Does radiofrequency ablation of the lower oesophagus allow for clonal expansion of highly mutated neosquamous epithelium?

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ABSTRACT

Objective In Barrett’s oesophagus (BE), after radiofrequency ablation (RFA), the oesophagus can be repopulated with a stratified ‘neosquamous epithelium’ (NeoSE). While histologically normal, the origin and clonal make-up of this NeoSE is unknown. An increased understanding of NeoSE is important as some studies suggest that NeoSE is biologically abnormal. The aim of this study was to determine whether there were major differences in the mutational landscape or clonal size in NeoSE versus normal squamous epithelium and determine whether NeoSE shares any pathogenic mutations with BE.

Methods and analysis 10 patients who underwent RFA and 10 samples from 8 control patients were sequenced using a clinical targeted sequencing platform (cohort 1). An additional, eight patients with paired preablation BE and postablation NeoSE were also sequenced (cohort 2). Patient advocates will be used to disseminate the findings of this study.

Results NeoSE samples had a mean of 2.2 pathogenic mutations per sample, including 50% of samples with an NOTCH1 and 30% of samples with a TP53 mutation. The normal oesophageal squamous epithelium samples had 1.5 mutations per sample, including 40% of samples with NOTCH1 and 10% of samples with TP53 mutations. There was no difference in mutational allele fractions between NeoSE and normal squamous samples. When we compared paired BE and NeoSE samples, no shared mutations were identified.

Conclusion While there was a trend for more TP53 mutations in NeoSE, overall, the mutational profile and clonal sizes (allele fractions) were very similar, suggesting NeoSE is genetically similar to the normal oesophageal squamous epithelium.

INTRODUCTION

Barrett’s oesophagus (BE) is defined as the replacement of the distal oesophageal squamous epithelium by a metaplastic columnar epithelium, which is the major risk factor for the development of oesophageal adenocarcinoma (EAC). As BE progresses to cancer, a series of histologic changes can be recognised, from the non-dysplastic epithelium through dysplastic epithelium (low-grade dysplasia (LGD) or high-grade dysplasia (HGD)) and eventually invasive adenocarcinoma. Endoscopic surveillance of non-dysplastic BE on set intervals of 3–5 years is recommended to detect the development of dysplasia, with possible endoscopic therapy for patients who progress to dysplasia. Several endoscopic treatments are widely used for the treatment

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The normal squamous epithelium of the oesophagus can contain small clones of cells that contain mutations in NOTCH1 genes as well as in TP53.
⇒ These mutations can be detected using special, high-sensitivity sequencing techniques.
⇒ Studies have shown that while neosquamous epithelium is generally considered histologically normal, there can be functional defects.

WHAT THIS STUDY ADDS

⇒ Mutations in NOTCH1 and TP53 can be detected in the normal squamous oesophagus at a low allele fraction using standard sequencing techniques.
⇒ Similarly, mutations in NOTCH1 and TP53 can be found in the neosquamous epithelium at a similar allele fraction.
⇒ However, there was a trend for an increased number of TP53 mutations in the neosquamous epithelium.
⇒ The study also confirms that neosquamous epithelium does not usually share any mutations with the ablated Barrett’s oesophagus.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ A major question has been how ‘normal’ the neosquamous epithelium is and what its mutational profile looks like.
⇒ This study shows that there are no major genomic differences between postradio frequency ablated neosquamous and normal squamous epithelium, which suggests that the abnormalities in neosquamous tissue are more functional than genomic.
⇒ This study should begin to alleviate clinical concerns that the neosquamous epithelium is more genetically advanced towards neoplasia than normal oesophageal squamous epithelium.
of dysplasia or early cancer in BE, including radiofrequency ablation (RFA), argon plasma coagulation and various forms of cryotherapy. These procedures aim to destroy the dysplastic BE epithelium, while leaving deeper oesophageal structures intact. After the mucosal ablation, the ablated oesophagus heals on acid suppressive therapy and is generally repopulated with a stratified squamous epithelium, referred to as ‘neosquamous epithelium’ (NeoSE).

