Loss of KAI1 Expression in the Progression of Colorectal Cancer

Donald P. Lombardi, Joseph Geradts, Julie F. Foley, Chia Chiao, Pattie W. Lamb, and J. Carl Barrett

ABSTRACT

The transmembrane 4 superfamily member KAI1 (CD82) has been shown to inhibit pulmonary metastases in experimental metastasis models of prostate cancer and melanoma. KAI1 expression is decreased in the progression of common solid epithelial tumors of adulthood, including lung, prostate, breast, esophageal, gastric, pancreatic, and bladder cancers. The purpose of our study was to investigate KAI1 expression in the progression of human colorectal cancer. We first analyzed 20 colorectal cancer cell lines by immunoblot techniques. KAI1 was expressed heterogeneously, with the tumor cell lines having a more complex degree of glycosylation compared with that of the normal colonic tissue. KAI1 was highly expressed in the primary SW480 colon cancer cell line but was down-regulated 15-fold in the matched metastatic SW620 cell line. We also investigated KAI1 protein expression by immunohistochemistry in tissues from 84 patients with colorectal cancer. Each tissue section was assigned a KAI1 mean score (KMS) from 0 to 300 based on the product of the percentage of cells that stained for KAI1 and the intensity of the stain (1, 2, or 3). In 84 patients with colorectal cancer, KAI1 was expressed at high levels in normal colonic mucosa (KMS 226) but was expressed at lower levels in the primary tumors (KMS 65; P < 0.0001). In a subset of 12 patients with stage IV metastatic disease, we observed a progressive down-regulation of KAI1, from the normal adjacent colonic mucosa (KMS 193) to the primary tumor (KMS 72; P = 0.0001) to the liver metastasis (KMS 25; tumor compared with metastasis, P = 0.0135). We found no correlation between loss of KAI1 expression and stage of disease. In 10 patients, we also noted loss of KAI1 expression in the transition from normal colonic mucosa (KMS 237) to adenoma (KMS 174) to carcinoma (KMS 62; P < 0.0167 for all three comparisons). We conclude that the down-regulation of KAI1 occurs early in the progression of colorectal cancer.

INTRODUCTION

Colorectal cancer is the fourth most common cancer diagnosed and the second leading cause of cancer death in the United States (1). In 1999, there will be ~129,400 new cases (2). The majority of colorectal cancers arise from a series of somatic genetic changes (3) that involve activation of ras oncogenes and inactivation of tumor suppressor genes on chromosomes 5q (APC3), 17p (p53), and 18q (uncertain gene(s)). The delineation of the molecular genetic and biological changes that accompany the pathogenesis of colorectal cancer will hopefully improve patient outcome in the future. Unfortunately, the overwhelming majority of the 56,600 patients (2) estimated to die with colorectal cancer this year will die from metastatic disease (4). Unlike the molecular events described for the pathogenesis of primary colon carcinomas (3), the genes responsible for metastasis in these tumors have not been well characterized. The 5-year survival of stage IV colorectal cancer is <5% and has not changed in 50 years (4). However, because treatment of patients with 5-fluorouracil-based chemotherapy in stage III disease (that has spread to local-regional lymph nodes) improves survival (5), the identification of markers for potential metastatic disease in stages I–II that predict subsequent metastasis and poor outcome is crucial. These patients could be enrolled into clinical trials designed to ask whether adjuvant chemotherapy after surgery could improve survival.

Several candidate antimetastasis or anti-invasion genes have been studied in colorectal carcinoma, including nm23 (6), E-cadherin (7, 8), CD44 (9, 10), and others, but no consistent findings have been reported. For example, in separate studies, nm23 expression has been found to directly correlate (11), not to correlate (12, 13), or inversely correlate (14, 15) with metastatic potential in colorectal cancer. KAI1 was originally isolated as a gene that suppressed metastasis of rat prostate tumor cells to the lungs in an experimental metastasis assay (16). The isolation of this gene had its origin in the observation that the fusion of a tumorigenic, nonmetastatic cell to a tumorigenic, metastatic cell resulted in a somatic cell hybrid that was nonmetastatic (17, 18). This finding supported the view that metastasis is a recessive phenotype, and that genes in the nonmetastatic cell complement the loss of genes in the metastatic cell. Human chromosome 11 introduced by microcell-mediated chromosome transfer into a highly metastatic rat prostate cancer cell line suppressed lung metastases in a murine experimental metastasis model (19). KAI1 was positioned cloned at chromosome 11p11.2 and was also shown to suppress metastasis (16).

