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Overexpression of the receptor for hyaluronic acid mediated motility is an independent adverse prognostic factor in colorectal cancer

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RHAMM, a member of the microtubule-associated protein family that interacts with the mitogen-activated protein kinase pathway, is associated with tumor progression, aggressive disease and shortened survival in several tumor types. This study aimed to determine the prognostic value of RHAMM in colorectal cancer (CRC). A series of 1420 unselected, nonconsecutive CRC resections were subdivided into three groups: (1) DNA mismatch repair (MMR)-proficient, (2) MLH1 negative and (3) presumed Lynch syndrome. Immunohistochemical analysis of RHAMM expression (0 *vs* >0%), increasing expression (increasing percentage positivity) and complete expression (100 *vs* <100%) was performed using tissue microarray technique and the results were correlated with clinicopathological parameters. Fifty-seven tissue samples of normal colonic mucosa were included as a control group. In a univariate analysis increasing and complete expression of RHAMM were associated with higher N stage ($P=0.023$ and 0.021) and worse survival ($P<0.0001$) in MMR-proficient CRC. Complete expression of RHAMM was associated with worse survival in presumed Lynch syndrome ($P=0.016$). In MLH1-negative CRC there was no association between RHAMM expression and the clinicopathological features. In a multivariate analysis, increasing RHAMM expression was an independent adverse prognostic factor in MMR-proficient CRC ($P<0.0001$) and complete expression in MMR-proficient CRC and presumed Lynch syndrome ($P<0.0001$ and $P=0.031$, respectively). Nuclear pERK expression was associated with increasing RHAMM expression in MMR-proficient CRC ($P=0.012$) and with complete RHAMM expression in presumed HNPCC ($P=0.03$). Increasing and complete RHAMM expressions are independent adverse prognostic factors in MMR-proficient CRC and presumed Lynch syndrome.
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The receptor for hyaluronic acid mediated motility (RHAMM; CD168, intracellular hyaluronic acid binding protein) has a cell surface and intracellular distribution.[1] RHAMM binds hyaluronan,[2] interacts with both mictotubules and microfilaments,[3,4] localizes to the centrosome maintaining the spindle integrity[5] and is suggested to represent a member of the MAP family.[3] RHAMM is involved in cell motility and signaling[6] as well as oncogenic events.[7]

The mitogen-activated protein kinase (MAPK) pathway includes several families of signal transduction cascades which mediate information provided by extracellular stimuli.[8] The MAPK pathway is considered to be important for cellular growth, development and differentiation and regulates cell proliferation, apoptosis, cell differentiation and tissue development.[9] The Raf-MEK-ERK pathway belongs to the MAPK pathways and represents one of the best characterized Ras signaling pathways.[10] The molecule ERK is activated by a cascade of phosphorylation events downstream from the ras proto-oncogene[8] and plays a role in differentiation, secretion, proliferation and hypertrophy.[11] RHAMM binds ERK kinase[12] and controls expression levels of ERK.[13]

There is evidence that RHAMM influences tumor progression and metastasis in different tumor types including pancreatic cancer,[14] stomach cancer,[15] endometrial carcinomas,[16] breast cancer,[4,13,17]

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transitional cell carcinomas of urinary bladder,[18] aggressive fibromatosis (desmoid tumor),[19] lung cancer,[20] B-cell malignancies[1,21–23] and melanoms.[24] However, the prognostic significance of RHAMM in colorectal cancer (CRC) is poorly understood. The aim of this study was therefore to determine the prognostic significance of expression, increasing expression and complete expression of RHAMM assessed by means of immunohistochemistry (IHC) in 1420 tissue microarray (TMA) specimens stratified into mismatch repair (MMR) proficient, MLH1 negative and presumed Lynch syndrome/hereditary nonpolyposis colorectal cancer (HNPCC) and to in vestigate the interaction between RHAMM and pERK.

Materials and methods

Tissue Microarray Construction

A TMA of 1420 unselected, nonconsecutive CRCs was constructed as described previously.[25] Formalin-fixed, paraffin-embedded tissue blocks of CRC resections were retrieved from the archives of the Institute of Pathology, University Hospital of Basel, Switzerland, the Institute of Clinical Pathology, Basel, Switzerland and the Institute of Pathology, Stadtspital Triemli, Zürich, Switzerland. One tissue cylinder with a diameter of 0.6 mm was punched from morphologically representative tissue areas of each 'donor' tissue block and brought into one recipient paraffin block (3×2.5 cm) using a homemade semiautomated tissue arrayer. Failure of analysis including missing samples or fractions containing only a few tumor cells was related to tissue microarray technology.

