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Original Paper

Expression of Metastasis-related Genes in Surgical Specimens of Human Gastric Cancer can Predict Disease Recurrence

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It was determined whether the expression level of several genes that regulate different steps of metastasis in formalin-fixed paraffin-embedded archival specimens of human gastric cancers correlated with disease recurrence and metastasis. The steady-state mRNA expression level for epidermal growth factor receptor (EGF-R), basic fibroblast growth factor (bFGF), E-cadherin, type IV collagenase and multidrug resistance (*MDR*-1) were examined by a colorimetric *in situ* hybridisation (ISH) technique, concentrating on reactivity at the periphery of the lesions. All patients were operated on for cure. 15 cases were disease-free and 10 had disease recurrence by 4.5 years after resection of the primary tumours. The expression of EGF-R and bFGF type IV collagenase was higher and expression of E-cadherin was lower in the disease-recurrence cases than in the disease-free cases. The ratio between the expression level of collagenase type IV and E-cadherin at the periphery of the surgical specimens differed significantly between the disease-free cases and the recurrent-metastatic cases. These data show that multiparametric ISH analysis for several metastasis-related genes may allow prediction of disease recurrence of gastric cancer. © **1998 Published by Elsevier Science Ltd. All rights reserved.**

Key words: molecular diagnosis, *in situ* hybridisation, gene expression, metastasis
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INTRODUCTION

THE PROGNOSIS and design of therapy for patients with gastric cancer, as well as the determination of disease recurrence and metastasis, are based on clinical staging and histopathological classification [1–4]. However, routine microscopic examination of tissue sections from the primary neoplasm and lymph nodes cannot always identify the patients at highest risk of recurrence [5]. Recent advances in the understanding of the molecular regulation of cancer metastasis and the design of molecular diagnostic tools have provided new procedures with which to predict the malignant potential of individual human cancers [6].

The process of metastasis consists of sequential and selective steps that include proliferation, induction of angiogenesis, detachment, motility, invasion into the circulation, aggregation and survival in the circulation, cell arrest in distant capillary beds and extravasation into the organ parenchyma, response to local growth factors, induction of angiogenesis, and proliferation [6]. The outcome of metastasis is determined by multiple interactions between metastatic tumour cells and host factors [7, 8]. To produce clinically relevant metastases, tumour cells must complete all steps in the metastatic cascade [6–8]. Thus, the failure to produce a metastasis can be due to different single or multiple deficiencies [9].

The metastatic potential of neoplasms has been correlated with the expression level of several independent genes that regulate cell growth: epidermal growth factor receptor (EGF-R) [10–18], basic fibroblast growth factor (bFGF) [19, 20], invasion, type IV collagenase genes [21–26] and multidrug resistance (*MDR*-1) [27–31]. There have also been several reports that the expression of E-cadherin, which is directly related to cell-to-cell cohesion, is inversely correlated with tumour progression and metastasis [32–37]. Most of these correlative studies reached the inevitable conclusion that the expression of a given gene is necessary but insufficient to account for the multistep process of metastasis [38].

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Because each of the discrete steps in the pathogenesis of metastasis is regulated by one or several independent genes, the identification of cells with metastatic potential in heterogeneous primary human tumours requires multiparametric-multivariate analysis of gene expression [6].

We have recently developed a rapid colorimetric *in situ* hybridisation (ISH) technique for the evaluation of gene expression in formalin-fixed paraffin-embedded surgical specimens [39–43]. This technique was used to study the expression level of several genes that regulate different steps in cancer metastasis. Analysis of 25 archival specimens of human gastric cancers suggests that the expression level of some metastasis-related genes at the periphery (invasive edge) of the lesions can predict disease recurrence and metastasis.

