DNA-bound STAT3.

These findings suggest that the tumor microenvironment plays an important role in inducing oncogenic signaling pathways. IL-6 levels are elevated in serum and tumor samples of multiple cancers, and IL-6 and STAT3 activation constitute an important pathway in tumorigenesis in colitis associated cancers (19, 20). Moreover, miR-135b expression was elevated in tumors in mouse models of APC mutations as well as inflammatory bowel disease (21). If miR-135b expression is in fact tightly regulated by STAT3 in a variety of cancers, *in vitro* studies may be under-representing the degree of upregulation and oncogenicity that miR-135b contributes *in vivo*, particularly in colon cancer and the subpopulation of colitis associated cancers. miR-135b upregulation may be the common initiating pathway between malignancy derived from somatic mutations and those derived from inflammatory backgrounds where Wnt derangement is typically a later event. *In vivo* overexpression of miR-135b may help elucidate the role in tumorigenesis, particularly in the context of existing *APC* mutations and colitis models.

miR-135b IN CANCER STEM CELLS

The role of miR-135b in cancer stem cells (CSCs) is also emerging and supported by the potential role of STAT3 in the same context. Through a screen for microRNAs deregulated in CSC in pediatric solid tumor lines, investigators identified miR-135b as highly expressed in the stem-cell enriched cell population (22). A stemness assay confirmed that *in vitro* blockade of miR-135b demonstrated a markedly diminished ability of the neuroblastoma and medulloblastoma cells to form neurospheres, thereby alluding to the role of miR-135b in self-renewal and proliferation (22).

In colon cancer cell lines, investigators utilized CD133 and aldehyde dehydrogenase 1 (ALDH1) as selective markers for CSC and demonstrated increased ability of this selected cell population in producing subcutaneous tumors in xenograft models (23). This selected cell population demonstrated increased levels of phosphorylated STAT3 compared to the unsorted cells. Furthermore, immunohistochemistry/immunofluorescence analysis of over 100 colon cancer microarrays demonstrated that co-localization of phosphorylated STAT3 and ALDH1 or CD133 occurred in over 60% of the cases. An anchorage-independent assay showed that pharmacological inhibition of STAT3 in the CSCs markedly decreased tumorsphere formation. Furthermore, treatment of the cell lines with STAT3 inhibitors decreased the percentage population of ALDH1+/CD133+ cells in contrast to treatment with doxorubicin or 5-flurouracil which increased the ALDH1+/CD133+ subpopulation, suggesting that STAT3 plays a unique role in CSC propagation.

The parallel findings of miR-135b suppression in pediatric CSC and STAT3 blockade in colon CSC strongly allude to miR-135b as being an important driver in maintaining cell oncogenicity, particularly in the self-renewing behaviors of CSCs. Further experimentation to assess miR-135b levels with better defined markers of stemness in colon cancer would be needed to provide conclusive evidence, and would be supported by differential expression of miR-135b along the crypt-villus axis.

CONCLUSION

With the knowledge that we have about the regulation of miR-135b, its important downstream targets, and the oncogenic impact produced from its dysregulation, the data begs for more conclusive studies, particularly *in vivo* assessment of its role in colon cancer. miR-135b emerges as one example of a microRNA that may deepen our understanding of resistance to current cancer therapy, and possibly serve as an adjunct to guiding anti-EGFR treatment in colon cancer patients, particularly as its role is better explored in *KRAS* mutants. Direct blockade of miR-135b may itself serve as a therapeutic intervention against a network of events driving oncogenesis and even target the self-renewing CSCs. Understanding this valuable molecular target and placing it in our diagnostic and therapeutic armamentarium will enhance our options against colon and other cancers in an era of personalized medicine.

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Toward a molecular classification of colorectal cancer: the role of MGMT

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O^6-methylguanine DNA methyltransferase (MGMT) is a DNA repair enzyme with the ability to protect cells from DNA mutations by removing alkyl groups from the O^6 position of guanine. Colon mucosa is exposed to the direct effects of environmental carcinogens and therefore maintaining a proficient DNA repair system is very important to stay protected against DNA mutagenesis. Loss of MGMT expression is almost exclusively associated with methylation of CpG islands in the *MGMT* gene promoter region which is found in approximately 40% of colorectal cancers. The role of MGMT loss in colorectal tumorigenesis is complex but numerous studies have documented methylation of this gene even in the normal appearing mucosa as well as in aberrant crypt foci, suggesting that MGMT methylation can be regarded as an early event or "field defect" in colon cancer neoplasia. The focus of this perspective is the role of MGMT in different pathways of colorectal carcinogenesis as well as the implication of this molecule in treatment decisions in colorectal cancer patients.

