Basic Fibroblast Growth Factor (FGF-2) Overexpression Is a Risk Factor for Esophageal Cancer Recurrence and Reduced Survival, which Is Ameliorated by Coexpression of the FGF-2 Antisense Gene

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Abstract

Purpose: The basic fibroblast growth factor (FGF-2) gene is bidirectionally transcribed to generate overlapping sense and antisense (FGF-AS) mRNAs. FGF-AS has been implicated in the post-transcriptional regulation of FGF-2 expression. The aim of this study was to characterize FGF-2 and FGF-AS in esophageal cancer and to correlate their expression with clinicopathologic findings and outcome.

Experimental Design: Reverse transcription-PCR was used to study FGF-2 and FGF-AS mRNA expression (normalized to glyceraldehyde-3-phosphate dehydrogenase) in 48 esophageal cancers relative to matched histologically normal esophageal epithelia (internal control). We used Cox proportional hazards analysis to calculate hazard ratios for recurrence and survival of patients with underexpression relative to the overexpression of FGF-2 and/or FGF-AS.

Results: Overexpression of FGF-2 mRNA, by comparison with tumors underexpressing FGF-2, was associated with significantly increased risk for tumor recurrence (hazard ratio, 3.80; 95% confidence interval, 1.64-8.76) and reduced overall survival (hazard ratio, 2.11; 95% confidence interval, 1.0-4.58). When the effects of FGF-2 and FGF-AS were considered simultaneously, the association of FGF-2 mRNA overexpression with recurrence and mortality was even more pronounced, whereas FGF-AS mRNA overexpression was associated with reduced risk for recurrence and improved survival.

Conclusions: Overexpression of FGF-2 mRNA is associated with tumor recurrence and reduced survival after surgical resection of esophageal cancer and that these risks are reduced in tumors coexpressing the FGF-AS mRNA. These data support the hypothesis that FGF-AS is a novel tumor suppressor that modulates the effect of FGF-2 expression and may have potential clinical application to the development of novel therapeutic strategies.

Cancer of the esophagus, one of the ten most frequent malignancies worldwide, is a relatively uncommon tumor in North America (1). However, over the past three decades, there has been a marked change in the epidemiology of this disease.

Although the incidence of esophageal squamous cell carcinoma has remained steady, incidence rates for adenocarcinomas of the lower esophagus and esophagogastric junction have increased in excess of any other human solid tumor (2, 3). Furthermore, despite recent advances in multimodality therapy, the prognosis for patients with invasive esophageal malignancy remains generally poor (4). Substantial progress in the treatment of esophageal malignancy requires a clearer understanding of esophageal tumor biology and the incorporation of molecular biomarkers into clinically relevant treatment strategies.

Basic fibroblast growth factor (FGF-2) is the prototypic member of a family of related genes encoding heparin-binding proteins with growth, antiapoptotic, and differentiation promoting activity (5). FGF-2 is expressed in esophageal squamous cell carcinoma cell lines (6) and is elevated in esophageal adenocarcinoma (7), suggesting an autocrine or paracrine role in esophageal tumorigenesis. The FGF-2 gene maps to chromosome 4q26. This region is a site of frequent gain or loss in esophageal adenocarcinoma and its premalignant lesion, Barrett’s esophagus (8–10), suggesting a possible structural basis for dysregulation of FGF-2 function.
Although the control of FGF-2 expression is poorly understood, one intriguing possibility is regulation by an endogenous antisense RNA, FGF-AS (11). Like the majority of *cis*-antisense transcript pairs characterized to date, FGF-2 and FGF-AS are transcribed as 3′-to-3′ overlaps; the sense and antisense RNAs are fully complementary over extensive regions of their 3′ untranslated regions and have been shown to form stable double-stranded RNA duplexes in *vivo* (12). The organization and sequence of the FGF-2 and FGF-AS genes has been highly conserved from amphibian to human, indicating a functional importance for this structural relationship (reviewed in ref. 13). The inverse association of FGF-2 and FGF-AS mRNA expression observed in a variety of species supports the hypothesis that FGF-2 may be regulated by interaction with the antisense RNA (14–18). We have shown recently that forced overexpression of the FGF-AS mRNA can effectively suppress FGF-2 levels in stably transfected cells in *vivo* (11). In addition to its putative role as a regulatory RNA, FGF-AS also encodes the protein GFNa;NIUDL6, a member of the nudix family of nucleoside phosphohydrolases (19, 20). The nudix motif is characteristic of a diverse group of phosphohydrolase enzymes active on nucleoside diphosphates linked to another moiety (x). Several nudix motif proteins, including the human homologues of MutT and MutY, have been shown to play important roles in prevention and repair of DNA transversion mutations (21). Although the physiologic function of FGF is unknown, we have shown recently that it can partially complement MutT function in *mutT* *Escherichia coli* (20).

