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Simultaneous measurement of soluble carcinoembryonic antigen and the tissue inhibitor of metalloproteinase TIMP1 serum levels for use as markers of pre-invasive to invasive colorectal cancer

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Abstract Matrix metalloproteinases (MMP) are members of a multigene family of zinc-dependent enzymes involved in the degradation of extracellular matrix components. Cancer research suggest that MMP and tissue inhibitors of metalloproteinases (TIMP) may be involved in disease progression; these enzymes could therefore be used as markers in cancer prevention programmes and for clinical monitoring. To establish whether MMP and TIMP can be used effectively as markers we determined serum levels of MMP1 and TIMP1, and studied the relationships between these enzymes and the stage of disease. The potential diagnostic and prognostic value of serum level measurements of MMP1 and TIMP1 was evaluated by comparing them with serum levels of soluble carcinoembryonic antigens (sCEA) and p53 antibodies. Our overall results indicate that simultaneous measurements of serum sCEA and TIMP1 in patients with colorectal cancer could be used as prognostic and diagnostic markers for disease progression from the pre-invasive nodal phase to the invasive phase (stages I, II to III, IV). In addition, serum levels of TIMP1 could be used as a selective marker for metastatic disease (stage III to IV). In fact, the 95% confidence interval of the serum levels of sCEA at stage III ($18.4 \leq$ sCEA ≤ 68.6 ng/ml) and TIMP1 at stage IV ($1620 \leq$ TIMP1 ≤ 3906 ng/ml) identified statistically significant ranges of values (sCEA $P = 0.02$, TIMP1 $P = 0.02$), which may be useful in the monitoring of patients at these disease phases. More specifically, our data suggest that, when the serum level of sCEA is below 18.4 ng/ml and the level of TIMP1 below 1620 ng/ml, there is a 95% probability that the disease is in the pre-invasive nodal phase; when the serum level of sCEA falls between 18.4 ng/ml and 68.6 ng/ml and the level of TIMP1 is below 1620 ng/ml, there is a 95% probability that the disease is in the phase when lymph node infiltration occurs; when the level of sCEA is above 68.6 ng/ml and the level of TIMP1 is at least 1620 ng/ml, there is a 95% probability that the disease is in the metastatic phase.

Key words TIMP1 · sCEA · Colorectal cancer · Prognostic and diagnostic indices

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Introduction

The matrix metalloproteinases (MMP) play an important role in physiological and pathological processes such as wound healing, angiogenesis, tumour invasion and metastasis [2, 12, 23, 26]. Matrix metalloproteinase 1 (MMP1), a member of the MMP family, is a membrane-anchored enzyme [7] that can cleave collagen helixes to yield characteristic one-quarter to three-quarter products. It is secreted as a latent pro-enzyme of 52 kDa, which is *N*-glycosylated to a minor form of 57 kDa and can be activated, in vitro, by proteinases (e.g. trypsin and plasmin), mercurials (e.g. 4-aminophenylmercuric acetate) and, in vivo, by plasmin and MMP3 (stromelysin) [20]. MMP1 activity is subject to regulation by cytokines at gene level [23] and post-translationally by inhibitors in the extracellular space. One of these inhibitors is TIMP1, a member of the family of tissue inhibitors of metalloproteinases (TIMP) [8].

TIMP1 is an 184-amino-acid glycoprotein of 28.5 kDa, showing 41% sequence homology with the non-glycosylated 21.5 kDa TIMP2 [28]. TIMP1 inhibits the activity of all active MMP by binding reversibly to form a 1:1 complex [9]. TIMP1 is not cleaved by this

