

MOYENS POUR LE DIAGNOSTIC IN VITRO DE CELLULES MALIGNES ORIGINAIRES
DU TUBE DIGESTIF

The invention relates to in vitro diagnostic agents for the presence or absence in man of proliferations of malignant cells, more particularly of the adenocarcinoma type, originating from tumor cells of the digestive tract. These agents relate to both in vitro diagnostic procedures and material elements likely to be used in these procedures, more particularly DNA fragments which can be used as probes or also antibodies, preferably monoclonal antibodies, directed against human villin.

The invention also extends its effects to the various uses of these monoclonal antibodies, for example to the purification of human villin from cell extracts containing it. Consequently, the invention also relates to purified human villin itself.

It has already been suggested that recourse be had to the detection of structural proteins as specific markers for the identification of some types of higher eukaryotic cells and for the study of the sequence of the different stages of their development, in particular, during the course of their differentiation. As examples of known markers mention should be made of filament proteins such as vimentin, keratins or neurofilaments proteins which have made possible the identification of some types of cells, the study of their development from the stage of embryogenesis up to the final stage of cellular differentiation, as well as their behaviour towards the surrounding medium (E. LAZARIDES, Nature, 283, 249 (1980) and R. MOLL et al., Cell 31, 11 (1982).

The keratins are often regarded as particularly effective markers, which make it possible to distinguish epithelial cells from non-epithelial cells. This being so, it has been found that this type of distinction did not always enable the origin of the epithelial cells in question to be established unambiguously.

However, the need to establish the origin of certain types of cells with certainty assumes particularly great importance in a large number of cases, for example when, on examination of a biological sample showing signs of a proliferation of tumor cells, it is required to identify the primary tumor cells which are at the origin of this proliferation or again to detect the existence of metastases which might originate in a cancer of the digestive region or again to verify the efficacy of chemotherapy or other types of treatment of cancerous metastases. It is well known that the efficacy of an anti-cancer treatment may depend on the exact identification of the tumor cells which are the primary cause of the disease.

These observations apply with particular force to the preferably early detection of proliferations of tumor cells of digestive origin (responsible for a large proportion of the cancers observed in the clinic) or kidney cells, or cells derived from the former, more particularly to the identification of characteristic markers of these cells.

The invention is hence more particularly concerned with the detection of tumor cells which have preserved some of the essential phenotypes of the epithelial cells normally present in the mucous membranes of the digestive apparatus, more particularly the intestinal mucosa, and the major differentiated types of which are constituted by the absorbent cells or enterocytes.

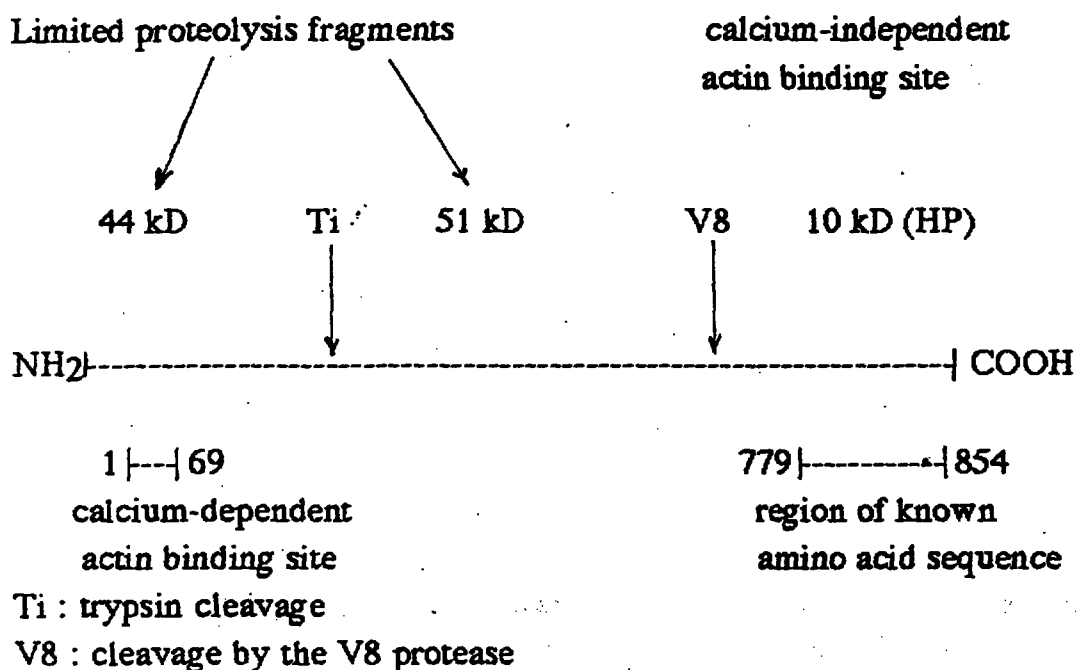
More particularly again, the invention relates to specific markers capable of being detected whether borne by tumor cells or not.

In particular, the invention aims to make possible the detection of these markers, even when they are detached from the said tumor cells and released into the blood circulation, for example, in particular when these cells are necrosed. The marker used in the framework of the present invention is constituted by all or part of villin. It is a protein having a molecular weight of the order of 95,000 Daltons and which is normally present in the villus of the digestive mucosa, more especially the intestinal mucosa. In the presence of calcium ions villin is capable of binding to actin.

In an article published in 1984 (J. Submicrosc. Cytol. 16 (1) : 159-160) Coudrier et al., studying a carcinoma cell line, described the presence of villin in differentiated cells and in non-differentiated cells. Villin was detected in this cell line by means of polyclonal antibodies.

The amino acid sequence of villin may vary slightly from one species to another but the functional properties are the same. The presently available purification procedures allow chicken and pig villin to be obtained in a practically pure state. On the other hand, these procedures do not lead to purified human villin.

