Immunohistochemical Detection of K-sam Protein in Stomach Cancer¹

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ABSTRACT

The K-sam gene, originally isolated as an amplified gene from the stomach cancer cell line KATO-III, is characterized by its preferential amplification in the undifferentiated type (diffuse type) of stomach cancer and encodes one of the receptors for heparin-binding growth factors or fibroblast growth factors. The K-sam gene has been isolated by different methods and has been designated BEK, TK14, and Cek2. The receptor for keratinocyte growth factor was also found to be encoded by the same gene. To examine the expression of the K-sam protein in stomach cancer, polyclonal antibody pK1–2 was raised against the extracellular domain of the gene product. This antibody detected K-sam proteins by Western blot and flow cytometry analyses in stomach cancer cell lines KATO-III and HSC39, in which the K-sam gene is amplified and overexpressed. This antibody detected K-sam proteins by Western blot and flow cytometry analyses in stomach cancer cell lines KATO-III and HSC39, in which the K-sam gene is amplified and overexpressed. By immunohistochemical analysis, 20 of 38 cases of the undifferentiated type of stomach cancer showed K-sam staining. The K-sam product was observed predominantly in diffusely infiltrative lesions. In one autopsy case, the K-sam protein was detected only focally in the primary tumor, whereas markedly increased staining for the K-sam product was detected diffusely in the metastasized tumor in the lymph node and liver. These results suggest that K-sam overexpression is associated with the malignant phenotype of the undifferentiated type of stomach cancer, such as infiltrative growth and metastasis.

INTRODUCTION

Although stomach cancer is the most common malignant disease in the world after lung cancer, information on the genetic changes of stomach cancer has just begun to be accumulated (1, 2). Little has been known about the correlation between clinicopathological characteristics and genetic information. Stomach cancers are histopathologically classified into two types: undifferentiated (diffuse type) and differentiated (intestinal type). These two types are also divergent in terms of biological behavior (3). For example, undifferentiated carcinoma is more frequently observed in the younger generation than is the differentiated type, demonstrates diffusely infiltrative growth, readily causes peritoneal dissemination, and is thought to be unfavorable in prognosis (4). From the clinicopathological point of view, it is important to clarify the molecular mechanism that determines the biological features of the undifferentiated type of stomach cancer.

The K-sam gene was originally identified as an amplified gene in a stomach cancer cell line, KATO-III, by the in-gel DNA renaturation method (5, 6). This gene is preferentially amplified in the undifferentiated type of stomach cancer, including poorly differentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous adenocarcinoma (6). K-sam is also known to encode one of the heparin-binding growth factor receptors or FGFRs (7). The K-sam gene has been isolated by different methods and has been designated BEK, TK14, and Cek2 (8, 9). The receptor for KGF was also found to be encoded by the same gene (10). The K-sam protein showed selective affinity for KGF or basic FGF by mutually exclusive alternative splicing of exons in the ligand-binding domain (11). The K-sam gene is capable of transforming NIH3T3 cells, and the transformed cells are tumorigenic in nude mice (12). Interestingly, truncation of the COOH terminus of the K-sam gene product potentiates transforming activity and is predominantly expressed in stomach cancer cell lines of undifferentiated type (12).

In the present paper, we studied the expression of K-sam protein in stomach cancer. We also immunohistochemically examined the expression of the K-sam protein in surgical and

¹ The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; AMeX, acetone, methyl benzoate, and xylene.
postmortem samples of stomach cancer. It was found that K-sam is preferentially expressed in the undifferentiated type, and that the expression is associated with malignant phenotypes such as infiltrative growth and remote metastasis.

PATIENTS AND METHODS

Cell Culture. Stomach cancer cell lines KATO-III, HSC39, and MKN1 were cultured in RPMI 1640 supplemented with 10% FCS as described previously (13, 14). For establishment of K-sam and N-sam transfectants to NIH3T3 cells, K-sam-IIC1/KGFR, K-sam-IIC3/KGFR with truncated COOH terminus, and N-sam/KGFR cDNAs were cloned into pcDNA1neo expression vector (Invitrogen, San Diego, CA) and were transfected by the calcium phosphate method as described previously (12, 15, 16). After two weeks of coculture with G418, independent colonies were isolated and were used for...
further analysis. For Western blotting and flow cytometry, culture mediums were changed one day before the experiments to obtain logarithmically growing cells.