Keywords: MGMT, MSI-H, MSS, MSI-low, CIMP, methylation, DNA repair

INTRODUCTION

O^6-methylguanine DNA methyltransferase (MGMT) is a ubiquitously expressed DNA repair enzyme with a unique ability to directly remove alkyl groups from the O^6 position of guanine. O^6-alkylguanine adducts cause damage by mispairing with thymine during replication leading to G:C to A:T transitions (1). Therefore MGMT protects normal cells from exogenous carcinogens. For example it has been shown that MGMT protects body against N-nitroso compounds, known to induce colon cancer by methylating the DNA (2). The downside is that MGMT with the same mechanism can protect cancer cells from alkylating chemotherapeutic agents. Each MGMT molecule can only engage in one enzymatic reaction since the active site of MGMT cannot be regenerated. Therefore, upon performing its enzymatic reaction, MGMT is targeted for ubiquitination and degradation (1). Because of this "suicide" mechanism, a cell will have only limited resources to repair abnormal adducts depending on the available numbers of MGMT molecules and the rate of MGMT synthesis. This concept raised many efforts to find an inhibitor for MGMT to be used in clinical practice to overcome resistance to alkylating chemotherapy; however, none of the inhibitors that have been identified showed a clinical advantage in different clinical trials (3). This is partly because of the exacerbation of the toxic side effects of the alkylating drugs due to inactivation of MGMT in normal tissues.

MGMT protein is encoded by *MGMT* gene located at chromosome locus 10q26 (4). The *MGMT* gene has a CpG island containing promoter and thus its expression is significantly regulated by DNA methylation which leads to epigenetic silencing of the gene and loss of MGMT protein expression (1). The most reliable method to evaluate MGMT methylation is a matter of controversy. Methylation specific polymerase chain reaction (MSP) is the most widely used technique with relatively high sensitivity and specificity (5). However, the reliability of MSP is dependent on good quality DNA, which is not typically obtainable from formalin-fixed, paraffin-embedded (FFPE) specimens (6). On the other hand, MSP fails to provide quantitative measurements on MGMT methylation. These limitations constrain the implication of MSP in the clinical setting. Pyrosequencing, combined bisulfite restriction analysis (COBRA), MethyLight, Methylation Sensitive – High Resolution Melting (MS-HRM), Methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) are other semiquantitative or quantitative methods that have been used to evaluate MGMT promoter methylation (7, 8). A recent study investigating the association between MGMT methylation and protein expression showed that MGMT protein expression assessed by immunohistochemistry (IHC) did not correlate with methylation status of MGMT (assessed by MSP) suggesting that MSP and IHC should not be used interchangeably (9).

There are 97 CpG sites present on the promoter region of *MGMT*. Interestingly, these CpG sites do not equally contribute to gene silencing as it has been shown that methylation among these sites is not uniform. Extensive studies have been conducted to map the specific CpG sites that can best predict gene silencing. In one the recent studies, Everhard et al. found six isolated CpG sites (CpGs −228, −186, +95, +113, +135, and +137) as well as two CpG regions (−186 to −172, and +93 to +153), each with a minimum of 81.5% of concordant results between methylation and expression (10). Furthermore, an association between MGMT methylation and the germline C to T SNP (rs16906252) within the first exon of *MGMT* is observed in colorectal cancer and normal colonic mucosa (11, 12).

The impact of MGMT loss in carcinogenesis was first reported in 1999 by Esteller et al. (5). Loss of MGMT expression due to aberrant promoter methylation was shown in 40% of colorectal cancers and gliomas and 25% of non-small cell lung carcinomas, lymphomas, and head and neck carcinomas (5). One year later, the same group documented a link between loss of MGMT and G to A mutations in *K-ras* gene in colon cancer (13), which was followed by a report showing the similar findings in gastric cancers (14). Two other groups described an association between loss of MGMT and G to A mutations in *p53* gene in astrocytomas and non-small cell lung cancers (15, 16). The link between MGMT loss and G to A mutagenesis has been confirmed in subsequent studies (17–19). However, the results of other studies did not support this sequence of changes (12, 20, 21).

MGMT AND COLON CANCER

The role of MGMT loss in colorectal tumorigenesis is complex and not well characterized. *MGMT* methylation has been detected in the aberrant crypt foci, which are the earliest precursor lesions in colon cancer development (22) suggesting that *MGMT* methylation is an early event in neoplastic pathway. Furthermore, low level methylation of *MGMT* has been reported in normal appearing colon mucosa in patients with a correspondingly *MGMT* methylated tumor, as well as individuals without colon cancer (12, 18, 23–25). This finding is suggestive of a role for *MGMT* methylation as a "field defect" in sporadic colon cancer carcinogenesis which is defined as an area of molecularly abnormal tissue that precedes and predisposes to the development of cancer (18). Therefore, it has been proposed that *MGMT* status might be a useful marker for early detection and risk assessment in sporadic colon cancers.