The primary objective of this study was to evaluate the expression of FGF-2 and FGF-AS mRNAs and their cognate proteins in a well-characterized series of surgically resected human esophageal tissues and cell lines and to correlate these findings with clinicopathologic features and outcome.

**Materials and Methods**

**Patients and tumors.** The study population comprised a consecutive series of 48 patients with esophageal malignancy treated by a single surgeon (A.G.C.) between February 1997 and February 2003. All patients had a histologic diagnosis of esophageal carcinoma from biopsies obtained at esophagogastroscope. Preoperative staging comprised computer tomography of the chest and upper abdomen, and all patients were considered to have locally resectable tumors, with no clinical evidence of distant visceral metastases. No patient received induction chemotherapy or radiotherapy. Subtotal esophagectomy was done using a right thoracotomy (31 patients) or transthoracic (17 patients) approach. A potentially curative resection was done, completely resecting all macroscopic tumor, with the thoracic and abdominal esophagus and the lesser curvature of the stomach, to achieve a minimum 5 cm distal resection margin as reported previously (22). Regional lymph node stations were resected extensively (two-field) and mapped to document the resection margin as reported previously (22). Regional lymph node stations were resected extensively (two-field) and mapped to document the resection margin as reported previously (22).

**Reverse transcription-PCR.** Reverse transcription-PCR was used to study FGF-2 and FGF-AS mRNA expression [normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] in tumors, relative to matched histologically normal esophageal epithelia (internal control), using techniques reported previously (11). RNA was extracted from each esophageal tissue specimen using the TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) according to the manufacturer’s instructions. Briefly, the tissue was homogenized in 1 mL TRIzol per 0.1 g tissue. The homogenate was centrifuged and RNA in the supernatant was purified using sequential washing with chloroform, isopropanol, and 75% ethanol. The isolated RNA was resuspended using RNase-free water and stored at −80°C. Total RNA yield was determined spectrophotometrically. Reverse transcription of 5 μg RNA was done using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. Amplification was done using EnzyPlus 2000 polymerase (EnzyPol Ltd., London, Ontario, Canada) in a 25 μL reaction composed of 10 pmol of each primer, 0.5 mmol/L deoxynucleotide triphosphates, 2.2 mmol/L MgCl₂, and 1.5 units polymerase in 10-fold buffer. Thermal cycling conditions followed an initial denaturation at 94°C for 3 minutes and 29 (GAPDH) or 39 (FGF-2/FGF-AS) cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 35 seconds followed by a final extension at 72°C for 5 minutes. PCR primers for FGF-2 were 5′-GGCTCTTCCGCCCATCCA-3′ (forward) and 5′-GGCTCTTACGACATTGGAA-3′ (reverse). PCR primers for FGF-AS were 5′-CTGACATGACATTGGCA-3′ (forward) and 5′-CCATCTTTGATGTAAGCATATC-3′ (reverse). PCR primers for GAPDH were 5′-GGGACCTTGAGGGAAACCATCCAGG-3′ (forward) and 5′-CCATTTGGAATGAGATCCGGGA-3′ (reverse). Amplification products (FGF-2, 356 bp; FGF-AS, 198 bp; GAPDH, 321 bp) were resolved on 1.8% agarose gels and stained with ethidium bromide.

