KAII Is Unchanged in Metastatic and Nonmetastatic Esophageal and Gastric Cancers


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ABSTRACT

Down-regulation of KAII mRNA expression has been shown to be associated with the formation of metastases or disease progression in pancreatic cancer. Whether KAII possesses similar characteristics in other malignancies of the gastrointestinal tract is not known.

Here, we compared the patterns of KAII mRNA expression in 41 esophageal cancers and 35 stomach cancers to assess whether KAII might also be of biological relevance in the metastatic ability of these tumors. By Northern blot analysis, KAII mRNA levels ranged widely in both normal and cancerous esophageal and gastric tissue samples, with no significant differences. No association between KAII mRNA expression and tumor stage or tumor differentiation was seen in these tumors. In addition, KAII mRNA expression was similar in primary esophageal and gastric cancer samples with or without metastases. By in situ hybridization, KAII mRNA expression was evident in the cytoplasm of most squamous epithelial cells in the normal esophagus and in nonmucosal epithelial cells of the normal stomach. The staining intensity in the esophageal and gastric cancer cells was similar to that in the normal controls.

This differential pattern of KAII mRNA expression in esophageal and gastric cancers in comparison to pancreatic cancer indicates that KAII seems to influence the potential of gastrointestinal cancer cells to metastasize differently. In esophageal and gastric cancers, the formation of metastases is not dependent on the reduction of KAII in the cancer cells.

INTRODUCTION

The esophagus and the stomach are major sites of human cancers (1). The high mortality associated with esophageal and gastric cancers stems, in part, from the fact that many tumors are not detected until the disease has progressed to an advanced stage in which lymph node or distant metastases are already present. Even when the primary tumor is resectable, surgery alone is often unable to provide a cure in these tumor stages. Therefore, the overall 5-year survival rates for patients with esophageal cancer and gastric cancer who undergo resection are still unsatisfactory (2, 3). Standard oncological regimens such as chemotherapeutic agents and radiotherapy have not had a major impact on the prognosis of these malignancies to date, and therefore, new approaches, including early diagnosis, preventive strategies, and innovative treatment, are still sorely needed.

In the past few years, many biological studies have analyzed genetic and molecular events occurring in cancers to identify genes that are involved in their initiation, progression, and suppression. The search for genes promoting or suppressing tumor spread has led to the identification of several factors that are associated with metastases (4, 5). nm23-H1, which is located on the long arm of chromosome 17, has been proposed as a metastasis suppressor gene. Down-regulation of its expression during tumor development and progression has been reported in several in vitro and in vivo systems (6, 7). Recent molecular cloning experiments identified a new gene named KAII, which suppresses the ability of prostate cancer cells to metastasize (4). The KAII gene belongs to a structurally distinct family of cell surface membrane glycoproteins (8–14). These glycoproteins have a similar structure, with integral membrane proteins consisting of four transmembrane domains and one large extracellular domain, which is N-glycosylated. Recent studies indicate that members of this gene family may influence the growth behaviors of cancer cells. However, their physiological and pathological functions are generally unknown.

Recently, we reported enhanced KAII mRNA expression in early-stage primary pancreatic cancers but decreasing levels in primary pancreatic cancer samples in which lymph node or distant metastases were present (15). This observation, together with the findings in prostate cancer, suggests that the presence of KAII might influence the ability of cancer cells to metastasize (4). However, it is not known whether KAII possesses similar characteristics in other gastrointestinal malignancies, such as esophageal and gastric cancers, which would support the hypothesis that this glycoprotein is generally important for the metastatic ability of cancer cells. Therefore, here, we analyzed and compared the patterns of KAII mRNA expression in esophageal and gastric cancers to determine whether KAII might also be of biological relevance in the metastatic capability of these tumors.

PATIENTS AND METHODS

Patients. Tumor specimens were taken from 41 patients with nonmetastatic (n = 12) and metastatic (n = 29) primary esophageal cancers (8 female and 33 male; mean age, 59.8 years; range, 42–76 years) and 35 patients with nonmetastatic (n = 13) and metastatic (n = 22) primary stomach cancers (14 female and 21 male; mean age, 67 years; range, 49–80 years) and were classified (Table 1) according to the TNM classification of the International Union Against Cancer (16). For comparison, normal tissue specimens of the esophagus (n = 15) and stomach (n = 14) were obtained from 16 previously healthy multiorgan donors (7 female and 9 male; mean age, 39 years) from whom other organs were obtained for transplantation. All studies were approved by the Ethics Committee of the University of Bern (Bern, Switzerland).

