Neosquamous Epithelium Does Not Typically Arise from Barrett's Epithelium  
Thomas G. Paulson, Lianjun Xu, Carissa Sanchez, Patricia L. Blount, Kamran Ayub, Robert D. Odze, and Brian J. Reid

Abstract  
Purpose: Neosquamous epithelium (NSE) can arise within Barrett’s esophagus as a consequence of medical or surgical acid reduction therapy, as well as after endoscopic ablation. Morphologic studies have suggested that NSE can develop from adjacent squamous epithelium, submucosal gland ducts, or multipotent progenitor cell(s) that can give rise to either squamous or Barrett’s epithelium, depending on the luminal environment. The cells responsible for Barrett’s epithelium self-renewal are frequently mutated during neoplastic progression. If NSE arises from the same cells that self-renew the Barrett’s epithelium, the two tissues should be clonally related and share genetic alterations; if NSE does not originate in the self-renewing Barrett’s, NSE and Barrett’s esophagus should be genetically independent.  
Experimental Design: We isolated islands of NSE and the surrounding Barrett’s epithelium from 20 patients by microdissection and evaluated each tissue for genetic alterations in exon 2 of CDKN2A or exons 5 to 9 of the TP53 gene. Nine patients had p16 mutations and 11 had TP53 mutations within the Barrett’s epithelium.  
Results: In 1 of 20 patients, a focus of NSE had a 146 bp deletion in p16 identical to that found in surrounding Barrett’s epithelium. The NSE in the remaining 19 patients was wild-type for p16 or TP53.  
Conclusion: Our mutational data support the hypothesis that, in most circumstances, NSE originates in cells different from those responsible for self-renewal of Barrett’s epithelium. However, in one case, NSE and Barrett’s epithelium seem to have arisen from a progenitor cell that was capable of differentiating into either intestinal metaplasia or NSE.
unclear whether squamous reepithelialization is associated with a reduction in cancer risk (15).

The origin of NSE is unknown. NSE may simply represent an alternate epithelial phenotype of the Barrett's epithelium. It is unlikely that differentiated Barrett's epithelium directly trans-differentiates into NSE, given that most ablative therapies regenerate NSE although most of the Barrett's has been eliminated. The cells that generate the self-renewing Barrett's epithelium may be able to undergo separate differentiation pathways depending on the environmental conditions in the esophagus (Fig. 1A). According to this model, a self-renewing squamous epithelium is generated under normal conditions and self-renewing Barrett's epithelium under chronic reflux conditions. Alternatively, the two epithelia may arise from independent progenitors, leading to establishment of either squamous or intestinal-type epithelium depending on luminal conditions (Fig. 1B). Development of NSE islands within a region of the esophagus previously covered with genetically altered Barrett's epithelium provides an opportunity to investigate the relationship between these epithelia (Fig. 2). If NSE derives from the same cells that self-renew the Barrett's epithelium, but has a different phenotype, then NSE will share a common genetic background with the Barrett's esophagus. Alternatively, if NSE arises independently of the cells that self-renew the Barrett's epithelium, then NSE will be genetically independent of the Barrett's esophagus.

Barrett's epithelium has similar proliferation and renewal characteristics to those of the small intestine (17, 18), with cells differentiating as they move up the crypt where they are eventually sloughed off into the lumen. We have taken advantage of the fact that development of esophageal adenocarcinoma is characterized by genetic instability, selection of genetic variants, and expansion of clonal populations early in progression (19) to characterize the relationship between Barrett's esophagus and NSE. Alterations in the tumor suppressor genes CDKN2A (p16; 9p loss of heterozygosity, mutation, and promoter hypermethylation) and TP53 (17p loss of heterozygosity, mutation) are frequently found in patients with Barrett's esophagus (20). The resulting clonal expansion indicates cells with a given genetic alteration have spread from a single crypt to cover thousands or hundreds of thousands of crypts in the Barrett's epithelium. We examined Barrett's esophagus patients having (a) clonally expanded populations containing mutations in either p16 or TP53 and (b) NSE completely within the clonally expanded region. Detection of the same mutation in the NSE and surrounding Barrett's epithelium would indicate that both derive from the cells that self-renew the Barrett's epithelium. Our results indicate that in the majority of cases, NSE has an origin independent of the surrounding self-renewing Barrett's epithelium.