Two major pathways have been described in sporadic colorectal cancers: the chromosomal instability (CIN) pathway and CpG Island Methylator Phenotype (CIMP) pathway. The strong association of MGMT loss with CpG methylation links MGMT to the CIMP pathway, which is associated with BRAF-V600E mutation and MSI-high status (26, 27). In fact *MGMT* methylation has been documented in their precursor lesions, sessile serrated adenoma/polyp (SSA/SSP) (28–31). It has been shown that serrated adenomas with dysplasia are more associated with MGMT methylation compared to hyperplastic polyps and serrated adenomas without dysplasia (31). A recent study reported *MGMT* methylation in 46.7% of microvesicular hyperplastic polyps (MVHP), 60% of SSA/SSP without dysplasia, and 75% of SSA/SSP with dysplasia (32). In supporting of the contribution of MGMT protein in MSI-H pathway of CRC neoplasia, Svrcek et al. reported that field defects resulted from MGMT loss are more frequently associated with MSI-H than microsatellite-stable (MSS) colorectal cancers and concluded that methylation tolerance may represent a crucial initiating step prior to MMR deficiency in the development of MSI-H CRC (24).

On the other hand, the association of *MGMT* loss with G to A transition in *K-ras* and *p53* mutated genes, links MGMT to the CIN pathway of colorectal cancers which is characteristically MSS or -low (MSI-L) and CIMP-low (17, 33–35). The association of MGMT with K-ras in the context of MSS/MSI-L CRCs are not straight forward. For example, a recent study on 776 CRCs revealed that K-ras mutated carcinomas that are associated with *MGMT* methylation, more frequently develop in contiguity with a residual polyp and are associated with different MSI status (36). Jass has suggested a "fusion pathway" with overlapping features from the two major colorectal cancer pathways in which MGMT serves as a "cross-over" point (37). He hypothesized that the "fusion" of the hyperproliferation and crypt fission that characterize adenomas with the inhibition of apoptosis that has been linked with serrated polyps may generate lesions with enhanced aggressiveness. The presence of p53 mutation (likely associated with *MGMT* methylation) in some of the serrated polyps with dysplasia provides an example of this link (37). Another possible link between these two pathways is villous adenoma which, on one hand, is thought to represent an advance lesion in CIN pathway and is frequently associated with K-ras mutation (38). On the other hand, this lesion has morphologic resemblance to the traditional serrated adenoma (TSA) and also harbors K-ras mutation in a subset of cases, likely in association with MGMT methylation (35, 37, 39). Therefore, it has been suggested that villous adenoma may represent a bridge between the two pathways. Despite evidence for involvement of MGMT in colon cancer carcinogenesis, previous studies fail to show any prognostic significance of MGMT methylation (or loss of MGMT) in colorectal cancers (33, 40, 41).

MGMT IN TREATMENT OF COLORECTAL CANCERS

The role of MGMT in response to alkylating chemotherapeutic agents is well studied in glioma patients treated with temazolamide (42, 43). Based on these studies, it is well established that the patients with promoter methylation and loss of MGMT expression have much better response to chemotherapy and also longer progression free and overall survival while the intact expression of MGMT is predictive of a poor response to treatment and worse overall survival (7, 44, 45). As it discussed earlier (see above) this effect is most likely due to the protective function of MGMT against alkylating agents in cancer cells. The significance of MGMT expression in colorectal cancers is less investigated. One of the early studies revealed that CRC patients with unmethylated MGMT promoters who had been treated with chemotherapy were found to have a 5.3-fold greater risk of recurrence than those who had no exposure to chemotherapy (46). The exact mechanism for this finding is not understood as 5FU is an antimetabolite and does not function through alkylation of DNA. Regardless, this finding suggests that CRC patients with intact MGMT expression are not good candidates for 5FU adjuvant chemotherapy. Prior clinical studies did not show a benefit for using alkylating agents in treatment of colorectal cancer. However, given the effect of MGMT loss in sensitizing cancer cells to alkylating agents, recently several attempts were made to select suitable patients for these medications. In a phase II clinical trial study with dacarbazine in metastatic CRC patients who had failed standard therapies, objective clinical response was limited to those patients with *MGMT* methylation (47). Similar findings were seen in metastatic patients with MGMT methylation who were treated with single agent Temozolomide (48). This data opens a new window for an effective treatment in patients with colon cancer who are deficient in MGMT and represent an example of a personalized approach in treatment of cancers.

CONCLUSION

Colorectal cancer is a heterogeneous disease arising in association with abnormalities in different molecular pathways. The fine dissection of molecular events is necessary to establish molecular signatures that can correctly classify CRCs and reliably predict tumor behavior and prognosis. This article is a part of an attempt to put together our current knowledge about molecular mechanisms in CRC under the title of "Toward molecular classification of colorectal cancer." The role of MGMT protein in colorectal carcinogenesis is rather complex and poorly understood. However, based on the available data there are grounds to believe that MGMT plays an important role in development of CRC and may represent a bridge between different molecular pathways. Further studies are required to shed light on the contribution of this molecule in colorectal neoplasia.