KAI1 is a member of the TM4SF, many of which, including KAI1, are CD antigens present on the surface of leukocytes (20). At least three TM4SF members are implicated in metastasis, including CD9/ MRP-1 (21), CD63/ME491 (22), and CD82/KAI1 (16). KAI1 mRNA is ubiquitously expressed, with abundant expression on the surface epithelium of the major epithelial tissues, including lung, breast, prostate, and gastrointestinal tract (23, 24). KAI1 and other TM4SF members have been demonstrated to bind to each other (20), integrins (25, 26), and E-cadherin (27). Recently, KAI1 has been shown to suppress invasion and motility and enhance homotypic cell adhesion in human colon cancer cell lines (28). Therefore, KAI1 may associate with other TM4SF members, integrins, E-cadherin, and other surface molecules to relay extracellular signals to signal transduction pathways that are important in cellular adhesion, invasion, motility, and metastasis suppression.

In this study, we hypothesized that KAI1 protein expression would be down-regulated in the progression of colorectal cancer. We analyzed 20 tumor cell lines and 84 patient samples that represented all four stages of disease. We also examined a subset of 12 stage IV patients that had procured samples from normal adjacent colonic mucosa, primary colorectal tumors, and metastatic lesions in the liver to assess KAI1 expression in the progression to metastatic disease.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The RKO cell line was a kind gift from Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD).
NIEHS. centrifuged at 4°C for 5 min at 1200 rpm. The supernatant was aspirated, and five ml of cold PBS were then added to the monolayer, and the cells were reported previously (29). Cell monolayers were washed twice in cold PBS.

The Western blot protocol was based on methods previously published (29). Protein cell lysates were prepared from cell lines, size-fractionated by SDS-PAGE, transferred to nylon membranes, incubated with C33 KAI1 or actin antibodies, and exposed to film.

The other cell lines (DLD-1, HCT-15, HT-29, WiDr, SW48, SW480, SW1116, HCTT16, LS180, LS174T, SW620, LoVo, T84, SNU-C2A, SNU-C2B, NCI-H716, NCI-H747, SW837, and SW1463) were obtained from American Type Culture Collection (Manassas, VA). Cultured cells were grown in media and conditions according to the recommendations of American Type Culture Collection. RKO was grown in DMEM/F12/10% fetal bovine serum. All cells were derived from human tumors and were maintained in a 37°C humidified incubator. The cell lines were grown in the presence of 5% CO2, except those (SW48, SW480, SW1116, SW620, SW837, and SW1463) that were grown in Leibovitz’s L15 medium, which specifically required incubation in a CO2-free environment. All cells tested negative for Mycoplasma contamination at the NIHES.

Immunoblot Analysis. The Western blot protocol was based on methods reported previously (29). Cell monolayers were washed twice in cold PBS. Five ml of cold PBS were then added to the monolayer, and the cells were scraped into a 15-ml conical tube (Sarstedt, Inc., Newton, NC). The cells were centrifuged at 4°C for 5 min at 1200 rpm. The supernatant was aspirated, and the pellet was gently vortexed to briefly disperse the cells. Cell proteins were solubilized in 150–400 μl of lysis buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 3 mM MgCl2, 0.5% NP-40, and 2 mM phenylmethylsulfonyl fluoride] for 10 min on ice. The lysates were then centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was removed, and the protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). An equal volume of Laemmli’s sample buffer without 2-mercaptoethanol was added to the soluble protein and boiled for 5 min. Twenty μg of cellular protein were size fractionated by 17.5% SDS-PAGE, transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA), and incubated with KAI1 C33 hybridoma supernatant (1:100 dilution; a kind gift from Dr. Osamu Yoshie, Shionogi Institute for Medical Science, Osaka, Japan). Bound antibody was measured by the ECL Western blotting analysis system (Amersham Life Sciences, Buckinghamshire, England).

To ensure equal loading of protein, actin control experiments were performed as above, with the following modifications. Solubilized proteins were mixed with an equal volume of Laemmli’s sample buffer supplemented with 10 mM DTT. The proteins were separated on a 10% SDS-PAGE gel, transferred to a nylon membrane, and incubated with an antihuman actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO).

We compared the relative amount of KAI1 protein expression on Western blots by densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA). For each cell line, the density of the KAI1 band was divided by the density of the actin band. NCI-H716 had the lowest quotient (0.0085), and therefore all other cell line quotients were divided by 0.0085 for normalization to NCI-H716.

Patient Samples. The patient samples were obtained from Dr. Stanley R. Hamilton (The Johns Hopkins Hospital, Baltimore, MD), who procured the specimens from 1985 to 1995. “Normal” colon mucosa adjacent to the primary colon tumor, primary tumors, and liver metastases were immediately snap frozen after surgical resection. Tissue procurement was approved by the Joint Committee on Clinical Investigation and the Institutional Review Board.

Immunohistochemistry. Frozen human colon tissue was sectioned and fixed in Rapid Fixx (Shandon-Lipshaw, Pittsburgh, PA) for 7 s at room temperature. Active ingredients in Rapid Fixx include methanol (75%), formic acid (20%), and glacial acetic acid (5%). The slides were immersed in 1× AB (Biomed, Foster City, CA) until all sections were cut. After the last section was cut, the slides remained in buffer for 5 min and were washed again in another 5 min 1× AB wash. Endogenous peroxidases were blocked by incubating with 0.3% H2O2 for 30 min at room temperature. The sections were subsequently blocked with 5% normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature.

