Biomarkers, 2010; 15(4): 315–324

informa
healthcare

ORIGINAL ARTICLE

Improvement of malignant serous effusions diagnosis by quantitative analysis of molecular claudin 4 expressison

Fauzia Mohamed[1], Nadine Vincent[1], Michèle Cottier[2], Michel Peoc'h[3], Yacine Merrouche[4], Brigitte Patouillard[5], Stéphane Paul[1], and Christian Genin[1]

[1]*Departments of Immunology,* [2]*Cytology,* [3]*Pathology and* [5]*Gastroenterology, University Hospital Center, and* [4]*Cancerology Institute of Loire, Saint-Etienne, France*

Abstract

Claudin gene expression is frequently altered in human cancers. Our aim was to improve the cytology diagnosis of malignancy in serous fluids with the quantification of claudins compared with various classic molecular markers using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method. Peritoneal or pleural effusions of 56 patients were assessed as malignant from histological analysis and 19 as benign. Claudin 4 was the most significantly upregulated (68%) marker in patients with malignant effusions. In cytologically negative malignant effusions, claudin 4 was found increased in 8/18 fluids. Quantitative RT-PCR is a sensitive method for the detection of free cancer cells in serous effusions.

Keywords: *Biomarkers; Claudin 4; effusions; quantitative RT-PCR*

Abbreviations:RT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; CLDN, claudin; TJ, tight junction; CLDN3, claudin 3; CLDN4, claudin 4; CLDN7, claudin 7; CLDN1, claudin 1; CLDN10, claudin 10; CLDN18, claudin 18; CEA, carcinoembryonic antigen; Ep-CAM, epithelial cell adhesion molecule; CK19, cytokeratin 19; CK20, cytokeratin 20; MUC1, mucin 1; MUC16, mucin 16; RPMI, RPMI-1640 medium; FCS, fetal calf serum; DMSO, dimethylsulfoxide; cDNA, complementary DNA; NTC, non-template control; RPLPO, ribosomal protein PO; Ct, threshold cycle; mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICC, immunocytochemistry; MUC5AC, mucin 5AC.

Introduction

The differential diagnosis between benign and malignant effusions represents a critical clinical problem. Cytological analysis is the method usually adopted to identify malignant cells in peritoneal and pleural fluids, but it seems not to be sensitive enough (40–60%). Molecular study of biomarkers could represent an interesting complementary approach to increase the efficiency of the diagnosis in these biological samples.

The claudin (CLDN) genes encode a family of 24 proteins essential in tight junction formation and function (TJ) in epithelial and endothelial cells (Tsukita & Furuse 2000, Lim et al. 2008, Krause et al. 2008, Kulka et al. 2009). Immunohistochemical localization of CLDN indicated that CLDN were expressed along the entire length of the lateral plasma membranes between epithelial cells, including apical areas containing TJ structures (Kulka et al. 2009). It is thought that various claudin family members can confer different properties to epithelial cell permeability and account for some of the selective variability of different barriers. Two distinct pathways are known for this transport: the transcellular and paracellular pathways in which materials move across plasma membranes and TJs, respectively (Spring 1998, Tsukita et al. 2000, Hou et al. 2008). The exact combination of claudin proteins within a given tissue is thought to determine the selectivity and strength of the TJs. Recent gene

Address for Correspondence: C. Genin, Department of Immunology, CHU de Saint Etienne, 42055 Saint Etienne Cedex 2, France. Tel: 33 (0) 4 77 82 83 79. Fax: 33 (0) 4 77 82 84 93. E-mail: Christian.Genin@chu-st-etienne.fr

(Received 27 November 2009; revised 27 January 2010; accepted 27 January 2010)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547501003658114

expression profiling analyses have shown that claudin gene expression is frequently altered in various cancers (Morin 2005, Swisshelm et al. 2005). Several studies have been done by immunohistochemistry showing an expression of CLDN in tumour tissues. For example, CLDN3 and CLDN4 are frequently upregulated in ovarian, breast, prostate and pancreatic tumours (Michl et al. 2001, Hough et al. 2000, Long et al. 2001, Rangel et al. 2003, Kominsky et al. 2004, Tokes et al. 2005). CLDN7 is downregulated in breast and head and neck cancer, but elevated in gastric cancer (Kominsky et al. 2003, Johnson et al. 2005). On the other hand, loss of CLDN7 appears to be associated with a more aggressive behaviour of breast carcinoma (Kominsky et al. 2003, Sheehan et al. 2007). CLDN1 is typically downregulated in various cancers, but has also been reported to be elevated in other studies. CLDN1, CLDN10 and CLDN18 have been reported to have prognostic value in colon cancer, hepatocellular carcinoma and in gastric cancer, respectively (Hewitt et al. 2006, Sanada et al. 2006, Resnick et al. 2005, Hoevel et al. 2002, Kramer et al. 2000, Cheung et al. 2005). Moreover, claudins are surface proteins and for this reason may represent useful target for various therapeutic strategies. However, the expression patterns of the whole family of claudins in cancer and normal tissues have not been well characterized.

In this study, we evaluated the quantitative expression of CLDN1, CLDN4 and CLDN18 in comparison with a panel of molecular classic markers for the detection of cancer cells in serous effusions, and to assess their usefulness as differential diagnostic markers of primary tumour tissues. It is likely that the use of multiple markers may be required to achieve optimal correlation with histology of distinct original malignant diseases. For these reasons, we have also decided to evaluate in our cohort the quantitative expression of carcinoembryonic antigen (CEA) which is one of the most common specific markers of tumours (Passebosc-Faure et al. 2005), the epithelial cell adhesion molecule (Ep-CAM) which is expressed in most of epithelial cells and epithelial tumours and usually overexpressed in the latter (Zhang et al. 2007, Winter et al. 2003), two members of the cytokeratin family (CK19, CK20), and two of the mucin family (MUC1, MUC16) which are usually preserved in neoplastic cells, and may be useful to classify the primary sites of digestive or gynaecological cancers and may be useful in predicting the primary sites of digestive cancers (Backus et al. 2005, Lee et al. 2003, Yu et al. 2001). Despite several studies, the overall quantitative expression of these markers in cancer patients remains conflicting and inconclusive and is still not used to detect tumour cells in serous effusions.