binding and has been recovered with full activity [21]. To gain a better understanding of the physiological role of MMP1 and TIMP1 in the progression of cancer, which could be used to create prognostic and diagnostic indices for disease evaluation, we determined the relationships between the stage of the disease and serum levels of these enzymes, in patients with colorectal cancer. The potential diagnostic and prognostic value of serum level measurements of MMP1 and TIMP1 was also assessed by comparing these measurements with serum levels of soluble carcinoembryonic antigen (sCEA) and p53 antibodies. The sCEA molecule is the soluble form of a tumour-associated antigen. Its expression has been studied to establish its use as a phenotypic marker for various cancer diseases. It has been identified as a member of the Ig superfamily [29] and has been shown to act as a homotypic [3] and heterotypic [30] cell-adhesion molecule. sCEA is frequently produced in great quantities by human epithelial tumour cells, particularly of colorectal origin [25]. This production was found to be directly related to the degree of differentiation [25] and it has been studied for use as a prognostic index in correlation to tumour progression and patient survival [22]. The *p53* tumour-suppressor gene, located on chromosome 17p13, encodes a nuclear 393-amino-acid phosphoprotein that is believed to act as a tumour suppressor [15], blocking the cell-cycle progression in G1 and, in some cells, precipitating entry into apoptosis [15]. Cellular accumulation of the p53 protein in tumour cells is supposed to initiate a self-immune response characterised by generation of autoantibodies directed against this protein. p53 antibodies have been detected in the sera of patients with various digestive cancers including colorectal malignancies [1, 13] and p53 antibody can indicate poor rates of survival in some types of cancer [13, 24]. Furthermore the simultaneous assessment of serum p53 antibody and sCEA appears to be effective in monitoring high-risk and post-operative patients [6]. These substances form, at a physiological level, a highly regulated, complex and integrated system. The relationships between parameters were therefore studied using multivariate statistical procedures, as mathematical modelling is an effective way of studying a system as a whole [19].

Materials and methods

Patients

A group of 41 patients (26 men and 15 women), who were diagnosed for the first time as having colorectal cancer and who had to undergo colectomy, were studied. Men were aged from 44 to 90 years (mean, 64.8 ± 12.2 years), and women from 45 to 81 years (mean, 65.5 ± 12 years). No statistical difference was found between male and female age (Student's test, $P = 0.9$). No significant variances (ANOVA test) of the serum levels of MMP1 ($P = 0.1$), TIMP1 ($P = 0.7$), sCEA ($P = 0.9$) and p53 antibody ($P = 0.2$) between male and female patients were found. Hence data on male and female patients were pooled in the analyses carried out. Clinical diagnosis was confirmed histopathologically and patients were subtyped using the pTNM classification according to the diagnostic criteria of the American Joint Committee on Cancer and the Committee of the International Union Against Cancer. After this classification the patients were divided into four groups, which corresponded to the four stages shown in Table 1.

Blood samples

A 5-ml sample of venous blood was collected from patients 1 h prior to anaesthesia and centrifuged within 1 h of withdrawal. The serum obtained was stored frozen in aliquots at −80 °C until use. Plastic disposable material (plates, test-tubes, cryotubes, flasks etc.) was used in all experimentation and was made by Nunc-Inter Med plastics, Denmark.

Assay of MMP1, TIMP1, sCEA and anti-p53 antibody

The serum levels of MMP1, TIMP1 and p53 were determined, using commercially available, enzyme-linked, immunosorbent assay (ELISA) kits in accordance with the manufacturer's instructions. The method involved has been described in detail elsewhere [4]. The sensitivity of these ELISA assays was as follows: MMP1 < 1.7 ng/ml, TIMP1 < 1.25 ng/ml (Amersham International) p53 = 0.16 U/ml (Oncogene research products). Values for enzymes, cytokines and soluble molecules were obtained on-line using a specific software programme (ELISA-AID, Eurogenetics).

Table 1 Clinical and histopathological data on patients with colorectal cancer

Characteristic	Stage			
	I	II	III	IV
Rectum/sigmoid colon[a]	2	–	1	1
Transverse colon	–	3	1	–
Descending colon	2	2	1	1
Ascending colon	–	4	1	2
Sigmoid	1	3	2	3
Rectum	4	5	2	–
PTNM	T1N0M0 3 T2N0M0 6	T3N0M0 14 T4N0M0 2 T4N1M0 1	T3N1M0 1 T3N2M0 4 T3N3M0 1 T4N3M0 2	T1N1M1 1 T3N1M1 2 T3N3M1 1 T4N1M1 1 T4N3M1 2
Number of patients	9	17	8	7

[a] Location of colorectal cancer

The patients' sCEA levels were taken from their case notes. The presence of sCEA in human serum was determined using a chemiluminescent microparticle immunoassay. The assay for sCEA is a two-step immunoassay. In the first step, sample and anti-CEA-coated paramagnetic microparticles are combined, and CEA present in the sample binds to the anti-CEA-coated microparticles. After washing, acridinium-labeled anti-CEA is added in the second step. Pre-trigger and trigger solutions are then added to the reaction mixture and the chemiluminescence generated in the resulting reaction is measured as relative light units (RLU). A direct relationship exists between the amount of CEA in the sample and the chemiluminescence. The sensitivity is 0.5 ng/ml (Abbott Lab. Diagnostics Division).

