Presence of Villin, a Tissue-specific Cytoskeletal Protein, in Sera of Patients and an Initial Clinical Evaluation of Its Value for the Diagnosis and Follow-up of Colorectal Cancers


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ABSTRACT

Villin is an actin-binding protein found in a few normal adult epithelia, namely epithelial cells in the digestive and urogenital tracts. Moreover, villin production is maintained in malignant cells. We assumed that cell lysis and necrosis of solid tumors producing villin might result in villin release into blood. We analyzed the villin content of sera from 788 patients and controls using an enzyme-linked immunosorbent assay. Patients and controls were classified into healthy donors, patients with benign diseases of the gastrointestinal tract, patients with colorectal cancers, and patients with malignant nondigestive diseases. In the panel of sera analyzed, the sensitivity of the assay for colorectal cancers was 50.5%, and its overall specificity for malignant digestive tumors was 94.5%. Results were statistically analyzed comparing each group of sera with each other. We conclude that the presence of villin is indicative of a pathological state in the gastrointestinal tract ($P < 0.001$). Finally, we followed villin levels after tumor resections (60 patients). We found that the villin level in sera remains low in remissions but is raised in recurrences.

We suggest that the villin assay may have clinical utility as a diagnostic adjunct for adenocarcinoma of the gastrointestinal tract. It may also have some value in monitoring patients with advancing colorectal carcinomas after resection of these tumors.

INTRODUCTION

Enterocytes, the epithelial cells of the intestinal mucosa, exhibit a functional and morphological polarity. Their apical plasma membrane shows an ordered array of microvilli forming the brush border. Each microvillus is supported by a cytoskeletal based on actin microfilaments. These actin microfilament bundles are associated with a few actin-binding proteins (1). Among them, villin, a $M_r$ 95,000 protein, is able to bind actin in a calcium-dependent process. Production of villin and its regulation were previously studied in our laboratory in vivo and in cell cultures (2–4). During ontogenesis of the human digestive tract, villin is found in immature intestinal cells as early as the eight wk of gestation of the human fetus, before the final stages of histogenesis. Similarly, villin is found in adult undifferentiated crypt cells of the intestinal mucosa. In the adult human, high levels of villin were primarily found in epithelial cells with a well-organized brush border, namely the epithelial cells in the small and large intestine and cells lining the proximal tubules of the kidney.

This restricted tissue specificity of villin is also conserved during neoplastic processes. In this respect, it is particularly worth recalling that villin continues to be produced in colorectal tumors at all stages of epithelial cell transformation, but that villin is conspicuously absent from tumors arising from cell types in which it is not normally found (5, 6).

These observations are in contrast with the more generalized production of CEA, a tumor marker that is routinely assayed for screening and follow-up of patients with tumors of the digestive tract. For example, it is now well established that CEA is found in several normal cells of numerous origins (7–9).

The above mentioned properties of villin prompted us to determine whether it is released into the blood circulation, due to lysis of malignant cells or by means of another unknown mechanism, which could indicate the existence of a lesion in the digestive tract. We therefore investigated the presence of villin in the sera of 788 patients and controls with an ELISA developed for this study in our laboratory. Our initial data showed that the occurrence of villin in human sera corresponded to the pathological state of the gastrointestinal tract. Moreover, the presence of villin in sera was very often associated with a malignant digestive tumor. This is the first report of the presence of villin in human sera. We discuss the value of villin as a diagnostic adjunct for the diagnosis and follow-up of patients with colorectal carcinomas.

