

Original Article

Prognostic value of the density of Wilms tumour protein 1 (WT-1) positive microvessels in stage IIA colorectal cancer

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Abstract: Stage IIA colorectal cancer (CRC) displays 5-year survival rate around 85%; hence it is not currently submitted to any adjuvant treatment. However a percentage of stage IIA CRCs undergoes disease progression and would benefit from additional therapies. In the aim to investigate whether Wilms Tumor-1 (WT-1) expression might represent a prognostic factor in stage IIA CRC, we analyzed its immunohistochemical expression in 90 stage IIA CRCs divided into two groups according to the evidence of disease progression. While WT-1 staining in the neoplastic cells was not significantly associated with any of the clinico-pathological parameters, the density of intra-tumoral microvessels positive for WT-1 (WT-1 MVD) was significantly higher in stage IIA CRCs characterized by disease progression compared to non-recurring tumours and it was significantly and independently associated with shorter disease-free survival. This study is the first to demonstrate that WT-1 MVD may be useful to discriminate high risk patients with stage IIA CRCs and who may benefit from adjuvant treatment. WT-1 expression in the tumor vessels, but not in the vessels of normal colorectal mucosa, suggests its possible role in tumor neo-angiogenesis and it may represent a target for novel anti-angiogenic therapies in stage IIA CRC at high risk of progression.

Keywords: Colorectal cancer, WT-1, microvessel density, prognosis, stage IIA, poorly differentiated clusters

Introduction

According to the current (7th) edition of the post-surgical (p) TNM (tumor, node metastasis) staging of malignant tumours [1, 2], stage II colorectal carcinomas (CRCs) are those spreading into the subserosa or non peritonealized pericolic or perirectal tissue (pT3), or perforating visceral peritoneum and/or directly invading other organs or structures (pT4), in the absence of nodal (N0) and distant metastasis (M0). On the whole, stage II CRC has good prognosis, with 5-year overall survival rate ranging between 75% and 95% after surgery alone [3, 4]. Hence the benefit of adjuvant chemotherapy in patients affected by stage II CRC is controversial [5, 6] and, according to the current international guidelines, only patients at high risk of progression should receive additional treatments [3, 7, 8]. At present, high-risk patients are identified by reason of several pathological

parameters such as pT4 stage, lymphatic/vascular invasion, positive margins, poorly differentiated histology and a number of harvested lymph nodes lower than 12 [3, 7, 8]. Hence, among stage II CRCs, stage IIA (pT3N0M0) tumors do not receive adjuvant chemotherapy because of their significantly better prognosis compared to stage IIB (pT4aN0M0) and stage IIC (pT4bN0M0) ones [5]. However, around 10% of patients affected by stage IIA CRC undergo disease progression for causes still to be determined [8]. For this reason, there is the need to identify potential immunomarkers which could allow to discriminate patients with stage IIA CRCs at high risk of progression, who could benefit from adjuvant chemotherapy.

Wilms tumour protein-1 (WT-1) is a transcription factor which controls cell growth and differentiation, tumorigenesis and normal and neoplastic angiogenesis through the transcriptional or

post-transcriptional regulation of gene expression [9-16]. WT-1 was firstly identified in Wilm's tumour [17] and it was initially considered to be an oncosuppressor due to its ability to inhibit GC-rich gene promoters [16]. Recently, with the advent of commercially available antibodies (clone 6F-H2) against the N-terminus of the protein, WT-1 has been identified in the cytoplasm of several human developing tissues (skeletal muscle tissues; endothelial cells, neuroblasts; radial glia) [18] as well as in the neoplastic cells of several pediatric tumors, such as infantile fibrosarcoma, ganglioneuroblastoma and rhabdomyosarcoma [19, 20]. The demonstration of WT-1 over-expression in breast, lung and thyroid carcinomas [9, 10, 13-15] suggested that this protein may also have a pro-tumorigenic role and that it behaves as an oncosuppressor or as an oncogene depending upon the cellular context [21]. Of note, WT-1 was defined as the most important cancer antigen in a ranking based on specificity, oncogenicity, immunogenicity and therapeutic function [22]. In addition, it seems to be one of the most promising target antigens for tumour immunotherapy [23].