Statistical analysis

The statistical analyses were carried out using the Statgraphics software systems (full system 5.25 version 4.0; graphics system by Statistical Graphics Corporation, USA 1989). To study the relationships between disease progression and levels of MMP1, TIMP1, sCEA and p53 antibody in the serum, multivariate statistical analyses were used as they allow a simultaneous evaluation of all variables. Serum levels of the parameters being studied were analysed in relation to disease progression as a generic parameter, and in relation to specific stage transitions (I to II, II to III, III to IV) in order to highlight possible progression markers for these phases of disease. Using principal component analysis, we evaluated the network of relationships between parameters and disease stage by plotting the vector of each variable. The length of each vector is proportional to the weight of the variable in the network and the angle between any two is inversely proportional to the correlation between them. To study the network of parameter relationships we also used Spearman's rank correlation analysis, which creates a matrix of correlation coefficients for a set of observed values by forming linear combinations of variables. Statistically significant differences between serum levels of parameters at various stages of disease were determined using (as appropriate) the Mann–Whitney *U*-test and Student's *t*-test; the ANOVA test was used to study variance.

Results

MMP1, TIMP1, sCEA and p53 antibody

Table 2 shows serum levels of MMP1, TIMP1, sCEA and p53 antibody for all patients and for patients grouped by stage (I, II, III, IV). Only levels of TIMP1 and sCEA proved to be statistically significant among the parameters studied in relation to the stage of the disease. The level of sCEA differed significantly between stages II and III as did the level of TIMP1 between stages III and IV. These significant differences were also observed when the correlation method was used.

Study of the network of relationships between MMP1, TIMP1, sCEA and p53 antibody and stage of the disease using statistical multivariate analyses

Figure 1a shows the results of the principal-component analysis of the serum parameters MMP1, TIMP1, sCEA and p53 antibody in relation to disease progression, taken as a generic parameter. It can be seen from Fig. 1a that serum levels of TIMP1 and sCEA are both associated with progression of disease. The degree of association is practically the same (TIMP1 and sCEA vectors form a similar angle with the stage vector) but the vectors are independent (they are located in opposite positions with respect to the stage vector). The MMP1 and p53 antibody vectors form larger angles with the stage vector than do the TIMP1 and sCEA vectors. This suggests that measurement of serum levels of MMP1 and p53 antibody is of less importance in the clinical monitoring of disease progression. The sCEA vector is the one most closely associated to the stage progression vector in the passage from stage I to stage II (Fig. 1b) and from stage II to stage III (Fig. 1c). The p53 antibody vector was also found to be associated to the stage vector in the transition from stage II to stage III but was independent of the sCEA vector (the p53 antibody and sCEA vectors are not close and are in opposition with respect to the stage vector). The TIMP1 and MMP1 vectors are the most closely associated to disease progression in the passage from stage III to stage IV (Fig. 1d) but are independent of each other.

Significant values were only observed for levels of sCEA (§$P = 0.030$) in the passage from stage II to stage III, and for levels of TIMP1 (§$P = 0.015$) in the passage from stage III to stage IV (Table 2) when correlation procedures were used.

These results indicate that serum level ranges of sCEA and TIMP1 can be used as markers in the above-mentioned stages of disease.

Table 2 Serum levels of matrix metalloproteinase 1 (*MMP1*), tissue inhibitor of metalloproteinase 1 (*TIMP1*), soluble carcinoembryonic antigen (*sCEA*) and p53 antibody. Values are expressed as means ± SE. The statistical evaluations were done using the most appropriate statistical test, which depended on the population distribution

Protein	Level in serum (ng/ml)					Stage correlations
	All patients	Stage I	Stage II	Stage III	Stage IV	
MMP1	16.2 ± 2.0	17.6 ± 2.6	15.2 ± 3.6	16.9 ± 3.7	25.2 ± 5.4	
TIMP1	446.5 ± 254.0	532.1 ± 114.4	634.2 ± 301.7	320.3[*1] ± 447.6	2763.1[*1] ± 1040.3	III to IV[*3]
sCEA	3.6 ± 6.0	2.3 ± 0.6	3.0[*2] ± 1.1	18.4[*2] ± 24.5	39.4 ± 17.9	II to III[*4]
p53 antibody[a]	8.4 ± 8.9	18.2 ± 6.6	15.7 ± 3.8	5.4 ± 44.9	7.7 ± 2.2	