MATERIALS AND METHODS

Surgical specimens

Twenty-five formalin-fixed, paraffin-embedded archival surgical specimens of primary gastric cancers from patients treated at Saitama Medical School were chosen at random. The specimens were staged according to the UICC TNM pathological classification of malignant tumours [1–4]. All stage II and III tumours were operated on 'for cure'. The margins of these neoplasms were free of tumour cells. The neoplasms were evaluated to determine if the expression profile of the metastasis-related genes correlated with the development of recurrent disease or metastasis at any point in the 4.5 years after resection of the primary tumour. None of these patients received adjuvant therapy. Only specimens with intact mRNA were evaluated for expression of the metastasis-related genes.

Oligonucleotide probes

Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts of EGF-R [44], bFGF [45], collagenase type IV [46], E-cadherin [47] and *MDR*-1 [48], based on published reports of the cDNA sequences. The specificity of the oligonucleotide sequences was initially determined by a GenEMBL database search

Table 1. *Sequence of oligonucleotide probes*

Probe	Sequence 5′–3′ (GC content)	Working dilution	[Ref.]
EGFR	GGA′GCG′CTG′CCC′CGG′CCG′TCC′CGG (87.5%)	1:800	[24]
bFGF	CGG′GAA′GGC′GCC′GCT′GCC′GCC (85.7%)	1:200	[25]
Type IV collagenase	TGG′GCG′ACG′GCG′CGG′CGG′CGG′CGT′GGC (88.9%)	1:200	[26]
E-cadherin	TGG′AGC′GGG′CTG′GAG′TCT′GAA′CTG (62.5%)	1:200	[27]
(mixture)	GAC′GCC′GGC′GGC′CCC′TTC′ACA′GTC (75.0%)		
MDR-1	CAG′ACA′GCA′GCT′GAC′AGT′CCA′AGA′ACA′GGA′CT (53.1%)	1:200	[28]
(mixture)	GCA′TTC′TGG′ATG′GTG′GAC′AGG′CGG′TGA′G (60.7%)		
Poly $d(T)_{20}$		1:1000	

Table 2. *Expression level of metastasis-related genes in human colon carcinomas determined by* in situ *hybridisation*

Case no.	Stage*	Histology/ differentiation	Follow-up (months)	mRNA expression index†					Met‡	Rec§
				EGF-R	bFGF	Collagenase	E-cadherin	*MDR*-1		
1	II	Moderately	54	7	4	24	54	46	—	—
2	II	Moderately	62	3	78	57	62	73	—	—
3	II	Moderately	61	62	68	48	82	54	—	—
4	II	Well	59	38	31	34	41	30	—	—
5	II	Well	60	63	29	35	37	52	—	—
6	II	Poorly	64	82	75	46	54	59	—	—
7	II	Poorly	52	52	38	33	30	33	—	—
8	III	Well	63	17	46	44	50	38	—	—
9	III	Moderately	56	38	25	38	38	46	—	—
10	III	Moderately	63	51	47	56	49	67	—	—
11	III	Poorly	61	64	60	55	55	42	—	—
12	III	Well	61	7	28	83	83	86	—	—
13	III	Poorly	60	58	62	38	38	44	—	—
14	III	Poorly	62	44	67	64	80	36	—	—
15	III	Poorly	54	47	69	50	97	59	—	—
16	III	Poorly	6‖	56	61	46	38	41	—	Peritoneum
17	III	Poorly	6‖	51	51	47	49	54	—	Liver
18	III	Poorly	17‖	56	77	60	47	53	—	Bone
19	III	Well	45‖	58	66	65	59	46	—	Peritoneum
20	III	Moderately	12‖	96	73	96	27	67	—	Peritoneum
21	III	Poorly	29‖	58	17	50	48	69	—	Peritoneum
22	IV	Moderately	3‖	97	82	41	27	25	Liver	—
23	IV	Well	6‖	87	67	54	36	59	Liver	—
24	IV	Poorly	6‖	61	86	39	38	39	Peritoneum	—
25	IV	Poorly	13‖	78	89	67	58	67	Peritoneum	—

