Patients with localized and disseminated tumors have reduced but measurable levels of ADAMTS-13 (von Willebrand factor cleaving protease)

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Background and Objectives. Patients with disseminated malignancies have been noted to have a deficiency of von Willebrand factor (VWF) cleaving protease, ADAMTS-13. The very low or undetectable plasma levels of this protease are said to be similar to those found in patients with thrombotic thrombocytopenic purpura (TTP). This observation, which challenges the paradigm that severe ADAMTS-13 deficiency is a specific diagnostic marker for TTP, remains so far unconfirmed.

Design and Methods. We measured the protease and VWF antigen (VWF:Ag) in parallel in 49 Iranian patients with solid tumors, which in 29 cases were localized (stages I and II) and in 20 disseminated (stage IV). Forty-nine healthy individuals matched with cases for sex, age and smoking habits were taken as controls.

Results. Patients with disseminated tumors had lower mean plasma levels of ADAMTS-13 than those with localized tumors, but these differences did not reach the level of statistical significance (p=0.059). However, in no patient was the level of ADAMTS-13 below 18% of normal, at variance with previous findings of lower or unmeasurable levels (<15%). The level of ADAMTS-13 was significantly lower in patients with localized tumors than in controls (p=0.0003), but higher than in patients with disseminated disease (p=0.0001 vs controls).

Interpretation and Conclusions. Malignancy, whether localized or disseminated, is another condition associated with low ADAMTS-13 levels not accompanied by signs and symptoms of TTP and other thrombotic microangiopathies.

Key words: tumors, ADAMTS-13, von Willebrand factor, thrombotic thrombocytopenic purpura.

Haematologica 2003;88:454-458
http://www.haematologica.org/2003_04/454.htm

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Von Willebrand factor (VWF) is a large multimeric glycoprotein present in plasma, platelets and vascular endothelial cells.[1] The main roles of VWF in hemostasis are to support platelet adhesion to the damaged vessel wall, platelet-to-platelet interactions and stabilization of coagulation factor VIII in plasma.[1] Under high shear stress conditions, such as those occurring in arterioles and in large stenotic arteries, plasma VWF is changed from a globular configuration into a filamentous one,[2] becoming susceptible to the action of a plasma metalloprotease that cleaves VWF to smaller forms at the bond between tyrosine 1605 and methionine 1606 (residues numbered according to a recently proposed convention).[3-6] The deficiency or dysfunction of this enzyme, which is the thirteenth member of the ADAMTS (A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif) class of metalloproteases and is therefore called ADAMTS-13, is often associated with the presence in plasma of uncleaved ultralarge VWF multimers,[7] not usually present in normal plasma. Ultralarge multimers, normally present only in vascular endothelial cells and platelets, are more effective than the largest plasma multimers at sticking to platelets and supporting the formation of aggregates in vessels under conditions of elevated fluid shear.[8-10] The clinical epitome of the association between the presence of ultralarge VWF multimers, inherited or antibody-induced ADAMTS-13 deficiency and intravascular platelet aggregation is thrombotic thrombocytopenic purpura (TTP), a condition characterized by microangiopathic hemolytic anemia associated with the formation in the terminal circulation of platelet-rich thrombi containing large amounts of VWF but little or no fibrin, which cause ischemic manifestations in several organs.[11-13]

Other conditions besides TTP are associated with ADAMTS-13 deficiency or dysfunction.[14-18] Oleksowicz *et al.*[19] compared 20 adults with disseminated tumors with 15 adults with localized tumors and found that the levels of ADAMTS-13 were very low or undetectable (<15%) in patients with disseminated tumors not associated with microangiopathic hemolytic anemia, whereas the protease was normal in those with localized tumors. ADAMTS-13 deficiency was accompanied by a concomitant increase of plasma VWF levels and the presence of ultralarge VWF multimers in plasma.[19] The authors postulated that the abnormalities of plasma VWF and its cleaving metalloprotease might play a key role in the adhesive interactions between tumor cells,

circulating platelets and the vascular endothelium that lead to the formation of metastases.[19] On the basis of these observations we examined whether deficiencies of were present and to what degree in a population including children and adults with localized or disseminated solid tumors.

Design and Methods

All patients originated from Southern Iran and were sequentially enrolled at Shiraz University Hospital, the main academic hospital in Southern Iran, where they were referred and diagnosed in hospital wards or in outpatient clinics over a period of 6 months. Complete staging work-up and evaluation of the extent of tumor dissemination using biopsies and imaging studies and, when appropriate, biochemical markers were carried out in all patients. Blood samples were obtained prior to any surgical or medical treatment.

