‘MCC’ protein interacts with E-cadherin and beta-catenin strengthening cell-cell adhesion of HCT116 colon cancer cells.

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Abstract

E-cadherin and beta-catenin are key proteins that are essential in the formation of the epithelial cell layer in the colon but their regulatory pathways that are disrupted in cancer metastasis are not completely understood. *Mutated in colorectal cancer* (*MCC*) is a tumour suppressor gene that is silenced by promoter methylation in colorectal cancer and particularly in patients with increased lymph node metastasis. Here, we show that MCC methylation is found in 45% of colon and 24% of rectal cancers and is associated with proximal colon, poorly differentiated, circumferential and mucinous tumours as well as increasing T stage and larger tumour size. Knockdown of MCC in HCT116 colon cancer cells caused a reduction in E-cadherin protein level, which is a hallmark of epithelial-mesenchymal-transition (EMT) in cancer, and consequently diminished the E-cadherin/β-catenin complex. MCC knockdown disrupted cell-cell adhesive strength and integrity in the dispase and transepithelial electrical resistance assays, enhanced hepatocyte growth factor (HGF)-induced cell scatter and increased tumour cell invasiveness in an organotypic assay. The Src/Abl inhibitor dasatinib, a candidate anti-invasive drug, abrogated the invasive properties induced by MCC deficiency. Mechanistically, we establish that MCC interacts with the E-cadherin/β-catenin complex. These data provide a significant advance in the current understanding of cell-cell adhesion in colon cancer cells.

Keywords: MCC, adhesion, invasion, EMT, colorectal cancer

Introduction

The *mutated in colorectal cancer* (*MCC*) gene is located close to the *adenomatous polyposis coli* (*APC*) gene on chromosome 5. It is a tumour suppressor that regulates a number of cellular processes, including cell cycle checkpoints and the DNA damage response [1-3]. Unlike *APC* and despite its name, *MCC* is not frequently mutated in colorectal cancer (CRC) or other cancers, only up to 6-8% [4, 5]. However, *MCC* is frequently silenced by promoter methylation in up to 50% of sporadic CRCs and 80% of serrated polyps [6 7]. This suggests that *MCC* silencing is an important early event in colon carcinogenesis and particularly in the serrated neoplasia pathway [6, 8, 9]. Decreased expression of MCC is also found in hepatocellular carcinoma [10, 11] and loss of heterozygosity in lung cancer [12]. *MCC* mutation is a driver of colorectal and liver carcinogenesis in the Sleeping Beauty mouse model of carcinogenesis [13, 14], strengthening its significance as a *bona fide* tumour suppressor.

MCC methylation is associated with the spread of cancer to lymph nodes in CRC patients [6], indicating that *MCC* silencing has a role in promoting metastasis. Carcinomas metastasize via a multistep process called epithelial-mesenchymal transition (EMT), which leads to the invasion of tumour cells through the basement membrane and their dissemination through the lymphatic system (reviewed in Thiery et al [15]). Epithelial cells have a particular set of characteristics that distinguishes them from mesenchymal cells [16]. They are arranged in sheets, held together by intercellular junctions and protein complexes that maintain their structural integrity. E-cadherin is a key cell-cell adhesion molecule, and its loss of expression is considered a hallmark of EMT [17, 18]. The cytoplasmic domain of E-cadherin forms a complex with β-catenin at the adherens junctions [19-21]. During EMT, E-cadherin expression decreases and this

releases β-catenin that can become hyperactivated in cancer, and drive a transcriptional program in the nucleus further promoting cell invasiveness. MCC interacts with β-catenin [8], and can function as a suppressor of Wnt-stimulated β-catenin-dependent transcription [8, 22]. However, the mechanism is not known and the subcellular localization of MCC/β-catenin interaction has not been addressed.

Epithelial cells lose their apical-basal polarity as cell-cell junctions become obsolete during EMT [23]. MCC also interacts with the polarity protein SCRIB (Scribble), which localizes to the lateral membrane of epithelial cells and regulates the ability of cells to migrate [24, 25]. MCC binds SCRIB through its class I PDZ-binding motif, which constitutes the last four amino acids of the protein [24]. Increasing evidence suggests that polypeptides containing PDZ-binding motifs play a significant part in assembling transmembrane and intracellular protein complexes [26, 27]. As MCC binds β-catenin and SCRIB through different domains, MCC may serve as a scaffold, which positions key proteins at the cell membrane. Therefore, we hypothesized that given its proposed role in lymph node metastasis, *MCC* may also interact with E-cadherin through β-catenin at the cell membrane.

