Photodynamic Therapy of Barrett's Esophagus: Ablation of Barrett's Mucosa and Reduction in p53 Protein Expression after Treatment

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Abstract. *Background: The effectiveness of photodynamic therapy (PDT) for ablation of high grade dysplasia (HGD) in Barrett's esophagus (BE) is typically reported histologically. Following successful PDT, Barrett's mucosa is replaced with neosquamous mucosa. The objective of this study was to compare the expression of p53 protein in neosquamous mucosa as compared to that in HGD samples not treated with PDT. Patients and Methods: The patients were divided into two groups. Group I patients (n=12) had been treated with PDT for HGD and provided 23 biopsy samples of neosquamous mucosa. Group II patients (n=10) had not received any ablative therapies for BE and provided 14 HGD samples. The immunohistochemical (IHC) staining for p53 protein was performed using mouse anti-human monoclonal antibody DO-1. The degree of p53 protein expression in the cell nuclei was scored using an established IHC scoring system (0 for negative samples and range of 2 to 8 for positive samples). Results: The HGD samples showed diffuse strong p53 staining. The median IHC score for HGD was 7.0. The median IHC score for neosquamous mucosa following PDT was 4.0, with positive scores indicating weak staining in the basal layer of the neosquamous samples. There was significantly lower p53 expression in the neosquamous samples compared to that in the HGD samples (p<0.001). Conclusion: Significantly lower p53 protein expression was detected in neosquamous mucosa of patients who had received PDT for HGD, suggesting a decreased risk for neoplastic progression after treatment.*

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Barrett's esophagus (BE) is a premalignant condition associated with chronic acid reflux. A diagnosis of BE with high grade dysplasia (HGD) places the patient in a high risk category for developing adenocarcinoma of the esophagus. Photodynamic therapy (PTD) using porfimer sodium, is a new endoscopic technique for the ablation of HGD in BE (1, 2). Successful PDT treatment, in the presence of effective acid suppression therapy, results in ablation of Barrett's mucosa with replacement by squamous (neosquamous) mucosa over the treated area. Evaluation of the response to PDT is through an aggressive follow-up biopsy protocol with histological examination by an expert pathologist. The histological documentation of neosquamous mucosa following PDT may be interpreted as reduction in risk of progression into cancer. Although PDT can promote the replacement of HGD with neosquamous mucosa in BE, histological documentation alone may not necessarily indicate a lower risk of cancer.

Tumor markers have been suggested as powerful tools for the early detection of potential cancer risk in BE. The p53 tumor suppressor gene is involved in cell growth control, DNA repair, as well as in modulating apoptosis (3-6). The expression of a mutant p53 protein has been linked to neoplastic progression of Barrett's metaplasia (3, 7-13). The immunohistochemical (IHC) technique used for detection of p53 expression is technically simple, economical, quick and correlates well with gene mutation and aneuploidy (4, 8, 10, 11, 14, 15). Wild-type and mutant p53 proteins differ significantly in their nuclear stability. While the half-life of wild-type protein is 20-30 minutes, that of mutant p53 protein is about 12 hours. This results in intranuclear accumulation of the mutant protein to levels that can be detected by IHC. This technique may, therefore, be used to detect the reduction in p53 expression (mutation) after PDT of BE.

The main objective of this study was to measure the expression of p53 protein in neosquamous mucosa (following PDT ablation) and compare it to that of HGD

samples before treatment. Additional samples were analyzed from untreated patients, including normal squamous mucosa, intestinal metaplasia (IM), and low grade dysplasia (LGD).

Patients and Methods

Patient population. The study was approved by the Institutional Review Board of the Thompson Cancer Survival Center, and all the patients signed informed consent for the study. Tissue samples were collected during upper endoscopy procedures of BE patients. Four quadrant biopsies were collected at every 2 cm of treated or untreated Barrett's mucosa. To assure deep biopsy sampling, jumbo biopsy forceps were used in all cases.