*UICC TNM classification (stage III including stage IIIA and IIIB). †The intensity of the cytoplasmic staining was quantitated by an image analyser and compared with the maximal intensity of poly d(T) staining in each sample defined as 100. ‡Metastasis at the time of diagnosis (organ site). §Site of disease recurrence. ‖Death.

using the Genetics Computer Group sequence analysis program (GCG, Madison, Wisconsin, U.S.A.) based on the FastA algorithm [49], which showed 100% homology with the target gene and minimal homology with non-specific mammalian gene sequences. The specificity of each sequence was also confirmed by Northern blot analysis. A $d(T)_{20}$ oligonucleotide was used to verify the integrity of the mRNA in each sample [39, 40]. All DNA probes were synthesised with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry [TAG-BBB-(TGA)-BBB] (Research Genetics, Huntsville, Alabama, U.S.A.) [50]. The lyophilised probes were reconstituted to a 1 μg/μl stock solution in 10 mM Tris (pH 7.6) and 1 mM EDTA. The stock solution was diluted with Probe Diluent (Research Genetics) immediately before use. The probes and working dilutions are shown in Table 1.

In situ *hybridisation (ISH)*

ISH was performed as described previously [39–41] with a minor modification. ISH was carried out according to the Microprobe manual staining system (Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.) [51]. Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on Silane-treated ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed (10 min in 60°C oven), and dehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin [52]. Hybridisation of the probe was carried out for 45 min at 45°C. The samples were then washed three times with 2× SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labelled avidin for 30 min at 45°C, briefly rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer

