Survivin, a potential biomarker in the development of Barrett’s adenocarcinoma

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Background. Survivin, a member of the inhibitor-of-apoptosis family, is reported to be overexpressed in esophageal cancer but no data are available about its status in the metaplastic/dysplastic sequence. The aim of this study was to measure survivin gene expression in normal squamous/columnar epithelium and in the various stages of development of Barrett’s adenocarcinoma.

Methods. Endoscopic biopsy or operative specimen samples from 5 tissue types were analyzed: (1) squamous epithelium from 3 cm above the gastroesophageal junction in patients with a negative pH study and no histologic injury (n = 17, pH- control); (2) antral tissue from patients with no evidence of Barrett’s, dysplasia, or cancer (n = 29, antral control); (3) specialized intestinal metaplasia from patients with Barrett’s esophagus (n = 16; Barrett’s group); (4) low- or high-grade dysplasia (n = 12, dysplasia group), and (5) adenocarcinoma (n = 45 cancer group). After laser-capture microdissection cellular RNA was extracted from each tissue and reverse transcribed to complementary DNA. Expression levels of survivin were measured by reverse-transcription polymerase chain reaction.

Results. Survivin gene expression was greater in columnar (antral) compared with squamous (pH-) control tissues (P = .03). Expression in quiescent Barrett’s epithelium was similar to both control tissues. Expression levels in dysplastic epithelium were greater than in squamous control (P = .01) and Barrett’s tissues (P = .04), but not higher than columnar control tissues, whereas expression in adenocarcinoma was greater than all tissues except dysplasia (P < .001).

Conclusions. Survivin expression may be a biomarker in the development of Barrett’s adenocarcinoma that is able to distinguish between quiescent Barrett’s, dysplastic Barrett’s, and Barrett’s adenocarcinoma. (Surgery 2005;138:701-7.)

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BARRETT’S ESOPHAGUS, in which the normal squamous epithelium of the distal esophagus is replaced with metaplastic specialized intestinal-type epithelium, is an acquired condition secondary to chronic gastroesophageal reflux disease. 1,2 Its significance lies in the fact that it is the major predisposing factor to esophageal adenocarcinoma. The development of esophageal adenocarcinoma is known to be a multistep process that arises from intestinal metaplasia of the distal esophagus through a metaplasia-dysplasia-adenocarcinoma sequence. 1,2 Recent biotechnologic advances have elucidated many of the molecular and cytologic events associated with the development of Barrett’s adenocarcinoma including alterations in p53 and p16 genes, decreased apoptosis, cell-cycle abnormalities, and aneuploidy. 3 The potential to use these genetic changes to predict which patients with Barrett’s esophagus will develop malignancy before histologic changes are apparent remains a major, although as yet elusive, goal.

Survivin, a member of the inhibitor-of-apoptosis gene family, has important functions in both cell
division and the upstream initiation of mitochondrial-dependent apoptosis.4,5 Although it virtually is undetectable in most terminally differentiated normal tissues, recent studies have shown that survivin is overexpressed in several cancer types, including esophageal.5,6 Studies of survivin in squamous cell esophageal cancer have suggested it may be a prognostic factor.6,9 Limited data in esophageal adenocarcinoma indicate that overexpression correlates with more aggressive neoplasms.4,10 Overexpression of survivin has been shown to correlate with shorter survival times, accelerated recurrence rates, and resistance to chemotherapy. Given these observations, it has been suggested that survivin participates in tumor progression rather than in the initial steps of carcinogenesis.5

Survivin expression in the metaplasia-dysplasia-adenocarcinoma sequence and whether it may be a potential biomarker to predict the development of esophageal adenocarcinoma is unknown. The purpose of the present study was to investigate expression levels of survivin at different stages of the development of esophageal adenocarcinoma using 2 recent technologic methods—laser-capture microdissection and quantitative real-time polymerase chain reaction (PCR).