The patients (n=22) were divided into two groups. Group I (n=12) included those Barrett's patients who had previously been treated with Photofrin® balloon (Axcan Pharma, Birmingham, AL, USA) PDT for HGD and were being followed for evaluation of their response to the treatment. In one patient, possible superficial adenocarcinoma could not be ruled out before PDT. The follow-up period after PDT ranged from 6 to 47 months with a mean of 20 months. Neosquamous samples were collected from the PDT-treated areas in the Group I patients. All the Group I patients received aggressive proton-pump inhibitors (PPI) therapy to maintain their gastric pH above 4.

Group II included Barrett's patients (n=10) who had not received any ablative therapy and presented with different degrees of dysplasia. All the untreated Barrett's samples with and without dysplasia were obtained from the Group II patients. In addition, normal squamous mucosa samples were collected from Group II patients, all at 20 cm from the dental margin. The Group II patients were also on PPI therapy at the time of their endoscopy to control their acid reflux symptoms. However, gastric pH was not checked routinely in those patients.

A total of 97 samples was analyzed,, 23 samples of neosquamous mucosa from the Group I patients and 14 samples of HGD, 21 samples of LGD, 22 samples of IM, and 17 samples of normal squamous mucosa from the Group II patients. More specimens were collected from the patients in Group II to potentially obtain samples for each dysplastic category of Barrett's mucosa and normal squamous samples.

Sample preparation. Each specimen was fixed in formalin overnight, embedded in paraffin, and stained with hematoxylin and eosin (H&E) (Richard-Allan Scientific, Kalamazoo, MI, USA) using standard histological techniques. Three-micron serial sections were cut and mounted on positively charged slides. After deparaffinization and rehydration, mouse anti-human monoclonal antibody (DO-1) directed against an amino terminal epitope (residues 37-45) of the human p53 protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100) was applied to the tissues using the avidin-biotin-peroxidase complex (IHC) method (Vectastain Elite ABC Kit, Vector, Burlingame, CA, USA), at room temperature. Antigen retrieval was performed by microwaving (1100W) the slides for four periods of 5 minutes each in 500 ml of 0.01 M citrate buffer at pH 6.0. Blockage of endogenous peroxidase and the prevention of non-specific background staining were achieved by incubating the slides with 3% aqueous hydrogen peroxide for 10 minutes. After washing with phosphate-buffered saline (PBS) for 5 minutes, the tissues were blocked with normal serum for 20 minutes, and incubated with each of the antibodies for 60 minutes. After treatment with a biotinylated secondary antibody the slides were incubated with avidin-biotin complex for 30 minutes and washed. The chromogen was developed with 10 mg of 3,3 diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) diluted in 12 ml of Tris buffer at pH 7.6 for 2 minutes. All the slides were lightly counterstained with modified Mayer's hematoxylin for 30 seconds before dehydratation and mounting. Positive controls and nonimmune protein-negative controls were used with each antibody.

Quantification of p53 protein. The immunostained slides were examined by light microscopy. The degree of p53 protein expression in the cell nuclei was scored using an established IHC scoring system, reported by Allred *et al*. (16). Using this scoring system, each sample was assigned a proportion score and an intensity score. The proportion score represented the estimated fraction of positively staining cell nuclei (0=none, 1=less than 1/100, 2=1/100 to less than 1/10, 3=1/10 to less than 1/3, 4=1/3 to less than 2/3, and 5=greater than 2/3). The intensity score represented the estimated average staining intensity of positively stained cell nuclei (0=none, 1= weak, 2=intermediate, 3=strong). The overall amount of p53 protein was expressed as the sum of the proportion score and intensity score for each sample (IHC score). This scoring system assigns a zero to stain negative samples and a score ranging from 2 to 8 for positive samples.

Statistical analysis. The non-parametric two-sample Wilcoxon signed-ranks test was used to compare the median IHC scores of the different tissue types. The statistical routine was provided by Analyse-it for Microsoft Excel software (Analyse-it Software, LTD, Lees, England, UK).