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Loss of Cdx2 expression in primary tumors and lymph node metastases is specific for mismatch repair-deficiency in colorectal cancer

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Background: Approximately 20% of all colorectal cancers are hypothesized to arise from the "serrated pathway" characterized by mutation in *BRAF*, high-level CpG Island Methylator Phenotype, and microsatellite instability/mismatch repair (MMR)-deficiency. MMR-deficient cancers show frequent losses of Cdx2, a homeodomain transcription factor. Here, we determine the predictive value of Cdx2 expression for MMR-deficiency and investigate changes in expression between primary cancers and matched lymph node metastases.

Methods: Immunohistochemistry for Cdx2, Mlh1, Msh2, Msh6, and Pms2 was performed on whole tissue sections from 201 patients with primary colorectal cancer and 59 cases of matched lymph node metastases. Receiver operating characteristic curve analysis and Area under the Curve (AUC) were investigated; association of Cdx2 with clinicopathological features and patient survival was carried out.

Results: Loss of Cdx2 expression was associated with higher tumor grade ($p = 0.0002$), advanced pT ($p = 0.0166$), and perineural invasion ($p = 0.0228$). Cdx2 loss was an unfavorable prognostic factor in univariate ($p = 0.0145$) and multivariate [$p = 0.0427$; HR (95% CI): 0.58 (0.34–0.98)] analysis. The accuracy (AUC) for discriminating MMR-proficient and –deficient cancers was 87% [OR (95% CI): 0.96 (0.95–0.98); $p < 0.0001$]. Specificity and negative predictive value for MMR-deficiency was 99.1 and 96.3%. One hundred and seventy-four patients had MMR-proficient cancers, of which 60 (34.5%) showed Cdx2 loss. Cdx2 loss in metastases was related to MMR-deficiency ($p < 0.0001$). There was no difference in expression between primary tumors and matched metastases.

Conclusion: Loss of Cdx2 is a sensitive and specific predictor of MMR-deficiency, but is not limited to these tumors, suggesting that events "upstream" of the development of microsatellite instability may impact Cdx2 expression.

Keywords: colorectal cancer, Cdx2, mismatch repair, microsatellite instability

INTRODUCTION

Colorectal cancer is a heterogeneous disease at the clinical, histopathological, and molecular level (1). Several molecular classifications of colorectal cancer based on features such as chromosomal instability, point mutations (*APC*, *KRAS*, *BRAF*), microsatellite instability (MSI), and CpG island methylation have been proposed (2–4). It is now generally accepted that approximately 20% of all colorectal cancers arise from serrated adenomas that have undergone a series of genetic changes (5). In the earliest phase of this "serrated pathway" it is hypothesized that mutational activation of *BRAF* leads to an initial burst in proliferation within the normal colonic epithelium followed by p16-induced cell senescence (oncogene-activated senescence) (6, 7). Escape from senescence would be achieved by methylation of p16INK4A, loss of p53 function, or silencing of insulin-like growth factor binding protein 7 (IGFBP7). Responsible for this silencing is the CpG Island Methylator Phenotype (CIMP), a state of aberrant methylation of promoter region CpG islands associated with transcriptional inactivation of tumor suppressor genes (8). These changes lead to the development of sessile serrated adenomas (SSA) that may eventually progress to colorectal cancers (4).

Importantly, among the relevant tumor suppressor genes frequently silenced by CIMP is *MLH1*, a critical gene involved in DNA mismatch repair (9, 10). When hypermethylated, *MLH1* contributes to the development of MSI, a feature observed in 15% of all cases. Defects in the DNA mismatch repair system can be observed by immunohistochemistry for microsatellite instability/mismatch repair (MMR) proteins, such as Mlh1, Msh2, Msh6, and Pms2 (11, 12) with negativity in any one of these proteins a sign of MMR-deficiency.

Interestingly, some studies have observed that MMR-deficient colorectal cancers show a frequent loss of Cdx2, a tumor suppressor gene and homeodomain transcription factor that functions to regulate intestinal epithelial cell differentiation (13–15). Reduced Cdx2 expression has additionally been associated with increased migration and invasion of cancer cells and may play a role in the epithelial mesenchymal transition (EMT) by disrupting WNT pathway signaling (16–21).

The aim of this study is to determine the predictive value of Cdx2 expression for MMR-deficiency, the association with clinicopathological features and patient survival as well as to investigate changes in Cdx2 expression between primary cancers and matched lymph node metastases.

PATIENTS AND METHODS

PATIENTS

The patient cohort consisted of 201 non-consecutive patients treated at the Visceral and Transplantation Surgery department the Insel Hospital in Bern, Switzerland between 2002 and 2011. Gender and age information was available for all patients. Histopathology was systematically re-reviewed. TNM staging was performed in accordance with the seventh edition of the AJCC/UICC staging manual. Clinical metastasis staging (cM) information was available for 190 patients. Lymphatic, venous, and perineural invasion could be reviewed on a majority of cases. Information on adjuvant therapy was available for 197 patients and survival time for 93 patients. No patients received neoadjuvant therapy. Median overall survival time was 54.6 months.