In the present study, we have analysed the expression of CLDN1, CLDN4, CLDN18 and CEA, Ep-CAM, CK19, CK20, MUC1 and MUC16 by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) in peritoneal or pleural effusions in patients with cancer assessed by cytological analysis or with hepatic cirrhosis or cardiac failure as controls.

Methods

Patients

Serous effusions of 75 patients (43 female patients, age range 42–90 years and 32 male patients, age range 48–86 years), (67 ascitic and eight pleural fluids) were collected prospectively from the Outpatients Department, Cancerology Institute of Loire and Gastro-Enterology, University Hospital Center of Saint Etienne and from the Department of Internal Medicine, Hospital Center of Saint-Chamond. The effusion fluids were collected in 2 l autoclaved containers with 5000 units of heparin.

All effusions had a reliable diagnosis documented by histological analysis of primary tumours which is the standard in this study, and follow-up of the patients could connect the effusion with the primary tumour. The pleural or peritoneal biopsies were performed only for the diagnosis of mesothelioma or if the primary tumour was not detected. Effusions from 19 patients with non-malignant disease caused by hepatic cirrhosis (15) or cardiac failure (4) were used as controls.

Among the 56 malignant effusions, 18 (32%) were cytologically negative. The cancer primary sites of patients with malignant serous effusions are detailed in Table 3. This study was approved by the institutional ethics committee.

Preparation of cells from serous effusions

Each fluid sample was centrifuged in 50 ml tubes at 300 g for 10 min at room temperature. Then, pellets were pooled together, washed once and resuspended in RPMI 1640 with 10% FCS. Fractions of 900 µl were distributed in 1.5 ml cryotubes containing 100 µl DMSO and stored in liquid nitrogen until use. When needed, aliquots were thawed in a 37°C water bath and then washed once with Ca^{2+}/Mg^{2+}-free NaCl solution (0.01 mol l^{-1}), and a cell count was done.

RNA extraction

Total RNA was extracted by using an RNeasy Mini Kit (Qiagen, Courtaboeuf, France) accordingly to the manufacturer's instructions. Briefly, 600 µl of RLT solution was used to lyse the cells. The lysed cells were homogenized by the use of a column arise, and then 600 µl of ethanol 70% was added and the RNA was treated by the RNase-free DNase Set (Qiagen). The solution was then washed with 600 µl RW1 buffer and 500 µl of REP buffer. RNA was

eluted in 30 µl of RNase-free water. Purified RNA were quantified by spectrophotometry at 260 nm and stored at -80°C before use.

Reverse transcription PCR

Reverse transcription was conducted with the Omniscript RT Kit for RT-PCR (Qiagen) following the manufacturer's instructions; 2 µl of DNTP 5 mM, 2 µl of buffer mix 10x, 1 µl of Oligo DT, 1 µl of ORT and 0.5 µg total RNA in 20 µl final volume were incubated for 1 h at 37°C, then the obtained cDNA was stored at –20°C before use.

The primers were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). Primers for RPLPO were also used to check RNA integrity and the efficiency of the reverse transcription step. The sequences of the primers were designed to span splice junctions or in two different exons so that genomic DNA contamination could be monitored (Table 1).

Quantitative real-time PCR

PCR reactions were performed with the ABI Prism 7500 (Applied Biosystems). PCR amplifications were performed in a total volume of 25 µl comprising 5 µl of cDNA template diluted 1:50 and 20 µl Master Mix Syber Green PCR (Applied Biosystems) and primers at 200 nM. The program included a PCR initial denaturation of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each experiment included non-template control (NTC) for which the cDNA was replaced by pure water. All PCR reactions were performed in duplicate or triplicate. Each pair of primers was tested by adding a dissociation curve as a result of PCR. The presence of a single peak for each sample confirmed the specificity of the PCR.

Preparation of the standard curve

The plasmids RPLPO, CLDN1, -4, -18, CEA, Ep-CAM, CK19, -20, MUC1 and -16 were obtained by cloning the amplified DNA sequence in the vector pDrive (3850 bp). The RPLPO reference plasmid was used to standardize and determine the number of RPLPO copies compared with the concentration of plasmid allowing us to define a standard range of gene RPLPO (897 ng µl^{-1}). For the standard range, a series of dilutions of 10^6 copies to 500 copies were used. NTC controls were also included where DNA has been replaced by pure water.

Standard curve

RPLPO was chosen in this study as a housekeeping gene. A standard curve was calculated using linear regression analysis. The standard curve showed a linear relationship between the values of Ct and the logarithm of the initial plasmid copy number. The amount of product in a sample was determined by interpolation from a standard curve of Ct values generated by the serial dilution plasmid ranging from 10^6 to 500 copies of each plasmid.

Statistical analysis

The comparison of RPLPO mRNA levels among all the patients examined was performed using the non-

Table 1. Sequences and features of the primers used for RT-PCR.