It is not uncommon for BE to recur after ablation therapy. Recurrence of BE following successful ablation by RFA has been reported in 6%–25% of patients within 1 year. Since there is still a risk of recurrence and progression to adenocarcinoma after these techniques, endoscopically treated patients are maintained under endoscopic surveillance. Given the high recurrence rate of frank BE, with occasional development of dysplasia or oesophageal adenocarcinoma, it is unclear whether the regenerated NeoSE is truly ‘normal’. Even though endoscopically NeoSE looks similar to squamous epithelium in patients with no known disease, multiple studies have shown that NeoSE is abnormal based on the barrier function and microRNA profile.

Studies using highly sensitive techniques have shown that pathogenic mutations can be identified in the normal oesophageal squamous epithelium, suggesting that mutant squamous cells are already present within the oesophagus. The origin and clonal make-up of NeoSE are unknown. Having a better understanding of this process is especially important as there is some controversy in the field to whether this NeoSE may have a higher incidence of developing squamous cell carcinoma compared with the normal oesophagus. The origin of BE cells has also been debated, and different candidate precursor cells have been suggested. A recent study suggests that undifferentiated gastric cells from the cardia give rise to BE. Also, given the debate on the origin of BE, it is unknown how this NeoSE may be related to the previously ablated BE. With the commonplace nature of ablative treatments for BE, it is imperative to understand the malignant potential of NeoSE.

Therefore, to determine whether clonal populations of mutations large enough to detect with standard sequencing are present in NeoSE and how those mutations compare to BE and true normal oesophagus, we performed targeted massively parallel sequencing on a cohort of NeoSE and true normal squamous oesophagus tissue. To understand the potential relationship between NeoSE and the pretreatment BE, we similarly sequenced paired pretreatment BE and post-treatment NeoSE. Our hypothesis was that by ablating the epithelium through RFA, mutated squamous epithelial cells that have a growth and survival advantage can expand and re-epithelialize the ablated region as breaks on clonal growth such as cell-to-cell contact would be removed, potentially allowing any proliferating cell with an advantage (such as an advantageous mutation) to expand and cover a larger surface area. Thus, pathogenic mutations in the NeoSE would have larger allele fractions allowing easy detection of these expanded clones by standard bulk sequencing. In contrast, in the normal oesophagus these mutated cells do not have the ability to expand to larger clones due to growth restraints of the surrounding epithelial cells. When using standard bulk sequencing techniques, the mutations will be at a lower allele fraction and harder to detect. Our aim was to determine whether clonal populations of mutations large enough to detect with standard sequencing are present in the NeoSE and how those mutations compare to the normal squamous oesophagus. To determine whether pathogenic mutations found in the NeoSE may be shared with the prior pretreatment BE, a small cohort of pretreatment BE samples was then compared with their paired NeoSE.

MATERIALS AND METHODS
Patient selection
After IRB approval, patients who underwent successful endoscopic RFA and attained complete eradication of intestinal metaplasia, and then had subsequent endoscopic surveillance and biopsy of unambiguous areas of NeoSE as well as patients with a normal upper endoscopy were identified. Based on sample availability, the primary study (cohort 1) consisted of 10 endoscopic biopsies with NeoSE from 10 patients and 10 normal oesophageal biopsies from eight patients with no known oesophageal pathology. NeoSE samples were taken from patients with no evidence of recurrent or persistent BE. The mean time between the RFA and the biopsy for the NeoSE samples was 3.5 years (1–8 years), (table 1). Additionally, to compare the mutational profile of NeoSE to prior BE, samples from eight patients with paired BE (from prior to ablation) and NeoSE after ablation were sequenced (cohort 2). Samples were selected based on matched endoscopic location within the oesophagus. Two of these eight patients had residual non-dysplastic BE at the time the NeoSE samples were taken (Pt 19 and 21), and one patient later developed low-grade dysplasia (LGD) (Pt 22). All patients were adult (27–86 years of age, cohorts 1 and 2). The Institutional Review Board for human subjects’ research at the UCSF Medical Center has exempted the study (IRB # 19–27460).