SPECIMEN CHARACTERISTICS

Formalin fixed (10% neutral buffered formalin) paraffin-embedded tumor blocks were retrieved from the Institute of Pathology, University of Bern, Switzerland. One representative tumor block of primary cancer and lymph node metastases was identified for immunohistochemistry. Ethical consent was obtained from the local ethics commission for both groups.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was carried out on whole tissue sections, cut at 4 μm, for all primary colorectal cancers and lymph nodes (Cdx2, Mlh1, Msh2, Msh6, and Pms2). Negative controls were tested with omission of the primary antibodies. An automated Bond III instrument was used along with the following antibodies and protocols: Cdx2, Leica-Novocastra, NCL-Cdx2, 1:200, Tris 95°, 30 min; MLH1, Leica-Novocastra, NCL-MLH1, 1:200, Tris 95°, 30 min; MSH2, Leica-Novocastra, NCL-MSH2, 1:200, Tris 95°, 30 min; MSH6, Leica-Novocastra, MSH6-L-CE, 1:1600, Tris 95°, 30 min; PMS2, Leica-Novocastra NCL-L-PMS2, 1:75, Tris 95°, 30 min. For Cdx2 expression, the percentage of positive tumor cells was estimated. For MMR proteins, any tumor cell expression was defined as positivity for that marker. MMR-deficiency was assigned to cases showing loss of any of the four proteins. Since information on family history was unavailable, no attempt was made to further subdivide patients into Lynch syndrome or sporadic MSI.

STATISTICS

The association between Cdx2 expression as continuous variable and MMR status (proficient versus deficient) was investigated using simple logistic regression analysis. Odds ratio (OR) and 95% confidence intervals (CI) were used to determine effect size. The area under the receiver operating characteristic (ROC) curve (AUC) was used to determine the discriminatory ability of Cdx2 expression for MMR-deficiency, with values closer to 1.0 indicating a better discrimination. Cutoffs for Cdx2 focal and diffuse expression were also assessed by ROC curve analysis, by selecting the point on the curve giving the highest sensitivity and specificity for MMR-deficiency. For the association with age, a Wilcoxon's Test was used and to test the difference in expression between tumor and lymph node, a Wilcoxon's Signed Rank Test for matched pairs. Univariate survival analysis was performed using the log-rank and Wilcoxon's tests. Multivariable survival analysis was carried out using Cox regression analysis, with "loss" of Cdx2 used as a baseline. Hazard ratios and 95% CI were used to determine the effect of Cdx2 expression on overall survival. p-Values <0.05 were considered statistically significant. All analyses were carried out using SAS V9.2 (The SAS Institute, Cary, NC, USA).

RESULTS

PATIENT CHARACTERISTICS

Patient characteristics are shown in **Table 1**. Whole tissue sections from 201 patients were evaluated for Cdx2 expression. Of these, 59 patients had available lymph node metastases that underwent Cdx2 staining as well. Representative photomicrographs are shown in **Figures 1A,B**.

ASSOCIATION OF Cdx2 IN TUMOR AND LYMPH NODES WITH CLINICOPATHOLOGICAL FEATURES

Focal Cdx2 expression was significantly more frequent in colorectal cancers with mucinous histology ($p = 0.0053$), higher tumor grade ($p = 0.0002$), more advanced pT stage ($p = 0.0166$), with perineural invasion ($p = 0.0228$), and in those receiving adjuvant therapy ($p = 0.0058$). In addition, there was a significant and adverse effect of Cdx2 loss on patient survival ($p = 0.0145$; **Figure 2A**). This result was maintained in multivariable analysis with pT and pN classifications [$p = 0.0427$; HR (95% CI): 0.58 (0.34–0.98)] but not when clinical metastasis staging was included in the model. Although not statistically significant, possibly due to a smaller number of patients, loss of Cdx2 seemed to occur more frequently in tumors with lymphatic invasion ($p = 0.0809$), and in patients with metastasis ($p = 0.0887$).

Table 2 shows the associations between lymph node expression of Cdx2 and clinicopathological features of the primary cancers. Indeed, only tumor location was linked to loss of Cdx2 expression, which occurs more frequently in the right-side of the colon ($p = 0.0088$). Of the 59 patients with evaluable lymph nodes, information on survival was only available in 26. Loss of Cdx2 in lymph node metastasis was marginally associated with overall survival ($p = 0.0512$).

Evaluating the matched lymph nodes and primary colorectal cancers, average expression was 66.7% in lymph nodes and 71.0% in primary tumors. Using a matched pairs analysis, this difference was not significant ($p = 0.5801$).

Table 1 | Patient characteristics ($n = 201$) and association with Cdx2 expression in tumor.