**Patients and Histological Characteristics.** Surgical specimens from 49 Japanese patients with advanced gastric cancer were used in this study. All of the patients were admitted to and operated on at the National Cancer Center Hospital in Tokyo between May 1990 and October 1991. Pathological diagnoses and clinical staging were based on the Japanese Classification of Gastric Carcinoma by the Japanese Research Society for Gastric Cancer (17). Based on the Japanese Classification of Gastric Carcinoma or on Borrman’s classification (18), tumors were macroscopically divided into four types: type 1, polypoid tumors; type 2, ulcerated carcinomas with sharply demarcated and raised margins; type 3, ulcerated carcinomas infiltrating the surrounding wall; and type 4, diffusely infiltrating carcinomas without marked ulceration. Surgical and autopsy specimens were fixed by acetone and were embedded in paraffin by the AMeX method described previously (19).

**Western Blotting and Dot-Blot Analysis.** The anti-human K-sam antibody pK1–2 was raised by the inoculation into rabbits of the synthetic oligopeptide conjugated with bovine thyroglobulin, consisting of residues 23–38 of the K-sam type II product P23SFSLVEDTTEPEDA38, which corresponds to just downstream of the signal peptide in the extracellular domain of K-sam protein (7). Immunoaffinity purification of the antisera was carried out by protein A and immunogen synthetic oligopeptide. In the same way, the anti-human N-sam/FGFR1 polyclonal antibody pN1–1 was raised by the inoculation of synthetic oligopeptide conjugated with bovine thyroglobulin, I81TGEEVEQVSVPADSGLYA190, and was purified by immunoaffinity column (20).

For Western blot analyses, cell lysates from cell lines and paraffin-embedded sections of surgical and autopsy specimens were prepared by a radioimmunoprecipitation assay buffer [RIPA buffer: 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, and 0.2 units/ml aprotinin]. Cell lysates (50 μg) were electrophoresed in 7.5%
SDS-polyacrylamide gel under reducing conditions and were transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA), using blotting apparatus (Trans-Blot CELL; Bio-Rad, Richmond, CA). Filters were incubated with pKl-2 at a dilution of 1:100 with or without immunogen oligopeptide (0.1 μg/ml) for 1 h at room temperature. The detection of K-sam protein was done by the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom). The peroxidase-conjugated donkey anti-rabbit IgG was used as the secondary antibody at a dilution of 1:5000.

DNA purification from the paraffin-embedded sections of these tumors was also described elsewhere (21). DNAs (10 μg) were blotted on Nitroplusmembrane (Micron Separations, Westborough, MA) and were hybridized to the K-sam-specific probe RA0.7 under high-stringency conditions as described previously (7).

Flow Cytometry. Cells were treated with or without pKl-2 antibody at a 1:100 dilution in PBS with 2% FCS for 30 min on ice. Cells were then incubated with fluorescein-conjugated anti-rabbit IgG (Cappel, West Chester, PA) at a 1:50 dilution in PBS with 2% FCS for 30 min on ice. K-sam or N-sam expression in stained cells was analyzed by using the Becton Dickinson FACScalibur (San Jose, CA).
RESULTS

The specificity of this antibody was examined by Western blot analysis in NIH3T3 cells transfected with K-sam cDNAs. As shown in Fig. 1A, K-sam-IIIC3 (KGFR with a truncated COOH terminus)/NIH3T3 cells demonstrated a Mr, 125,000 protein (Lane 3), and K-sam-IIIC1 (KGFR with an intact COOH terminus)/NIH3T3 cells demonstrated a Mr, 135,000 protein (Lane 4). These results are consistent with those reported previously (23).

At least four members of the sam/FGFR family have been reported, including FGFR1/N-sam, FGFR2/K-sam/KGFR, FGFR3/sam3, and FGFR4 (7–10, 20, 24). The comparison of deduced whole amino acid sequences showed that N-sam was the molecule most closely related to K-sam. The amino acid sequence of the immunogen oligopeptide of K-sam was 31% homologous to the corresponding amino acid sequence of N-sam, 0% homologous to that of sam3/FGFR3, and 6% homologous to that of FGFR4. To examine the cross-reaction of

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<th>Table 1</th>
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<td>No. (%) of patients</td>
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<td></td>
<td>K-sam positive (n = 20)</td>
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<td>Histological type</td>
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<tr>
<td>Undifferentiated type</td>
<td>20 (53)</td>
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<tr>
<td>Por1</td>
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<td>Por2</td>
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<td>Pap</td>
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a Por1, poorly differentiated adenocarcinoma, solid type; Por2, poorly differentiated adenocarcinoma, non-solid type; Sig, signet-ring cell carcinoma; Muc, mucinous adenocarcinoma; Pap, papillary adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