Slide preparation and gene sequencing
Twelve unstained slides were cut for each sample and the first and last slides were stained with H&E to guide macrodissection of the samples. DNA was purified using the ReliaPrep FFPE gDNA kit (Promega) from the middle 10 slides. 20–100 ng of DNA was fragmented (Covaris sonicator) to 250 bp and then ligated to specific adapters using automated library preparation (KAPA Hyper KK8504) using the Beckman FXp liquid handling robot. Libraries were pooled and sequenced on an Illumina MiSeq nano flow cell to estimate each library’s concentration based on the number of barcode reads per sample. All samples had sufficient DNA for hybrid capture. The
libraries were pooled and captured using a custom bait set that includes all exons from 300 cancer-associated genes (cohort 1) covering ~0.85 exonic Mb or 243 gastroesophageal cancer-associated genes (cohort 2) covering ~0.75 exonic Mb (online supplemental table 1). Captures were performed using the Agilent Sureselect XT HS Hybrid Capture kit. Captures were further pooled and sequenced on a HiSeq 2500 in Rapid Run mode as previously described.13

Pooled samples were demultiplexed using Picard tools. Read pairs were aligned to the hg19 reference sequence using the Burrows-Wheeler Aligner,14 and data were sorted and duplicate marked using Picard tools. The alignments were further refined using the Genome Analysis Toolkit (GATK)15 16 for localised realignment around indel sites (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_gatk_tools_walkers_indels_IndelRealigner.php). Recalibration of quality scores was also performed using the GATK (http://gatkforums.broadinstitute.org/discussion/44/base-quality-score-recalibration-bqsr). Mutation analysis for single-nucleotide variants (SNV) was performed using MuTect V.1.1.4 and annotated by Variant Effect Predictor (VEP). We used the SomaticIndelDetector tool that is part of the GATK for indel calling.

SNVs and Indels are annotated for gene and amino acid impact using VEP V.79, afterwards OncoAnnotate determines the presence of the variant in external data sources such as the Exome Sequencing Project (ESP), gnomAD and COSMIC to allow flagging common SNPs in samples as described below.

**Criteria for filtering mutations**

To identify likely pathogenic somatic mutations, all non-coding mutations were removed unless they were predicted to affect a splice site. For missense variants, variants identified at greater than 0.1% in any population in either the gnomAD or ESP database were removed. Additionally, any variant identified in previously sequenced normal control samples were removed. Genes were separated into tumour suppressors or oncogenes based on COSMIC consensus cancer gene list. For the oncogenes, we kept only recurrent mutations that are unlikely to lead to loss of the protein and which were recurrently found in cancer (previously reported in COSMIC≥five times). For the tumour suppressor genes, mutations that may lead to loss of function were kept, such as frameshift mutations, non-sense mutations, splice site mutations (within +1, –1,+2, –2), missense mutations reported in COSMIC≥5 and inframe insertions or deletions reported in COSMIC≥5 or 3 amino acids or larger (ie, nine base pairs or larger).

A clinically validated, in house algorithm, RobustCNV, was used for copy number assessment. RobustCNV relies on localised changes in the mapping depth of sequenced reads in order to identify changes in copy number at the loci sampled during targeted capture. This strategy includes a normalisation step in which systematic bias in mapping depth is reduced or removed using robust regression to fit the observed tumour mapping depth against a panel of normals sampled with the same capture bait set. Observed values are then normalised against predicted values and expressed as log2 ratios. A second normalisation step is then done to remove GC bias using a loess fit. Finally, log2 ratios are centred on segments determined to be diploid based on the allele fraction of the loci sampled during targeted capture. This strategy includes a normalisation step in which systematic bias in mapping depth is reduced or removed using robust regression to fit the observed tumour mapping depth against a panel of normals sampled with the same capture bait set. Observed values are then normalised against predicted values and expressed as log2 ratios. A second normalisation step is then done to remove GC bias using a loess fit. Finally, log2 ratios are centred on segments determined to be diploid based on the allele fraction of the loci sampled during targeted capture. This strategy includes a normalisation step in which systematic bias in mapping depth is reduced or removed using robust regression.
RESULTS