To the best of our knowledge, only few studies investigated the expression of WT-1 in CRC [14, 24-26]. According to those, WT-1 is over-expressed in CRC surgical tissue and cell lines compared to normal colonic mucosa [14, 24-26] and it is significantly associated with worse prognosis in this tumor [26]. In addition, experiments *in vitro* suggest that WT-1 protein may represent a potential target for antigen-specific immunotherapy in human colon cancer [26].

Given this premise, the goal of the present study was to investigate the possible prognostic value of WT-1 expression in the specific subgroup of pTNM stage IIA (pT3N0M0) CRCs. As WT1 expression was previously demonstrated in the vessels of CRC [25], we also assessed the density of WT-1 stained microvessels (WT-1 MVD) and its prognostic value for recurrence risk in stage IIA CRC.

Materials and methods

Study subjects and clinical data

43 pTNM Stage IIA (pT3N0M0) consecutive CRCs which had been surgically resected between 2002 and 2004 and which had under-

gone to disease progression were taken from our files and included in the present study. Then, 47 stage IIA CRCs occurred in the same years and with no evidence of disease progression in a follow-up time longer than five years were selected. Finally, the cohort in the study included a total of 90 cases (age range: 42-90 years; mean age: 69 years).

All cases were anonymously collected and all procedures were performed in accordance with the Helsinki Declaration. All relevant ethical issues were identified and discussed with the local Ethical Committee. No further ethical approval was necessary to perform immunohistochemistry in the cases included in the study herein.

None of the patients had received chemotherapy for their cancer. In all the cases, pathological staging had been performed according to the pTNM system and at least 12 lymph nodes had been retrieved from the peri-visceral adipose tissue.

According to their location, the tumors were divided into three groups: 1) CRC located in the right colon, including caecum, ascending and transverse colon, 2) CRC located in the left colon, including descending and sigmoid colon, and 3) CRC located in the rectum.

Pathological analyses

All tumors had been fixed in neutral buffered formalin and paraffin embedded at 56°C for the histological evaluation with haematoxylin and eosin (H&E) stain. For each case, we revised the H&E-stained slides and assessed the histological grade according to the WHO criteria [2] as well as the tumor budding and tumor border configuration. Tumor budding was defined as isolated single cancer cells or clusters of cells composed of less than five elements in the stroma of the actively invasive margin of the tumor. In detail, after choosing one field where the budding was the most intensive, a budding count was made under $\times 200$ magnification, with a count of less than five foci considered as negative and a count of five or more as positive [27].

For each case we also evaluated the histological grade based on the count of poorly differentiated clusters (PDC) of cancer cells in tumor

Table 1. Clinico-pathological characteristics and WT-1 MVD in the 90 stage IIA CRCs in the study

Age (mean \pm SD)	69 \pm 9.9		
Site (n)	Right colon (45)	Left colon (40)	Rectum (5)
WHO Grade (n)	Low (66)	High (24)	
PDC grade (n)	G1 (61)	G2 (16)	G3 (13)
TB (n)	Absent (57)	Present (33)	
Tumour border configuration	Expansive (42)	Infiltrative (48)	
WT-1 expression neoplastic cells (n)	Absent (71)	Present (19)	
WT-1 MVD (mean \pm SD)	33.51 \pm 25.12		
CD34 MVD (mean \pm SD)	68.83 \pm 25.63		
Recurrences (n)	Absent (47)	Present (43)	

tissue, as previously described [28-30]. PDC grade was determined according to the original definition provided by Ueno and coll [31]. who described PDC as cancer clusters composed of ≥ 5 cancer cells lacking a gland-like structure within the tumor stroma (at the invasive front and within the tumor). The whole tumor was first scanned at a lower power magnification to identify the area with the highest number of PDC. Then the clusters were counted under the microscopic field of a $\times 20$ objective lens (i.e., a microscopic field with a major axis of 1 mm), using a Zeiss microscope. Cancers with < 5 , 5 to 9, and ≥ 10 clusters were classified as grade 1 (G1), grade 2 (G2) and grade 3 (G3), respectively, as previously suggested [31].