Clinicopathological Data and Tumors

CRC resections were subdivided into three CRC subsets: (1) DNA MMR-proficient (expressing MLH1, MSH2 and MSH6), (2) MLH1 negative and (3) presumed Lynch syndrome/HNPCC (MSH2 and/or MSH6 negative at any age or MLH1 negative and <55 years of age). These immunohistochemical groupings presented a good fit to the known clinicopathological features associated with these groups of CRC. While a small proportion of presumed sporadic MSI-H and HNPCC cases may have been incorrectly assigned, the overall findings are likely to be valid in view of the large numbers of samples and the good fit with clinicopathological features.

One pathologist (L.Te.) systematically re-evaluated the clinicopathological data with respect to CRCs. The clinicopathological data of the different CRC subsets are summarized in Table 1. Any disagreement between the clinicopathological features and numbers of available tissue punches shown in Table 1 is due to missing clinicopathological data.

Immunohistochemistry of TMA

Sections (4 μm) of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ, USA) to facilitate the transfer of tissue microarray sections to slides and to minimize tissue loss. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vector Laboratories, Burlingame, CA, USA). 1420 CRCs and 57 normal colonic mucosa samples were immunostained for RHAMM (clone 2D6; dilution 1:25, Novocastra, UK), MLH1 (clone MLH-1; dilution 1:100; BD Biosciences Pharmingen, San Jose, CA, USA), MSH2 (clone MSH-2; dilution 1:200; BD Biosciences Pharmingen, San Jose, CA), MSH6 (clone 44; dilution 1:400; BD Biosciences Pharmingen, San Jose, CA). After dewaxing and rehydration in dH2O, sections for immunostaining were subjected to heat antigen retrieval in a microwave oven (1200 W, 15 min) in 1 mM EDTA buffer pH 9.0 for RHAMM, 0.001 mol/l ethylenediaminetetraacetic acid, pH 8.0 for MLH1 and MSH2 and 0.01 mol/l citrate buffer pH 6.0 for MSH6. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. After transfer to a humidified chamber, the sections were incubated with 10% normal goat serum (Dako Cytomation) for 20 min and incubated with primary antibody at 4°C overnight for hMLH1, hMSH2 and hMSH6 and at room temperature for RHAMM (1 h). Subsequently, the sections were incubated with peroxidase-labeled polymer (K4005, EnVision+ System-HRP(AEC); DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole + substrate-chromogen (K4005, EnVision+ System-HRP (AEC); DakoCytomation) for 30 min, and counterstained lightly with Gill's haematoxylin.

RHAMM immunoreactivity was evaluated using the percentage of positive cells ranging from 0 to 100%. RHAMM expression was defined as 0 *vs* >0%, increasing expression as increasing percentage positivity and complete expression as 100 *vs* <100%. Normal colonic mucosa was considered as baseline to determine RHAMM expression in CRC and cells were scored as positive when the intensity of baseline expression was clearly exceeded. Immunohistochemistry for MLH1, MSH2 and MSH6 was scored as negative when no staining (0%) was observed and as positive when any immunoreactivity (>0%) was found.

Statistical Analysis

Clinicopathological characteristics across CRC groups were analyzed using the Kruskal–Wallis and χ^2 tests. Univariate analysis of 5-year survival rates across CRC groups and according to RHAMM expression and complete expression was carried out using the Kaplan–Meier method and log-rank test. The distribution of RHAMM expression across

