A9 Region in *EPHB2* Mutation Is Frequent in Tumors with Microsatellite Instability. Analysis of Prognosis

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Abstract. *Aim: The aim of the present study was to determine the relation of EPH tyrosine kinase receptor B2 (EPHB2) A9 region mutation and microsatellite instability (MSI); and to analyze their influence in prognosis of patients with sporadic colorectal cancer (CRC). Patients and Methods: A total of 481 patients with CRC were examined. MSI (NCI criteria) and EPHB2 were analyzed using PCR and fragment analysis software. Results: EPHB2 mutation was detected in 3.1% of patients. Mutation of EPHB2 was associated with location and with MSI status. We considered low instability (L-MSI) when only one marker showed instability, high instability (H-MSI) when two or more markers were positive and microsatelllite stable (MSS) when no instability was detected. The stratified analysis of overall survival (OS) and disease-free survival (DFS) in MSI according to EPHB2 status revealed no statistically significant differences. However, the risk of recurrence of H-MSI tumors with EPHB2 mutation carriers was 3.6-times higher than in non-mutation carriers. Conclusion: The frequency of EPHB2 mutation is higher in patients with H-MSI than MSS tumors. Promising results were found regarding the prognostic influence of EPHB2 in H-MSI.*

Since the theory of the adenoma carcinoma sequence was enunciated by Volgestein *et al*., the genetic mechanisms of tumorigenesis in colorectal cancer (CRC) have been extensively studied (1). Two different genetic pathways have been described in colorectal tumorigenesis: the most frequent is the chromosomic instability (CIN) pathway, characterized by alterations in proto-oncogenes and tumor-suppressor genes; secondly, the microsatellite instability (MSI) pathway, associated with 15% of sporadic CRC and with most cases of hereditary non-polyposis colorectal cancer (HNPCC) (2).

The two genetic pathways give rise to different clinical phenotypes of colorectal tumors, which differ in characteristics and outcome. The MSI pathway has been associated with proximal tumors, mucinous tumors and with better prognosis in CRC (3-8).

Ephrin type-B receptor-2 (*EPHB2*) is a member of the ephrin tyrosine kinase receptor family (9). This is a family of transmembrane glycoproteins implicated in the regulation of cell growth, differentiation and motility (10). *EPHB2* is becoming more important in tumorigenesis, its role as a tumor-suppressor gene in CRC has been demonstrated (11).

The *EPHB2* gene is located in chromosome 1p35-p36.1 and different mechanisms of its de-regulation have been described in tumor progression (12). This gene contains a microsatellite region A9 in exon 17 that has been described as a target of mutation. For this reason, mutations in the A9 region have been mainly studied in patients with high MSI (H-MSI), instead of overall population of sporadic CRC (13).

The aim of this study was to analyze the relation of mutation in the A9 region of *EPHB2* gene and the MSI pathway. Their influence on the prognosis of patients with sporadic CRC was also evaluated.

Patients an Methods

Study population. The study cohort comprised of 481 patients undergoing surgery consecutively for primary CRC at the San Carlos Hospital, Madrid (Spain) between March 1995 and March 2007.

This was a prospective cohort study. All patients were operated on by the same surgeon. Patients with metachronic carcinoma, familial polyposis, HNPCC, or inflammatory bowel disease were excluded from the study. None of the patients had received neoadjuvant treatment. Informed consent was obtained from each patient, and the project was approved by the Clinical Research and Ethics Committee of this hospital. Follow-up was performed according to the protocol designed by the Authors (14).

The considered variables in the study were sex, age, stage, tumour site, differentiation grade and histological type. Tumors were staged according to Duke classification. Patients with stage B and C

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disease received adjuvant treatment with 5-fluorouracil (5-FU) and leucovorin (76.0% of patients included). For patients with stage D, different protocols were applied according to the Oncology Service criteria.