Immunohistochemistry

4 μ m consecutive tissue sections were cut from one representative paraffin block of each tumor for the immunohistochemical reactions against WT-1 and CD34.

The intrinsic endogenous peroxidase activity was blocked with 0.1% H_2O_2 in methanol for 20 min; then, normal sheep serum was applied for 30 min. to prevent unspecific adherence of serum proteins. WT-1 antigen was unmasked by microwave oven pre-treatment at 750 W in 10 mM, pH 6.0 sodium citrate buffer for 3 cycles \times 5 min. Sections were successively incubated with the primary antibodies against WT-1 (clone 6F-H2; Dako Cytomation, Glostrup, Denmark; working dilution 1:50) and CD34 (clone QBEnd; Dako Cytomation, Glostrup, Denmark; working dilution 1:50) by using Dako Autostainer. The bound primary antibodies were visualized by using the LSAB kit (Dako Cytomation, Glostrup, Denmark) according to

the manufacturer's instructions. To reveal the immunostaining, the sections were incubated in darkness for 10 min. with 3-3' diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline solution (PBS).

Nuclear counterstaining was performed by Mayer's haemalum. Sections of Wilms' tumour tissue were used as positive controls for WT-1 immunohistochemical reaction.

Two independent pathologists, blinded to the clinico-pathological data, examined the whole immunostained sections. In case of disagreement, consensus was reached by examination using a double-headed microscope.

Staining for WT-1 was evaluated in the neoplastic cells and in the tumor vessels in all the cases.

WT-1 immuno-expression in the tumor cells was scored semi-quantitatively by taking into account the intensity of staining (IS) and the area of staining positivity (ASP). The IS was graded as weak (1), moderate (2), strong (3), while ASP was classified as 0 (absence of stained cells), 1 (1-50% stained cells), 2 ($> 50\%$ stained cells). Then an intensity distribution (ID) score was obtained for each case by multiplying the value of IS and ASP. Cases showing an ID score of 0 were considered to be negative for WT-1.

The quantification of WT-1 positive microvessels was performed as previously described [32-37]. Briefly, the three most vascularized areas detected by WT-1 staining were initially selected (so-called hot spots) under $40\times$ field. Then WT-1 positive microvessels were counted in each of these areas under a $400\times$ field. Only vessels with a clearly defined lumen were counted. The assessment was done within the cancerous mass, excluding all the necrotic and the ulcerated areas. The mean value of three \times 400 field (0.30 mm^2) counts was recorded as

