ORIGINAL ARTICLE

Expression of KAI1 and tenascin, and microvessel density are closely correlated with liver metastasis of gastrointestinal adenocarcinoma

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J Clin Pathol 2007;60:50–56. doi: 10.1136/jcp.2006.036699

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Accepted 8 March 2006
Published Online First
12 May 2006

Aim: To seek good markers to predict invasion and metastasis of gastrointestinal adenocarcinoma (GIA).

Methods: Expression of KAI1 and tenascin were examined on tissue microarrays containing gastric adenocarcinoma (n=98), colorectal adenocarcinoma (n=125), gastric adjacent non-cancerous mucosa (n=95) and colorectal adjacent non-cancerous mucosa (n=112) by immunostaining. Microvessel density (MVD) in GIA was labelled using anti-CD34 antibody by immunostaining. Expression of KAI1 and tenascin, and MVD were compared with clinicopathological features of tumours, including PTEN (phosphatase and tensin homology deleted from human chromosome 10) and EMMPRIN (extracellular matrix metalloproteinase inducer) expression.

Results: KAI1 expression was higher in GIAs than in their adjacent non-cancerous mucosa ($p<0.05$). KAI1 and tenascin expression showed a significantly negative association with liver metastasis of GIA ($p<0.05$), but not with depth of invasion, venous invasion or lymph node metastasis ($p>0.05$). A significantly negative relationship was observed between EMMPRIN and tenascin expression in GIA ($p<0.05$). MVD was positively correlated with depth of invasion, venous invasion, lymph node metastasis and liver metastasis of tumours ($p<0.05$), whereas it was negatively correlated with PTEN expression ($p<0.05$).

Conclusions: Up-regulated KAI1 expression may play an important part in malignant transformation of gastrointestinal epithelial cells. Reduced expression of KAI1 and tenascin might underlie the molecular basis of liver metastasis of GIA. Angiogenesis is a key event in the invasion and metastasis of GIA. These markers might be used to indicate liver metastasis of GIA in clinicopathological practice.

The liver is the most common site of distant metastasis from gastrointestinal adenocarcinoma (GIA) because the liver filters the venous drainage from the intra-abdominal viscera and is occupied by numerous cell types, capable of providing a rich milieu for tumour cell growth.¹ Liver metastasis of GIA, which is a common malignant disease all over the world, is the most critical impediment to the patient's survival.^{2–3} Therefore, it is of importance to look for good markers to reflect liver metastasis of GIA.

KAI1 (CD82/C33/R2/IA4) was initially identified as a tumour metastasis suppressor gene on human chromosome 11p11.2, and encodes transmembrane glycoproteins of the tetraspanins family (TM4SF).⁴ TM4SF proteins have cytoplasmic N-terminus and C-terminus and traverse the cell membrane four times, forming one small and one large extracellular loop with residues susceptible to post-translational phosphorylation or N-linked glycosylation. KAI1 interacts with integrin $\alpha 4 \beta 1$, other TM4SF proteins and cell surface molecules including CD4, CD8, CD19, CD21 and major histocompatibility complex class I and II, forming "the tetraspanin web".⁵ Several recent reports suggested that a complex combination between KAI1 and specific proteins was involved in several biological processes, such as cellular adhesion, mobility, proliferation and apoptosis.^{6–7} When KAI1 was introduced into metastatic cancer cells, it was able to suppress their metastatic ability.⁸

Tenascin is a large extracellular matrix (ECM) glycoprotein with a six-armed disulphide-bonded macromolecular structure, consisting of tenascin-C (formerly known under various synonyms, such as cytotactin and hexabrachion), tenascin-R (for restrictin), tenascin-X, tenascin-Y and tenascin-W. All family members share a modular structure, consisting of a

cysteine-rich N-terminal domain involved in oligomerisation of tenascin-C, tenascin-R and possibly tenascin-X, as well as a series of epidermal growth factor-like repeats, followed by a number of fibronectin type III-like domains and a C-terminal fibrinogen-like domain.⁹ Tenascin has many biological functions likely to influence tumour development, such as regulation of tumour cell-cell interaction, proliferation, invasion and metastasis, and involvement in angiogenesis.^{10–11}

