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Intra-tumor Genetic Heterogeneity in Squamous Cell Carcinoma of the Oral Cavity (OCC)

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<u>Abstract</u>

Background: We sought to evaluate intra tumor heterogeneity in OCC and specifically determine the effect of physical separation and histologic differentiation within the same tumor.

Methods: We performed whole exome sequencing on five biopsy sites – two from well-differentiated, two from poorly-differentiated regions and one from normal parenchyma – from five primary OCC specimens.

Results: We found high levels of intra tumor heterogeneity and, in four primary tumors, identified only 0-2 *identical* mutations in all sub-sites. We found that the heterogeneity inversely correlated with physical separation and that pairs of well-differentiated samples were more similar to each other than analogous poorly-differentiated specimens. Only TP53 mutations, but not other purported "driver mutations" in HNSCC, were found in multiple biopsy sites.

Conclusion: These data highlight the challenges to characterization of the mutational landscape of OCC with single site biopsy and have implications for personalized medicine.

Introduction

Squamous cell carcinoma of the oral cavity (OCC) is a world-wide health problem with an incidence in males of 6.6/100,000 in the United States and 16.4/100,000 in India.[1] Unlike Human papilloma virus (HPV)-associated oropharyngeal carcinomas,[2] which have exceptionally good outcomes with current treatment modalities,[3] survival for patients with advanced OCC remains poor.[4]

Improved outcomes for patients afflicted with OCC will likely be predicated on a detailed understanding of the genetic changes that drive tumor proliferation/ survival and the correlation of these changes with routine histopathologic findings that are the backbone of current staging

systems. For instance, understanding the variability in mutations amongst select OCC sub-sites is an important consideration for molecular staging systems, such as the Mutant Allele Tumor Heterogeneity (MATH) score, which are poised for integration into clinical care. [5-7] In addition, insight into the *mutational heterogeneity* of OCC is critical to the development of personalized treatment strategies for patients afflicted with this disease. For example, the NCI-MATCH trial relies upon whole exome and mRNA analysis of a single biopsy sample from a patient's tumor to determine if that individual might be successfully treated with an approved drug. Similarly, based on the recent insight that responses to PD-1:PD-L1 axis blockade are not only dependent on a high mutational burden, [8, 9, 10, 11] but also, upon conservation of specific mutations throughout the tumor, [12] understanding of mutational heterogeneity may provide a useful adjunct to prospective immunotherapy trials. Finally, knowledge of the distinct mutations that encode for putative neoantigens shared amongst all and/or the majority of tumor sub-sites is a prerequisite for the development of neoantigen specific cancer vaccines.[13]

Initial large collaborative whole exome sequencing efforts of squamous cell carcinomas of the head and neck (SCCHN) revealed extensive *inter*-tumor heterogeneity, with the involved genes generally conserved in less than 10% of specimens -- notable exceptions including TP53, CDKN2A, FAT1, NOTCH1, SYNE1, and MLL2.[14, 15] Subsequent analysis of data in the **C**ancer **G**enome **A**tlas (TGCA) validated and extended these initial observations, confirming the transcription of these genes harboring mutations in 86% of cases. [16, 17] Unfortunately, with few exceptions, the published studies that define the somatic mutations in OCC and SCCHN do not

speak to the issues of *intra*-tumor heterogeneity nor do they correlate mutational burden with tumor grade. Given that other tumor types such as renal cell carcinoma and pancreatic cancer demonstrate profound intra tumor heterogeneity in both the primary tumor and regional lymph node metastasis, this information is likely critical to our understanding of disease pathogenesis.[18-22]

In this report, we utilized whole exome sequencing (WES) to explore the inter- and intra-tumor mutational landscape of primary OCC. We describe the development and validation of a method that considers three parameters to assess the probability that a non-synonymous mutation in an OCC exon is valid -- (i) identification of a DNA sequence variant with two mutation caller systems, (ii) the extent of DNA sequence coverage for each variant, and (iii) the normalized allele frequency. Using this approach, we mapped the exomes of four unique sites -- two from well and two from poorly differentiated regions -- from five primary OCCs.

Materials and Methods

Pathology: Representative sections (2-3 mm in thickness; at least one section/cm of tumor) were obtained from five randomly selected patients who had undergone surgical resection of their OCC. Sections were fixed in 10% formaldehyde, processed, and embedded in paraffin blocks, from which 5 micrometer sections were cut and stained with Hematoxylin & Eosin (H&E). Selected areas were categorized as "well-differentiated" or "poorly-differentiated" by a

head and neck pathologist. We selected two areas of well-differentiated (e.g. W1 and W2) and two areas of poorly-differentiated carcinoma (e.g. P1 and P2), as well as one area of normal mucosa (N) from each primary tumor. These areas were circled on the H&E stained slides and the distances between all of these selected areas were recorded (Figure 1).

