MIC-1 Serum Level and Genotype: Associations with Progress and Prognosis of Colorectal Carcinoma[1]

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ABSTRACT

Purpose: **Macrophage inhibitory cytokine-1 (MIC-1) is a divergent member of the tumor growth factor β (TGF-β) superfamily. Several observations suggest that it plays a role in colorectal carcinoma (CRC). In particular, MIC-1 is markedly up-regulated in colorectal cancers as well as in premalignant adenomas. This study examines the relationship of serum MIC-1 levels and genotypes to clinical and pathologic features of colonic neoplasia.**

Experimental Design: **We confirmed the presence of MIC-1 in CRC tissue and the cell line CaCo-2. The normal range for serum MIC-1 levels was defined in 260 healthy blood donors, and the differences between normal subjects and 193 patients having adenomatous polyps or CRC were then determined. In a separate cohort of 224 patients, we evaluated the relationship of MIC-1 serum level and genotype to standard tumor parameters and outcome measures.**

Results: **MIC-1 was expressed in CRC tissue and the cancer cell line CaCo-2. There was a progressive increase in serum MIC-1 levels between normal individuals [mean (M) = 495 pg/ml, SD = 210), those with adenomatous polyps (M = 681 pg/ml, SD = 410), and those with CRC (M = 783 pg/ml, SD = 491)]. Serum MIC-1 level was correlated with the extent of disease so that the levels were higher in patients with higher Tumor-Node-Metastasis stage. There were significant differences in time to relapse and overall survival between subjects with different MIC-1 levels and genotypes.**

Conclusions: **This study identifies a strong association between MIC-1 serum levels and neoplastic progression within the large bowel. We suggest that the measurement of serum MIC-1 levels and determination of MIC-1 genotype may have clinical use in the management of patients with CRC.**

INTRODUCTION

Epithelial cells are subject to important regulatory influences, prominent among which are those of the TGF-β[3] superfamily of cytokines. They are angiogenic and immunomodulatory and have been shown to regulate epithelial growth, cell motility, and adhesion (1). Multiple alterations to TGF-β, its receptor, and signaling pathways have been described in epithelial neoplasms such as those of the breast, prostate, and colon. In the case of the latter, the role of TGF-β, its receptors, and signaling pathways have been extensively reviewed (1, 2). In CRC, both *in vitro* and *in vivo* studies have shown that TGF-β plays a role in regulation of cancer cell growth and subsequent apoptosis (3–5). Accordingly, mutations that inactivate the TGF-β pathway, involving either the TGF-β receptor II or its downstream signaling component Smad4, often occur during colorectal tumorigenesis (6, 7). Additionally, TGF-β itself has significant modulatory effects on colon derived lymphocytes, indicating a direct role in tumor immunity (8). Although the role of TGF-β in CRC has been extensively examined, the part played by other TGF-β superfamily cytokines is less well studied.

Another cytokine from this family with links to CRC is MIC-1 (9). This is a divergent member of the TGF-β superfamily originally identified on the basis of increased expression associated with macrophage activation (10). It has also been subsequently reported under a wide variety of names such as placental TGF-β (11), prostate-derived factor (12), growth/differentiation factor 15/MIC-1 (13), placental bone morphogenetic protein (14), and as nonsteroidal anti-inflammatory drug-regulated gene-1 (15). The major function of this protein is still uncertain, although it has been variously described as being able to inhibit tumor necrosis factor α production from lipopolysaccharide-stimulated macrophages (10), induce cartilage formation in the early stages of endochondral bone formation (12),

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[3] The abbreviations used are: TGF-β, transforming growth factor β; MIC-1, macrophage inhibitory cytokine-1; CRC; colorectal carcinoma; M, mean; CEA, carcinoembryonic antigen; OR, odds ratio; 95% CI, 95% confidence interval; PTL, peritumoral lymphocyte; TNM, Tumor-Node-Metastasis.

inhibit proliferation of primitive hemopoietic progenitors (16), and act as a neurotrophic factor (17, 18). It may also play a role in embryo implantation and placental function (19).

The development of sensitive immunoassays for MIC-1 has made it possible to study MIC-1 levels in human serum and other body fluids (20). Unlike TGF-β, MIC-1 is not produced in significant quantity by circulating platelets or other blood cells and can therefore be readily and reliably measured in serum or plasma (20, 21). Markedly elevated serum levels of MIC-1 occur during pregnancy, suggesting a generalized function in this process (19).

As with many cytokines, polymorphisms of MIC-1 have also been documented. The MIC-1 gene has at least two major allelic variants with the more common variant having a histidine at position 6 (designated *H*) of the mature MIC-1 peptide. In the less common variant, a single nucleotide change leads to the replacement of the histidine with an aspartic acid (designated *D*; Ref. 22). The markedly differing properties of these two amino acids suggest that this substitution may have functional consequences.

A number of direct and indirect lines of evidence suggest a link between MIC-1 and cancer. The MIC-1 promoter region is a target for the p53 tumor suppressor gene product, which increases MIC-1 expression (23–25). MIC-1 has been found to be up-regulated in colonic carcinoma cell lines treated with nonsteroidal anti-inflammatory drugs, indicating a possible role in the antitumor effect of these drugs (26). This up-regulation of MIC-1 was associated with apoptosis in which MIC-1 appeared to be involved (27). These authors also reported that transfection of a colon carcinoma line with MIC-1 cDNA, followed by implantation of cells into nude mice, led to a decrease in tumor size compared with the untransfected controls.