**Immunohistochemistry.** A modified indirect immunoperoxidase assay was used to study FGF-2 and GFG protein expression and distribution in serial unstained formalin-fixed, paraffin-embedded tissue sections (4 μm thickness). Affinity-purified anti-FGF-2 polyclonal antibodies raised against a synthetic peptide corresponding to amino acids 40 to 63 of human FGF-2 were obtained from Oncogene Research Products (Cambridge MA). The polyclonal antibody against the COOH-terminal normal esophageal epithelium adjacent to the proximal resection margin were snap frozen in liquid nitrogen and stored in our esophageal tumor bank at −80°C for subsequent molecular analysis. All remaining esophageal tissues were processed according to standard protocol by the Department of Pathology of the Faculty of Medicine of Dalhousie University (Halifax, Nova Scotia, Canada). Representative sections were stained with H&E and examined by an independent consultant histopathologist. Serial unstained formalin-fixed, paraffin-embedded tissue sections were used for subsequent immunohistochemical analysis. All tumors were staged according to the International Union Against Cancer (IIIIC) classification based on pTNM subsets (24). Strict clinicopathologic criteria (25) were used to define primary esophageal adenocarcinomas (Siewert type I), thereby excluding adenocarcinomas of the cardia (Siewert type II) or proximal (subcardia) gastric tumors (Siewert type III).

All participating patients gave full informed consent, and collection and storage of resected esophageal tissues was in accordance with the 1998 Canadian Tri-Council Policy “Statement on Ethical Conduct for Research Involving Humans.” Approval to study banked esophageal tissues was approved by the Health Sciences and Humanities Research Ethics Board at Dalhousie University (2002-539).
domain of the FGF-AS protein (GFg) has been characterized previously (19). Briefly, after deparaffinization in xylene, sections were hydrated through a series of ethanol solutions of graded concentration. Endogenous peroxidase activity was quenched with 3.0% hydrogen peroxide, and sections were heated in 10 mmol/L citrate buffer at 95°C for 10 minutes (antigen retrieval). Sections were incubated overnight at 4°C with primary antibodies against FGF-2 at 1:50 dilution and GFg at 1:100 dilution in a high-humidity chamber. Subsequent steps were done using Universal LSAB plus and 3,3′-diaminobenzidine plus kits according to the manufacturer’s protocols (DAKO Corp., Carpinteria, CA). After counterstaining with hematoxylin and dehydration, coverslips were applied. Laboratory controls were run in parallel with test sections and included known positive and negative tissues (tissue controls) and sections stained without the primary antibody (reagent controls).

Interpretation of coded tissue sections was done by two investigators at a double-headed microscope, blinded to mRNA expression or associated clinicopathologic data. To overcome the issue of tissue heterogeneity, a composite score based on intensity of immunoreactivity (0, no staining; 1, weak; 2, intermediate; 3, strong) and proportion of immunopositive cells (0, none; 1, less than one-hundredth; 2, one-hundredth to one-tenth; 3, one-tenth to one-third; 4, one-third to two-thirds; 5, greater than two-thirds) was assigned to each tissue section as reported previously (25). Overall accumulation of FGF-2 or GFg protein was then expressed as the sum of the intensity and proportion scores (range, 0 and 2–8). Tissues were considered negative with a composite score of 0, 2, or 3 (thereby avoiding a false-positive result from occasional cells with weak immunoreactivity). Tissues were considered positive with a composite score of 4 to 8. The subcellular distribution of each protein (cytoplasmic, nuclear, or both) was recorded.

Cell lines and culture. Two human esophageal adenocarcinoma cell lines (Bic-1, Seg-1; a generous gift from David Beer, Ph.D., University of Michigan, Ann Arbor, MI) were grown as monolayers in DMEM with l-glutamine (Life Technologies/Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and maintained in an humidified atmosphere with 5% CO₂ at 37°C. Cells were grown to semiconfluence, and after extraction of RNA, reverse transcription-PCR was used to evaluate expression of FGF-2 and FGF-AS mRNA as described above.