For each of the selected areas, a tissue core (2.0 x 2.0 x 2.5 mm) was obtained with a metallic biopsy needle from the corresponding paraffin block and re-embedded in a new paraffin block. A 5 μm thick section was cut from this new block in the vertical plane and embedded/sectioned in the horizontal plane. The resulting slides were stained with H&E and used to validate the assigned tumor differentiation category, and to determine the tumor purity throughout the core (i.e. % of tumor/core tissue, "biopsy of the biopsy") (Figure 1 and supplemental table 1). The remaining tissue, which represented most (i.e. >90%) of the punched-out core, was submitted for molecular studies. In addition to histologic evaluation, we employed the recently described informatics tool, Sequenza, as an additional means to evaluate tumor purity.[23]

Fresh frozen and FFPE DNA extractions: Genomic DNA was extracted from FFPE sections and biopsy cores using Covaris truXTRAC FFPD DNA Kit (Covaris, Woburn, MA). In brief, FFPE sections less than 25 µm thick or biopsy cores smaller than 1.2 mm in diameter were loaded into Covaris microTUBEs, supplemented with buffer and processed in Covaris E210 instrument to dissociate the paraffin and rehydrate the tissue. Tissue was then lysed with Protease K overnight at 56°C followed by a 1-hr incubation at 80°C to reverse formaldehyde

crosslinks. Genomic DNA was extracted through a spin column provided in the kit, and further purified with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA).

Library construction: Genomic DNA libraries were constructed for sequencing on the Illumina platform using the KAPA Library Preparation Kit (Kapa Biosystems, Wilmington, MA). First, DNA was fragmented with the Covaris E210. Then libraries were prepared using a modified version of manufacturer's protocol. The DNA was purified between enzymatic reactions and the size selection of the library was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA). The PCR amplification step was performed with primers containing an index sequence seven nt in length.

Exome capture & sequencing: Exome capture was performed using the Agilent SureSelect Human All Exon v4 kit according to manufacturer's protocol. Multiplexed sequencing was performed on the Illumina HiSeq 4000 platform using 151bp paired-end runs. An average of 518 million reads were generated for each sample.

Data preprocessing & alignment: Illumina reads were aligned to human reference v37 with decoy sequences using BWA [24] version 0.7.12-r1039. Picard [25] tools (version 1.140) CleanSam, FixMateInformation, and MarkDuplicates were used to clean up any mapping artifacts and mark duplicate read pairs. Indel realignment and base quality recalibration were performed using GATK [26]version 3.3-0. All the tumor and normal samples derived from the same patient were co-realigned. The resulting alignments had an average on-target depth of 284 reads. The median

on-target depth was 260x with a range of 142x -- 479x. On average >99% of the targeted bases were covered at 30x or more with a range of 96% - 100% (Supplemental Table 2).

Variant calling, annotation, and filtering: Somatic SNVs were called using Mutect[27] version 1.1.7 and VarScan[28] version 2.4.1 to obtain a set of high confidence variants. Independent variant calling was done for each sample analyzed. MuTect applies several variant filters to detect any false positive SNVs generated due to mapping and sequencing errors. A variant was identified as a false positive if: 1. it was observed in the matched normal sample, 2. there was a proximal gap, 3. a site exhibited poor mapping or strand bias, 4. alternate alleles were clustered at a consistent distance from the read ends, or 5. the site was tri allelic. The VarScan call set was also processed to obtain a high confidence set. Default allele frequency thresholds were applied to variants classified as somatic. If the variant allele frequency was >5% in tumor and <5% in normal, it was marked as high confidence. Furthermore, variants were filtered to identify false positives due to poor mapping, strand bias, read position bias, etc.

The resulting somatic variants were annotated for functional effect using Annovar[29] (version 2015-06-17). Annovar was also used to annotate the variants with allele frequencies in the 1000 Genomes Project, NHLBI-ESP 6500 exomes, CLINVAR, COSMIC, NCI60 and to calculate scores for prediction algorithms including, SIFT, PolyPhen, and MutationAssessor. The 1000 Genomes Project alternate allele frequency threshold was set to 0.01 to filter out common variants.

To evaluate the purity of the normal samples, we calculated the allele frequency distribution of each sample. An average of 83% of variant AF were heterozygous ($0.4 <= AF <= 0.6$) or homozygous ($AF > 0.9$), with a range of 77% - 86% (Supplemental Table 3, Supplemental Figure 1).

Because FFPE data carries a risk of artefactual mutations due to cytosine deamination, we evaluated the prevalence of C->T and G->A mutations in each sample (Supplemental Figure 2). Though a number of samples showed significant enrichment for these mutation types, after removing all C->T and G->A mutations from the final call set, the rate of overlap among the samples changed by less than 1% (Supplemental Figure 3, Supplemental Table 4).

Somatic variant validation: All non-synonymous, stop loss and stop gain variants that were called with high confidence by both callers were used to pick a set of variants for validation. These were prioritized using MutationAssessor[30] predictions and cross-referenced with genes known to be associated with cancers (specifically head and neck cancers) using COSMIC [31] and tumorportal.org[32], and a final validation set was chosen. PCR primers for each variant were selected using a combination of Primer3 and manual primer design. Resulting PCRs were sequenced using barcoded, multiplexed runs on a PacBio RS II with P6-C4 chemistry. The resulting PacBio CCS reads were aligned to the human reference with BLASR and the alignments and consensus calls were manually inspected using IGV.

Statistical Analysis: The Jaccard similarity coefficient *J(A,B)* was used for quantifying the co-occurrence of SNVs in two samples from the same patient, A and B:

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|} = \frac{|A \cap B|}{|A| + |B| - |A \cap B|}$$

For constructing dendrograms the Jaccard distance or Jaccard dissimilarity defined as *1-J(A,B)* was used as the distance metric between samples. Dendrograms were produced using Wolfram Mathematica ver. 11.0.0. A forest plot of the fraction of shared SNVs in individual samples was generated using the 'meta' library in the R software.[33] Partial correlation was used to test the significance of the association between increasing Jaccard similarity and decreasing physical distance between samples with the effect of parent tumor removed. Likewise, the uni-directional hypothesis that Jaccard similarity increases with decreasing physical distance between paired samples in individual tumors were tested using the non-parametric Spearman's rank correlation coefficient, R_s. The corresponding P-value is for rejecting the hypothesis $R_s=0$. These analyses were conducted using IBM SPSS Statistics ver. 24.0.0.