Sample processing. Tumoral and non-tumoral tissue samples were obtained during the surgical procedure and immediately immersed in liquid nitrogen for storage in a freezer at –80˚C. Specimens were independently examined by two pathologists, who confirmed the samples had over 80% tumor cells. For genetic analyses, genomic DNA was isolated from tumoral and non-tumoral tissue samples using the DNasy® Blood&Tissue Kit (Qiagen. Germantown, Maryland, USA) according to the manufacturer's instructions.

MSI determination. The microsatellites analyzed were BAT25, BAT26, D17S250, D5S346 and D2S123 in accordance with the NCI criteria (15). The amplification was performed following a protocol described previously (4). The size of the amplified products in tumoral and non-tumoral tissue was compared using an ABI 310 PRISM (Applied Biosystem. Foster City, California, USA) sequencer and analyzed using Genescan and Genotyper software(Applied Biosystem).

EPHB2 mutation analysis. The amplification was performed following a protocol described previously (16). The size of the amplified products was also determined using an ABI 310 PRISM (Applied Biosystem. Foster City, California, USA.) sequencer and Genescan and Genotyper software (Applied Biosystem).

All samples with deletion were confirmed by sequencing using the same primers without labeling and analyzed in an ABI 310 PRISM sequencer (Applied Biosystem. Foster City, California, USA.).

Statistical analysis. Qualitative variables were provided with their corresponding frequency distributions. Associations between qualitative variables were evaluated using the χ^2 test or Fisher's exact test when 25% of expected frequencies fell below a value of 5. Overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan-Meier method and compared between groups using Breslow's exact test. Data were fitted into a Cox's proportional risk regression model. The hazard ratio (HR) is given with a 95% confidence interval (95% CI). All statistical tests were performed using the SSPS 11.5 software (SPSS Inc. Chicago, IL, USA).

Results

The study cohort comprised of 481 patients. The surgery was defined as curative when there was no evidence of macroscopic residual tumor. Using this criterion, the surgeon performed a curative resection in 408 patients (84.8%) and resected the primary tumor in 73 patients (15.2%) as palliative treatment. Clinicopathological variables are shown in Table I. In 60 patients, differentiation grade could not be established. *EPHB2* mutation was detected in 15 out of the 473 patients (3.1%) and all cases were heterozygotes T9/8. The relationship between the *EPHB2* mutation and clinicopathological variables is summarized in Table I. We found a statistically significant relationship of *EPHB2* and location; 14 out of the 15 tumors with mutation were located in the proximal colon and only one in the rectum ($p<0.001$). We also analyzed the association of *EPHB2* mutation in the A9 region and the MSI status (Table I). We found a strong relationship between these two genetic parameters ($p<0.001$). In the 473 patients analyzed for the two features, 14 out of 15 patients with mutation of *EPHB2* were H-MSI, which means that 38.9% of this subgroup had mutations vs. 61.1% of H-MSI group who were non-carriers.

Table I. *Clinicopathological variables of the 481 patients with colorectal carcinoma. Relationship between Ephrin type-B receptor 2 (EPHB2) mutation and these variables.*

Variable	n (%)	Mutant n (%)	WT n (%)	*p*-Value
Gender				0.52
Male	264 (54.9)	7 (1.5)	257 (53.4)	
Female	217 (45.1)	8 (1.7)	209 (43.4)	
Age				0.37
≥71 years	267 (55.5)	10 (2.1)	257 (53.4)	
<71 Years	214 (44.5)	5 (1.0)	209 (43.5)	
Dukes				0.6
A+B	263 (54.7)	9 (1.9)	254 (52.8)	
C	125 (26)	4 (0.8)	121 (25.2)	
D	93 (19.3)	2 (0.4)	91 (18.9)	
Site				<0.001
Proximal	143 (29.7)	14 (2.9)	129 (26.8)	
Distal	165 (34.3)	0 (0.0)	165 (34.3)	
Rectum	173 (36)	1 (0.2)	172 (35.8)	
Differentiation grade[a]				0.51
I	146 (34.7)	4 (1.0)	142 (33.7)	
II+III	275 (65.3)	11 (2.6)	264 (62.7)	
Histological type				0.36
Adenocarcinoma	441 (91.7)	13 (2.7)	428 (89.0)	
Mucinous	40 (8.3)	2 (0.4)	38 (7.9)	
MSI[b]				<0.001
MSS	396 (83.7)	1 (0.2)	395 (83.5)	
L-MSI	41 (8.7)	0 (0.0)	41 (8.6)	
H-MSI	36 (7.6)	14 (3.0)	22 (4.7)	