Endogenous avidin and biotin were blocked by tandem 15-min incubations, with the samples washed with 1× AB between reactions. All incubations were carried out in a humidified chamber for 30 min (excluding the primary antibody) at room temperature using an indirect IHC staining procedure (30). Localization of KAI1 protein expression was investigated using a monoclonal antibody against KAI1 (C33; a kind gift from Dr. Yoshie). The KAI1 and normal mouse serum antibodies were diluted with 1% BSA (Sigma) in 1× AB. KAI1 staining was completed using a mouse IgG kit containing the block, as well as secondary and label antibodies, according to the manufacturer’s instructions (Vectastain Mouse IgG ABC Elite kit; Vector Laboratories). Mouse anti-KAI1 antibody was applied at a dilution of 1:100 for 1 h. Normal mouse serum, at the same concentration of the primary antibody, was used as the negative control in place of KAI1. Visualization of the antibody complex was completed using a 10-μg diaminobenzidine tablet (Sigma) dissolved in 20 ml of 1× AB, containing 12 ml of 30% H2O2 for 6 min in the dark. Slides were then rinsed in running tap water, counterstained with Harris hematoxylin (Harelco, Gibbstown, NJ), dehydrated through a series of graded alcohols to xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

The slides were scored for KAI1 protein expression by D. P. L. and J. G., who were blinded to the clinical and pathological stage of the patients. We determined the KAI1 score by estimating the percentage of cells that had membrane staining for KAI1 and multiplying by the assessment of the intensity of the stain on a 1+ to 4+ scale. The theoretical limits of the scores ranged from 0 (0% of cells staining) to 300 (100% of the cells staining at 3+ intensity).

Statistical Analysis. SAS JMP software (version 3.2.2, 1997) was used to perform the calculations (SAS Institute Inc., Cary, NC). For all analyses, the Shapiro-Wilk W test was performed to examine whether the data were normally distributed. The comparison of relative KAI1 expression of the MMR-deficient and wild-type cell lines was determined by the Wilcoxon rank-sum test, and allowing the SDs to be unequal, was adjusted with the Welch ANOVA test. Student’s t test was used to compare the KMS of the normal colonic mucosa and the tumors of the 84 patients. To compare the KMS of the normal mucosa, tumor, and differences between the normal and tumor, we used the Wilcoxon rank-sum, the Welch ANOVA, and the Bonferroni procedures. We made six comparisons between stages I through IV and
Therefore set $\alpha = 0.05/6 = 0.0083$. We analyzed the question of whether the tumor stage was associated with increasing KMS by the Wilcoxon rank-sum and the Welch ANOVA tests. Each of the 84 patients had a normal adjacent colonic mucosa sample as a matched control tissue to the tumor sample. The difference between the KMS of the normal mucosa and the tumor was calculated and declared to be significant if it was different from 0. In the normal-adenoma-carcinoma sequence, we set the $\alpha = 0.05/3$ comparisons = 0.0167 and used the Tukey HSD procedure.

RESULTS

KAI1 Expression in Human Colorectal Cancer Cell Lines. We first examined whether KAI1 protein expression was decreased in colorectal metastasis by analyzing normal colon tissue (Fig. 1A, Lane 1) and a series of primary (Fig. 1A, Lanes 2–12) and metastatic (Fig. 1B, Lanes 2–4, 7, and 8) colon carcinoma cell lines by immunoblot analysis. A summary of the Western blot data is provided in Table 1, with a brief clinical history of the patients from whom the cell lines were derived. Relative KAI1 expression was calculated as the ratio of the densitometry of the KAI1 band:actin band on Western blot (Fig. 1); lowest ratio was calculated in NCI-H716 (0.0085). Other cell lines were normalized to NCI-H716 by dividing their respective ratios by 0.0085.