More direct evidence for links between MIC-1 and cancer have been obtained from serial analysis of gene expression, which has indicated that MIC-1 is one of a very limited set of genes for secreted proteins in which the expression is up-regulated in both adenomatous polyps and CRC (9). Similar findings, made using microarray technology, have also been reported in prostate cancer (28). We have also documented an association between elevated serum MIC-1 and metastatic colorectal, prostate, and breast cancer (21). Examination of tumor tissue by immunohistochemistry confirmed increased expression of MIC-1 by the neoplastic cells (21).

In this article, we show that MIC-1 is produced by a colonic tumor cell line and tumor tissue. Additionally, serum MIC-1 and CEA levels are correlated in a small sample of patients with advanced metastatic disease. We also provide the first evidence demonstrating a significant relationship between serum MIC-1 levels, clinical stage, presence of metastasis, and progression of CRC. In addition, we provide data indicating that the MIC-1 genotype is correlated with disease-free survival and overall survival of CRC. This suggests that measurement of MIC-1 levels and determination of MIC-1 genotype may have clinical use.

MATERIALS AND METHODS

Patient Serum Samples

Normal Subjects. A total of 260 normal serum samples was obtained from the Red Cross Blood Bank (Adelaide, South Australia) for the purpose of defining the normal range for serum MIC-1 level. The age distribution was 17–71 years (M = 48, SD = 13) with 139 males and 122 females. All donors met the selection criteria for blood donation, as per the Australian Red Cross blood services South Australia, and samples were collected with informed consent, as approved by St. Vincent's Hospital Ethics Committee.

Colorectal Carcinoma Cohorts. Serum was collected from two cohorts. The first, from individuals who entered into the CRC registry at The Johns Hopkins Hospital, consisted of 301 individuals presenting for colonoscopy. The second, from 224 individuals (141 males and 86 females (ages 32–93 years; M = 66.6 ± 12.4 years) with a diagnosis of adenocarcinoma of the colon or rectum at St. Vincent's Hospital (Sydney, Australia) between 1993 and 1998. Ethics approval was obtained from respective institutions for study of both cohorts.

In both cohorts, informed consent was obtained from individuals before entering the study. Participants were without history of inflammatory bowel disease, familial adenomatous polyposis, or hereditary nonpolyposis colorectal cancer. Serum was centrifuged in Vacutainer glass tubes and then transferred to 1.5-ml tubes. These tubes were put into liquid nitrogen and then into −80°C freezers. Adenomas and cancers were diagnosed according to standard criteria after surgical or endoscopic removal and histopathological analysis of tumors.

For all tumors in the Sydney cohort, the stage of the tumor was determined independently by a histopathologist within the Department of Anatomical Pathology, St. Vincent's Hospital. The tumor grade as well as the presence of a Crohn's-like inflammatory infiltrate, intraepithelial lymphocytes, or peritumoral lymphocytes were determined prospectively. The extent of peritumoral and Crohn's-like lymphoid reactions was classified according to the method of Jass (29). Intraepithelial lymphocytes were identified by light microscopy on H&E sections as cells with the morphology of lymphocytes, seen wholly within tumor epithelium. They were classified as conspicuous when >30 were present/10 high power fields. Patient were followed at three monthly intervals for the first 2 years and then every 6 months for the next 3 years. Follow-up included history, physical examination, and endoscopy, with positive findings investigated by routine blood testing and symptom-directed imaging.

Patient serum samples from 10 subjects who had stage IV disease, according to United Union Internationale Contre le Cancer/American Joint Committee on Cancer classification 1997, were selected for CEA measurement using the Abbott, Axsym enzyme immunoassay system. The 10 patients with CRC consisted of 9 males and 1 female with an age of 59 ± 12 years (M ± SD).

In the Sydney cohort, individuals were excluded where preoperative radiotherapy or chemotherapy had been administered. Additionally, incomplete primary tumor resection was an exclusion criterion. All patients had MIC-1 serum level determination performed. There was sufficient serum to determine the genotype of 187 patients.

Cell Culture

The colonic cancer cell line, CaCo-2, was cultured to confluence in DMEM high glucose (Life Technologies, Inc.,

Grand Island, NY) and 10% FCS (Life Technologies, Inc.) in a 75-cm^2 flask. Conditioned medium was obtained by the addition of fresh medium to a confluent monolayer of cells after washing with PBS. The medium was incubated with the cells for an additional 24 h, then decanted and assayed in the MIC-1 ELISA. Unconditioned media were used as an additional control.