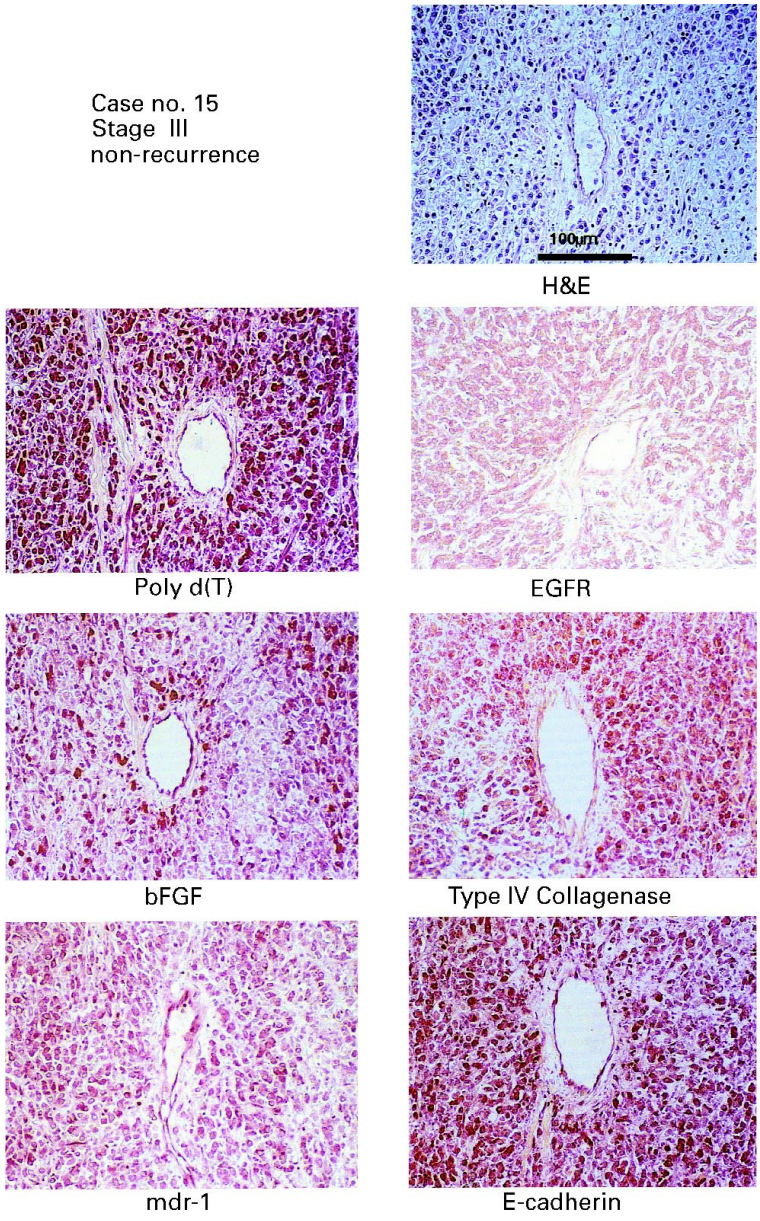

Figure 1. ISH analysis of metastasis-related genes in a non-recurrent stage IIIB gastric carcinoma. Hybridisation with a hyperbiotinylated poly $d(T)_{20}$ probe confirmed the integrity and lack of mRNA degradation. A positive reaction in this assay stains red. The expression intensity of the poly $d(T)_{20}$ probe was assigned the value of 100. The expression intensity values for EGF-R, bFGF, type IV collagenase, *MDR*-1 and E-cadherin were 47, 69, 50, 59, and 97, respectively.

(Biomedia Corp., Foster City, California, U.S.A.) for 1 min, and finally incubated with the chromogen substrate Fast Red (Research Genetics) for 30 min at 45°C. A positive reaction in this assay stains red. Control for endogenous alkaline phosphatase included treatment of the samples in the absence of the biotinylated probe and use of chromogen in the absence of any oligonucleotide probes.

To check the specificity of the hybridisation signal, the following controls were used: (a) RNase pretreatment of tissue sections; (b) substitution of the antisense probe with a biotin-labelled sense probe; and (c) competition assay with unlabelled antisense probes. A markedly decreased or absent signal was obtained after each of these treatments.

Image analysis to quantify intensity of colour reaction

Stained sections were examined in a Nikon photomicroscope (Nikon, Tokyo, Japan) equipped with a three-chip-charged coupled device colour camera (HOC205, Hitachi Corp., Tokyo, Japan). The images were analysed using the Luzex-SF image analyser (Nireco, Tokyo, Japan). The slides were prescreened by one of the investigators to determine the range of staining intensity of the slides to be analysed. Images covering the range of staining intensities were captured electronically, a colour bar was created, and a threshold value was set in the red, green and blue mode of the colour camera. All subsequent images were quantified based on this threshold. The integrated optical density (OD) of the selected fields was determined based on its equivalence to the mean log inverse grey value multiplied by the area of the field. As the samples were not counterstained, the OD was due solely to the product of the ISH reaction. Three different fields from the periphery