Tissue Sampling. For RNA extraction and Northern blot analysis, normal and cancerous specimens were immediately frozen in liquid nitrogen after removal in the operating room and were stored at −80°C until use. In addition, freshly removed normal and cancerous tissue samples were immediately fixed in buffered formaldehyde solution for 24 h and embedded in paraffin for in situ hybridization.

Northern Blot Analysis. Total RNA was isolated by the single-step guanidinium isothiocyanate method (17, 18) and size-fractionated (20 μg/lane) on 1.2% agarose-1.8 M formaldehyde gels (17, 18). The gels were stained with ethidium bromide for verification of RNA integrity and loading equivalency. Fractionated RNA was electrophoretically transferred onto nylon membranes (GeneScreen; DuPont International, Regensdorf, Switzerland) and cross-linked by UV irradiation.

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The blots were prehybridized for 1–2 h at 65°C in a buffer containing 50% formamide, 5X SSC (sodium chloride/sodium citrate buffer), 2% blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany), and 0.1% N-lauroylsarcosine. After addition of the DIG-labeled KAII antisense probe, hybridization was carried out at 65°C for 18 h. The filters were washed for 5 min in 2X SSC-0.1% SDS at room temperature, followed by two washes in 0.1X SSC-0.1% SDS at 68°C for 15 min. Afterward, the filters were incubated in 20 ml of blocking buffer (1% blocking reagent in 100 mmol/liter maleic hydroxide) containing 1 fil of anti-DIG alkaline phosphatase antibodies (Boehringer Mannheim) for 30 min, washed with blocking buffer for 15 min, and incubated with 4 µl of CDP-Star (25 ITIM; Boehringer Mannheim). The membranes were then exposed to X-ray films for 15 s at room temperature, as described previously (17–19).

To assess equivalent RNA loading, the membranes were rehybridized with the 32P-labeled 7S cDNA probe (17, 19). The blots were prehybridized for 4–8 h at 42°C in a buffer containing 50% formamide, 1% SDS, 0.75 m sodium chloride, 5 mM EDTA, 5X Denhardt’s solution, 100 µg/ml denatured salmon sperm DNA, 10% dextran sulfate, and 50 mM sodium phosphate buffer (pH 7.4). The hybridization was carried out at 42°C for 12 h with the 32P-labeled cDNA probe (1 X 105 cpm/ml) in the same buffer. Washing was started by rinsing the blots twice (50°C) in 2X SSC. The blots were, subsequently, washed three times at 55°C in 0.2X SSC and 2% SDS. The blots were then exposed at −80°C to Fuji X-ray films with intensifying screens for 4–8 h. The intensity of the radiographic KAII and 7S signals was calculated for each sample and statistically analyzed.

**In Situ Hybridization.** In situ hybridization was performed as reported previously (15, 20). Tissue sections of normal and cancerous tissue samples were always processed simultaneously. In addition, we processed consecutive slides, one slide was incubated with the sense probe, and the other slide was incubated simultaneously with the antisense probe. Tissue sections (4 µm) were deparaffinized, rehydrated, and incubated in 0.2 mol/liter HCl for 20 min. After washing with 2X SSC, the tissues were permeabilized with proteinase K (Boehringer Mannheim) at concentrations of 30 µg/ml in esophageal tissues and 40 µg/ml in gastric tissues for 15 min at 37°C. After postfixation with 4% paraformaldehyde in phosphate buffer solution (5 min) and washing in 2X SSC, the sections were prehybridized for 1 h at 37°C for esophageal tissue slides and at 55°C for gastric tissue slides) in a buffer containing 50% (v/v) formamide, 4X SSC, 2X Denhardt’s reagent, and 250 µg/ml RNA. Hybridization was performed overnight at the same temperatures in 50% (v/v) formamide, 4X SSC, 2X Denhardt’s reagent, 500 µg/ml RNA, and 10% (v/v) dextran sulfate. The final concentration of the DIG-labeled KAII probes (antisense and sense) was approximately 0.5 ng/µl. After hybridization, excess probe was removed by washing in 2X SSC and by RNase treatment as follows: 100 units/ml RNase T1 (Boehringer Mannheim) and 0.2 µg/ml DNase-free RNase (Boehringer Mannheim) at 37°C for 30 min. Washings were performed at 55°C for esophageal tissue slides and at 60°C for gastric tissue slides in 2X SSC for 10 min and twice in 0.2X SSC for 10 min each. Afterward, the sections were incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim). For the following color reaction, we used 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma, Buchs, Switzerland).