Immunofluorescent confocal microscopy was used to study FGF-2 and GFg protein expression as described previously (26) with minor modification. Briefly, cells at ~50% confluence in culture flasks were trypsinized and reseeded into dishes of equal area containing microscope slides and allowed to attach for 18 hours before further...
manipulation. The cells were washed twice with cold PBS, fixed with −20°C acetone for 2 minutes, air-dried, and kept at −70°C until further processing. The slides were treated with ice-cold 2% paraformaldehyde (with 9 mg/mL disodium hydrogen orthophosphate and 6 mg/mL 1-lysine; pH 7.4) for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, and then blocked with 3% bovine serum albumin in PBS for 60 minutes. Double staining was by sequential exposure to primary antibodies and their corresponding fluorescently tagged secondary antibodies each for 1 hour at room temperature. All antibodies were diluted in 0.1% bovine serum albumin-PBS as follows: anti-FGF-2, 10 μg/mL; anti-GFG, 350 μg/mL; anti-nucleolin, 1.0 μg/mL; Alexa Fluor 488 (green) or 594 (red) anti-rabbit and anti-goat fluorescent F(ab')2 fragment IgG conjugates, 40 μg/mL; Control staining to eliminate antibody nonspecificity was done by application of secondary antibodies without prior exposure of the cells to the primary antibodies or following incubation with the primary antibody's preimmunizing peptide. Slides were mounted in a drop of glycerol-PBS (Citifluor, Marivac, Halifax, Nova Scotia, Canada). Image analysis used the standard operating software on the Zeiss LSM 510 microscope.

**Data collection and statistical analysis.** Clinicopathologic data and outcomes were prospectively collected and recorded in a research database. Follow-up was complete for all patients until February 2005. Differences in the frequency of FGF-2 and FGF-AS mRNA and protein expression, according to demographic and clinicopathologic factors (age, gender, tumor differentiation, pT stage, pN stage, and UICC stage), were tested with a χ² test, with a Fisher exact test used if a cell contained <5 patients. The prognostic importance of FGF-2 and FGF-AS mRNA expression in tumors (underexpressed versus same versus overexpressed, underexpressed/same versus overexpressed, and under-expressed versus same/overexpressed) for disease-free and overall survival was examined in a univariate analysis with Kaplan-Meier survival methods and tested with the log-rank test. Multivariate analysis using Cox proportional hazards was then used to adjust for the effects of age, gender, tumor grade, pT stage, pN stage, and overall UICC stage. Statistical significance was set at \( P = 0.05 \) and all analyses were done using SPSS for Windows 9.0 (SPSS, Inc., Chicago, IL).

### Results

**Clinicopathologic features, staging, and outcome.** The series comprised 41 males and 7 females ranging in age from 38 to 81 years (median, 63 years). Tumor histology was squamous cell in 10 patients and primary esophageal adenocarcinoma (Siewert type I) in 38 patients. Fourteen (29%) tumors were well differentiated, 12 (25%) were moderately differentiated, and 22 (46%) were poorly differentiated. Tumor stage is summarized in Table 1, with associated 5-year disease-free and overall survival. As expected, significant correlations between tumor stage and outcome were seen. At the time of last follow-up, 16 (33%) patients remain disease free, with a median follow-up of 31.7 months. Overall survival and disease-free survival for all patients are presented in Fig. 1.

**Fibroblast growth factor mRNA expression in human esophageal tissues.** Representative gels illustrating expression of FGF-2 and FGF-AS mRNAs are shown in Fig. 2. Using criteria defined \( a \ priori \) to stratify levels of FGF-2 mRNA expression in tumors (relative to matched histologically normal esophageal squamous epithelia), FGF-2 mRNA was found to be underexpressed in 19 (40%) tumors, overexpressed in 17 (35%) tumors, and unchanged in 12 (25%) tumors. From the scatter graph (Fig. 2) illustrating the range of FGF-2 mRNA expression, we identified two groups of tumors: those underexpressing FGF-2 mRNA (n = 19) and those with the same/overexpression of FGF-2 mRNA (n = 29) relative to matched normal epithelia.