Table 1 Clinicopathological characteristics of 1420 CRC patients

	Total (%)	*MMR-proficient (%)*	*MLH1 negative (%)*	*Presumed Lynch syndrome (%)*	P-*value*
Number (*n*)	1420	1197 (84.30)	141 (9.93)	82 (5.77)	<0.0001
Age, median (min, max) (years)	71 (30, 96)	71 (30, 96)	76 (56, 93)	60 (37, 82)	<0.0001
Sex					
Male	673 (47.60)	595 (49.96)	41 (29.08)	37 (45.12)	<0.0001
Female	741 (52.40)	596 (50.04)	100 (70.92)	45 (54.88)	
Anatomic site of the tumor					
Right sided	489 (34.90)	344 (29.20)	112 (79.43)	33 (40.24)	<0.0001
Left sided	912 (65.10)	834 (70.80)	29 (20.57)	49 (59.76)	
Tumor size, median (min, max)	4.5 (0.4–17.0)	4.5 (0.4–15.0)	5.5 (1.5–17.0)	5.0 (2.5–13.0)	<0.0001
T stage					
T1	62 (4.47)	60 (5.15)	0	2 (2.44)	<0.0001
T2	203 (14.64)	189 (16.22)	6 (4.29)	8 (9.76)	
T3	899 (64.82)	740 (63.52)	97 (69.29)	62 (75.61)	
T4	223 (16.08)	176 (15.11)	37 (26.43)	10 (12.20)	
N stage					
N0	711 (52.16)	587 (51.40)	79 (56.83)	45 (54.88)	0.593
N1	358 (26.27)	308 (26.97)	29 (20.86)	21 (25.61)	
N2	294 (21.57)	247 (21.63)	31 (22.30)	16 (19.51)	
Tumor grade					
G1	31 (2.24)	27 (2.32)	2 (1.44)	2 (2.47)	<0.001
G2	1177 (84.98)	1010 (86.70)	103 (74.10)	64 (79.01)	
G3	177 (12.78)	128 (10.99)	34 (24.46)	15 (18.52)	
Vascular invasion					
No	1002 (72.35)	834 (71.53)	104 (75.36)	64 (79.01)	0.244
Yes	383 (27.65)	332 (28.47)	34 (24.64)	17 (20.99)	
5-year survival, mean ± s.e. (months)	31.90 ± 0.77	31.87 ± 0.81	28.40 ± 2.56	20.39 ± 1.62	<0.0001

CRC groups was evaluated using the χ^2 test. The association of clinicopathological characteristics and expression, increasing expression and complete expression of RHAMM were performed using univariate regression analysis. To determine whether RHAMM was a prognostic indicator of 5-year survival independent of T stage, N stage, tumor grade and vascular invasion, the Cox-proportional hazard method was used. *P*-values <0.05 were considered statistically significant. All analyses were carried out using SAS (Version 9.1, The SAS Institute, NC, USA).

Results

Normal Colonic Mucosa

In normal colonic mucosa RHAMM was diffusely but weakly expressed in the cytoplasm of columnar cells of the crypts, but apparently not in the goblet cells (Figure 1a). RHAMM was less strongly expressed as compared to the cancer cell population. A more detailed analysis was limited by the small number of samples and the lack of the surface epithelium in most of the tissue microarray samples.

Distribution of Cytoplasmic RHAMM Expression in the Different CRC Subsets (Table 2)

A different percentage of cytoplasmic RHAMM-positive tumors occurred across MMR proficient (95.7%), MLH1 negative (91.7%) and presumed Lynch syndrome (79.2%) subgroups ($P<0.001$) (Figure 1b–d).

MMR-Proficient CRC (Table 3)

In a univariate analysis increasing and complete expression of RHAMM was associated with higher N stage ($P=0.023$ and $P=0.021$) and worse survival ($P<0.0001$) in MMR-proficient CRC (Figure 2).

MLH1-Negative CRC (Table 4)

In a univariate analysis there was no association between expression, increasing expression and complete expression of RHAMM and the clinicopathological features including T stage, N stage, tumor grade, vascular invasion and survival.

Presumed Lynch Syndrome (Table 5)

In a univariate analysis RHAMM expression was associated with lower tumor grade ($P=0.004$) and