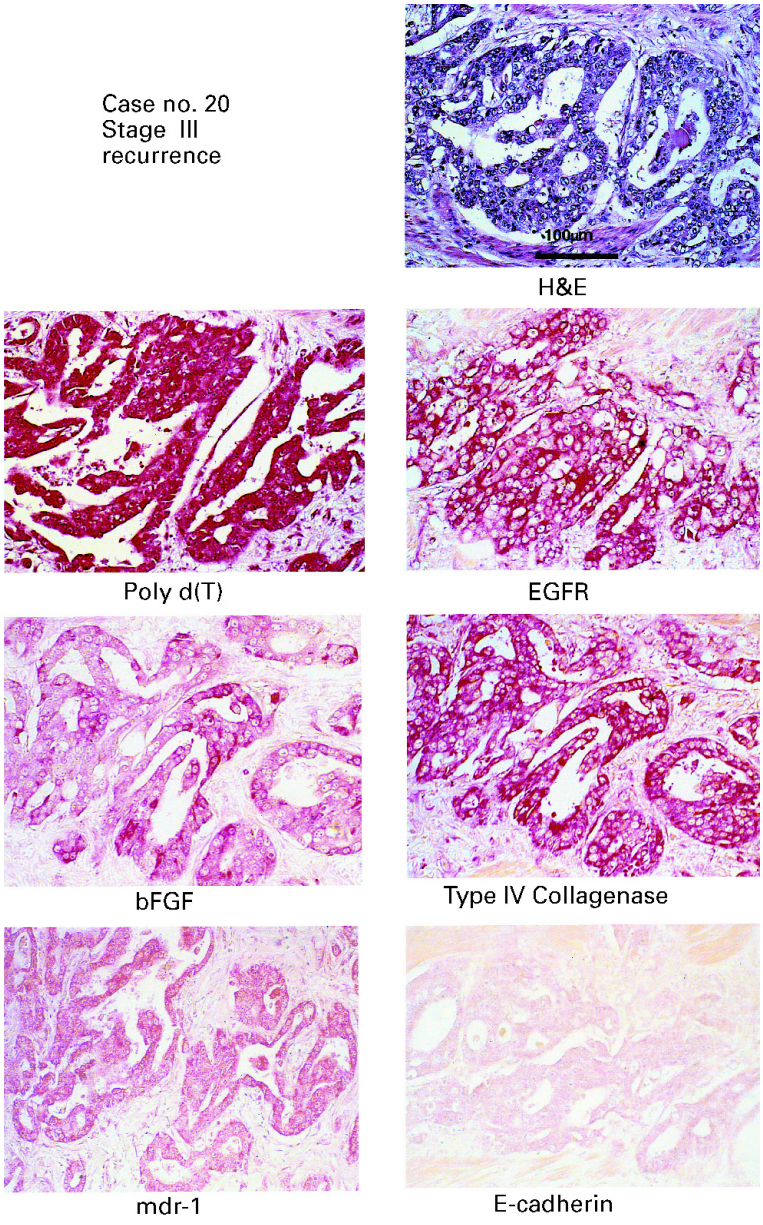

Figure 2. ISH analysis of metastasis-related genes in a recurrent stage IIIB gastric carcinoma. Hybridisation with a hyperbiotinylated poly $d(T)_{20}$ probe confirmed the integrity and lack of mRNA degradation. A postive reaction in this assay stains red. The expression intensity of the poly $d(T)_{20}$ probe was assigned the value of 100. The expression intensity values for EGF-R, bFGF, type IV collagenase, *MDR*-1 and E-cadherin were 96, 73, 96, 67, and 27, respectively.

of each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated OD of poly $d(T)_{20}$, which was set to 100 [43].

Statistical analyses

TNM stage III patients were classified as having recurring (metastasis) or non-recurring disease according to the disease status 4 years after surgery. The Mann–Whitney test was used to compare the level of gene expression in metastasising (stage III recurring and IV) versus non-metastasising (stages II and III non-recurring) tumours for each of the genes individually (univariate analyses). Discriminant analysis [53] was performed to obtain the linear discrimination function using a commercial statistical analysis programme (SAS Institute, Cary, North Carolina, U.S.A.) [54].

RESULTS

Integrity of mRNA in archival human surgical specimens

Paraffin blocks of 25 specimens were cut into 4 μm sections and stained with haematoxylin and eosin for histopathological evaluation, or processed for ISH analysis. The integrity of the mRNA in each sample was verified using a poly $d(T)_{20}$ probe. All 25 samples had an intense histochemical reaction, indicating that the mRNA was not degraded. The specimens consisted of samples from seven stage II, 14 stage III (A and B) and four stage IV tumours. All the specimens were adenocarcinomas with different levels of differentiation. Neither the location of the primary neoplasm nor the state of differentiation correlated with production of metastasis. All patients were followed-up for a minimum of 4 years. The clinicopathological data of these tumours are summarised in Table 2.