MATERIALS AND METHODS

Clinical Specimens. Age- and sex-matched healthy controls were provided by a serum bank from the Centre National de Transfusion sanguine (Hôpital Cochin, Paris, France). The mean age of these donors was $37 \pm 15$ yr. They included a roughly equal proportion of men and women. The mean age of donors from whom sera contained detectable amounts of villin was $39 \pm 11$ yr. Coded serum collections from patients bearing gastrointestinal tumors, adenocarcinoma of other organs, or benign diseases were obtained from three hospitals (Departments of Internal Medicine and Surgery). For this study, the diagnosis and follow-up of patients bearing digestive tumors were carried out with routine clinical and endoscopic examinations. All specimens (healthy donors' sera, benign and malignant disease patients' sera) were coded and collected during the same period of time. All of them were aliquoted and stored in the same conditions at $-20^\circ$C. We have observed that villin is stable in these storage conditions. Provided that the assay was performed on frozen and thawed samples, there was no significant variation in the measured villin concentration. Medical histories of patients were checked to validate this information and to secure additional data about the serum withdrawal date and about the ultimate

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2. To whom requests for reprints should be addressed.

3. The abbreviations used are: CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
course of the disease. Laboratory assays, such as that for CEA, were determined in parallel on the same sample and were available for correlation with the results of the villin assays.

Determination of Villin Levels. Sera were analyzed by an ELISA using a mouse monoclonal anti-villin (BD1D1C3) antibody obtained and characterized as described in Ref. 4. This monoclonal antibody recognizes a well-conserved epitope of villin across species. This epitope is located in an actin-binding domain at the COOH-terminal end of the molecule.

Primary sequence analysis of this domain carried out by our group (10) has revealed that the carboxy-terminal end of villin is villin specific and displays no sequence homologies with other actin-binding proteins nor with any other known proteins currently listed in a protein data library.

In addition, cell extracts prepared with various cell lines established from cells not expressing villin in vivo, when investigated for villin content with this ELISA, proved to be negative (2, 11).

Moreover, if a known amount of exogenous villin were added to these extracts, villin could be quantitatively assayed in these conditions. Altogether, these data illustrate the specificity of the antibody for villin and the lack of cross-reactivity for ubiquitous cytoskeletal proteins, such as gelsolin, tropomyosin, calpactin, actinin, as well as the lack of interference with the assay of structural and cellular proteins unrelated to villin.

Polystyrene microtiter plates were coated with 10 µg/ml of purified monoclonal anti-villin IgG in 50 mM potassium phosphate buffer (pH 8), using 50 µl per well. Coating was carried out at 37°C for 2 h and then at 4°C overnight. After five washes with 0.1% Tween 20 in PBS, plates were saturated for 30 min at 4°C with 1% (w/w) BSA in PBS-Tween 20. Serial 2-fold dilutions (from 1/2 to 1/32) of the sera in PBS-Tween 20 were added to the wells and incubated for 2 h at 37°C. Plates were washed five times with PBS-Tween 20 buffer, and the secondary antibody, a β-galactosidase-labeled polyclonal rabbit anti-villin IgG diluted in PBS-Tween 20-BSA, was added, and the incubation was continued for 3 h at 4°C. After five more washes with PBS-Tween 20 buffer, hydrolysis of p-nitrophenyl β-D-galactopyranoside was determined as described (12) and nitrophenol was measured at 414 nm, using a MK 700 ELISA spectrophotometer (Dynatech, South Windham, ME).

Each plate contained a serial dilution of pure porcine villin (a generous gift from Dr. V. Gerke and Dr. K. Weber, Max-Planck Institute, Göttingen, West Germany) diluted in villin-free human serum to serve as a standard. False-positive assays due to nonspecific binding to the plate were detected by the following assay. Sera containing over 20 ng of villin per ml of serum were incubated with an excess of monoclonal anti-villin IgG (35 µg/ml) for 3 h at room temperature in order to saturate specific villin binding sites prior to incubation with the plate. Control experiments carried out with dilutions of purified villin added to normal human sera showed a 98% inhibition in these conditions. Sera for which this competition was ineffective were considered as false-positive and scored as negative.

Determination of CEA Levels. To compare villin and CEA as markers from malignant digestive diseases before and after surgery, CEA levels were assayed in some sera with a solid phase enzyme immunoassay (Abbott diagnostic kit). Levels of CEA over 5 ng/ml of serum were considered to be abnormally high.