Results

MCC methylation is associated with adverse prognostic markers

We previously reported that *MCC* methylation was associated with high tumour grade and metastasis to four or more lymph nodes in a patient cohort [6]. Here we analysed the clinicopathological features of an independent larger cohort of 365 colon and rectal cancer patients. *MCC* methylation was found in 58% of cancers in the right colon, 28% in the left colon and 24% of rectal cancers. It was more common in poorly differentiated, circumferential or mucinous tumours, and correlated with increasing T stage and larger tumour size (Table 1). Many of these features are associated with a more aggressive tumour phenotype [28, 29].

MCC is a membrane-associated protein that interacts with the E-cadherin/β-catenin adhesion complex

As E-cadherin is the main binding partner of β-catenin at the cell membrane and is regarded as the master controller of cell-cell adhesion in epithelial cells, we tested if MCC also interacts with E-cadherin in addition to β-catenin. We found MCC co-localising with E-cadherin at the linear adherens junctions (AJ) at cell-cell contacts and with β-catenin (Figure 1A) in HCT116 colon cancer cells, which express MCC endogenously. Most MCC localised to the cytoplasm, consistent with other studies. To further validate the interaction, β-catenin and E-cadherin were co-immunoprecipitated (co-IP'd) with MCC in the same cell line (Figure 1B and confirmed by Proximity Ligation Assay, Supplementary Figure S4).

To examine whether MCC binds E-cadherin and β-catenin in the same protein complex, we extracted total native proteins from HCT116 cells, which

were then resolved by Blue-Native polyacrylamide gel electrophoresis (BN-PAGE). Both E-cadherin and β-catenin were found in protein complexes of similar size to MCC, ~720 kDa (Figure 1C). MCC also formed a ~600 kDa complex (Figures 1C) that was similar in mass to the complex formed by the polarity protein SCRIB. The ~720 kDa complex was found only in the membrane fraction (Figure 1D), compatible with MCC being co-localised with E-cadherin at the linear AJ at cell-cell contacts (Figure 1A). E-cadherin and β-catenin were also found in a 480 kDa complex without MCC.

Following the co-IP using E-cadherin, β-catenin or MCC antibody, the unbound protein complexes were analyzed by BN-PAGE and Western blot to detect the level of E-cadherin complex. As expected, E-cadherin complexes were present in the IgG control (Figure 1E, lane 1) but undetectable following E-cadherin or β-catenin depletion (Figure 1E, lanes 2-3). When MCC was depleted, the level of 720 kDa E-cadherin adhesion complex was significantly reduced while the 480 kDa complex remained unchanged (lane 4), relative to IgG control (lane 1). Collectively, these data strongly suggest that MCC associates with the E-cadherin/β-catenin in the same 720 kDa complex.

The assembly process of the proteins at the adherens junction is still poorly understood and we subsequently focused on the formation of the E-cadherin/β-catenin assembly with MCC in HCT116 cells. We quantified the abundance of 720 kDa E-cadherin complex using BN-PAGE and Western blot. HCT116 cells were grown to 20% confluency and then synchronised to the G0/G1 phase following serum starvation [30]. The abundance of the 720 kDa E-cadherin complex increased when cells were growing, with a marked rise observed at 32 hours serum release onwards (Supplementary Figure S1A). Its abundance was significantly reduced when cell-cell contacts were disrupted

following treatment with EGTA (Supplementary Figure S1B). The protein assembly was re-established after removal of EGTA from culture followed by supplementation of full serum media (Supplementary Figure S1B).

Loss of MCC weakens the interaction between β-catenin and E-cadherin in colon epithelial cells

As *MCC* is typically silenced by promoter methylation in CRC, we next tested the impact of MCC knockdown on the E-cadherin/β-catenin complex. *MCC* was stably knocked down in clonal HCT116 cells with two independent short hairpin RNA (shRNA). A non-targeting (NT) shRNA with a scrambled sequence was used as a control. Both shRNA lines exhibited a significant reduction in *MCC* mRNA and protein expression compared with NT cells (Figures 2A-B). Importantly, densitometric analysis showed that MCC-shRNA cells had a decrease in E-cadherin protein levels and a more delicate decrease in β-catenin protein levels relative to β-actin levels (Figures 2B). However, there was no significant alteration in *CDH1* (E-cadherin) or *CTNBB1* (β-catenin) at the mRNA level (Figure 2A). When E-cadherin was immunoprecipitated from HCT116 cells, the interaction of E-cadherin with β-catenin was diminished in MCC-deficient cells compared with NT control cells (Figure 2B,C). This is likely due to the reduced amount of E-cadherin protein following MCC knockdown resulting in a reduced overall abundance of the E-cadherin/β-catenin complex. The reduction in E-cadherin/β-catenin complex formation after MCC knockdown was also shown using a Proximity Ligation Assay (Supplementary Figure S2). Thus, MCC can interact with the E-cadherin/β-catenin complex and may promote cell-cell adhesion in colon epithelial cells.