One of the best known villins is that of chicken. It is composed of a sequence of 854 amino acids. It can be cleaved by limited proteolysis with trypsin and the V-8 protease at specific sites to yield fragments of 44 kD, 51 kD and 10 kD, the relative positions of which are shown on the map of villin according to Paul MADSUDAIRA, MRC Laboratory of Molecular Biology, Cambridge, U.K. and John GLENNEY et al., J. B. C., 1981, 256, 8156-8161, which is established as follows :



The amino acid sequence of the COOH-terminal fragment (779-854) or the head piece or "HP peptide" for chicken villin is the following :

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1      10 11
Val-Phe-Thr-Ala-Thr-Thr-Thr-Leu-Val-Pro-Thr-Lys-Leu-Glu-
      20
Thr-Phe-Pro-Leu-Asp-Val-Leu-Val-Asn-Thr-Ala-Ala-Glu-Asp-
      30 31      40 41
Leu-Pro-Arg-Gly-Val-Asp-Pro-Ser-Arg-Lys-Glu-Asn-His-Leu-
      50 51
Ser-Asp-Glu-Asp-Phe-Lys-Ala-Val-Phe-Gly-Met-Thr-Arg-Ser-
      60 61      70 71
Ala-Asn-Leu-Pro-Leu-Trp-Lys-Gln-Gln-Asn-Leu-Lys-Lys-Glu-
Glu-Lys-Gly-Leu-Phe

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In a general manner, villin occurs more particularly in the brush borders which are observed at the surface of the enterocytes, in particular when the latter have reached their final stage of differentiation on the walls of the intestinal lumen. The microvillousities are organelles assembled in the final stage of differentiation of the enterocytes. Villin is localised more particularly in the bundles of axial microfilaments of these microvillousities. It contributes to the constitution of their cytoskeleton. By examination of frozen slices in immunofluorescence, it has been possible to observe that the villin is present at the apical pole of the elongated cells forming columns which are flush with the surface of the internal wall of the intestine, and also in the vicinity of the cells of the proximal tubules of the

kidney. In both cases regions juxtaposed to the brush border of the cells are concerned. However, villin is not detected in various other types of villousities of various other types of epithelia, when the latter lack a very well organized brush border (A. BRETSCHER et al., Exp. Cell Res., 135, 213 (1981) and also the article by H. REGGIO et al., published in the monograph entitled "Membranes in Growth and Development" by A. LISS, New York ((1982) pp. 89-105).

Furthermore, it has been observed that villin is present in the enterocytes at an early stage of their development, well before the brush border is formed.

The invention is the result of a double discovery. Villin remains systematically detectable throughout the course of the tumorigenesis of the tissues of the digestive tract, and more particularly in cancers of the colon, one of the most widespread forms of cancer and also in certain types of renal cancer, whereas villin has never been detected in primary tumor cells resulting from the malignant transformation occurring initially in tissues localized in other organs (liver, ovaries, lungs, etc).

Furthermore, villin can be detected in vivo at all levels of development of the tumor cells in the digestive region, both in the early and at the latest stages of tumorigenesis, irrespective of the state of differentiation of the cells in question.

The diagnostic procedure according to the invention, which is designed for the in vitro detection of tumor cells formed initially in the digestive region and/or derived from the latter in a biological sample is carried out by placing this biological sample in contact with reagents which exhibit a specific affinity for villin or recognize the gene coding for this protein.

The diagnostic procedure according to the invention can be applied to any biological sample likely to contain tumor cells of digestive origin or tumor cells possibly formed in other regions of the organism under the influence of primary cells of digestive or renal origin, or also to any biological sample likely to contain degradation products of these various tumor cells, including the marker villin, should the said cells undergo necrosis. This biological sample may consist of a sample in any form : fragments of tissues or solid tumors, such as those obtained by biopsy, or also samples of biological fluids, more particularly whole blood or serum, which are capable of transporting malignant cells or fragments originating from them.

According to one embodiment of the invention, the reagent used in the diagnostic procedure of the invention is constituted by a DNA, the sequence of which comprises a region coding for an amino acid sequence of human villin, more especially the COOH-terminal part.

According to another embodiment of the invention, the procedures used in the diagnostic procedure make use of immunological techniques, the reagent exhibiting a specific affinity for villin, being constituted in that case by antibodies capable of recognizing villin.

These antibodies are themselves labelled in any known manner, or can be recognized in their turn when they are in the state of being bound to villin or the tissues which bear it by labelled immunoglobulins or other labelled proteins (for example, the protein A of Staphylococcus aureus).

Particularly suitable procedures are those which have been used in assays which will be described below, and which have led to the observations mentioned above.

The aim of the invention is also to provide specific novel agents for the detection of villin in the microvillousities of the enterocytes or similar cells (it being understood that the use of these novel agents is not necessarily limited to the implementation of the diagnostic procedure in conformity with the invention).

According to one feature of the invention, these agents are constituted by the DNA corresponding to the complete mRNA of human villin or by a DNA fragment, the nucleotide sequence of which comprises a region coding for an amino acid sequence of human villin capable of hybridizing specifically with a nucleotide sequence coding for villin.

The invention relates more specifically to the use in the above procedure of a DNA corresponding to the complete mRNA of human villin or a cDNA fragment characterized by the fact that it contains at least a part of the nucleotide sequence coding for the amino acids of the COOH terminus of human villin. This nucleotide sequence and the corresponding amino acid sequence are shown below :

