

MAPT promoter CpG island hypermethylation is associated with poor prognosis in patients with stage II colorectal cancer

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Background: The methylation of microtubule-associated protein tau (*MAPT*) was first described in patients with Alzheimer's disease. In this study, we aim to determine if *MAPT* promoter CpG island is hypermethylated and whether this signature could work as a prognostic marker for patients with stage II colorectal cancer (CRC).

Methods: *MAPT* methylation level and CpG island methylator phenotype (CIMP) status were examined. The prognostic value of *MAPT* methylation was analyzed using Cox regression analysis.

Results: Amongst stage II CRC patients (n=107), hypermethylation of *MAPT* promoter CpG island was seen in 23.4% of them. *MAPT* methylation was much more frequent in patients with age ≥ 60 compared to age < 60 ($P < 0.001$). *MAPT* were preferentially methylated among proximal colon tumors or CIMP high tumors (both $P < 0.001$). Five-year overall survival (OS) rates were 57.1% and 79.4% for patients with and without *MAPT* hypermethylation, respectively, HR=2.33 (95% CI, 1.19–4.57; $P = 0.014$). *MAPT* hypermethylation remained an important prognostic variable for OS in multivariate analysis with a HR of 2.29 (95% CI, 1.01–5.18; $P = 0.047$).

Conclusion: Our findings suggest that *MAPT* is frequently methylated and hypermethylation is associated with worse prognosis in patients with stage II CRC.

Keywords: colorectal cancer, methylation, *MAPT*, prognosis

Introduction

Colorectal cancer (CRC), the second and third most common cancer in women and men, respectively, causes 832,000 deaths in 2015 worldwide.¹ CRCs develop as a result of the accumulation of genetic and epigenetic alterations. CpG island methylator phenotype (CIMP) is one of the main mechanisms underlying CRC progression, which is characterized by the simultaneous methylation of cytosine residues in CpG islands from the promoter regions of multiple cancer-specific genes.² Widespread changes in DNA methylation are common in CRC and may confer to oncogenesis through transcriptional silencing of tumor-suppressor genes.³

Microtubule-associated protein tau (*MAPT*) is a gene located on chromosomal subband 17q21.⁴ Tau protein encoded by *MAPT* is among the best characterized microtubule-associated protein that promotes microtubule assembly and reduces microtubule instability.⁵ In addition to neurons, it is expressed at low levels in several non-neuronal cells. It has been shown to be involved in a number of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease,

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and progressive supranuclear palsy, etc. The ability of tau to induce microtubule polymerization⁶ and its interaction with actin⁷ indicates a possible role for tau in tumor cell migration and invasion. However, the function of tau in cancer remains relatively unexplored.

Recently, methylation of the *MAPT* promoter has been reported in a number of human diseases, including Alzheimer's disease,⁸ Parkinson's,⁹ and prostate cancer.¹⁰ However, the methylation status of *MAPT* and its influence on prognosis in CRC remains unclear. The goal of this study was to assess the methylation of *MAPT* promoter CpG island to determine its prognostic value in a cohort with 107 stage II CRC patients.

Materials and methods

Subjects for methylation analysis

Colorectal tissue samples were acquired by surgical resection from 107 patients (47 male and 60 female) with stage II CRC at the Johns Hopkins Hospital and the Johns Hopkins Bayview Hospital between 1995 and 2009. The median age was 67.8 years (range, 37–90 years). Tissue samples were available from all patients for molecular analysis; in 50 of these samples, a matched nontumor colorectal tissue was also obtained that was at least 2 cm distant from the tumor and in which cancer cell infiltration was ruled out by histologic review. Patients who were willing to participate provided written informed consent. This study was with approval from the Institutional Review Board and in accordance with Health Insurance Portability and Accountability Act regulations.

MAPT methylation analysis by quantitative real-time methylation-specific PCR (Q-MSP)

Q-MSP was used to assess *MAPT* methylation in primary tumors. Primers were designed using MSPprimer.¹¹ Sequences are listed in Table 1. DNA was extracted and 1 µg was used for bisulfite conversion with the EZ DNA methylation Kit (Zymo Research). The Q-MSP was carried out in duplicates in 96-well plates using a total reaction volume of 20 µL including 10.0 µL of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 2.5 pmol each of forward and reverse primers, and 2 µL of DNA template. Fragments were amplified at 95°C for 10 mins, and 40 cycles of 95°C for 15 s followed by 60°C for 60 s using the Applied Biosystems 7500 One-Step Plus Real-Time PCR System. Dissociation curve analysis was performed to confirm the specificity of

Table 1 Primers for quantitative real-time methylation-specific PCR of MAPT

Unmethylation		Methylation	
Forward primer sequence	Reverse primer sequence	Forward primer sequence	Reverse primer sequence
GTTTTGTGGAGTTGTGTGTTG	ACCAACAAAAAACACACATTCTTAAACCAACA	CGGAGTTCGCGTTGTTT	AAACGCGTTCTTAAACCGACG

amplicons. Cycle threshold (Ct) values were used to calculate methylation index (MI) using the following formula: $MI=100/[1+2^{(C_{tm}-C_{tu})}]$. C_{tm} and C_{tu} indicate Ct value of the probes specific for the methylated and unmethylated states, respectively.