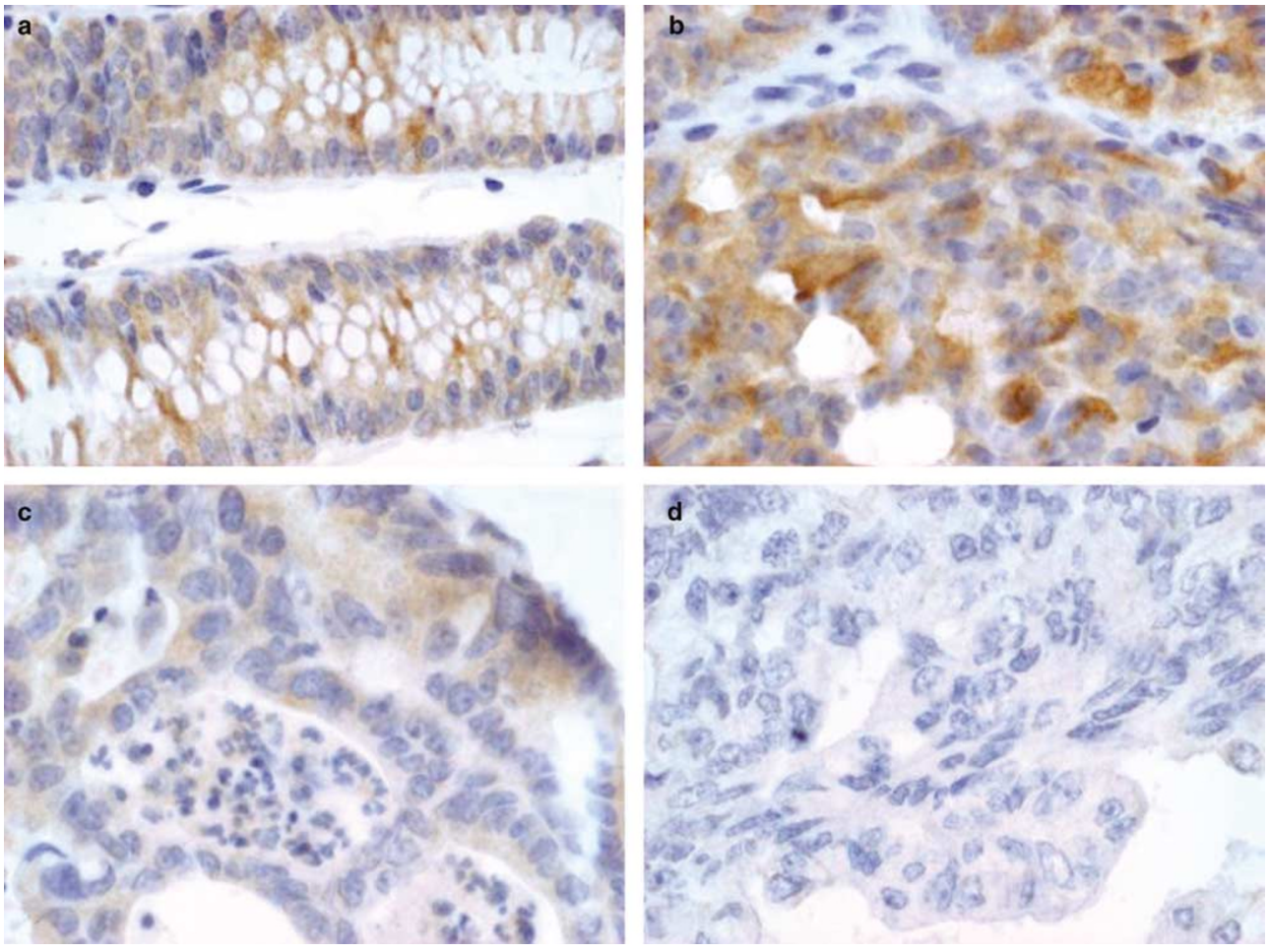

Figure 1 Cytoplasmic expression of RHAMM in normal colonic mucosa (**a**) (40 ×). Complete (**b**), focal (**c**) and no (**d**) RHAMM expression in a moderately differentiated MMR-proficient CRC (40 ×).

Table 2 Distribution of cytoplasmic RHAMM positivity (>0%) in the different CRC subsets

	MMR-proficient		*MLH1 negative*		*Presumed Lynch syndrome*		P-*value*
	N	%	N	%	N	%	
RHAMM (c) 0%	42	4.3	11	8.3	15	20.8	<0.0001
RHAMM (c) >0%	925	95.7	121	91.7	57	79.2	

N, number; c, cytoplasmic.