Pretreatment of the slides with RNase abolished the hybridization signals, and sense probes corresponding to the antisense probes failed to produce a signal.

The results of in situ hybridization were semiquantitatively analyzed and scored as previously reported (15, 21). The in situ hybridization signals were evaluated by two independent pathologists who were blinded to patient status, followed by resolution of any differences by joint review and consultation with a third observer.

**Preparation of DIG-labeled KAII Probes.** To prepare DIG-labeled cRNA probes for Northern blot analysis and in situ hybridization, a 500-bp XbaI/HindIII fragment of human KAII cDNA was subcloned into the pCR-II vector (Invitrogen, San Diego, CA), which contains promoters for DNA-dependent SP6 and T7 RNA polymerases. After linearization of the plasmid with XbaI, the antisense KAII probe was transcribed using SP6 polymerase and the Ribomax System (Promega Biotechnology, Madison, WI). The transcription resulted in a DIG-labeled antisense riboprobe that was specific for the KAII mRNA. To evaluate the specificity of the in situ hybridization reaction, a DIG-labeled sense probe was generated after linearization of the plasmid with HindIII and in vitro transcription with T7 polymerase and the Ribomax System (Promega Biotechnology, Madison, WI; Ref. 22). For the in situ hybridization experiments, the KAII antisense and sense probes were shortened to a length of approximately 150 bases, according to the procedure of Cox et al. (23).

**Preparation of a 32P-labeled 7S cDNA Probe.** To verify equivalent RNA loading on Northern blot membranes, all filters were rehybridized with a murine 0.19-kb 7S cDNA probe that cross-hybridizes with human 7S RNA, as reported previously (17, 18, 20). The 7S cDNA probe was radiolabeled with [α-32P]dCTP (3000 Ci/mmol; DuPont, Boston, MA) using a random primer labeling system (Pharmacia Biotech AG, Dübendorf, Switzerland; Refs. 17–19).

**Statistical Analysis.** Results were expressed as median and range or as mean ± SE. For statistical analysis, the Mann-Whitney U test, the Student’s t test, or the χ2 test was used. Significance was defined as P < 0.05.

**RESULTS**

**KAII mRNA Expression by Northern Blot Analysis**

Northern blot analysis was performed using 20 µg of total RNA extracted from normal and cancerous tissue samples.

**Esophagus.** In the normal esophagus, moderate to strong KAII mRNA signals were detectable in 60% of the samples (Fig. 1). In the remaining samples, KAII mRNA levels were weak or too low to be detected by Northern blot analysis. In esophageal cancers, 49% of the samples exhibited KAII mRNA expression levels 1.6-fold higher than the mean values for normal controls. In the remaining esophageal cancer samples, KAII mRNA expression was similar to that of the normal controls. However, the mean KAII mRNA levels of the esophageal cancers were not statistically different from normal controls (Table 2 and Fig. 2). Tumor differentiation had no influence on KAII mRNA expression levels. In addition, the presence of lymph...

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**Table 1** TNM classification of the investigated tumor samples according to the International Union Against Cancer

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Tumor grade</th>
</tr>
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<tbody>
<tr>
<td>Esophageal cancer (n = 41)</td>
<td>4 13 22 2 4 20 16 1</td>
</tr>
<tr>
<td>Gastric cancer (n = 35)</td>
<td>12 3 13 7 2 16 16 1</td>
</tr>
</tbody>
</table>

The abbreviation used is: DIG, digoxigenin.
node or distant metastases had no influence on KAI1 mRNA levels in the primary esophageal tumor samples (Table 2 and Fig. 2). Primary esophageal tumors in which lymph node (n = 29) or distant metastases (n = 2) were present at the time of resection exhibited KAI1 mRNA expression levels similar to those of primary esophageal tumors in which no lymph node metastases (n = 12) were found at the time of tumor resection (Table 2 and Fig. 2).