Determination of Sensitivity and Specificity Scores. The sensitivity of a marker, defined here as the ability to identify true-positive cases in patients with colorectal cancer, is calculated by dividing the number of patients with above-normal concentrations of the marker by the total number of patients with colorectal cancer.

The specificity of a marker, defined here as the ability to identify true-negative cases in patients without colonic malignancy, is calculated by dividing the number of patients with normal concentrations of the marker by the total number of healthy donors, patients with benign colorectal disease, and patients with extradigestive disease.

Statistical Analysis. The classical χ² test was used to analyze the statistical significance of the presence of villin in sera of patients and controls.

RESULTS

Sensitivity and Specificity of the Villin ELISA Assay. The ELISA assay for villin designed in our laboratory uses a highly specific monoclonal antibody raised against villin and able to recognize a unique epitope found only in villin (4). The assay for villin used in this study is a sandwich ELISA (see "Materials and Methods"). Fig. 1 shows a typical standard curve, made with villin concentrations ranging from 2 ng/ml to 150 ng/ml. The slope of the curve depends upon the villin-free medium used for the pure villin dilution. The minimal detectable amount is as low as 0.5 ng/ml in PBS and up to 4 ng/ml in some villin-free sera obtained from healthy donors. In all cases, a linear response up to 75 ng/ml was obtained. Such differences may be due to interference by serum proteins and were not investigated further. Specificity was checked on every serum sample with villin levels above 20 ng/ml by preincubation with an excess of monoclonal antibody against villin as described in "Materials and Methods." Only samples exhibiting a total inhibition after preincubation with the monoclonal antibody were considered to contain villin.

Determination of Villin Levels in Sera from Healthy Donors and from Patients with Benign Digestive Diseases and Malignant Digestive and Extradigestive Diseases. The distribution of serum villin concentrations in healthy donors and in patients with benign diseases is shown in Fig. 2. Among the healthy donors (421 cases), 408 displayed serum villin levels below 20 ng/ml, while sera from 13 donors (3%) contained more than 20 ng/ml of villin (Fig. 2A). Among the sera from patients with benign diseases of the digestive tract (132 cases), 17 were found to contain significant amounts of villin (12.9%) (Fig. 2B). Among 19 samples obtained from patients with duodenal ulcers, only 2 contained levels of villin slightly above the threshold (22 and 46 ng/ml, respectively). Villin assays were also carried out on sera of 20 patients with gastric ulcers: 3 patients had serum villin levels above the threshold. Interestingly enough, one of these patients had an ulcer with a severe dysplasia (50 ng of villin/ml of serum), and the other two had an ulcer with intestinal metaplasia (76 and 107 ng of villin/ml of serum). Among 40 sera from patients with Crohn's disease, 5 patients (12.5%) showed...
One patient of 19 (5%) had levels above 20 ng/ml (Lane D). 46 (10.8%) had villin levels above threshold (Lane C). For colorectal polyposis, cancer (Fig. 4).

We also found that serum villin levels above threshold (range, from 20 to 3000 ng of villin/ml of serum). Among the 31 patients with colorectal cancers (95 cases) were analyzed before surgery and at Stages A to D according to a modified Dukes' classification (Stage A, cancer confined to the mucosa; Stage B, cancer penetrating deeper, even through the bowel wall; Stage C, cancer penetrating as in Stage B but with involvement of regional lymph nodes; Stage D, cancer with distant metastases). The three Stage A patients had undetectable serum villin levels (below 2 ng/ml). Among the 31 patients at Stage B, 16 (51%) exhibited elevated serum villin levels (range, 20 to 1250 ng/ml), while 6 of 13 Stage C patients (46%) were positive (range, 20 to 2000 ng/ml). We also found that among 48 patients at Stage D, 26 (54%) had values above threshold (range, from 20 to 3000 ng of villin/ml of serum). When all stages were taken together, 50.5% of samples were above the threshold (Figs. 3 and 4).