MCC regulates cell-cell adhesion in colon epithelial cells

The impact of MCC deficiency on cell-cell adhesion was measured with two complementary assays for intercellular cohesive strength and integrity. In the dispase assay [31], a confluent cell monolayer is disrupted with dispase and mechanical force. MCC knockdown in HCT116 cells produced a significant increase in the percentage of single detached cells in both MCC shRNA1 (*P*=0.005) and shRNA2 (*P*=0.0005) lines compared to MCC-expressing control cells (Figure 2E), indicating that loss of MCC had weakened intercellular adhesive strength. In reverse, stable MCC expression induced by doxycycline treatment in HCT15 cells increased E-cadherin protein expression and E-cadherin/β-catenin interaction (Figure 2D, Supplementary Figure S3) and strengthened cell-cell adhesion showing fewer detached single cells (*P*=0.0194) (Figure 2E).

Cell-cell junction integrity and permeability were quantified using the EVOM2 Volt-Ohm meter to measure transepithelial electrical resistance (TEER). This is a rapid and sensitive technique specifically designed to measure the resistance across epithelial cell monolayers without affecting the membrane voltage or capacitance (Figure 2F) [32]. Higher resistance readings were detected for MCC expressing NT control cells compared with MCC shRNA1 (*P*=0.0016) and shRNA2 (*P*<0.0001) knockdown cells (Figure 2G). When MCC was stably expressed in HCT15 (+dox), electrical resistance increased significantly (*P*=0.0048) (Figure 2G). These data suggest that MCC plays a role in maintaining cell-cell cohesion and integrity in colon cancer cells.

MCC deficiency enhanced hepatocyte growth factor (HGF)-induced cell scatter

A cell scatter assay with live imaging was used to investigate whether loss of MCC expression caused other EMT-associated changes apart from a reduction in E-cadherin. HCT116 cells were allowed to grow as discrete colonies for 48-72 hours, then serum-starved and HGF was added. HGF induces EMT by activating the Met-signaling pathway in epithelial cells, diminishing cell-cell adhesion, promoting cell motility and enhancing invasion and metastasis [33]. MCC-deficient shRNA1 and shRNA2 cells scattered significantly more than MCC-expressing NT cells (*P*=0.0001 at 24 hours) (Figure 3A). 24 hours post-HGF treatment, MCC-deficient cells became more elongated and exhibited a more fibroblast-like morphology consistent with an EMT phenotype (Supplementary Movies 1-3). These cells also showed a strong loss of E-cadherin expression and an increase in both the cytoplasmic and nuclear localization of β-catenin at 8 hours post-HGF treatment (Figure 3B), when the cells were actively scattering (Figure 3A).

We then chose small molecule inhibitor dasatinib as a candidate anti-invasive drug to determine whether the invasive phenotype of MCC-deficient cells can be reverted. Dasatinib stabilizes cell-cell junctions by preventing E-cadherin degradation, mobilisation or cleavage [34-36]. The cells were treated with HGF alone (30 ng/mL) or both HGF and dasatinib (200 nM) for 24 hours. Dasatinib prevented the enhanced scattering caused by MCC deficiency following HGF treatment (Figures 3C-D). MCC-deficient cells also exhibited finger-like projections resembling filopodia (Figure 3C, Supplementary Movies 4-5), suggesting that these cells responded to HGF morphologically but were prevented from scattering by dasatinib. Furthermore, dasatinib increased the adhesiveness of both NT control cells (*P*<0.0001) and MCC-deficient cells (*P*=0.0033) in the TEER assay (Figure 3E). This suggests that dasatinib can

restore epithelial strength and integrity and reverse HGF-induced cell scatter in both MCC-depleted and MCC-expressing cells.

MCC deficiency promotes the invasiveness of colon cancer cells which can be prevented by dasatinib treatment

For tumour cells to invade, they must be able to move through and degrade the extracellular matrix (ECM). An organotypic 3-dimensional culture of HCT116 cells was established to determine the impact of MCC knockdown on cell invasion *in vitro.* ECM composed of collagen and fibroblasts can recapitulate aspects of the tumour stroma [37]. During invasion, the organotypic cultures were either treated with 200 nM dasatinib or vehicle (DMSO). Sections of the matrix were then stained with multi-cytokeratin (Multi-CK) to identify epithelial cells and differentiate them from fibroblast cells within the matrix. MCC-NT control cells exhibited very weak invasion, whereas MCC knockdown cells showed a higher number of cells invading into the matrix (P<0.0001). This was reversed by concurrent treatment with dasatinib (Figure 4A).