Neovascularisation is essential for the invasion and metastasis of solid tumours. A rich and extensive vascularisation network provides tumour cells with a channel into the circulation system, which plays a vital part in the progression of malignancies.³ Recently, EMMPRIN (extracellular matrix metalloproteinase inducer) was found to involve degradation of ECM by regulating metalloproteinase (MMP) expression.¹² It was previously reported that deletion or mutation of PTEN (phosphatase and tensin homology deleted from human chromosome 10) could enhance the expression of vascular epithelial growth factor (VEGF), which in turn closely correlated with tumour angiogenesis.³ In our study, microvessel density (MVD) and KAI1 and tenascin expression were examined in GIA on tissue microarray and compared with aggressive parameters of tumours to search for good markers to

Abbreviations: DAB, 3,3'-diaminobenzidine; ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer; GIA, gastrointestinal adenocarcinoma; H&E, haematoxylin and eosin; MMPs, matrix metalloproteinases; MVD, microvessel density; PTEN, phosphatase and tensin homology deleted from human chromosome 10; TMA, tissue microarray; TM4SF, transmembrane glycoproteins of tetraspanins family; VEGF, vascular epithelial growth factor

Figure 1 Immunostaining of KAI1 in gastrointestinal adenocarcinomas (GIAs) and their adjacent non-cancerous mucosa. KAI1 was localised to cytoplasm and membrane. KAI1 showed positive expression in superficially hyperplastic epithelium, mononuclear cells in lymph follicle and infiltrating inflammatory cells including macrophages in gastrointestinal mucosa (A,D). KAI1 was highly expressed in the gastrointestinal cancer cells (B,E), but negatively in the tumours with liver metastasis (C,F).

reflect invasion and metastasis of GIA. We also compared PTEN expression with MVD, and EMMPRIN expression with tenascin expression, to explore the regulatory factors of tenascin expression and angiogenesis.

PATIENTS AND METHODS

Pathology

Samples of gastric adenocarcinoma ($n = 98$), colorectal adenocarcinoma ($n = 125$), gastric adjacent non-cancerous mucosa ($n = 95$) and colorectal adjacent non-cancerous mucosa ($n = 112$) were collected from our hospital and related institutes between 1999 and 2005, from 136 men and 87 women (31–99 years, mean 68.9 years). Among them, 116 patients also had lymph node metastasis and 46 patients had liver metastasis. All tissues were fixed in 4% neutralised formaldehyde, embedded in paraffin wax and cut into 4 μ m thick sections. These sections were stained by haematoxylin and eosin (H&E) to confirm their histological diagnosis. Depth of invasion, venous invasion, lymph node metastasis and liver metastasis were also determined.

Tissue microarray

H&E-stained sections of the selected tumour cases were re-examined, and representative areas of solid tumour from each case were identified for sampling. A 2 mm diameter tissue core per donor block was punched off and transferred to a recipient block with a maximum of 48 cores using a tissue microarrayer (AZUMAYA KIN-1, Japan). Sections of 4 μ m thickness were consecutively cut from the recipient block and transferred to poly-lysine-coated glass slides. H&E staining was performed on tissue microarray (TMA) for the following evaluation.