**Antisense fibroblast growth factor mRNA expression in human esophageal tissues.** Relative to matched normal esophageal squamous epithelia, FGF-AS mRNA was underexpressed in 17 (35%) tumors, overexpressed in 17 (35%) tumors, and unchanged in 14 (30%) tumors. As noted for FGF-2 (see above), based on the range of FGF-AS mRNA expression (scatter graph; Fig. 2), two groups of tumors were identified: those underexpressing FGF-AS mRNA (n = 17) and those with the same/overexpression of FGF-AS mRNA (n = 31) relative to matched normal epithelia.

**Fibroblast growth factor and antisense fibroblast growth factor mRNA and protein expression in human esophageal cell lines.** Figure 3 illustrates the relative expression of FGF-2 and FGF-AS mRNAs in the Bic-1 and Seg-1 human adenocarcinoma cell lines. Whereas FGF-2 mRNA was not expressed in Bic-1, the FGF-2 transcript was expressed in Seg-1. Both cell lines expressed FGF-AS mRNA. Immunofluorescent microscopy showed that FGF-2 protein was localized only to the cytoplasm, whereas GFG was localized to the cytoplasm and nucleus, specifically the nucleolus (Fig. 4).

**Fibroblast growth factor and GFG protein expression in human esophageal tissues.** Using immunohistochemistry, FGF-2 protein was not detected in any normal esophageal squamous epithelia (Fig. 5A) but was overexpressed in 83% (40 of 48) of tumors, where immunoreactivity was localized exclusively to the cytoplasm (Fig. 5B and C).

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**Fig. 2.** Left, representative gels illustrating FGF-2 mRNA overexpression and FGF-AS mRNA underexpression in the same esophageal tumor (T) relative to matched normal (N) esophageal squamous epithelium. Right, range of FGF-2 and FGF-AS mRNA expression in esophageal cancers (normalized to GAPDH) relative to matched normal epithelia. Underexpression was defined \( a \ priori \) by a relative mRNA level of <0.7; the same was defined as between 0.71 and 1.5, and overexpression was defined as ≥1.50. From this scatter graph, two groups of tumors were identified based on FGF-2 and FGF-AS mRNA expression: underexpressors and tumors with the same/overexpression. Horizontal bars, means for underexpression and overexpression for FGF-2 and FGF-AS.

**Fig. 3.** Representative gels illustrating relative FGF-2 and FGF-AS mRNA expression in two human esophageal adenocarcinoma cell lines, Seg-1 and Bic-1.
In normal esophageal squamous epithelia, weak immunoreactivity for GFG protein was seen only in the cytoplasm of cells in the middle third layer but not in cells of the basal or parabasal layers. However, occasional cell nuclear immunoreactivity was seen in proliferating basal and parabasal cells (Fig. 5D). In contrast, GFG protein was detected in the cytoplasm of all tumors and in 63% (30 of 48) of tumor cell nuclei (Fig. 5E and F).

**Associations between fibroblast growth factor and antisense fibroblast growth factor mRNA expression, survival, and clinicopathologic findings.** Associations between levels of expression of FGF-2 and FGF-AS mRNA in esophageal cancer (relative to matched normal epithelia) and disease-free survival (reflecting tumor recurrence) and overall survival (mortality) are summarized in Table 2. Patients with tumors overexpressing FGF-2 mRNA had a significantly increased risk for tumor recurrence [hazard ratio (HR), 3.80; 95% confidence interval (95% CI), 1.64-8.76; \( P = 0.0009 \)] and reduced overall survival (HR, 2.11; 95% CI, 1.0-4.58; \( P = 0.050 \)). Representative survival curves are shown in Fig. 5A, illustrating improved disease-free survival for patients with tumors underexpressing FGF-2 mRNA.

When the effect of FGF-2 and FGF-AS mRNA expression in tumors was considered simultaneously (Table 2), the effect of FGF-2 overexpression on risk for tumor recurrence was even more pronounced (HR, 5.45; 95% CI, 1.99-14.91; \( P = 0.001 \)). In contrast, FGF-AS mRNA overexpression was associated with a trend toward reduced risk for tumor recurrence (HR, 0.53; 95% CI, 0.21-1.32; \( P = 0.170 \)) and improved overall survival (HR, 0.81; 95% CI, 0.34-1.90; \( P = 0.620 \)).