Table 3 Association of RHAMM positivity and the clinicopathological parameters in MMR-proficient CRC (*P*-values)[a]

	Expression	*Increasing expression*	*Complete expression*
T stage	0.372	0.894	0.569
N stage	0.25	0.023	0.021
Tumor grade	0.902	0.123	0.443
Vascular invasion	0.976	0.98	0.221
Survival	0.773	<0.0001	<0.0001

[a]Univariate analysis.

complete expression with worse survival ($P=0.016$). Increasing expression of RHAMM was not associated with the clinicopathological parameters.

Multivariate Analysis of Survival

Increasing RHAMM expression was an independent adverse prognostic factor in MMR-proficient CRC ($P<0.0001$) and complete expression in MMR-proficient CRC and presumed Lynch syndrome ($P<0.0001$ and $P=0.031$, respectively).

Association between Cytoplasmic RHAMM and Nuclear pERK (Table 6)

In MMR-proficient CRC increasing RHAMM expression was associated with nuclear pERK expression ($P=0.012$), whereas complete expression of RHAMM was associated with pERK expression in presumed Lynch syndrome ($P=0.03$). In MLH1-negative CRC RHAMM was not associated with pERK.

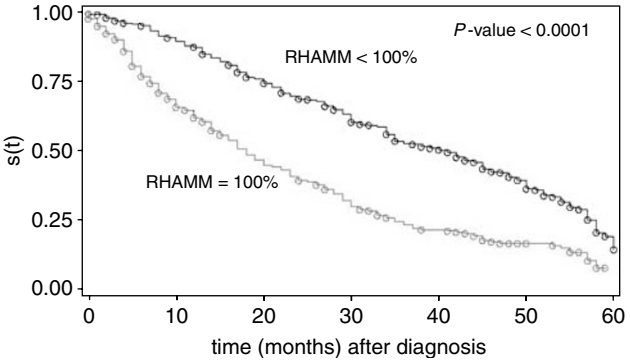

Figure 2 Kaplan–Meier 5-year survival curve for complete RHAMM expression (100%) in MMR-proficient CRC. Median survival time for RHAMM = 100%: 18 months (CI 95%: 15–22 months) and for RHAMM <100%: 41 months (CI 95%: 35–45 months).

Table 4 Association of RHAMM positivity and the clinicopathological parameters in MLH1-negative CRC (*P*-values)[a]

	Expression	*Increasing expression*	*Complete expression*
T stage	0.305	0.399	0.273
N stage	0.132	0.531	0.077
Tumor grade	0.789	0.708	0.704
Vascular invasion	0.212	0.473	0.314
Survival	0.646	0.467	0.555

[a]Univariate analysis.

Table 5 Association of RHAMM positivity and the clinicopathological parameters in presumed Lynch syndrome (*P*-values)[a]

	Expression	*Increasing expression*	*Complete expression*
T stage	0.204	0.622	0.673
N stage	0.345	0.793	0.402
Tumor grade	0.004	0.22	0.157
Vascular invasion	0.136	0.961	0.418
Survival	0.878	0.337	0.016

Discussion

In this study, we used TMA technology and IHC on a large number of unselected CRC cases ($n=1420$), stratifying these according to MMR repair status, and describing cytoplasmic RHAMM-positive tumor cells in every punch sample. This 'descriptive' evaluation system has the advantage of defining marker expression (0 *vs* >0%), increasing expression (percentage of positive tumor cell staining) and complete expression (100 *vs* <100%) avoiding an 'interpretative' and often complex composite scoring system.

In normal colonic mucosa RHAMM was diffusely expressed in the cytoplasm of columar cells in the crypts, but apparently not in the goblet cells. RHAMM expression was weaker and quantitatively less extensive as compared to cancer cells.

Our results point to biological differences between expression (0 *vs* >0%), increasing expression (increasing percentage positivity) and complete expression (100 *vs* <100%) of RHAMM in CRC. RHAMM expression was not associated with tumor progression and worse survival in all three CRC subgroups, which can be explained by the fact that RHAMM expression was found in normal colonic mucosa. This suggests that increasing RHAMM expression is needed to induce tumor progression. Indeed, in MMR-proficient CRC increasing and complete expression of RHAMM were correlated with higher N stage and worse survival in a univariate analysis and were independent adverse prognostic factors in a multivariate analysis, whereas in presumed Lynch syndrome complete RHAMM expression was associated with worse survival in univariate and multivariate analysis. These findings fit with the results obtained in a recent study in which using RT-PCR on tissue specimens of patients with CRC RHAMM mRNA levels were higher in tumor tissue when compared to adjacent normal tissue.[26]