The slight differences between the groups have no statistical significance ($\chi^2 = 3.2; P = \text{not significant}$).

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The slight differences between the groups have no statistical significance ($\chi^2 = 0.52; P = \text{not significant}$).

Among 80 patients with various extradigestive cancers, only 4 cases (5%) corresponding to advanced cancers had levels of serum villin above threshold; these cases are: one primary epidermoid tumor of the esophagus; one breast cancer; one ovary tumor with peritoneal carcinomatosis; and one larynx cancer (Fig. 4).

Statistical Analysis—Sensitivity and Specificity Scores. Control donors and patients were divided in four groups: healthy donors; donors with benign digestive diseases; donors with malignant digestive diseases; and donors with malignant non-digestive diseases. Each group was compared with each other, and the values obtained were subjected to the $\chi^2$ test. The analysis of the results obtained is summarized in Table 1.

Our data show that the occurrence of villin in the serum correlated strongly with a pathological benign or malignant state in the digestive tract. It is particularly noteworthy that, when healthy donors are compared to donors bearing malignant digestive diseases, the statistical parameters $\chi^2$ and $P$ were found to be particularly significant. Similarly, the comparison donors; donors with benign digestive diseases; donors with malignant digestive diseases; and donors with malignant non-digestive diseases. Each group was compared with each other, and the values obtained were subjected to the $\chi^2$ test. The analysis of the results obtained is summarized in Table 1.
of patients with malignant digestive diseases and patients with malignant nondigestive diseases is also significant (Table 1).

From the results reported in this study, we have also determined figures for the sensitivity and specificity of the villin as a tumoral marker. We found a sensitivity of 50.5% (48 of 95) and a specificity of 94.5% (598 of 633) for villin using this particular panel of sera.

A Comparison of Villin and CEA as Markers of Malignant Digestive Diseases before Surgery. Sera obtained from the majority of patients with malignant digestive diseases could be compared before surgery for villin and CEA levels (75 cases). All of these patients had colorectal tumors and were classified as being at one of the three Dukes' stages (B, C, or D), as defined above. Among the 27 patients of Stage B, 12 (44.5%) exhibited serum villin levels significantly above threshold, and 10 (37%) had elevated levels of CEA. When both villin and CEA were assayed, the sensitivity of the analysis was improved since 17 patients of 27 (63%) were positive for at least one marker. Similarly, among 13 patients at Stage C, 9 (69.3%) were positive for at least one marker as compared with 6 for the villin test above and 8 if only CEA were assayed. At Stage D, 17 of the 35 patients (48.6%) exhibited serum villin levels above 20 ng/ml, and 31 (88.6%) had CEA levels above 5 ng/ml. When both villin and CEA were assayed, the existence of colorectal cancers was correctly predicted in 32 cases (91.3%). Taken altogether, these observations suggest that patients in whom colorectal cancer has escaped diagnosis by CEA analysis might be positive for villin. These preliminary data suggest that a simultaneous search for villin and CEA in sera may be useful, particularly in the case of patients at Stage B, when tumor excreses give a better prognosis. It should be mentioned that the comparison of the figures reported here for villin and CEA measured separately and those obtained when both markers were assayed simultaneously is promising but not statistically significant. Therefore, further analysis including a larger number of patients should be performed in the future. These data are summarized in Table 2.

Villin and CEA Levels during Postsurgery Follow-up. For over 3 to 60 mo, 60 patients bearing colorectal cancers were monitored after surgery for villin and CEA serum levels and villin and CEA before surgery were only available for 12 patients. Among those, 7 were positive for at least one marker before surgery. This included 5 patients positive for both villin and for CEA. After surgery, 10 patients had remission and were negative for both markers. The other two patients were positive for both villin and CEA.

