RESEARCH ARTICLE

Evidence for Enhanced Telomerase Activity in Barrett's Esophagus with Dysplasia and Adenocarcinoma

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Abstract

Background: Dysplasia and adenocarcinoma developing in Barrett's esophagus (BE) are not always endoscopically identifiable. Molecular markers are needed for early recognition of these focal lesions and to identify patients at increased risk of developing adenocarcinoma. The aim of the current study was to correlate increased telomerase activity (TA) with dysplasia and adenocarcinoma occurring in the setting of BE. Materials and Methods: Esophageal mucosal biopsies were obtained from patients (N=62) who had pathologically verified BE at esophagogastroduodenoscopy (EGD). Mucosal biopsies were also obtained from the gastric fundus as controls. Based on histopathology, patients were divided into three groups: 1) BE without dysplasia (n=24); 2) BE with dysplasia (both high grade and low grade, n=13); and 3) BE with adenocarcinoma (n=25). TA was measured by a PCR-based assay (TRAPeze® ELISA Telomerase Detection Kit). Statistical analyses were performed using one-way ANOVA and post-hoc Bonferroni testing. Results: TA was significantly higher in biopsies of BE with dyplasia and BE with adenocarcinoma than in BE without dysplasia. Subgroup analyses did not reveal any significant correlations between TA and patient age, length of BE, or presence of gastritis. Conclusions: Telomerase activity in esophageal mucosal biopsies of BE may constitute a useful biomarker for the early detection of esophageal dysplasia and adenocarcinoma.

Keywords: Barrett's - telomerase activity - esophagus - dysplasia - adenocarcinoma

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Introduction

Barrett's esophagus (BE) is characterized by the replacement of the normal squamous epithelium of the esophagus with metaplasia of columnar epithelium as a result of chronic gastroesophageal reflux disease (GERD). It is generally accepted that metaplastic cells in BE progress via a multistep process of genetic alterations, through histologic changes from low-grade dysplasia (LGD) to high-grade dysplasia (HGD), and finally to invasive adenocarcinoma (Wang et al., 2008; 2011; Milind et al., 2012). While metaplastic BE cells have a malignant predisposition and confer a significantly increased risk of developing adenocarcinoma (Wang et al., 2008; Milind, 2012), only a small subset of patients with BE will progress to high-grade dysplasia and esophageal cancer.

Current management of patients with BE includes routine endoscopic surveillance with random biopsies of esophageal columnar epithelium to identify patients with neoplastic transformation at an earlier, potentially more curable stage. Several methods, including standardized surveillance and biopsy protocols (Levine et al., 2000; Wang et al., 2008) and the use of methylene blue chromoendoscopy (Canto et al., 2000; Ngamruengphong et al., 2009), have been proposed to enhance the detection of dysplasia and adenocarcinoma in patients with BE. Most investigators agree that a defined protocol of obtaining biopsies in all four quadrants, every 1-2 cm, and in areas of irregular mucosa is optimal (Wang et al., 2008). However, despite these standardized techniques, 31-38% of patients still have a missed diagnoses of adenocarcinoma in the setting of HGD and BE (Falk et al., 1999). This ellipsis may result in part from the clonal nature of neoplastic transformation in BE. Although only a subset of patients with high-grade dysplasia progress to develop invasive adenocarcinoma, our inability to accurately identify such patients has led to recommending esophagectomy in HGD patients to remove all at-risk mucosa as well as any occult adenocarcinoma (Luna et al., 2012). Clearly, additional biomarkers of neoplastic transformation are needed in order to detect these changes earlier in the natural history of BE.

Telomeres, which are located at the ends of chromosomes, comprise over 1000 short base sequences

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(TTAGGG) that play a role in chromosomal protection and replication. During normal somatic cell division, telomeres shorten with each round of DNA replication. After many replicating cycles, the telomeric ends lose their protective function, and cells are unable to divide and may die as a result of apoptosis (Blackburn et al., 2010; Shay et al., 2011). Non-senescent germline or stem cells maintain their replicative ability by means of telomerase. Telomerase is a ribonucleoprotein that adds nucleotide repeats to the ends of telomeres, counteracting the progressive loss of DNA that occurs during replication (Harley et al., 1996; Shay et al., 2011). Activation of telomerase results in maintenance of telomere length, immortalization of cells, and the eventual development of a malignant clone (Harley et al., 1996; Blackburn 2010). Telomerase activity is low or undetectable in normal somatic tissues in which telomeres are not extended and, therefore, undergo progressive shortening with cell division (Shammas et al., 2008). Telomerase activity is seen in 85-95% of biopsies of various tumor types but is absent in normal somatic cells and in normal tissue adjacent to cancers (Kim et al., 1994). Several studies have shown that the detection of telomerase activity can be used to distinguish malignant from normal tissue in various tumor types (Dalbagni et al., 1997; Takubo et al., 1997; Pearson 2000; Zhang et al., 2000; Zheng et al., 2000; Shay, 2011). It has also been suggested that high telomerase activity produces immortal clones in Barrett's and may lead to its progression from dysplasia to Barrett's associated adenocarcinoma through accumulation of other mutations (Meyerson et al., 1997).