MIC-1 Immunohistochemistry

Tissue samples were fixed and paraffin embedded. Four μm sections were placed on silane-coated slides. Sections were brought to water and microwaved for 20 min in 0.01 M citrate (Univar, Sydney, Australia) buffer (pH 6.0). To block nonspecific peroxidase activity, sections were incubated in hydrogen peroxide (Analar, Kilsyth, Australia), 3 ml/100 ml of PBS for 30 min at 21°C. Nonspecific antibody binding was blocked using blocking buffer (2 g of BSA and 2 ml of FBS/100 ml of PBS) for 30 min at 21°C. Slides were then incubated with the sheep anti-human MIC-1 polyclonal antibody 233B3 or normal sheep IgG and diluted 1:1000 in blocking buffer overnight at 4°C. After washing, slides were treated with biotinylated donkey antisheep IgG (Jackson Immunoresearch, West Grove, PA) diluted to 1:200 in blocking buffer and was incubated with slides for 45 min at 21°C. Streptavidin-horseradish peroxidase (large volume kit; Dako, Carpinteria, CA) was incubated with samples for 10 min at 21°C and the slides developed using 3,3′-diaminobenzidine (Pierce, Rockford, IL) 1:10 (v/v) in stable peroxide buffer (Pierce). The slides were counterstained with hematoxylin and coverslipped. Three 10-min washes with wash buffer (0.5 ml Triton-X/100 ml of PBS) were performed between all steps after the primary antibody incubation until counterstaining.

Determination of Serum MIC-1 Levels

MIC-1 serum levels were determined using a MIC-1 sandwich ELISA with the 26G6H6 antibody as described previously (19, 20). Briefly, ELISA plates (Maxisorb; Nunc) were coated for 24 h at 4°C with 80 μl, 1:500 of 26G6H6 in bicarbonate buffer (pH 9.4–9.8). Samples were diluted 1:10–50 in sample buffer [1% w/v BSA (Progen)] and 0.05% v/v Tween (Sigma) in PBS (pH 7.2). A MIC-1 standard (1 μg/ml recombinant MIC-1 in 1% BSA w/v, 3 mM HCl) was diluted 1:1000 in sample buffer, and eight doubling dilutions were then performed (1000–7.8 pg/ml) and used as the standard curve for each plate in the assay. Coated plates were washed three times with wash buffer (0.05% v/v Tween in PBS) 300 μl/well. The plates were then blocked by incubation with 250 μl of 1% BSA w/v at 37°C for 1 h. Blocking buffer was removed, and 100 μl/well of standards or samples were added without intervening washing for 1 h at 37°C. The detection antibody, 233-BP, 1:25,000, in sample buffer v/v, was added at 100 μl/well and incubated for 16 h at 4°C. After washing, visualization was achieved with donkey, antisheep, biotinylated IgG (Jackson's Laboratories), Streptavidin-large volume kit conjugate (Genzyme), and *o*-phenylenediamine substrate (Sigma). The reaction was terminated with 100 μl/well 2 N H_2SO_4. Absorbance was measured at 490 nm, and a standard curve was constructed using a single binding site hyperbole using the Graph Pad Prism program (GraphPad Software, Inc., San Diego, CA). Sample values were extrapolated from this curve. Each sample was assayed at minimum in duplicate and the coefficients of variations were always <10%.

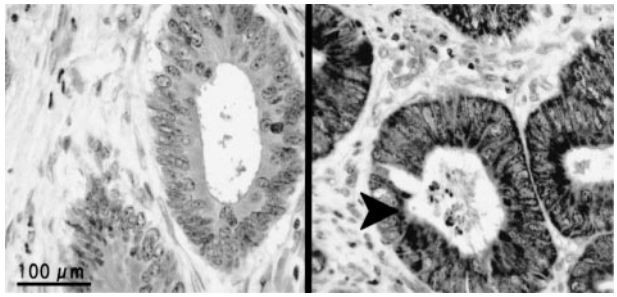

Fig. 1 Immunoperoxidase and hematoxylin counterstain of MIC-1 in colon cancer. The *left panel* represents staining with control antibody. *Arrows* show MIC-1 present in tumor. Magnification is as shown.

Determination of MIC-1 Genotype

Genotyping of MIC-1 for the *H* and *D* allelic variants was undertaken using a serum-based ELISA assay (20). This assay is based on the difference of affinity of two monoclonal antibodies for the *H* and *D* variants of MIC-1. The 13C4H4 antibody has a much lower affinity for the *D* allele, compared with the H allele of MIC-1 protein, whereas the 26G6H6 antibody binds equally to both MIC-1 alleles. This difference in affinity leads to a reduction in the measured MIC-1 serum level in samples containing the *D* allele when the 13C4H4 antibody is used. The genotype of a serum sample is represented by the ratio of the 13C4H4 assay value to the total MIC-1 serum concentration as determined by the 26G6H6 assay. A homozygous *H* MIC-1 serum sample will have a ratio in the range 0.6–1.4, and the heterozygous protein (*HD*) will have a value between 0 and 0.45. The ratio is <0 for the homozygous *D* protein. The reason for values < zero in homozygous *D* subjects is extensively explained in the methods article describing this assay (20).