Study Approval: All studies were conducted under the auspices of a protocol approved by the Institutional Review Board, University of Maryland, Baltimore. As determined by the IRB the protocol did not require written informed consent.

Results

Tumor Site and Stage

Four study participants had SCC of the oral tongue (Patients 5280, 1646, 6803, 7157), while one tumor originated in the lower gingiva (9464). Patient's ages ranged from 49-89 years. Tumors were pathologically staged TNM classification as: T2N0, T1N2c, T3N2c, T2N0, and T1N0. None of the patients had distant metastatic disease at time of resection.

Validation of Somatic Mutations Correlates with both Allele Frequency and Sequence Coverage

We analyzed our WES data using two widely employed caller systems, MuTect and VarScan, and only validated mutations called by both systems (Table 1).[27, 28] We performed 190 PCR reactions for overlapping SNVs and successfully validated 139 (73%). The frequency of allele detection and sequence coverage directly correlated with successful validation (Table 1, Figure 2). With sequence coverage greater than 500, the validation rate was 79% and for allele frequency greater than 0.5, the validation rate was 86%. Combining both of these criteria resulted in a validation rate of 100%. Lowering the coverage to >250 with allele frequency >0.5 still produced a 92% validation rate. Collectively, these data define the likelihood that a mutation identified by our approach *in silico* is valid based on set parameters.

Primary OCCs have Variable Mutational Burdens

In order to determine if the mutational burden of OCC was consistent throughout primary OCCs, we evaluated high impact SNVs -- defined as non-synonymous or stop mutations-- in four samples from each primary tumor (Table 2). We identified 635 SNVs, with each primary tumor having variable tumor wide and sub-site specific mutational burdens (range: 60-223/ patient tumor) (Figure 3A). For example, in tumors 5280 and 1646, two of the sub-sites had high and two had low mutational burden indices. In contrast, two tumors (9464 and 6803) displayed high mutational burdens with internal consistency. Finally, the mutational burden index was approximately equal in three of the four sub-sites from tumor 7157. These data demonstrate that OCCs have variable levels of *intra* and *inter* tumor mutational burdens that contributes to their overall genetic diversity.

Primary OCCs have high Level of *Intra* Tumor Heterogeneity

In general, these tumors had high levels of mutation and gene specific intra tumor heterogeneity. For example, in four primary tumors, we identified only 0-2 *identical* mutations in all sub-sites -- a finding that correlated with a 0.7% median SNV overlap per patient. For the three patients that did share at least one common SNV in all four sub sites, each SNV corresponded to one gene (supplemental table 5). Interestingly, in two of these patients, we found a relatively high percentage of identical mutations, in three of the four sub-sites. Similarly, in tumor 9464, despite the discovery of 41 identical mutations found in all four sub-sites, individual regions showed high numbers of mutations that were either unique or shared by only one other area. (Figure 3B, supplemental table 5).

To quantify the mutational variability among samples from within a tumor, we computed dendrograms with the Jaccard Dissimilarity coefficient as the measure of mutational distance between pairs of samples (Figure 3C). The Jaccard Dissimilarity coefficient is the proportion of all somatic mutations present in one of the two samples in a pair that are present in both samples. These data demonstrate considerable tumor-to-tumor variation in the mutational relatedness among samples. As an example, tumor 1646 shows a relatively higher similarity within the poorly-differentiated and the well-differentiated samples than between well versus poorly-differentiated samples. The independent sample Kruskal-Wallis test showed that in this small sample, the distribution of the Jaccard coefficient was not statistically significantly different among the tumors (P=0.19).

Intratumor Heterogeneity is Partially Constrained by Physical Distance and the Degree of Histologic Differentiation

Within each tumor, the relationship between the distance between samples and their mutational profiles was variable. However, combined analysis of four tumors (6803 was not included because P2 had no SNVs called by both calling systems) showed samples that were physically closer together shared more common SNVs (p=0.03). Furthermore, well-differentiated samples within each tumor had a higher percentage of shared SNVs (median 80% overlap) compared to poorly-differentiated specimens (Median overlap 1.3%) (p=0.06) (Table 2). This trend was not explained by physical distance between samples as there was no difference in proximity between the well and poorly-differentiated samples (p = 0.1). Importantly, as

highlighted in Figure 3C, in some tumors (5280 (W1 and P1), 9464 (P2 and W2/W1), and 7157 (P1 and W2), well-differentiated samples shared more SNVs with poorly-differentiated samples, and in all of these cases tumor samples were of short distance from each other (each 3mm) (Supplemental table 6).

The Majority of SNVs Identified are in Genes Not Typically Associated with SCCHN

Previous WES studies have identified a group of mutated genes that are conserved in a relatively small number of SCCHN specimens.[17, 34-36] Using the two-caller approach, we only found only one gene, TP53, which was mutated in more than one patient (Table 5). NOTCH1 and 2, CASP8, and SYNE1 were detected in 2-4 of the primary tumor specimens but, generally, not by both callers. Furthermore, unlike TP53 and CDKN2A, in which conserved tumor specific SNVs were identified by both calling systems in all 4 sub-sites throughout an *individual* tumor (patient 9464), when present, NOTCH1 and 2, CASP8, and SYNE1 were only found in 1-2 primary tumor sub-sites, even when considering calls made by only one calling system. Finally, even with TP53, we did not identify any common SNVs between patients.