wt: Wild-type; MSS: microsatellite stable; L-MSI: low microsatellite instability; H-MSI: high microsatellite instability. [a]Not determined in 60 patients. [b]Not determined in 8 patients.

Prognosis. The median follow-up period was 61.6 months with an interquartile range of 40.8-90.4 months. In our patient population, the OS at five years was 68.8%. All survival analyses were adjusted to the median follow-up period. Univariate analysis of OS is shown in Table II. Patients with mutated *EPHB2* had a relative but not significant reduction in the risk of death of 47% (HR=0.53, 95% CI=0.13-2.15; $p=0.32$). MSI significantly positively influenced the OS of patients with CRC ($p=0.018$).

Table II. *Univariate analysis of overall survival in the 481 patients with colorectal cancer. Cox analysis.*

Variables	Category	OS at 62 months (%)	HR	95% CI	*p*-Value	DFS at 62 months (%)	HR	95% CI	*p*-Value
Gender	Male	68.2	1.3	0.9-1.87	0.15	73.4	1.1	0.72-1.64	0.67
	Female	75	Ref.			74.2	Ref.		
Age	≥71 Years	67.4	1.36	0.94-1.95	0.10	72.8	1.16	0.77-1.74	0.47
	<71 Years	75.6	Ref.			74.9	Ref.		
Dukes Class	A+B	91.5	Ref.		<0.001	84.4	Ref.		<0.001
	C	69.6	4.2	2.4-7.4		58.3	3.13	2-4.89	
	D	14.4	24.29	14.4-40.7		33.5	5.74	3.12-10.54	
Site	Proximal	65.8	1.65		0.08	79.2	0.97		0.03
	Distal	71.4	1.19	1.06-2.57		65	1.72	0.55-1.72	
	Rectum	76	Ref.	0.76-1.87		78.3	Ref.	1.08-2.75	
Differentiaion grade	I	67.6	Ref.		0.11	65.9	1.45		0.11
	II + III	72.7	0.72	0.49-1.07		76.7	Ref.	0.93-2.27	
Histological type	Adenocarcinoma	73.2	0.61		0.09	75.1	0.6		0.14
	Mucinous	52.6	Ref.	0.35-1.05		58.9	Ref.	0.32-1.12	
EPHB2	Mutated	77.9	0.53	0.13-2.15	0.32	80.8	0.59		0.42
	Wild-type	71.1	Ref.			73.5	Ref.	0.14-2.4	
MSI	MSS	68.8	Ref.		0.02	71.6	Ref.		0.04
	L-MSI	82.9	0.49	0.22-1.13		80.2	0.61	0.27-1.4	
	H-MSI	86.3	0.37	0.13-1		89.6	0.32	0.1-1.01	

OS: Overall survival; DFS: disease-free survival; EPHB2: Ephrin type-B receptor 2; HR: hazard ratio; 95% CI: 95% confidence interval; MSI: microsatellite instability; MSS: microsatellite stable; L-MSI: low microsatellite instability; H-MSI: high microsatellite instability.