**Stomach.** An expression pattern similar to that in the esophagus was found in gastric tissue samples (Fig. 3). In normal gastric tissues, KAI1 mRNA expression was detectable in all samples. The expression levels of KAI1 mRNA in normal and gastric cancer samples were comparable. In esophageal tissues, a wide range in KAI1 mRNA expression levels was seen in the normal as well as in the gastric cancer samples. The KAI1 mRNA levels in primary gastric cancer tissues in which lymph node (N1 or N2, n = 22) or distant (n = 4) metastases were present at the time of resection were not different from the KAI1 mRNA levels of primary gastric cancer tissues in which no lymph node metastases (n = 13) were found at the time of tumor resection (Table 2 and Fig. 4). Furthermore, we found no influence of tumor differentiation on KAI1 mRNA levels in gastric carcinomas (Table 2 and Fig. 4).

**KAI1 mRNA Expression Analysis by in Situ Hybridization**

To localize the exact site and cellular distribution of KAI1 mRNA expression in the normal and cancerous tissue samples, in situ hybridization was performed. For in situ hybridization the same tissue samples were studied as for Northern blot analysis. The results obtained by in situ hybridization in regard to the expression levels were comparable to the Northern blot data.

**Esophagus.** In the normal esophagus, KAI1 mRNA staining was present in the cytoplasm of most squamous epithelial cells (Fig. 5A). Lymphocytes in the submucosal areas of the normal esophagus exhibited moderate expression of KAI1 mRNA. There was no difference in KAI1 mRNA between normal esophageal tissue that was adjacent to the tumor tissue and normal esophageal tissue obtained from organ donors. In the esophageal cancer samples, KAI1 mRNA staining intensity in the cancer cells was comparable to that of normal esophageal epithelial cells (Fig. 5B). In the esophageal cancer samples, there was some variation in the intensity of KAI1 mRNA staining within the cancer cells of the same tumor. In addition, fibroblasts of the connective tissue around esophageal cancer cells showed weak KAI1 mRNA expression.

**Stomach.** In the cytoplasm of nonmucosal epithelial cells of the normal stomach, KAI1 in situ hybridization signals were detectable at moderate intensity (Fig. 5C). Submucosal and muscular cells in the normal stomach were devoid of KAI1 mRNA staining. No difference was found between normal stomach tissue that was adjacent to the tumor tissue and normal stomach tissue obtained from organ donors. In gastric cancers, KAI1 mRNA in situ hybridization signals were of

![Bar graph](image)

**Fig. 2.** KAI1 mRNA expression levels in the normal esophagus and in esophageal cancer, in terms of tumor stage and tumor differentiation. *, normal esophagus; ●, primary esophageal cancer without metastasis; ●, primary esophageal cancer with lymph node or distant metastasis.

**Table 2.** KAI1 mRNA expression in normal and primary tumor samples of the esophagus and stomach, dependent on the presence (M+) or absence (M−) of lymph node or distant metastases.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Cancer</th>
<th>Cancer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M−</td>
<td>M+</td>
</tr>
<tr>
<td>Esophagus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>15</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>KAI1 expression (mean)</td>
<td>0.55</td>
<td>0.53</td>
<td>0.59</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
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<tr>
<td>Stomach</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>14</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>KAI1 expression (mean)</td>
<td>0.33</td>
<td>0.31</td>
<td>0.35</td>
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<td>P</td>
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* M−, primary tumor without lymph node or distant metastases; M+, primary tumor with lymph node or distant metastases; NS, not significant.

[a] In relative densitometric units.

[b] Normal vs. M−.

c Normal vs. M+.

d M− vs. M+.

[e] Grades I–II vs. grades III–IV.

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moderate to strong intensity (Fig. 5D). In the cancer samples, there was some variation of KAI1 mRNA expression within the cancer cells. The signals were located mainly in the cytoplasm and were distributed with uniform intensity. Some lymphocytes and a few fibroblasts exhibited weak KAI1 mRNA staining, whereas the stroma surrounding the cancer was devoid of KAI1 mRNA staining.

**DISCUSSION**

A cascade of cellular, biochemical, and genetic events are known to occur in the development and progression of tumors, leading to malignancy and, ultimately, to tumor spread. The presence of metastases is one of the most significant problems facing patients with disseminated gastrointestinal cancers because almost all are incurable with currently available therapy. The mechanisms that contribute to the formation of tumor metastases, such as detachment of cancer cells from the primary tumor mass, invasion by these cells of blood and lymph vessels, and their ability to transverse capillaries, are not completely understood (24, 25).

The discovery of genetic alterations in oncogenes and tumor suppressor genes has encouraged the search for new genes that may promote or suppress tumor growth, cancer cell spread, and metastasis. The epidermal growth factor receptor, epidermal growth factor, transforming growth factor α, and amphiregulin have been implicated as regulatory factors in the progression of gastrointestinal cancers, especially in pancreatic cancers (19, 26). However, no relationship has been found between the presence of these factors in the cancer cells and their ability to metastasize (27).