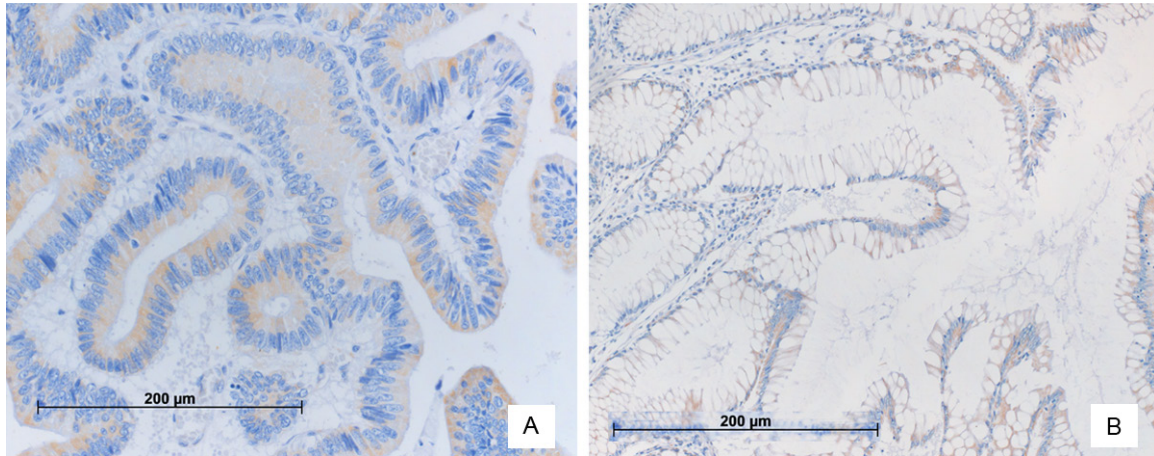


Figure 1. A. Cytoplasmic staining for WT-1 in the neoplastic cells of CRC (WT-1 stain; original magnification, $\times 200$). B. Weak cytoplasmic staining for WT-1 in the epithelial cells of normal colonic mucosa adjacent to a CRC (WT-1 stain; original magnification, $\times 200$).

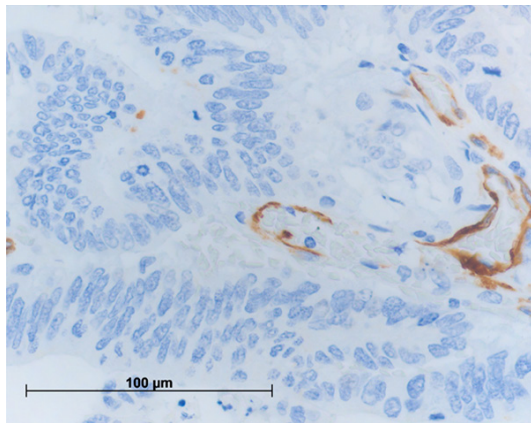


Figure 2. WT-1 stain in the cytoplasm of the intra-tumor vessels in CRC (WT-1 stain; original magnification, $\times 400$).

WT-1 MVD of the section. Finally the MVD value was converted into the mean number of microvessels/ mm^2 for the statistical analyses. The vessels were counted using a Zeiss microscope by two independent observers blinded to the clinico-pathological data. The same procedure was carried out on consecutive slides stained by anti-CD34 antibody.

Statistical analyses

The statistical association between WT-1 expression in the tumor cells and the various clinico-pathological variables was analyzed by using Chi-squared test, while Mann-Whitney and Kruskal-Wallis tests were applied to investigate the correlation between WT-1 MVD and

the clinico-pathological features of the CRCs. Spearman correlation test was carried out to verify the correlation between WT-1- and CD34-MVD.

Disease free survival (DFS) was assessed by the Kaplan-Meier method, with the date of primary surgery as the entry data and length of survival to the detection of a recurrent tumor as the end point. The Mantel-Cox log-rank test was applied to assess the strength of association between DFS and each of the parameters (site, WHO grade, tumor budding, PDC grade, WT-1 MVD, CD34 MVD) as a single variable. Successively, a multivariate analysis (Cox regression model) was utilized to determine the independent effect of each variable on DFS.

The median values in the cohort (WT-1-MVD median value: 26.6; CD34 median value: 64.95) were used as cut-off values for the DFS analyses according to WT-1-MVD and CD34-MVD of the tumors. A probability (P) value lower than 0.05 was considered to be statistically significant. Data were analyzed using the SPSS package version 6.1.3 (SPSS Inc., Chicago, IL, USA).

Results

The clinico-pathological characteristics and immunohistochemical data of the analyzed cases are summarized in **Table 1**.