Feature		Frequency *N* (%)	Frequency *N* (%)	Frequency *N* (%)	*p*-Value
		Total	Focal Cdx2	Diffuse Cdx2	
Gender ($n = 201$)	Male	125 (62.2)	51 (59.3)	74 (64.4)	0.4655
	Female	76 (37.8)	35 (40.7)	41 (35.7)	
Age (years) ($n = 201$)	Median (range)	72.0 (19–91)	70.9 (19–90)	73 (48–91)	0.0825
Tumor location ($n = 200$)	Left	76 (38.0)	28 (32.9)	48 (41.7)	0.3806
	Rectum	29 (14.5)	12 (14.1)	17 (14.8)	
	Right	95 (47.5)	45 (52.9)	50 (43.5)	
Histological subtype ($n = 200$)	Non-mucinous	162 (81.0)	62 (72.1)	100 (87.7)	0.0053
	Mucinous	38 (19.0)	24 (27.9)	14 (12.3)	
Tumor grade ($n = 199$)	G1–2	140 (70.4)	48 (56.5)	92 (80.7)	0.0002
	G3	59 (29.6)	37 (43.5)	22 (19.3)	
pT ($n = 201$)	pT1–2	47 (23.4)	13 (15.1)	34 (29.6)	0.0166
	pT3–4	154 (76.6)	73 (84.9)	81 (70.4)	
pN ($n = 200$)	pN0	92 (46.0)	32 (37.2)	60 (52.6)	0.0303
	pN1–2	108 (54.0)	54 (62.8)	54 (47.4)	
Metastasis ($n = 190$)	cM0	133 (70.0)	50 (63.3)	83 (74.8)	0.0887
	cM1	57 (30.0)	29 (36.7)	28 (25.2)	
Perineural invasion ($n = 111$)	Absence	97 (87.4)	38 (79.2)	59 (93.7)	0.0228
	Presence	14 (12.6)	10 (20.8)	4 (6.4)	
Venous invasion ($n = 132$)	Absence	59 (44.7)	21 (37.5)	38 (50.0)	0.1534
	Presence	73 (55.3)	35 (62.5)	38 (50.0)	
Lymphatic invasion ($n = 128$)	Absence	32 (25.0)	10 (17.5)	22 (31.0)	0.0809
	Presence	96 (75.0)	47 (82.5)	49 (69.0)	
Adjuvant therapy ($n = 197$)	None	135 (68.5)	48 (57.8)	87 (76.3)	0.0058
	Treated	62 (31.5)	35 (42.2)	27 (23.7)	
Mismatch repair status ($n = 201$)	Proficient	174 (86.6)	60 (69.8)	114 (99.1)	<0.0001
	Deficient	27 (13.4)	26 (30.2)	1 (0.9)	
Overall survival ($n = 93$)	Median (95%CI)	54.6 (28–72)	26.4 (10–61)	68.7 (44–101)	0.0145

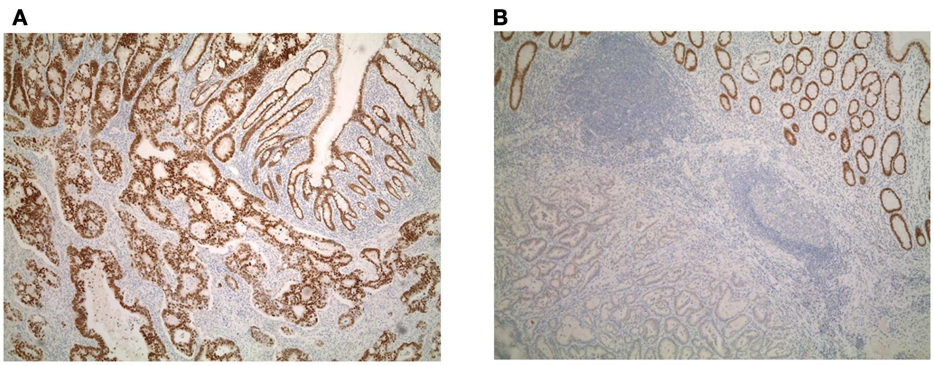

FIGURE 1 | (A) Diffuse and **(B)** focal expression of Cdx2 in colorectal cancers.