CIMP methylation status analysis by methylight

A 5-gene signature including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* was used to assess CIMP methylation status in tumor tissues.¹² Methylation was quantified using MethyLight.¹² Alu was used as a normalization control. A 5' FAM fluorophore, a 3' IBFQ quencher, and an internal ZEN quencher (Integrated DNA Technologies) were used for all CIMP probes.¹³ DNA methylation was calculated as percent of methylated reference (PMR)= $100 \times [(\text{methylated reaction}/\text{Alu})_{\text{sample}}/(\text{methylated reaction}/\text{Alu})_{\text{M.SssI-reference}}]$. Genes were considered methylated when $PMR \geq 0$. When at least three of the five studied markers were methylated, the samples were considered CIMP high.¹²

Statistical analysis

For statistical analyses, the SPSS 18.0 (SPSS, Chicago, IL, USA) was used. Determination of cutoff values was made by the receiver-operator characteristics (ROC) curve. The area under the curve and the best sensitivity and specificity were then computed. The comparison of clinicopathologic factors was analyzed using the χ^2 tests. Results were considered significant when $P < 0.05$. The Kaplan–Meier method and log-rank test were used to estimate survival. Univariate and multivariate hazard ratios (HRs) were determined by using cox proportional hazard regression models. Overall survival (OS) was defined as the time from cancer diagnosis until death of all causes.

Results

Methylation analysis

DNA extraction and methylation analyses were successful in all 107 patients. Cutoff value of MI to separate all 107 cases into two groups (low-risk group and high-risk group) was calculated by using ROC analysis. Optimal cutoff was determined by maximizing the sensitivity and specificity. Therefore, tumors were dichotomized into both a unhypermethylated group ($MI < 10$) and a hypermethylated group ($MI \geq 10$). Samples were hypermethylated in 25 cases (23.4%) of the 107 patients. To confirm that these methylation

consequences were specific to tumor tissue, we re-measured methylation levels of *MAPT* in 50 tumor samples in parallel with their paired normal colonic mucosa tissues. *MAPT* was hypermethylated in 11 of these tumors and no methylation was found in matched normal sample. The MI values of tumor were 28.4, 18.4, 74.7, 14.6, 10.0, 27.2, 31.0, 13.9, 28.3, 34.7, and 93.7.

Methylation and clinicopathological features

Associations between *MAPT* methylation status and patient clinicopathological features were examined. No significant difference was found in the distribution of patients with positive or negative hypermethylation of *MAPT* in terms of gender, lymph nodes examined, tumor differentiation, or pT4. However, *MAPT* hypermethylation was much more common in patients with age ≥ 60 ($P < 0.001$, Table 2) and in proximal colon tumors ($P < 0.001$, Table 2). *MAPT* hypermethylation was also strongly associated with CIMP status ($P < 0.001$, Table 2).

Methylation and survival

Survival analyses were conducted to assess the prognostic role of *MAPT* methylation. Among 107 eligible patients with adequate follow-up, there were 40 deaths (37.4%). Five-year OS was significantly lower in *MAPT*-hypermethylated cases than *MAPT*-unhypermethylated cases (57.1% vs 79.4%; log-rank $P = 0.011$) in Kaplan–Meier analysis (Figure 1). *MAPT* hypermethylation was associated with a significant decrease in OS (HR: 2.33; 95% CI: 1.19–4.57; $P = 0.014$) in univariate Cox regression analysis. *MAPT* hypermethylation remained significantly associated with OS (HR: 2.29; 95% CI: 1.01–5.18; $P = 0.047$) in multivariate analysis (Table 3).