In 19 cases WT-1 staining was observed in the cytoplasm of the neoplastic cells (ID score > 0). Of those cases, 10 had WT-1 ID score 1, 6 ID

Table 2. Statistical correlation between WT-1 MVD and the various clinico-pathological parameters of stage IIA CRCs assessed by Mann-Whitney and Kruskal-Wallis tests

Variable	n	Mean rank	P
Site			
Right colon	45	42.21	0.241
Left colon	40	50.43	
Rectum	5	35.7	
WHO Grade			
Low grade	66	44.7	0.561
High grade	24	47.5	
PDC grade			
G1	61	40.4	0.028
G2	16	55.2	
G3	13	57.1	
Tumour budding			
Absent	57	42.3	0.136
Present	33	50.8	
Tumour border configuration			
Expansive	42	39.4	0.038
Infiltrative	48	50.8	
Recurrences			
Not	47	37.2	0.0017
Yes	43	54.5	

score 2 and 3 ID score 3 (**Figure 1A**). The percentage of stained cells was always lower than 50% (ASP < 2) in all of the positive cases. WT-1 staining in the neoplastic cells was observed with higher frequency in WHO low-grade CRCs, although statistical significance was not reached ($P=0.0673$). No significant association was evidenced with the other clinico-pathological features ($P > 0.05$). In 3 out of the 90 analyzed cases WT-1 immuno-reactivity was also appreciable in the glands of normal colonic mucosa adjacent to the tumors (**Figure 1B**).

In all the analyzed cases WT-1 staining was noted in the cytoplasm of the endothelial cells of tumor vessels (**Figure 2**), with variable number of stained vessels. Specifically, WT-1 MVD ranged between 1.11 and 156.6 (mean: 33.51 ± 25.12) in the CRCs of our cohort. Statistical analyses showed that the density of WT-1 positive vessels was significantly higher in CRCs showing high PDC grade ($P=0.028$), infiltrative tumor border configuration ($P=0.038$), and disease progression ($P=0.0017$) (**Table 2; Figure 3**).

In 38 cases WT-1 expression was also evidenced in the endothelia of some of the vessels present within the normal mucosa adjacent to the tumors. In those cases, WT-1 MVD in normal mucosa ranged between 1.11 and 20 (mean: 7.49 ± 7.3) and it was significantly lower compared to that assessed in the neoplastic tissue ($P < 0.0001$).

CD34 immunoreaction stained all kinds of vessels present in all CRCs. In the case by case analysis, the number of microvessels stained by WT-1 was lower than the one revealed by CD34 staining (27.87 ± 26.41 versus 68.83 ± 25.63) (**Figure 4**); by Spearman's test, there was a highly significant correlation between WT-1-MVD and CD34-MVD ($r=0.785$, $P < 0.0001$).

The follow-up time ranged between 9 and 162 months. Univariate analysis revealed that high PDC grade ($P < 0.0001$) the presence of tumor budding ($P=0.0001$), and high (≥ 26.6 vessels/ mm^2) WT-1 MVD ($P=0.0001$) (**Figure 5; Table 3**) were significant negative prognostic parameters associated with shorter DFS in stage IIA CRC. All of those were independent prognostic variables in multivariate analysis by using stepwise selection method (**Table 3**).

Discussion

In this study we investigated the immunohistochemical expression of WT-1 in a series of stage IIA CRCs. WT-1 expression was evidenced in the neoplastic cells of 21% CRCs. This percentage is significantly lower compared to those (80% and 91.1%, respectively) demonstrated in previous papers [14, 26]. Disagreement between our data and those achieved by other authors [14, 26] may depend upon the use of different antibodies for the detection of WT-1. Indeed we used WT-1 monoclonal antibody clone 6F-H2, while clone WT49 [26] and the polyclonal antibody C19 [14] were used in the other immunohistochemical analyses. The two monoclonal antibodies 6F-H2 and WT 49 both react with the N terminus of WT-1 protein, while polyclonal antibody C-19 binds to the C terminus [14]. It was shown that the sensitivity of C-19 and 6F-H2 antibodies differs greatly among tumours and this difference may depend upon aberrant or deregulated splicing and alterations of the WT-1 gene [25]. Indeed WT1 gene encodes for at least 24 isoforms, which originate from alternative splicing, RNA editing,