Forty-nine pediatric and adult patients with localized (n=29) or disseminated (n=20) solid tumors were included in the study (Tables 1 and 2). Patients with localized tumors had disease at one site or at contiguous local sites (stages I or II). Patients with disseminated tumors were required to have metastatic disease through a hematogenous route as opposed to local spread (stage IV). Ages ranged from 2 to 76 years (median 36) and there were 32 males and 17 females (Tables 1 and 2). As controls, 49 healthy individuals matched for sex, age, ethnic background and smoking habits with patients were enrolled among hospital personnel, spouses and friends of the patients.

Blood sampling. Citrated blood specimens for ADAMTS-13 and VWF antigen (VWF:Ag) assays were centrifuged at 2,000 g for 20 min at 20°C, after which aliquots were snap-frozen and stored at –70°C. Case and control samples were dispatched with dry ice to the Milan Hemophilia and Thrombosis Center, where the ADAMTS-13 functional assay and VWF:Ag assay were carried out after thawing plasma at 37°C. A pool of plasma obtained from 50 healthy women (not pregnant and not taking contraceptives) and 50 healthy men was used as the reference for the assays and was arbitrarily defined to contain 100% of the ADAMTS activity and VWF:Ag.

Assay of ADAMTS-13 activity. The enzyme immunoassay described by Gerritsen *et al*.[20] was carried out with no substantial modification. The source of VWF used as substrate for the protease was pooled normal plasma in which protease activity was neutralized by the addition of both EDTA and an inhibitor of serine proteases (4 (2-aminoethyl-benzenesulfonyl fluoride hydrocloride; Pefabloc SC, Roche, Mannheim, Germany). The substrate was then dialyzed to eliminate EDTA. Serial dilutions in a buffer containing urea of the plasma samples to be tested were incubated with $BaCl_2$ to activate ADAMTS-13 and achieve partial degradation of endogenous VWF, particularly of larger VWF multimers that might interfere with the assay by preferentially binding to type III collagen. Digested samples were then incubated for 2 h with the VWF substrate and centrifuged at 2,500 g for 3 min. Supernatants were added to microtiter plates coat-

Table 1. Demographic and clinical characteristics of the 29 patients with localized solid tumors.

Sex/age	*Diagnosis*	*Site of tumor*
F/3	Astrocytoma (grade I)	Brain
M/12	Medulloblastoma	Brain
M/12	Neuroblastoma	Abdominal cavity
F/15	Small cell tumor	Spine
M/17	Seminoma	Testis
M/24	Astrocytoma (grade I)	Brain
F/26	Liposarcoma	Retroperitoneum
M/30	Squamous cell carcinoma	Anus
M/36	Adenocarcinoma	Colon
F/37	Adenocarcinoma	Ovary
F/37	Ductal carcinoma	Breast
F/39	Ductal carcinoma	Breast
M/42	Mucoepidermoid carcinoma	Parotid gland
F/43	Ductal carcinoma	Breast
M/44	Adenocarcinoma	Stomach
F/48	Squamous carcinoma	Uterus
M/49	Adenocarcinoma	Pancreas
F/50	Carcinoma	Nasopharynx
M/54	Adenocarcinoma	Rectum
F/54	Ductal carcinoma	Breast
F/58	Ductal carcinoma	Breast
F/60	Follicular carcinoma	Thyroid
M/63	Squamous cell carcinoma	Ethmoid sinus
M/64	Adenocarcinoma	Stomach
F/66	Ductal carcinoma	Breast
M/67	Hypernephroma	Kidney
M/67	Squamous cell carcinoma	Esophagus
M/69	Adenocarcinoma	Colon
M/73	Squamous carcinoma	Lung

Table 2. Demographic and clinical characteristics of the 20 patients with disseminated solid tumors.