Results

p53 expression in HGD vs. neosquamous mucosa following PDT. All the HGD samples showed strong and widespread staining for p53. Figure 1 (a) shows the H&E-stain of a case of BE with HGD from Group II. Figure 1 (b) shows the corresponding immunoreactivity for p53 protein. In the neosquamous samples, 17.5% were negative for p53 while the rest exhibited weak staining which was confined to the basal layer of the sample. Figure 2 (a) shows the H&E-stained neosquamous mucosa and 2 (b) shows the corresponding p53 staining displaying no reactivity.

Table I shows the IHC scores of the HGD and neosquamous mucosa samples. All the HGD samples exhibited high p53 IHC scores (median 7.0), while the majority of neosquamous samples showed low p53 IHC scores (median 4.0), with all positive scores corresponding to weak basal layer staining. This represented statistically significant lower ($p<0.001$) p53 protein expression in the neosquamous samples compared to HGD.

Out of the 12 patients who received PDT, in 9 patients the entire Barrett's was eliminated and replaced with neosquamous mucosa. In two patients, there was a residual island of Barrett's without dysplasia. In one patient there were

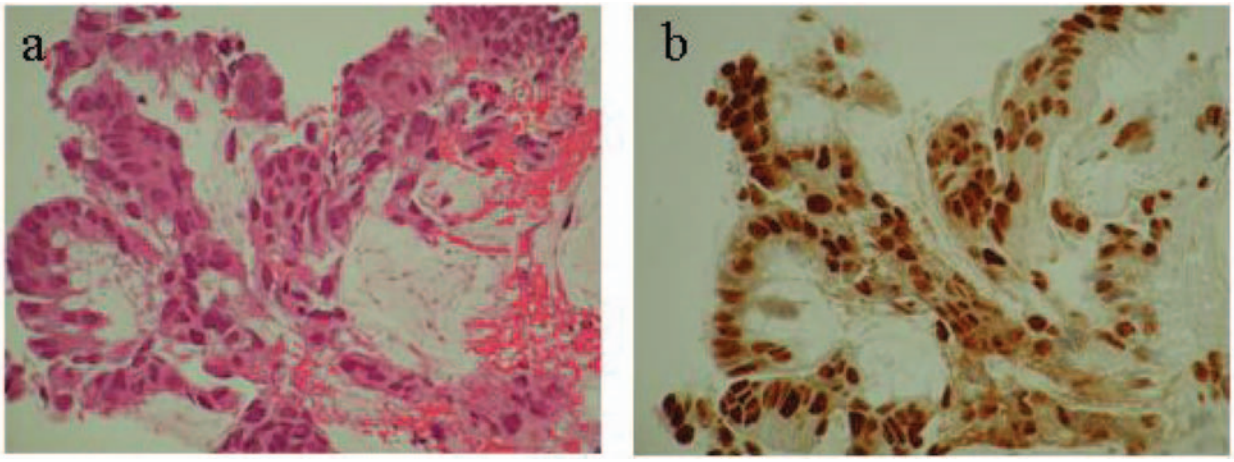

Figure 1. *High grade dysplasia in Barrett's esophagus. (a) H&E stain; (b) strong nuclear immunoreactivity for p53 (both at x250).*