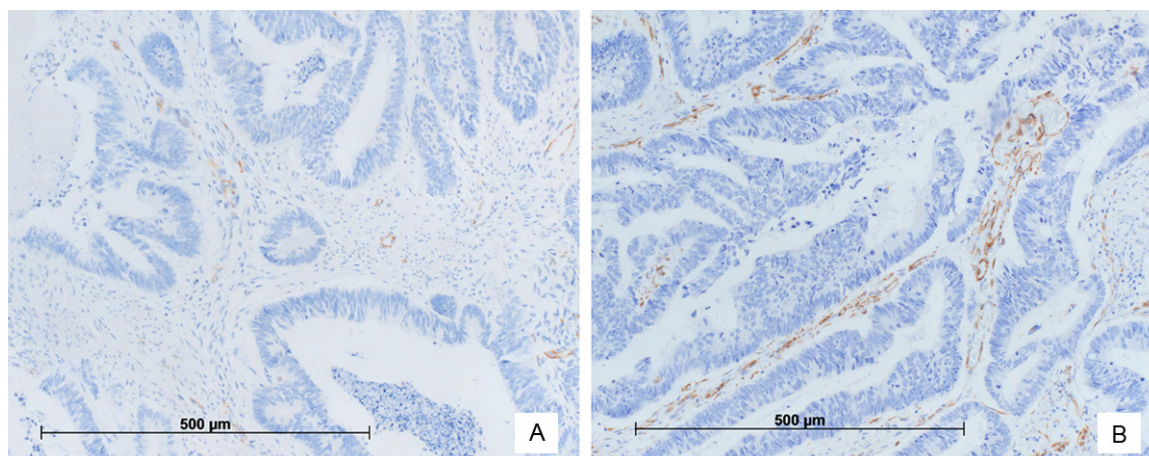


Figure 3. Density of the intra-tumour vessels positive for WT-1 in a case of non-recurring CRC (A), compared to a CRC which recurred in the follow-up (B) (WT-1 stain; original magnification, $\times 200$).

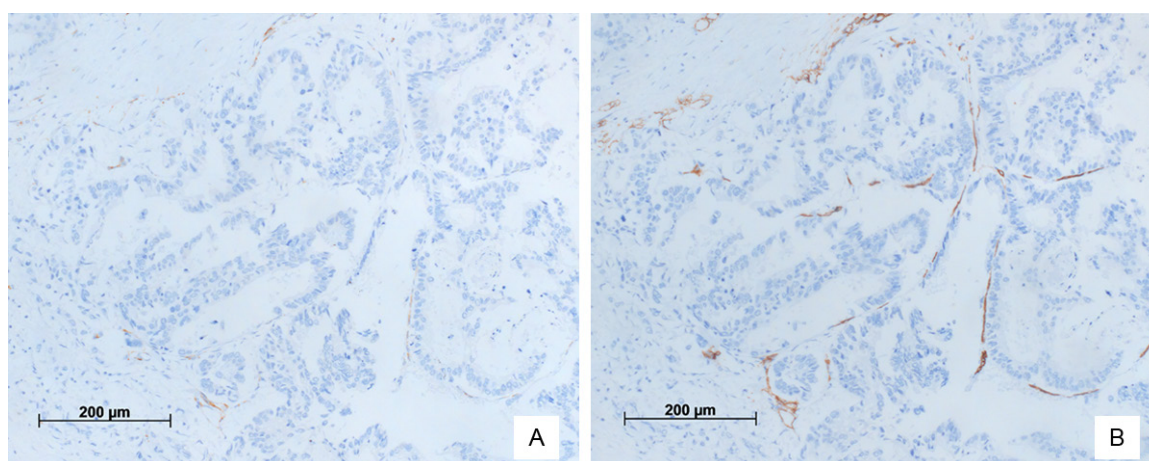


Figure 4. Comparison of the density of vessels stained by WT-1 (A) (WT-1 stain; original magnification, $\times 100$) and by CD34 (B) (CD34 stain; original magnification, $\times 100$) in a case of stage IIA CRC.

