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E-selectin gene S128R polymorphism is associated with poor prognosis in patients with stage II or III colorectal cancer ☆

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ABSTRACT

Some host-related factors may predict the risk of metastasis after surgery of colorectal cancer (CRC). The endothelial adhesion molecule E-selectin is implicated in the metastatic spread of CRC. We postulated that some polymorphisms within the E-selectin gene, especially the S128R polymorphism, may increase the risk of metastases by facilitating adhesion of tumour cells to the endothelium. We collected blood samples for DNA extraction from 264 patients treated for stage II or III CRC and from 310 healthy controls in order to assess three polymorphisms within the E-selectin gene (S128R, G98T and L554F) and one within the P-selectin gene (V640L). Genotypes were analysed by the allelic discrimination TaqMan real-time PCR assay. The S128R polymorphism was detected in 59 patients (22.3%) and was strictly correlated with the G98T polymorphism. In multivariate analysis, the S128R polymorphism was associated with shorter event-free survival (EFS) and overall survival (OS) in the whole population (EFS: $P = .003$, HR 1.82, 95% CI 1.23–2.70; OS: $P < 10^{-4}$, HR 4.31, 95% CI 2.46–10.99), in patients with stage II CRC(EFS: $P = .04$, HR 1.92, 95% CI 1.02–3.60; OS: $P = .02$, HR 4.44, 95% CI 1.16–17.03), and in patients with stage III CRC (EFS: $P = .04$, HR 1.68, 95% CI 1.01–2.80; OS: $P = .001$, HR 4.04, 95% CI 1.73–9.46). L554F and V640L polymorphisms had no prognostic value. The S128R polymorphism is a constitutional factor associated with a higher risk of relapse and death in

☆ This study shows a relationship between the constitutional E-selectin S128R polymorphism and the prognosis of patients with stage II or III colorectal cancer (CRC). This result highlights new ways to explore in order to reduce mortality by CRC. Firstly, patients carrying the S128R variant could systematically receive adjuvant chemotherapy, which is of special importance for patients with stage II disease. Secondly, more intensive strategies of follow-up may be explored after surgery of patients harbouring the S128R variant. Thirdly, screening for this genotype could be useful for the detection of subjects at high risk of relapse and death in the case of CRC.

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patients treated for CRC. This polymorphism detection may permit better selection of patients suitable for adjuvant therapy, especially among those with stage II disease.

1. Introduction

Colorectal cancer (CRC), a leading cause of mortality, is responsible for more than 500,000 deaths worldwide every year.[1] In 70–80% of patients, the tumour appears to be localised at diagnosis, usually at UICC stage II (T3N0 or T4N0) or stage III (nodal involvement).[2] After resection of the tumour, 40–50% of patients will relapse, generally with distant metastases, and most of them will die from the disease. To date, the main prognostic factor used in clinical practice is the tumoural stage. Neoadjuvant or adjuvant radiotherapy or chemoradiotherapy is the standard treatment for most of the rectal cancers.[3] Adjuvant chemotherapy (fluoropyrimidine compound with or without oxaliplatin) is indicated for stage III colon cancer.[4,5] The decision of whether to use adjuvant therapy is more controversial for stage II colon cancer. In this situation, the benefit of adjuvant chemotherapy over follow-up has not been clearly demonstrated, although chemotherapy may be proposed for patients with some of the following factors: tumour revealed by occlusion or perforation, presence of vascular emboli, neural involvement or fewer than 12 analysed nodes on pathology.[6,7] There is clearly a need for more accurate prognostic factors.

Among the biological factors implicated in cancer metastasis, one of the main determinants is E-selectin, which is an adhesion molecule expressed by endothelial cells that is involved in adhesion and extravasation of leucocytes carrying the ligands sialyl-Lewis^{x} or sialyl-Lewis^{a}. Carcinoma cells, especially of breast or colorectal origin, may express these ligands. The above-mentioned mechanism is implicated in the occurrence of distant metastases.[8–12] Additionally, the main biomarker of CRC, carcinoembryonic antigen (CEA), has been demonstrated to be an E-selectin ligand.[13]

Several single nucleotide polymorphisms have been identified within the E-selectin gene. The most common polymorphism, S128R, is present in 10–15% of the Caucasian population.[14] This polymorphism in exon 4 results in the substitution of a serine by an arginine within the extracellular domain of the receptor, which increases its affinity for ligands.[15] More specifically, this genetic variant enhances the adhesion of lymphocytes and myeloid cells to endothelial cells.[16,17] Moreover, S128R-transduced endothelial cells support significantly more rolling and adhesion of neutrophils and mononuclear cells, as compared with endothelial cells transduced with wild-type E-selectin.[18] In humans, the S128R polymorphism has been found to be associated with a higher risk of atherosclerosis and myocardial infarction, both of which exhibit an increased interaction between endothelial cells and leucocytes.[19,20]