Immunostaining

TMA slides were deparaffinised with xylene, dehydrated with alcohol and immunostained using intermittent irradiation as described previously.¹³ Mouse anti-human KAI1 (Novocastro, Newcastle Upon Tyne, UK; 1:50), mouse anti-human tenascin (Chemico, Temecula, California, USA; 1:300), mouse anti-human CD34 (Dako, Carpinteria, California, USA; 1:50), mouse anti-human EMMPRIN (Novocastro; 1:50), and mouse anti-human PTEN (Novocastro; 1:150) antibodies were used for the detection

Table 1 KAI1 expression in gastrointestinal non-cancerous mucosa and adenocarcinoma

	n	KAI1 expression				Positive rate (%)	R	p Value
		–	+	++	+++			
Non-cancerous mucosa	207	120	49	23	15	42.0	0.188	<0.001
Adenocarcinoma	207	78	77	23	29	62.3		

–, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Table 2 Relationship between KAI1 expression and clinicopathological features of gastrointestinal adenocarcinomas

Clinicopathological features	n	KAI1 expression				Positive rate (%)	R	p Value
		–	+	++	+++			
Depth of invasion								
T _{is} –T ₁	60	26	23	9	2	56.7	0.123	>0.05
T ₂ –T ₄	153	55	55	14	29	64.1		
Venous invasion							0.130	>0.05
–	107	46	39	11	11	57.0		
+	106	35	39	12	20	67.0		
Lymph node metastasis							0.028	>0.05
–	97	32	43	11	11	67.0		
+	116	49	35	12	20	57.8		
Liver metastasis							0.275	<0.001
–	167	52	65	22	28	68.9		
+	46	29	13	1	3	37.0		

T_{is}, carcinoma in situ; T₁, lamina propria and submucosa; T₂, muscularis propria and subserosa; T₃, exposure to serosa; T₄, invasion into serosa; –, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Figure 2 Immunostaining of tenascin in gastrointestinal adenocarcinomas (GIAs) and their adjacent non-cancerous mucosa. Tenascin was strongly expressed in the submucosal muscularis of stomach (A) compared with the colorectum (E). Tenascin was mainly expressed in extracellular matrix (B,F) and seldom in gastrointestinal cancer cells (C,G). The GIA with liver metastasis displayed negative expression of tenascin (D,H).

Table 3 Relationship between tenascin expression and clinicopathological features of gastrointestinal adenocarcinomas

Clinicopathological features	n	Tenascin expression				Positive rate (%)	R	p Value
		–	+	++	+++			
Depth of invasion								
T _{is} –T ₁	69	17	11	16	25	75.4	0.068	>0.05
T ₂ –T ₄	154	37	48	23	46	75.8		
Venous invasion							0.101	>0.05
–	114	33	28	21	32	71.1		
+	109	21	31	18	39	80.7		
Lymph node metastasis							0.013	>0.05
–	107	27	23	24	33	74.8		
+	116	27	36	15	38	76.7		
Liver metastasis							0.296	<0.001
–	177	35	43	32	67	80.2		
+	46	19	16	7	4	58.7		
EMMPRIN expression							0.138	<0.05
–	37	6	7	8	16	83.8		
+	26	6	7	5	8	76.9		
++	31	7	8	7	9	77.4		
+++	108	28	33	18	29	74.1		

T_{is}, carcinoma in situ; T₁, lamina propria and submucosa; T₂, muscularis propria and subserosa; T₃, exposure to serosa; T₄, invasion into serosa. –, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

of the respective proteins. Anti-mouse Envison-PO (Dako, Carpinteria, California, USA) was used as the secondary antibody in our study. All slides were coloured with 3,3'-diaminobenzidine (DAB) and counterstained with Mayer's haematoxylin. Omission of the primary antibody was used as a negative control, and appropriate positive controls were used as recommended by the manufacturers.

Evaluation of immunostaining

The specific cellular membranous, cytoplasmic or nuclear brown staining is considered to be positive immunoreactivity. Immunoreactivity to KAI1 and EMMPRIN was localised in the cytoplasm or membrane, but tenascin was mainly in the ECM and rarely in cancer cells of a few patients, and PTEN was in the nucleus. For KAI1, EMMPRIN and PTEN, 100 cells were randomly selected and counted from 5 representative fields of

each section. The positive percentage of counted cells was graded semiquantitatively into one of the four-tier scoring system: negative (–), 0–5%; weakly positive (+), 6–25%; moderately positive (++), 26–50%; and strongly positive (+++), 51–100%. For tenascin, the positive degree was evaluated according to the percentage of positive area in the hot field of tenascin: negative (–), 0–5%; weakly positive (+), 6–10%; moderately positive (++), 11–15%; and strongly positive (+++), 16–100%. The evaluation was performed blindly by two independent observers.