Ten patients with 1 NeoSE sample each and 8 patients with a total of 10 normal oesophageal epithelium samples (two patients had two samples each) were included in the study with mean ages of 65.7 and 54.5, respectively (cohort 1). There was no significant age difference between the two groups, p=0.25. All samples selected for cohort 1 were successfully sequenced, with a mean target coverage over 400× for all samples. Within the group of the patients with NeoSE, there were multiple examples of samples containing pathogenic mutations in the known oesophageal adenocarcinoma and squamous cell carcinoma-associated genes, \(TP53\) and \(NOTCH1\). In total, we found 22 likely pathogenic mutations in the NeoSE samples (22 likely pathogenic mutations per NeoSE sample). The most common mutation was in \(NOTCH1\) (15, 68% of all mutations), followed by \(TP53\) (6, 27%) and one sample contained a mutation in \(PRKDC\) (1, 4%) (table 1, online supplemental table 2). Interestingly, when a mutation was present in \(TP53\) or \(NOTCH1\), there were often more than one mutation identified in the same gene. Overall, 5/10 (50%) samples contained a \(NOTCH1\) mutation, and 3/10 (30%) samples contained a \(TP53\) mutation. The mean allele fraction of these mutations was low at 0.07 (range 0.03 to 0.11), suggesting small somatic clones contained the mutations but of a size large enough to be confidently called by our sequencing platform. On copy number analysis, no copy number alterations were identified.

In the group of 8 patients with 10 normal oesophageal epithelium samples, we identified 15 mutations (1.5 likely pathogenic mutations per sample) at similar allele fractions as the NeoSE samples (mean 0.06, range 0.02–0.13). The most common mutation was again in \(NOTCH1\) (12, 80% of total mutations) (table 2, online supplemental table 2). Interestingly, one patient (patient 2) with two samples showed the majority of the total mutations in \(NOTCH1\) (12, 80% of total mutations). We identified a \(TP53\) mutation in only one of our normal samples (1/15 mutations, 7% of total mutations). We also identified two likely pathogenic mutations of \(KMT2D\). Two normal samples from two different sites of the oesophagus were used for patient #2 and patient #6. Despite patient 2 having the majority of mutations, there were no shared mutations between samples in either patient with more than one sample. In total, 4/10 (40%) samples in 3/8 (37%) patients contained a \(NOTCH1\) mutation and 1/10 (10%) samples in 1/8 (12.5%) patients contained a \(TP53\) mutation (table 2). Overall, the mutational profile between NeoSE and normal squamous epithelium was similar, though there was a trend for increased \(TP53\) mutations in the NeoSE (p=0.17). Similar to the NeoSE, no copy number alterations were identified in the normal squamous samples.

As prior sequencing of the normal oesophagus showed the number of mutations correlated with patient age, we next looked to see if a similar pattern was seen in the NeoSE samples. Interestingly, we did not see a positive correlation between age and the number of pathogenic mutations in the NeoSE samples, though this could be secondary to a small number of samples, online supplemental figure 1A. While it was driven by a single patient, there was a weak positive correlation between age and the number of pathogenic mutations in the normal squamous samples (\(R^2=0.28\)), online supplemental figure 1B. The patient who showed the majority of the total mutations in the normal squamous group was the oldest patient in our cohort (patient in mid-80s, table 2).

To determine whether pathogenic mutations found in the NeoSE may be shared with the pretreatment BE, we next identified and sequenced an additional eight patients with both a preablated BE sample and a postablation NeoSE sample. Pretreatment and post-treatment samples were selected from the same general region of the oesophagus. For this data set, the mean target coverage was lower at 147×. Similar to the squamous samples in cohort 1, no copy number alterations were seen in the NeoSE samples. Despite not being shared with the NeoSE samples, multiple copy number changes common in BE such as amplifications in \(CCNE1\) and

<table>
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<th>Patient</th>
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KRAS were identified in the BE samples, suggesting copy number analysis performed as expected. Of the mutations identified, three of the NeoSE samples contained an NOTCH1 mutation, one NeoSE sample contained an NOTCH2 mutation, and one NeoSE sample contained a TP53 mutation. None of the likely somatic/pathogenic mutations identified in the NeoSE samples was shared with the paired pretreatment BE samples (online supplemental table 3).