Among the patients for whom the information for villin and CEA was not available before surgery but available for a postsurgical follow-up, we found that, among 49 patients clinically in complete remission, 46 of these displayed undetectable villin levels (below 2 ng/ml) and, in 47 cases, the CEA level was also under threshold. Among the 11 recurrences, 9 were positive in the serum villin test, and 7 had an elevated level for CEA.

Table 1 Statistical analysis of villin detection in healthy donors, patients with benign digestive diseases, or those with malignant extradigestive and malignant digestive diseases

<table>
<thead>
<tr>
<th>Comparison of clinical variables</th>
<th>No. of patients' sera with villin levels above threshold (1)*</th>
<th>%</th>
<th>No. of patients' sera with villin levels above threshold (2)*</th>
<th>%</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors (1) and benign digestive diseases (2)</td>
<td>13</td>
<td>3% (13/421)</td>
<td>17</td>
<td>12.9% (17/132)</td>
<td>16.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Healthy donors (1) and malignant digestive diseases (2)</td>
<td>13</td>
<td>3% (13/421)</td>
<td>48</td>
<td>50.5% (48/95)</td>
<td>162.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Benign digestive diseases (1) and malignant digestive diseases (2)</td>
<td>17</td>
<td>12.9% (17/132)</td>
<td>48</td>
<td>50.5% (48/95)</td>
<td>36.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Malignant extradigestive diseases (1) and malignant digestive diseases (2)</td>
<td>4</td>
<td>5% (4/80)</td>
<td>48</td>
<td>50.5% (48/95)</td>
<td>40.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Healthy donors (1) and malignant extradigestive diseases (2)</td>
<td>13</td>
<td>3% (13/421)</td>
<td>4</td>
<td>5% (4/80)</td>
<td>0.280</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* NS, not significant.
* The numbers (1) and (2) correspond to the donor and patient populations listed in column "Comparison of clinical variables".
Villin, a Cytoskeletal Protein in Sera of Patients with Colorectal Cancers

In this study, we have investigated the occurrence of villin in 788 sera. We show that the presence of villin in human serum can often be correlated with the existence of a malignant tumor in the gastrointestinal tract. In contrast to existing biological assays for colorectal carcinoma, villin concentrations above threshold indicate a pathological state in the gastrointestinal tract in almost 95% of cases. We have focused this first study of villin in human sera on colorectal cancers, since they are more frequent in Western countries than other cancers which might release villin into sera, such as pancreatic cancers, cholangioma, or renal cancers. Indeed, further studies should be carried out to evaluate the usefulness of the villin assay for these cancers. The sensitivity score of the villin test is 50%, regardless of the stage of invasion of the tumor. This indicates that the villin assay has a sensitivity equivalent to existing biological assays for colorectal cancers. Thus, taking into account the specificity score mentioned above, it would appear that the main advantage of the villin assay in diagnosis as well as for follow-up patients after surgery lies in its high specificity for digestive cancers. The villin test would indeed be primarily advantageous for patients bearing resectable colorectal tumors (such as Stage B tumors). Moreover, significant improvement for all stages of colorectal tumors may be obtained when both CEA and villin are assayed, but the latter proposal remains to be rigorously tested on a larger number of patients.