Materials and Methods

Patients. Participants were selected from the Seattle Barrett's Esophagus Study, which has been approved by the University of Washington Human Subjects Division and/or the Fred Hutchinson Cancer Research Center Institutional Review Board continuously since 1983. Endoscopic and histologic records were reviewed to identify 20 patients who had a previously characterized area of specialized intestinal metaplasia with a clonally expanded population of cells with either a p16 or TP53 gene mutation (as detected previously), and also had endoscopically and histologically confirmed islands of neosquamous mucosa arising within the area containing the mutated Barrett's epithelium.

Isolation of NSE. Biopsies containing NSE were fixed in 10% buffered formalin and sectioned. The first and last sections were cut at a thickness of 5 μm and stained with H&E to allow morphologic evaluation of the type of epithelium present in the biopsy. Microdissections of the biopsies were done using a new no. 10 scalpel blade on 10-μm-thick tissue sections at ×20 magnification using an American Optical dissecting microscope. Microdissected epithelia were placed in sterile microcentrifuge tubes for processing. Cutting blades were changed between specimens and between different epithelia. Microdissected sections were deparaffinized using Histoclear (National Diagnostics, Atlanta, GA) and washed sequentially in 100%, 75%, and 50% ethanol. DNA was isolated by a protocol developed by Frank et al. (21). Briefly, dehydrated sections were resuspended in 50 to 100 μL buffer [50 mmol/L Tris-HCl (pH 8.3), 0.2 mg/mL proteinase K] and digested at 55°C overnight. Digests were spun at 14K in an Eppendorf microcentrifuge for 5 minutes to pellet debris. Five microliters

![Image](329x513 to 525x642)
of the supernatant were used in subsequent whole genome reactions. The remaining tissue on the microdissected slides was stained with H&E and examined by a gastrointestinal pathologist (R.D. Odze) to verify the nature of the microdissected tissue (i.e., squamous, columnar, etc.).

**Flow sorting of NSE.** Biopsies of NSE were minced to release nuclei (22), stained with the DNA binding dye 4',6-diamidino-2-phenylindole, and nuclei from NSE cells were identified on the basis of side scatter and 4',6-diamidino-2-phenylindole fluorescence as described previously (23). Briefly, this sorting strategy is based on the retention of dense cytoplasm around the nuclei of squamous cells that nonspecifically binds 4',6-diamidino-2-phenylindole. Thus, these nuclei have both a high degree of side scatter due to the complexity of the cytoplasm around the nucleus, and fluoresce brightly with 4',6-diamidino-2-phenylindole, in contrast to the Barrett's intestinal epithelial nuclei, which take up less 4',6-diamidino-2-phenylindole and have significantly lower side scatter. In experiments with lung bronchial sputum samples, such a sorting procedure was able to generate populations with <1% columnar epithelium (23). DNA from the flow-sorted cells was isolated as described previously (22).

**Genetic analyses.** Given the small size of the microdissected sections, we first did whole genome amplification before sequencing. Primer extension preamplification was done as previously reported (22). p16 exon 2 (24, 25) or TP53 exons 5 to 6, 7, and 8 to 9 (26–28) were amplified by PCR from primer extension preamplification products. All PCR products were purified with MultiScreen PCR Filtration System (Millipore, Bedford, MA), and sequenced using BigDye Terminator cycle sequencing (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA sequencer. All mutations were confirmed with at least two independent primer extension preamplification, PCR, and sequencing reactions. Wild-type sequences were confirmed for all patients using constitutive samples. Mutations in TP53 for the 11 patients in this study have been previously reported (28).