Name	Accession number	Sequence of sense and antisense primers	Localization	Length
RPLPO	NM-001002	5′-GGCGACCTGGAAGTCCAACTAC-3′	Exon 2-3	98 bp
		5′-AGACAATGTGGGCTCCAAGC-3′		
CLDN1	NM-021101	5′-GCAACCCGTGCCTTGATG-3′	Exon 2	63 bp
		5′-GGTGGCCACAAAGATTGCTATC-3′		
CLDN4	NM-001305	5′-GCTGGCCAGGATAGCTTAACC-3′	Exon 1	54 bp
		5′-GCCAACGCCGATGCA-3′		
CLDN18	NM-016369	5′-CACCTCCGTGTTCCAGTACGA-3′	Exon 1	70 bp
		5′-GCATTCGGTGAAGCC TGAA-3′		
CEA	NM-001712	5′-GCCCTGGTTGCTCTGATAGC-3′	Exon 6	58 bp
		5′-CGGTCTTCCCGAAATGCA-3′		
Ep-CAM	NM-002354	5′-CTGAATTCTCAATGCAGGGTCTAA-3′	Exon 7	68 bp
		5′-AACTGCTATCACCACAACCACAA-3′		
CK19	NM-002276	5′-GAACCAAGTTTGAGACGGAACAG-3′	Exon 2-3	64 bp
		5′-CGCAGGCCGTTGATGTC-3′		
CK20	NM-019010	5′-CTGCAAAATGCTCGGTGTGT-3′	Exon 2	61 bp
		5′-TGAAGTCCTCAGCAGCCAGTT-3′		
MUC1	NM-002456	5′-ACGGGTTCTGGTCATGCAA-3′	Exon 2	59 bp
		5′-TGGGTAGCCGAAGTCTCCTTT-3′		
MUC16	NM-024690	5′-CGCTGGACATAAACTTGGAATCT-3′	Exon 3	69 bp
		5′-TTCTCTCATATGGGCTGCTTTTC-3′		

parametric Kruskal–Wallis test. Considering that we defined a positivity threshold in copy number for each marker, values measured by quantitative RT-PCR were expressed as positive or negative. mRNA values from the various markers studied in groups of patients with or without serous malignant fluid were compared using the χ^2 test. A p-value < 0.05 was considered statistically significant.

Results

Normalization of the method

To measure gene expression quantitatively in biological specimens, normalization was necessary to correct expression data due to variations introduced during sample preparation such as the quality of RNA or PCR efficiency. In most previous studies, a single housekeeping gene was used for normalization.

In 15 samples of peritoneal fluids, the expression of three different genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ribosomal protein (RPLPO) frequently used as housekeeping genes to study the stability and the homogeneity were measured by quantitative RT-PCR. We found that RPLPO was the most adapted gene in such samples, while GAPDH and β-actin were demonstrated as quite variable housekeeping genes (data not shown).

We then checked that the results were reproducible over time and with different preparations of total RNA for each patient (data not shown).

Real-time PCR standard curves obtained for the different genes studied

Quantitative real-time PCR monitoring the fluorescent signal of each cycle allowed sensitive and specific detection of CLDN1, CLDN4, CLDN18, CEA, Ep-CAM, CK19, CK20, MUC1 and MUC16 mRNA to determine the absolute number in the different samples of serous fluids from patients. For each of these genes, the assay limiting value for sensitivity was found to be 500 copies. Each curve was obtained after three experiments repeated and constructed using data by plotting the Ct (threshold cycle) value against the input cDNA concentration (serial dilutions of pDrive-vector) of samples. The calculated threshold cycle (Ct) reflects the quantity of the starting targets, with lower Ct values reflecting a greater amount of starting target molecules (Oki et al. 2002). The Ct value decreased linearly with increasing target quantity from 500 copies to 1 million copies. For the different genes studied, the values for patient samples were calculated with reference to the standard curve (Figure 1 and Table 2). All plasmids were verified by sequencing. The real-time PCR was performed on dilutions of plasmids for each gene studied. The parameters of calibration lines obtained indicate that the PCR conditions were very efficient ($R^2 = 0.99$) and the measured fluorescence was proportional to the quantities present in the samples (theoretical slope = –3.32). We were able to establish optimal conditions for the quantitative study of several tumour markers by real-time PCR (Table 2).

Amplification and subsequent data analysis without post-amplification procedures such as gel electrophoresis could be achieved using a sequence detector (ABI Prism; Applied Biosystems). This will theoretically reduce the possibility of laboratory contamination and false positivity.

Expression level of different markers in serous fluids

RT-PCR using CLDN1, CLDN4, CLDN 18, CEA, Ep-CAM, CK19, CK20, MUC1 and MUC16 specific primers were

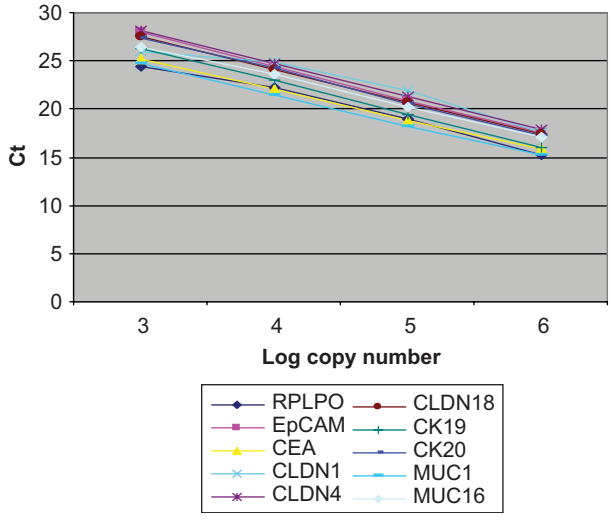

Figure 1. Standard curves obtained with plasmids encoding a panel of markers. Each curve was obtained with various plasmid concentrations. They were constructed using data from three experiments repeat by plotting the Ct (threshold cycle) value against the input cDNA concentration (serial dilutions of pDrive-vector) of samples.

Table 2. Parameters of the standard lines obtained for each tumour marker.

Plasmids	Slope	Intercept	R^2
RPLPO	–3.3	37.74	0.99
CLDN1	–3.31	37.66	0.99
CLDN4	–3.39	38.24	0.99
CLDN18	–3.39	37.75	0.99
CEA	–3.37	37.67	0.99
Ep-CAM	–3.12	36.48	0.99
CK19	–3.43	36.65	0.99
CK20	–3.4	37.67	0.99
MUC1	–3.16	34.14	0.99
MUC16	–3.09	35.69	0.99

performed with all of the 75 samples obtained in this study. Relative quantification using RPLPO as the reference gene was also applied for each sample. No significant difference for the relative RPLPO gene copy numbers was observed in patients with benign or malignant effusions ($p > 0.05$). The RPLPO copy number was used to normalize the biomarker gene copy number in all patients (data not shown).