<table>
<thead>
<tr>
<th>Cell line origin</th>
<th>Carcinoma cell line</th>
<th>Relative KAI1 expression</th>
<th>APC</th>
<th>p53</th>
<th>MMR</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary colon</td>
<td>DLD-1</td>
<td>16</td>
<td>mut</td>
<td>mut</td>
<td>mut</td>
<td>hMSH6</td>
</tr>
<tr>
<td></td>
<td>HCT 15</td>
<td>10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>hMSH6</td>
</tr>
<tr>
<td></td>
<td>HT 29</td>
<td>683</td>
<td>mut</td>
<td>mut</td>
<td>mut</td>
<td>wt</td>
</tr>
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<td></td>
<td>WiDr</td>
<td>463</td>
<td>--</td>
<td>mut</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td></td>
<td>SW 48</td>
<td>46</td>
<td>wt*</td>
<td>--</td>
<td>--</td>
<td>hMLH1</td>
</tr>
<tr>
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<td>SW 480</td>
<td>587</td>
<td>mut</td>
<td>mut</td>
<td>mut</td>
<td>wt</td>
</tr>
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<td></td>
<td>SW 1116</td>
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<td>mut</td>
<td>wt</td>
<td>--</td>
<td>wt</td>
</tr>
<tr>
<td></td>
<td>RKO</td>
<td>17</td>
<td>wt*</td>
<td>wt</td>
<td>RER</td>
<td>wt</td>
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<tr>
<td>Metastatic colon</td>
<td>HCT 116</td>
<td>20</td>
<td>wt</td>
<td>--</td>
<td>--</td>
<td>hMLH1</td>
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<td></td>
<td>LS180</td>
<td>44</td>
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<td>--</td>
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<td>LS174T</td>
<td>38</td>
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<td>RER</td>
<td>+</td>
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<tr>
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<td>SW 620</td>
<td>39</td>
<td>mut</td>
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<td>wt</td>
</tr>
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<td></td>
<td>LoVo</td>
<td>28</td>
<td>mut</td>
<td>wt</td>
<td>hMSH2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T84</td>
<td>153</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>Lung nodule</td>
</tr>
<tr>
<td>Metastatic cecum</td>
<td>SNU-C2A</td>
<td>370</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>43-yr Korean F; grade II; 3rd passage nude mouse xenograft; loosely adherent and floating aggregates in vitro</td>
</tr>
<tr>
<td></td>
<td>SNU-C2B</td>
<td>515</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>Same patient as SNU-C2A; 4th passage nude mouse xenograft; adherent monolayer in culture</td>
</tr>
<tr>
<td></td>
<td>NCI-H716</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>33-yr M with poorly differentiated tumor; isolated from ascites after treatment with 5-fluorouracil; floating aggregates in vitro</td>
</tr>
<tr>
<td>Primary rectum</td>
<td>SW 837</td>
<td>272</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>69-yr M with grade II tumor; isolated from a common bile duct lymph node</td>
</tr>
<tr>
<td></td>
<td>SW 1463</td>
<td>767</td>
<td>mut</td>
<td>--</td>
<td>--</td>
<td>wt</td>
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</tbody>
</table>

Relative KAI1 expression = densitometry of KAI1 band:actin band by Western blot (Fig. 1); lowest ratio was calculated in NCI-H716 (0.0085). Other cell lines were normalized to NCI-H716 by dividing their respective ratios by 0.0085.

* Relative KAI1 expression = densitometry of KAI1 band:actin band by Western blot (Fig. 1); lowest ratio was calculated in NCI-H716 (0.0085). Other cell lines were normalized to NCI-H716 by dividing their respective ratios by 0.0085.

** mut, mutation; wt, wild type; --, unknown.

Data from Dr. Eric R. Fearon, personal communication.

We next examined whether KAI1 was decreased in colon cancer metastasis by analyzing the SW480 and SW620 cell lines. SW480 was isolated from a high-grade primary colon tumor, and SW620 was isolated from a metastatic lymph node from the same patient 1 year later at the time of clinical relapse. KAI1 protein expression was high in SW480 but reduced 15-fold in SW620 (Fig. 1B, Lanes 1 and 2; Table 1). Two other colon cancer cell lines derived from metastatic lesions, LoVo and T84, exhibited low and intermediate expression, respectively (Fig. 1B, Lanes 3 and 4; Table 1).

The structure of KAI1 suggests a role in cellular adhesion (16); therefore, we compared protein expression in a pair of cell lines that differ in their adhesion properties (Fig. 1B, Lanes 5 and 6). The SNU-C2A and SNU-C2B cell lines were established from the same patient, but the former grows as floating aggregates in serum-free medium, whereas the latter grows as an adherent monolayer in serum-containing medium in cell culture. Although the KAI1 expression is comparatively similar in both lines, the glycosylation pattern of SNU-C2A contains higher molecular weight species of KAI1 protein relative to that of SNU-C2B.

The metastatic cecum cell lines differed in their levels of KAI1 expression. NCI-H716, a 5-fluorouracil-resistant cell line isolated from ascites, expressed a low level of KAI1 and grew as floating aggregates in suspension (Fig. 1B, Lane 7; Table 1). NCI-H747 is an adherent cell line isolated from a patient with a metastatic common bile duct lymph node and expressed a moderate amount of KAI1 (Fig. 1B, Lane 8; Table 1). The rectal carcinoma cell lines SW837 and SW1463 are both high-grade tumors and expressed abundant levels of KAI1 (Fig. 1B, Lanes 9 and 10; Table 1).
Low KAI1 Expression Is Associated with MMR Status in Several Colorectal Cancer Cell Lines. Several key genes are lost or mutated in the progression of colorectal cancer; therefore, we next compared KAI1 expression with the genotype of the APC, p53, and DNA MMR genes (Table 1). APC mutations are common events in these cell lines, but no relationship was found between KAI1 expression and APC mutations. APC mutations exist in DLD-1 (31), HT-29 (32), SW480 (33), SW620 (34), and LoVo (35, 36), but KAI1 protein expression varied considerably in these cell lines (Table 1; Fig. 1). SW48, RKO, HCT116 (31, 37), and LS174T (34) are all wild type for APC but express low amounts of KAI1.