Sex/age	*Diagnosis*	*Metastatic sites*
M/2	Rhabdomyosarcoma	Both kidneys
M/2	Germ cell tumor	Bones
M/3	Wilms' tumor	Liver
M/3	Germ cell tumor	Lung, liver
F/5	Wilms' tumor	Both lungs
F/5	Medulloblastoma	Spine
M/5	Neuroblastoma	Bones
M/6	Neuroblastoma	Bones, liver, both kidneys
M/6	Histiocytosis	Bones
M/7	Ewing sarcoma	Lung
M/10	Neuroblastoma	Bones
M/11	Ewing's sarcoma	Lung, bones
M/11	Neuroblastoma	Liver
F/12	Histiocytosis	Bones
F/12	Wilms' tumor	Both kidneys
M/33	Gastric adenocarcinoma	Liver
M/48	Gastric adenocarcinoma	Liver
M/61	Gastric adenocarcinoma	Liver
M/70	Hypernephroma	Cervical spine
M/76	Small cell lung carcinoma	Bones

ed with human collagen type III, and VWF bound to collagen was quantified using a peroxidase-labeled rabbit anti-human VWF polyclonal antibody (Dako, Glostrup, Denmark). ADAMTS-13 values were read from a dose-response curve obtained for each assay run by testing, as described above, serial dilutions from 1:5 to 1:320 of the reference plasma. Within-assay (n=18) coefficient of variation was 8%, between-assay (n=74) coefficient of variation was 14%, and the lower limit of sensitivity of the method was 6% of the normal protease levels. The mixing procedure used to evaluate the presence of an inhibitor of the protease in plasma heated at 56°C for 60 min was that described by Gerritsen *et al.*[20] The presence of an

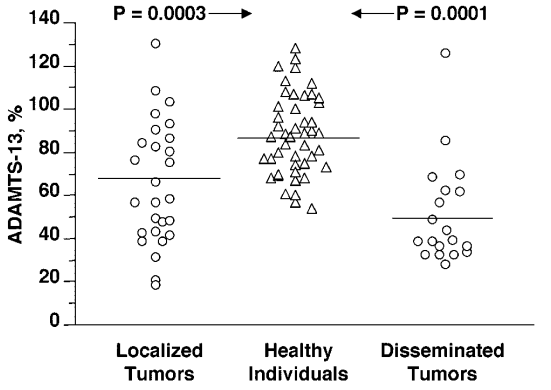

Figure 1. Values of ADAMTS-13 in patients with localized and disseminated tumors compared with values in the control group of healthy individuals.

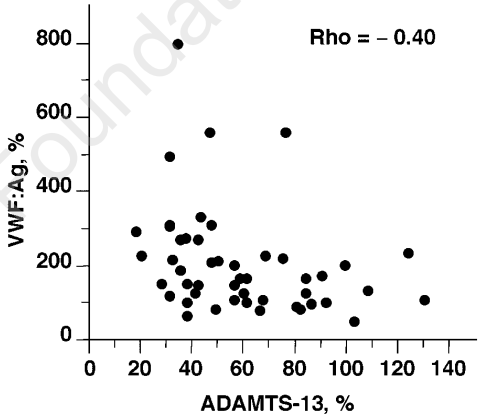

Figure 2. Spearman rho correlation coefficient between levels of ADAMTS-13 and von Willebrand factor antigen in patients with tumors (disseminated and localized).

Table 3. Values of ADAMTS-13 and von Willebrand factor antigen in patients with localized and disseminated tumors.

Group	*No.*	*ADAMTS-13 %*	*VWF antigen %*
Localized tumors	29	64±28 (18-130)	170±103 (52-560)
Disseminated tumors	20	50±23 (28-124)	261±177 (64-800)
Controls	49	87±18 (54-128)	114±37 (54-184)

Values are given as means±standard deviations and ranges (between parentheses). Statistical analysis: for ADAMTS-13, controls vs localized, p=0.0003; controls vs disseminated, p=0.0001; localized vs disseminated, p=0.059; for von Willebrand factor antigen; controls vs localized p=0.002, controls vs disseminated p=0.0007, localized vs disseminated, p=0.03.

inhibitor was also indirectly evaluated by checking whether the dose-response curves of the test plasmas were parallel to those of reference plasma. To assay plasma VWF antigen (VWF:Ag), an enzyme immunoassay using rabbit anti-human VWF polyclonal antibodies as first and second antibodies (DAKO, Glostrup, Denmark) was performed.

Data analysis. For descriptive purposes, the values of ADAMTS-13 and VWF:Ag are given as means ± standard deviations and ranges for each subgroup of cases or controls. Spearman's correlation coefficient (rho) was used to determine the relationship between ADAMTS-13 activity and VWF:Ag. Student's t test for unpaired samples was used to compare the groups of patients with and without metastases and to compare these two groups with the controls. The level of statistical significance was set at $p<0.05$ (2-tailed).