NT control cells exhibited a higher Ki67 percentage positivity score compared to MCC shRNA1 (P=0.0017) and shRNA2 (P=0.0026) (Figure 4B). This suggests that invasion in MCC-deficient cells is not due to increased proliferation. Dasatinib treatment did not alter the proliferative capacity of MCC-expressing or MCC-deficient cells (Figure 4B), while cleaved caspase 3 analysis was inconclusive for apoptosis (Figure 4C). Taken together, this demonstrates that loss of MCC promotes cell invasiveness. Dasatinib abrogated the invasive phenotype caused by loss of MCC without causing any alterations in cell proliferation.

Discussion

E-cadherin is the master regulator of cell-cell adhesion and junction formation in epithelial cells. It recruits β-catenin and other catenins to the cell membrane to form an adhesion complex between cells. The E-cadherin/β-catenin complex is essential for maintaining a functioning epithelium and cell-cell integrity [38]. Here, we have identified MCC as a membrane-associated tumour suppressor protein that interacts with E-cadherin and β-catenin.

Importantly, knockdown of MCC decreased the level of E-cadherin, a hallmark of EMT. This describes a novel tumour suppressor role for MCC in the regulation of E-cadherin expression. MCC-deficient cells did not exhibit changes in *CDH1* (E-cadherin) or *CTNBB1* (β-catenin) mRNA levels, suggesting that MCC is likely to regulate E-cadherin expression through post-translational modifications of the protein. Accordingly, we confirmed the weakening of cell-cell junctions following MCC knockdown by two complementary adhesion assays. Moreover, lower levels of E-cadherin render cells more susceptible to EMT induced by growth factors [39, 40], involving both intrinsic changes and altered tissue microenvironment [41]. When EMT was induced in these cells by HGF, MCC knockdown cells scattered significantly more than MCC-expressing control cells, which in primary tumours promotes invasion [41] and represents the first step in metastasis [42]. MCC-deficient cells also exhibited increased invasiveness in an organotypic invasion assay. This suggests that both the expression of E-cadherin and its interplay with its microenvironment can contribute to the invasive phenotype observed in MCC-deficient cells [36, 43, 44]. However, the role of MCC may be context-dependent and can be compensated for by other factors, because colorectal cancer lines with no MCC expression, such as HT-29, LS174T and Caco-2 form E-cadherin-containing cell-adhesion complexes.

Dasatinib, a candidate anti-invasive drug prevented both MCC-deficient and MCC-expressing cells from scattering when exposed to HGF and potently inhibited their invasion in organotypic assays. Dasatinib is a dual inhibitor of Bcr/Abl and Src family kinases, which stabilizes cell-cell junctions and prevents E-cadherin mobilization, degradation or cleavage [35, 36, 45]. Targeting Src family kinases by dasatinib has been shown to sensitise cancer cells to chemotherapy in preclinical models and dasatinib is currently being trialled in CRC in combination with 5-FU, leucovorin and oxaliplatin [46]. We previously reported that MCC methylation is associated with increased lymph node metastasis in CRC [6]. Here we show that MCC promoter methylation is associated with higher T stage, mucinous or circumferential tumours and the poorly differentiated tumour phenotype. Dasatinib prevented the increased invasiveness and enhanced HGF-induced scattering caused by MCC knockdown *in vitro*. These findings encourage the further evaluation of dasatinib as an anti-invasive therapy in CRC.

In summary, we have described a novel tumour suppressor function of MCC in the regulation of E-cadherin mediated cell-cell adhesion in CRC cells. Our study provides a significant advance in the current understanding of the protein-protein interactions that control cell-cell adhesion and suppress metastasis. Potential therapies exploiting our findings may include strengthening cell-cell junctions with dasatinib or other anti-invasive combination therapies or restoring the critical protein interactions that are lost when MCC is silenced in this highly aggressive disease.

Materials and methods

Patient cohort. A consecutive series of 366 patients who underwent surgery for stage I-IV colorectal cancer at the Sydney South West Area Health Service in 2000-2003 was selected for the study. The Australian National Statistical Service Sample Calculator was used for sample size and power calculations. Patients were only excluded if insufficient pathology tissue was available for DNA extraction. MCC methylation analysis was carried out as previously described [6, 47-49]. This project was approved by the Human Research Ethics Committee of Sydney Local Health District, X08-0224 & HREC 08/RPAH/376. The requirement to obtain informed consent was waived by the Committee for these archival specimens.