Recently, investigators discovered a putative p53 binding site within the promoter region of KAI1 and suggested that p53 directly activates the expression of KAI1 (38). We found no relationship between KAI1 expression and p53 mutation status in our colorectal cancer cell lines. For example, SW48, RKO, HCT 116, LS174T (39), and LoVo (36) are wild type for p53 but expressed little KAI1 protein. Mutant p53 cell lines were associated with both high KAI1 expression (HT-29, WiDr (39, 40), SW480 (39, 41), SW1116, SW837 (39), and low KAI1 expression [DLD-1 (39) and SW620 (39); Table 1].

However, we did observe a correlation between loss of KAI1 expression and the presence of MMR mutations. In the MMR-deficient (MMR−) or replicative error-positive cell lines DLD-1 (42), HCT-15 (43), SW48 (44), RKO, HCT116 (43), LS180, LS174T, and LoVo (44, 45), KAI1 expression was low (median relative KAI1 expression, 19). In contrast, in the MMR wild-type cell lines HT29, SW480, SW1116, SW837, and SW1463, KAI1 was highly expressed (median relative KAI1 expression, 587; P = 0.003; Wilcoxon rank-sum; Welch ANOVA testing means equal, allowing SDs unequal: P = 0.04).

KAI1 Is Down-Regulated in the Progression of Stage IV Colorectal Cancer Patients. Because we found that KAI1 expression is lower in the metastatic SW620 compared with that in the primary SW480 cell line, we next asked whether KAI1 was decreased in the progression to metastatic disease in colorectal cancer patients. In Fig. 2, we show KAI1 IHC in one representative patient with stage IV disease in which we have samples from the “normal” adjacent colonic mucosa, primary colon tumor, and colon metastasis in the liver. KAI1 was strongly expressed in the normal colonic epithelium (Fig. 2A), lymphoid aggregates, and tissue macrophages (Fig. 3B). Because KAI1 is expressed in activated B and T lymphocytes and macrophages (46, 47), its expression in lymph nodes and tissue macrophages was used as an internal positive control in the patient samples. Normal mouse serum was substituted for the C33 KAI1 antibody in the protocol to serve as a negative control (Fig. 2B).

Although KAI1 was strongly expressed in the normal mucosa, it was expressed heterogeneously in the primary tumor (Fig. 2, C–E), with both KAI1-positive (Fig. 2D) and KAI1-negative (Fig. 2E) portions of the tumor. In sections from the colon tumor metastasis in the liver, the hepatocytes and sinusoids were KAI1 positive, but the tumor KAI1 expression was clearly down-regulated (Fig. 2F). In the majority of the metastatic tumor, KAI1 expression was absent (Fig. 2G).

We analyzed 12 stage IV patients and found that KAI1 was down-regulated in all patient samples, in both the primary tumors and in the hepatic metastases. We were able to quantify KAI1 protein by using IHC to calculate the product of the intensity of the stain by the

Fig. 2. Immunohistochemical detection of KAI1 in samples from a stage IV colon cancer patient. Frozen sections were incubated with the C33 mAb (A and C–G) or normal mouse serum control antibody (B). A, normal colonic epithelium, strongly KAI1 positive; B, normal colonic epithelium, normal mouse serum control; C–E, primary colon tumor; C, heterogeneous (KAI1+ and − portions); D, KAI1+ portion; E, KAI1− portion (note KAI1+ tumor stroma); F, metastatic colon tumor in liver; KAI1+ hepatocytes and sinusoids; mainly KAI1− tumor; G, metastatic colon tumor in liver, KAI1−, which represented 60% of the tumor specimen.
percentage of cells that had membrane staining (see “Materials and Methods”). In Table 2, we present the KAI1 IHC scores for the normal colon, primary tumor, and liver metastasis in terms of the mean score, SD, and SE. The difference between the mean normal colon and the mean tumor score \((P < 0.0001)\), as well as that between the mean tumor score and the mean metastasis score, was significant \((P < 0.01)\). Therefore, the loss of KAI1 expression is associated with both tumor formation and the development of liver metastasis.