Results

Even though several patients had mild thrombocytopenia (defined as 70,000-150,000 cells/μL) and/or anemia (hemoglobin 10-13 g/dL in men and 9-12 g/dL in women) none had clinical or laboratory signs of disseminated intravascular coagulation or microangiopathic hemolytic anemia. To compare patients with localized tumors and those with disseminated tumors and to compare these two groups with matched controls, adults and children were considered together as single groups. Table 3 and Figure 1 show that patients with both disseminated and localized tumors had significantly lower plasma levels of ADAMTS-13 than did controls (p=0.0003 and 0.0001, respectively). ADAMTS-13 were lower in patients with disseminated tumors than in localized tumors but the difference did not reach statistical significance (p=0.059) (Table 3). VWF:Ag levels were significantly higher (p=0.002 and p=0.0007, respectively) in patients with localized and disseminated tumors than in controls (Table 3). There was a significant inverse correlation between VWF:Ag and ADAMTS-13 (all tumor patients: $r = -0.40$, $p = 0.005$) (Figure 2).

Discussion

Low plasma levels of ADAMTS-13 were proposed by Furlan *et al.*[12] and Tsai and Lian[13] to be a specific diagnostic marker of TTP, but we and others have shown that this is not always the case. The protease level is low or undetectable in a number of patients with hemolytic uremic syndrome not associated with *Escherichia coli* 0157-H7 infection[21] and in many other physiologic or pathologic conditions unrelated to thrombotic microangiopathies (liver cirrhosis, renal failure, inflammatory states, the post-operative state, the neonatal period, disseminated intravascular coagulation, pregnancy and various thrombocytopenic states other than TTP).[14,15,18] Oleksowicz *et al.*[19] were the first to show that the level of the protease was very low or undetectable (<15%) in 15 adults with disseminated tumors not accompanied by signs of microangiopathic hemolytic anemia, whereas it was normal (> 88%) in adults with localized tumors.[19] Our study confirms that the level of ADAMTS-13 is low in adult and pediatric patients with metastatic tumors, but the measured values, ranging from 18 to 130%, were much higher than in Oleksowicz's study.[19] Perhaps the type of tumors investigated and/or the different protease assay methods account for these differences. Another feature that might explain the different results between the two studies is that Oleksowicz *et al.*,[19] unlike us, matched patients with localized and disseminated tumors by size and site of the primary tumor. At variance with Oleksowicz[19] we found that also in patients with localized tumors the level of ADAMTS-13 was significantly lower than in healthy individuals (Figure 1). Hence, cancer is another condition in which low protease levels are found in plasma. None of our patients with low ADAMTS-13 levels had clinical or laboratory signs of tumor-associated thrombotic microangiopathy (severe thrombocytopenia, schistocytosis, focal neurologic symptoms, renal failure).[22]

As in previous studies of patients with chronic diseases other than tumors,[14,15] protease levels were negatively correlated with the plasma levels of VWF antigen. The mechanism of this negative relationship and its pathophysiologic significance remain to be established by studies on the synthesis and catabolism of the protease. Although we have not formally tested this hypothesis, ADAMTS-13 levels in cancer patients may be decreased because of impaired protein synthesis as a result of direct tumor involvement of the liver and/or the catabolic action of tumor-related cytokines. Whether the low levels of the protease affect the interactions between VWF, tumor cells, platelets and endothelial cells in the process of metastasis formation remains to be determined by animal experiments. It would be of interest, for instance, to see whether or nor experimental abrogation of the *ADAMTS 13* gene has an effect on the metastatic process in animal models. Prospective studies relating outcome to baseline protease levels in cancer patients are also warranted.

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Pre-Publication report & Outcomes of Peer Review

Contributions

KM and AM were responsible for the enrollment of the patients, MTC for the laboratory techniques, and FP for analysis and interpretation of the data. PMM wrote the article and was responsible for the conception and planning of the study. KM was recipient of a training fellowship of the World Federation of Hemophilia at the Hemophilia and Thrombosis Center and of the Thrombosis Vascular Training Centers of the World Heart Foundation. PMM, MTC and FP were funded through the Fondazione Italiana per la Ricerca sul Cancro.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received September 16, 2002; accepted January 31, 2003.

In the following paragraphs, the Deputy Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

Thrombotic thrombocytopenic purpura (TTP) has been related with ADAMTS-13 deficiency or dysfunction. Recently, other clinical conditions, including malignancies, have been associated with this defect.

What this study adds

The authors confirm that malignancy is a clinical condition associated with low ADAMTS-13 levels. However, no signs and symptoms of TTP were observed in these patients.