MVD counting

CD34 expression in the cytoplasm and membrane of vascular epithelial cells was selected for MVD counting, although it was also seldom localised in the tumour cells and fibroblasts. A modified Weidner's method was used to calculate the MVD of GIA by anti-CD34 immunostaining.¹³ The observers selected five such

Figure 3 Relationship between EMMPRIN (extracellular matrix metalloproteinase inducer) and tenascin expression in gastrointestinal adenocarcinomas (GIAs). EMMPRIN was expressed in the cytoplasm and membrane. The GIA with strong EMMPRIN expression (A) displayed low tenascin expression (C), whereas the GIA with weak EMMPRIN expression (B) showed high tenascin expression (D).

Figure 4 CD34-labelled microvessel in gastrointestinal adenocarcinomas (GIAs). CD34 distributed to cytoplasm and membrane (A–D). The GIAs with liver metastasis displayed higher microvessel density (B,D) compared with those that were not accompanied by any distal metastasis (A,C).

areas and counted individual microvessels on a $\times 400$ field ($0.1885 \text{ mm}^2/\text{field}$) after the area of highest neovascularisation was identified. Any brown-staining endothelial cell or endothelial cell cluster that was clearly separated from the adjacent microvessel, tumour cells and other connective tissue elements was considered to be a single, countable microvessel. The counts were performed independently by two investigators.

Statistical analysis

Statistical evaluation was performed using Spearman's correlation test to analyse the rank data and the Mann–Whitney U test or Kruskal–Wallis test to differentiate non-parametric means of different groups; $p < 0.05$ was considered as significant. SPSS V.10 software for Windows was used to analyse all data.

RESULTS

KAI1 displayed positive expression in superficially hyperplastic epithelium, mononuclear cells in the lymph follicle and

infiltrating inflammatory cells including macrophages in gastrointestinal mucosa (fig 1A,D). Table 1 shows KAI1 expression, which was higher in GIAs than in their adjacent non-cancerous mucosa ($p < 0.05$; fig 1A,B,D,E). KAI1 expression showed a significantly negative association with liver metastasis of GIA ($p < 0.05$; fig 1B,C,E,F), but not with depth of invasion, venous invasion or lymph node metastasis ($p > 0.05$; table 2). Tenascin was strongly expressed in the submucosal muscularis of stomach compared with the colorectum (fig 2A,E). Tenascin was mainly expressed in ECM (fig 2B,F) and seldom in cancer cells of some patients (fig. 2C,G). Tenascin expression was negatively correlated with liver metastasis and EMMPRIN expression of GIA ($p < 0.05$; figs 2 B–D,F,G and 3), but not with depth of invasion, venous invasion or lymph node metastasis ($p > 0.05$; table 3). MVD was positively correlated with depth of invasion, venous invasion, lymph node metastasis and liver metastasis of tumours ($p < 0.05$), but negatively with PTEN expression ($p < 0.05$; figs 4, 5 and table 4).

Figure 5 Relationship between PTEN (phosphatase and tensin homology deleted from human chromosome 10 expression and microvessel density (MVD) in gastrointestinal adenocarcinomas (GIAs). PTEN expression was located in the nucleus. The GIA with negative PTEN expression (A) displayed high MVD (C), whereas the GIA with strong PTEN expression (B) showed low MVD (D).