Immunohistochemical Staining. Immunocytochemical and immunohistochemical analyses were performed as follows: 5-μm-thick AMeX sections of stomach cancer cell line and surgical or autopsy specimens were cut and deparaffinized. NIH3T3 transfectants were cultured in chamber slides (Nunc, Roskilde, Denmark) and were washed with PBS. After blocking endogenous peroxidase activity and nonspecific binding, the anti-human K-sam antibody pK1-2 was applied to the sections at a 1:250 dilution with or without immunogen oligopeptide (0.1 μg/ml) overnight at 4°C. Subsequently, biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Inc., Burlingame, CA) was added at a 1:100 dilution and was incubated for 1 h. Finally, 0.02% H2O2 were used to visualize immunoreactivity, and counterstaining was performed with methyl-green.

Statistical Analyses. Clinicopathological features of K-sam product-positive and -negative cases of stomach cancer were compared by Fisher’s exact test for dichotomous variables or Wilcoxon’s rank-sum test for polychotomous variables. Overall survival was defined as the time from surgical operation to death. Survival distributions were estimated by the Kaplan-Meier method, using the Statistical Analysis System (SAS) procedure (22). The significance of the difference in survival rates was tested by use of the log-rank test.
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Fig. 5 Immunohistochemical detection of K-sam protein in stomach cancer cells. A, a 50-year-old male with poorly differentiated adenocarcinoma, stage IV. Tumor cells that invaded the lymphatic ducts were predominantly stained. B, same case as in Fig. 2. C, a 51-year-old female with poorly differentiated adenocarcinoma, stage IV. Tumor cells diffusely infiltrated the muscle layer and subserosa. Scirrhous reaction was clear. A, ×200; B, ×200; C, ×200.

pK1–2 with the N-sam product, Western blot analysis was conducted with pK1–2. On N-sam-transfected NIH3T3 cell lysates, no N-sam signal was detected, although the N-sam gene product was overexpressed in these transfectants (Fig. 1A, Lanes 2 and 6). In KATO-III cells, a broad, strong band with a Mr of 125,000–135,000 was observed by Western blotting (Fig. 1A, Lane 7). The strong signal was also detected by immunoprecipitation of the metabolically labeled KATO-III lysates (23) and was likely to correspond to the translational product of the 3.5-kb mRNA detected by Northern blot analysis (Fig. 1B, Lane 1). The K-sam 4.7-kb and 4.0-kb mRNAs were also overexpressed in the human stomach cancer cell line HSC39 (Fig. 1B, Lane 2). In this cell line, two bands (Mr, 145,000 and Mr, 130,000) of proteins were observed by Western blot analysis (Fig. 1A, Lane 8). The Mr, 125,000–135,000 strong signal in KATO-III cells and the Mr, 145,000 and Mr, 130,000 signals in the HSC39 cells were all completely absorbed by incubation with immunogen oligopeptides (Fig. 1A, Lanes 10 and 11). The different sizes of the K-sam products were probably generated by the alternative splicing of the K-sam transcripts (12, 25) and by the genomic rearrangement in the course of K-sam gene amplification. In MKN1 cells from differentiated adenocarcinoma, there was no K-sam mRNA or protein (Fig. 1A, Lane 9; Fig. 1B, Lane 3).

The peptide antiserum was directed against the amino terminus of the receptor. Therefore, it did not distinguish K-sam-II/KGFR from the K-sam-I/BEK isoforms. We have shown previously that the amount of the K-sam-I/BEK type of mRNAs was significantly less than that of K-sam-II/KGFR in all the stomach cancer cell lines examined (15). Furthermore, all of the 24 K-sam cDNA clones obtained from the cDNA library of KATO-III cells contained K-sam-II/KGFR-type cDNAs (12). These results suggest that the K-sam/FGFR2 proteins detected by this antibody in stomach cancer cells are very likely to be mainly K-sam-II/KGFR proteins, whereas the amounts of K-sam-I/BEK proteins detected are likely to be small.

Expression of the K-sam protein was also examined by flow cytometry with pK1–2 antibody (Fig. 2). The intensity of FITC staining was consistent with the results obtained by Western and RNA blotting. High K-sam expression was observed in K-sam-transfected NIH3T3 cells, KATO-III, and HSC39, but no K-sam signal was detected in NIH3T3 cells transfected with vector or MKN1 cells.