The expression of CLDN1, CLDN4, CEA, Ep-CAM, CK20 and MUC1 expressed in copy number is plotted in Figure 2.

We set up a threshold of positivity for each marker defined as the value of the copy number just below the highest one measured by real-time PCR in benign fluid controls for each marker (significant 95%). Fluids with higher copy numbers than these values were considered as malignant ($p < 0.05$).

Our study demonstrates that the limit copy number was 95 725 for CLDN1, 28 908 for CDLN4, 33 191 for CLDN18, 43 755 for CEA, 81 475 for Ep-CAM, 56 367 for CK19, 21 430 for CK20, 22 962 for MUC1 and 34 755 for MUC16.

The percentage of positive fluids for all markers examined, except CLDN18 and CK19, were significantly higher in the patients with malignant effusions than those with benign effusions ($p < 0.05$) (Table 3).

The rate of patients positive for CLDN4 was 68% ($p < 0.0000001$) in all malignant effusions group, 74% ($p < 0.0000001$) in the gynaecological cancers group and 61% ($p = 0.000006$) in the digestive cancers group, and for Ep-CAM 57% ($p = 0.000042$), 70% ($p = 0.000003$) and 43% ($p = 0.004$), respectively (Table 3). Only four malignant effusions with negative markers were detected by cytology (Table 4).

The addition of the percentage of patients positive for CLDN4, Ep-CAM and CK20 molecular markers combined with the percentage of patients positive for cytology could enhance the detection of malignant effusions 48/56 (86%), gynaecological cancers 20/23 (87%) and 19/23 (83%) digestive cancers, respectively, as shown in Tables 4 and 5. Ten patients were cytologically negative but positive with molecular markers.

The rates of patients positive only in gynaecological cancer effusions were 43% ($p = 0.004$) for CLDN1,

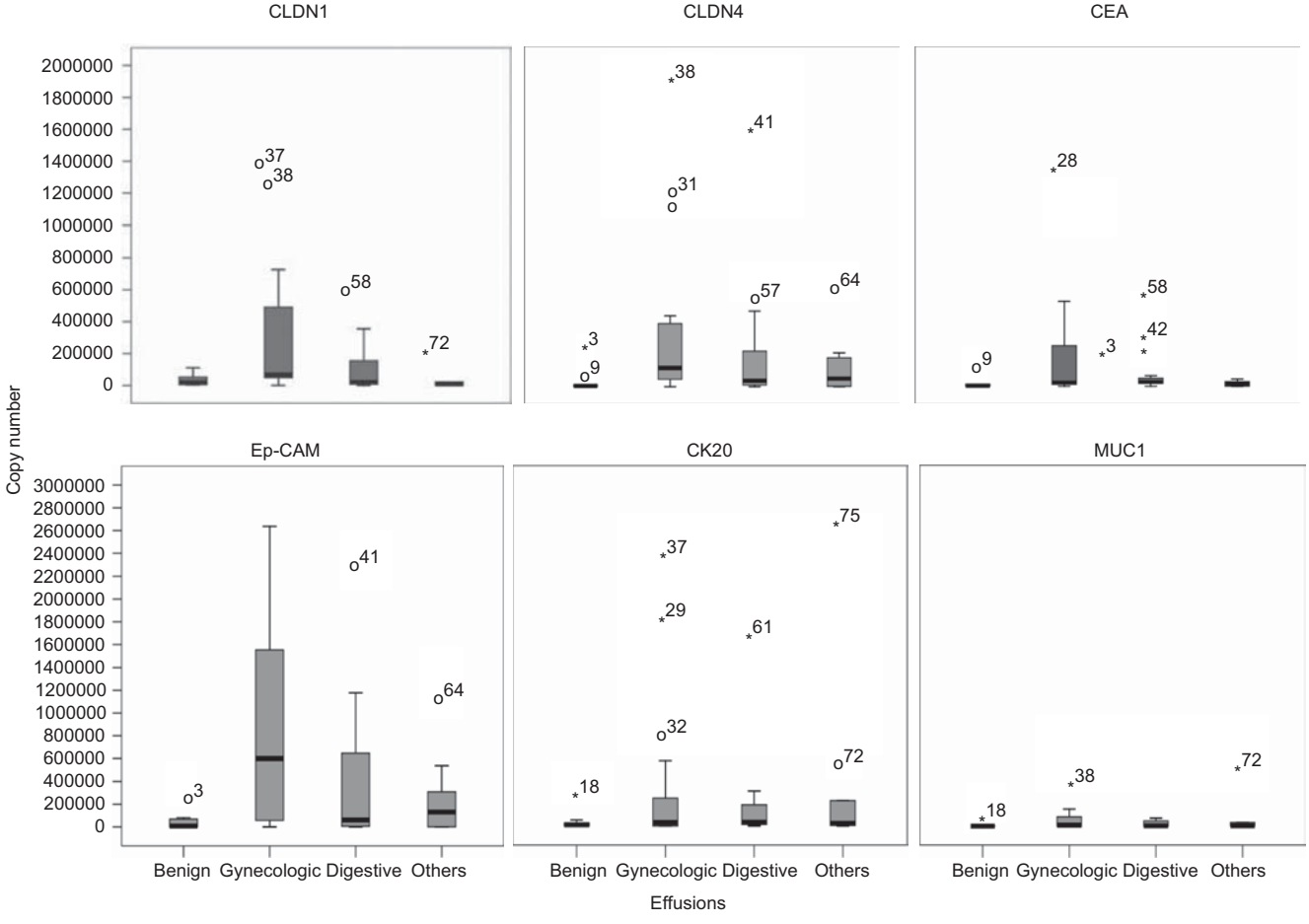

Figure 2. Box-plot showing the expression of CLDN1, CLDN4, CEA, Ep-CAM, CK20 and MUC1 by real-time PCR in serous fluids. The *x*-axis indicates the tissue origin of effusions, the *y*-axis indicates the copy number of each gene. Median value and interval limits are indicated for all the markers studied in each group of patients.