**Table 2** KAI1 IHC scores in 12 stage IV colorectal cancer patients

<table>
<thead>
<tr>
<th>Tissue of origin</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colon</td>
<td>193</td>
<td>51</td>
<td>15</td>
</tr>
<tr>
<td>Primary tumor</td>
<td>72</td>
<td>48</td>
<td>14</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>25</td>
<td>43</td>
<td>12</td>
</tr>
</tbody>
</table>

*KAI1 IHC scores = (% of cells KAI1+) \times (intensity of stain: 1, 2, or 3).

Comparison of normal colon and primary tumor; \(P = 0.0001\).

Comparison of primary tumor and liver metastasis; \(P = 0.01\).

**KAI1 Expression Is Reduced in Early and Late Stage Colorectal Cancer.** Having demonstrated that KAI1 is down-regulated in the progression of colorectal cancer in stage IV patients, we next analyzed 84 patients from stages I to IV to determine whether the down-regulation was related to stage progression and metastatic potential (Table 3; Fig. 4). Essentially all of the patient samples had lower KAI1 expression in the primary tumor compared with the normal colon (82 of 84; 98%). The only two cases that did not show a difference between the normal and the tumor scores were two stage I patients. These two patients account for the high end of the range of scores in stage I tumor scores (Fig. 4).

The overall mean KAI1 score for the normal mucosas of the 84 patients was 226 (SD, 46; SE, 5), compared with 65 (SD, 61; SE, 7) for the primary tumors \((P < 0.0001; \text{Student's} \ t \text{ test})\). Analyzing the data for all four stages, we observed that the normal and tumor KAI1 scores overlapped, but the means and medians were clearly different (all \(P < 0.0001\); Wilcoxon rank-sum test; Table 3; Fig. 4).

In Table 3 and Fig. 4, we note that there is an apparent trend for the

**Table 3** Stage distribution of KAI1 IHC scores in 84 patients with colorectal cancer

<table>
<thead>
<tr>
<th>Stage I ((n = 19))</th>
<th>Stage II ((n = 27))</th>
<th>Stage III ((n = 15))</th>
<th>Stage IV ((n = 23))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Normal</td>
<td>250</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>Tumor</td>
<td>92</td>
<td>94</td>
<td>21</td>
</tr>
</tbody>
</table>

*Includes the 12 stage IV patients from Table 2. Only the normal and primary tumor KAI1 scores from these patients are included here. Difference = KMS (normal colon – tumor).*

Fig. 3. Immunohistochemical detection of KAI1 in a stage III colon cancer patient with normal colonic epithelium-adenoma-carcinoma sequence. C33 KAI1 mAb. A, nonmalignant colonic mucosa; adenoma; carcinoma (note KAI1+ epithelial cells in normal > adenoma > carcinoma); B, normal colonic mucosa adjacent to the tumor (note KAI1+ colonic epithelium, tissue macrophages, and lymphoid follicle); C, adenoma (note heterogeneous KAI1 expression that is greater in the upper left and decreases in the lower right of the panel; D, carcinoma (note KAI1– epithelia and KAI1+ tumor stroma).
normal KAI1 scores to decrease, as the stage increased from I to IV
\((P = 0.0052, \text{Wilcoxon rank sum}; \text{Welch ANOVA testing means}
\text{equal, allowing SDs unequal: } P = 0.006)\). However, only stage I was
statistically different from stage IV (Bonferroni \(i\) test, \(P < 0.0083\)).
We did not observe an inverse relationship between increased tumor
stage and decreased tumor mean KAI1 score \((P = 0.16, \text{Wilcoxon}
\text{rank sum test}; \text{Welch ANOVA testing means equal, allowing SDs not}
\text{equal: } P = 0.13\)).

The use of normal controls allowed us to calculate the differences
between the scores of the normal mucosa and the tumors for each
patient. Although we found that the differences were significant for
each stage \((P < 0.0083)\), we did not observe increased differences as a
function of increasing stage \((\text{for } \alpha = 0.05/6 = 0.0083; \ P = 0.07, \text{Wilcoxon}
\text{rank sum test}; \text{Welch ANOVA, } P = 0.05; \text{both not significant)}\).

Reduced KAI1 Expression in the Normal Colonic Epithelium-
Adenoma-Carcinoma Sequence. We had originally hypothesized
that the loss of KAI1 expression would be related to increasing stage.
With evidence that loss of KAI1 occurred in stage I patient
samples, we then asked whether KAI1 was down-regulated in precur-
sor lesions (adenomas). In 10 of 84 colorectal carcinoma specimens,
we noted the presence of adenomas admixed with the primary tumors.
The “normal” colonic epithelium, adenoma, and carcinoma varied in
degree of KAI1 immunostain as shown in Fig. 3 for one representative
patient. The normal colonic epithelium stained strongly in nearly all
cells (Fig. 3B), but the adenoma had heterogeneous staining with
some KAI1 protein loss (Fig. 3C), whereas the carcinoma was absent
for KAI1 expression (Fig. 3D). We determined the KAI1 IHC scores
in the 10 patients and found that KAI1 expression was decreased in the
progression from normal colon to adenoma to carcinoma (Table 4).