Statistical Analysis

Survival was measured from the date of resection of CRC until death or until the censor date of March 1, 2000. Time to recurrence was the period from resection to medical documentation of tumor recurrence. Survival and time to recurrence were censored at time of death for patients dying of causes other than CRC. Survival curves were prepared according to the method of Kaplan and Meier, and statistical significance between curves was tested using the log-rank test. The MIC-1 serum levels and genotypes were compared with the data collected as outlined above, using ANOVA, parametric and nonparametric correlates, regression analysis, Kaplan-Meier analysis, as well as χ^2 analysis. Where parametric testing was performed, the $\log_{10}$ of MIC-1 levels was used. *P* of <0.05 was considered significant. All data were analyzed using the Statview v5.0 statistical software (SPSS, Inc., Cary, NC).

RESULTS

Distribution of MIC-1 Serum Levels and Genotype in a Normal Population. By examining a population of 260 ambulant blood donors, we have defined the normal serum level of

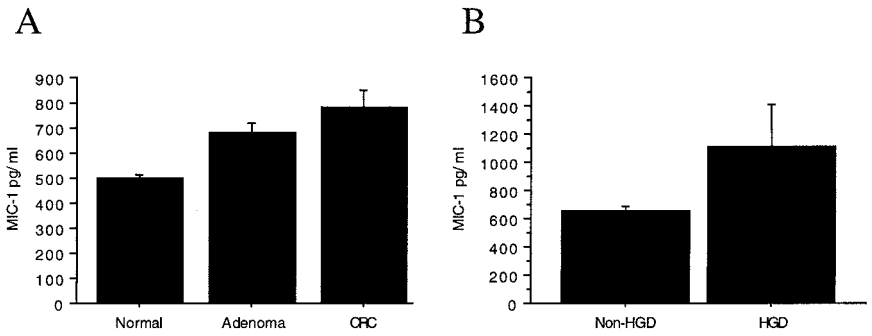

Fig. 2 *A*, in patients with CRC, serum MIC-1 increase with the TNM tumor stage. *B*, increased serum MIC-1 levels are associated with the presence or absence of metastasis.

MIC-1 as 200-1150 pg/ml (M ± 2 SDs). Two samples were excluded as they had MIC-1 serum levels more than three SDs from the M. We have also previously defined the distribution of MIC-1 genotypes in this group as homozygous *H* (*HH*) 54%, heterozygous (*HD*) 39%, and homozygous *D* (*DD*) 7% (20).

We have also estimated the serum MIC-1 levels in a group of 20 United States individuals who had recently undergone a normal colonoscopy. The serum levels were all within the normal MIC-1 range, thereby confirming that there were no differences in sample collection, storage, and transport, which could have influenced the United States data. On this basis, the Australian cohort was used as the reference normal population for all analysis.

MIC-1 Is Expressed in Colon Cancer Tissue and the Colon Cancer Cell Line CaCo-2. MIC-1 was detected by immunohistochemistry in all of the 10 specimens of carcinoma of the colon examined. Nine were strongly positive for MIC-1, whereas 1 exhibited only weak staining. The staining for MIC-1 in the malignant colonic epithelium cells was predominantly cytoplasmic with apical and basal accentuation (Fig. 1). Conditioned medium from the colonic cancer cell line CaCo-2 cell line examined contained 523 pg/ml MIC-1.

Serum MIC-1 Level Are Raised in Patients with Colonic Polyps or CRC. The United States cohort was used to investigate the alteration in serum MIC-1 levels associated with adenomatous polyps or CRC. Colonoscopy of 301 patients identified 135 patients with colonic polyps and 58 with CRC. The remainder was free of disease. The CRC and colonic polyp groups were compared with each other and the normal population. As seen in Fig. 2*A*, serum MIC-1 levels were significantly elevated in patients with adenomatous polyps (M = 681 pg/ml, SD = 410; $P < 0.0001$, ANOVA) and patients with CRC (M = 783 pg/ml, SD = 491; $P < 0.0001$, ANOVA) compared with normals (M = 495 pg/ml, SD = 210). Serum MIC-1 levels in CRC patients were higher than those of patients with adenomatous polyps ($P < 0.05$). This is indicative of an associated rise in serum MIC-1 levels with progression from normal to adenomatous polyps and finally CRC (Fig. 2*A*).

Increasing Dysplasia in Colonic Polyps Is Associated with a Relative Increase in Serum MIC-1 Levels. If colonic polyps are left untreated, there is the risk that they will progress to invasive colonic carcinoma. This transition from polyp to frank invasive carcinoma is heralded by an increase in dysplasia (30). Although there were only 6 patients with histologically documented high-grade dysplasia (M = 1114 pg/ml, SD = 726), they had significantly higher serum MIC-1 levels than other patients with polyps (M = 661 pg/ml, SD = 382; $P < 0.05$, ANOVA; Fig. 2*B*).

Elevated MIC-1 Serum Level Is Associated with Progressive Stage and Course of CRC. Analysis of the Sydney cohort of 224 patients was used to define the associations between MIC-1 serum level and specific parameters of CRC. All subsequent data are from this cohort.