In the present study, we hypothesised that, if a CRC occurs in a subject carrying the 128R variant, the adhesion of circulating tumour cells to the endothelium at distant sites may be facilitated, leading to a higher risk of metastatic seeding. To explore this hypothesis, we analysed the S128R polymorphism in a population of patients that had been surgically treated for stage II or III CRC, focusing on the correlations with event-free survival (EFS) and overall survival (OS). As genetic controls, we analysed two common polymorphisms present in other regions of the E-selectin gene, one in an untranslated region (G98T) and one in the transmembrane domain (L554F), as well as a polymorphism in the P-selectin gene (V640L). All genetic analyses were also performed in a group of healthy subjects.

2. Patients and methods

2.1. Patients

Patients followed after resection of a histologically documented stage II or III colorectal adenocarcinoma in three institutions of northern France were enrolled in the study. Patients with a family history suggestive of hereditary CRC were not included. In each patient, a blood sample was collected after signing a written informed consent for the purposes of DNA extraction and genotyping. Each patient was followed every 3–6 months for the first five years after the diagnosis of CRC, and every year subsequently, in accordance with national guidelines.[21] The biological and radiological follow-up consisted of measurement of the serum CEA level and abdominal ultrasonography every 6 months and chest radiography every year. A colonoscopy was performed every 2–3 years. Results of the genetic analyses obtained in these patients were compared to results obtained in a population of healthy blood donors (blood samples provided by the Etablissement Français du Sang, Lille, France). The study was approved by the university ethics committee of Lille, France.

2.2. DNA extraction

Genomic DNA was extracted from EDTA-treated blood samples (400 μl) using the MagNA Pure Compact instrument (Roche Diagnostics, Meylan, France) and the MagNA Pure Compact Nucleic Acid Isolation Kit according to the manufacturer's instructions.

E-selectin (S128R: rs5361, G98T: rs1805193, L554F: rs5355) and P-selectin (L640 V: rs6133) polymorphisms were genotyped using the allelic discrimination TaqMan real-time PCR assay.[22] The assay includes a sequence-specific fluorogenic minor groove binder probe for each allele. Each probe is 5′-labeled with a different reporter fluorescent dye (VIC and 6-carboxyfluorescein: FAM) in order to differentiate the amplification of each allele. Primer-probe sets for the detection of the polymorphisms were purchased from Applied Biosystems (Courtaboeuf, France). Each reaction mixture (20 μl) contained 20 ng of DNA, 900 nM of each forward and reverse primer, 200 nM of each allele-specific probe and 2X (10 μl) TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). PCR was carried out on an ABI PRISM 7700 Sequence

Detection System as follows: 95 °C for 10 min followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min. Four non-template controls as well as four positive controls for each allele were included in each assay run. For each polymorphism, 20 randomly selected DNA samples were genotyped twice to confirm the results.

2.3. Statistical methods

For statistical analyses, we pooled all patients carrying the minor alleles (heterozygous and homozygous) and compared them to patients homozygous for the wild-type alleles. All genetic data were tested for the Hardy-Weinberg equilibrium. Frequencies of the different genotypes in the patients with CRC and in healthy subjects were compared using the chi-square test.

The EFS was defined as the time between resection of the tumour to relapse or death, whichever occurred first. EFS and OS curves were drawn using the Kaplan–Meier method. Univariate survival analysis using the Cox proportional hazard model was used to determine the predictive factors of relapse or death. The parameters with a P value less than 10 were introduced in a Cox proportional hazards multivariate regression analysis of survival, with stepwise selection. The validity of the proportional hazard assumption was checked using the scaled Schoenfeld residuals. Age was tested as a continuous variable. The following other features were tested as dichotomous variables: gender M versus F, primary colon versus rectum, UICC II versus III, T classification for patients with UICC stage II disease (T3 versus T4), number of involved nodes for patients with stage III disease (<4 versus >4), revelation by bowel occlusion or perforation versus no, neoadjuvant or adjuvant therapy versus no such therapy, and presence of genetic polymorphisms versus absence of genetic polymorphisms (homozygous for the wild-type allele versus presence of the minor allele). The prognostic analyses were performed in the whole population and subgroup analyses were performed separately for patients with stage II and patients with stage III disease. No sample size calculation has been performed in this initial retrospective study. The analyses were performed with SAS software (SAS Institute Inc., Cary, NC 25513).