DISCUSSION

In this study, the progression of colorectal cancer was associated
with decreased expression of KAI1 [Tables 1–4; Fig. 1B (Lanes 1 and
2), 2–4]. KAI1 was consistently and highly expressed in normal
colonic epithelium, was down-regulated in nearly all of the primary
tumors, and was further down-regulated in hepatic metastases. There-
fore, colorectal cancer joins a growing list of cancers in which the
down-regulation of KAI1 is associated with tumor progression, in-
cluding non-small cell lung (48), prostate (49), breast (50), esophageal
(51), gastric (52), hepatocellular (53), pancreas (54), and bladder (55)
cancers. However, KAI1 expression is down-regulated to the same
extent in stages I–IV colorectal cancer (Table 3; Fig. 4), where the
metastatic potential varies from <10% in stage I to 100% in stage IV
disease. Because KAI1 loss of expression is not associated with
increased stage, KAI1 loss of expression is unlikely to predict metas-
tasis and poor clinical outcome in colorectal cancer patients. In
comparison, the loss of KAI1 expression is correlated with increased
tumor stage, lymph node involvement, distant metastasis (54), and
poor survival (56) in pancreatic cancer. Therefore, KAI1 loss of
expression is an early event in colorectal cancer and may occur later
in lung, breast, and pancreatic cancer.

We found that KAI1 expression is decreased at several points along
the progression of colorectal cancer. In all 10 cases in which adenom-
as were associated with carcinomas, KAI1 was abundantly ex-
pressed in the normal colonic epithelium but was down-regulated in
the adenomas and further down-regulated in the carcinomas (Fig. 3;
Table 4). E-cadherin, located at intercellular adherens junctions and
involved in homotypic adhesion and the suppression of tumor inva-
sion, was also demonstrated to have reduced expression in the tran-
sition from colonic adenoma to carcinoma (57). Furthermore, loss of
E-cadherin expression was associated with increased degree of
dysplasia in adenomas. Because KAI1 and E-cadherin physically
interact (27), the down-regulation of KAI1/E-cadherin/catenin com-
plex may be important in the progression of colorectal cancer. We
recently observed loss of KAI1 protein expression in a subset of
high-grade squamous intraepithelial lesions of the cervix (58). There-
fore, the loss of cell adhesion molecules, such as E-cadherin and
KAI1, may occur in the progression from normal epithelium to
premalignant lesion to carcinoma in a variety of malignant diseases.

However, the down-regulation of KAI1 is not limited to only the early
stages of progression. In this study, the 12 cases with hepatic
metastases had a median KAI1 score of 7.0, whereas five cases had no
KAI1 staining in the colon metastases to the liver (Fig. 4). Therefore,
although most of the KAI1 loss of expression seems to occur early in
colorectal cancer progression, further down-regulation of KAI1 and/or
selection of low or absent KAI1 expresser colon carcinoma cells
occurs along the progression of colorectal cancer to liver metastases
(Tables 2–4).

We observed that KAI1 protein expression in normal colonic mu-
cosa adjacent to the tumor was less in stage IV than in stage I
colorectal cancer. We speculate that the low KAI1 expression may be
the result of paracrine factors produced by the tumor that not only
decrease KAI1 expression in the tumor but also act on nonmalignant
mucosa to down-regulate KAI1 expression. This concept will need to
be tested in controlled experiments.

The membrane localization of KAI1, as well as its extensive gly-
cosylation, suggests a role in cellular adhesion. In transfection studies,
KAI1 enhanced the calcium-independent aggregation of colon carci-
noma cell lines \textit{in vitro} (28). We studied the nonadherent SNU-C2A
and the adherent SNU-C2B by Western blot to compare KAI1 protein
levels and glycosylation patterns (Table 1; Fig. 1B, Lanes 5 and 6).
We found that although the protein levels were comparable, the