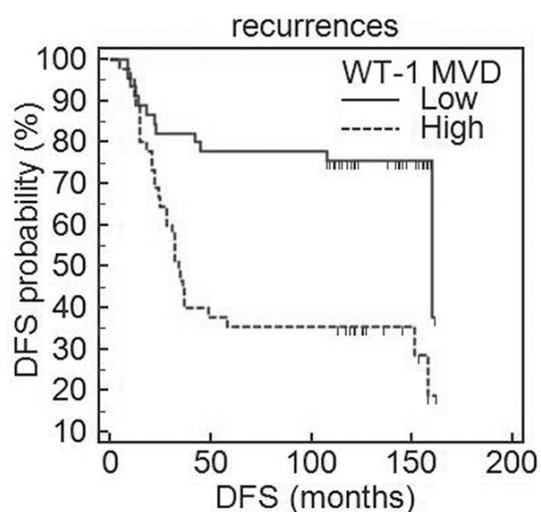


Figure 5. Kaplan-Meier curves for DFS in stage IIA CRC according to WT-1 MVD. The DFS of patients with WT-1 MVD above or equal to the cut-off value (WT-1 MVD ≥ 26.6 vessels/ mm^2) was significantly shorter than that of the patients with WT-1 MVD below the cut-off.

and alternative sites for translation initiation [38]. Initiation of translation upstream or downstream to the original site generates WT-1 proteins which are extended or shortened at the N terminus, with consequent altered binding to 6F-H2 antibody which recognizes the N terminus of WT-1 [25]. On the other hand, a comparative study between wt 49 and 6F-H2, showed that the former is less specific than the latter antibody [39].

WT-1 MVD in CRC

Table 3. Univariate and multivariate analysis for DFS in stage IIA CRCs according to the various clinico-pathological parameters and WT-1 MVD

Variables	Category	Total (recurrent cases)	Univariate		Multivariate	
			HR (95% CI)	P	HR (95% CI)	P
Site	Right	45 (20)	1			
	Left	40 (20)	1.2 (0.6-2.2)			
	Rectum	5 (3)	1.5 (0.3-6.1)	0.679		
	LG	66 (32)	1			
WHO grade	HG	24 (11)	1 (0.5-2.1)	0.801		
	G1	61 (20)	1		1	
	G2	16 (14)	4.5 (1.7-11.6)		2.9 (1.4-5.7)	0.001
PDC grade	G3	13 (9)	2.4 (1-5.9)	< 0.0001		
	Absent	57 (17)	1		1	
TB	Present	33 (26)	3.1 (1.6-5.8)	0.0001	2.2 (1.1-4.2)	0.015
Tumor border Configuration	Expansive	42 (17)	1			
	Infiltrative	48 (26)	1.3 (0.7-2.4)	0.353		
WT-1 MVD	Low (< 26.6 v/mm ²)	45 (12)	1		0.4 (0.1-0.8)	
	High (≥ 26.6 v/mm ²)	45 (31)	3.3 (1.8-6.1)	0.0001	1	0.0144
	Low (64.95 v/m ²)	46 (19)	1			
CD34 MVD	High (≥ 64.95 v/mm ²)	44 (24)	1.5 (0.8-2.7)	0.170		

V: vessels. HR: hazard ratio. CI: confidence interval.

The higher frequency of poorly differentiated CRCs in our series may also account for the discrepancy with previous studies [25, 26]. Indeed, as we also observed, WT-1 expression in CRC tumor cells is more frequent in differentiated CRCs [25, 26]. The correlation between WT-1 expression and differentiation of CRC may depend upon the role of WT-1 in the up-regulation of e-cadherin expression [39] and consequently in the induction of tumor differentiation.

Differently from that previously reported by Bejnaranda and co-workers [26], we did not find any correlation between WT-1 expression in the tumour cells and the recurrence-free survival of stage IIA CRCs, which may presumably depend on the low frequency of WT-1 positive cases in our series.