Loss of heterozygosity analyses were done as previously described (22). Briefly, products from the primer extension preamplification reactions were amplified using primers for the single tandem repeat loci D9S925, D9S932, and D9S1118, desalted and concentrated using Microcon-100 sample filters (Millipore), and run on an ABI 3730 Capillary Electrophoresis System (Applied Biosystems). Loss of heterozygosity was determined by comparing the ratio of the peak heights of each allele with normal controls run previously for each patient.

**Results**

We identified 20 patients having islands of NSE within a region of the Barrett’s segment that contained mutations in either p16 or TP53. The characteristics of these patients are described in Table 1. Islands of NSE (two to seven biopsies per patient) and surrounding Barrett’s epithelium (one to six biopsies per patient) were obtained from all patients. Endoscopic and histologic examples of NSE arising in a region surrounded by Barrett’s epithelium are shown in Fig. 2A and B, and the experimental design illustrated in Fig. 3.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>17:3</td>
</tr>
<tr>
<td>Mean age</td>
<td>73.1 y</td>
</tr>
<tr>
<td>Mean segment length</td>
<td>8.2 cm</td>
</tr>
<tr>
<td>Patients with p16 mutation</td>
<td>9/20</td>
</tr>
<tr>
<td>Patients with p53 mutation</td>
<td>11/20</td>
</tr>
<tr>
<td>No. neosquamous samples</td>
<td>77 (two to seven per patient)</td>
</tr>
<tr>
<td>No. Barrett’s samples</td>
<td>56 (one to six per patient)</td>
</tr>
</tbody>
</table>

Sequencing results were obtained from 74% of the samples and from all patients; samples that failed to provide adequate sequencing results were associated with specific patients (e.g., samples from five patients accounted for 75% of the failures) and likely reflected an artifact of fixation that resulted in poor-quality DNA for PCR. All microdissected tissues were verified to be entirely neosquamous or intestinal epithelium (Fig. 4), but included some stromal cells.

We confirmed the previously identified p16 or TP53 mutations in the microdissected Barrett’s epithelium in 18 of 20 patients (90%; Table 2). The Barrett’s epithelium present in the biopsies from the other two patients was small and had >75% stromal cell contamination, making unambiguous identification of the mutations difficult; however, these mutations had been previously verified in adjacent Barrett’s esophagus biopsies from the same patients. All mutations were identical to those identified in tissue obtained from prior endoscopies (28). Islands of NSE were found to be wild type at the loci mutated in the adjacent Barrett’s epithelium in 19 of 20 patients examined (95%). However, one patient (patient 13) was found to have an identical p16 mutation, a 146 bp deletion in the NSE and in the adjacent intestinalized epithelium (Fig. 5). Stromal cells present in the microdissected Barrett’s and NSE contribute the wild-type background sequence. In this patient, the alteration was found in one biopsy containing NSE out of seven NSE biopsies examined from three separate levels of a 15 cm Barrett’s segment. The patient had a wild-type p16 DNA sequence in normal constitutive tissue, confirming that the mutation was not inherited. This mutation has not been reported in the literature and it is unlikely to have arisen independently in the two tissues. This result was verified by sequencing independently isolated microdissected tissue from another section from the same biopsy. In addition, the patterns of loss of heterozygosity at informative markers on chromosome 9p were consistent between the NSE and surrounding Barrett’s epithelium, further indicating a clonal relationship (data not shown).
We further verified that the NSE from patient 13 contained a mutation in p16 by using a flow cytometric sorting method that discriminates squamous from columnar epithelium on the basis of side scatter and fluorescence. This protocol purifies the NSE cells from the underlying stromal cells that are present after mechanical microdissection. The sequence of the p16 gene obtained from the flow-purified NSE cells contained only the identical 146 bp deletion (Fig. 5) found in the microdissected samples.