LYS TRP SER ASN THR LYS SER TYR GLU ASP LEU LYS ALA GLU
 AAG TGG AGT AAC ACC AAA TCC TAT GAG GAC CTG AAG GCG GAG

 SER GLY ASN SER ARG ASP TRP SER GLN ILE THR ALA GLU VAL
 TCT GGC AAC TCT AGG GAC TGG AGC CAG ATC ACT GCT GAG GTC

 THR SER PRO LYS VAL ASP VAL PHE ASN ALA ASN SER ASN LEU
 ACA AGC CCC AAA GTG GAC GTG TTC AAT GCT AAC AGC AAC CTC

 SER SER GLY PRO LEU PRO ILE PHE PRO LEU GLU GLN LEU VAL
 AGT TCT GGG CCT CTG CCC ATC TTC CCC CTG GAG CAG CTA GTG

 ASN LYS PRO VAL GLU GLU LEU PRO GLU GLY VAL ASP PRO SER
 AAC AAG CCT GTA GAG GAG CTC CCC GAG GGT GTG GAC CCC AGC

 ARG LYS GLU GLU HIS LEU SER ILE GLU ASP PHE THR GLN ALA
 AGG AAG GAG GAA CAC CTG TCC ATT GAA GAT TTC ACT CAG GCC

 PHE GLY MET THR PRO ALA ALA PHE SER ALA LEU PRO ARG TRP
 TIT GGG ATG ACT CCA GCT GCC TTC TCT GCT CTG CCT CGA TGG

 LYS GLN GLN ASN LEU LYS LYS GLU LYS GLY LEU PHE *
 AAG CAA CAA AAC CTC AAG AAA GAA AAA GGA CTA TTT TGA GAAG
 AGTAGCTGTGGTTGTAAAGCAGTACCCTACCCTGATTGTAGGGTCTCATTTTCTCA
 CCGATATTAGTCCTACACCAATTGAAGTGAAATTTTGCAGATGTGCCTATGAGCAC
 AAAC TTCTGTGGCAAATGCCAGTTTTGTTTAATAAATGTACCTATTCCTTCAGAAA
 GATGATACCCCAAAAAAAAAA

The nucleotide sequence implemented is used as probe for the detection of the mRNAs or DNAs coding for human villin.

A suitable probe for this type of detection is advantageously labelled by means of a radioactive element or any other group making possible its recognition in the hybridized state with the preparation containing the mRNA or the DNA to be studied.

In accordance with standard procedures, these probes are placed in contact with a biological sample containing the cells to be studied or directly with their nucleic acids under conditions permitting the possible hybridization of the nucleotide sequence of the probe with a complementary sequence possibly contained in the sample tested.

The reproducibility of the detection was tested by using a probe containing an insert of 200 base pairs.

These nucleotide probes, which constitute novel products which are included in the framework of the invention, are also used, in accordance with another feature, for the study of the expression of the mRNAs of villin in each of the states of differentiation of the enterocytes and/or cells expressing villin, and in the different stages of maturation of the cells of renal or intestinal origin.

The probes obtained thus make it possible to determine the number, the organization and the structure of the gene(s) for villin as isolated from a human genomic library.

In conformity with the invention, the nucleotide sequence coding for the extremity of human villin is isolated by carrying out the following steps:

- the total mRNAs (messenger RNAs) are prepared according to known procedures from a cell line expressing villin ;
- a cDNA (complementary DNA) library is constructed ;
- the cDNAs are inserted in a vector capable of expressing the protein encoded by the insert;
- a bacterial strain is transformed with the aid of the recombinant vectors obtained, then conditions are defined which make possible the expression of the desired protein in the bacterium;
- the recombinant clones containing the specific villin clone is selected with the aid of antibodies which recognize villin.

The step involving the construction of the cDNA library is carried out with the aid of a vector capable of expressing the protein encoded by the cDNA inserted.

Cloning vectors which are particularly useful on account of their high expression yield are constituted by plasmids of the pEX type. Such plasmids are described by Stanley and Luzio in EMBO Journal, 1984, 3, 1429-1434. They are derived from plasmids containing the cro-lacZ fusion gene, the expression of which is under the control of the promoter PR of the bacteriophage lambda. This expression is temperature-inducible.

A polynucleotide containing several unique restriction sites is inserted in each of the reading frames as are the signals for the termination of transcription and translation. The cDNAs inserted at the unique restriction sites are expressed with high efficiency in the form of the corresponding cro-beta-gal-peptide hybrid protein. This hybrid protein is only slightly soluble and this advantageously reduces the risks of proteolysis in the bacterium and facilitates its immunological detection by means of specific antibodies.

In order to carry out the insertion of the cDNA in the correct orientation with respect to the cro-lacZ gene in order to obtain the expression of the corresponding polypeptide in the bacterium a procedure of sequential addition of different linkers to the two ends of the cDNA is used according to the technique described by Helfmann et al. in P.N.A.S. USA, 1983, 80, 31-35. The linkers used correspond to the BamHI and Sall cleavage sites.

These recombinant plasmids are used to transform according to standard procedures the bacterial strain employed for the expression of the protein so that the desired sequence may be expressed. Bacterial strains of E.coli are particularly preferred, more particularly the E. coli strain pop 2135 which contains the clts857 allele of the cro repressor.

A cDNA library is thus obtained in the bacterial strain which corresponds to the mRNA of the cells expressing a high level of villin and which contains about 30,000 recombinant clones.

The expression of the hybrid protein can be induced by temperature. Hence induction occurs at a temperature at which the repressor of the cro gene is no longer active. For this purpose, it is satisfactory to incubate the strains for about 2 hours at a temperature higher than room temperature, in particular at about 40-42°C.

The clones recovered after lysis of the bacteria and renaturation of the proteins according to standard procedures are selected by immunological screening.

An initial screening of the cDNA library is preferably carried out by at least one type of polyclonal anti-villin antibody. The clones which react specifically with the polyclonal antibody or antibodies are rescreened with the aid of at least one type of monoclonal antibody directed against COOH-terminal epitopes.

A satisfactory screening is carried out with the aid of a polyclonal anti-villin antibody such as a polyclonal antibody directed against intact

porcine villin, then by one or more monoclonal antibodies which recognize epitopes situated in the COOH-terminal region of the molecule.

The screening with the aid of the polyclonal antibody is advantageously followed by a secondary screening using another polyclonal antibody, in particular a polyclonal antibody directed against the COOH-terminal fragment of chicken villin.

The invention relates more particularly a clone bearing a cDNA corresponding to human villin characterized in that it reacts specifically with the polyclonal antibody directed against intact villin, with the polyclonal antibody directed against the COOH-terminal peptide of chicken villin and with the two monoclonal antibodies directed against COOH-terminal epitopes.

The invention also relates to a clone bearing a cDNA corresponding to villin comprising an insert with a polyA sequence and a polyadenylation site.

According to another feature of the invention, the agents used for the detection of villin are constituted by monoclonal antibodies.