Table 3. Quantitative analysis of molecular marker expression by real-time RT-PCR in effusions

			Number of positive fluids. **(% of positive fluids)**								
			p-Value								
Diagnosis	**Effusions** (*n*)	**Tumour cells negative** (*n*)	**CLDN1** 95725[a]	**CLDN4** 28908[a]	**CLDN18** 33191[a]	**CEA** 43755[a]	**Ep-CAM** 81475[a]	**CK19** 56367[a]	**CK20** 21430[a]	**MUC1** 22962[a]	**MUC16** 34755[a]
Benign	19	19	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)	1 (**5**)
Malignant	56	18	20 (**36**)	38 (**68**)	13 (**23**)	20 (**36**)	32 (**57**)	11 (**20**)	23 (**41**)	16 (**29**)	15 (**27**)
			*0.01**	*0.0000001**	*0.08*	*0.01**	*0.000042**	*0.14*	*0.003**	*0.03**	*0.04**
Gynaecological	23	7	10 (**43**)	17 (**74**)	7 (**30**)	10 (**43**)	16 (**70**)	5 (**22**)	10 (**43**)	8 (**35**)	10 (**43**)
			*0.004**	*0.0000001**	*0.03**	*0.004**	*0.000003**	*0.13*	*0.004**	*0.01**	*0.004**
Breast	15	6	5 (**33**)	10 (**67**)	3 (**20**)	5 (**33**)	11 (**73**)	3 (**20**)	5 (**33**)	4 (**27**)	6 (**40**)
Ovary	8	1	5 (**62**)	7 (**87**)	4 (**50**)	5 (**62**)	5 (**62**)	2 (**25**)	5 (**62**)	4 (**50**)	4 (**50**)
Digestive	23	9	6 (**26**)	14 (**61**)	5 (**22**)	7 (**30**)	10 (**43**)	3 (**13**)	10 (**43**)	6 (**26**)	3 (**13**)
			0.07	*0.00006**	*0.13*	*0.03**	*0.004**	*0.4*	*0.004**	*0.07*	*0.4*
Colon	8	3	2 (**25**)	5 (**62**)	2 (**25**)	1 (**12**)	3 (**37**)	0 (**0**)	5 (**62**)	1 (**12**)	1 (**12**)
Pancreas	7	0	3 (**43**)	5 (**71**)	2 (**28**)	4 (**57**)	4 (**57**)	1 (**14**)	3 (**43**)	3 (**43**)	2 (**28**)
Liver	5	5	1 (**20**)	2 (**40**)	1 (**20**)	2 (**40**)	2 (**40**)	2 (**40**)	2 (**40**)	2 (**40**)	0 (**0**)
Small intestine	1	1	0	0	0	0	0	0	0	0	0
Stomach	1	0	0	1	0	0	1	0	0	0	0
Gall bladder	1	0	1	1	0	0	0	0	0	0	0
Others	10	2	4 (**40**)	7 (**70**)	1 (**10**)	3 (**30**)	6 (**60**)	3 (**30**)	3 (**30**)	2 (**20**)	2 (**20**)
			*0.01**	*0.00003**	*0.6*	*0.07*	*0.0004**	*0.07*	*0.07*	*0.23*	*0.23*
Lung	4	0	0 (0)	4 (100)	0 (0)	1 (25)	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Mesothelioma	3	0	2 (66)	1 (33)	1 (33)	1 (33)	1 (33)	2 (66)	2 (66)	2 (66)	2 (66)
Kidney	1	1	0	0	0	0	0	0	0	0	0
Prostate	1	0	1	1	0	1	1	1	1	0	0
Myeloid leukaemia	1	1	1	1	0	0	0	0	0	0	0

[a]Limit copy number was the value just below the highest one measured in benign fluid controls for each marker by real-time PCR (significant 95%).
*Significant 95%

35% ($p = 0.01$) for MUC1 and 43% ($p = 0.004$) for MUC16 (Table 3).

The quantitative analysis of molecular marker expression in control effusions is shown in Table 6.

Two patients with benign effusions (cardiac failure and hepatic cirrhosis) were positive for CLDN4, CEA and Ep-CAM markers and for CK20 and MUC1, respectively (Table 6). Although the copy number was moderately increased, it cannot be excluded in those patients who we did not have the opportunity to follow, who developed a cancer or who presented an overexpression of markers in a chronic inflammatory effusion.

Analysis of biomarker expression in cytologically negative malignant effusions

Among the 56 effusions diagnosed as malignant by histological analysis and patient clinical data, cytological examination was negative in 18 (32%). Markers demonstrated as able to discriminate significantly malignant versus benign effusions (all except CLDN18 and CK19), were measured in these fluids. At least one marker was found increased in 10 out 18 effusions (Table 5). CLDN4 and Ep-CAM and CK20 were the markers most frequently found to be increased.

Discussion

To improve the detection of cancer cells in the effusions, very sensitive and specific new methods appear to be useful. The presence of cancer cells in effusions is a straightforward issue when a large number of overtly atypical cells are present but may pose more difficulty when these cells are few and less conspicuous. The detection of malignancy is hampered by the problem of differentiating malignant cells from reactive mesothelial cells. Therefore, it has been the practice to supplement morphological examination of cytological specimens with ancillary studies, of which immunocytochemistry has become the most frequently applied (Bedrossian 1998). These techniques provide new insight regarding the molecular characteristics of cancer cells in effusions, and therefore are of potential interest from a biological and therapeutic standpoint (Fetsch & Abati 2001). The results of previous studies have suggested that a panel of different molecular markers in the effusions can improve the diagnostic power of cytological analysis in detecting malignant cells (Brock et al. 2005, Mohanty & Dey 2003). Recent studies using RT-PCR suggested the usefulness of this method in the detection of cancer cells in effusions. The RT-PCR test for some markers such as mammoglobin

Table 4. Quantitative analysis of molecular marker expression by real-time PCR in cytologically positive effusions.