Table 4 Relationship between microvessel density and clinicopathological features of gastrointestinal adenocarcinomas

Clinicopathological features	n	MVD	p Value
Depth of invasion			<0.05
T _{is} -T ₁	46	38.8 (16.8)	
T ₂ -T ₄	163	47.0 (22.0)	
Venous invasion			<0.05
-	98	43.2 (23.0)	
+	111	46.9 (19.4)	
Lymph node metastasis			<0.05
-	96	41.1 (18.2)	
+	113	48.6 (23.0)	
Liver metastasis			<0.001
-	166	40.5 (17.8)	
+	43	63.0 (23.8)	
PTEN expression			<0.001
-	63	56.3 (26.0)	
+	30	40.8 (14.1)	
++	41	38.6 (16.8)	
+++	70	40.6 (17.0)	

MVD values are mean (SD).

MVD, microvessel density; T_{is}, carcinoma in situ; T₁, lamina propria and submucosa; T₂, muscularis propria and subserosa; T₃, exposure to serosa; T₄, invasion into serosa; -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

DISCUSSION

Cancer metastasis is a highly complex process that involves aberrations in gene expression by cancer cells, leading to transformation, growth, angiogenesis, invasion, dissemination, survival in the circulation, and subsequent attachment and growth in the organ of metastasis.¹⁴ However, comparatively¹⁵ little is known about the intricate pathways that govern the complex phenotypes associated with metastasis.

KAI1 belongs to a structurally distinct family of cell surface glycoproteins—that is, TM4SF—which has four hydrophobic transmembrane domains and one large extracellular N-glycosylated domain.¹⁶ KAI1 was suggested to function via cell-cell and cell-ECM interactions, thereby potentially influencing the ability of cancer cells to invade and metastasise. In this study, we found that KAI1 expression was distributed in the gastric hyperplastic gland and was increased in GIA. Maurer *et al*¹⁷ reported up-regulated expression of KAI1 in colon cancer at the level of both mRNA and protein. These results suggested that KAI1 might be involved in a physiological process in the gastrointestinal mucosa. One explanation of KAI1 overexpression is that mucosal epithelial cells undergo malignant transformation, with some changed biological events. The other explanation for KAI1 overexpression is up-regulation of transcriptional regulators of KAI1 in the cancer cells.

Additionally, a negative relationship between KAI1 expression and liver metastasis of GIA was found in our study, indicating that reduced KAI1 expression might underlie the molecular basis of liver metastasis of GIA and be regarded as a good marker to predict liver metastasis of GIA. As our results showed, KAI1 expression tended to be positively correlated with depth of invasion and venous invasion, but this relationship was not statistically significant. Taken together, we speculated that the GIA with down-regulated expression of KAI1 had the capacity to metastasise to the liver. In vitro studies have shown that reduced KAI1 expression was associated with altered adhesion to specific components of the ECM, such as fibronectin, reduced cell-cell interactions and

increased cell motility, leading to a more invasive and metastatic ability.⁴ Another report showed that KAI1 could induce apoptosis through reactive oxygen intermediates.⁷ Therefore, we might believe that the involvement of low KAI1 expression in liver metastasis of GIA was possibly attributable to reduced adhesion or apoptosis of cancer cells.

Dynamic and reciprocal communication between epithelial and stromal compartments is critical during cancer progression. Tenascin, an extracellular component, has a role in cell adhesion and mobility, and guidance along cell migration pathways, and may suppress tumour progression.¹⁸⁻¹⁹ In our study, tenascin immunoreactivity was detected in the ECM and submucosal muscularis, but undetectable in gastrointestinal non-cancerous epithelium, which was consistent with previous reports.¹⁹⁻²⁰ More tenascin expression was detected in the gastric submucosal muscularis and submucous area than the colorectal area, suggesting that gastric submucosal muscularis has greater ability to resist the invasion of early cancer.