Table III. *Survival analysis according to microsatellite instability (MSI) status stratified by Ephrin type-B receptor 2 (EPHB2) gene status in 473 patients with colorectal cancer.*

Variable	Category	*EPHB2*	OS at 62 months	HR‡	95% CI	*p*-Value	DFS at 62 months	HR	CI 95%	*p*-Value
MSI	MSS	Mutated	100%	0.05	0-101366.73	0.55	100%	0.049	0-202289.51	0.57
		Wild-type	68.7%	Ref.			71.5%	Ref.		
	L-MSI	Mutated	--	--	--	--	--	--	--	--
		Wild-type	82.9%				80%			
	H-MSI	Mutated	75%	1.9	0.26-13.65	0.74	78.6%	3.6	0.32-40.36	0.35
		Wild-type	90%	Ref.			95%	Ref.		

OS: Overall survival; DFS: disease-free survival; HR: hazard ratio; 95% CI: 95% confidence interval; MSI: microsatellite instability; MSS: microsatellite stable; L-MSI: low microsatellite instability; H-MSI: high microsatellite instability.

In this population DFS after five years was 73.3%. The univariate analysis of DFS is summarized in Table II. Patients with mutated *EPHB2* showed a relative non-significant reduction of 41% in the risk of recurrence (HR=0.59; 95% CI=0.14-2.40; p=0.42). MSI significantly positively influenced the DFS of patients with CRC (p=0.038).

In the stratified analysis of OS and DFS by MSI no significant differences were observed according to *EPHB2* gene status (Table III). However, in the subgroup of patients with H-MSI tumors, DFS was 78.6% in *EPHB2* mutation carriers and 95% in non-carriers (HR=3.6; p=0.35).

Discusion

Two genetic mechanisms have been described in the tumorigenesis of CRC: CIN and, MSI (17). It has been described that tumors developing through MSI confer a better prognosis on OS and on DFS (3, 4, 6, 8). Nevertheless, there are some genetic factors that could modulate the effect of MSI on prognosis and improve the discrimination of different subgroups of patients with CRC (4).

Inactivation of WNT signaling, associated with CIN, mostly occurs as a consequence of mutations in the adenomatous polyposis coli (*APC*) tumor-supressor or in

β-catenin genes (18). *EPHB2* is also a downstream effector of the WNT pathway (11). This gene has a microsatellite region in exon 17 that is a target for mutation in MSI and has been mainly studied in patients with tumors developing through this pathway (13).

The *EPHB2* mutation in the A9 region was found at a frequency of 2.7% in our cohort of patients of sporadic CRC; tumors in 7.6% of patients were H-MSI and in this subgroup, 38.9% of patients had *EPHB2* mutation. These data are in agreement with the frequency of *EPHB2* mutation in MSI CRC reported in the literature. Alazzouzi *et al*. found the A9 mutation in 37.5% of MSI cell lines and 41% of patients studied (13); similar percentages were described in MSI gastric cancer (19).

As we have previously described, *EPHB2* mutation was related to the presence of proximal tumors (16). The association of MSI phenotype has also been widely demonstrated with this location (3). Although the relation of *EPHB2* mutation has not been demonstrated by others, we can assume that MSI and *EPHB2* mutation are implicated in proximal tumor development.

Prognosis studies of the A9 region of *EPHB2* are rare in the literature. *EPHB2* mutation was found to be associated with DFS in patients with non-mucinous adenocarcinomas (16). However, loss of expression of *EPHB2* protein was associated with tumor progression, advanced stage and reduced OS and DFS (11, 20, 21). In the sub-population of patients with H-MSI, we observed that the presence of *EPHB2* mutation confers increased risk of death and recurrence, especially for DFS, in which the risk was found to be 3.6-times higher in patients with H-MSI tumors with *EPHB2* mutation than in non-mutation carriers. However, data were not statistically significant, probably due to the reduced number of events in this subgroup; nevertheless, it is an important finding from the clinical point of view.

The frequency of mutation of the A9 region located in the exon 17 in *EPHB2* gene was found to be higher in H-MSI than in MSS. Promising results regarding the prognostic influence of *EPHB2* in H-MSI were found here. It would be very interesting to increase the H-MSI population size to verify our results. If these findings are confirmed, *EPHB2* could help discriminate patients with MSI tumors with different prognosis and to improve the selection of the most suitable treatment in each case.

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