Cell lines. HCT116 and HCT15 (American Type Culture Collection, ATCC) were maintained in RPMI 1640 (Thermo Fisher, Waltham, MA) and 293T cells (ATCC) in DMEM medium, both supplemented with 10% FBS and 50 µg/mL gentamicin (Invitrogen, Carlsbad, CA). Cell authentication was carried out in December 2015 using the Multiplex STR system with the Promega 18D Powerplex Kit (Promega, Madison, WI). MCC was stably expressed in HCT15 cells using a doxycycline-inducible pCLX-pTF-DEST-EBR backbone lentiviral vector containing MCC.

EGTA treatment. HCT116 cells were grown to ~20% confluency and then synchronized by serum starvation for 24 hours. The cells were grown in full serum media for 40 hours and then treated with 2 mM EGTA (Santa Cruz, Dallas, TX) for 2, 8 and 24 hours, washed and fed with full serum media for 16 hours.

Blue-Native PAGE. Cells were grown in 6-well plates to ~90% confluency. Cells were scraped and washed in PBS and then lysed in 100 µL lysis buffer (20 mM Hepes, 10 mM KCl, 2 mM MgCl2, 1% NP-40) on ice for 15 minutes. The supernatant containing native proteins was collected following centrifugation at 15,000 x g for 10 minutes at 4^{o}C. Equal amount (10 µg) of proteins from each sample was loaded on NativePAGE^{TM} Novex^{TM} 4-16% Bis-Tris gel (Invitrogen, Carslbad, CA). Separation of native protein complexes was performed according to the manufacturer's instructions. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) Immobilon-P membrane (EMD Millipore, Billerica, MA) for Western Blot analysis.

Generating MCC shRNA lentiviruses. MCC Mission shRNA Human Gene Family Set (SHCLNG-NM_002387, Sigma-Aldrich, St Louis, MO) was used. Each clone was constructed in pLKO.1-Puro vector and contained an average of 3 MCC targeting constructs. A non-targeting scrambled shRNA with a non-human or mouse shRNA insert in a TRC1/1.5 vector backbone (SHC002, Sigma-Aldrich) was used as a control. The sequence for the control shRNA is CGAGTTGGTGCTCTTCATCTTGTTGTTTTT. Lentiviruses for each of the shRNAs were produced in 293T cells using the calcium phosphate method [50]. Best MCC knockdown was achieved with MCC-shRNA1 (CCGGGCTCCAATATCCAAGAGATTTCTCGAGAAATCTCTTGGATATTGGAG CTTTTTG) and MCC-shRNA2 (CCGGCACCAATGAAACTTCGCTTTACTCGAGTAAAGCGAAGTTTCATTGGT GTTTTTG).

Western blotting. The cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with cOmplete Protease Inhibitor Cocktail Tablet (Roche Applied Science, Penzburg, Germany) for whole cell lysates. SDS-PAGE was used to separate the cell lysates containing equal amounts of protein before they were transferred on a PVDF membrane. MCC (1:2000, sc-135982, Santa Cruz), E-cadherin (1:2000, 610181, BD Biosciences, Franklin Lakes, NJ), β-catenin (1:2000, 610153 BD Biosciences), Scrib (1:2000, C-20 sc-11049, Santa Cruz) and β-actin (1:10000, ab-6276, Abcam, Cambridge, UK) primary antibodies were used to probe the membrane. Bands were visualized by enhanced chemiluminescence (ECL) HRP substrate (Western Lightning Plus ECL, PerkinElmer, Waltham, MA).

Real-time PCR. RNA was extracted from exponentially growing cells using the RNeasy Mini Kit (Qiagen), and cDNA synthesis carried out using the QuantiTect Reverse Transcription Kit (Qiagen). Taqman assays (Life Technologies) were used for *MCC* (Hs01058597_m1) and *GAPDH* (Hs99999905_m1), and Roche UPL assays were used for B-catenin (*CTNNB1*) and E-cadherin (*CDH1*).

Immunofluorescence. 1 x 10^4 cells were seeded in a 24 well plate. Next day, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 minutes at room temperature (RT). The cells were then permeabilized with 0.1% Triton X-100 for 20 minutes at RT, washed with PBS and blocked with 10% FBS in PBS. The cells were then incubated in primary antibodies at RT for 1 hour. Primary antibodies used are E-cadherin (1:500), β-catenin (1:500), β-actin (1:500), MCC (BD Biosciences, 610740 1:500) and MCC (Proteintech, Rosemont, IL 11525-1-AP, 1:500). The appropriate secondary