On multivariate analysis, HRs did not substantially alter when adjusted for age, gender, tumor histology, tumor differentiation, pT stage, pN stage, and overall (UICC) stage and when FGF-2 and FGF-AS mRNA expression in tumors (relative to matched normal) was stratified as same/underexpressed versus overexpressed or as underexpressed versus same versus overexpressed. The relationship between FGF-2 and FGF-AS mRNA expression and selected clinicopathologic features of esophageal cancers in this series is shown in Table 3.

As seen in Fig. 5C, based on coexpression of FGF-2 and FGF-AS mRNA in tumors, four subgroups of patients with different outcomes were identified, with FGF-2 underexpression/FGF-AS overexpression representing the best disease-free survival and with FGF-2 overexpression/FGF-AS underexpression representing the worst disease-free survival.
In this present study, we first characterized the expression of FGF-2 and its natural antisense mRNA (FGF-AS) in a well-defined series of surgically resected human esophageal tumors, each with matched histologically normal epithelia. This series is unique as no patient received preoperative chemotherapy or radiation therapy, which is now current practice in many North American centers and which may potentially confound molecular studies. We have shown, for the first time, that...
overexpression of FGF-2 mRNA is a significant predictor of tumor recurrence and mortality in a subset of patients with esophageal cancer and that coexpression of FGF-AS mRNA ameliorates this risk (Fig. 6C; Table 2). We also show that FGF-2 protein was overexpressed in the Seg-1 esophageal adenocarcinoma cell line (Fig. 3) and in the majority of primary tumors (83%) where it was localized exclusively to the cytoplasm (Fig. 5).

Ectopic expression of FGF-2 has been shown previously to transform normal cells into a malignant phenotype (27), and inhibition of FGF-2 expression with antisense oligonucleotides can reverse the transformed phenotype (28). The endogenous antisense RNA (FGF-AS) has been implicated in the post-transcriptional regulation of FGF-2 expression (11), but its possible role in tumor progression has not been investigated previously. The sense and antisense mRNAs are fully complementary over a span of several hundred nucleotides at their 3' ends and have been shown to form double-stranded RNA duplexes in vivo. In Xenopus oocytes, where this phenomenon was first identified (12), the duplex was believed to be a target for ADAR-directed RNA editing and degradation. However, this sense-antisense interaction may also contribute to the post-transcriptional regulation of FGF-2 by several other possible mechanisms, including nuclear retention of the mRNA (29, 30), inhibition of pre-mRNA splicing, polyadenylation, RNA transport, or translation initiation (31–34). The inverse association of FGF-2 and FGF-AS mRNA levels in avian, rat, and human tissues, tumors, and cell lines has tended to corroborate the hypothesis that FGF-AS negatively regulates FGF-2 expression (14, 17, 35–37). We have shown previously that transfection and overexpression of FGF-AS in rat glioma cells resulted in post-transcriptional suppression of FGF-2 and inhibition of cell proliferation (11). It is therefore possible that the expression of the FGF-AS mRNA under basal conditions is sufficient to suppress FGF-2 gene expression by transcriptional or post-transcriptional mechanisms.

We also showed here that GFG, the protein product of the FGF-AS mRNA, is expressed in esophageal tumor cells (Figs. 4 and 5). GFG/NUDT6 is a highly conserved protein belonging to the MutT/nudix family of nucleotide phosphohydrolases. The nudix box motif is a signature sequence characteristic of a family of enzymes that catabolize potentially toxic compounds in the cell (38, 39). The founding member of this family, the prokaryotic MutT protein, is responsible for removing 8-oxo-dGTP from the nucleotide pool, thus preventing transversion mutations caused by misincorporation of 8-oxo-guanine residues into DNA (reviewed in ref. 40). At least 20 distinct nucleoside diphosphates and triphosphates to the corresponding over a span of several hundred nucleotides at their 3' ends and have been shown to form double-stranded RNA duplexes in vivo. In Xenopus oocytes, where this phenomenon was first identified (12), the duplex was believed to be a target for ADAR-directed RNA editing and degradation. However, this sense-antisense interaction may also contribute to the post-transcriptional regulation of FGF-2 by several other possible mechanisms, including nuclear retention of the mRNA (29, 30), inhibition of pre-mRNA splicing, polyadenylation, RNA transport, or translation initiation (31–34). The inverse association of FGF-2 and FGF-AS mRNA levels in avian, rat, and human tissues, tumors, and cell lines has tended to corroborate the hypothesis that FGF-AS negatively regulates FGF-2 expression (14, 17, 35–37). We have shown previously that transfection and overexpression of FGF-AS in rat glioma cells resulted in post-transcriptional suppression of FGF-2 and inhibition of cell proliferation (11). It is therefore possible that the expression of the FGF-AS mRNA under basal conditions is sufficient to suppress FGF-2 gene expression by transcriptional or post-transcriptional mechanisms.