			CLDN1	CLDN4	CEA	Ep-CAM	CK20	MUC1
No.	Sex/age (years)	Diagnosis	95725[a]	28908[a]	43755[a]	81475[a]	21430[a]	22962[a]
1	F/86	Breast	+	−	−	+	−	−
2	F/75	Breast	−	+	−	+	−	−
3	F/79	Breast	−	+	−	+	−	+
4	F/54	Breast	−	+	−	+	−	−
5	F/58	Breast	+	+	+	+	+	−
6	F/56	Breast	+	+	+	+	+	−
7	F/46	Breast	+	+	+	−	+	+
8	F/61	Breast	−	+	+	+	+	−
9	F/56	Breast	−	+	−	+	−	+
10	F/73	Ovary	+	+	+	+	+	+
11	F/56	Ovary	+	+	+	+	+	−
12	F/73	Ovary	+	+	+	+	+	+
13	F/82	Ovary	+	+	+	+	+	+
14	F/66	Ovary	+	+	+	+	+	+
15	F/88	Ovary	−	+	−	−	−	−
16	F/59	Ovary	−	−	−	−	−	−
17	F/85	Pancreas	+	+	−	+	−	+
18	F/64	Pancreas	+	+	+	+	+	−
19	F/69	Pancreas	−	−	−	−	−	−
20	M/57	Pancreas	−	−	−	−	−	−
21	M/56	Pancreas	−	+	+	−	+	−
22	F/76	Pancreas	+	+	+	+	+	+
23	M/65	Pancreas	−	+	+	+	−	+
24	M/67	Colon	+	+	+	+	+	−
25	F/52	Colon	−	−	−	−	−	−
26	F/61	Colon	−	−	−	+	−	−
27	M/66	Colon	−	+	−	+	+	+
28	F/85	Colon	−	+	−	−	+	−
29	F/56	Stomach	−	+	−	+	−	−
30	M/73	Gall bladder	−	+	−	−	−	−
31	M/69	Lung	−	+	+	+	−	−
32	F/78	Lung	−	+	−	+	−	−
33	M/59	Lung	−	+	−	+	−	−
34	M/56	Lung	−	+	−	+	−	−
35	M/60	Mesothelioma	−	+	−	+	−	−
36	F/80	Mesothelioma	+	−	−	−	+	+
37	M/72	Mesothelioma	+	−	+	−	+	+
38	M/77	Prostate	+	+	+	+	+	−

[a]Limit copy number was the value just below the highest one measured in benign fluid controls for each marker by real-time PCR (significant 95%).

was found to be of higher sensitivity than routine cytology in the detection of carcinoma cells (Grunewald et al. 2002). The expression of the glycoprotein genes MUC1 and MUC5AC was found to be a discriminator between malignant and benign effusions using quantitative RT-PCR (Yu et al. 2001).

In the present study, our aim was to demonstrate that the quantitative expression analysis of several molecular markers is a more sensitive and rapid method and that it could improve the yield of cytological analysis for cancer diagnosis in serous effusions. We have developed a real-time RT-PCR technique to quantify the mRNA copy numbers of three members of the claudin family (CLDN1, -4 and -18) and we compared them with a panel of usual cancer-specific molecular markers in a large number of benign and malignant serous fluids using plasmid dilutions of cloned PCR fragments to obtain more reliable and reproducible quantification of these marker transcripts.

Even if these markers have been studied at the mRNA level, one could expect them to be functional at the protein level according to the numerous publications that have studied them by immunocytochemistry in tissues or serous effusions (Kulka et al. 2009, Tokes et al. 2005, Monte et al. 1987, Facchetti et al. 2007). Our results indicate that the markers studied are highly expressed in serous effusions of tumoral origins except CLDN18 and CK19, which were not significantly increased compared with benign fluids.

Table 5. Quantitative analysis of molecular marker expression by real-time PCR in cytologically negative malignant effusions.

			CLDN1	CLDN4	CEA	Ep-CAM	CK20	MUC1
N°	Age (years)/sex	Diagnosis	95725[a]	28908[a]	43755[a]	81475[a]	21430[a]	22962[a]
1	42/F	Breast	–	–	–	–	–	–
2	49/F	Breast	–	+	–	+	–	–
3	70/F	Breast	–	–	–	–	–	–
4	63/F	Breast	+	+	+	+	+	+
5	83/F	Breast	–	–	–	–	–	–
6	83/F	Breast	–	–	–	+	–	–
7	90/F	Ovary	–	+	–	–	–	–
8	65/M	Colon	–	+	–	–	–	–
9	80/M	Colon	+	+	–	–	+	–
10	82/F	Colon	–	–	–	–	+	–
11	77/M	Liver	–	–	–	–	–	–
12	72/M	Liver	–	–	–	–	–	–
13	67/M	Liver	–	+	+	+	+	+
14	58/M	Liver	–	–	–	–	–	–
15	76/M	Liver	+	+	+	+	+	+
16	79/F	Small intestine	–	–	–	–	–	–
17	61/M	Kidney	–	–	–	–	–	–
18	49/M	Myeloid leukaemia	+	+	–	–	–	–

[a]Limit copy number was the value just below the highest one measured in benign fluid controls for each marker by real-time PCR (significant 95%).

Table 6. Quantitative analysis of molecular marker expression by real-time PCR in control effusions.