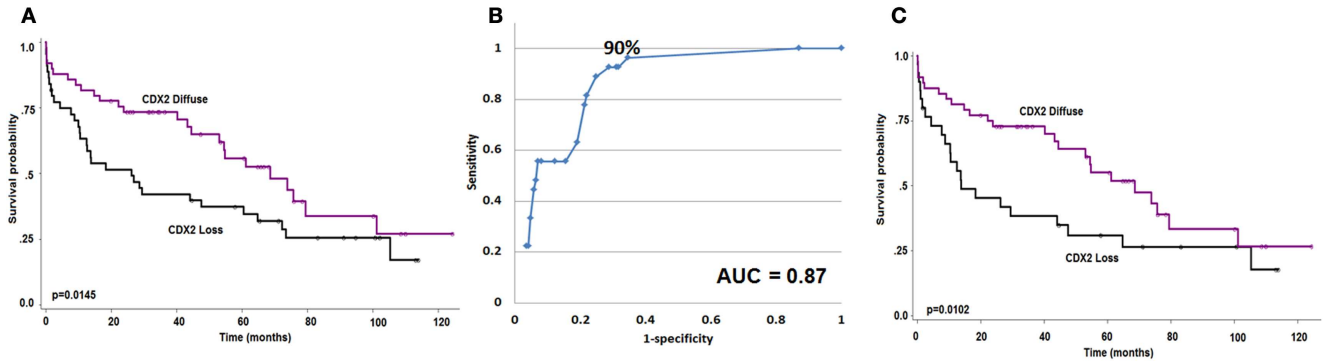

FIGURE 2 | (A) Kaplan–Meier survival curve showing effect of Cdx2 expression on overall survival ($n = 93$). **(B)** ROC curve highlighting the strong predictive effect and specificity of loss of Cdx2 expression for MMR-deficiency. **(C)** Kaplan–Meier survival curve showing effect of Cdx2 expression on overall survival in MMR-proficient colorectal cancer patients only ($n = 78$). Wilcoxon's test.

Table 2 | Association of Cdx2 loss in tumor and lymph nodes with clinicopathological features ($n = 59$).

Feature		Lymph node metastases ($n = 59$)		
		Cdx2 focal	Diffuse	*p*-Value
Gender ($n = 59$)	Male	9 (64.3)	29 (64.4)	1.0
	Female	5 (35.7)	16 (35.6)	
Age (years) ($n = 59$)	Median (range)	71 (19–87)	74 (30–91)	0.1169
Tumor location ($n = 58$)	Left	1 (7.1)	20 (45.5)	0.0088
	Right	5 (35.7)	4 (9.1)	
	Rectum	8 (57.1)	20 (45.5)	
Histological subtype ($n = 59$)	Non-mucinous	10 (71.4)	34 (75.6)	0.7376
	Mucinous	4 (28.6)	11 (24.4)	
Tumor grade ($n = 59$)	G1–2	7 (50.0)	32 (71.1)	0.3156
	G3	7 (50.0)	13 (28.9)	
pT ($n = 59$)	pT1–2	2 (14.3)	4 (8.9)	0.6204
	pT3–4	12 (85.7)	41 (91.1)	
Metastasis ($n = 56$)	cM0	9 (75.0)	23 (52.3)	0.1585
	cM1	3 (25.0)	21 (47.7)	
Perineural invasion ($n = 33$)	Absence	6 (75.0)	20 (80.0)	1.0
	Presence	2 (25.0)	5 (20.0)	
Venous invasion ($n = 41$)	Absence	2 (18.2)	8 (26.7)	0.7004
	Presence	9 (81.8)	22 (73.3)	
Lymphatic invasion ($n = 44$)	Absence	1 (8.3)	1 (3.1)	0.4757
	Presence	11 (91.7)	31 (96.9)	
Adjuvant therapy ($n = 59$)	None	7 (50.0)	23 (51.1)	0.9421
	Treated	7 (50.0)	22 (48.9)	
Mismatch repair status ($n = 59$)	MMR-proficient	7 (50.0)	43 (95.6)	<0.0001
	MMR-deficient	7 (50.0)	2 (4.4)	

Cdx2 EXPRESSION AND MISMATCH REPAIR STATUS

There was a major significant association between reduced Cdx2 expression and MMR-deficiency. The AUC value for Cdx2 expression in tumor was 0.87 indicating 87% accuracy for discriminating MMR-proficient and – deficient cancers (**Figure 2B**). The OR (95% CI) was 0.96 (0.95–0.98); $p < 0.0001$. The ROC

curve was used as a basis for the identification of an optimal threshold value for considering tumors with "focal" and "diffuse" expression and determined to be 90%. Of the 115 patients with diffuse expression of Cdx2, 114 were MMR-proficient (99.1% specificity) and of the 27 MMR-deficient patients 26 had only focal expression (96.3% negative predictive value). There were 174 patients with MMR-proficient cancers, of which 60 (34.5%) indeed showed loss of Cdx2. Cdx2 loss among patients with MMR-proficient cancers was significantly and unfavorably related to survival ($p = 0.0102$; **Figure 2C**). Again, in multivariable analysis, Cdx2 loss was associated with worse outcome after adjusting for pT and pN [$p = 0.0414$; HR (95% CI): 0.54 (0.3–0.98)], but not when clinical metastasis stage was added.

The AUC for Cdx2 expression in lymph nodes and MMR status was 0.943 indicating 94% discriminatory ability of the protein. The OR (95% CI) was 0.93 (0.87–0.99); $p = 0.037$. Using the ROC curve for the selection of a threshold value, tumors with <30% staining were considered "focal" and >30% considered "diffuse" for Cdx2 expression. Of the 45 cases with diffusely expressing Cdx2, 43 were MMR-proficient (95.6% specificity), whereas 7/9 MMR-deficient cancers showed focal expression of Cdx2 (77.8% NPV).