In GIA, tenascin mainly existed in the ECM around cancer cells and only rarely in cancer cells of a few patients, as reported in breast cancer.²¹ It was suggested that tenascin in tumour tissue is synthesised by stromal fibroblasts, induced by the tumour cells. Therefore, tenascin might play a part in tumour propagation as a paracrine effect.²² In addition, we found that tenascin expression was negatively correlated with the liver metastasis of GIA. Some investigators reported that melanoma cells inoculated in tenascin knockout mice might facilitate invasion and metastasis, and lack of tenascin expression led to the high ability to invade and metastasise in malignancies.²²⁻²³ These findings indicated that low expression of tenascin in the liver metastasis of GIA was probably because cancer cells lost the defensive effect of a tenascin-deposited fibrous stroma on invasion and metastasis of tumours.

Angiogenesis facilitates metastasis by providing a mechanism to increase the likelihood of tumour cells entering the circulation and to provide nutrients and oxygen for growth at the metastatic site.²⁴ In cancer cells, overexpression of angiogenic factors (eg, VEGF) will lead to secretion into the ECM, stimulating the proliferation and mobility of vascular epithelial cells by a paracrine effect to promote tumour angiogenesis. High MVD in tumour tissue is not only an aggressive phenotype of malignancies but also largely contributes to the progression of tumours. We examined MVD in GIA and found that MVD was closely correlated with each step of haematogenous metastasis, including primary venous invasion and distal liver metastasis. These results suggested that angiogenesis was involved in invasion and metastasis by constructing a channel for cancer cells of GIA to metastasise distally, and MVD might be regarded as a good marker to predict invasion and metastasis of GIA, especially liver metastasis.

To investigate the regulatory factors of tenascin expression and angiogenesis, we examined EMMPRIN and PTEN expression in GIA. The results indicated that tenascin expression was inversely correlated with EMMPRIN expression. EMMPRIN was reported to act in a paracrine fashion on tumour stromal cells to synthesise MMP1, MMP2 and MMP3, which consequently contributed to tumour invasion and metastasis because MMPs could degrade the ECM of the tumour.¹²⁻²⁵ Taken together with our result, we speculated that EMMPRIN expression participated in tenascin degradation, which was closely linked to liver metastasis of GIA. Additionally, we found a negative relationship between PTEN expression and MVD in GIA. Several studies showed that PTEN could inhibit angiogenesis by: (1) lowering VEGF expression by decreasing expression of hypoxia-inducible factor-1 α , which is required for VEGF transactivation via binding to the VEGF promoter; (2) restraining activation of phosphatidylinositol-3-kinase, which

Take-home messages

- Up-regulated KAI1 expression may play an important role in malignant transformation of gastrointestinal epithelial cells.
- Reduced expression of KAI1 and tenascin might underlie the molecular basis of liver metastasis of GIA.
- Angiogenesis is a key event in the invasion and metastasis of GIA. Higher MVD in GIA with liver metastasis might be attributable to the lower PTEN expression.
- EMMPRIN expression might participate in the tenascin degradation, which was closely linked to liver metastasis of GIA.
- KAI1 and tenascin expression and microvessel density might be employed to indicate liver metastasis of GIA in clinicopathological practice.

is also targeted by the VEGF pathway.³⁻²⁶ In combination with our study, it was suggested that higher MVD in GIA with liver metastasis might be attributable to the lower PTEN expression in some way.

In summary, up-regulated KAI1 expression may play an important part in the tumorigenesis of GIA. Reduced expression of KAI1 and tenascin might underlie the molecular basis of liver metastasis of GIA. Angiogenesis is a key event in the invasion and metastasis of GIA. KAI1, tenascin and MVD may be good markers to predict liver metastasis of GIA in clinicopathological practice.

ACKNOWLEDGEMENTS

We thank Tokimasa Kumada and Hideki Hatta for their technical help and Yukari Inoue for her secretarial assistance.

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Funding: This work was partially supported by the Japanese Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research 14770072 and 15922084 and the 21st Century COE Program in Japan.

Competing interests: None.

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