			CLDN1	CLDN4	CEA	Ep-CAM	CK20	MUC1
No.	Sex/age (years)	Diagnosis	95725[a]	28908[a]	43755[a]	81475[a]	21430[a]	22962[a]
1	F/76	Cardiac failure	–	–	–	–	–	–
2	M/78	Cardiac failure	–	–	–	–	–	–
3	M/54	Cardiac failure	–	+	+	+	–	–
4	F/90	Cardiac failure	–	–	–	–	–	–
5	M/73	Hepatic cirrhosis	–	–	–	–	–	–
6	F/86	Hepatic cirrhosis	–	–	–	–	–	–
7	M/48	Hepatic cirrhosis	–	–	–	–	–	–
8	F/76	Hepatic cirrhosis	–	–	–	–	–	–
9	M/75	Hepatic cirrhosis	–	–	–	–	–	–
10	F/46	Hepatic cirrhosis	–	–	–	–	–	–
11	M/54	Hepatic cirrhosis	–	–	–	–	–	–
12	M/64	Hepatic cirrhosis	–	–	–	–	–	–
13	M/60	Hepatic cirrhosis	–	–	–	–	–	–
14	M/86	Hepatic cirrhosis	–	–	–	–	–	–
15	F/46	Hepatic cirrhosis	–	–	–	–	–	–
16	M/56	Hepatic cirrhosis	–	–	–	–	–	–
17	F/82	Hepatic cirrhosis	–	–	–	–	–	–
18	M/57	Hepatic cirrhosis	+	–	–	–	+	+
19	M/61	Hepatic cirrhosis	–	–	–	–	–	–

[a]Limit copy number was the value just below the highest one measured in benign fluid controls for each marker by real-time PCR (significant 95%).

Only CLDN4 and Ep-CAM were found increased in all types of cancer tested and appeared to be the most discriminant markers. CLDN4 appeared as the most sensitive and discriminant marker for cancer diagnosis. The combined use of CLDN4 and/or Ep-CAM and/or CK20 and cytology increased the efficiency of the diagnosis of malignant effusions from 68% to 86% of patients. CLDN1, MUC1 and MUC16 were increased only in gynaecological cancers. In cytologically negative malignant effusions, at least one molecular marker was found increased in 50% of patients and CLDN4 was the most sensitive. CLDN4 exhibited several advantages compared with other markers studied for optimal specificity and sensitivity (Facchetti et al. 2007). Many explanations can be expected for marker expression in cytologically negative effusions. First, the malignant cells could be too few to be visualized by microscopy, their morphological abnormalities are not characteristic enough to set up the diagnosis of

malignancy and the disturbance of gene transcription can explain why molecular markers can appear very early in the malignant process. False-positive results can be excluded because the analysis was repeated two times with a conserved copy number very increased.

The histological type of the primary tumours was very heterogeneous and without common features concerning tumour size and grade in these 18 cases. Unfortunately, we could not analyse by immunochemistry the original tumour tissues concerning their CLDN4 and Ep-CAM expression.

In conclusion, our study indicates that RT-PCR quantification of different markers in serous effusions is a valuable tool for the detection of free cancer cells in the fluids. Claudin family genes appeared of great interest as CLDN4 was identified as the most sensitive and discriminant marker for cancer diagnosis and CLDN1 was increased only in gynaecological cancers. From quantification of molecular markers in cytologically negative malignant effusions, CLDN4 could be interestingly associated with Ep-CAM and CK20 in a RT-PCR multiplex setting to improve the efficiency of diagnosis.

Our study demonstrated that the ability to malignancy in effusion specimens from cancer patients can be markedly improved by the use of molecular techniques such as quantitative real-time RT-PCR in addition to the standard cytopathological examination. If these molecular bioassays could improve the early diagnosis of cancer and/or the ability to predict a metastatic development from primary tumours this would be very useful in the future.

Acknowledgements

We thank C. Ruesch MD, D. Mille MD, B. Hacquard MD, S. Kawaye MD and W. Mazloum for providing serous effusions, M. Rehailia for statistical analysis, A. Buchmuller and F. Rancon from DRCC for methodology assistance, F. Duplat for technical assistance and C. Moulin for secretary assistance. This work was supported by a grant from la Ligue Contre le Cancer, Comité de la Loire and the Clinical Research Direction of University Hospital Center.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Backus J, Laughlin T, Wang Y, Belly R, White R, Baden J, Justus Min C, Mannie A, Tafra L, Atkins D, Verbanac KM. (2005). Identification and characterization of optimal gene expression markers for detection of breast cancer metastasis. *J Mol Diagn* 7:327–36.

Bedrossian CW. (1998). Diagnostic problems in serous effusions. *Diagn Cytopathol* 19:131–7.

Brock MV, Hooker CM, Yung R, Guo M, Han Y, Ames SE, Chang D, Yang SC, Mason D, Sussman M, Baylin SB, Herman JG. (2005). Can we improve the cytologic examination of malignant pleural effusions using molecular analysis? *Ann Thorac Surg* 80:1241–7.

Cheung ST, Leung KL, Ip YC, Chen X, Fong DY, Ng IO, Fan ST, So S. (2005). Claudin-10 expression level is associated with recurrence of primary hepatocellular carcinoma. *Clin Cancer Res* 11:551–6.

Facchetti F, Lonardi S, Gentili F, Bercich L, Falchetti M, Tardanico R, Baronchelli C, Lucini L, Santin A, Murer B. (2007). Claudin 4 identifies a wide spectrum of epithelial neoplasms and represents a very useful marker for carcinoma versus mesothelioma diagnosis in pleural and peritoneal biopsies and effusions. *Virchows Arch* 451:669–80.

Fetsch PA, Abati A. (2001). Immunocytochemistry in effusion cytology: a contemporary review. *Cancer* 93:293–308.

Grunewald K, Haun M, Fiegl M, Urbanek M, Muller-Holzner E, Massoner A, Riha K, Propst A, Marth C, Gastl G. (2002). Mammaglobin expression in gynecologic malignancies and malignant effusions detected by nested reverse transcriptase-polymerase chain reaction. *Lab Invest* 82:1147–53.

Hewitt KJ, Agarwal R, Morin PJ. (2006). The claudin gene family: expression in normal and neoplastic tissues. *BMC Cancer* 6:186.

Hoevel T, Macek R, Mundigl O, Swisshelm K, Kubbies M. (2002). Expression and targeting of the tight junction protein CLDN1 in CLDN1-negative human breast tumor cells. *J Cell Physiol* 191:60–8.