[*1] Differences in TIMP1 levels in serum between stages III and IV (Mann–Whitney *U*-test, $P = 0.018$)
[*2] Difference in sCEA levels in serum between stages II and III (*U*-test, $P = 0.03$)
[*3,*4] Correlation between TIMP1 and sCEA levels in serum and disease stage. Significant values were only observed for levels of TIMP1 ([*3]$P = 0.015$) in the passage from stage III to stage IV, and levels of sCEA ([*4]$P = 0.030$) in the passage from stage I to stage III
[a] U/ml

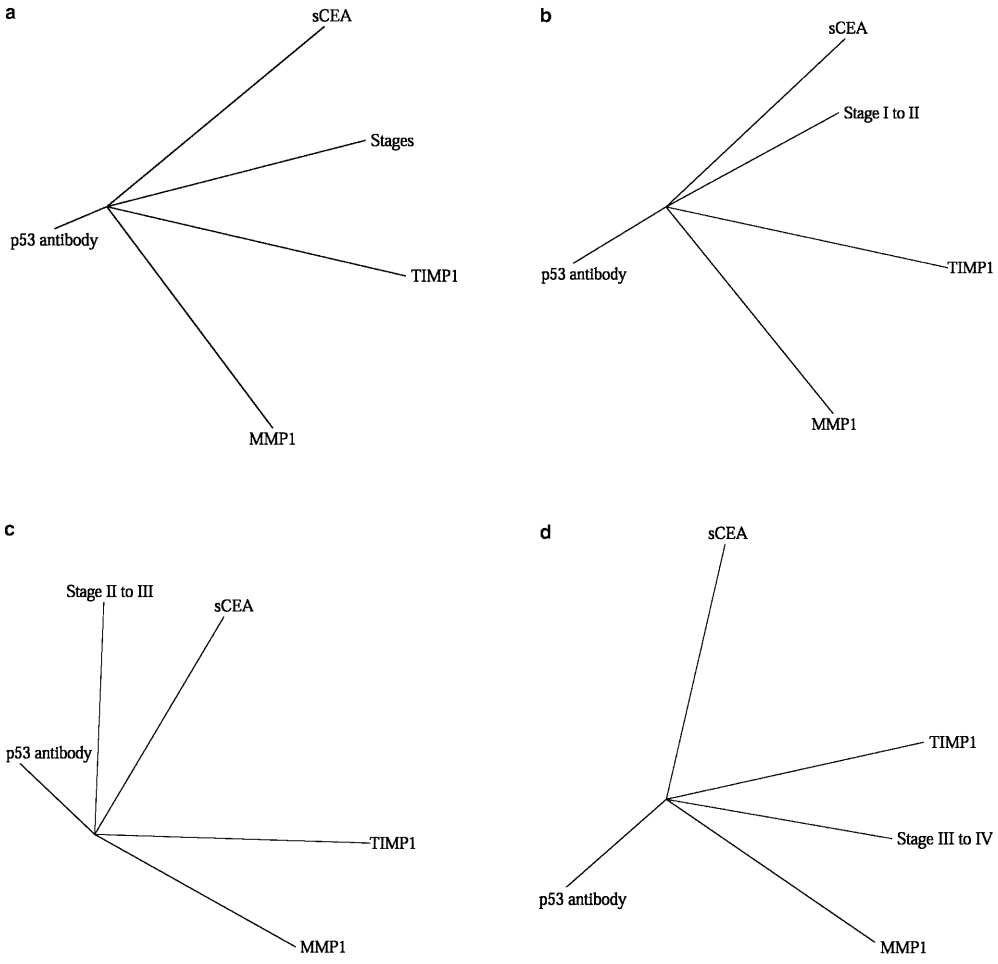

Fig. 1a–d Principal-component analysis plots of the network of relationships between matrix metalloproteinase 1 (*MMP1*), tissue inhibitor of metalloproteinase 1 (*TIMP1*), soluble carcinoembryonic antigen (*sCEA*), p53 antibody and disease progression. Serum parameters were analysed in relation to stage as a generic parameter (**a**) and in the passage from stage I to II (**b**), from stage II to III (**c**), and from stage III to IV (**d**)

Variation of MMP1, TIMP1, sCEA and p53 antibody serum levels in the differing stages

Only levels of sCEA (*U*-test, $P = 0.03$) in the passage from stage II to III; and levels of TIMP1 (*U*-test, $P = 0.018$) in the passage from stage III to IV (Table 2) were found to be statistically significant. No statistically significant variance of the MMP1 ($P = 0.6$) and p53 antibody ($P = 0.4$) serum levels in the differing stages was found, using the ANOVA test (Table 3); sCEA and TIMP1 levels, on the other hand, were found to be significant in the passage from stage II to stage III (ANOVA, $P = 0.02$) and from stage III to stage IV (ANOVA, $P = 0.02$) respectively (Figs. 2, 3).