<table>
<thead>
<tr>
<th>Tissue of origin</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colon</td>
<td>237\textsuperscript{a}</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>Adenoma</td>
<td>174\textsuperscript{b}</td>
<td>51</td>
<td>16</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>62\textsuperscript{c}</td>
<td>50</td>
<td>16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Comparison 1: normal colon and adenoma.
\textsuperscript{b} Comparison 2: adenoma and carcinoma.
\textsuperscript{c} Comparison 3: normal colon and carcinoma. \(P < 0.0167\) for all three comparisons, Tukey HSD procedure.
glycosylation pattern of SNU-C2A had higher molecular weight species than that of SNU-C2B. Structural changes in oligosaccharide side chains of glycoproteins are associated with the progression toward invasive and metastatic phenotypes in tumors (59). In a study of colorectal carcinoma patients, β1,6-branched oligosaccharides were associated with lymph node status and was an independent prognostic indicator for tumor recurrence and overall survival (60). The tumor cell lines had more complex glycosylation patterns (Fig. 1) relative to that of normal colon tissue (Fig. 1A, Lane 1), and we speculate that these changes may be important in tumor development and metastasis. KAI1 protein expression was down-regulated 15-fold in the metastatic cell line SW620, compared with that of the primary SW480 cell line (Fig. 1B). Furthermore, the highly variable glycosylation pattern of SW480 contrasts dramatically with the focused glycosylated bands of \( M_r \approx 46,000 \) in SW620. Recently, a study with a Chinese hamster ovary mutant cell line 1d1D deficient in UDP-Glc 4-epimerase that was transfected with either KAI1 or CD9 and grown in the presence of galactose supplemented medium was shown to inhibit cellular motility and cause massive cell death after a latent period (61). Additional work is necessary to clarify the role of posttranslational modifications, such as glycosylation, of KAI1 in tumor cells.

In the colorectal cancer cell lines studied, we noted a correlation between low KAI1 protein expression and the replicative error or MMR-deficient phenotype (Table 1). The functional significance for the association is not immediately apparent, because KAI1 is not commonly mutated in prostate cancer (49). The mutation frequency of KAI1 is not known in colorectal cancer. The patient database presented in this study has not been characterized for MMR status at this time. To ask whether MMR genes directly regulate KAI1 expression, we are studying KAI1 expression in several cell lines that contain wild-type MMR genes by either chromosome transfer or transfection studies.

Masih et al. (38) recently found a putative p53 consensus-binding site within the promoter region of KAI1 and demonstrated that p53 directly activated KAI1 expression in prostate carcinoma cells. In 177 prostate cancer patients, they observed a direct correlation between p53 and KAI1 expression in formaldehyde-fixed, paraffin-embedded sections in much less sensitive than that observed in frozen tissues. We have recently improved the detection in archival tissues using the C33 mAb in squamous and lymphoid neoplasms (58), but to our knowledge no progress has been made in prostate cancer. We did not observe a relationship between p53 status and KAI1 protein expression in the colorectal cancer cell lines studied (Table 1). Wild-type p53 cell lines SW48, RKO, HCT116, LS174T, and LoVo are all low KAI1 expressers. Mutant p53 cell lines demonstrated both high and low KAI1 expression.

The potential shortcomings of this work include the difficulty of quantifying KAI1 protein expression by IHC and the lack of survival data in the patient group. IHC is the preferred method for detecting KAI1 because the gene is expressed in stromal elements such as lymphoid follicles, fibroblasts, and tissue macrophages. We tried to be quantitative by calculating the KAI1 scores by multiplying the percentage of cells stained by the intensity of the stain. Although these quantitative by calculating the KAI1 scores by multiplying the percentage of cells stained by the intensity of the stain. Although these quantitative scores by multiplying the percentage of cells stained by the intensity of the stain.

with which the KAI1 tumor scores could be compared. The patient database is not mature enough to provide survival information, but we do not anticipate that KAI1 expression will have prognostic significance, because it is lost to the same degree in all four stages of colorectal cancer in the patient population studied.

In conclusion, we found that the progression of colorectal cancer, from the nonmalignant, “normal” adjacent colonic epithelium, to the premalignant adenoma, to the primary tumor, to the liver metastasis was associated with the progressive loss of KAI1 protein expression. The loss of KAI1 expression was not correlated with higher stage, a surrogate marker for metastatic potential. The down-regulation of KAI1 that is observed in “normal” colonic mucosa adjacent to the primary tumors in stage IV disease and in adenomas demonstrates that loss of KAI1 expression occurs earlier in cancer progression than understood previously. The selection of cells that have the ability to spread from the primary tumor to the liver metastasis may favor those cells that have lost KAI1 expression. Those cells would be expected to be less adhesive, more invasive, and more motile (28), three characteristics that are necessary for metastasis.

ACKNOWLEDGMENTS

This work is dedicated to Dr. Harold Amos (Harvard Medical School, Boston, MA) by D. P. L. We are indebted to Dr. Stanley R. Hamilton (M. D. Anderson Cancer Center, Houston, TX) for providing the colorectal samples. We are grateful to Dr. E. Fearon (University of Michigan, Ann Arbor, MI) for sharing data in Table 1. We appreciate the comments of Drs. J. Risinger, P. Blackshear, and C. Afshari (NIHES) on the manuscript. We had expert biostatistics support by Dr. Paul Stewart at the University of North Carolina (Chapel Hill, NC). We appreciate the work of C. Rahj Robinson and Kathy Romans in the GI Pathology Tissue Procurement Division at Johns Hopkins Hospital (Baltimore, MD). J. Fleming (NIEHS) provided excellent computer support.

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Loss of KAI1 Expression in the Progression of Colorectal Cancer

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