Immunocytochemical Staining of K-sam Products. Results of the immunocytochemical staining of NIH3T3 transfected and the stomach cancer cell line KATO-III with pK1–2 are shown in Fig. 3. The cytoplasm and membrane of NIH3T3 transfected of K-sam-IIC3/KGFR with truncated COOH terminals and K-sam-IIC1/KGFRs with intact COOH terminals were clearly stained, but in-vector transfecteds demonstrated no K-sam staining. In KATO-III cells, the periphery of the cells was clearly stained, indicating the presence of K-sam products on the cell membrane. The specificity of immunocytochemical staining was also confirmed by immunogen absorption test and by negative staining without pK1–2 incubation (data not shown).

Expression of K-sam Protein in Surgical Specimens of Gastric Cancer. The staining pattern with pK1–2 was compared between cancerous and noncancerous samples (Fig. 4). In a slice-section from a noncancerous area, only the fundic glands in the stomach wall were weakly stained in the cytoplasm (Fig. 4D). The signals were absorbed by incubation with immunogen oligopeptide, suggesting K-sam-specific staining. In contrast, tumors were stained more intensely than noncancerous cells (Fig. 4A). The tumor cells were judged as K-sam-staining positive when the following conditions were fulfilled: (a) the tumor cells were stained much more strongly than the noncancerous portion of the same sample; and (b) the staining was absorbed by immunogen oligopeptides (Fig. 4B). A total of 49 AMeX samples of advanced stomach cancer (including 38 samples of undifferentiated type and 11 samples of differentiated type) obtained at surgical resection were studied. Table 1 summarizes the results. Of the 49 samples, 20 were positive, and all of these positive samples were diagnosed as undifferentiated type, including 15 cases of poorly differentiated adenocarcinoma, 2 cases of signet-ring cell carcinoma, and 3 cases of mucinous adenocarcinoma. Eighteen undifferentiated-type samples were negative. In contrast, none of the 11 cases of differentiated...
Fig. 6 Postmortem specimens of a stomach cancer patient with mucinous adenocarcinoma. A, dot-blot analysis of K-sam with RA 0.7 probe. 1, primary stomach tumors; 2, metastasized tumors in the liver; 3, metastasized tumors in the para-aortic lymph nodes. B, immunohistochemical staining of K-sam. Numbers of the photographs correspond to 1–3 of A. 1a, primary site of tumors in the stomach. Tumor cells were not clearly stained with pK1–2 antibody. 1b, small group of stomach cancer cells that invaded the stomach serosa were selectively stained with K-sam antibody. 2, liver metastasis; 3, para-aortic lymph node metastasis. 1a, ×100; 1b, ×400; 2, ×100; 3, ×100.

Fig. 7 Kaplan-Meier survival analysis of patients with K-sam immunohistochemical staining-positive and -negative cells of undifferentiated type of stomach cancer in the primary lesion. Left, patients in all stages (log-rank, \( P = 0.155 \)); right, patients in stage IV (log-rank, \( P = 0.102 \)).

Adenocarcinoma showed positive staining, although 8 of them were in advanced stage IV. The representative cases are shown in Fig. 5. In case 1, tumor cells that invaded the lymphatic duct were predominantly stained. In case 2, the K-sam gene was found to be amplified by dot-blot hybridization of DNAs isolated from a paraffin-embedded section (data not shown). In case 3, tumor cells that invaded diffusely into the muscle layer were strongly stained. The presence of K-sam protein was confirmed by Western blot analysis (data not shown).

The correlation of K-sam-positive staining with various clinicopathological characteristics of patients with the undifferentiated type of stomach cancer is summarized in Table 2. Macroscopically, Borrmann type IV or diffusely infiltrative stomach cancer was frequently stained compared to other types (\( P = 0.050 \)). The infiltrative growth pattern examined by microscopic observation was classified into three types in the Japanese Classification of Gastric Carcinoma by the Japanese Research Society for Gastric Cancer (17). Namely, in IFN-\( \alpha \), tumor cells
grow, constructing a tumor nest, and tumors are bordered clearly from the noncancerous area. IFN-β has characteristics between IFN-α and IFN-γ. In IFN-γ, tumors demonstrate an infiltrative growth pattern, and the difficulty is to show a clear margin between the tumor mass and the noncancerous portion. The P-value of association with infiltrative growth pattern IFN-γ was 0.087.