Discussion

Patients with Barrett’s esophagus frequently develop islands of NSE during acid suppression therapy and the goal of ablative therapies is to completely replace the Barrett’s epithelium with a regenerated squamous epithelium. However, it has been difficult to determine if the development of NSE affects the risk of developing cancer because little is known about the origin or genetics of NSE. We have shown that NSE and the surrounding Barrett’s do not contain common genetic alterations and, therefore, the NSE is not derived from the self-renewing Barrett’s epithelium in most cases. However, in one case, the presence of the same p16 mutation and pattern of loss of heterozygosity in both NSE and the surrounding Barrett’s epithelium indicate that they can be clonally related and share a common precursor. In this case, we find evidence for a progenitor that is capable of generating both types of epithelia. Because the progenitor cell(s) of the esophageal epithelium are unknown, it is impossible to determine where in the pathway of epithelial progenitors this common precursor lies. Future studies using progenitor cell markers, as opposed to the tumor suppressor gene alterations used here, may clarify this question.

Although many forms of surgical or medical antireflux therapy are able to induce some degree of squamous reepithelialization, there is no evidence that neosquamous regrowth during antireflux therapy reduces the risk of developing esophageal adenocarcinoma (15, 29). Most antireflux treatments are able to induce a moderate level of neosquamous regeneration, but only rarely will this completely eliminate the Barrett’s epithelium (8, 12, 15). Ablative therapies, such as photodynamic therapy or multipolar electrocoagulation, can successfully eradicate most of the Barrett’s epithelium in a patient, and the results presented here suggest the NSE developing after these treatments may frequently be wild type, although none of the patients we examined had undergone ablative therapies. However, concerns still remain that residual Barrett’s hidden under the regenerated squamous poses a risk of cancer that may be difficult to detect (30, 31), or that the residual Barrett’s after ablation may contain mutations (32) and may progress rapidly after treatment (33).

Our results that a minority of the NSE we sampled contained mutations present in the surrounding Barrett’s esophagus are consistent with a previous study examining p53 overexpression in NSE that developed in patients treated by multipolar electrocoagulation and proton pump inhibitor therapy. Garewal et al. (13) found regenerated NSE was negative for p53 by immunostaining in 11 of 11 patients with total squamous reepithelialization, but p53 protein expression was elevated in 6 of 14 cases who only developed squamous islands. In addition, they reported anecdotally that the Barrett’s esophagus adjacent to the NSE seemed to express more p53 than elsewhere. Similarly, another report described TP53 mutations in two cases of NSE arising after photodynamic therapy, but did not characterize the preexisting Barrett’s epithelium (32). The approach used in the present study specifically examined genetic alterations that (a) frequently occur in Barrett’s esophagus and (b) were already known to exist in large fields of clonal populations surrounding the islands of NSE. Thus, the Barrett’s epithelium surrounding the NSE, as well as the self-renewing cells that generated the Barrett’s, should have contained a p16 or TP53 mutation in our cases. It is unlikely that the one case in

![Figure 4](image-url)  
*Fig. 4. Examples of microdissection of NSE. Mosaic images of (A) a Barrett’s esophagus biopsy containing both Barrett’s epithelium (BE) and NSE and (B) a section adjacent to that shown in (A), after microdissection of the Barrett’s epithelium and NSE.*