Although there was no significant differences between TNM tumor stage 1 (M = 783 pg/ml, SD = 574) and stage 2 (M = 686 pg/ml, SD = 416) tumors, there were increases in serum MIC-1 levels between TNM tumor stages 2 and 3 (M = 1403 pg/ml, SD = 1893; $P = 0.037$), as well as between stages 3 and 4 (M = 1965 pg/ml, SD = 2514; $P = 0.04$; Fig. 3*A*). There is an increasing likelihood of more distant disease as the primary tumor invades and, consequently, the TNM tumor stage increases. Therefore, the rise in serum MIC-1 levels with increasing tumor stage indicates a relationship to the extent of the tumor. The presence of metastasis leads to increased serum MIC-1 levels compared with nonmetastatic disease ($P = 0.0089$; Fig. 3*B*). As would be expected from this finding, there was also a significant association between an abnormal MIC-1 level (>1150 pg/ml) and the presence of metastasis ($P = 0.0052$, $\chi^2 = 7.9$).

Using logistic regression analysis, an abnormal (>1150 pg/ml) serum MIC-1 level was associated with a 2.8-fold likelihood of distant metastasis (95% CI, 1.7–4.7; $P = 0.0001$; Table 1). A high MIC-1 serum level remained an independent predictor of the presence of metastasis when included with TNM tumor stage, presence of positive lymph nodes and MIC-1 allele, using multivariate analysis (OR = 2.7, 95% CI, 1.2–6.5; $P = 0.0191$; Table 2).

MIC-1 and CEA Level Are Correlated. Metastatic colorectal carcinoma patients had serum MIC-1 levels elevated above normal in 8 of 10 patients (M = 3.0×10^3 pg/ml, SD = 2.5×10^3). There was a strong positive correlation between the serum MIC-1 level and CEA ($P < 0.01$; $r = 0.765$ using log serum MIC-1 and CEA, correlation-Z test; data not shown).

The *D* Allele of MIC-1 Is Associated with Increased Risk of Metastasis. Overall, there was no significant difference in the MIC-1 serum levels among subjects with different genotypes. Additionally, there were no significant differences in genotype frequency between the normal and the CRC groups. It should be noted that the numbers in the groups are relatively

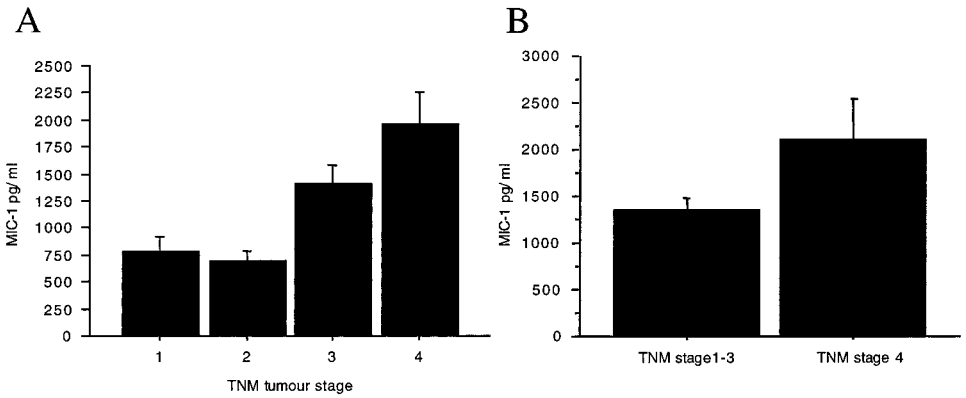

Fig. 3 *A*, serum MIC-1 levels are raised in patients with CRC compared with adenoma and normals. *B*, serum MIC-1 level is increased in patients with high-grade dysplastic (HGD) polyps when compared with those patients with other adenomatous polyps (non-high-grade dysplastic).

Table 1 Univariate logistic regression for Sydney cohort

Risk	*n*	OR	95% CI	*P*[a]
Presence of metastasis at presentation				
Presence of lymph nodes	105	3.9	1.8–8.9	0.0004
Abnormal serum MIC-1	71	2.8	1.4–5.8	0.0053
Presence of *H* MIC-1 allele	106	0.5	0.2–1.0	0.0637
Likelihood of relapse				
TNM metastasis	38	32.8	9.6–112	<0.0001
TNM tumor				
1	17	1.0		
2	22	4.7	0.5–44.8	
3	150	5.5	0.7–43.1	
4	72	28.3	3.5–225.9	<0.0001[a]
Dukes stage				
A	27	1.0		
B	83	2.0	0.4–9.7	
C	78	9.3	2.0–42.0	
D	38	132.0	20.4–852.2	<0.0001[a]
Lymph nodes present	105	5.4	2.7–8.8	<0.0001
Sex male	140	2.1	1.2–3.8	0.0113
Abnormal serum MIC-1	71	1.6	0.9–2.9	0.1184
Homozygous *H* MIC-1	106	0.9	0.5–1.7	0.8671
Death from cancer				
TNM tumor				
1	17	1.0		
2	22	3.3	0.3–33.1	
3	150	3.4	0.4–27.3	
4	72	16.3	2.0–130.3	<0.0001[a]
TNM metastasis	38	15.4	6.4–36.7	<0.0001
Dukes stage				
A	27	1.0		
B	83	2.8	0.3–23.8	
C	78	5.0	1.4–85.1	
D	38	84.0	9.8–720.7	<0.0001[a]
Lymph nodes present	105	5.0	2.5–9.7	<0.0001
Abnormal serum MIC-1	71	2.1	1.1–4.0	0.0182
Sex male	140	2.0	1.0–4.0	0.0310
Homozygous *H* MIC-1	106	0.9	0.5–1.8	0.8231

[a] *P* for group trend.