METHODS

Tissue samples for semiquantitative real-time PCR. Tissue samples (n = 142) were obtained from endoscopy or operative specimens from 100 patients with foregut symptoms and were snap-frozen immediately in liquid nitrogen. Samples containing Barrett’s metaplasia, dysplasia (high- or low-grade), or cancer were separated into 3 different groups based on routine histology: (1) Barrett’s group: 23 samples from 16 patients showing intestinal metaplasia; (2) dysplasia group: 18 samples from 12 patients showing intestinal metaplasia and either low- (n = 4) or high-grade dysplasia (n = 8); and (3) carcinoma group: 55 samples from 45 patients showing esophageal adenocarcinoma. The gene expression levels in patients with multiple biopsy examinations were averaged.

Both squamous and columnar epithelium was collected as normal controls. Endoscopic biopsy specimens were classified as follows: (1) pH-squamous control group: 17 endoscopic biopsy samples of squamous epithelium were taken 3 cm above the gastroesophageal junction from 17 patients with a negative pH study and no histologic evidence of mucosal injury; (2) antral control group: 1 endoscopic biopsy sample of normal columnar antral tissue was obtained from each of 29 patients without evidence of Barrett’s, dysplasia, or cancer. No patients had prior foregut operations or prior neoadjuvant therapy.

Approval for this study was obtained from the Institutional Review Board of the University of Southern California Keck School of Medicine and written informed consent was obtained from participating patients.

Histologic definitions of tissue samples analyzed in this study. All pathologic examinations were performed by a single pathologist (P.T.C.) with specialized expertise in gastrointestinal pathology and Barrett’s-associated adenocarcinoma. The following criteria were used to define each tissue type we analyzed. The first criterion was normal squamous epithelium, which was defined by the absence of criterion for the diagnosis of reflux esophagitis, and when there was no other pathologic lesion such as infection, dysplasia, or malignancy. Second, columnar epithelium of the antrum: antral mucosa was characterized by shorter, more tortuous, and less densely packed glands in comparison with the gastric body and fundus, lined by a single cell type that is similar or identical to the mucous cells of fundic glands. Third, intestinal metaplasia, which was defined by the presence of a cardiac mucosa that contained well-defined goblet cells on a hematoxylin-eosin-stained section. We did not include Alcian blue positivity in the absence of goblet cells in our definition of intestinal metaplasia. Fourth, dysplasia: dysplastic Barrett’s was defined by the presence of cytologic abnormalities of dysplasia in the columnar epithelium involving both surface and foveolar/glandular regions. For low-grade dysplasia, the cytologic changes exceeded those associated with repair but did not satisfy criterion of high-grade dysplasia. High-grade dysplasia was defined by the presence of severe cytologic abnormality associated with either gland complexity or loss of nuclear polarity. The final criterion was adenocarcinoma, which was defined by the presence of invasive malignant glands.

Microdissection. Frozen samples were embedded in optimal cutting temperature compound (OCT; Sakura Finetek U.S.A., Inc., Torrance, Calif) and cut into serial sections with a thickness of 20 μm. Sections were mounted on uncoated glass slides and stored at −80°C. Representative sections from the beginning, middle, and end of sectioning were stained with hematoxylin-eosin for histologic analysis. Each section was air-dried, fixed in 70% ethanol for 3 minutes, and washed in H2O for 2 minutes. They were stained with nuclear fast red (NFR; American MasterTech Scientific, Inc., Lodi, Calif) for 10 seconds and again washed in
H₂O for 30 seconds. Samples then were dehydrated in a stepwise manner with 70% ethanol, 95% ethanol, and 100% ethanol for 30 seconds each, followed by incubation in xylene for 5 minutes and complete air-drying. Normal esophageal samples or normal gastric samples were dissected from the slides using a scalpel if the histology was homogeneous and contained more than 90% tissue of interest. All other sections were isolated selectively by laser capture microdissection (P.A.L.M. Microsystem; Leica, Wetzlar, Germany) according to the standard procedure.¹¹