antibody was added (1:500). Nuclei was stained with DAPI (1:1000). Cells were mounted on slides and images were collected using a Leica DMI 6000 SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Proximity Ligation Assay (PLA). PFA fixed cells were subjected to the Duolink Proximity Ligation Assay (Sigma) as described by the manufacturer. Primary antibodies were used at a 1:100 dilution (mouse E-cadherin, 610181, BD Biosciences; rabbit β-catenin, ab32572, Abcam; mouse β-catenin, 610153, BD Biosciences; mouse MCC, sc-135982, Santa Cruz; rabbit MCC, 11525-1-AP, Proteintech; rabbit IgG, sc2027, Santa Cruz). For E-cadherin/β-catenin, mouse and rabbit β-catenin were subjected to PLA for positive control and mouse E-cadherin and rabbit IgG (negative control IgG) or mouse E-cadherin alone (primary) were subjected to PLA for negative controls. PLA assays were counterstained with FITC-Phalloidin and DAPI and repeated 3 times. 5 z-stacks per condition per repeat were acquired on a Leica SP8 Confocal microscope. For quantification of foci a central image of each z-stack was quantified by counting the number of foci per cell. Qualitative assessment of MCC/E-cadherin and MCC/β-catenin interaction via PLA (2 repeats) was performed as described before but with mouse MCC and rabbit MCC as positive control.

Cell adhesion quantification. TEER was measured on cells seeded at high density on clear polyester Transwell permeable supports (Corning, NY) in growth medium described above. When cells reached 100% confluency, EVOM epithelial volt/ohmmeter with STX electrodes (World Precision Instruments, Sarasota, FL) was used to measure ohmic TEER. A minimum of 12 readings

were recorded per condition. The dispase assay was done on confluent cells as previously described [31, 36, 51].

Organotypic assay. $0.8x10^5$ of cells were seeded onto contracted collagen I matrices [36] in complete RPMI media. Cells were allowed to invade for 21 days [36]. The cultures were fixed using 10% formalin and processed by standard methods for haematoxylin and eosin (H&E) staining. Multi-cytokeratin, Ki67 and cleaved caspase 3 were stained using the Leica Bond RX system. Heat-induced epitope retrieval (HIER) was performed for 30 minutes with ER2 (EDTA pH9, DAKO) at 93^{o}C for Multi-cytokeratin and Ki67, and for 20 minutes with ER2 at 93^{o}C for cleaved caspase 3. Anti-Ki67 (1:500, clone SP6, Thermo Scientific) and anti-cleaved caspase 3 (1:500, #9661, Cell Signaling) primary antibodies were incubated for 60 minutes. Ki67 and cleaved caspase 3 staining was quantified by calculating the percentage of positively stained cells. For all scoring, ten different fields of view were counted.

Cell scattering. 5 x 10^3 cells were seeded per well in a 24 well plate. 2-3 days later when the cells started to form discrete colonies, the media was replaced with RPMI containing 2% FBS with either 30 ng/mL HGF (Sigma-Aldrich, St Louis, MO) alone or with 200 nM dasatinib (Sigma-Aldrich, St Louis, MO). The experiment was terminated after 24 hours and 100 colonies were scored as either “scattered” or “not scattered” per condition.

Co-immunoprecipitation. Immunoprecipitation of MCC from HCT116 was performed on cells resuspended in cold TNE buffer and from HCT15 cells from TNE buffer with 1% NP-40. Lysates were pre-cleared with G beads (Invitrogen)

and incubated with the appropriate primary antibody for 1 h at 4°C. Immunoprecipitates were resolved on 10% SDS-PAGE gel. Proteins were analyzed by Western blotting.

Statistical analysis. Unless otherwise indicated, t tests were performed using Graphpad Prism software (La Jolla, CA, USA). Two tailed Chi-Square testing was used to analyse correlations with clinicopathological variables. All error bars indicate standard error. Real-time PCR was quantified using the ΔCt method, normalized to the housekeeping gene GAPDH using Graphpad Prism. The P value level set for statistical significance is $P \leq 0.05$. All error bars indicate standard error.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

Conflict of interest

The authors declare no conflict of interest.

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672 **Table 1.** *MCC* methylation is associated with an aggressive tumour phenotype in
673 colorectal cancer patients.
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Characteristic		***MCC* Methylation (n=365)**		***P* value**
		Methylated n=131 (36%)	Unmethylated n=234 (64%)	
Site	Right colon	**67 (58%)**	**49 (42%)**	<0.0001
	Left colon	27 (28%)	68 (72%)	
	Rectum	37 (24%)	117 (76%)	
Tumour size	<20 mm	3 (17%)	15 (83%)	
	20-50 mm	78 (32%)	161 (68%)	
	>50 mm	**48 (48%)**	**53 (52%)**	0.007
Histology	Adenocarcinoma	106 (33%)	215 (67%)	
	Mucinous Ca	**22 (58%)**	**16 (42%)**	0.004
Differentiation	Well	2 (25%)	6 (75%)	
	Moderate	96 (32%)	202 (68%)	
	Poor	**31 (66%)**	**16 (34%)**	<0.0001
Pathological T stage	TIS,T1,T2	23 (24%)	73 (76%)	
	T3,T4	**108 (40%)**	**161 (60%)**	0.005
Circumferential tumour	Yes	**57 (45%)**	**70 (55%)**	0.008
	No	72 (31%)	162 (69%)	

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Figures

Figure 1. MCC is a membrane-associated protein that localizes and interacts with E-cadherin and β-catenin in colon cancer cells.