Table 2 Multivariate logistic regression for Sydney cohort

Risk	*n*	OR	95% CI	*P*
Presence of metastasis at presentation when TNM tumor stage >2[a]				
Presence of lymph nodes	93	4.2	1.7–10.5	0.0009
Abnormal serum MIC-1	64	2.7	1.2–6.5	0.0191
Homozygous *H* MIC-1	87	0.4	0.2–0.8	0.0170
Likelihood of relapse				
TNM tumor >2	185	3.0	1.0–8.5	0.0310
Lymph nodes present	105	4.3	2.2–8.3	<0.0001
Abnormal serum MIC-1	71	1.5	0.8–2.9	0.2449
Homozygous *H* MIC-1	106	1.0	0.5–1.9	0.9537
Death from cancer				
TNM tumor >2	185	1.5	0.5–4.3	0.4860
TNM mets	38	9.5	3.4–26.7	<0.0001
Lymph nodes present	105	2.5	1.2–5.3	0.0122
Abnormal serum MIC-1	71	2.1	1.0–4.4	0.0516
Sex male	140	1.2	0.7–4.3	0.5276
Homozygous *H* MIC-1	106	0.9	0.4–1.8	0.4806

[a] As there were no cases of distant metastasis at presentation in patients with a TNM tumor stage of ≤2, multivariate analysis was performed using the above variables to predict metastasis on presentation with a TNM tumor stage >2 (n = 185).

small. This study is inadequately powered to determine whether MIC-1 genotype contributes to disease pathogenesis.

The MIC-1 genotype, although not associated with CRC incidence, did influence disease outcome. Using multivariate logistic regression analysis with TNM tumor stage, the presence of the *HH* genotype was associated with a decreased risk of metastasis at presentation (OR = 0.4, 95% CI, 0.2–0.8; P = 0.0170; Table 2). Although numbers in the *DD* genotype were too small for formal statistical analysis, there was a suggestion of a gene dose effect with 29% of *DD* subjects presenting with metastasis, whereas 22% of *HD* and 13% of *HH* patients had metastasis on presentation. This suggests that the presence of at least one *D* allele is associated with a greater likelihood of metastasis being present. This may indicate that the different genotypes of MIC-1 are associated with changes in tumor behavior such as alteration in growth and/or ability to metastasis. Additionally, MIC-1, like TGF-β, may also be involved in the modulation of immune function, leading to alteration in tumor immunity.

High Serum MIC-1 Levels and *D* Allele Are Associated with Decreasing Relapse-free Survival. Patients with high serum MIC-1 levels relapsed sooner than those who had serum MIC-1 levels in the normal range [P = 0.032; Log rank (Mantel-Cox); Kaplan-Meier; Fig. 4*A*]. This effect was independent of disease stage at diagnosis. In those patients who died of cancer and consequently were likely to have a greater tumor bulk at presentation, *HH* genotype was associated with a longer relapse-free survival [P < 0.02; Log rank (Mantel-Cox);

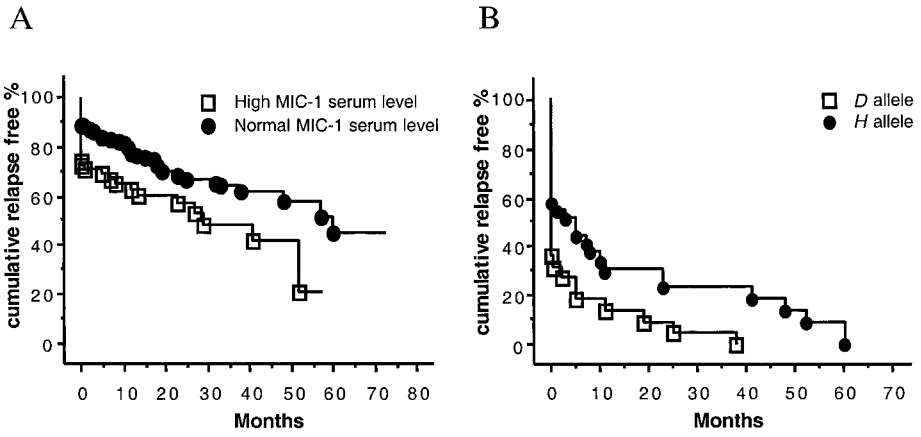

Fig. 4 *A*, the presence of a high serum MIC-1 level at diagnosis of CRC predisposes to earlier relapse of disease after initial treatment. *B*, the presence of a *D* allele is associated with earlier relapse of disease. In patients who died of CRC, those with at least a single *D* allele of MIC-1 relapsed earlier than those who were *HH*.