**Table I. Primers and probes sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gen Bank accession number</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Taqman probe (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_001101</td>
<td>GAGCGCGGCCTACACGCTT</td>
<td>TCCTTAATGTCAAGCACGATT</td>
<td>ACCACACGCGCGGAGCGG</td>
</tr>
<tr>
<td>Survivin</td>
<td>NM_001168</td>
<td>TGCCCCGAGGTTGCC</td>
<td>CAGTTCTTGAAAGTGAAGATGC</td>
<td>CCTGGCAGCCCTTCTCAAGGACC</td>
</tr>
</tbody>
</table>

Quantitative PCR in Table I

RNA isolation and complementary DNA synthesis. Tissue samples to be extracted were placed in a 0.5-mL, thin-walled tube containing 400 μL of 4 mol/L dithiothreitol-guanidinium isothiocyanate (GITC)/sarc (4 mol/L guanidinium isothiocyanate, 50 mmol/L Tris-HCl, pH 7.5, 25 mmol/L ethylenediaminetetraacetic acid) (15577-018; Invitrogen Carlsbad, Calif). The samples were homogenized and an additional 60 μL of GITC/sarc solution was added. They were heated at 92°C for 30 minutes and then transferred to a 2-mL centrifuge tube. A total of 50 μL of 2 mol/L sodium acetate was added at pH 4.0, followed by 600 μL of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 seconds, placed on ice for 15 minutes, and then centrifuged at 13,000 rpm for 8 minutes in a chilled (8°C) centrifuge. The supernatant was poured off carefully and placed in a 1.5-mL centrifuge tube. Glycogen (10 μL) and 300 to 400 μL of isopropanol were added and the samples were vortexed for 10 to 15 seconds. The tubes were placed at −20°C for 30 to 45 minutes to precipitate the RNA. The samples then were centrifuged at 13,000 rpm for 7 minutes in a chilled (8°C) centrifuge. The supernatant was poured off and 500 μL of 75% ethanol was added. The tubes were centrifuged at 13,000 rpm for 6 minutes in a chilled (8°C) centrifuge. The supernatant was poured off carefully so as not to disturb the RNA pellet and the samples were quickspun for 15 seconds at 13,000 rpm. The remaining ethanol was removed with a 20-uL pipette and the samples were air-dried for 15 minutes. The pellet was resuspended in 50 μL of 5 mmol/L Tris.

**Real-time PCR quantification of messenger RNA expression.** Quantification of survivin and an internal reference gene (β-actin) was performed using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, Calif). The PCR reaction mixture consisted of 1,200 nmol/L of each primer, 200 nmol/L probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol/L each deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxymethylidine triphosphate, 3.5 mmol/L MgCl₂, and 1x TaqMan Buffer A containing a reference dye, to a final volume of 20 μL (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The primers and probes used are listed in Table I.

TaqMan measurements yield cycle threshold (Ct) values that are inversely proportional to the amount of complementary DNA in the tube (ie, a higher Ct value means that more PCR cycles are required to reach a certain level of detection). Gene expression values (relative messenger RNA [mRNA] levels) are expressed as ratios (differences between the Ct values) between the gene of interest and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

**Statistical analysis.** Survivin mRNA expression levels in the pH control, antral control, Barrett’s, dysplasia, and cancer groups were compared by using the Kruskal-Wallis test to identify significant differences among all histologic groups. The Mann-Whitney U test was used to identify significant differences between 2 groups. To assess the associations between the expression level of survivin and different clinicopathologic variables (sex, T stage, lymph node involvement, histologic grading, International Union against Cancer [UICC]-stage) the Mann-Whitney U test was performed. Statistical significance was set at a P level of .05.
RESULTS

The study population included 29 women and 71 men. The median age of the patients was 57 years (range, 23-86 y). The median value and range of expression levels of survivin determined by semi-quantitative reverse-transcription PCR analysis of the 5 tissue groups are listed in Table II (142 tissue samples from 100 patients) and shown in the figure.