This aspect of the invention is based on the fact that the villins derived from different species possess common epitopes, accessible to monoclonal antibodies which recognize specifically not only the particular villin which was used to immunize the animals, and whose spleen cells had been fused with myeloma cells suitable for the production of hybridomas secreting the said monoclonal antibodies, but which recognize equally specifically villins originating in other species, including the human species.

A preferred monoclonal antibody according to the invention is thus characterized by the following properties :

- It recognizes purified porcine villin (Western blotting)
- It recognizes chicken villin (Western blotting)
- It recognizes villin extracted from cells of the line derived from an adenocarcinoma of the human colon : HT29 (Western blotting).
- It recognizes rat villin in immunocytochemistry (frozen sections (cryostat) of rat intestinal mucosa fixed with formaldehyde).

A preferred monoclonal antibody among those which comply with the preceding definition also recognizes an epitope contained in the "HP peptide" of chicken villin, recognition of the HP peptide being possible both after its isolation and when it still forms part of the 51 kD peptide ;

this antibody recognizes neither the 44 kD peptide nor the 44 kD peptide of chicken villin.

Furthermore, the preferred antibody of the invention recognizes the HP protein both in the denatured state, in particular after treatment with sodium dodecyl sulfate (SDS), and on epithelial cells bearing villin. This result demonstrates that the epitope borne by the HP peptide is not masked by other proteins in the cellular environment.

Naturally, the invention also relates to the hybridomas secreting these monoclonal antibodies. A useful procedure for the production of these hybridomas, particularly by fusion of spleen cells of mice immunised against purified porcine villin and suitable myeloma cells will, moreover be described below. The preferred hybridoma thus obtained (strain BD-D 2C3) was deposited with the Collection Nationale des Cultures de Microorganismes (CNCM) at the Pasteur Institute in Paris on 29 April 1985 under the No. I-440.

In accordance with a useful feature of the invention, the immunogenic agent used to form antibodies may be expressed in large amounts in bacteria. This immunogenic agent corresponds to the peptide encoded in the nucleotide sequence defined above which corresponds to the DNA of human villin or to a fragment thereof.

The peptide is injected into animals in accordance with standard procedures, the antisera are recovered, then, if necessary, the antibodies are recovered from the antisera, for example by means of affinity chromatography. The production of antibodies according to this alternative assumes an importance all the greater because the HP region encoded by the cDNA fragment defined above is both the most immunogenic and the most specific region of villin.

It should be noted that the protein encoded in the nucleotide sequence of the villin complementary DNA or of a fragment of this DNA also constitutes a source of antigens which make it possible to carry out competitive radioimmunoassays (either by means of the direct ELISA method, or by the ELISA method by competition).

However, it will be obvious that any other immunogenic agent containing the same immunogenic sequence can be used; for example, the HP peptide itself, if necessary grafted beforehand on to a carrier molecule such as bovine serum albumin, in order to augment its immunogenic properties.

It will be clear to the person skilled in the art that having on hand the preferred monoclonal antibody according to the invention, it is possible to define the epitopic region characteristic of the HP peptide. In order to identify it more precisely, it would be possible in particular to have recourse to a procedure comprising the following steps :

- synthesis of a polynucleotide sequence coding for the HP peptide (or for that part of the HP peptide which can be reasonably supposed to contain the desired epitope),
 - linearisation of the said plasmid defined above containing the nucleotide sequence which codes for the COOH terminus of villin, at a restriction site external to the said sequence,
 - limited trimming of the linearised plasmid with an exonucleolytic enzyme such as the enzyme Bal 31,
 - recircularisation of the plasmid by means of a DNA ligase,
 - transformation of a suitable microorganism, itself transformable by the corresponding plasmid and capable of expressing the insert contained in the latter,
 - placing of the products expressed by this microorganism in contact with the antibody concerned,
- the cycle of operations which has just been defined being repeated until the said immunogenic peptide can no longer be detected among the products expressed by the said microorganism transformed by the last recircularised plasmid.

It is possible at the end of each of the cycles of the procedure defined above, in particular the sequencing of the ends of the plasmid before and after the last trimming operation which led to the loss of the capacity of the intermediate recircularised plasmid to be recognized, to locate the epitope on the peptide encoded by the nucleotide sequence eliminated during this last trimming operation. That is to say that the fact that the person skilled in the art has at his disposal the monoclonal antibody indicated above is equivalent to placing at his disposal the chemically defined peptide sequence containing this epitope.

The invention also relates to almost biologically pure human villin which reacts with the preferred monoclonal antibody defined above and which gives essentially only a single band when allowed to migrate in polyacrylamide gel electrophoresis, in particular SDS-PAGE.

In fact, human villin can be extracted from a lysate of human enterocytes, in particular a lysate obtained by treatment of these latter with

an aqueous solution containing a suitable detergent, and purified with the aid of the above-mentioned monoclonal antibody. A purification procedure of this type makes use of the monoclonal antibody advantageously immobilised on a solid support, preferably adapted to operations of affinity chromatography. For example, the monoclonal antibody is fixed to a three-dimensionally cross-linked agarose network marketed under the trade name SEPHAROSE by the Swedish company PHARMACIA A.G., for example by the cyanogen bromide method.

Thus the invention relates more particularly to a separation procedure for human villin characterised by the operations which consist of passing a solution containing it through an affinity column bearing the above-mentioned monoclonal antibody in order to bind human villin selectively, then of recovering the latter by dissociation of the antigen-antibody complex either by an acidic glycine buffer at pH 2-4 or by a basic methylamine buffer, pH 11, then of dialysing against an ammonium acetate buffer.

It will be obvious that the invention also relates to polypeptides of lower molecular weights consisting of fragments of human villin. It will be clear to the person skilled in the art that the fragments can be obtained by cleavage of human villin by enzymes which cleave polypeptides at specific sites. As examples of such proteins mention should be made, in the first instance, of trypsin and the V8 protease of Staphylococcus aureus, alpha chymotrypsin, mouse submaxillary gland protease marketed by the BOEHRINGER company, the collagenase of Vibrio alginolyticus chemovar iophagus, which specifically recognizes the said peptides Gly-Pro and Gly-Ala, etc.

It goes without saying that the hybridomas and monoclonal antibodies, species-specific or not, which can be produced starting from human villin or its fragments also form part of the invention.