(**A**) Endogenous MCC co-localises with E-cadherin at linear adherens junctions (AJ) at cell-cell contacts (arrow) and with β-catenin. Indirect immunofluorescence assay was performed using anti-MCC, anti-E-cadherin and anti-β-catenin antibodies on colon epithelial HCT116 cells. DAPI stains the nucleus blue. Scale bar, 25 μM. Representative images are from 3 independent experiments (*n*=3).

(**B**) Endogenous MCC forms a complex with E-cadherin and β-catenin. Co-immunoprecipitation assays were performed on protein lysate of HCT116 cells. IgG antibody (IP IgG) used as a negative control. Asterisk denotes a non-specific second band.

(**C**) MCC is found in a ~720 kDa complex similar to E-cadherin and β-catenin (arrow); and a 600 kDa complex similar to SCRIB. Blue-Native PAGE and Western Blot were performed to detect native protein complexes extracted from HCT116 cells in a non-denaturing condition. Antibodies were used to detect MCC, SCRIB, E-cadherin and β-catenin.

(**D**) Similar to the 720 kDa E-cadherin/β-catenin complex, the 720 kDa MCC complex was identified in the cellular membrane fraction. Membrane (M) and cytosolic (C) proteins were extracted from HCT116 cells by fractionation lysis according to [52]. BN-PAGE and Western Blot were performed on the protein fractions to detect the level of E-cadherin, β-catenin and MCC protein complexes.

(**E**) MCC was found in the same 720 kDa complex with E-cadherin and β-catenin. BN-PAGE analysis of the E-cadherin adhesion complex immunodepleted with either E-cadherin, β-catenin or MCC antibodies (lane 2, 3, 4). IgG was also used as control depletion (lane 1). E-cadherin protein complexes were detected by

Western Blot using antibody against E-cadherin. Immunodepletion assays were performed on native proteins extracted from HCT116 cells. Arrow indicates reduced level of 720 kDa E-cadherin complex after depletion of MCC, relative to IgG control.

Figure 2. MCC knockdown disrupts the E-cadherin/β-catenin complex and cell-cell adhesion in colon cancer cells.

(**A**) MCC knockdown did not affect *CDH1* or *CTNBB1* mRNA expression. mRNA level of *MCC*, *CDH1* and *CTNNB1* in NT control, MCC shRNA1 and MCC shRNA2 lines (* indicates P≤0.05 & ** indicates *P*=0.001-0.01, t-test, (*n*=3). RT-PCR values were normalized to the housekeeping gene *GAPDH*.

(**B**) MCC knockdown reduced the abundance of the E-cadherin/β-catenin complex. Non-targeting (NT) scrambled control, *MCC* shRNA1 and shRNA2 were delivered by lentiviruses to stably knock down MCC in isogenic HCT116 cells. E-cadherin, β-catenin and MCC protein expression was measured in the total lysate by Western Blot. On the same gel, immunoprecipitation of the interaction between E-cadherin, β-catenin and MCC was tested. E-cadherin was IP'd from NT control, MCC shRNA1 and MCC shRNA2 lines. GAPDH was used for loading control.

(**C**) MCC knockdown reduced E-cadherin protein expression in HCT116 cells. Densitometric analysis used to quantify protein expression relative to β-actin from three independent experiments (*n*=3), expressed as fold change. P values were calculated using the t-test.

(D) MCC overexpression via doxycycline induction (+Dox) increased the abundance of the E-cadherin/β-catenin complex. A pCLX-pTF-DEST-EBR backbone lentiviral vector containing MCC was introduced into HCT15 cells for

stable expression. Treating these cells with 1 μg/mL of doxycycline induced MCC expression and increased E-cadherin/β-catenin interaction as shown by IP. E-cadherin was IP'd from HCT15 cells in the absence (-Dox) and presence of doxycycline (+Dox). GAPDH was used for loading control.

(**E-G**) MCC modules cell-cell adhesion in colon epithelial cells.

(**E**) Monolayers of HCT116 cells were lifted from culture dishes by using dispase II, a neutral protease that cleaves the basement membrane while preserving the viability of epithelial cells. NT control, *MCC* shRNA1 and shRNA2 cells were allowed to grow to 100% confluency. Following treatment with dispase, the cells were mechanically disrupted and the number of single cells was expressed as a percentage of all detached cells (control) and is represented as mean±S.E.M., *n*=9. Dispase measurements were also recorded on untreated and doxycycline-treated HCT15 cells as described above.. All P-values were calculated using the t-test.