Survivin gene expression levels differed among all histologic groups analyzed (P < .001). Survivin mRNA levels were lowest in squamous epithelium from pH- patients (median, 4.22) and highest in patients with adenocarcinoma (median, 15.19).

When compared with normal squamous epithelium, survivin expression was greater in dysplasia and cancer tissue (P < .001). There was a stepwise increase in survivin expression throughout the metaplasia-dysplasia-adenocarcinoma sequence in that expression levels in intestinal metaplasia were less than dysplasia (P = .04), and dysplasia expression levels were less than adenocarcinoma (P = .06). Antral control tissue had survivin expression less than cancer (P < .001), but not different than Barrett’s or dysplasia.

No significant correlation was found between intratumoral survivin gene expression levels and different clinico-pathologic variables (sex, T stage, lymph node involvement, histologic grading, UICC stage; Table III). There was a trend for patients with well-differentiated to moderately differentiated neoplasms to have lesser survivin gene expression than patients with poorly differentiated neoplasms (median, 13.74 vs 24.86; P = .11). Because of the varied treatment regimens of the cancer patients included in this study, analysis of survivin gene expression levels and clinical outcome were not performed.

DISCUSSION

The balance between cell division and cell loss is important for the maintenance of normal human tissue. An imbalance promoting either increased proliferation and/or decreased cell loss results in the uncontrolled cell growth characteristic of carcinogenesis. Recent studies have shown that the
deregulation of apoptosis is an essential event in the development of Barrett’s-associated adenocarcinoma and that the anti-apoptotic gene survivin is upregulated in esophageal cancer.3,5-10

This study analyzed gene expression levels of survivin, a member of the inhibitor-of-apoptosis family, in the esophageal metaplasia-dysplasia-adenocarcinoma sequence. Two recent technologic methods were used: laser-capture microdissection and quantitative real-time PCR, with the aim of maximizing the tissue-specific accuracy and reliability of the gene expression data. We identified a 3-fold increase in median survivin mRNA levels in esophageal adenocarcinoma when compared with normal squamous epithelium. These results are consistent with 3 previous studies, all using quantitative reverse-transcription PCR, in which survivin gene expression levels were found to be 1.5- to 4.0-fold higher in esophageal squamous cell cancer compared with adjacent normal squamous epithelium.6,8,9

The data also show a stepwise increase in survivin expression from metaplastic to dysplastic to neoplastic tissue, suggesting survivin may be an effective biomarker in this carcinogenic sequence. These findings are consistent with recent studies describing the reduction of apoptotic indices in dysplasia and adenocarcinoma compared with intestinal metaplasia of the distal esophagus.12,13 Whittles et al12 reported a decrease of apoptosis and conversely an increase of proliferation in the progression of Barrett’s adenocarcinoma. Van der Woude et al13 reported an increase of the anti-apoptotic factor Bcl-xl and a decrease of the pro-apoptotic factor Bax in the Barrett’s metaplasia-dysplasia-carcinoma sequence, and suggested that the balance in this malignant progression switches to an anti-apoptotic phenotype.

Unlike most studies to date, we also used gastric antral biopsy specimens as a columnar epithelial control tissue. This helps to ascertain that any increase in survivin mRNA levels are not simply caused by a change in the epithelial type (squamous to columnar). Although antral tissue had significantly greater survivin expression than squamous epithelium, a 2-fold increase in survivin gene expression levels in adenocarcinoma remained, as did the progressive increase from metaplasia to dysplasia to adenocarcinoma. We believe this indicates that the upregulation of this anti-apoptotic gene in esophageal carcinogenesis is not mainly a reflection of a change in the type of epithelium. The reason for the difference in survivin expression between normal squamous epithelium and normal columnar epithelium is unknown.