Table 2. Sequencing results for Barrett’s epithelium and NSE

<table>
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<th>Patient</th>
<th>Gene</th>
<th>Barrett’s epithelium</th>
<th>NSE</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>p53</td>
<td>C388T</td>
<td>Wild type</td>
</tr>
<tr>
<td>2</td>
<td>p53</td>
<td>C388T</td>
<td>Wild type</td>
</tr>
<tr>
<td>3</td>
<td>p53</td>
<td>C455T</td>
<td>Wild type</td>
</tr>
<tr>
<td>4</td>
<td>p53</td>
<td>G518A</td>
<td>Wild type</td>
</tr>
<tr>
<td>5</td>
<td>p53</td>
<td>C535T</td>
<td>Wild type</td>
</tr>
<tr>
<td>6</td>
<td>p53</td>
<td>T650G</td>
<td>Wild type</td>
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<tr>
<td>7</td>
<td>p53</td>
<td>A659G</td>
<td>Wild type</td>
</tr>
<tr>
<td>8</td>
<td>p53</td>
<td>G743A</td>
<td>Wild type</td>
</tr>
<tr>
<td>9</td>
<td>p53</td>
<td>G743A</td>
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<td>10</td>
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<tr>
<td>11</td>
<td>p53</td>
<td>G818T</td>
<td>Wild type</td>
</tr>
<tr>
<td>12</td>
<td>p16</td>
<td>174 2bp del</td>
<td>Wild type</td>
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<tr>
<td>13</td>
<td>p16</td>
<td>198 146bp del</td>
<td>198 146bp del</td>
</tr>
<tr>
<td>14</td>
<td>p16</td>
<td>235 11bp del</td>
<td>Wild type</td>
</tr>
<tr>
<td>15</td>
<td>p16</td>
<td>NR (C238T)</td>
<td>Wild type</td>
</tr>
<tr>
<td>16</td>
<td>p16</td>
<td>C238T</td>
<td>Wild type</td>
</tr>
<tr>
<td>17</td>
<td>p16</td>
<td>C247T</td>
<td>Wild type</td>
</tr>
<tr>
<td>18</td>
<td>p16</td>
<td>C247T</td>
<td>Wild type</td>
</tr>
<tr>
<td>19</td>
<td>p16</td>
<td>G262T</td>
<td>Wild type</td>
</tr>
<tr>
<td>20</td>
<td>p16</td>
<td>NR (G330A)</td>
<td>Wild type</td>
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</table>

NOTE: Sequencing results for the TP53 and p16 genes for Barrett’s epithelium and NSE. Mutation locations are given as the nucleotide mutated or the beginning base of the indicated deletion.
which we found a p16 mutation in NSE was the result of undetected contamination by Barrett's cells because the mutation was found in two independent microdissections, and purification of NSE by flow sorting resulted in a pure population of cells containing the mutation. The lack of genetic alterations in the NSE in most cases indicates that the cells that self-renew the mutated Barrett's epithelium do not generate NSE.

The single patient with mutated NSE had only a single focus with mutation of seven biopsies with NSE taken from three different levels in the esophagus. Development of NSE from the same precursor that generates the surrounding Barrett's epithelium does not seem to depend on the specific p16 mutation examined, because the same mutation was found throughout the 15 cm Barrett's segment. This finding suggests that NSE can arise from multiple sources even within the same patient. Because mutation in the NSE was only found in 1 of 20 patients, one can speculate that mutation of a progenitor cell capable of generating both NSE and Barrett's epithelium is not a common event.

NSE has been hypothesized to originate from three sources: encroachment of adjacent normal squamous epithelium from the squamocolumnar junction, outgrowth from an existing pool of squamous cell progenitors in the esophageal epithelium, or from multipotent progenitor cells that also generate the Barrett's epithelium (8, 9). In the current study, we eliminated the possibility that NSE is derived from adjacent normal squamous epithelium by only including patients who developed islands of NSE completely surrounded by a field of Barrett's epithelium. Submucosal esophageal gland ducts, which are partially lined with squamous epithelium and found throughout the esophageal epithelium, may provide a reservoir for regrowth of an unaltered squamous epithelium (8, 34–37).

An earlier study examining the characteristics of Barrett's epithelium found underneath squamous islands not those with NSE alone. It is likely the ultimate fate of the esophageal epithelium, whether it be squamous or Barrett's epithelium, is dependent on complex interactions between the luminal environment of the esophagus and its different progenitor cell populations. Further studies to characterize these interactions may allow development of interventions to promote regeneration of genetically normal squamous epithelium in Barrett's esophagus patients or even prevent development of Barrett's epithelium in patients with gastroesophageal reflux disease.

Acknowledgments

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