Based on these findings, we postulated that the detection of telomerase activity could be used to identify neoplastic transformation in esophageal mucosal biopsies of BE. The purpose of our study was to determine whether telomerase activity as a biomarker, correlated with the occurrence of dysplasia or adenocarcinoma in BE.

Materials and Methods

Esophageal tissue biopsies were obtained and snap-frozen during EGD from 62 patients with a known diagnosis of BE. Using a standardized protocol, four quadrant mucosal biopsies were obtained at 2 cm intervals along the entire length of BE. At esophagogastroscopy, additional biopsy specimens were taken from the gastric fundus to serve as columnar epithelial cell controls. All specimens were examined by a pathologist considered to be an expert in BE.

Specimens were retrieved for analysis of telomerase activity. Clinicopathologic data were obtained from available medical records which included patient's age, length of BE segment, presence of inflammation in BE, presence of gastritis in the stomach, and use of proton pump inhibitors (PPIs).

After evaluation, patients were divided into three groups: 1) BE without dysplasia (n=24); 2) BE with dysplasia (n=13); and 3) patients with adenocarcinoma (n=25).

Extract preparation

80 mg of tissue were homogenized in 200 µl 1X CHAPS lysis buffer containing 0.15 μl ribonuclease inhibitor. The homogenized sample was incubated on ice for 30 minutes and then centrifuged at 1500 rpm for 20 min at 4°C. Supernatants were transferred to fresh tubes and protein concentrations were measured using a protein assay kit from Bio-Rad based on a modified Lowry assay (Hercules, CA cat. # 500-0111).

Telomerase assay

Telomerase activity was determined using the TRAPezeTM ELISA telomerase detection kit from Intergen (Purchase, NY, cat. #S7750 kit). With some modifications, the methodology used was followed as described in the manufacturer's protocol. Each PCR reaction consisted of 1µg protein, 1 X TRAP Reaction mix, dH_2O and 2 units Taq polymerase in a final volume of 50 µl.

All tubes were placed into a Perkin-Elmer Gene Amp PCR system 2,400 and incubated at 30°C for 30 minutes. 35 cycles of PCR were then performed, each cycle at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds following a final extension at 72°C for 3 minutes. The ELISA portion of the assay was followed exactly as described in the included protocol. The final absorbance was measured at 450 nm and 650 nm.

Controls

For each PCR reaction, controls were included. One tube was a primer-dimer/PCR contamination control to which only 1X CHAPS buffer was added. Two tubes were telomerase-positive controls, one containing 1,000 positive control cells supplied by the manufacturer as part of the kit, and the other consisting of 1,000 LS 180 tissue culture cells. The cells had been previously extracted as described above under "extract preparation," aliquotted, and frozen. A third control was a PCR/ELISA-positive sample included with the kit and containing 1µl of TSR8, a synthetic oligonucleotide with 8 telomeric repeats.

Data analysis

For each PCR and ELISA assay, data was analyzed with controls. Background absorbance at 650 nm was subtracted from 450 nm absorbance. The mean absorbance of 1X CHAPS buffer was also subtracted from each sample (n=33; mean value=0.251).

Statistical analysis

Measurements of telomerase activity between groups was analyzed by one-way ANOVA and post-hoc Bonferroni testing. To identify significant relationships of variables such as age or length of BE to telomerase activity a Pearson bivariate correlation was performed. Analyses of the relationship of the presence of inflammation, gastritis or the use of PPIs to telomerase activity were performed by independent t-test sampling. A p-value of <0.05 was considered significant.

Results

There were 62 patients with BE who had esophageal mucosal biopsy specimens obtained for evaluation. These

specimens were separated into three groups: BE without dysplasia (n=24); BE with dysplasia (n=13); and BE with adenocarcinoma (n=25). The comparisons of patient's age, length of BE, presence of gastritis, and use of PPIs for each group is shown in Table 1.

Telomerase activity in esophageal biopsies (Table 2 and Figure 1): Telomerase activity as significantly higher in BE with dysplasia (p=0.015) than in BE without dysplasia. Further, patients with BE with adenocarcinoma also had significantly higher telomerase activity than patients with BE without dysplasia (p=0.023). However, telomerase activity in BE with dysplasia patients was not statistically different from those with BE and adenocarcinoma.
Telomerase activity in gastric fundic biopsies was significantly lower than telomerase activity in the corresponding esophageal mucosal biopsies in each group (Figure 1). However, as seen in Figure 1, telomerase activity in gastric fundic biopsies from patients with BE and adenocarcinoma was significantly higher compared to patients with BE without dysplasia (p<0.03). No significant differences in telomerase activity were seen between BE patients with or without dysplasia (p=0.35) or between BE with dysplasia patients and BE with adenocarcinoma patients (p=0.40).