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an antimitor role in mammalian cells (48). Although the physiologic substrate of GFG (NUDT6) is not yet known, we have established that the nudix domain of GFG is enzymatically active and has antimitor activity that can partially complement MutT-deficient E. coli (20). In human leukemic cells, GFG/NUDT6 is rapidly translocated from cytoplasm to nucleus in response to mitogenic stimulation or stress, suggesting a key role in the regulation of cellular proliferation (26). We showed here that, although GFG protein was localized to the nucleus of occasional cells of the proliferative basal/parabasal layers of normal esophageal squamous epithelium, GFG protein was detected in 63% of esophageal tumor cell nuclei. Although we cannot rule out the possibility that the protective effect of FGF-AS is mediated by actions of GFG, the lack of association between GFG protein expression and recurrence or survival suggests that the protective effect is likely to be the result of the known post-transcriptional effects of the antisense RNA on FGF-2 expression (11).

Despite the striking changes reported recently for the epidemiology of esophageal cancer (1–3), esophageal cancer is a relatively uncommon tumor in North America, with incidence rates generally of <10 per 100,000 population. Given the rarity of this malignancy, the 48 tumors studied is a relatively large series, which is representative of the provincial population. Additional strengths of this study include that all tumors were treated in a consistent manner by a single university-based surgeon and that no patient received preoperative chemotherapy or radiation therapy, that all tumors were well staged pathologically with primary esophageal adenocarcinomas defined according to strict clinicopathologic criteria (22, 23, 25), and that follow-up and outcomes data were complete for all patients.

Because of high rates of distant metastatic failure (89% in our series; ref. 25) after potentially curative esophageal resection, there has been considerable recent interest in the use of systemic chemotherapy in combination with surgery or radiation therapy (49). However, conflicting results have been obtained from a limited number of randomized controlled clinical trials (50–53), with the conclusion that only modest survival advantage has been achieved using this approach (54, 55). As FGF-2 has been shown recently to modulate sensitivity of tumor cells to various cytotoxic agents (56, 57), we have evaluated recently the effect of FGF-2 on chemosensitivity of human esophageal adenocarcinoma cell lines exposed to cisplatin, a chemotherapeutic agent widely used in current clinical practice. Our preliminary data indicate that exogenous FGF-2 confers resistance to cisplatin in Bic-1, which normally does not express FGF-2 (Fig. 2), whereas no effect was seen in Seg-1 (58).

In summary, we characterized the expression of FGF-2 and its natural antisense mRNA in a well-defined series of surgically resected human esophageal cancers and have shown that overexpression of FGF-2 mRNA, by comparison with tumors underexpressing FGF-2, was associated with significantly increased risk for tumor recurrence and reduced survival. These observations have potential clinical application to improve the accuracy of tumor staging, for prognosis, and to develop a rational basis for use of chemotherapy in the treatment of esophageal malignancy (58). When the effects of FGF-2 and FGF-AS were considered simultaneously, the association of FGF-2 mRNA overexpression with recurrence and mortality was even more pronounced, whereas FGF-AS mRNA overexpression, but not its cognate protein product, ameliorates this risk. These data support the hypothesis that FGF-AS is a novel tumor suppressor that modulates the effect of FGF-2 expression. Lack of association between GFG protein expression and recurrence or survival suggests that the protective effect may be the result of post-transcriptional effects of the antisense mRNA on FGF-2 expression.

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