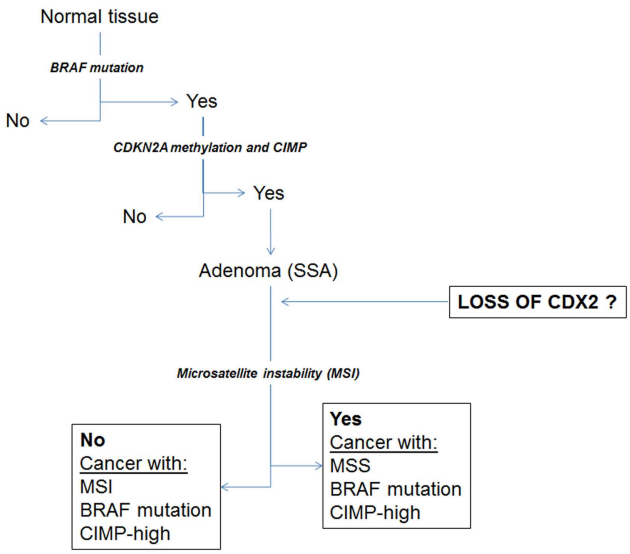

FIGURE 3 | Schematic diagram illustrating a proposal for the involvement of Cdx2 during progression of cancers through the serrated pathway.

DISCUSSION

The findings of this study suggest that reduced expression of Cdx2 in primary tumors and lymph node metastases is an accurate predictor of MMR-deficiency in colorectal cancer. Moreover, loss of Cdx2 is a poor prognostic factor, even among patients with MMR-proficient cancers.

In a first step, we examined the specificity of Cdx2 for MMR status. The ROC curve for this analysis underlines the major discriminatory power of reduced Cdx2 expression for MMR-deficiency in both colorectal cancers and lymph nodes. Previous reports by our group and others have highlighted similar findings. Using a tissue microarray containing more than 600 patient tissues, Baba and colleagues showed a high specificity of reduced Cdx2 expression for MSI-high colorectal cancers (22). The protein expression of Cdx2 in MMR-proficient versus deficient cancers has been reported at 84 versus 61% on average, again using tissue microarrays (14). Our study goes one step further and uses whole tissue sections for the establishment of both MMR status and Cdx2 expression. Indeed, all MMR-deficient cancers with the exception of one case showed only focal positivity for Cdx2 expression.

Despite this observation, a subgroup of MMR-proficient cancers also shows focal positivity for Cdx2. Our hypothesis is that Cdx2 loss may be an important marker of other molecular changes associated with the serrated pathway to colorectal cancer, including *BRAF* mutation and high-level CIMP. Indeed, we could previously show using a cohort of more than 300 patients, that loss of Cdx2 was nearly 100% specific for *BRAF* mutation, and found in 23/24 mutated cases (23). Baba and colleagues as well as Walsh et al. found loss of Cdx2 in *BRAF* mutated tumors and a significantly more frequent number of cases in tumors with CIMP-H (22, 24). Loss of Cdx2 has also been found to be an independent predictor of the CIMP-H phenotype (25). **Figure 3** illustrates some of the changes hypothesized to occur during the serrated pathway. We believe that loss of Cdx2 expression occurs prior to the establishment of MSI and only after the development of both *BRAF* mutation and CIMP. Although the evidence fits well for an involvement of Cdx2 in the serrated pathway, whether this molecule is actually functionally involved as a cause rather than a consequence of progression of tumors within this pathway has not yet been established.

Next, we evaluated the association between focal expression of Cdx2 and clinicopathological features. Our results point toward an association of Cdx2 with an array of important and adverse prognostic features including unfavorable overall survival. Our findings are in line with previous work from our group using a single punch tissue microarray showing strong correlations between Cdx2 loss and pT, pN, tumor grade, and vascular invasion on more than 1000 tumors. Baba and colleagues showed similar results of Cdx2 loss with more advanced TNM stage, higher tumor grade, mucinous, or signet ring cell histology (22). These results are in agreement with Choi et al. who show loss of Cdx2 expression associated with advanced Dukes' stage and more poorly differentiated cancers (26). Unfavorable survival times are reported by several groups upon reduced Cdx2 expression (22, 27). In addition, the predilection for female gender and more right-sided tumor location has also been observed in other studies (22, 28). We also show that the unfavorable impact of Cdx2 is maintained in patients with MMR-deficient cancers.

Thirdly, we evaluated for the first time Cdx2 expression in matched lymph node metastases. We found no differences in expression between lymph nodes and primary colorectal cancers. These results appear to indicate that a further "evolution" leading to loss of Cdx2 after lymph node spread is unlikely.

To conclude, Cdx2 is significantly reduced in patients with MMR-deficient colorectal cancers, but is not limited to these tumors. It is an unfavorable prognostic factor, even among

patients with MMR-proficient cancers. Taken together with previous reports on *BRAF* and CIMP, we hypothesize that Cdx2 loss may play an early role in the progression of cancers arising through the serrated pathway.

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