Discussion

Whole exome sequencing of four spatially separated primary tumor samples from each of five patients with OCC demonstrated significant inter and intra-tumor variability in both mutational burden indices and specific somatic SNVs. Interestingly, the physical distance between samples had a significant inverse correlation with their genetic similarity. Furthermore,

pairs of well-differentiated samples trended towards sharing greater numbers of conserved SNVs than their poorly differentiated analogues.

There is limited information on the mutational burden and the mutational heterogeneity of OCC and SCCHN in general. In the most comprehensive study to date, SNV heterogeneity of OCC was analyzed with a 202-gene "cancer gene" panel using 2-3 distinct samples from each primary tumor and 0-2 samples from one lymph node/patient. The findings from this study indicate a high degree of intratumor concordance amongst the distinct areas sampled from the four patients with oral tongue cancer, with dramatically higher levels of heterogeneity in the one patient with a floor of mouth malignancy.[37] The differences observed between this report and our study may be as a result of the small samples sizes examined and/or by the use of closed versus open data sets, respectively – the latter a supposition reinforced by our observation that the majority of SNVs found in our study occurred in genes not commonly associated with SCCHN. Taken in concert, these data suggest that while multiple sub-sites from individual OCCs share common mutations in the exomes of a defined group of cancer relevant genes, this overlap does not reflect the profound heterogeneity captured by WES. Similar to our study, for example, a high level of intratumor heterogeneity was identified using whole genome sequencing of an HPV positive oropharyngeal cancer patient.[38]

Previous WES studies have shown that certain genes are preferentially targeted for mutations in SCCHN.[17, 34-36] Based on these data, there is speculation that some of these genes e.g. TP53 and NOTCH1, are integral to the pathophysiology of these tumors in select patients.

We found that TP53 was mutated in a large number of tumor sub-sites in more than one patient. These findings are consistent with recent studies in non-small cell lung cancer, demonstrating that TP53 driver mutations are generally clonal.[21] However, while NOTCH1 and 2, CASP8, and SYNE1 were detected in 2-4 of the primary tumor specimens, they were only identified in 1-2 sub-sites, even when we liberalized our detection approach to the use of a single caller system. While the interpretation of these findings is debatable, they appear to suggest that these genes may not be required in OCC -- at least in certain tumor sub-sites -- and, unlike in squamous cell lung cancer, might have occurred later in the process of tumor progression.[21, 39]

In addition to carefully characterizing the mutational heterogeneity of OCC, we asked whether the degree of overlap observed correlated with either the distance between samples and/or histologic differentiation. As anticipated, the distance between samples was inversely correlated with the number of shared SNVs. The fact that these findings were not absolute suggests that OCCs may adhere to both linear as well as non-linear genetic models of progression.[40-42] In the long term, it will be important to determine whether, like other tumors, the molecular pathways used for tumor growth and survival in these distinct regions, move towards concordance, as is observed in other tumors.[43-45]

To our knowledge, this is the first evaluation of the effect of histologic differentiation on intra tumor genetic heterogeneity. Well-differentiated areas within the same patient's tumor trended towards sharing a higher percentage of common SNVs than poorly differentiated regions. This effect was not explained by distance; as well-differentiated samples were not

significantly closer to each other than poorly differentiated samples. Also, this observation does not appear to be related to the absolute number of SNVs identified, as both poor and well-differentiated samples varied in mutational burden. While the small sample size of our study mandates that these data be interpreted with caution, they suggest that mutational heterogeneity might correlate with the degree of histologic differentiation in OCC. Further studies will be required to validate or refute this finding.

There are several limitations to our study. First, because of the relatively small sample size, our observations will need to be validated in a larger patient cohort. Second, while we developed and validated a "two caller" screening algorithm for mutant allele detection that may prove useful for estimating the frequency of "valid calls" in cases where concomitant RNA Seq is not planned, the requirement that both callers identify the same mutation, almost certainly decreased the sensitivity of SNV detection, and may have led to a higher false negative rate. However, as shown in supplemental figure 4 and 5 the use of one calling system only decreased the intratumor heterogeneity slightly or not at all when comparing all samples. Additionally, as highlighted in table 3, in only one patient for one gene (TP53 in 5280 in P1 called by Mutect but not Varscan) was there a discrepancy where the mutation was not found in all samples as a result of using two calling system. We analyzed our validation data set, and found only 10 SNV positions where the validation PCRs detected the variant in a tumor sample where it was not predicted (i.e. the variant was present but missed by our algorithm). Of these, 4 were found by neither Mutect of VarScan, 1 was found by Mutect but not VarScan, and 5 were found by Mutect

but rejected as poor-quality or likely contamination. Therefore, we do not feel that the use of a two-caller screening algorithm significantly affected our results.

A third limitation of our study, and all genomics studies comparing samples, is the variability in tumor purity between samples. In order to better understand this inherent variability, tumor purity was defined using both "2-dimensional" H & E staining and analysis with the recently developed informatics tool, Sequenza.[23] The fact that these methods had variable concordance highlights the fact that better analytic tools are required to evaluate tumor purity.