Our findings are also in agreement with previous studies that assessed RHAMM expression and the

Table 6 Expression, increasing expression and complete expression of cytoplasmic RHAMM associated with expression and increasing expression of nuclear pERK in different CRC subsets (*P*-values)

	MMR proficient			*MLH1 negative*			*Presumed Lynch syndrome*		
	E	*IE*	*CE*	*E*	*IE*	*CE*	*E*	*IE*	*CE*
pERK expression (>0%)	0.063	0.012	0.216	0.967	0.779	0.338	0.962	0.106	0.03
pERK increasing expression (increasing %-positivity)	0.238	0.755	0.92	0.503	0.208	0.087	0.895	0.074	0.195

E, expression; IE, increasing expression; CE, complete expression.

[a]Univariate analysis.

associations with clinicopathological parameters in different tumor types. In endometrial carcinoma Rein *et al*[16] found a significant correlation between RHAMM expression and lymph node metastasis and Assmann *et al*[4] showed an association between trabecular (trabeculae = single cells in the stroma which appear to be budding off from the main tumor mass) RHAMM expression and worse survival in breast cancer. RHAMM expression was also associated with tumor progression in transitional cell carcinomas of urinary bladder[18] and stomach cancer.[15]

In a recent study, we analyzed the association between expression (0 *vs* >0%) and increasing expression (increasing percentage of positivity) of nuclear pERK and different clinicopathological parameters including T stage, N stage, tumor grade, tumor budding, vascular invasion and survival in MMR proficient, MLH1 negative and presumed Lynch syndrome.[27] Only nuclear pERK expression was associated with tumor budding in the MMR-proficient CRC subgroup, whereas no association was observed between nuclear pERK expression/increasing expression and the clinicopathological features in the MLH1 negative and the presumed Lynch syndrome subgroups. Tumor budding is defined as the presence of isolated single cells or small cell clusters (up to 4) scattered in the stroma at the invasive tumor margin and is established as an adverse prognostic indicator.[28–33] Tumor budding is at least in part driven by the wnt signaling pathway as attested by the fact that nuclear β-catenin accumulates in the nuclei in tumor buds (dedifferentiated cancer cells) at the invasive tumor border.[34–37]

In the present study, nuclear pERK expression was correlated with increasing RHAMM expression in MMR proficient ($P = 0.012$) and with complete RHAMM expression in presumed Lynch syndrome ($P = 0.03$), whereas an association was not found in MLH1-negative CRC. This finding leads to the hypothesis that pERK is involved in the mechanism of tumor progression of MMR-proficient CRC and Lynch syndrome by interacting with the wnt signaling pathway and RHAMM: (1) KRAS mutation is found in approximately 35% of unselected CRCs, whereas it is mutated at a particularly low frequency in sporadic MSI-H cancers.[38–42] (2) The molecule ERK, a member of the MAPK pathway, is activated by a cascade of phophorylation events downstream from the ras proto-oncogene.[8] (3) Intracellular and cell surface RHAMM isoforms are important for the activation of ERK by PDGF (platelet-derived growth factor) and mutant (activated) RAS, respectively, while intracellular RHAMMv4 overexpression activates ERK.[12] (4) ERK interacts with the wnt signaling pathway by inactivating GSK3β which is a part of a complex (with APC and axin) responsible for the degradation of β-catenin.[43,44] (5) Dysregulation of the wnt signaling pathway (activating mutation of β-catenin and inactivating mutation of APC) is more likely to occur in MMR proficient than in MLH1-negative CRC.[45,46] (6) Although oncogenic mutation of β-catenin has been linked with MSI-H CRC, this association is only with Lynch syndrome (and only around 20% of these) and not in MLH1-negative CRC.[41,46]

Therefore, pERK may not function in isolation as a prognostic factor in CRC (the same observation was made by by Wang *et al*[13] in breast cancer), but may be implicated in CRC progression through its interactions with the wnt signaling pathway and RHAMM.

In summary, our study has shown that complete expression of RHAMM is an independent adverse prognostic factors in MMR-proficient CRC and presumed Lynch syndrome. Additionally, RHAMM expression is correlated with nuclear pERK in both groups of CRC.

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