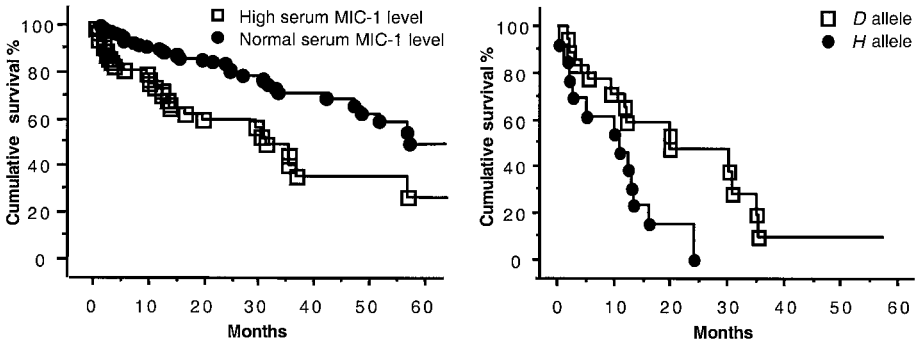

Fig. 5 *A*, the presence of a high serum MIC-1 level at diagnosis of CRC is associated with decreased overall survival. *B*, the presence of at least a single MIC-1 *D* allele is associated with longer overall survival in those patients that presented with metastatic disease.

Kaplan-Meier] when compared with those patients with at least one MIC-1 *D* allele (Fig. 4*B*).

These results indicate that serum MIC-1 level is not only associated with tumor bulk, but there may also be an association with altered disease course. Supporting this proposition is the difference in clinical characteristics at presentation of disease, with subjects having at least one *D* allele having a greater likelihood of having metastasis. Additionally, patients with at least one *D* allele develop metastasis or local recurrence sooner than those with only the *H* allele.

High MIC-1 Serum Level and *H* Allele Are Associated with Decreased Survival Time. A high serum MIC-1 level was associated with a significantly shorter survival time [$P = 0.0002$; Log rank (Mantel-Cox); Kaplan-Meier; Fig. 5*A*]. Using Cox proportional analysis, a high MIC-1 serum level was associated with an elevated risk of death (2.2; 95% CI, 1.3–3.7; $P = 0.0034$; Table 3). This would be expected because the MIC-1 level is associated with the TNM stage of disease, as well as the presence of metastasis. Homozygous *D* and heterozygous *HD* combined had an overall survival advantage compared with homozygous *H*. Again, a gene dose effect was discernible, but this did not reach statistical significance in the group as a whole. When subjects were stratified according to Dukes stage, there was an increased survival advantage associated with the presence of a *D* allele in all groups, but this only reached statistical significance in the stage 4 group indicating those subjects with metastatic disease. [$P < 0.02$; Log rank (Mantle-Cox); Kaplan-Meier; Fig. 5*B*].

The effect of MIC-1 allele on disease-free survival and overall survival are in opposition. The presence of at least one *D* allele was associated with earlier relapse of disease but overall longer survival. The reverse is true for those who have only the *H* allele present.

MIC-1 Serum Level and Genotype Are Associated with the Patterns of Tumor Lymphocyte Infiltration. Crohn's type and intraepithelial lymphocyte- and PTL-type infiltration are strongly related and hence not independent of each other ($P = 0.0005$; logistic regression). Assessment of Crohn's infiltrate was available 137 subjects. There was a trend for increasing MIC-1 serum level to be associated with absence of a Crohn's pattern of mononuclear cell infiltrate, but this was not statistically significant. Additional investigation suggested that this effect was significant but confined to those with an *HH* MIC-1 genotype ($P = 0.0236$, ANOVA; Fig. 6). Although there was no significant change in the intraepithelial lymphocyte or PTL pattern infiltrate when compared with MIC-1 serum level, the relationship to the MIC-1 genotype was similar to that seen with the Crohn's pattern of infiltrate. This may indicate that the

Table 3 Cox proportional hazards for Sydney cohort

Variable	*n*	OR	95% CI	*P*[a]
Time to relapse				
Sex male	140	0.6	0.4–1	0.0612
TNM nodes				
0	117	1.0		
1	51	4.4	2.5–7.6	
2	47	2.5	1.4–4.5	
3	9	5.2	2.0–14.0	<0.0001[a]
TNM tumor				
1	17	1.0		
2	22	2.9	0.3–24.6	
3	150	3.8	0.5–28.4	
4	72	12.2	1.7–88.4	<0.0001[a]
Dukes stage				
A	27	1.0		
B	83	1.9	0.4–8.6	
C	78	6.2	1.5–25.0	
D	38	43.2	10.0–185.3	<0.0001[a]
Abnormal serum MIC-1	71	1.7	1.1–2.8	0.0241
Homozygous *H* MIC-1	106	1.1	0.7–1.8	0.6717
Time to death				
Sex male	140	0.6	0.4–1	0.0612
TNM nodes				
0	117	1.0		
1	51	3.0	1.5–6.2	
2	47	5.5	2.8–10.9	
3	9	4.6	1.5–14.3	<0.0001[a]
TNM tumor				
1	17	1.0		
2	22	1.7	0.2–15	
3	150	2.0	0.3–15.3	
4	72	7.2	1.0–52.0	<0.0001[a]
Metastasis	38	11.4	6.5–20.4	<0.0001
Dukes stage				
A	27	1.0		
B	83	1.9	0.2–15.8	
C	78	7.7	11.0–56.9	
D	38	36.1	4.9–266.9	<0.0001[a]
Abnormal serum MIC-1	71	2.2	1.3–3.7	0.0034
Homozygous *H* MIC-1	106	1.1	0.7–1.8	0.7599

[a] *P* for group trend.

higher levels of *HH* MIC-1 are associated with decreased numbers of tumor-related lymphocytes.