Table 3 The 95% confidence intervals for mean values for MMP1, TIMP1, sCEA and p53 antibody levels, determined using the confidence interval range test. No statistically significant variance of the MMP1 ($P = 0.6$) and p53 antibody ($P = 0.4$) serum levels in the differing stages was found. Only sCEA (in the passage from stage II to stage III $P = 0.02$) and TIMP1 (in the passage from stage III to stage IV $P = 0.02$) variance was found to be significant (ANOVA test in each case)

Stages	95% confidence interval for mean values (ng/ml)			
	MMP1	TIMP1	sCEA	p53 antibody[a]
I	9–26.3	−475.9–1540	−22.8–27.4	−19.9–57.1
II	12.5–25.1	244.3–1711.1	−11.5–20.7	−12.3–43.7
III	7.7–26	−307.6–1830.6	18.4–68.6	13.7–95.4
IV	15.3–35	1620–3906	12.3–66.5	−36–51.3

[a] U/ml

Discussion

In cancer the extent to which the disease spreads (the stage) is probably the most important factor determining patient prognosis and must be given prime consideration in evaluating and comparing different therapeutic regimes. The need for practical and non-invasive methods in the early screening and clinical monitoring of patients to establish disease stage is therefore clear. We evaluated the possibility of creating reliable indices for tumour disease progression using range values of blood parameters, as we have proposed in previous papers [5, 10]. The potential diagnostic and prognostic value of MMP1 and TIMP1 serum level measurements was assessed in the peripheral blood of a group of colorectal cancer patients. The serum level of these enzymes was studied in relation to the progression of the disease and serum levels of sCEA and p53 antibody. sCEA is frequently produced in great quantities by human epithelial tumour

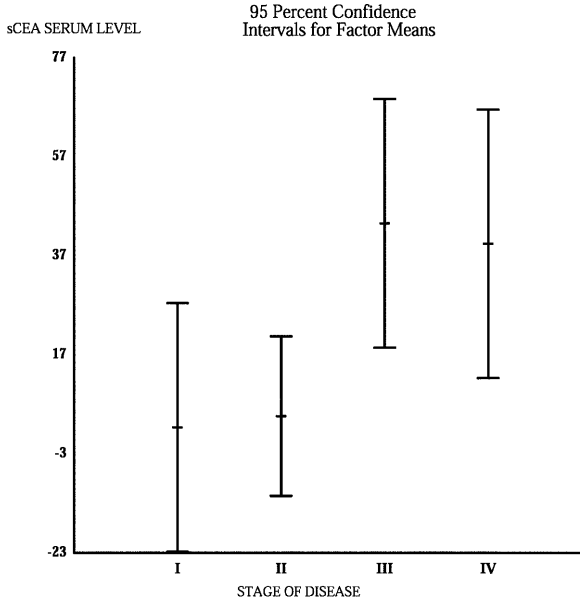

Fig. 2 Plot of the sCEA level mean for each stage of disease and the range (95% confidence intervals) for the means using the ANOVA test of analysis of variance ($P = 0.02$)

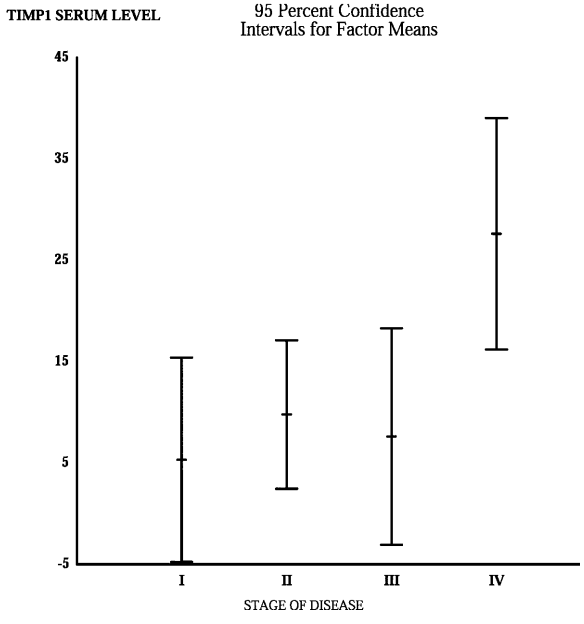

Fig. 3 Plot of the TIMP1 level mean for each stage of disease and the range (95% confidence intervals) for the means, using the ANOVA test of analysis of variance ($P = 0.02$)

cells, particularly of colorectal origin [25]; it has also been found to be directly related to the degree of differentiation [2, 25] and studied for use as a prognostic index for tumour progression and patient survival [6, 22].