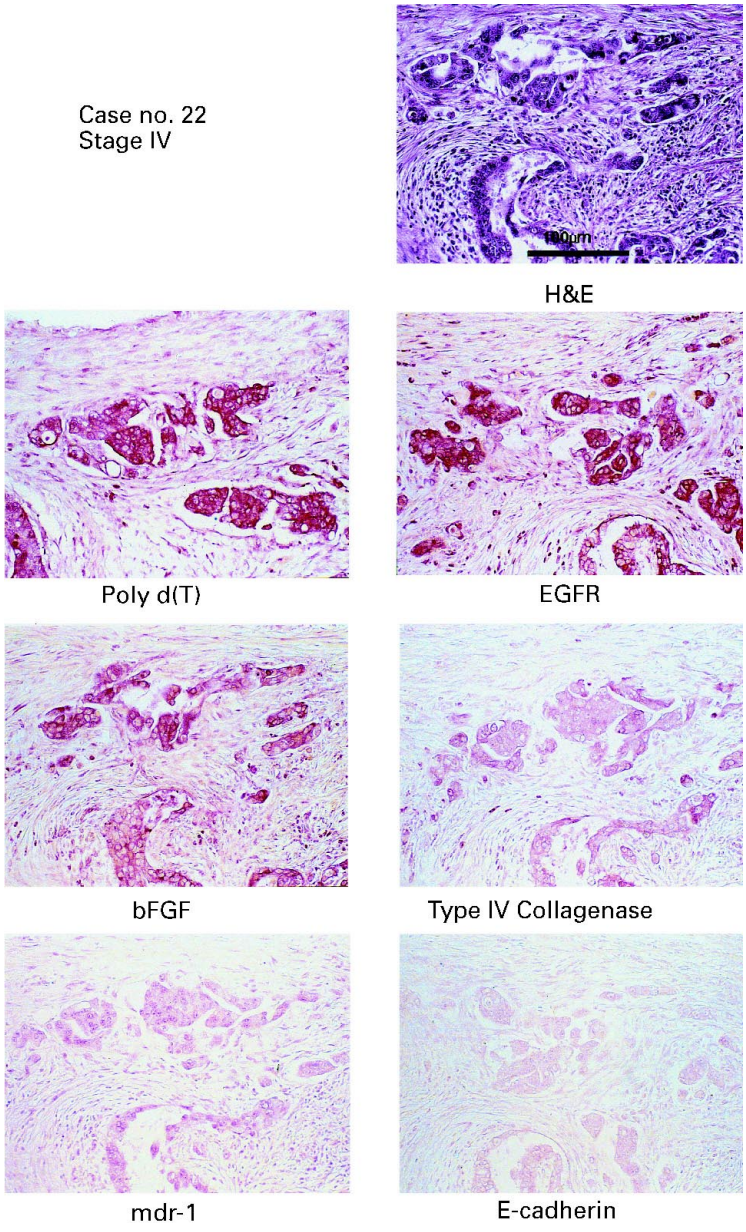

Figure 3. ISH analysis of metastasis-related genes in stage IV gastric carcinoma. Hybridisation with a hyperbiotinylated poly $d(T)_{20}$ probe confirmed the integrity and lack of mRNA degradation. A positive reaction in this assay stains red. The expression intensity of the poly $d(T)_{20}$ probe was assigned the value of 100. The expression intensity values for EGF-R, bFGF, type IV collagenase, *MDR*-1 and E-cadherin were 97, 82, 41, 25, and 27, respectively.