Other characteristics and advantages of the invention will also become apparent in the description which follows of the particular conditions, indicated as examples, for the detection of human villin, the production of hybridomas and monoclonal antibodies and their use in assays.

A procedure for the production of a clone bearing a portion of human villin cDNA is also reported.

EXAMPLE 1 : DETECTION OF HUMAN VILLIN

The expression of villin can be studied at different stages of the proliferation of tumor or other cells originating in the digestive or renal regions, on the one hand, and in other regions of the organism, on the other. Starting from the tissues concerned, these latter are frozen, fixed and prepared under conditions giving rise to cryosections according to the procedure described by BROWN and FARQUHAR, Cell 36, 295 (1984).

Sections having thicknesses of 5 μ m were obtained at -25°C and placed on glass slides coated with polylysine. After being soaked in a buffer solution constituted of a phosphate salt solution and containing 0.2% of gelatin (PBS-gelatin), the sections were treated with the aid of a solution containing 50 μ l of rabbit anti-villin antiserum diluted to 1/800 in PBS-gelatin, then incubated for 25 minutes at room temperature in a humid chamber in order to detect villin.

The sections were then washed three times for 10 minutes in PBS-gelatin.

Rabbit anti-IgGs labelled with rhodamine (produced by the Dutch company Nordic Immunological Laboratories) were then added and the sections were incubated in the presence of these anti-IgGs at room temperature for 25 minutes. The sections were then washed completely in PBS-gelatin, then mounted for examination under a fluorescence microscope (Photomicroscope ZEISS) fitted with an immersion lens in Plan Neofluar oil and a set of suitable filters.

The control tissues or tumor tissues of different origin were treated in the same manner.

It was possible to make the following observations :

When the tissues were taken from adenocarcinomas of the colon, 11 observations out of 12 were found to be positive with respect to the expression of villin. Human tumors of other origins such as lymphomas, in particular lymphomas of mesenteric nodules and various types of carcinomas of other origins resulting, in some cases but not in others, from metastases of the pancreas, oesophagus did not express the protein.

In a general manner, the observations have shown a close correlation between the primary intestinal origin of the treated tumors and the expression of villin. The very many assays carried out on tumors of clearly different origin were found to be negative with respect to the expression of the protein in practically all cases.

It is to be noted that villin has always been observed in tissues of intestinal origin irrespective of the degree of proliferation of the said cells.

The foregoing assays were carried out with polyclonal antibodies. Much clearer results can be obtained with the preferred monoclonal antibody which has been deposited with the CNCM.

The conditions under which the hybridomas secreting these monoclonal antibodies were isolated will be described below as examples. A preferred embodiment of the conditions under which the diagnosis of the presence or absence of villin can be detected in immunological assays of the ELISA type will also be given.

EXAMPLE 2 : PREPARATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST VILLIN

A) IMMUNIZATION PROTOCOL

- Mice : Balb/C 6 to 8 weeks old
- Antigen : purified porcine villin (10 mg/ml) (homogeneous band with molecular weight of 95 kD after electrophoresis of the protein on polyacrylamide gel (10%) in the presence of SDS) :
 - . 10 mM imidazole
 - . 75 mM HCl
 - . 1 mM EGTA
 - . 0.1 M DTT
 - . 50% glycerol.
- Protocol :
 - . Day 0 : i.p. injection of 50 ug of pure villin in a 50/50 emulsion (PBS-Freund's complete adjuvant).
 - . Day 14 : i.p. booster dose of 50 ug of villin (PBS-Freund's incomplete adjuvant emulsion).
 - . Day 21 : i.p. booster dose of 50 ug of villin (PBS-Freund's incomplete adjuvant emulsion).
 - . Day 28 : i.m. injection of 20 ug of pure villin dissolved in PBS.
 - . Day 29 : i.v. injection of 10 ug of pure villin dissolved in PBS.
 - . Day 32 : fusion

B) FUSION

I) Parental cells

a) Myeloma cells :

- . Sp2/O-Ag14 line (8-azaguanine resistant)
- . Sterile culture in DMEM medium 10% FCS

b) Spleen cells :

Origin : spleen of Balb/C mice hyperimmunised against porcine villin.

II) Cell fusion procedure according to Köhler G and Milstein C

(Continuous culture of fused cells secreting antibody of predefined specificity, Nature 1975, 256, 495).

- Fusion in DMEM medium without serum under sterile conditions in the presence of a 50 % solution of polyethylene glycol (PEG 1000 - Merck 9729) in a DMEM medium without serum. After counting of the two types of parental cells, the suspension of myeloma cells in culture is mixed with the suspension of spleen cells in the proportion of 1 to 5.
- 2' 30" contact.
- The action of PEG is stopped by dilution with complete DMEM medium.
- Final dilution of the cell suspension (2×10^5 cells/ml) in the selective medium DMEM-HAT.
- Distribution in the wells of a 24 x 1 ml wells COSTAR plate (COSTAR Tissue Cluster 24, Cat. No. 3524, COSTAR 205 Groadway, Cambridge, Ma., USA).

III) Selection of the clones

The clones are identified for their capacity to secrete antibodies directed against purified porcine villin.

C) METHOD OF SELECTION

I) ELISA assay : This assay makes it possible to detect anti-villin monoclonal antibodies in the culture supernatants after fusion.

- Binding of the antigen to the support (ELISA plate)
Concentration : 5 ug/ml in 50 mM potassium phosphate buffer, pH 8.1, overnight at 4°C.
- Saturation with PBS-Tween 20 - BSA.
- Incubation I with undiluted hybridoma supernatant, 3 hours at 4°C.
- Incubation II with mouse anti-IgG labelled with beta-galactosidase, 2 hours at 4°C.

All of the washes between each step are carried out with PBS-0.1% Tween 20.

- Substrate : o-nitrophenyl-beta-D-galactosidase (Sigma N11-27) in 0.1 M phosphate buffer, pH 7.0, 10^{-3} M $MgSO_4$ 2×10^{-3} M $MnSO_4$ 2×10^{-3} M magnesium Tritriplex anhydrous (Merck 8409).