The nm23 gene appears to influence metastasis in gastrointestinal cancers in different ways (6, 28, 29). For example, it is up-regulation of nm23 that is positively associated with lymph node metastasis and poor prognosis in human pancreatic cancers (28), whereas in gastric cancer, it is down-regulation of nm23 that seems to contribute to tumor invasion and metastasis (29). These findings indicate that the effects of nm23 on metastasis depend on the underlying malignancy.

It has been suggested that the KAI1 gene may function as a tumor metastasis suppressor. The gene is localized on chromosome 11p11.2 and encodes a M, 29,600 nuclear and cytoplasmic protein consisting of 267 amino acids (4). Although the exact functions of the KAI1 gene have not yet been elucidated, it belongs to a structurally distinct family of membrane glycoproteins (14). A characteristic feature of this family is a polypeptide chain containing four hydrophobic, presumably membrane-spanning segments and, presumably, a single, major, extracellular, and usually N-glycosylated loop. The highest degree of sequence similarity is observed within the putative transmembrane region (14). Currently, available data strongly suggest that this gene superfamily plays a role in the regulation of cell development, cell proliferation, and cell mobility (30). KAI1 is highly expressed in normal prostate tissue and greatly reduced in at least five human metastatic prostate cell lines (4). Recently, we reported that reduction of KAI1 mRNA expression in primary human pancreatic cancers is associated with advanced tumor stage and the presence of lymph node or distant metastases (15). Similar findings were made in lung cancers, in which the loss of KAI1 was associated with faster tumor growth and shorter survival (31). Furthermore, in bladder cancer, grade 3 transitional cell carcinomas and invasive transitional cell carcinomas have been found to exhibit lower KAI1 mRNA expression than do normal bladder, inflammatory bladder, or noninvasive papillary transitional cell carcinomas with high-grade differentiation (32).

Here, we sought to determine whether loss of KAI1 mRNA expression in metastatic cancer is a common event in gastrointestinal cancers or if it occurs only in some tumor types. To evaluate this, we chose esophageal and gastric cancers, which both metastasize as early as pancreatic cancer. Using Northern blot analysis, KAI1 expression was studied in various tumor stages in both malignancies to evaluate if metastasis formation is promoted by the loss of KAI1 expression. In normal, nonmetastatic and metastatic esophageal and gastric tissue samples, KAI1 mRNA expression levels were comparable. In situ hybridization confirmed the Northern blot results. KAI1 mRNA expression was localized in the cancer cells, and the intensity was comparable to the normal controls. In addition, the hybridization signals in metastatic and nonmetastatic primary esophageal and gastric cancer samples were comparable. Our findings indicate that esophageal and gastric cancers exhibit a different KAI1 mRNA expression pattern than do prostate or pancreatic cancers (4, 15). Furthermore, the divergent expression patterns of KAI1 in the investigated cancer tissues suggest that KAI1 possesses a different role in the formation of metastases in these malignancies than in previously analyzed tumors of the prostate, the pancreas, or the lung (4, 15, 31). Therefore, KAI1 cannot simply be considered to act in the prevention of tumor metastasis formation, as was first reported in prostate cancers, in which the loss of KAI1 mRNA expression is strongly associated with the formation of metastases when tumor cells are transplanted into nude mice (4). However, by in situ hybridization, there was found to be some heterogeneity in KAI1 mRNA expression levels within the cancer cells of esophageal and gastric cancers. Lower expression of KAI1 in some cancers cells might increase their metastatic potential, as generally occurs in prostate and pancreatic cancer.
Although our findings in esophageal and gastric cancer are negative, they indicate that the influence of KAI1 in the formation of metastases seems to be dependent on the phenotype of the cancer cells. Furthermore, the differences between the expression patterns of KAI1 mRNA in esophageal and stomach cancer and in other tumors strongly suggest that factors other than KAI1 are involved in the formation of metastases in these gastrointestinal cancers. Therefore, one of our interests in the future will be to detect additional metastasis-promoting genes by comparative analysis of these tumors and to understand the mechanisms by which KAI1 influences the ability of some cancer cells to leave the primary tumor mass.

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KAI1 Is Unchanged in Metastatic and Nonmetastatic Esophageal and Gastric Cancers

Xiao-Zhong Guo, Helmut Friess, Christoph Maurer, et al.


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