Discussion

In this study, we examined the methylation status of *MAPT* promoter CpG island and its possible prognostic value in patients with stage II CRC. We demonstrate here for the first time that *MAPT* CpG island of promoter is hypermethylated in 23.4% of patients with stage II CRC and *MAPT* methylation was absent from a subgroup of paired normal colorectal mucosa, confirming that this methylation is tumor specific. Importantly, this event was unequally distributed among different ages; *MAPT* methylation was much more common in patients with age ≥ 60 than in patients with age < 60 . Furthermore, *MAPT*

Table 2 Differential clinicopathologic features of colorectal cancer according to *MAPT* methylation status

Variables		Total n=107 (%)	<i>MAPT</i> -M (%)	<i>MAPT</i> -U (%)	P-value
Age (years)	<60	33 (30.8)	1 (4.0)	32 (39.0)	0.000
	≥60	74 (69.2)	24 (96.0)	50 (61.0)	
Sex	Male	47 (43.9)	8 (32.0)	39 (47.6)	0.170
	Female	60 (56.1)	17 (68.0)	43 (52.4)	
Location	Proximal	55 (51.4)	21 (84.0)	34 (41.5)	0.000
	Distal	52 (48.6)	4 (16.0)	48 (58.5)	
Lymph nodes examined	≥12	74 (69.2)	20 (80.0)	54 (65.9)	0.180
	<12	33 (30.8)	5 (20.0)	28 (34.1)	
Differentiation	Well to moderate	89 (83.2)	20 (80.0)	69 (84.1)	0.628
	Poor	18 (16.8)	5 (20.0)	13 (15.9)	
pT4	No	96 (89.7)	24 (96.0)	72 (87.8)	0.452
	Yes	11 (10.3)	1 (4.0)	10 (12.2)	
CIMP	Low	83 (77.6)	9 (36.0)	74 (90.2)	0.000
	High	24 (22.4)	16 (64.0)	8 (9.8)	

Abbreviations: CIMP, CpG island methylator phenotype; *MAPT*-U, *MAPT*-unhypermethylated; *MAPT*-M, *MAPT*-hypermethylated.

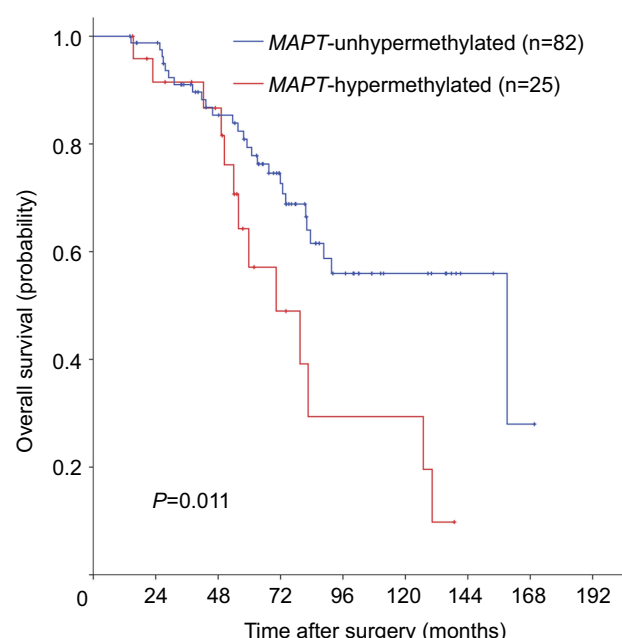


Figure 1 Kaplan-Meier survival estimates of overall survival between groups classified by *MAPT* methylation status in patients with stage II colorectal cancer. P-values were based on the log-rank test.

methylation was strongly associated with tumor location and CIMP status. *MAPT* appears to be preferentially methylated among proximal colon tumors or CIMP high tumors. Most important is that we highlight the negative and independent prognostic effect of *MAPT* hypermethylation on prognosis in patients with CRC using qMSP.

Pusztai et al previously assessed tau protein expression in primary tumors from 1942 patients with breast cancer by using tissue microarrays. They found 43% of patients were tau positive. Tau positivity was correlated with lower histologic grade and associated with better disease-free survival and OS both in univariate and multivariate analyses.¹⁴ Hristodorov et al cloned tau in-frame with EGF using EGF as a binding component to test the efficacy of tau. The tau-mediated and proliferation-dependent antitumor activity was demonstrated in vitro using EGF receptor-overexpressing breast cancer cell lines and in vivo using a mouse xenograft model.¹⁵ This holds true in lymphomas by using similar technique, in which study they noted tau could induce apoptosis when delivered to rapidly proliferating cancer cells.¹⁶ Collectively, these observations provide strong evidence that *MAPT* is a candidate tumor-suppressor gene.