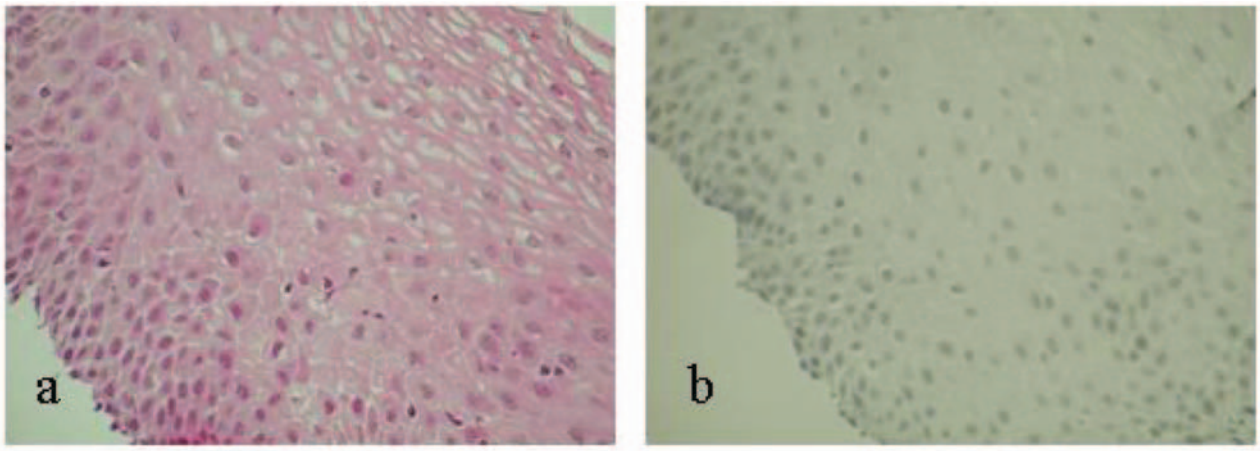

Figure 2. *Neosquamous mucosa. (a) H&E stain; (b) no evidence of p53 staining (both at x250).*

multifocal residual islands with LGD. Examination of the p53 IHC scores for the neosquamous mucosa in those three patients revealed no clear relationship between p53 expression and the presence of residual lesions after PDT. The median p53 IHC score for the neosquamous samples from the patients with complete reversal was 3.50. The median p53 IHC score for those with residual nondysplastic islands was 4.0. However, the median p53 IHC score for the neosquamous samples in one patient with residual LGD islands was zero.

p53 expression in untreated Barrett's mucosa. For comparison purposes, untreated Barrett's specimens were also analyzed from Group II patients. The median p53 IHC score for the LGD samples was 4.0, which was significantly lower than the median p53 IHC score for HGD in the same group ($p<0.033$). The median p53 IHC score for the IM samples was zero, which was significantly lower than the median p53 IHC score for LGD ($p<0.0034$). Significantly higher levels of p53 were expressed as the tissue progressed toward more severe dysplasia.

Table I. *Percentages of high grade dysplasia and neosquamous samples exhibiting IHC scores ranging from 0 to 8, indicating the highest level of positivity for p53 protein expression.*

	IHC Score							
	0	2	3	4	5	6	7	8
HGD					35.7%	7.1%	28.6%	28.6%
NEOSQ	17.5%	17.5%	13%	39%	13%			

HGD=high grade dysplasia; NEOSQ=neosquamous mucosa.

The examination of p53 expression in the normal squamous mucosa of patients in Group II revealed a dependence on the presence of HGD within their Barrett's segment. The normal squamous mucosa samples from patients with IM and LGD did not show any nuclear staining (all with a p53 IHC score of zero). However, the normal squamous mucosa samples obtained from the patients harboring HGD within their Barrett's segment showed weak staining in the basal layer of the samples, with a median p53 IHC score of 3.0.

The median p53 IHC scores for neosquamous mucosa and squamous mucosa in the two groups of patients was also compared. There was no statistically significant difference between the two groups ($p<0.17$). It should be noted that, there were some positive IHC scores in the squamous mucosa (due to staining at the basal layer) of patients harboring HGD, which may have affected this comparison analysis.

Most of the patients in Group II had mixed diagnoses of IM, LGD or HGD resulting from biopsies at different levels of the Barrett's segment. In 5 out of the 6 patients with HGD, all the HGD and LGD samples were positive for p53 protein. However, the presence of HGD in a patient did not correspond to positive p53 in the nondysplastic samples for that patient. Only 2 out of the 6 patients with HGD had positive staining within their nondysplastic specimens.

Discussion

This study reports the lower p53 protein expression in the neosquamous mucosa following PDT for HGD in BE.