O.D. reading :. 414 nm.

The clones selected are those for which the hybridoma supernatants give an O.D. 10 times higher than the background O.D.

II) Western blotting according to Burnette. Anal. Biochem. 1981, 112, 195-203.

This test enables those clones among the positive clones for the ELISA assay to be selected which secrete porcine anti-villin monoclonal antibodies and which also recognize human villin and chicken villin.

Different steps of the test :

a) Polyacrylamide gel electrophoresis of a cell extract of chicken intestinal mucosa and a cell extract of the line HT29 (adenocarcinoma of human colon) which express villin.

b) Electrophoretic transfer of the proteins separated on polyacrylamide gel to nitrocellulose paper.

c) Incubation overnight at 4°C of the nitrocellulose paper to which the cell proteins have been transferred with the hybridoma supernatants previously selected by means of ELISA.

d) Incubation for 1 hour at room temperature with mouse anti-IgGs labelled with peroxidase.

e) Substrate :

- 10 mg diaminobenzidine tetrahydrochloride
- 20 ml 0.1 M Tris HCl, pH 7.6
- 0.2 ml of 1% H₂O₂

All of the washings between each step are carried out with newborn calf serum - PBS - Triton X100.

f) Positive reaction : rapid appearance of brown band (molecular weight 95 kD) if the antigen-monoclonal antibody reaction has taken place.

D) CLONING OF THE HYBRIDS SELECTED

- Method of limited dilutions :

Dilution of the positive clones in selective medium so as to distribute only one cell per well (plates with 96 wells) to macrophages (obtained from 4 weeks old Balb/C mice) attached to the bottom of the well.

Addition of conditioned selective medium.

- Selection of the positive clones according to the previously described methods.

- Recloning if necessary.

E) PREPARATION OF ASCITES

- The clones selected are injected into mice (which had been given an injection of "PRISTANE" (Aldrich, 2, 6, 10, 14-tetramethylpentadecane)

4 to 5 days before).

- 1 to 2 x 10⁶ cells injected/mouse.
- Recovery after 10 to 15 days of the ascites fluid containing the cells of the clone which have proliferated. The ascites fluid has an anti-villin monoclonal antibody concentration higher than 1 mg/ml.
- = Cold storage of the clones selected in DMEM medium 10% FCS - 5% DMSO.

Storage in liquid nitrogen at -176°C.

DIRECT ELISA PROCEDURE FOR ASSAYING VILLIN

= Adsorption of the IgGs purified from the ascites BD-D2C3 on ELISA plates at constant concentration.

. The antibody was purified by ion exchange chromatography (DEAE-Tris acryl) and on a hydroxyapatite column.

. Concentration to be determined.

. Incubation for 2 hours at 37°C, then overnight at 4°C.

. Washing with PBS / 0.1% Tween 20.

= Saturation of the plates

Incubation for 30 minutes in the presence of PBS / 0.1% Tween 20 / 0.4% BSA.

= Addition of the antigen

. Concentration range of purified villin decreasing from 5 ug/ml to 1 or 0.1 ng/ml in the presence of 0.4% BSA.

. Incubation for 3 hours at 4°C

. Washings with PBS / 0.1% Tween 20.

= Addition of the IgGs purified from a polyclonal rabbit serum directed against villin and coupled to beta-galactosidase.

. Incubation for 2 hours at 4°C.

. Washings with PBS / 0.1% Tween 20.

= Detection of the beta-galactosidase

Addition of 20 mg of p-nitrophenyl-beta-D-galactopyranoside from a 4 mg/ml solution to 20 ml of 0.1 M phosphate buffer, pH 7, 10⁻³ M MgSO₄, 2 x 10⁻⁴ M MnSO₄, 2 x 10⁻³ M EDTA (Merck 8409).

. Incubation for x hours at 37°C.

. Reading of the optical density at 414 nm.

EXAMPLE 3 : OBSERVATIONS IN THE CLINIC.

Using the ELISA assay described below, the sensitivity of which enables very low concentrations of villin to be detected in a cell extract (0.5 ng of villin per mg of total proteins), measurements have been made to detect the amounts of villin which can be assayed in serum. In fact, since villin is an intracellular protein, expressed by normal and tumor cells of the digestive tract, the inventors have confirmed that under certain physiopathological conditions the necrosis of the cells containing villin can lead to its release into the blood.

A study in a population of blood donors (n=190) shows that villin can not be detected in a very large majority of sera (n=168); however, a small number of individuals (n=15) exhibit a detectable level of villin (5 to 10 ng/ml). This value is selected as the villin level which has no pathological significance. Finally, some individuals (n=7) have a villin level higher than the basal level (50 to 100 ng/ml). These so-called "normal" individuals (3.6%) have not undergone an additional clinical examination of their digestive tract (fibroscopy, colonoscopy).

The initial results obtained in different gastro-intestinal diseases are summarized in the table below :

Detection of measurable quantities of villin (values between 10 and 10,000 ng/ml) in the sera of patients suffering from :

* **Malignant diseases of the digestive tract :**

- Colorectal cancers
- Gastric cancers

* **Benign diseases of the digestive tract :**

- Villous tumors
 - Crohn's disease
 - Hemorrhagic rectocolitis
 - Ulcers of the duodenal, gastric or oesophageal bulb.
-

It is important to note that digestive polyadenomas ("polyps"), on the other hand, do not lead to the release of this protein into the blood.

It was possible to make some additional deductions from this study :

1/ There does not seem to be a correlation between the amount of circulating villin and the size or stage of development of the tumor.

2/ During the post-operative follow-up, the determination of this protein shows a fall of its level in the serum after complete excision. On the other hand, in cases in which tumoral tissue persists (incomplete excision, recurrences, metastases) the level of villin is observed to remain steady or to increase.

This study shows that villin can not usually be detected in the sera of normal subjects, whereas it is possible to determine it in the sera of patients suffering from malignant diseases (cancers of the colon or rectum) or who have benign ulcerations (ulcer, RCH, Crohn's disease) of the digestive tract causing inflammatory and necrotic processes of the digestive mucosa.