Table 3. Median level of gene expression (%) by disease stage and recurrence status

Gene	TNM stage*				*P* value†
	II ($n = 7$)	III ($n = 8$)	III (recurring) ($n = 6$)	IV ($n = 4$)	
EGF-R	52 (3–82)‡	46 (7–64)	57 (51–96)	83 (61–97)	0.013
bFGF	38 (4–78)	54 (25–69)	64 (17–77)	84 (67–89)	0.049
Collagenase	35 (24–57)	53 (38–83)	55 (46–96)	48 (39–67)	0.12
E-cadherin	54 (30–82)	53 (38–97)	43 (27–58)	37 (27–58)	0.071
MDR-1	52 (30–72)	45 (36–86)	54 (41–69)	49 (25–67)	0.68

*UICC TNM classification (stage III including stage IIIA and IIIB). †Mann–Whitney test for a difference in the median level of gene expression between stage II and III tumours that did not produce metastasis, and stage III (recurring) and IV tumours that produced metastasis. ‡The intensity of the cytoplasmic staining was quantitated by an image analyser and compared with the maximal intensity of poly d(T) staining in each sample defined as 100. The numbers in parentheses are the range of values.

Expression level of metastasis-related genes in human gastric cancers of different TNM stages

The expression level of the EGF-R, bFGF, type IV collagenase, E-cadherin and *MDR*-1 genes was examined at the periphery of each tumour [55]. The results of the analyses are summarised in Table 2. The ISH images for a representative case of stage III tumour from a disease-free patient (case 15) are shown in Figure 1 and for a patient with a representative stage III tumour that recurred (case 20) are shown in Figure 2. The ISH images for a representative case of stage IV tumour from a patient with metastasis (case 22) are shown in Figure 3.

The median level of expression of each of the metastasis-related genes is listed in Table 3 by disease stage and recurrence status. Univariate analysis revealed that expression of EGF-R and bFGF was correlated with recurrence-metastasis ($P < 0.013$ and 0.04, respectively). A trend towards an increase in level of expression for collagenase type IV and a decrease in level of E-cadherin expression was also found in the recurrent cases (Table 3). No discernible differences in expression of *MDR*-1 mRNA were detected between the disease-recurrence and disease-free cases.

We have previously reported an inverse correlation between the expression of E-cadherin and collagenase type IV in human neoplasms [55]. We therefore determined the ratio between the expression level of collagenase type IV and E-cadherin (C:E) at the periphery of all 25 surgical specimens (Figure 4). The C:E ratio differed significantly between the disease-free cases and the recurrent-metastatic cases (Mann–Whitney test, $P < 0.01$). The linear discrimination function was calculated by using discrimination analysis comparing the numerical value of collagenase type IV mRNA expression level with that of E-cadherin mRNA expression level. The sensitivity and specificity of discrimination between disease-free and recurrent cases were 87 and 70%, respectively.

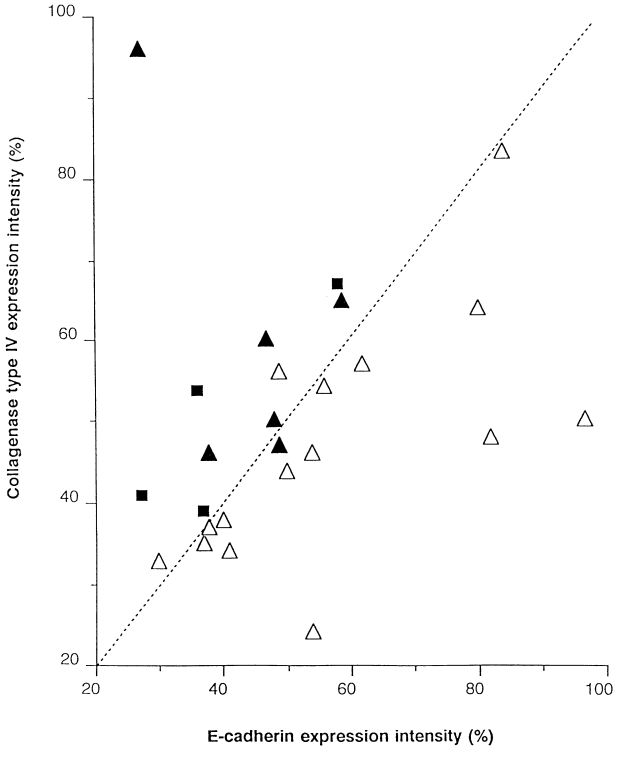

Figure 4. Expression intensity of collagenase type IV versus E-cadherin for the individual tumours in this study. Stage II and non-recurrent stage III (△); recurrent stage III (▲); stage IV (■). The broken line represents linear discrimination function: [Coll] = 1.0076649×[ECD] + 1.5743.