**DISCUSSION**

The goal of ablative therapy in BE is to generate histologically normal looking NeoSE in the area of previous BE following endoscopic treatment and acid suppression. Although NeoSE appears to be relatively normal with its intact squamous cell layer and endoscopically smooth surface, in addition to potential BE or adenocarcinoma recurrence, there is evidence in the literature of possible squamous defects and even squamous cell carcinoma after RFA.11

Martincorena et al sequenced normal oesophageal samples from nine patients using a specialised sequencing technique designed to detect very low allele fraction mutations.10 Using this highly sensitive technique, they were able to identify squamous cells with mutations in 14 different cancer-associated genes. They identified NOTCH1 and TP53 mutations at a high frequency, similarly to our results. Although clones carrying cancer-driver mutations were wide spread, the average number of driver mutations per cell in normal oesophagus was much lower than that in cancer cells, and in general, the size of each clone was quite small, a result that is consistent with the multistage theory of carcinogenesis. In a follow-up manuscript, utilising a mouse model, Colom et al suggested that the small clones were limited due to spatial competition.18 Therefore, we hypothesised that after RFA (which should remove the spatial competition), somatic clones containing advantageous mutations would be able to expand to re-epithelialize the denuded oesophageal surface. Thus, if true, ablation of epithelium might put this NeoSE with advantageous mutations at increased risk of neoplastic progression by allowing clones harbouring protumorigenic mutations (such as TP53) to expand. Our aim in this study was to compare the genomic makeup (and mutant allele fraction) of post-RFA NeoSE with normal squamous oesophageal epithelium. For this, we used a standard targeted sequencing approach of macro-dissected bulk samples in order to capture mutations in ‘larger’ clones encompassing at least 2% of the sequenced cells (0.01 allele fraction for a 2N cell).

In our study, we were able to identify multiple mutations in NOTCH1 and a TP53 mutation in the normal squamous oesophagus using these techniques, confirming even in the non-diseased setting clonal populations of mutant cells can be identified. In addition, we found a weak correlation between mutation rates in healthy oesophageal and advancing age, consistent with Martincorena’s study. NOTCH1 mutations have been identified in around 10%–20% of oesophageal squamous cell carcinomas but appear to be present in at least a small number of cells in most squamous epithelium from adults.10 NOTCH1 mutations are uncommon in the BE—oesophageal adenocarcinoma (EAC) spectrum of disease being rarely reported in BE or dysplastic BE and seen in approximately 7% of EACs.13 19 As discussed above, TP53 mutations were also found in normal squamous oesophagus although in a lower percentage of cells, estimated to be between 0% and 30% in the nine patients sequenced by Martincorena et al. Loss of functional p53 is more common in both oesophageal squamous cell carcinoma and adenocarcinoma, being present in 70%–90% of cases.10 19 The TP53 status varies considerably in BE depending on the progression status of the patient and whether or not the sample is dysplastic. In patients who will progress to high-grade dysplasia or cancer, loss of p53 can be seen in approximately 40%–50% of non-dysplastic BE and approximately 70%–90% of low-grade and high-grade dysplasia. In patients who do not progress beyond low-grade dysplasia, the frequency of p53 loss is 1%–5% in non-dysplastic BE and 20%–45% in low-grade dysplasia.20 21

A recent study explored the functional consequences of NOTCH1 mutations in the normal oesophagus and found that mutations reducing the function of one NOTCH1 allele confer a competitive advantage on mutant progenitors, making it likely they will form persistent, expanding clones. As the heterozygous mutant population grows, the probability that the remaining allele will be lost increases. When this happens, it confers a further increase in fitness. By driving wild-type cell differentiation, NOTCH1 null cells at the clone margins can divide, resulting in extensive colonisation of the epithelium. Our findings with NOTCH1 mutations in the normal oesophagus also support the findings of this recent study.22