Seven cases of postmortem AMeX specimens of stomach cancer and their metastasized tumor samples were available for analyses of the expression of K-sam protein (Fig. 6). In one case, K-sam products were found focally only in the primary tumor, which infiltrated deeply to the stomach subserosa. However, in this case, gene amplification was undetectable in the primary stomach tumor by dot-blot analysis. In contrast, metastasized tumors of the liver and para-aortic lymph nodes of the same patients were shown to be K-sam-product positive with the amplified gene.

To elucidate the clinical relevance of K-sam immunohistochemical positivity, we compared the survival of 20 K-sam product-positive cases and 18 patients with the undifferentiated type of stomach cancer by Kaplan-Meier methods (Fig. 7). The results showed that there was no statistically significant difference in survival between patients in all stages with K-sam-positive stomach cancer and those with K-sam-negative stomach cancer (P = 0.155). Among the patients in stage IV, the difference in survival between K-sam-positive and -negative cases (Fig. 7) was not statistically significant either (P = 0.102).

**DISCUSSION**

We reported previously that amplification of the K-sam gene preferentially occurs in the undifferentiated type of stomach cancer, whereas c-erbB-2 amplification occurs in the differentiated type (6, 26). In the present study, anti-human K-sam polyclonal antibody was raised and characterized to study the expression of K-sam protein in stomach cancer. Western blot and flow cytometry analyses using NIH3T3 cells transfected with K-sam and N-sam cDNAs revealed that this antibody specifically detected K-sam gene products and did not cross-react with N-sam protein, which is the most homologous to the K-sam protein. Both Western blotting and flow cytometry assay clearly demonstrated the expression of K-sam protein in the stomach cancer cell lines KATO-III and HSC39 with K-sam amplification. The sizes of K-sam protein were different in these two cell lines, and the difference was probably caused by various patterns of alternative splicing (12, 25), by genomic rearrangements during the process of the gene amplification (7), or by a different degree of glycosylation of the K-sam protein.

Immunohistochemical detection revealed that even noncancerous tissues were stained weakly by pK1-2 antibody. These signals were believed to represent K-sam proteins because (a) the staining was absorbed by incubation with immunogen oligopeptides; (b) pK1-2 antibody did not cross-react with other FGFR molecules; and (c) signals detected in Western blotting were considered to be the K-sam proteins. K-sam/KGFR is thought to be expressed selectively in epithelial cells and mediates proliferative signals in response to KGF stimulation (11, 27). Therefore, it is reasonable that epithelial cells of noncancerous stomach mucosa were stained with anti-human K-sam antibody. Immunohistochemical analyses clearly showed that K-sam overexpression was predominately observed in the undifferentiated type of stomach cancer, but not at all in differentiated type (P = 0.0018). Although the overexpression of K-sam protein was not always accompanied by gene amplification, the present results were consistent with our previous data, in which K-sam gene amplification was observed preferentially in the undifferentiated type (6). K-sam overexpression was also significantly associated with the macroscopically, diffusely infiltrative lesion Borrman type 4 (P = 0.050; Table 2). Also, in microscopic observation, an infiltrative tumor growth pattern tends to be more often observed in K-sam-positive cases (P = 0.081). Additionally, in one postmortem case, K-sam-positive cells existed focally only in the primary site but occupied the entire tumor cell in metastatic sites (Fig. 6). It is likely that tumor cells with an overexpression of K-sam product may have a growth advantage, may clonally evolve with more infiltrative capacity, and may have increased potential to metastasize. In fact, we often found cases in which tumor cells that invaded the lymphatic duct were significantly stained with pK1-2 antibody (Fig. 5A, case 1). It is possible that the putative K-sam ligand KGF may be expressed in the interstitial tissues in stomach or in metastatic lesions, and that tumor cells with the K-sam/KGFR overwhelmingly proliferate by paracrine mechanism (27). Many of the cases with overexpression of the K-sam protein caused a scirrhous reaction. In these cases, interstitial tissues were highly abundant by microscopic observation and may produce KGF. In **in vitro** study also showed that overexpression of the K-sam gene can transform NIH3T3 cells and can facilitate tumorigenesis (12). Therefore, these results suggest that K-sam overexpression is likely one of the determinants of a biologically malignant tendency of the undifferentiated type of stomach cancer rather than a simple tumor marker.

It will be worthwhile to further study whether or not K-sam-positive and -negative cases in the early stage take different clinical courses. These findings also suggest that K-sam may be a target molecule for the new approach of biotherapy of stomach cancer; for example, gene therapy or immuno-therapy. Further investigation will be needed to elucidate the more precise clinical relevance of studying the overexpression of K-sam gene products in stomach cancer.

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