The most relevant finding in our study concerned the expression of WT-1 in the endothelia of tumour vessels. Indeed not all of the tumour vessels were stained by anti-WT-1 antibody in our cases, although WT-1 positivity in endothelial cells was considered to be a universal phenomenon and used as internal positive control for WT-1 immuno-reaction for a long time [41]. A similar finding was previously

reported in endometrial carcinoma by other authors [42] and in meningiomas by our group [37]. Similarly to that observed in meningiomas [37], WT-1 MVD was significantly correlated with the development of recurrences and it was a negative independent prognostic parameter associated with shorter DFS in stage IIA CRC. In addition, cases showing pathological features suggestive of aggressive behaviour, such as infiltrative tumor border and high PDC grade, had significantly higher WT-1 MVD counts compared to CRCs with absence of those features. Although WT-1 stain was also noted in some of the vessels within the normal colonic mucosa adjacent to the CRC, the number and density of those vessels was significantly lower compared to those assessed in the neoplastic tissue. Besides, also other authors previously reported significant decrease in the number of WT-1 positive vessels moving from CRC to the surrounding normal colonic mucosa [43]. Although the meaning of WT-1 expression in the tumour vessels still needs to be clarified, we may speculate that the correlation between the density of WT-1 positive intra-tumoral vessels and the biological aggressiveness of CRC may depend upon the role of WT-1 in neo-angiogenesis. Indeed it was shown that WT-1 is required for

endothelial cell proliferation, migration and in vitro angiogenesis [16] and that its activation in vascular cells is mediated by HIF-1 under conditions of reduced oxygen supply [16]. Hence we may speculate that more aggressive CRCs undergo a hypoxic condition due to their rapidly increasing volume, with following WT-1 up-regulation in the endothelial cells via the hypoxia inducible factor-1 (HIF-1) [15] and consequent increase in WT-1 MVD. Then the nutrients provided by the newly formed vessels might favour tumour progression. In accordance with this hypothesis, the antibody against the pan-endothelial marker CD34 stained more microvessels than the WT-1 on parallel tissue sections. Hence it is likely that CD34 stains both newly formed and pre-existing vessels, while WT-1 is positive only in the former.

Although the prognostic value of MVD was already demonstrated in CRC by the use of other endothelial markers such as endoglin [34], the demonstration that CRCs with higher propensity to recur have higher density of vessels expressing WT-1 protein may have higher clinical relevance. Indeed, due to its low toxicity in clinical trials [44], WT-1 seems to be an ideal target for innovative immunotherapy against cancer. In addition, it was shown that WT-1 specific T cytotoxic lymphocytes may determine the lysis of WT-1 expressing colon cancer cells [24]. Our findings suggest that WT-1 based immunotherapy may be used in CRC regardless of WT-1 expression in the tumour cells. Indeed also WT-1 positive intra-tumoral vessels may represent an interesting therapeutic target for the reduction of tumour blood supply.

Interestingly we did not evidence any nuclear WT-1 staining, and the expression of this protein was exclusively cytoplasmic in normal and neoplastic epithelial cells as well as in the endothelia. Although cytoplasmic positivity was previously considered to be an artifact [45], it is currently widely accepted as being a true localization of the molecule [25]. Indeed WT-1 is not only involved in transcriptional regulation in the nucleus, but also in RNA metabolism and translational regulation in the cytoplasm [46].

In conclusion, in the present study we showed that a small percentage of stage IIA CRCs express WT-1 in their neoplastic cells, while all display WT-1 expression in the intra-tumoral vessels. If our findings are confirmed in larger

cohorts of patients, WT-1 MVD may be used as a tool to discriminate patients with stage IIA CRC at higher risk of progression. Finally, the meaning of WT-1 expression in the tumour blood vessels warrants further investigation as WT-1 endothelial expression may represent a target for immunotherapy in CRCs regardless of expression in the tumour cells.

Disclosure of conflict of interest

None.

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