Finally, to improve the functional relevance of our study, we only included non-synonymous SNV mutations in our analysis. Because somatic insertion/deletion calling carries a higher error rate, we elected to focus on SNVs to provide a more accurate call set from which to conduct intra-tumor comparisons. The exclusion of synonymous mutations and Insertions/Deletions (INDELs) from our analysis reduced estimates of mutational burden, with the effects on mutational heterogeneity being less certain. Finally, the observation in certain patients that large numbers of unique SNVs were found in only one of the four regions analyzed, suggests that our study likely under represents the mutational heterogeneity of OCC.

Conclusion

To the best of our knowledge, this is the first report to characterize the mutational burden and mutational heterogeneity of OCC using a whole exome sequencing approach.[38,37] Our

findings indicate that OCCs have variable levels of mutational burden and high levels of intratumor heterogeneity -- heterogeneity that is inversely correlated with the physical distance between specimens and is less variable in well versus poorly-differentiated regions. Our data also suggest, that some of the mutations e.g. NOTCH1, that were postulated to be drivers of tumorigenesis in select patients, may not be required in all tumor sub-sites. Finally, from a therapeutic perspective, our data highlight the challenges of using a single site biopsy for "precision medicine."

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Tables

Table 1. Validation by Allele frequency and Coverage[*]

Allele Freq	All validation attempts			PCR+ validation attempts		
>0.75	5/11 (45%)	3/5 (60%)	1/1 (100%)	5/6 (83%)	3/4 (75%)	1/1 (100%)
0.5 -- 0.75	14/19 (74%)	9/10 (90%)	11/11 (100%)	14/18 (78%)	9/10 (90%)	11/11 (100%)
0.25 -- 0.5	17/38 (45%)	17/32 (53%)	14/25 (56%)	17/30 (57%)	17/26 (65%)	14/21 (67%)
<0.25	11/36 (31%)	11/26 (42%)	16/24 (67%)	11/25 (44%)	11/18 (61%)	16/20 (80%)
Coverage	0 -- 250	250-- 500	>500	0 -- 250	250 -- 500	> 500

[*] "All validation attempts" refers to all validation attempts, including PCR failures. The "PCR+ validation attempts" set includes only those validation attempts where a PCR amplicon was obtained.

Table 2. Comparison of overlap of SNV's in tumor samples from all patients.[b]

Patient	Site Stage[h]	W1	W2	Distance (mm)[d]	Shared SNVs[e] n(%)	P1[c]	P2	Distance (mm)[d]	Shared SNVs[e] n(%)	Total Unique SNVs[f]	Total Shared SNVs[g] n(%)
5280	Tongue T2N0	29	81	2.5	22(25)	15	59	0.5	1(1)	149	1(0.7)
1646	Tongue T3N2c	58	68	5	56(80)	5	12	11	0(0)	81	0(0)
9464	Gingiva T1N2c	128	126	2	122(92)	131	128	1	42(19)	223	41(18)
6803	Tongue T2N0	57	56	10	55(95)	48	0	7	0(0)	60	0(0)
7157	Tongue T1N0	5	78	15	2(2)	84	93	1	58(49)	122	2(1.6)

[b]Variants are Non-synonymous exonic SNV's discovered by both calling systems

[c] W = well differentiated sample. P= Poorly differentiated sample. Numeric value represents the total number of SNVs in each sample.

[d]Distance between samples (W1 to W2 or P1 to P2)

[e] Found in both samples within a patients tumor (W1 and W2 or P1 and P2)

[f] Total number of unique SNVs in all 4 samples for each patient.

[g] Found in all 4 samples within a patient's tumor. Each SNV corresponds to one gene.

[h] Staging represents pathologic stage

Table 3. Mutations in commonly reported genes in SCCHN by patient and tumor sample[a]

Mutated Gene[b]	Patient 5280	Patient 1646	Patient 9464	Patient 6803	Patient 7157
TP53	W1,W2,P2 P1 (MuTect)	W2,W1, P2	All samples	None	W1 (Mutect) W2, P1, P2
NOTCH1	P2 (MuTect)	P2 (VarScan) W2	None	None	None
NOTCH2	None	W2 (MuTect)	W2 (VarScan) P2 (VarScan)	None	None
NOTCH3	None	None	None	P1, W1, W2	None
CDKN2A	None	P2 (MuTect) W1, W2	All samples	None	None
PIK3CA	None	None	None	None	None
FBXW7	W2	None	None	None	None
HRAS	None	P2 (VarScan)	None	None	None
NFE2L2	None	None	None	None	None
FAT1	P2 (Varscan)	None	None	None	None
CASP8	W1 (Mutect)	P1 (MuTect)	None	P1 (VarScan)	W1 (MuTect)
FGFR3	None	None	None	None	None
CEBPA	None	None	None	None	W2 (VarScan)
FES	P2 (Mutect)	None	None	None	None
TGFBR2	None	None	None	None	None
SYNE1	W2	W2 (VarScan), P2	P1 (Mutect)	P2 (MuTect) W1 (MuTect)	None
TP63	None	W2, W1	None	None	None
PTEN	None	P2 (VarScan) W1, W2	None	None	None
EGFR	P2	None	None	None	None

[a]Commonly reported genes = as reported by TCGA data

[b]Mutation was called by both Mutect and Varscan unless specified

Figure Legends

Figure 1. Approach for individual sample identification.