Clinical parameters

The relationships between each clinical parameters and telomerase activity were individually assessed. There was no correlation between patient age or length of BE and telomerase activity among the three groups (Tables 3 and 4). Similarly, the presence of gastritis did not correlate with telomerase activity in any of the three groups (Table 5).

Inflammation

Patients with BE without dysplasias were also analyzed for the presence of inflammatory cells within their esophageal mucosal and gastric fundic biopsies. In this group, 17 patients were found to have inflammatory cells in their esophageal biopsies, while 8 patients did not. Esophageal telomerase activity tended to be higher in patients with inflammation (1.28 nm) than in those without inflammation (0.78 nm), although this value did not achieve statistical significance (p=0.12). No difference in telomerase activity in gastric fundic biopsies was seen between patients with or without inflammatory cells in the esophagus (0.12 vs. 0.14, respectively; p=0.83).

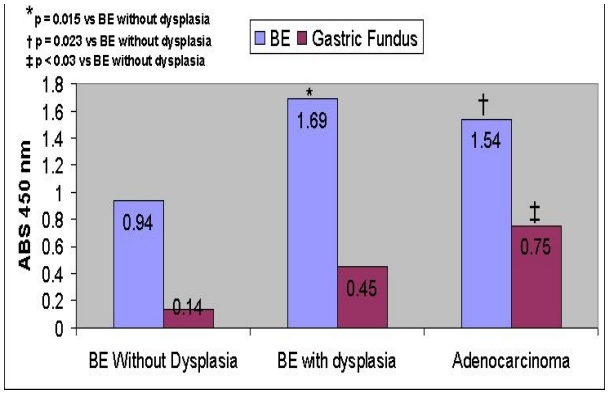

Figure 1. Telomerase Activity in Barrett's Esophagus and Gastric Fundus among the Three Subgroups

Table 1. Clinical Parameters of Three Groups

Groups	Age±SEM (years)	Use of PPI Yes:No (n=32)*	Gastritis Yes:No (n=32)*
BE without dysplasia (n=24)	62.3±2.7	16:01	7:10
BE with dysplasia (n=13)	65.6±2.3	8:01	6:03
Adenocarcinoma (n=25)	63.8±2.2	4:02	5:01

Table 2. Telomerase Activity in Esophageal Biopsies

Groups intervals	Telomerase Activity (Abs 450nm±SEM)	95% Confidence Lower	Upper
BE without dysplasia (n=24)	0.86±0.15	0.66	1.22
BE with dysplasia (n=13)	1.39±0.24	1.27	2.12
Adenocarcinoma (n=25)	1.58±0.52	1.18	1.89

Table 3. Telomerase Activity and Relationship

Clinical Parameter		BE without dysplasia (n=24)	BE with dysplasia (n=13)	BE and adenocarcinoma (n=25)
Age:				
Age±SEM (years)		62.3±2.7	65.6±2.3	63.8±2.2
Telomerase Activity		0.86±0.15	1.39±0.24	1.58±0.52
Correlation Coefficient		0.12	0.2	-0.78
p value		0.58	0.57	0.12
Length of Barrett's Esophagus (BE):				
Length (cms±SEM)		5.3±0.7	8.9±1.6	5.8±1.3
Telomerase Activity		0.86±0.15	1.39±0.24	1.58±0.52
Correlation Coefficient		0.27	-0.39	0.3
p value		0.21	0.26	0.62
Gastritis:	+	0.93±0.35	1.39±0.30	1.82±0.32
	-	0.81±0.21	1.91±0.49	1.99
	p value	0.76	0.37	0.84

Discussion

Mammalian telomerase is a holoenzyme consisting of three major subunits: the RNA subunit (human telomerase RNA, hTR), catalytic subunit (hTERT) and associated protein (human telomerase-associated protein 1, hTP1). Previous studies have attempted evaluating function of telomerase among patients with BE using these different components in order to detect early neoplastic transformation and/or dysplasia (Kim et al 1994; Morales et al., 1998; Lord et al., 2000; Barclay et al., 2005). For instance, Morales et al. (1998) used in-situ hybridization to detect human telomerase RNA (hTR) in formalin-fixed, paraffin-embedded esophageal biopsies from 48 patients with BE and in surgical resection specimens from 11 patients with esophageal cancer. Moderate hTR expression was noted in 70% of patients with BE without dysplasia and in 90% of BE patients with low-grade dysplasia (Morales et al., 1998). All patients with high-grade dysplasia or adenocarcinoma manifested strong expression of hTR in their study. The greatest magnitude of interval increase in hTR expression occurred between mucosal biosies with low-grade and high-grade dysplasia, suggesting that telomerase activation could reflect the early emergence of progression toward adenocarcinoma in patients with BE. However, the expression of hTR by in-situ hybridization