p53 antibody, on the other hand, develops in 10%–30% of most malignancies [16] and could serve as a prognostic marker of disease progression in some types of malignancy, including colorectal tumour [13, 24]. The simultaneous assessment of serum p53 antibody and sCEA would appear to be effective in monitoring high-risk and post-operative colorectal cancer patients [6]. Our study on the potential diagnostic and prognostic value of MMP1, TIMP1, sCEA and p53 antibody suggest that serum levels of both TIMP1 and sCEA are useful in the monitoring the disease in patients with colorectal cancer; sCEA could be used as a progression marker for the transition from stage II to stage III and TIMP1 as a marker for the transition from stage III to stage IV. We drew these conclusions from the results of our multivariate statistical analyses on the network of parameter relationships in relation to disease progression (Fig. 1, Table 2).

These conclusions were supported by a statistical study of the differences between serum parameters at the various stages, which showed that levels of sCEA and TIMP1 were only significant in the transitions from stage II to stage III and from stage III to stage IV respectively (Table 2).

Furthermore, variance studies analysing the quantitative variations of these parameters through the stages allowed us to identify range values for these potential markers (Table 3, Figs. 2, 3). In fact, our data show that the 95% confidence interval of sCEA serum levels at stage III ($18.4 \leq$ sCEA ≤ 68.6 ng/ml) and TIMP1 at stage IV ($1620 \leq$ TIMP1 ≤ 3906 ng/ml) identified statistically significant ranges of values (sCEA $P = 0.02$; TIMP1 $P = 0.02$) and so may be useful in monitoring patients in these phases. More specifically, our data suggest that, when the serum level of sCEA is below 18.4 ng/ml and the level of TIMP1 below 1620 ng/ml, there is a 95% probability that the disease is in the pre-invasive nodal phase; when the serum level of sCEA falls between 18.4 ng/ml and 68.6 ng/ml ($18.4 \leq$ sCEA $\leq$ 68.6 ng/ml) and the level of TIMP1 is below 1620 ng/ml, there is a 95% probability that the disease is in the phase where lymph node infiltration occurs; when the level of sCEA is above 68.6 ng/ml and the level of TIMP1 is at least 1620 ng/ml, there is a 95% probability that the disease is in the metastatic phase.

The principal-components analyses indicate that serum levels of p53 antibody together with those of sCEA could be used as a marker of progression from stage I to stage II, and levels of MMP1 and TIMP1 from stage III to stage IV. These findings were not confirmed by other statistical tests.

The results for MMP1 and TIMP1 would appear to be in conflict with the functional characteristics of these enzymes: MMP1 is responsible for the degradation of the extracellular matrix (an important phenomenon in the metastatic process) whilst TIMP1, as its inhibitor, should block this process. However our results, as well as those of other researchers [18], show that an increase in the serum levels of TIMP1 can be used as a prognostic and diagnostic index for the metastatic phase of the disease.

The preliminary results of the study we are currently engaged in, on the serum levels of a protein codified by the gene *bc12* (data not shown) and the parameters used here suggest that a relationship exists between serum

levels of TIMP1 and the protein encoded by *bc12*. *bc12* gene expression is important in the inhibition of apoptosis during the cell cycle and therefore also in cell survival. Consequently one conclusion that can be drawn is that TIMP are involved in protein processing and activation and are hence responsible for creating an environment that supports the initiation and maintenance of the metastatic phase of tumour disease.

Nevertheless we believe the role of p53 antibody and MMP1 enzyme should be investigated further, as p53 antibody and MMP1 serum levels could be indirect markers of disease progression and other parameters physiologically correlated to them could be used to clarify their role, for example the protein bcl2. bcl2 has also been shown to be associated with p53 in the progression of tumoral diseases [11, 14, 17, 27].

In conclusion, our results indicate that the simultaneous measurement of sCEA and TIMP1 serum levels in colorectal cancer patients may have prognostic and diagnostic value for the clinical monitoring of disease progression from the pre-invasive to invasive stages (from stages I and II to III and IV). Moreover, serum levels of TIMP1 could be used in the prognostic assessment of metastatic disease (from stage III to IV).

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