In contrast, an analysis of tau expression in 102 primary breast carcinomas and matched metastases reveals that 52% hold tau expression in metastases and 26% show significantly elevated tau expression during disease progression. They also demonstrated that endogenous tau localized to microtentacles and was both essential and adequate to promote microtentacle extension in detached breast tumor cells, and tau-induced microtentacles enhanced the suspended cells to reattach and the circulating tumor cells to retain in lung capillaries.¹⁷ These results

Table 3 Univariate and multivariate Cox proportional hazard analysis of overall survival

Variables	Total n	OS			
		Univariate		Multivariate	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)					
<60	33	1.00 (Referent)	0.072	1.00 (Referent)	0.234
≥60	74	2.04 (0.94–4.45)		1.68 (0.72–3.92)	
Sex					
Male	47	1.00 (Referent)	0.824		
Female	60	0.93 (0.50–1.74)			
Location					
Proximal	55	1.00 (Referent)	0.439	1.00 (Referent)	0.656
Distal	52	0.78 (0.42–1.46)		1.18 (0.56–2.48)	
Lymph nodes examined					
≥12	74	1.00 (Referent)	0.514	1.00 (Referent)	0.433
<12	33	1.24 (0.65–2.38)		1.33 (0.65–2.71)	
Differentiation					
Well to moderate	89	1.00 (Referent)	0.463	1.00 (Referent)	0.804
Poor	18	0.70 (0.28–1.80)		0.88 (0.31–2.49)	
pT4					
No	96	1.00 (Referent)	0.326	1.00 (Referent)	0.689
Yes	11	0.55 (0.17–1.80)		0.77 (0.21–2.83)	
<i>MAPT</i> hypermethylation					
No	82	1.00 (Referent)	0.014	1.00 (Referent)	0.047
Yes	25	2.33 (1.19–4.57)		2.29 (1.01–5.18)	

suggest that tau may augment, rather than inhibit tumor development.

Previous studies showed that tau was a multifunctional protein, whose role depended on its localization. For example, it mediates microtubule polymerization and stabilization in the cytoskeleton,¹⁸ while involved in DNA protection and the promotion of chromosomal stability within the nucleus.^{19,20} In addition, the *MAPT* primary transcript contains 16 exons, alternative splicing of these exons results in at least six tau isoforms.^{21,22} Each of these isoforms is likely to have specific physiological roles since they are differentially expressed over development. Moreover, the longest tau isoform have 80 putative Ser or Thr phosphorylation sites. Phosphorylation of these sites differentially influences its biological function.²³ Taken together, localizations, differential splicing, and phosphorylation may give rise to a complex pattern of interacting tau isoforms with tumor-suppressor or oncogenic functions. Therefore, for facilitating our comprehension of tau's function in cancer, further studies should

investigate its isoforms and the effect of phosphorylation of tau as the microarray probes or the antibody used were directed against shared domains and were not aimed at phosphorylation status.

Traditional risk factors for CRC include <12 lymph nodes examined, poor histologic differentiation, pT4 lesions, intestinal obstruction or perforation, and lymphovascular invasion. These high-risk features are also recommended for clinical decisions regarding adjuvant therapy in stage II CRC. However, these features have mainly been accepted from indirect evidence in stage III CRC studies and their clinical performance has limitations. As shown in our present study, none of the risk factors including <12 lymph nodes examined, tumor differentiation, or pT4 lesions, was associated with OS in patients with stage II CRC.

To our knowledge, the expression and methylation of *MAPT* in colon tissue or CRC has not been reported to date. However, a study analyzing tau levels in tissue samples from patients with Alzheimer's disease revealed that total tau protein has the highest levels in brain,

followed by submandibular gland, sigmoid colon, liver, scalp, and abdominal skin.²⁴ We searched the Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov/>) and found that 434 cases of primary CRC samples had both *MAPT* gene expression and methylation data. *MAPT* gene was differentially expressed and methylated (data not shown). In our series, *MAPT* promoter CpG island is frequently methylated in stage II CRC. Poorer prognosis is found in patients with *MAPT* hypermethylated tumors regardless of several clinicopathological parameters. It is also important to consider that the inclusion of other methylated genes in addition to *MAPT* in a multi-gene or multimolecular prediction score can improve its prognostic values. Further studies to evaluate the function of tau in CRC are currently in progress.

Abbreviations

MAPT, microtubule-associated protein tau; CRC, colorectal cancer; OS, overall survival; Ct, threshold cycles; FFPE, formalin-fixed and paraffin-embedded.

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Disclosure

Nita Ahuja has received grant funding from Cepheid and Astex and has served as consultant to Ethicon. She has licensed methylation biomarkers to Cepheid and has a patent Predicting Response to Epigenetic Drug Therapy pending to JHU, a patent Diagnostic Test for the Early Detection of Pancreatic Cancer with royalties paid to Cepheid, and a patent Markers for Improved Detection of Breast Cancer issued. The authors report no other conflicts of interest in this work.

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