The determination of villin in the blood also proves to be very useful for the monitoring of benign diseases (ulcerations and inflammatory diseases of the digestive tract). For the study of circulating villin, on the other hand, it is possible to use in combination with the antibodies of the invention other tumor markers such as the CEA (carcino-embryonic antigen) which is presently used for the monitoring of the progression of cancers but the principal disadvantages of which are : 1/ it has low specificity for the affected organ; 2/ it is observed in various benign diseases.

EXAMPLE 4 : PREPARATION OF cDNA CORRESPONDING TO HUMAN VILLIN

Preparation of the mRNAs.

The mRNAs are prepared from the cell line HT29 derived from a human colonic adenocarcinoma. The RNAs are extracted by the guanidinium chloride method described by Ullrich et al. in Science, 1977, 196, 1313-1319. The purification of the mRNA polyA is carried out by chromatography on an oligo dT-cellulose column according to the method described by Aviv and Leder in Proc. Natl. Acad. Sci. USA, 1972, 69, 1408-1412.

Preparation of the cDNAs

The first and second strand of the cDNAs are prepared according to the method of Fiddes and Goodman described in Nature, 1979, 281, 351-356. The sequential addition of the linkers Sall at the 3' end and BamHI at the 5' end is carried out by the method of Helfmann et al. reported in Proc. Natl. Acad. Sci. USA, 1983, 80, 31-35.

A cDNA library containing about 30,000 recombinant clones is obtained.

Vector

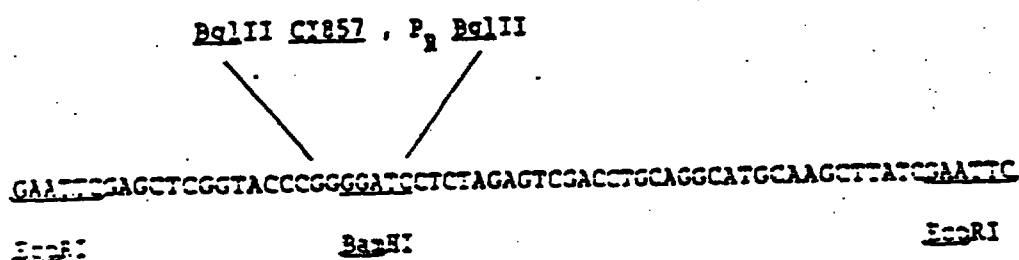
pEX 1-3 plasmids are used (see Stanley and Luzio, EMBO Journal, 1984, 3, 1429-1430, 1984). These vectors are derived from plasmids containing the cro-lacZ fusion gene, the expression of which is under the control of the PR promoter of the bacteriophage lambda. A polynucleotide containing several unique restriction sites has been inserted at the 3' end of the lacZ gene. The signals for the termination of transcription and translation are also inserted in the three reading frames.

The cDNAs were inserted in each of the pEX plasmids digested by the restriction enzymes BamHI and Sall. They are all thus found in the same orientation with respect to the cro-lacZ gene.

Transformation

The E. coli strain pop 2135 is used as bacterial strain and it is constructed as described by O. Raibaud in Nucleic Acid Research in the following manner :

The 2.3 kb BglII fragment of the phage lambda, bearing the allele C1857 and PR promoter are cloned into the BamHI site of a polylinker by operating according to the scheme indicated below :



The EcoRI fragment thus obtained is then cloned into the EcoRI site of pOM41 and transferred into the chromosome of the strain C600 (see Gene, 29, 231-241).

By means of cotransduction of P1' with a proximal marker aroB this structure is introduced into the chromosome of MM294 (F⁻ endA thi hsdR). E. coli pop 2135 is obtained : orientation maIT, C1857, PR, maIPO.

The transformation of the bacterial strain E. coli pop 2135 by the recombinant plasmids is performed according to the procedure using

rubidium described by Hanahan et al., J. Mol. Biol., 1983, 166, 557-580. This strain contains the allele *clt*s857 of the *cro* repressor.

The number of recombinants obtained is of the order of 10^3 to 10^4 ng of cDNA.

Selection of the clones by immunological detection.

The recombinant clones obtained are spread on nitrocellulose filters. The synthesis of the hybrid protein is induced after 2 hours of incubation at 42°C. After lysis of the bacteria by SDS and renaturation of the proteins in the absence of methanol, the clones are analysed by immunological screening. The renaturation of the proteins is carried out according to the procedure of Burnett reported in Anal. Biochem. 1981, 112, 195-203.

In the first step, the library is screened by means of a polyclonal antibody directed against intact porcine villin. A secondary screening is then carried out using a polyclonal antibody directed against a COOH-terminal fragment of chicken villin and two monoclonal antibodies (BDD2C3 and IID3H9) which recognizes the epitopes located in the COOH-terminal region of the molecule.

The clone pEXZ-V19 contains an insert of 510 base pairs. The coding part represents 330 base pairs. It is followed by a non-coding region of 200 base pairs.

The cloned cDNA codes for the 95 COOH-terminal amino acids of human villin and represents about 1/10 of the entire protein (molecular weight : 95 kD).

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CLAIMS

1/ Diagnostic procedure for the in vitro detection of tumor cells formed initially in the digestive region and/or derived from these latter in a biological sample suspected to have tumoral character, characterized by placing this biological sample in contact with a specific reagent for villin or its gene, constituted by anti-villin antibodies, these antibodies recognizing human villin, or by a DNA fragment coding for human villin.

2/ Monoclonal antibody directed against villin characterized by the following properties :

- it recognizes purified intact porcine villin (Western blotting)
- it recognizes the villin of a cell extract of the line derived from a human colon adenocarcinoma (Western blotting)-
- it recognizes rat villin in immunocytochemistry (frozen sections (cryostat) of rat intestinal mucosa fixed with formaldehyde),
- it is produced by the hybridoma deposited with the CNCM on 29 April 1985 under the No. I-440.