DISCUSSION

A clear association between MIC-1 serum levels and colon cancer has been demonstrated in this article, with a steady and progressive rise in MIC-1 levels with progression from normal to adenomatous polyps, CRC, and finally CRC with widespread metastatic disease. The major questions to be answered involve the role that MIC-1 may play in the pathogenesis of CRC and the value of estimation of serum MIC-1 levels as a clinical tool in this disease. For clinical and diagnostic use, MIC-1 has a number of significant practical advantages over other cytokines produced by epithelial tumors. There is no significant production of MIC-1 by blood elements such as platelets, negating the need for specialized collection protocols (21). However, although there are significant differences in MIC-1 serum levels among the populations of CRC, colorectal adenomas, and normals, the differences are not large enough to provide a discriminatory diagnostic test for the presence of these lesions on a

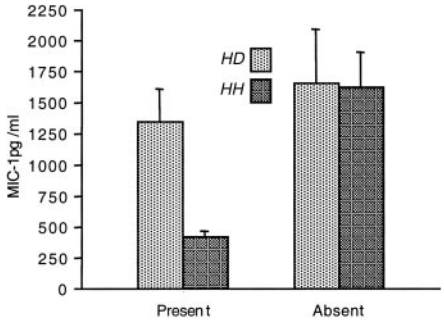

Fig. 6 Plotting the changes in MIC-1 serum level against the presence or absence of Crohn's type infiltration according to the MIC-1 genotype present shows that the homozygous *H* genotype has a significant effect on Crohn's lymphocytic infiltrate. Where homozygous *H* serum MIC-1 levels are low, lymphocytic infiltrate is present. Where lymphocytic infiltrate is absent, homozygous *H* MIC-1 serum levels are significantly higher. No such change is seen where a MIC-1 *D* allele is present.

single observation. However, serial measurements of MIC-1 seem to be relatively stable (unpublished observation), and prospective analysis of serial MIC-1 serum levels in at risk populations might prove to be of greater value. Additionally, the combined use of MIC-1 in concert with other markers also needs to be explored.

Measurement of MIC-1 serum level and genotype may add additional prognostic information independent of traditional markers. A high serum MIC-1 level at presentation of disease is associated with a doubling of risk of death and independently predicts the presence of metastasis. Additionally, patients with the MIC-1 *D* allele were at least twice as likely to present with metastatic disease and relapse sooner. However, although they relapse sooner, they survive longer, an apparent paradox that complicates the interpretation of the serum MIC-1 measurement and genotype determination.

A number of complex factors control the development, growth, local, and distant spread of tumors. These include factors directly controlling the growth rate of the tumor and those controlling the host response to the tumor. For example, in the Sydney cohort, the presence of a PTL pattern of infiltrate was associate with a ~3-fold decreased risk of dying from cancer. Additionally, lymphocytic infiltration of CRC, associated with microsatellite instability, is associated with better disease outcome (31, 32).

Data in this article suggests that MIC-1 may have an effect on tumor lymphocytic infiltration. This effect is independent of microsatellite instability status (data not shown), with strong correlation between the three patterns of tumor-lymphocytic infiltration; it is curious that only one pattern, Crohn's type infiltration, declines as MIC-1 serum levels increase. Although the reasons for this are uncertain, this is similar to findings previously observed with TGF-β and may be related to the differential sensitivity of lymphocyte subsets to the effects of TGF-β (8, 33). Higher homozygous *H* MIC-1 serum levels were associated with absence of Crohn's type infiltration. There was no significant effect on this compartment in those subjects with either heterozygous (*HD*) or homozygous *D* (*DD*) genotypes. This suggests that the *H* allele may be more biologically active

or functions differently to the *D* allele. Tumor growth and metastasis has previously been shown to be modulated by the cellular immune compartment (31, 32). Our data suggests a possible role for MIC-1 in modulation of at least one lymphocyte compartment.

Tumor growth is also directly regulated by factors that regulate normal epithelial growth. TGF-β secretion leads to suppression of epithelial cell growth and is important in normal epithelial layer homeostasis (1, 34). MIC-1 has been shown to inhibit human colonic tumor cell line growth in nude mice, independent of cell-mediated immunity (27). These experiments used the homozygous *H* form of MIC-1. In our cohort, those subjects with a *D* allele present had an earlier relapse of disease and were more likely to present with metastasis. This may indicate that homozygous *H* subjects had superior local tumor control compared with those with a *D* allele present. This may be mediated by direct effects of MIC-1 on tumor growth. However, as the tumor progresses and MIC-1 secretion increases, the positive effect on tumor control may be negated by decreased local tumor immunity, providing a mechanism for tumor escape. Consequently, a positive feedback mechanism might be established, leading to greater MIC-1 production, additional immunosuppression, dissemination of disease, and earlier death. This is one of several scenarios that might explain apparently paradoxical allele related differences in the course of CRC. Additional studies to define the roles of MIC-1 in tumor biology are clearly warranted.

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