The value in clinical practice of the CEA or of the CA 19.9 tests for the diagnosis or prognosis of digestive cancers is still under debate (13, 14). It is widely accepted, however, that none of the available assays is satisfactory. This is especially true for the early diagnosis of colorectal cancer when tumors are still resectable. The low specificity of these markers can be partly attributed to their wide distribution in malignant and normal cell types (15). On the contrary, the high specificity of the serological assay observed for villin can be easily explained by taking into account the tissue specificity of villin expression in normal or malignant epithelial cells in the gastrointestinal tract. Villin is a nonglycosylated intracellular protein which is produced early during embryogenesis in endodermic cells of the primitive gut (2–16). During adult life, villin continues to be produced at high levels in normal intestinal cells. This specific pattern of expression for villin is also maintained during neoplastic processes (2, 5). Thus, if villin is detected in sera of patients, one must suppose that it originates from one of a limited number of tissues. Moreover, our study strongly indicates that the release of villin into serum is very often associated with the existence of a neoplastic tissue in the gastrointestinal tract. Its entry into the general circulation presumably requires cell lysis. Our results suggest that, even if cell lysis occurs in ulcerative or inflammatory diseases of the large bowel, villin only rarely appears in the blood. This may be due to the fact that, under these circumstances, the blood supply and the cellular environment do not allow the entry of villin into the general circulation. In cases of highly vascularized colorectal adenocarcinomas, rapid necrosis and death of malignant cells are known to occur. Under these conditions one may assume that efficient release of cell content and discharge into the blood are facilitated. If such mechanism explains the release of villin into the blood, one would also expect a direct correlation between the size and stage of the tumor and the presence of villin in the serum. Indeed, our results indicate that there are no significant differences if one compares the percentage of patients positive for villin at Stages B and D. This implies that other factors must be taken into account to interpret our data, such as variability of villin stability in serum and large differences in the extent of tumor necrosis among patients. Alternatively, changes in villin organization in tumor cells may account for the release of villin into the blood. In support of this proposal one should recall the recent observation (6) reporting the localization of villin along the basement membrane in some colorectal adenocarcinoma. The physiopathological conditions leading to the appearance of villin in the blood are indeed important to analyze and remain to be studied in detail.

Our current study suggests that villin assays could be especially useful to follow the clinical course and diagnosis of recurrence of colorectal cancers in addition to CEA assays that
display low specificity. Indeed, serum villin levels drop after surgery and remain below threshold upon complete remission. In contrast, recurrence is accompanied by a significant increase in villin level, even before clinical evidence of the recurrence, especially in cases of liver metastases. Moreover, our preliminary observations strongly suggest that, in case of recurrence, a high level of villin in serum correlates with a rapid progression of the disease. It would therefore appear that villin may have a predictive value concerning the extent and the prognosis of the disease. However, these important findings require further investigation.

Since 15% of colorectal carcinomas do not produce CEA (15), this marker cannot be used during follow-up studies of some patients. Our work (5) (confirmed in Ref. 6) has established that villin production is maintained in all colorectal tumors so far studied (44 cases). Therefore, one would expect that the detection of tumor recurrence in patients with a negative CEA assay might be established using a villin assay. Our preliminary comparative study on CEA and villin before and after surgery supports this proposal.

In spite of the conspicuous production of villin in colorectal tumors, only 50% of patients bearing colorectal tumors at all stages had villin in their sera. We have observed by immunoprecipitation analysis that villin circulating in serum is proteolytically digested (data not shown). Moreover, the pattern of villin digestion by proteolytic enzymes may vary between different individuals. Thus, the epitope recognized by our monoclonal antibodies may be destroyed; consequently, even if villin is present in the serum, a negative result would be reported. This implies that further developments of our assay and/or new monoclonal antibodies for villin may improve the sensitivity of the test. Other parameters such as protein interference in sera is present in the serum, a negative result would be reported. The epitope recognized by our monoclonal antibodies may be destroyed; consequently, even if villin is present in the serum, a negative result would be reported.

Most studies on cancer markers are based upon the assumption that the transformed cell is capable of presenting at its surface new antigens, a dogma which is still widely disputed. Our rationale was the use of a differentiation marker such as villin, a well-characterized constituent of intestinal brush borders, that is produced very early during embryogenesis in primitive endoderm cells. Moreover, the profile of villin gene expression appears to be precisely modulated during the onset of the intestinal differentiation program (3, 16). The above findings are consistent with the proposal that molecules displaying these properties may in the future provide a new set of markers in cancer research. Such an approach should be assisted by our increasing knowledge of proteins playing a key role in cellular organization and differentiation.

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