Hou J, Renigunta A, Konrad M, Gomes AS, Schneeberger EE, Paul DL, Waldegger S, Goodenough DA. (2008). Claudin-16 and claudin-19 interact and form a cation-selective tight junction complex. *J Clin Invest* 118:619–28.

Hough CD, Sherman-Baust CA, Pizer ES, Montz FJ, Im DD, Rosenshein NB, Cho KR, Riggins GJ, Morin PJ. (2000). Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. *Cancer Res* 60:6281–7.

Johnson AH, Frierson HF, Zaika A, Powell SM, Roche J, Crowe S, Moskaluk CA, El-Rifai W. (2005). Expression of tight-junction protein claudin-7 is an early event in gastric tumorigenesis. *Am J Pathol* 167:577–84.

Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallioniemi OP, Sukumar S. (2003). Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. *Oncogene* 22:2021–33.

Kominsky SL, Vali M, Korz D, Gabig TG, Weitzman SA, Argani P, Sukumar S. (2004). Clostridium perfringens enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. *Am J Pathol* 164:1627–33.

Kramer F, White K, Kubbies M, Swisshelm K, Weber BH. (2000). Genomic organization of claudin-1 and its assessment in hereditary and sporadic breast cancer. *Hum Genet* 107:249–56.

Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. (2008). Structure and function of claudins. *Biochim Biophys Acta* 1778:631–45.

Kulka J, Szasz AM, Nemeth Z, Madaras L, Schaff Z, Molnar IA, Tokes AM. (2009). Expression of tight junction protein claudin-4 in Basal-like breast carcinomas. *Pathol Oncol Res* 15:59–64.

Lee MJ, Lee HS, Kim WH, Choi Y, Yang M. (2003). Expression of mucins and cytokeratins in primary carcinomas of the digestive system. *Mod Pathol* 16:403–10.

Lim TS, Vedula SR, Kausalya PJ, Hunziker W, Lim CT. (2008). Single-molecular-level study of claudin-1-mediated adhesion. *Langmuir* 24:490–5.

Long H, Crean CD, Lee WH, Cummings OW, Gabig TG. (2001). Expression of Clostridium perfringens enterotoxin receptors claudin-3 and claudin-4 in prostate cancer epithelium. *Cancer Res* 61:7878–81.

Michl P, Buchholz M, Rolke M, Kunsch S, Lohr M, McClane B, Tsukita S, Leder G, Adler G, Gress TM. (2001). Claudin-4: a new target

for pancreatic cancer treatment using Clostridium perfringens enterotoxin. *Gastroenterology* 121:678–84.

Mohanty SK, Dey P. (2003). Serous effusions: diagnosis of malignancy beyond cytomorphology. An analytic review. *Postgrad Med J* 79:569–74.

Monte SA, Ehya H, Lang WR. (1987). Positive effusion cytology as the initial presentation of malignancy. *Acta Cytol* 31:448–52.

Morin PJ. (2005). Claudin proteins in human cancer: promising new targets for diagnosis and therapy. *Cancer Res* 65:9603–6.

Oki E, Maehara Y, Tokunaga E, Shibahara K, Hasuda S, Kakeji Y, Sugimachi K. (2002). Detection of disseminated cancer cells in bone marrow of gastric cancer using real time quantitative reverse transcriptase polymerase chain reaction. *Cancer Lett* 188:191–8.

Passebosc-Faure K, Li G, Lambert C, Cottier M, Gentil-Perret A, Fournel P, Perol M, Genin C. (2005). Evaluation of a panel of molecular markers for the diagnosis of malignant serous effusions. *Clin Cancer Res* 11:6862–7.

Rangel LB, Agarwal R, D'Souza T, Pizer ES, Alo PL, Lancaster WD, Gregoire L, Schwartz DR, Cho KR, Morin PJ. (2003). Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clin Cancer Res* 9:2567–75.

Resnick MB, Konkin T, Routhier J, Sabo E, Pricolo VE. (2005). Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. *Mod Pathol* 18:511–18.

Sanada Y, Oue N, Mitani Y, Yoshida K, Nakayama H, Yasui W. (2006). Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. *J Pathol* 208:633–42.

Sheehan GM, Kallakury BV, Sheehan CE, Fisher HA, Kaufman RP, Jr., Ross JS. (2007). Loss of claudins-1 and -7 and expression of claudins-3 and -4 correlate with prognostic variables in prostatic adenocarcinomas. *Hum Pathol* 38:564–9.

Spring K. (1998) Routes and mechanism of fluid transport by epithelia. *Annu Rev Physiol* 60:105–19.

Swisshelm K, Macek R, Kubbies M. (2005). Role of claudins in tumorigenesis. *Adv Drug Deliv Rev* 57:919–28.

Tokes AM, Kulka J, Paku S, Szik A, Paska C, Novak PK, Szilak L, Kiss A, Bogi K, Schaff Z. (2005). Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. *Breast Cancer Res* 7:R296–305.

Tsukita S, Furuse M. (2000). Pores in the wall: claudins constitute tight junction strands containing aqueous pores. *J Cell Biol* 149:13–16.

Winter MJ, Nagtegaal ID, van Krieken JH, Litvinov SV. (2003). The epithelial cell adhesion molecule (Ep-CAM) as a morphoregulatory molecule is a tool in surgical pathology. *Am J Pathol* 163:2139–48.

Yu CJ, Shew JY, Liaw YS, Kuo SH, Luh KT, Yang PC. (2001). Application of mucin quantitative competitive reverse transcription polymerase chain reaction in assisting the diagnosis of malignant pleural effusion. *Am J Respir Crit Care Med* 164:1312–18.

Zhang KH, Cao F, Fu QB, Zhu JQ, Chen J, Lv NH. (2007). Detection of mRNAs of GA733 genes by RT-PCR in exfoliated cells of pleural and peritoneal effusions and its clinical values. *Intern Med* 46:1489–94.