3. Results

3.1. Patients and controls

We included 264 patients who were treated for CRC between March 2006 and March 2008. The tumour resections were performed between 1987 and 2007. The sex ratio (M/F) was 158/106, and the median age was 59.9 years (range 29.3–88.6). The primary tumour was colonic in 196 cases (74.2%), rectal in 67 cases (25.4%) and colonic plus rectal in one case (0.4%). The UICC stage was II in 101 patients (38.3%) and III in 163 patients (61.7%). Adjuvant chemotherapy was performed in 46 of the patients with stage II tumours (44.5%) (fluoropyrimidine monotherapy in 27 cases, oxaliplatin based in 19 cases). Adjuvant chemotherapy was performed in 136 of the patients with stage III disease (83.4%) (fluoropyrimidine monotherapy in 68 cases, oxaliplatin based in 68 cases). Among the 67 patients treated for a rectal cancer, 49 (67.1%) received adjuvant or neoadjuvant radiotherapy or chemoradiotherapy.

The population of healthy controls comprised 310 subjects with a sex ratio (M/F) of 210/100 and a median age of 40.0 years (range 19–65).

3.2. Genotyping of E-selectin and P-selectin polymorphisms

Table 1 shows the distributions of the different polymorphisms. The genotype distributions in the E-selectin and P-selectin genes were in accordance with the Hardy-Weinberg equilibrium in both the patient and healthy subject groups. E-selectin S128R and G98T genotypes were strictly correlated.

Table 1 – Distributions of the genotypes in patients with colorectal cancer and in healthy controls.

	Patients with colorectal cancer $n = 264$	Healthy controls $n = 310$	P value*
E-selectin S128R (%)			
S/S	205 (77.7)	265 (85.5)	.01
S/R + R/R	54 + 5 (22.3)	41 + 4 (14.5)	
Minor allele frequency (%)	64/528 (12.1)	49/620 (7.9)	
E-selectin G98T			
G/G	205 (77.7)	265 (85.5)	.01
G/T + T/T	54 + 5 (22.3)	41 + 4 (14.5)	
Minor allele frequency (%)	64/528 (12.1)	49/620 (7.9)	
E-selectin L554F			
L/L	254 (96.2)	275 (88.7)	.001
L/F + F/F	10 + 0 (3.8)	35 + 0 (11.3)	
Minor allele frequency (%)	10/528 (1.9)	35/620 (5.6)	
P-selectin V640L			
V/V	206 (78.0)	239 (77.1)	NS
V/L + L/L	53 + 5 (22.0)	63 + 8 (22.9)	
Minor allele frequency (%)	63/528 (11.9)	79/620 (12.7)	

NS, not significant.
* Chi-square test.

No correlation was found with the other tested polymorphisms (L554F in E-selectin and V640L in P-selectin). The groups of patients with the 128R variant and the wild-type S128S variant were well balanced for baseline features (Table 2). The frequency of the 128R variant was significantly higher in patients with CRC than in healthy controls ($P = .01$). Conversely, the frequency of the 554F variant was significantly lower in patients than in controls ($P = .001$).

3.3. *Prognostic analysis*

At the time of analysis, with a median follow-up of 36.3 months (range 2.6–246), 139 patients had relapsed (52.6%) and 39 had died (14.8%). Three deaths were not related to CRC. The relapse was local in four cases and distant in 135 cases.

In the univariate analysis, EFS was significantly shorter in patients carrying the 128R variant (Fig. 1), in patients with more than four involved nodes, and among those who did not receive adjuvant therapy. A prognostic value for OS was found for the S128R polymorphism (Fig. 2), bowel occlusion or perforation and age. All these parameters had an independent prognostic value in the multivariate analysis (Table 3). The prognostic value of the S128R polymorphism was maintained when only stage II or stage III patients were considered.