(**F**) Schematic diagram of TEER measurement apparatus.

(**G**) Cell-cell integrity was quantified by transepithelial electrical resistance (TEER) measurement. Electrical resistance (Ohms) was quantified on confluent monolayers in NT control cells, *MCC* shRNA1 and shRNA2 cells by EVOM2. 12 readings were recorded per experiment and the experiment was repeated 3 times (*n*=3). Results are represented as mean±S.E.M. TEER measurements were recorded as above on untreated and doxycycline-treated HCT15 cells. P-values were calculated using the t-test. For **(A, C, E, G)**, * indicates P ≤ 0.05, ** indicates *P* ≤ 0.01, *** indicates *P* ≤ 0.001, **** indicates *P* ≤ 0.0001. Error bars indicate standard error.

Figure 3. MCC knockdown accelerates HGF induced cell scatter and HGF-induced scattering is prevented by dasatinib in colon epithelial cells.

(**A**) MCC deficiency exacerbates HGF-induced cell scatter. MCC expressing NT control cells and *MCC* shRNA1 and shRNA2 cells were seeded at low confluency and allowed to form discrete colonies for 48 hours. Cell scattering was induced with 30 ng/mL HGF and scattering was monitored for 24 hours post-HGF treatment. Cell scattering was measured by live imaging (time-lapse) using the Incucyte Zoom. Images were taken every 2 hours. A colony was classified as scattered when at least 50% of its cells had lost contact with their neighbours as previously described [53]. The percentage of cell scattering for the three cell lines was plotted in a line graph. *P* value assessed at the 24 hour time point using the t-test (*n*=3).

(**B**) MCC deficiency exacerbates loss of E-cadherin and enhances β-catenin nuclear localization following HGF treatment. Immunofluoresence analysis of E-cadherin membrane staining and β-catenin subcellular localization 8 hours following 30 ng/mL HGF treatment in MCC-expressing (NT) and MCC-deficient (shRNA) HCT116 cells.

(**C**) Representative cell scatter images of MCC-deficient HCT116 cells (shRNA2) treated with HGF alone (30 ng/mL) or both HGF and dasatinib (200 nM) for 24 hours.

(**D**) Dasatinib prevented HGF-induced cell scattering in MCC-deficient cells. Colonies of MCC-deficient cells were administered either 30 ng/mL of HGF alone or HGF and 200 nM of dasatinib. Cell scattering was monitored by live imaging over a 24 hour period. The percentage cell scattering for the three cell lines was plotted in a line graph. *P* value was assessed at the 24 hour time point using the t-test (*n*=3).

(**E**) Measurements of electrical resistance with or without 200 nM of dasatinib for 4 hours were recorded for NT control and MCC-shRNA cells. 14 readings were recorded per experiment and the experiment was repeated 3 times (*n*=3). Results are represented as mean±S.E.M. For (**A, D & E**), * indicates P ≤ 0.05, ** indicates *P* ≤ 0.01, *** indicates *P* ≤ 0.001, **** indicates *P* ≤ 0.0001. Error bars indicate standard error and P values were calculated using the t-test.

Figure 4. MCC knockdown promotes cell invasion in a 3D organotypic invasion assay, and dasatinib inhibits invasion caused by MCC deficiency. Cells were seeded on fibroblast-contracted collagen I matrices and were allowed to invade for 21 days towards a chemoattractive gradient created by an air:liquid interface. During invasion, cells were treated with vehicle (DMSO) or 200 nM dasatinib. The matrices were fixed in formalin, embedded in paraffin and stained with multi-cytokeratin (Multi-CK), Ki67 and cleaved caspase 3 antibodies. (**A**) Multi-CK stains epithelial cells. The number of invading NT control, shRNA1 and shRNA2 cells were counted in 10 separate fields of view at 10X magnification. Cells were scored as invasive when they were completely surrounded by matrix. Ki67 (**B**) and cleaved caspase 3 (**C**) percentage (%) positivity was scored from 10 separate fields of view at 10X magnification. Three matrices were stained per condition. The experiment was repeated three times and the average was recorded. For (**A-C**), * indicates P ≤ 0.05, ** indicates *P* ≤ 0.01, *** indicates *P* ≤ 0.001, **** indicates *P* ≤ 0.0001. n.s. denotes *P* > 0.05 and hence a non-significance. The experiment was repeated 3 times (n = 3) with 3 technical replicates. Error bars indicate standard error. All P-values were calculated using the t-test.