Paulson et al first studied the genomic defects in NeoSE, analysing 20 patient samples for CDKN2A and TP53 gene mutations in NeoSE and surrounding BE epithelium.23 They concluded that typically the NeoSE and BE arise from separate clonal origins, however, in 1 of 20 patients a focus of NeoSE did show a mutation in CDKN2A identical to that found in the surrounding BE. In our study, we were able to analyse eight patients who had both NeoSE and pre-endoscopic therapy Barrett’s neoplasia by a more extensive targeted sequencing panel. We did not identify any shared mutations between the NeoSE and preablation BE tissue. While we tried to sequence paired samples that were in the same region of the oesophagus, given the sampling was performed at different time points, it is also possible that shared mutations were missed due to sampling. Importantly, the Paulson study used neosquamous islands that arose in patients who did not receive ablation therapy and, therefore, did not have a denuded epithelium. This and the fact we used a more sensitive method may explain why we found more frequent TP53 mutations in the NeoSE than they did. Given the lower sequencing depth (~147×) and different targeted panel in cohort 2, we did not combine this cohort...
with cohort 1 for analysis. In cohort 2, 1/8 (12.5%) patients had a TP53 mutation in their NeoSE sample. Whether this lower frequency was due to lower coverage or inherent to the samples is unknown. Further studies using larger cohorts with sufficient coverage (similar to cohort 1) are warranted to solidify the frequency of TP53 mutant clones in NeoSE. We found that, when a mutation was present in TP53 or NOTCH1, there were often more than one mutation identified in the same gene, suggesting either biallelic inactivation, multiple competing clones or a combination.

A couple of caveats to this study should be mentioned. First, this study was designed to analyse samples for clonal populations large enough to be detected by standard clinical tumour sequencing techniques (approximately 2%–3% allele percentage). As prior studies have already shown many mutations in the normal squamous oesophagus using highly sensitive techniques, we were interested in determining if post-RFA re-epithelialization may allow for more frequent and larger mutant clones to develop. It is highly likely that even smaller mutant clones exist in both normal squamous oesophagus and NeoSE that we were unable to detect. Second, the number of samples we were able to analyse was limited and, thus, definitive conclusions were difficult to make and caution is warranted not to over interpret the data. Specifically, the possibility of a type II error, with inability to detect relatively small, but significant, differences in mutation prevalence due to our small sample size, is present in these data. Follow-up studies using larger and more extensively analysed samples will be needed to further clarify the clonal makeup of NeoSE. Third, a field of BE within a patient can be comprised of multiple clones. While often sharing a set of mutations, this is not always the case. Given this potential mosaic distribution of mutations and targeted sequencing, our comparison of pre and post-RFA samples for shared mutations, while consistent with prior findings, does not rule out the possibility of a different pretreatment clone sharing a mutations/clonality with the NeoSE. This is especially true given no germline sample was sequenced and, thus, stringent filtering for pathogenic mutations was required. Finally, Martincorena et al suggested that NOTCH1 mutations were actually more common in normal squamous epithelium compared with squamous cell carcinoma. Even if RFA allows for clonal expansion of NOTCH1 and/or TP53 mutant clones, further studies will be needed to determine whether these expanded clones have a higher propensity for progressing to dysplasia and squamous cell carcinoma.

Acknowledging these limitations, this study identified NOTCH1 and TP53 mutations within both the NeoSE and normal oesophageal squamous epithelium at a similar allele fraction and suggested there were no major differences between the normal squamous oesophagus and NeoSE. A trend for an increased number of detectable TP53 mutations within the NeoSE was seen, which should be confirmed in larger, future studies making sure to control for patient age. Of note, finding these mutations in the normal squamous epithelium using standard clinical sequencing suggests that caution should be taken when sequencing tumour or other samples that are ‘contaminated’ with normal squamous epithelium as the mutations in known tumour suppressor genes could be coming from the squamous epithelium and not the tumour itself. These results suggest that, at least based on identified allele fractions of identified mutations, there does not appear to be large clonal expansions of mutated epithelium in NeoSE but there may be an overall increase in TP53 mutations.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not applicable.

Ethics approval University of California San Francisco (19-27460) IRB exempted the study as it used excess/discarded tissue (ie, archived pathology blocks) collected for an alternative use (clinical care), and no identifying patient information was retained after the samples were collected from the pathology archives.

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Supplementary Figure 1: Number of pathogenic mutations plotted against the age of the patient when the sample was taken. A) Neosquamous samples did not show a correlation with patient age and had multiple patients with more than one mutation. B) Normal squamous samples had a weak correlation with age driven by the two samples from the oldest patient (86 years old). No other sample had greater than one mutation.