Previously available data of survivin expression in metaplastic and dysplastic tissue of the distal esophagus are very limited. To our knowledge, the work of Grabowski et al7 assessing survivin expression by immunohistochemistry in squamous dysplastic epithelial cells is the sole report to date. Survivin staining was distinct in dysplastic tissue from normal squamous epithelium and esophageal squamous cell cancer. In cancer types other than esophageal, however, the role of survivin in premalignant epithelia is described in more detail and provides evidence for the importance of survivin in the early events of tumorigenesis. Kawasaki et al14 assessed the role of survivin in colorectal carcinogenesis, analyzing survivin expression in hyperplastic polyps, adenomas with low-grade dysplasia, adenomas with high-grade dysplasia, and carcinomas by immunohistochemistry. This study indicated that the immunoreactivity of survivin significantly increases during the transition from adenoma with low-grade dysplasia to high-grade dysplasia and carcinoma. In addition, Lin et al15 showed that the expression of survivin protein in human colorectal carcinogenesis is an essential event in the early stages and plays an important role in the transition to cancer. Kim et al16 described an increase in survivin protein expression in the progression from low-grade squamous intraepithelial to invasive uterine cervical cancer. Finally, Zhang et al17 investigated the survivin protein expression in the development of breast cancer.17 The immunohistochemical reactivity of survivin was 4.2% in normal mammary epithelium, 5.4% in cystic hyperplasia, 42.7% in...
atyypical hyperplasia, and 72.3\% in breast carcinoma.

The correlation with clinicopathologic characteristics and the prognostic significance of increased survivin expression remains controversial. We did not find a correlation between intratumoral survivin gene expression levels and most clinicopathologic parameters. The sole exception was a trend toward lower expression levels in patients with well-differentiated to moderately differentiated neoplasms when compared with poorly differentiated ones. These findings are in agreement with a recent study from Ikeguchi and Kaibara describing no correlation in esophageal cancer between survivin gene expression levels and histologic type of neoplasms, lymph node metastasis, and depth of tumor invasion. A similar lack of correlation between survivin expression and clinicopathologic parameters has been described in non–small-cell lung and breast cancer.\(^6,7,9\) This is in contrast to studies of esophageal cancer that reported correlations of survivin expression with lymph node metastasis and response to chemotherapy, lymph node metastasis and UICC stage, and overall survival and histologic type.\(^6,7,9\)

In summary, we have used laser-capture microdissection and real-time reverse-transcription PCR to suggest that survivin mRNA levels increase in a stepwise manner during the progression through the Barrett’s metaplasia-dysplasia-adenocarcinoma sequence. These data indicate that survivin has a promising role as a biomarker in disease progression. Its role as a prognostic factor of esophageal adenocarcinoma remains to be determined. However, numerous genetic pathways are known to be involved in esophageal carcinogenesis. In the future, survivin gene expression may be used as a member of a panel of genes that can be used to stratify patients for disease progression.

REFERENCES

DISCUSSION
Dr E. Christopher Ellison (Columbus, Ohio). The hypothesis that survivin expression correlates with progression from Barrett’s metaplasia/dysplasia to cancer is substantiated by the findings in this study. The observation is significant and may help us better understand the pathogenesis of Barrett’s related adenocarcinoma of the esophagus, but also has possible prognostic and therapeutic implications.

Since many patients with Barrett’s esophagus have reflux symptoms and are taking proton pump inhibitors, I am interested in learning whether you controlled for this variable.
It has been observed with other cancers that survivin expression may create resistance to chemotherapy. Did you notice a differential response rate with survivin overexpression in those patients receiving neoadjuvant or adjuvant chemotherapy?

Finally, could you speculate on the possibility of gene therapy directed at survivin?

Dr Daniel Vallbohmer. Our patients came in to 2 weeks after the stop of PPI therapy. However, we had very interesting findings from our working group in the last month showing the difference in gene expression before and after antireflux surgery that is allowing us to make a study in an animal model and in a human model to really look at the correlation between PPI therapy and the change in gene expression.

The small number of patients didn’t give us the opportunity to see a significant correlation between the effect of chemotherapy and survivin gene expression.

We think that survivin gene expression could be used in the future as more than a biomarker, although we have really to have a study more patients.