A, B, D. We harvested two areas of poorly-differentiated tumor (P) and two areas of well-differentiated tumor along with histologically normal tissue from the same site (N). For every core biopsy of tumor obtained, a 5-micron thick section was cut from this core (biopsy of the biopsy) and the resulting slides were stained with H&E to evaluate purity of differentiation in vertical direction of the sample. **C**. Distances between samples were mapped in two dimensions for statistical analysis.

Figure 2. Mutation validation as a factor of coverage and allele frequency.

Selected mutations were validated by PCR and PacBio sequencing from a range of coverages and allele frequencies. Tumor allele frequencies were normalized by expected tumor purity values. Validation attempts with strong, high-coverage support are labeled concordant or discordant. Those with low-coverage support are labeled inconclusive concordant or discordant.

Figure 3. OCC has high intra and inter tumoral heterogeneity.

A. Mutational burden index -- The percentage of mutations in a given sample/ total number of mutations for a given patient. Blue Diamond -- Average mutational burden for each primary tumor. W-well differentiated; P- poorly differentiated. Table shows tumor purity by Sequenza sequence-based analysis vs pathology assessment **B.** Venn Diagrams showing the overlap of mutations in shared genes between individual sites. **C.** Dendrograms created using the Jaccard

dissimilarity coefficient between pairs of samples from within each tumor. X-axis is the value of the Jaccard coefficient. The Jaccard Dissimilarity coefficient is the measure of mutational distance between pairs of samples. The Jaccard Dissimilarity coefficient is the proportion of all somatic mutations present in one of the two samples in a pair that are present in both samples.
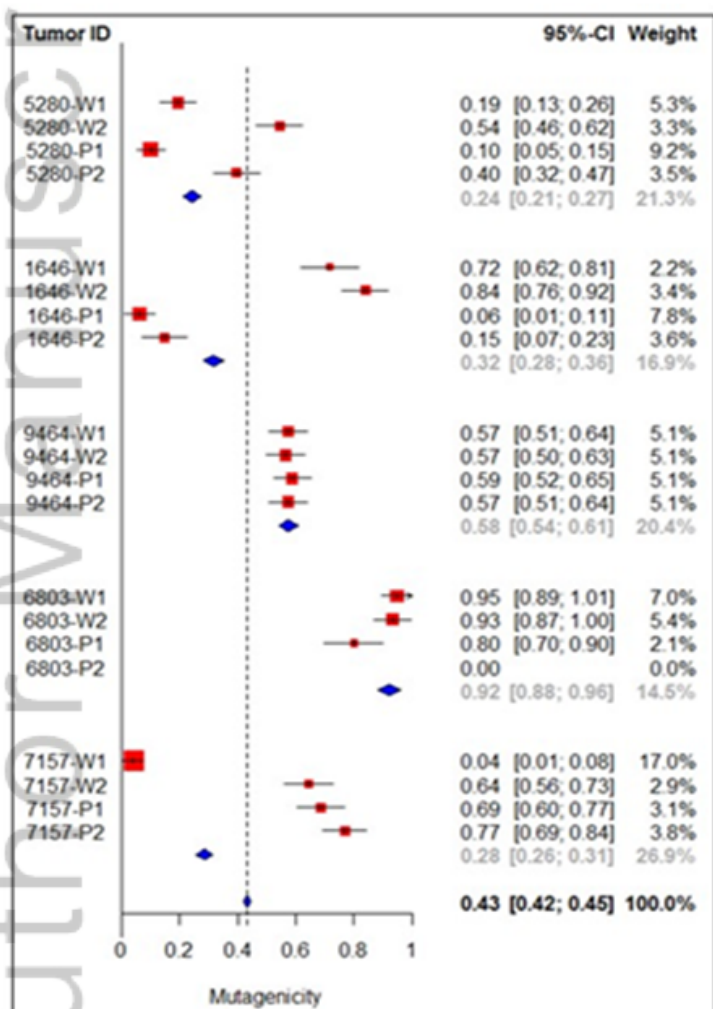
Figure 1.
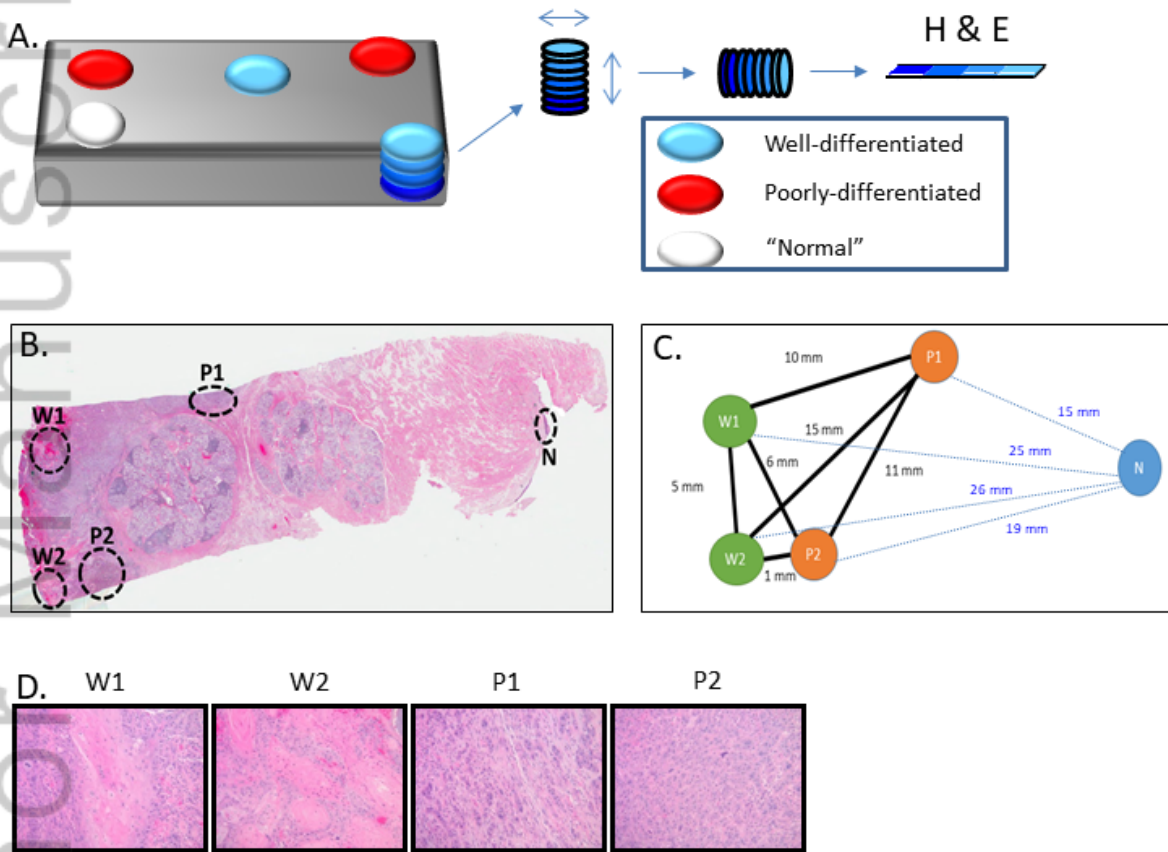
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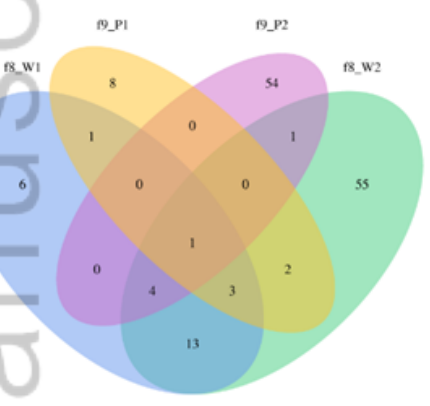
Figure 2.
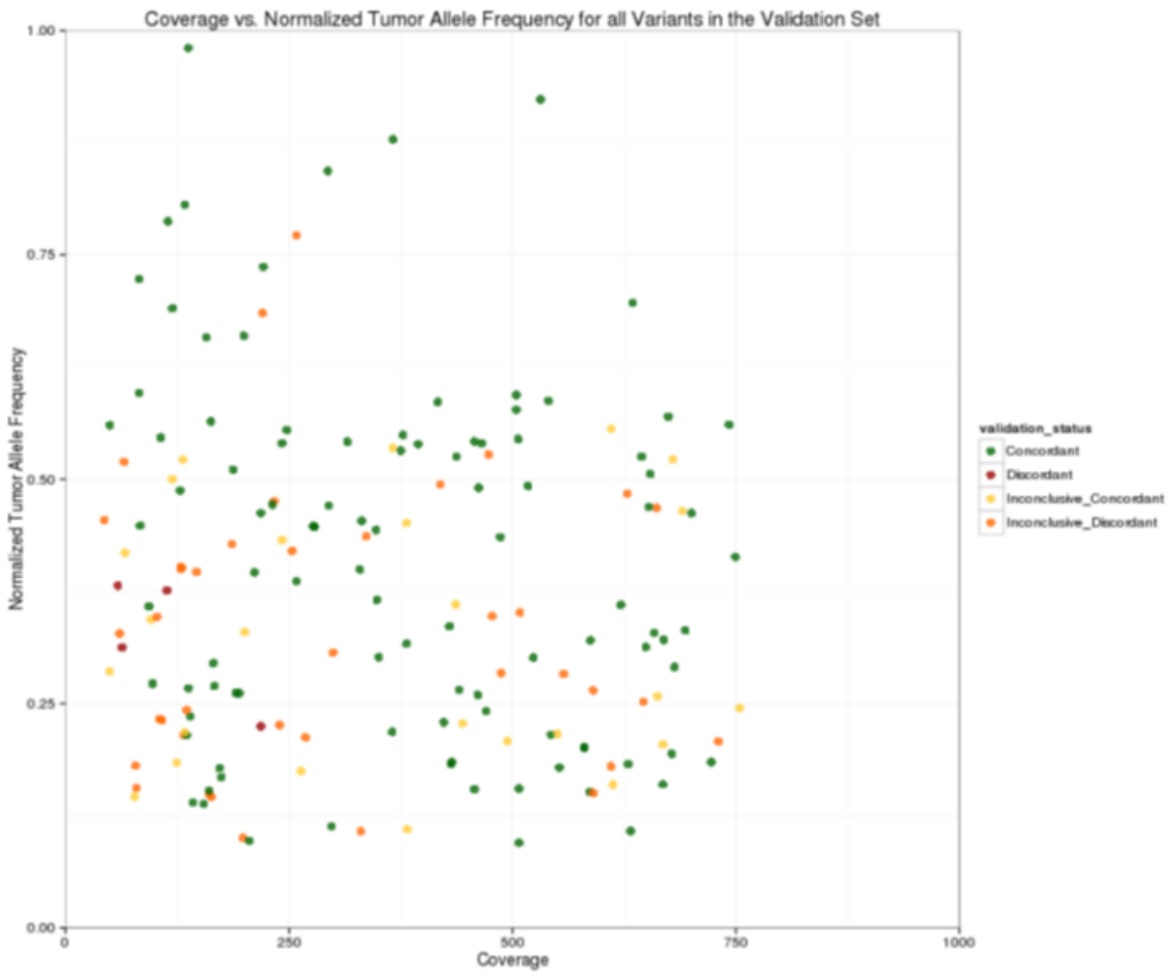
HED_25719_Slide2.TIF