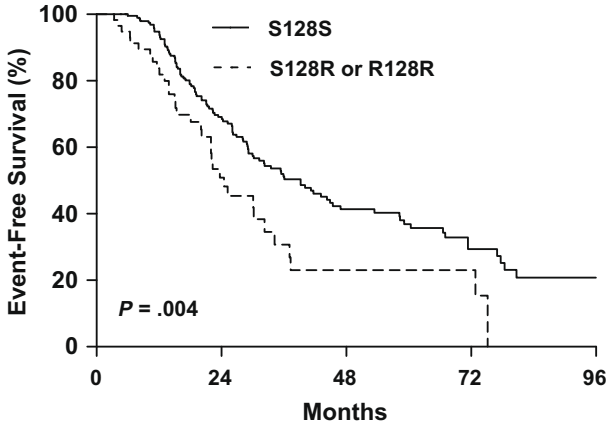

Fig. 1 – Kaplan–Meier estimates of event-free survival in patients carrying the E-selectin 128R variant ($n = 59$), and in patients with S128S genotype ($n = 205$). The number of events was 142 (139 relapses and 3 deaths before documented relapse), 34 in the 128R group and 108 in the S128S group. The hazard ratio for recurrence in the 128R group as compared to S128S group was 1.77 (95% confidence interval, 1.20–2.63; $P = .004$).

4. Discussion

Constitutional host-related biological features have long been suspected to explain, at least partly, why some patients treated for CRC will relapse and others will not despite similar baseline tumoural characteristics. Recently, Lurje et al. studied the prognostic value of polymorphisms in the genes involved in angiogenesis in 125 patients treated for stage III colon cancer. Polymorphisms in Vascular Endothelial Growth Factor (C936T) and in Interleukin-8 (T251A) were associated with shorter EFS, reinforcing the role of endothelial cells in the metastatic process.[23]

The E-selectin S128R polymorphism has been demonstrated to have functional implications in terms of adhesive-

Table 2 – Main patient's characteristics at baseline according to the E-selectin S128R genotype.

	E-selectin S128S	S128R or R128 R
Whole population (%)	205/264 (77.7)	59/264 (22.3)
Male gender	121 (59.0)	37 (62.7)
Median age (range)	59.9 (29.3–88.6)	59.9 (30.5–84.6)
Primary		
Colon	153 (74.6)	43 (72.9)
Rectum	52 (25.4)	16 (27.1)
Patients with stage II disease (%)	78/205 (38.0)	23/59 (39.0)
Male gender	46 (58.9)	13 (56.5)
Median age (range)	60.6 (29.3–88.6)	57.2 (30.5–83.1)
Primary colon	65 (83.3)	20 (86.9)
T4	20 (25.6)	8 (38.0)
Number of examined nodes (range)	16 (9/37)	16 (9–44)
Perforation and/or bowel obstruction	11 (14.1)	3 (14.2)
Adjuvant chemotherapy	36 (46.2)	10 (43.5)
Oxaliplatin-based	11	8
Fluoropyrimidine	25	2
Patients with stage III disease (%)	127/205 (62.0)	36/59 (61.0)
Male gender	75 (59.0)	24 (66.7)
Median age (range)	59.8 (33.7–84.7)	62.5 (39.0–84.6)
Primary colon	88 (69.3)	23 (63.9)
>4 nodes involved (N2)	29 (22.8)	9 (25.0)
Adjuvant chemotherapy	105 (82.6)	31 (86.1)
Oxaliplatin-based	48	20
Fluoropyrimidine	57	11

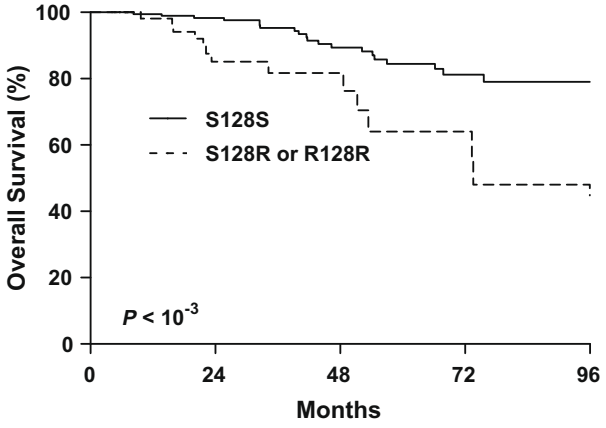

Fig. 2 – Kaplan–Meier estimates of overall survival in patients carrying the E-selectin 128R variant ($n = 59$), and in patients with S128S genotype ($n = 205$). The number of deaths was 39, 14 in the 128R group and 25 in the S128S group. The hazard ratio for death in the 128R group as compared to S128S group was 3.57 (95% confidence interval, 1.80–7.12; $P < 10^{-3}$).

ness to endothelial cells. In a recent study, S128R E-selectin-transfected endothelial cells supported increased adhesion and cellular signalling of T84 colon cancer cells.[24] The same study assessed the frequency of the S128R variant in a population of 172 patients with colon cancer, 82 with localised disease and 90 with metastatic disease. The minor allele was more frequent in patients with metastases but no prognostic analysis has been performed, especially in patients with localised disease.