3/ Monoclonal antibody according to Claim 2, characterized in that it recognizes an antigenic site contained in the C-terminal sequence (779-854) or the "head part" of chicken villin of formula :

1		10	11	
	Val-Phe-Thr-Ala-Thr-Thr-Thr-Leu-Val-Pro-Thr-Lys-Leu-Glu-			
	20			
	Thr-Phe-Pro-Leu-Asp-Val-Leu-Val-Asn-Thr-Ala-Ala-Glu-Asp-			
	30 31		40 41	
	Leu-Pro-Arg-Gly-Val-Asp-Pro-Ser-Arg-Lys-Glu-Asn-His-Leu-			
	50 51			
	Ser-Asp-Glu-Asp-Phe-Lys-Ala-Val-Phe-Gly-Met-Thr-Arg-Ser-			
	60 61		70 71	
	Ala-Asn-Leu-Pro-Leu-Trp-Lys-Gln-Gln-Asn-Leu-Lys-Lys-Glu-			
	Glu-Lys-Gly-Leu-Phe			



the said monoclonal antibody being capable of recognizing the said "head part" irrespective of whether it exists in isolation or forms an integral part of the 51 kD peptide contained in chicken villin.

4/ Hybridoma producing the monoclonal antibody according to Claim 2 deposited with the C.N.C.M. under the No. I-440 on 29 April 1985.

5/ Biologically pure human villin giving essentially only a single band of 95 kD molecular weight when allowed to migrate on polyacrylamide gel electrophoresis.

6/ Procedure according to Claim 1, characterized in that the reagent recognizing the gene coding for human villin is constituted by a DNA, the sequence of which comprises a region coding for an amino acid sequence of human villin, more particularly the COOH-terminal part.

7/ Nucleic acid, characterized in that it corresponds to the complete mRNA of human villin or to a DNA fragment, the nucleotide sequence of which comprises a region coding for an amino acid sequence of human villin capable of hybridizing specifically with a nucleotide sequence coding for villin.

8/ cDNA fragment characterized by the fact that it contains at least a part of the nucleotide sequence coding for the amino acids of the COOH terminus of human villin, the nucleotide sequence and that of the corresponding amino acids possessing respectively the following sequences :



LYS TRP SER ASN THR LYS SER TYR GLU ASP LEU LYS ALA GLU
AAG TGG AGT AAC ACC AAA TCC TAT GAG GAC CTG AAG GCG GAG

SER GLY ASN SER ARG ASP TRP SER GLN ILE THR ALA GLU VAL
TCT GGC AAC TCT AGG GAC TGG AGC CAG ATC ACT GCT GAG GTC

THR SER PRO LYS VAL ASP VAL PHE ASN ALA ASN SER ASN LEU
ACA AGC CCC AAA GTG GAC GTG TTC AAT GCT AAC AGC AAC CTC

SER SER GLY PRO LEU PRO ILE PHE PRO LEU GLU GLN LEU VAL
AGT TCT GGG CCT CTG CCC ATC TTC CCC CTG GAG CAG CTA GTG

ASN LYS PRO VAL GLU GLU LEU PRO GLU GLY VAL ASP PRO SER
AAC AAG OCT GTA GAG GAG CTC CCC GAG GGT GTG GAC CCC AGC

ARG LYS GLU GLU HIS LEU SER ILE GLU ASP PHE THR GLN ALA
AGG AAG GAG GAA CAC CTG TCC ATT GAA GAT TTC ACT CAG GCC

PHE GLY MET THR PRO ALA ALA PHE SER ALA LEU PRO ARG TRP
TIT GGG ATG ACT CCA GCT GCC TTC TCT GCT CTG CCT CGA TGG

LYS GLN GLN ASN LEU LYS LYS GLU LYS GLY LEU PHE *
AAG CAA CAA AAC CTC AAG AAA GAA AAA GGA CTA TTT TGA GAAG
AGTAGCTGTGGTTGTAAAGCAGTACCCTACCCTGATTGTAGGGTCTCATTTTCTCA
CCGATATTAGTCCTACACCAATTGAAGTGAAATTTTGCAGATGTGCCTATGAGCAC
AAACTTCTGTGGCAAATGCCAGTTTTGTTTAATAAATGTACCTATTCCTTCAGAAA
GATGATACCCCAAAAAAAAAAAAA



9/ Probe for the detection of the mRNAs or the DNAs coding for human villin, characterized in that it comprises at least a part of the C-terminal nucleotide sequence according to Claim 7, this sequence being labelled if necessary by a radioactive element or any other group making possible its recognition in the hybridized state with the preparation containing the mRNA or the DNA to be studied.

10/ Procedure for obtaining the nucleotide sequence coding for the C-terminus of human villin, characterized in that :

- the total mRNAs (messenger RNAs) are prepared from a cell line expressing villin according to known procedures;
- a cDNA (complementary DNA) library is constructed ; ,
- the cDNAs are inserted into a vector capable of expressing the protein encoded in the insert;
- a bacterial strain is transformed with the aid of the recombinant vectors obtained, then conditions are defined which allow the expression of the desired protein in the bacteria;
- the recombinant clones containing the clone specific for villin is selected with the aid of antibodies recognizing villin.

11/ Procedure according to Claim 10, characterized in that the construction of the cDNA library is achieved with the aid of a cloning vector capable of expressing the protein encoded in the inserted cDNA, more especially by plasmids of the pEX type.

12/ Procedure according to Claim 10 or 11, characterized in that a bacterial strain of the E. coli type, more particularly E. coli pop 2135, is transformed with the aid of the recombinant vectors containing a nucleotide sequence coding for the C-terminus of human villin.

13/ Procedure according to any one of the Claims 10 to 12, characterized in that a screening of the cDNA library is carried out using a polyclonal anti-villin antibody such as a polyclonal antibody directed against intact porcine villin, then one or more monoclonal antibodies which recognize epitopes located in the COOH-terminal region of the molecule, the screening with the aid of the polyclonal antibody being advantageously followed by a secondary screening using another polyclonal antibody, in particular a polyclonal antibody directed against the COOH-terminal fragment of chicken villin.

14/ Clone bearing a cDNA corresponding to human villin, characterized in that it reacts specifically with the polyclonal antibody directed against intact villin, with the polyclonal antibody directed against the COOH-



terminal peptide of chicken villin and with the two monoclonal antibodies directed against COOH-terminal epitopes.