DISCUSSION

The expression levels of several genes that regulate different steps in the process of gastric cancer metastasis—growth (EGF-R), angiogenesis (bFGF), invasion (type IV collagenase), cell adhesion-cohesion (E-cadherin) and multidrug resistance (*MDR*-1)—were examined in archival specimens of primary human gastric cancers. We have previously reported that the metastatic potential of human colon cancers can be identified by multiparametric ISH analysis for the expression of these genes [42, 43]. Our present data suggest that this analysis may also differentiate between gastric cancers that will produce recurrent and metastatic disease after surgery for cure and those that will not.

The prognosis for gastric cancer is routinely determined by the pathological and clinical TNM classification, which relies on criteria such as depth of invasion (of the primary cancer) and presence of regional or distant lymph node metastasis, respectively [2–4]. However, like other neoplasms, human gastric cancers are heterogeneous and consist of multiple subpopulations of cells with different metastatic potentials [6, 37, 56]. Metastasis can be produced from a small (< 1.0%) subpopulation of tumour cells with a unique set of properties, and presumably a unique genotype, that allow them to complete the sequential and selective steps of this process [6, 57, 58]. Histological observations can miss the presence of a small zone of malignant metastatic cells within a tumour [59] and, thus, fail to classify the lesion as potentially malignant. In contrast, the expression level of metastasis-related

genes in a tumour sample can be examined by the ISH technique, which can identify the cellular source of the mRNA as well as intratumoral heterogeneity in expression.

In general, stage IV tumours expressed a higher level of bFGF, EGFR and collagenase IV mRNA than stage II tumours, agreeing with an earlier report that the expression level of these genes is higher in metastatic human colon carcinomas than in non-metastatic lesions [42, 43].

E-cadherin is a cell surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion [60, 61]. E-cadherin is localised at the epithelial junction complex and is responsible for the organisation, maintenance, and morphogenesis of epithelial tissues [34–37]. Reduced levels of E-cadherin are associated with a decrease in cellular/tissue differentiation and increased grade in different epithelial neoplasms [31, 32, 62–66] and transfection of E-cadherin-encoding cDNA into invasive cancer cells has been shown to inhibit motility and invasion [67]. Since the adhesion properties of E-cadherin restrict the detachment of tumour cells and, hence, their migration–invasion, the use of E-cadherin expression as a prognostic factor has been widely investigated [68]. Our results showed a trend ($P < 0.071$) towards reduced expression in recurrent metastatic cases.

Invasion of the host stroma and degradation of the blood vessel basement membrane are necessary for metastasis [21–26]. The levels of collagenase type IV in human and rodent neoplasms directly correlate with invasion and metastasis and specific inhibitors of matrix metalloproteinases have been shown to inhibit tumour cell invasion [69–73]. Thus, a decrease in the expression of E-cadherin and an increase in collagenase type IV activity should enhance tumour cell invasion and metastasis. The present data and published data in human colon carcinomas [43] support this correlation.

In summary, the ISH technique described here examined the concurrent expression of metastasis-related genes in formalin-fixed, paraffin-embedded surgical specimens of human gastric cancers. The expression profile of EGF-R bFGF, collagenase type IV, and E-cadherin (but not *MDR*-1) at the periphery of the lesions may distinguish between neoplasms that will reoccur or produce metastasis and neoplasms that do not. The ratio between the expression level of collagenase type IV and E-cadherin at the periphery of the lesions identified most tumours with metastatic potential. The reliability of this technique should be tested in a larger prospective study of human gastric cancers.

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