does not always parallel telomerase activity and has been detected in cells that do not normally show telomerase activity (Feng et al., 1995; Avilion et al 1996; Blasco et al 1996; Barclay et al., 2005). Whereas many cell lines and tumors had both increased hTR and telomerase activity, Avilion et al. (1996) found that hTR was present in cell lines and tissues that lacked telomerase activity, indicating that the RNA is not limiting for telomerase activity, and that the RNA component is not a good predictor of the presence of enzyme activity. Consequently, hTR is a less reliable method for determining telomerase activity and hence did not gain widespread acceptance.

Studies have suggested that, in some cancers, human telomerase catalytic enzyme subunit (hTERT) mRNA expression is the main determinant of telomerase activity (Meyerson et al., 1997; Bodnar et al 1998; Nakayama et al., 1998; Going et al., 2004; Clement et al., 2006; Shammas et al., 2011), as expression of hTERT is restricted to cells with telomerase activity only (Avilion et al., 1996). Lord et al. (2000) evaluated hTERT expression in 13 patients with Barrett's metaplasia, 7 with Barrett's dysplasia, and 14 with esophageal adenocarcinoma. Their findings showed that hTERT expression was significantly higher in both adenocarcinoma and dysplasia groups as compared to patients without dysplasia. Furthermore, the authors noticed significantly higher levels of hTERT expression in normal esophageal mucosa from patients with adenocarcinoma versus controls without cancer, suggesting that an oncogenic telomerase "field" effect may exist. More recently, Barclay et al. (2005) evaluated telomerase activity along with hTERT and splice variants in BE and adenocarcinoma and suggested a significant increase in telomerase activity occurring in patients with BE and adenocarcinoma. However, neither hTERT mRNA levels nor hTERT mRNA splicing patterns correlate with telomerase enzyme activity, as it depends on posttranscriptional and posttranslational modification of hTERT, which includes phosphorylation of hTERT protein, assembly into telomerase holoenzyme, and its association with other proteins such as hsp90 and p23 etc (Shammas et al., 2008).

To avoid the limitations of hTR and hTERT expression, we directly measured telomerase activity using the telomere repeat amplification protocol (TRAPeze) (Kim et al., 1997), a highly sensitive polymerase chain reaction (PCR)-based assay. TRAPeze assay evaluates for telomerase products, thus justifying the description as a test of telomerase activity. The telomerase activity in our study was significantly higher in BE segments with dysplasia and/or adenocarcinoma, which supports the findings of increased expression of telomerase RNA (Morales et al., 1998) and hTERT (Barclay et al., 2005) expression by other authors. Furthermore, we measured telomerase activity in the gastric fundus as a means of assessing an "internal control" for tissue samples in each patient. In all cases, the telomerase activity in esophageal biopsies of BE patients was significantly higher than in the gastric fundus (Figure 2). In addition, our finding that gastric fundic telomerase activity in patients with adenocarcinoma was significantly higher than that in patients with BE without dysplasia suggests that this "field effect" may also extend to include the gastric mucosa, supporting a previous similar suggestion by Lord et al. (2000) with their hTERT studies. Given the limitations of visualizing areas of dysplasia or adenocarcinoma and inaccuracy of biopsy sampling, we suggest that increased gastric telomerase activity may serve as an additional biomarker to identify patients with early histologic changes of dysplasia or adenocarcinoma.

In our study, there was only one patient with high-grade dysplasia precluding any comparisons of telomerase activity between patients with high-grade and low-grade dysplasia. While such comparisons would be clinically relevant to identify patients at high risk of progressing to adenocarcinoma, our results did not show a significant increase in telomerase activity occurring between dysplasia and adenocarcinoma. Although very high levels of hTERT expression were seen only in adenocarcinoma patients in one previous study, no significant differences in hTERT expression were found between dysplasia and adenocarcinoma (p=0.17) (Lord et al., 2000). Even in our study, telomerase activity could not differentiate between dysplasia and adenocarcinoma.

In conclusion, our observations support measurement of telomerase activity in esophageal mucosal biopsies of patients with Barrett's esophagus as a potentially useful biological marker to detect early neoplastic transformation. Additional prospective studies are needed to determine the sensitivity and specificity of this biomarker in detecting or predicting dysplasia and adenocarcinoma in Barrett's patients.

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