In the present study, we found that the E-selectin gene S128R polymorphism was associated with a higher risk of relapse and death in patients with stage II or III CRC. This prognostic value was independent and not related to the main tumoural prognosticators. The study was partly retrospective, and most of the patients were followed in medical oncology departments and received systemic therapy for a metastatic relapse. These factors explain the high rate of relapse observed in comparison to recent prospective series: for example, in the MOSAIC study performed in patients with colon cancer (40% stage II and 60% stage III), the 3-year relapse rate was approximately 25%.[4] This selection bias may have contributed to the high frequency of the 128R variant observed in our CRC patients as compared to the frequency observed in the healthy controls. Moreover, the results on overall survival have to be confirmed after a longer follow-up duration.

Other E-selectin gene polymorphisms have been described, some of which display a correlation with the S128R polymorphism. We found a close correlation between S128R and G98T, which is consistent with the other studies that have reported correlation rates of 90–100%.[20,25] The G98T mutation occurs in the 5′-untranslated region of exon 2.[26] Conversely to S128R, the potential functional implications of this mutation are unknown.

The E-selectin L554F and the P-selectin V640L polymorphisms had no prognostic value. Notably, the frequency of the 554F variant was significantly lower in patients with CRC than in healthy controls. The explanation for this observation remains unclear. Since the L554F mutation occurs in exon 11, in the transmembrane domain of E-selectin, it has been hypothesised that it plays a role in correct membrane anchoring of the protein.[27]

Patients with the 128R variant allele are at risk of having higher tumoural adhesiveness to endothelial cells and are more likely to develop metastases. We anticipate that these findings may have implications for the management of patients with CRC. First, these results are of special importance to patients with stage II disease, in whom new predictive factors are needed to determine whether adjuvant chemotherapy is necessary. Additionally, more intensive strategies of follow-up may be explored in patients harbouring the S128R variant. Thirdly, screening for this genotype could be useful for the detection of subjects at high risk of relapse and death in the case of CRC. The frequency of the S128R genotype is about 10–15% in the general population, and the subjects dis-

Table 3 – Univariate and multivariate analyses for event-free survival and overall survival in the whole population, in patients with stage II CRC, and patients with stage III CRC. All the following parameters were tested: age (continuous), gender (M versus F), primary (colon versus rectum), stage (II versus III), T (3 versus 4), N (<4 nodes versus >4 nodes), revelation by bowel occlusion or perforation (Yes versus No), adjuvant therapy (Yes versus No), S128R, L554F and V640L polymorphisms. The G98T polymorphism has not been integrated into the analyses since it was strictly correlated with the S128R polymorphism. Only factors with a P < 0.10 are presented, with the corresponding hazard ratios. NA, not applicable; NS, not significant.

	Stages II and III		Stage II		Stage III	
	Univariate	Multivariate	Univariate	Multivariate	Univariate	Multivariate
Event-free survival						
N (<4 nodes versus >4 nodes)	.001 (2.04)	$<10^{-3}$ (2.33)	NA	NA	.001 (2.23)	$<10^{-3}$ (2.25)
Adjuvant therapy (Yes versus No)	.02 (0.68)	.006 (0.61)	$<10^{-3}$ (0.36)	$<10^{-3}$ (0.35)	NS	NS
S128S versus S128R or R128R	.004 (1.77)	.003 (1.82)	.04 (1.91)	.04 (1.92)	.05 (1.65)	.04 (1.68)
Overall survival						
Age (continuous)	.04 (1.03)	.02 (1.04)	NS	NS	NS	NS
Occlusion or perforation (Yes versus No)	.01 (3.03)	.001 (5.27)	NS	NS	.0002 (6.70)	$<10^{-4}$ (9.24)
T3 versus T4	NS	NS	.059 (3.47)	.06 (3.44)	NS	NS
S128S versus S128R or R128R	.0003 (3.57)	$<10^{-4}$ (4.31)	.02 (4.43)	.02 (4.44)	.005 (3.21)	.001 (4.04)

playing this genotype may be screened by methods that are more sensitive than the faecal occult blood test.

In conclusion, the present study is the first to clearly demonstrate a correlation between the constitutional S128R E-selectin polymorphism and the risk of relapse and death in patients with stage II or III CRC. This result may be of importance in the management of patients with CRC, and must now be confirmed in a larger series of consecutive patients. This study is ongoing and results of the present study have been used to perform the sample size calculation.

Conflict of interest statement

None declared.

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