Comparison of the p53 expression between the Group I and Group II patients' samples revealed a statistically significant ($p<0.001$) lower p53 protein expression following PDT of HGD. This is an important observation since HGD in BE is usually p53 positive. Our results were consistent with the report by Garewal *et al*. (17), who examined p53 and Ki-67 in the reversed squamous mucosa of BE. They suggested that completely reversed squamous epithelium (neosquamous) following thermal ablation was biologically similar to normal squamous epithelium and thus at a lower risk for cancer. In contrast, partially reversed Barrett's mucosa following prolonged PPI therapy was positive for both p53 and Ki-67. In another study, Krishnadath, *et al*. (18) indicated that p53 protein overexpression persisted after PDT in two patients where the dysplasia grade was down-staged, but the Barrett's mucosa persisted and was not replaced with neosquamous mucosa.

The negative or weak staining confined to the basal layer of the neosquamous epithelium, observed in this study, has previously been described in esophageal squamous epithelium associated to BE (19). When we compared the p53 staining IHC score in the neosquamous mucosa of patients with and without residual Barrett's islands we found no clear differences. It should be noted that limited data were available from neosquamous mucosa of patients with residual Barrett's islands and therefore, no definitive conclusions should be made. Our data as well as those from Garewal *et al*. (17) appeared to indicate that complete reversal of Barrett's mucosa is an important factor when using endoscopic ablative techniques for the management of BE. While the results of our study clearly showed lower p53 protein expression after PDT of HGD, a direct comparison of p53 expression before and after successful PDT in each patient may provide more direct evidence of reduction in p53 and warrants further investigation.

In our untreated patients most of the samples of IM did not stain for p53. In two patients whose nondysplastic samples stained positive for p53, high grade dysplasia was found within their Barrett's segment which stained strongly for p53. The LGD samples stained more frequently and more strongly, with a median p53 IHC score of 4.0, than the nondysplastic samples ($p<0.0034$). In two patients with p53 positive nondysplastic and HGD samples, strong staining in the LGD samples was also detected. The HGD samples were always p53 positive with a median p53 IHC score of 7.0 which was significantly higher than that of LGD ($p<0.033$). The increasing level of p53 expression as a function of the severity of dysplasia, in our study, was consistent with the published data (8-14).

Interestingly, we observed that in 83% of cases, the presence of HGD in one level correlated with the p53 positivity in LGD samples from another level of the Barrett's segment in the same patient. In two patients with HGD, there were nondysplastic and LGD specimens from other levels of the BE that were also p53 positive. The widespread staining at multiple levels of Barrett's segment in the same patient agrees with the reports of genetic alteration and "field effect" in BE patients (20). It seems that p53 mutant clones could expand throughout the length of Barrett's segment and 41% of patients had mutant clones at every evaluated level of the Barrett's segment (20). Galipeau *et al*. (21) studied distribution and heterogeneity of loss of heterozygosity (LOH) in the 9p and 17p chromosomes throughout Barrett's segment of patients who had HGD but not adenocarcinoma. In most patients, the Barrett's mucosa showed a mosaic of clones and subclones with different patterns of LOH, some involving extensive regions of Barrett's epithelium. Barrett *et al*. (22) reported widespread premalignant diploid clones with somatic genetic abnormalities in TP53 and CDKN2A affecting BE, 6 years before detection of cancer.

The theory of "clonal expansion" may explain our detection of p53 accumulation in the basal layer of the normal squamous mucosa of all the patients who had HGD within their Barrett's mucosa This finding may reflect the

hyperproliferative property within this region, and/or support the described "field effect" in Barrett's patients (20-23). Further studies are necessary to determine whether genetic abnormalities can expand into the normal squamous mucosa proximal to Barrett's segment (with HGD or cancer).

Conclusion

A significantly lower p53 protein expression was found in the neosquamous mucosa of patients who had received successful photodynamic therapy for high grade dysplasia in Barrett's esophagus. Given the overwhelming amount of data available implicating p53 expression as a marker for neoplastic progression of BE, the decreased expression of p53 in neosquamous mucosa following PDT may indicate reduced risk for neoplastic progression in those patients.

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