Figure 3A.

| Sample name	Sequenza Estimated Tumor Cellularity	Sequenza Estimated Ploidy	Estimated tumor purity by pathology
5280-W1	60%	2.1	40%
5280-W2	24%	6	100%
5280-P1	58%	2.1	35%
5280-P2	13%	2.3	35%
1646-P1	42%	2.1	20%
1646-P2	49%	2	70%
1646-W1	27%	4.1	80%
1646-W2	62%	3.7	100%
9464-W1	36%	4.1	70%
9464-W2	69%	4.1	95%
9464-P1	33%	3.6	25%
9464-P2	36%	4.1	65%
6803-P1	35%	3.9	100%
6803-P2	54%	2	15%
6803-W1	26%	4	60%
6803-W2	19%	4.2	85%
7157-W1	61%	1.9	10%
7157-W2	81%	1.9	80%
7157-P1	80%	1.8	85%
7157-P2	34%	3.7	45%

HED_25719_Slide3.TIF

Figure 3B.

Patient 5280

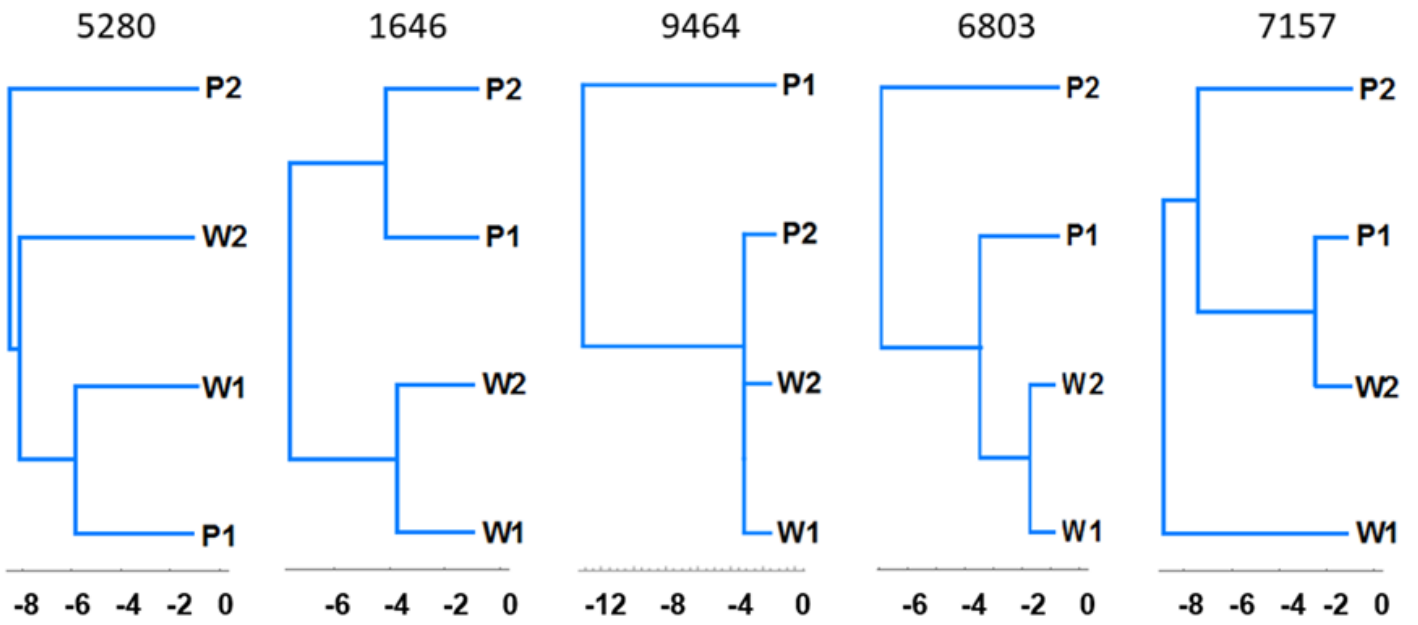Patient 1646
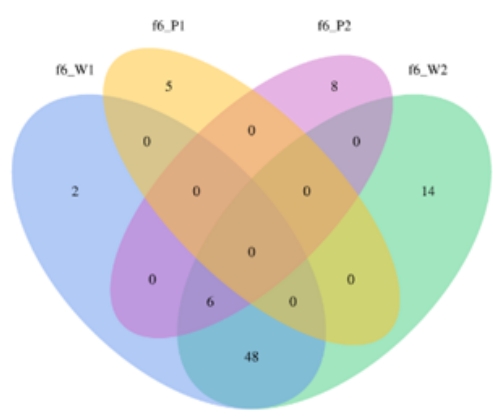
Patient 9464

Patient 6803

Patient 7157

HED_25719_Slide4.TIF

Figure 3C.